The Role of Type I Interferon in Vitiligo Pathogenesis and Melanoma Immunotherapy

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THE ROLE OF TYPE I INTERFERON IN VITILIGO PATHOGENESIS AND MELANOMA IMMUNOTHERAPY

A Dissertation Presented

By

Rebecca Lee Riding

Submitted to the Faculty of the

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Immunology and Microbiology Graduate Program
THE ROLE OF TYPE I INTERFERON IN VITILIGO PATHOGENESIS AND MELANOMA IMMUNOTHERAPY

A Dissertation Presented

By

Rebecca Lee Riding

This work was undertaken in the Graduate School of Biomedical Sciences

Immunology and Microbiology Program

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March 5, 2020
DEDICATION

I dedicate my thesis dissertation to my research mentors Estelle Hrabak PhD, Lenny Shultz PhD, Lisa Carney, Jillian Richmond PhD, Ann Rothstein PhD, and John Harris MD/PhD. To Dr. Hrabak, you taught me how to think critically, ask questions, be curious and fostered my love for science. To Dr. Shultz and Lisa, I learned so much from you about how to conduct science and gained skills that have brought me immense success throughout my PhD. To Jillian, you have been an incredible mentor even before joining the Harris lab. I thank you for your knowledge, advice, and for just listening. To Dr. Rothstein, you have been such a critical mentor and support for me during my PhD. Your experimental advice, knowledge, time, and help with my writing – you have grown me as a scientist in many ways. To Dr. Harris, I have learned an incredible amount from your leadership and mentorship. You have taught me about confidence, how to be curious and ask questions even when your question may be “stupid”, how to persevere through times when nothing is working. You have allowed me to grow as a scientist, supported me in my long term career goals and provided me with such a rich scientific experience. I thank you all for your unwavering support and advice over the years. I have taken it with me and will pass it down to others.
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I want to thank Dr. Tony Carruthers, Dr. Ken Knight, and Dean Mary Ellen Lane for the opportunities they provided me to grow and learn by serving on multiple committees and in leadership roles. These experiences have greatly enhanced my growth as a person and as a scientist and I am lucky to have been a part of these groups.

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ABSTRACT

Vitiligo is an autoimmune skin disease in which the pigment producing cells of the epidermis, melanocytes, are targeted for destruction by CD8$^+$ T cells specific for melanocyte/melanoma-shared antigens. Previous work has identified IFN$\gamma$ as the central cytokine driving disease pathogenesis in both human patients and in our mouse model of vitiligo. IFN$\gamma$ signaling induces production of the chemokines CXCL9 and CXCL10, which trigger autoreactive T cell migration into the epidermis where effector T cells can target and destroy melanocytes. However, both IFN$\gamma$ and type I IFN signaling through activation of STAT1 proteins can induce transcription of the chemokines CXCL9 and CXCL10. Therefore, it seems reasonable that type I IFN signaling may also contribute to disease pathogenesis.

The role of type I IFN in vitiligo is still unclear. Genome wide association studies identified multiple genes within the type I IFN pathway including TICAM1 and IFIH1 as susceptibility loci in vitiligo. One additional study reported increased epidermal staining of CD123, a marker expressed by pDCs, and the type I IFN induced gene MX1 in vitiligo patient skin. However, this study did not show any functional data to support the role of type I IFN signaling in vitiligo pathogenesis. Since the role of type I IFN in vitiligo is ill-defined, we used two different mouse models of vitiligo to functionally determine the role of type I IFN in disease by inducing vitiligo in hosts which lack the type I IFN receptor (IFNaR).
In the first model, we induced vitiligo by adoptive transfer of melanocyte-specific CD8 T cells, which are activated in vivo by infection with recombinant vaccinia virus (VACV) expressing their cognate antigen. Vitiligo induction in IFNaR-deficient mice led to the development of severe disease compared to wild type mice. Acceleration and severity of disease was characterized by increased early recruitment of melanocyte-specific CD8 T cells to the skin, increased production of effector cytokines TNFα and IFNγ, and reduced PD-1 expression. Increased production of IFNγ by CD8 T cells in the skin of IFNaR-deficient mice led to increased expression of the chemokines CXCL9 and CXCL10 driving disease progression. IFNaR-deficient mice also displayed significantly increased VACV titers compared to wild type hosts. This data reveals a role of type I IFN in the clearance of recombinant VACV. This data also suggests that persistent VACV infection and prolonged antigen exposure in IFNaR deficient hosts is likely driving enhanced activation of melanocyte specific CD8 T cells and the subsequent development of severe vitiligo.

Since melanocytes and melanoma cells express shared antigens that can be recognized by CD8 T cells, and because the development of vitiligo after melanoma immunotherapy is a positive prognostic factor for patients, we asked whether VACV vaccine therapy in IFNaR deficient mice would enhance the anti-tumor response to melanoma. B16-F10 inoculated wild type and IFNaR-deficient mice received adoptive transfer of melanocyte-specific CD8 T cells in combination with vaccinia virus expressing their cognate antigen to activate the cells in vivo. Treatment of adoptive T cell transfer and infection with VACV in IFNAR-deficient mice revealed significantly reduced tumor burden compared
to wild type mice. Improved tumor regression in IFNaR-deficient hosts was characterized by increased infiltrating cytotoxic T lymphocytes and reduced PD-1 expression. These results further demonstrate that in the absence of type I IFN, hosts mount a robust cytotoxic CD8 T cell response against melanocyte/melanoma antigens and this is likely a result of persistent VACV that leads to prolonged CD8 T cell priming. As a result, IFNaR deficient hosts kill tumor cells more efficiently.

To determine whether type I IFN regulates disease pathogenesis in the absence of virus infection, we generated a model of vitiligo in which bone marrow derived dendritic cells (BMDCs) pulsed with the cognate antigen were used to prime melanocyte-specific T cells in place of the viral vector. Induction of vitiligo in IFNaR-deficient hosts using BMDCs revealed no significant differences in disease score compared to wild type hosts. This data clearly demonstrates that type I IFN, in contrast to IFNγ, is not required during the effector stage of vitiligo pathogenesis in mice.

However, since we intentionally activate transferred melanocyte-specific CD8 T cells with VACV or BMDCs expressing their cognate antigen, our mouse models may circumvent the role of type I IFNs in initiating activation of autoreactive cells and driving autoimmunity. Type I IFN is critical for providing innate immune signals that drive the priming of autoreactive T cells through maturation of DCs by inducing antigen presentation, co-stimulatory molecule expression, and migration to the lymph nodes to encounter naïve T cells. Our mouse models of vitiligo may not capture this process. We have addressed this question by using a TLR ligand to activate BMDCs before transfer into hosts. In fact, activation of BMDCs before transfer leads to significantly enhanced
vitiligo in mice and this is partially a result of type I IFN signaling on host cells. Thus, we provide evidence that type I IFNs can enhance the activation of melanocyte-specific CD8 T cells and drive autoimmunity.

Collectively, our results show that type I IFN signaling has disparate effects on autoreactive T cell priming in a context dependent manner. We reveal that although type I IFN is not required for the effector phase of vitiligo in mice, maturation of DCs and subsequent type I IFN production can enhance the priming of autoreactive T cells and enhance vitiligo severity. Our studies also reveal that type I IFN is required to clear recombinant attenuated VACV infection and vaccine administration in IFNaR deficient hosts led to a robust autoreactive and anti-tumor response. These insights describing the role of type I IFN in autoimmunity and tumor immunology could have important implications for T cell dependent tumor immunotherapy.
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LIST OF ABBREVIATION USED IN THIS WORK

AGS: Aicardi Goutières syndrome
AIM2: Absent in Melanoma 2
APC: Antigen presenting cell
BMDC: Bone marrow derived dendritic cell
c-GAS: Cyclic GMP-AMP synthase
CCL: C-C motif ligand
CCR: C-C motif receptor
CD: Cluster of Differentiation
cDC: Conventional dendritic cell
cGAMP: Cyclic guanosine monophosphate-adenosine monophosphate
CLA: Cutaneous lymphocyte antigen
CTL: Cytotoxic T lymphocyte
CTLA-4: Cytotoxic T-lymphocyte associated protein 4
CXCL: C-X-C motif ligand
CXCR: C-X-C chemokine receptor
DAMP: Damage associated molecular pattern
DC: Dendritic cell
EAE: Experimental autoimmune encephalomyelitis
eIF2α: Eukaryotic initiation factor 2
EYFP: Enhanced yellow fluorescent protein
FADD: Fas associated protein with death domain
FASL: Fas ligand
Foxp3: Forkhead box P3
GAS: Gamma interferon activated site
GFP: Green fluorescent protein
GM-CSF: Granulocyte-macrophage colony stimulating factor
GWAS: Genome wide association studies
GZMB: Granzyme B
H2O2: hydrogen peroxide
HCV: Hepatitis C virus
hgp100: human gp100 peptide25-33
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigen
HSV: Herpes Simplex Virus
IDO: Indoleamine 2,3-dioxygenase
IFIH1: Interferon induced with helicase C domain 1-(also named MDA-5)
IFITM1: Interferon induced transmembrane protein 1
IFN: Interferon
IFNaR: Interferon alpha receptor
IFNGR: Interferon gamma receptor
IFNy: Interferon gamma
IKKe: Inhibitor of nuclear factor kappa B kinase subunit epsilon
IL-2R: Interleukin 2 receptor
IL: Interleukin
IRAK2: Interleukin 1 receptor associated kinase 2
IRF: Interferon regulatory factor
ISG: Interferon stimulated genes
ISGF3: Interferon stimulated gene factor 3
ISRE: Interferon sensitive response element
JAK: Janus kinase
LCMV: Lymphocytic choriomeningitis virus
LPS: Lipopolysaccharide
MAL: MyD88 adaptor like
MAPK: Mitogen-activated protein kinase
MART1/MelanA:
MAVS: Mitochondrial antiviral-signaling protein
MDA: Melanocyte differentiation antigen
MFI: Mean fluorescent intensity
MHC: Major histocompatibility complex
MS: Multiple sclerosis
mTOR: Mammalian target of rapamycin
MVA: Modified vaccinia virus Ankara
MX1: MX dynamin like GTPase 1
MyD88: Myeloid differentiation primary response 88
nbUVB: Narrow band ultraviolet B
NFκB: Nuclear factor kappa light chain enhancer of activated B cells
NK: Natural killer
NLRP: nucleotide binding domain (NOD) like receptor protein
OAS: 2,5-oligoadenylate synthetase
ODN: Oligodeoxynucleotide
P13K: Phosphoinositide 3 kinase
PAMP: Pathogen associated molecular pattern
PBMC: Peripheral blood mononuclear cells
PD-1: Programmed cell death protein 1
PD-L1: Programmed death-ligand 1
pDC: Plasmacytoid dendritic cell
PKR: Protein kinase R
PMEL/Pmel-1: Premelanosome protein constrained TCR (effector PMEL CD8 T cells)
PolyIC: Polynosinic:polycytidylic acid
PRR: Pattern recognition receptor
REX: Reporting expression of CXCR3 ligands
RIG-I: Retinoic acid-inducible gene 1
RIP: Receptor interacting protein
RLR: RIG-I like receptors
ROS: Reactive oxygen species
S1P: Sphingosine-1 phosphate
SARM: Sterile-alpha and Armadillo motif containing protein
SCF: Stem cell factor
SLE: Systemic lupus erythematosus
SOCS: Suppressor of cytokine signaling
STAT: Signal transducer and activator of transcription
STING: Stimulator of interferon genes
TBK-1: TANK-binding kinase 1
TCR: T cell receptor
Th: T helper
TICAM1: Toll like receptor adaptor molecule 1- (also named TRIF)
TLR: Toll like receptor
TNFα: Tumor necrosis factor alpha
TRAF6: Tumor necrosis factor receptor associated factor 6
TRAM: TRIF related adaptor molecule
Treg: Regulatory T cell
TREX1: Three prime repair exonuclease 1
TRM: Resident memory T cell
TRP: Tyrosinase related protein
TVEC: Talimogene laherparepvec
TYK2: Tyrosine kinase 2
VACV: Vaccinia virus
VSV: Vesicular stomatitis virus
WT: Wildtype mice
PREFACE TO CHAPTER 1

Chapter 1, which serves as the Introduction to the dissertation, includes new and original text written by Rebecca L. Riding that is incorporated with text from the manuscripts cited below. “Mouse Model for Human Vitiligo” was written by Rebecca L. Riding and edited by Jillian M. Richmond and Dr. John E. Harris. Figures were composed by Rebecca L. Riding and Jillian M. Richmond. “The Role of Memory CD8 T cells in Vitiligo” was written by Rebecca L. Riding and edited by Dr. John E. Harris.


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CHAPTER 1: INTRODUCTION

1.1: Global impact of vitiligo

Vitiligo is an autoimmune skin disease in which the pigment producing cells of the epidermis, melanocytes, are targeted for destruction. Death of melanocytes leads to the appearance of patchy depigmentation on the skin\(^1\). Vitiligo affects roughly 1% of the population, has no sex bias, and is chronic with the majority of patients developing disease before the age of 30. Two major types of vitiligo exist; non-segmental and segmental\(^2,3\). Non-segmental vitiligo appears in a symmetrical distribution on the body and follows an unpredictable disease course. Non-segmental vitiligo often spreads from the initial lesion throughout the body and is associated with other autoimmune diseases. Segmental vitiligo has a unilateral appearance and is stable with many patients observing stability of disease within the first year of vitiligo onset\(^4\). Segmental vitiligo is often associated with an earlier age of disease onset, and studies report that the prevalence of segmental vitiligo is between 5-30% of the total vitiligo population\(^5\). Non-segmental vitiligo is the most common form of the disease and therefore the work reported in this thesis focuses on pathogenesis in non-segmental vitiligo.

1.2: Psychological burden of vitiligo and associated comorbidities

Vitiligo is a severely disfiguring disease that negatively impacts patients’ quality of life and self-esteem\(^6,7\). The psychological burden of vitiligo varies depending on the patients’ skin phototype and location of depigmented spots. The history of social stigma associated with vitiligo has significantly increased the burden of those living with vitiligo. For
example, in Buddhist culture, patients with vitiligo cannot be ordained (Gauthier Y. and Benzekri L, 2010), and those of Islamic faith are seen as unfit for marriage and disease qualifies them for divorce. In Indian culture, patients with vitiligo as well as their children are unsuited to marry. The exclusion and alienation that occurs from these principals is devastating for patients. Since vitiligo often affects visible skin, patients avoid going out in public and participating in activities such as school or sports in fear of being discriminated against. Vitiligo also brings about anxiety, depression, suicidal thoughts, and other psychiatric morbidities to many patients. Vitiligo has a significant impact on patients’ quality of life and the disease burden is comparable to other skin inflammatory diseases such as psoriasis and eczema.

Patients may develop numerous adverse side effects from vitiligo and its systemic associations. Because melanocytes not only exist in the skin, but are also present in the eyes, inner ears, and brain, vitiligo patients can develop neurological, ocular and auditory abnormalities. In addition to these co-morbidities, multiple studies show that vitiligo patients have increased incidence, up to 25%, of other autoimmune or inflammatory diseases including Addison’s disease, type I diabetes, and thyroiditis, amongst others. A major contributing factor is the underlying genetic susceptibility that is shared between vitiligo and other autoimmune or inflammatory conditions.

1.3: Genetic contributions to vitiligo development

Vitiligo is a complex and multifactorial disease in which stochastic events, genetic susceptibility, and environmental triggers play a role. Genetics play a clear part in the susceptibility of vitiligo development from early studies that reported an increased
frequency of vitiligo in first degree relatives (11-38%) and studies that revealed a vitiligo concordance rate of 23% in monozygotic twins. In addition, vitiligo is epidemiologically associated with family members that have vitiligo or other autoimmune diseases including Addison’s disease, autoimmune thyroid disease, and type I diabetes. The association of vitiligo with multiple autoimmune diseases indicates that these autoimmune diseases share at least some genetic risk alleles. In fact early studies identified HLA-A2 and HLA-DR4 to be associated with vitiligo, both of which are associated with many other autoimmune diseases. Many genes have since been linked with vitiligo development through genome wide association studies (GWAS) including HLA-A, HLA-DRB1 and HLA-DQA1. About half of the identified genetic loci encode immune proteins consistent with vitiligo being autoimmune driven. Some of these genetic loci include proteins involved in cytotoxicity such as Fas Ligand (FasL) and Granzyme B (GZMB), which have predicted functional roles in disease pathogenesis, but the biological mechanisms of many others have not yet been identified.

1.4: Current therapies for vitiligo

Mature melanocytes in healthy human skin are present in both the epidermis and the hair follicle to which they provide pigment. The hair follicle also serves as a stem cell reservoir and houses epidermal precursor cells including melanocyte stem cells. In the majority of vitiligo patients, only the epidermal melanocytes are targeted for destruction, and melanocytes present in the hair follicles remain unaffected. The reasons for this remain unclear but mechanisms of immune privilege within the hair follicle may play a role. Because of this, vitiligo can be reversed by both suppressing the immune attack
and by stimulating melanocyte precursors that live in the hair follicle to proliferate, migrate and replenish lost epidermal melanocytes through a process known as perifollicular repigmentation. The standard treatment for vitiligo patients is narrow band ultraviolet B (nbUVB) therapy in combination with topical corticosteroids or calcineurin inhibitors. Topical corticosteroids and calcineurin inhibitors act to broadly suppress the local immune response in the skin and nbUVB, which also contributes to immune suppression, stimulates melanocyte regeneration from the hair follicles. Current therapy is effective for many patients with up to 100% repigmentation possible depending on the anatomical site that is affected but is often unpredictable, time consuming, and insufficient for many patients. Because hair follicles harbor the melanocyte precursors required for repigmentation, anatomical sites devoid of hair follicles, such as the fingertips, knuckles, ventral wrists, and elbows, often have poor treatment responses. Also, lesions in which the follicular melanocytes have been destroyed, resulting in white hair, often do not regain pigment following conventional treatment. Vitiligo is a chronic disease that requires lifelong therapy and about 40% of vitiligo patients relapse within 1 year after stopping treatment. Because of the few treatment options, limited access to nbUVB, slow and sometimes poor treatment efficacy, and only short-term benefits, there remains a significant need to develop targeted and durable treatments.

1.5: Evidence for innate immunity in vitiligo development

The role of innate immunity in vitiligo development is ill-defined but studies suggest that activation of the innate immune system may be involved in initiation of disease. Early
evidence suggests that melanocytes from vitiligo patients are intrinsically abnormal compared to healthy melanocytes. Melanocytes from vitiligo patients are difficult to culture *ex vivo* and have increased sensitivity to exogenous stress\textsuperscript{28}. Melanocytes from vitiligo patients also have dilated endoplasmic reticulum and have increased levels of H2O2 and other oxidative by-products, suggesting the presence of increased cellular stress\textsuperscript{29,30}. A study defining early gene expression during initiation of disease in a spontaneous vitiligo animal model, the Smyth line of chickens, revealed an increase in genes involved in melanocyte stress\textsuperscript{31}.

The innate immune system is designed to recognize danger signals, such as cellular stress through a variety of mechanisms. Molecular patterns released during sterile inflammation are known as damage associated molecular patterns (DAMPs) and are recognized by pattern recognition receptors (PRRs)\textsuperscript{32}. For example, the cytosolic nucleotide oligomerization domain- like receptor (NLR)-P3 is activated by reactive oxygen species and mitochondrial stress\textsuperscript{33,34}. Thus, DAMPs produced or released by melanocytes under stress may trigger the innate immune system to initiate inflammation in vitiligo\textsuperscript{35}. Infiltration of innate immune cells including macrophages, NK cells, and inflammatory dendritic cells have been found in vitiligo skin lesions\textsuperscript{36,37}. However, their role in disease pathogenesis requires further investigation.

Another clue into the contribution of innate immune activation in vitiligo is that multiple genetic loci encoding innate immune proteins have been identified as GWAS for vitiligo.
Both interferon induced with helicase C domain-1 (IFIH1)\textsuperscript{38}, also known as MDA-5, and NLRP1\textsuperscript{39}, which are intracellular PRRs were identified in GWAS studies to be associated with vitiligo\textsuperscript{40}. Activation of these receptors induces production of type I interferons and other pro-inflammatory cytokines triggering activation of adaptive immune responses. Another gene TICAM1, which encodes the toll like receptor adaptor protein TRIF is also associated with vitiligo\textsuperscript{41}. TRIF mediates signaling through TLR3 and TLR4, leading to type I interferon production\textsuperscript{42}. The potential activation of PRRs by melanocyte DAMPs may not only stimulates activation of innate immune cells but also modulate adaptive immune responses through dendritic cell maturation, which promotes T cell activation\textsuperscript{35}. Therefore, defining their role in vitiligo development is important.

1.6: Association of viruses in vitiligo

Development of autoimmunity is multifactorial and environmental insults including infection with viruses are associated with triggering autoimmune diseases such as type I diabetes, systemic lupus erythematosus (SLE), and multiple sclerosis (MS), but the role of viruses in the development of vitiligo is less clear\textsuperscript{43}. Multiple case studies have correlated the presence of human immunodeficiency virus (HIV) with vitiligo development\textsuperscript{44,45}. Even more interesting, treatment of the chronic virus with antiretroviral therapy led to reversal of vitiligo in two patients\textsuperscript{46,47}. Other viruses have also been associated with vitiligo including hepatitis C virus (HCV). In HCV patients, the development of vitiligo has been reported following treatment with PEGylated interferon alpha and retroviral therapy. Some HCV patients developed vitiligo at the injection site and others developed disease at distant sites of the body\textsuperscript{48-50}. In addition to these cases,
one study presented weak evidence of cytomegalovirus DNA in vitiligo lesional skin\textsuperscript{51}. The correlations between chronic virus infection and the development of vitiligo provide evidence that virus infections may stimulate pathogenic immune responses against melanocytes, but more information is required to conclude their causation of disease.

**1.7: CD8\textsuperscript{+} T cells are both necessary and sufficient for vitiligo development**

It is well established that CD8\textsuperscript{+} T cells are both necessary and sufficient to mediate human vitiligo. Early studies showed that the number of HLA-A2 melanocyte-specific CD8\textsuperscript{+} T cells in the blood of vitiligo patients correlated with disease severity and CD8\textsuperscript{+} T cells expressed high levels of the skin homing receptor, cutaneous lymphocyte-associated antigen\textsuperscript{52}. Furthermore, isolated melanocyte-specific CD8\textsuperscript{+} T cells from vitiligo patients were able to lyse HLA-A2 matched peptide pulsed cells and melanoma cells ex vivo whereas non-specific CD8\textsuperscript{+} T cells had no cytolytic ability. Examination of vitiligo patient skin cells using suction blistering found that the number of CD8\textsuperscript{+} T cells is significantly increased in active disease compared to stable, non-lesional, and healthy control skin\textsuperscript{53}. Furthermore, elegant studies showed that perilesional CD8\textsuperscript{+} T cells isolated from vitiligo skin could kill melanocytes from normal pigmented skin isolated from the same patient when cultured ex vivo, demonstrating that melanocyte-specific CD8\textsuperscript{+} T cells are both necessary and sufficient for the destruction of melanocytes\textsuperscript{54}.

**1.8: Transgenic mice used as hosts for mouse model of epidermal depigmentation**

In hopes to better understand mechanisms of vitiligo pathogenesis, a mouse model of disease was designed. Previous mouse models of vitiligo using C57BL/6 mice led to the development of hair depigmentation. This is because C57BL/6 mice have pink skin
resulting from the lack of melanocytes within the epidermis. The majority of melanocytes in C57BL/6 mice reside in the hair follicles. Since hair depigmentation is not representative of human disease, Harris et al. developed the first mouse model of vitiligo that displayed epidermal depigmentation.

Melanocytes require the expression of Kit protein, a receptor tyrosine kinase, for their development, migration, and survival. The ligand for Kit, also known as stem cell factor (SCF), is produced locally in human skin by multiple cell types including keratinocytes, fibroblasts, and endothelial cells. Expression of SCF by epidermal cells directs and positions melanocytes within the interfollicular epidermis in human skin. In contrast to human skin, the epidermis of adult C57BL/6 mice does not express SCF, and instead expression is restricted to the dermis and hair follicles, resulting in melanocytes restricted to the hair follicles with only a small number of melanocytes found in the tail and footpad epidermis.

SCF has two forms, a soluble and transmembrane form depending on alternative splicing of the protein. Previous studies of SCF identified two proteolytic domains required for the generation of soluble SCF and found that mutation of the proteolytic domains restricted the protein to the membrane while remaining biologically active. Transgenic mice were generated to express the membrane-bound form of SCF in keratinocytes, which make up the majority of cells within the epidermis. To accomplish this, engineered mice express SCF driven from the human keratin 14 promoter (KRT14-kitl*4XTG2Bjl). In contrast to wild type C57BL/6 mice that lack SCF expression in the epidermis, KRT14-kitl*4XTG2Bjl mice express SCF by epidermal keratinocytes and
have black appearing skin owing to the retention of melanocytes in the interfollicular epidermal basal layer and follicular epidermis. These host mice are used for all vitiligo mouse model experiments in this thesis and are named SCF mice.

![Figure 1.1: KRT14-kitl*4XTGbjl (SCF) mice.](image)

SCF mice have black skin owing to melanocyte retention in the epidermis. Images contrast wild-type C57BL/6 and SCF transgenic mouse tails, ears, nose and rear footpads (no vitiligo).

1.8.1: Melanocyte specific CD8 T cells

Induction of vitiligo in SCF mice requires CD8\(^+\) T cell effectors specific for melanocytes.

Multiple melanocyte-specific proteins recognized during the CD8\(^+\) T cell response to
melanoma had previously been identified. These proteins expressed by melanoma cells are also vitiligo autoantigens and are expressed by normal melanocytes and are known as melanocyte differentiation antigens. One identified protein is premelanosome protein (Pmel-1/gp100), which is highly enriched in the melanin-producing organelles of melanocytes, called melanosomes. Pmel-1 T cell receptor (TCR) transgenic mice were generated by the Restifo laboratory (National Institutes of Health (NIH)), and Pmel-1 T cells recognize the melanocyte differentiation antigen gp10025-33 presented on H-2Kb. For induction of vitiligo, Pmel-1 TCR transgenic mice are used as the source of effector CD8+ T cells and 1 million Pmel-1 are adoptively transferred into SCF recipients by retro-orbital injection. Pmel-1 CD8+ T cells are tracked in the SCF recipients by expression of the T lymphocyte Thy1a congenic marker or by fluorescent protein expression such as green fluorescent protein (GFP).

1.8.2: Infection with vaccinia virus to activate transferred Pmel-1 CD8+ T cells
Activation of transferred naïve Pmel-1 CD8+ T cells recognizing the peptide gp10025-33 is required for vitiligo development. The recombinant vaccinia virus used to express gp100 was generated from the modified vaccinia virus Ankara (MVA). This is an attenuated strain of vaccinia virus that was found to have several deletions that reduce its replication ability in human and mammalian cells. Recombinant vaccinia viruses (rVACV) encoding mouse gp100 and human gp100 were generated and tested in immunization experiments to target melanoma in mice. Studies showed that only immunization with rVACV encoding human gp100 (rVACV-hgp100) induced immune reactivity to both
mouse and human gp100 and resulted in a robust CD8\(^+\) T response. Recombinant VACV-hgp100 was a gift from the Restifo laboratory (NIH). Recipient SCF mice are infected the same day of Pmel-1 T cell transfer by intraperitoneal (i.p.) injection with rVV-hgp100 to induce activation of the transferred Pmel-1 CD8\(^+\) T cells in vivo. The infection induces the proliferation, expansion, and activation of Pmel-1 CD8\(^+\) T cells\(^{65}\).

### 1.8.3: Summary of the mouse model

This model of vitiligo is a modification of the adoptive transfer model developed for melanoma immunotherapy\(^{65}\). Induction of vitiligo in SCF mice is dependent on sublethal irradiation, Pmel-1 CD8\(^+\) T cell transfer, and infection with rVACV-hgp100 to activate Pmel-1 CD8\(^+\) T cells \textit{in vivo}. The day before Pmel-1 CD8\(^+\) T cell transfer, SCF recipient mice are sublethally irradiated (500 rad). This step is critical for the synchronization of vitiligo and may work by causing lymphocyte cell death to make space for the transferred Pmel-1 cells, although the exact role of irradiation in disease initiation is still not clear. The following day, 1 million purified Pmel-1 CD8\(^+\) T cells are transferred to SCF recipient mice by retro-orbital injection. On the same day, SCF recipients are infected with 10\(^6\) pfu rVACV-hgp100 i.p., which causes the activation and expansion of Pmel-1 CD8\(^+\) T cells \textit{in vivo}\(^{65}\). Induction of vitiligo in SCF mice results in epidermal white spots of depigmentation on the tail, ears, footpad, and nose\(^{57}\). The number of infiltrating Pmel-1 in wild type mice peaks at 5 weeks after vitiligo induction. Infiltration of Pmel-1 to the epidermis correlates with depigmentation and melanocyte death. Melanocyte transcripts slowly decrease over time and significantly drop by 5 weeks after vitiligo induction\(^{57}\). Spots of depigmentation can appear as early as 3 to 4 weeks and continue to develop in
severity over time. Wild type SCF mice reach a plateau of vitiligo around 7 to 8 weeks, after which the disease stabilizes, and the majority of epidermal Pmel-1 adopt a resident memory phenotype defined as CD103^+ CD69^+ CD44^+ CD62L^-67.

![Vitiligo Development Diagram](image)

**Figure 1.2: Summary of Vitiligo Induction in Mice using vaccinia virus.** Host SCF mice are sublethally irradiated on Day -1 followed by transfer of Pmel-1 CD8 T cells, and infection with recombinant vaccinia virus expressing their cognate antigen. SCF mice develop epidermal depigmentation over the course of 7 weeks. i.p., intraperitoneal; r.o. retro-orbital.

**1.9: IFNγ and downstream Th1 induced chemokines in disease pathogenesis**

Studies using the vitiligo mouse model, as well as studies using vitiligo patient skin, have greatly enhanced our understanding of the pathways driving vitiligo pathogenesis as well as the signals responsible for the recruitment, positioning, and survival of melanocyte-specific CD8^+ T cells in the skin. Early clinical studies showed that production of the pro-inflammatory cytokines IFNγ and TNFα by CD8^+ T cells isolated from perilesional vitiligo skin positively correlated with disease severity and could predict the success of nbUVB therapy68. Likewise, gene expression analysis of vitiligo lesional skin revealed an IFNγ-specific gene signature and no upregulation of IL-17 transcripts69,70. These human studies point to IFNγ as the central cytokine in disease, and mechanistic studies in mice
support this hypothesis\textsuperscript{57,71}. The IFN\textsubscript{γ} signature in mice parallels that seen in human patient skin. Affected skin of mice induces a significant increase in IFN\textsubscript{γ}, and Pmel-1 CD8\textsuperscript{+} T cells from vitiligo mice produce IFN\textsubscript{γ} after stimulation with melanocyte antigen. Most importantly, vitiligo progression is dependent on IFN\textsubscript{γ} as a therapeutic blockade of IFN\textsubscript{γ} significantly reduced the severity of disease\textsuperscript{57}.

IFN\textsubscript{γ} stimulates transcription of the chemokine ligands CXCL9, CXCL10, and CXCL11, which drive the migration of immune cells into tissues in many type 1 inflammatory diseases and infections. All three chemokine ligands bind to the shared receptor, CXCR3, which is expressed on activated immune cells, including effector CD4\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells, and NK cells\textsuperscript{72,73}. Expression of CXCR3 on melanocyte specific CD8\textsuperscript{+} T cells in vitiligo is required for skin tissue homing because CXCR3-deficient CD8\textsuperscript{+} T cells are unable to mediate vitiligo in mice\textsuperscript{70}. This CXCR3-dependent migration is a result of the upregulation of CXCL9 and CXCL10 in the skin of vitiligo mice\textsuperscript{74}. In fact, treatment of mice with CXCR3 depleting antibodies both prevented and reversed disease\textsuperscript{75}. In vitiligo patients, the majority of CD8\textsuperscript{+} T cells in lesional skin express CXCR3\textsuperscript{67,76,77}, and the chemokine ligands CXCL9 and CXCL10 are enriched within lesional skin compared with non-lesional and healthy control skin\textsuperscript{70,78}. These studies establish the IFN\textsubscript{γ}-CXCR3-CXCL9/10 axis as the central pathway in mediating vitiligo and for the recruitment of CD8\textsuperscript{+} T cells into mouse and human skin.

\textbf{1.10: The functional roles of CXCL9 and CXCL10 in vitiligo}

Mice that report the expression of CXCL9 and CXCL10 were used to determine the kinetics and source of CXCR3 ligands in epidermal vitiligo skin\textsuperscript{79}. Global epidermal
expression of CXCL9 followed a bimodal pattern; CXCL9 was maximally upregulated early after disease induction and then again at the peak of disease. Epidermal CXCL10 expression gradually increased over the duration of disease with the highest expression at the peak of the immune response in vitiligo. Induction of vitiligo in CXCL9-deficient mice led to a significant reduction of melanocyte-specific CD8 T cells in both the epidermal and dermal skin compartments, suggesting the importance of CXCL9 for early recruitment of CD8 T effector cells to the skin and the development of new lesions. However, CXCL9-deficient mice still developed vitiligo as the few melanocyte-specific CD8 T cells that did make it into the skin were evidently sufficient to induce disease. Interestingly, CXCL10-deficient mice and those treated with CXCL10 antibody did not show significant defects in bulk recruitment of melanocyte-specific CD8 T effector cells into the dermis, but CD8 T cells did not efficiently reach the epidermis, which may be partly due to low CD44 expression. CD44 is important for memory T cell survival, activation, and directed migration through the basement membrane. As a result, CXCL10-deficient mice were protected from vitiligo progression. In addition, vitiligo could be reversed in mice by treatment with a CXCL10-neutralizing antibody. These results suggest that CXCL10 is critical in both disease progression and maintenance, playing an active role in the directed migration and tethering of CD8 memory T cells in the epidermis as well as possibly modulating their effector function. A recent study by our group used suction blistering of human vitiligo skin to measure IFNγ-induced chemokine expression in situ. We found that active vitiligo patient skin contains significantly higher levels of CXCL9 protein than stable skin from vitiligo patients, non-
lesional skin, and healthy control skin. In fact, the presence of CXCL9 protein in the skin is sensitive and specific for disease activity and may serve as a biomarker of early treatment responses\textsuperscript{53}.

1.11: Interferons: an overview

Interferons (IFNs) are a family of cytokines that aid in host defense against pathogens and facilitate communication between immune cells. Type I IFNs are evolutionary conserved and all vertebrates that have NK, T and B cells, also have one gene encoding the type I IFN-β and at least two that encode IFN-α\textsuperscript{81}. Interferons were first discovered in 1957 to interfere with viral replication\textsuperscript{82}. Interferons are categorized into 3 main classes of interferons; type I, type II and type III. In humans, type I IFNs include 13 different IFNα genes, 1 IFN-β gene, and several other structurally similar subtypes\textsuperscript{83}. Type II IFN is comprised of a single type, IFNγ while type III interferons include 4 different IFN-λ subtypes\textsuperscript{84,85}. Each class of IFNs binds to a unique receptor and thus can initiate different immune responses.

Type I IFNs are induced following pathogen infection. Host PRRs are equipped to detect a variety of pathogen associated molecular patterns (PAMPs) including bacterial and viral DNA and RNA products\textsuperscript{81}. Detection of pathogen components leads to production of type I IFNs. Type I IFNs induce a robust anti-viral state through the upregulation of hundreds of IFN-stimulated genes (ISGs)\textsuperscript{86}. ISGs function to inhibit all aspects of the viral life cycle and although the mechanism of some have been identified the role of others remains unknown.
1.12: Type I IFNs in host defense

Type I IFNs can be produced by almost all cell types in the body following PRR activation. Several types of PRRs have been identified including Toll like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) to name a few\(^\text{85,87}\). These receptors detect a variety of different PAMPs on viral, bacterial, and fungal pathogens. Here we will focus on those receptors activated by viral components. Two main classes of receptors recognize viral pathogens. The first are cytosolic receptors including cGAS-STING, RIG-I, and MDA5, which are ubiquitously expressed to detect viral nucleic acids upon infection. RLRs including both RIG-I and MDA5 recognize RNA products\(^\text{88}\), whereas cGAS recognizes DNA components\(^\text{89}\). TLRs are the second class of receptors that detect viral nucleic acid components. TLRs are located both at the cell membrane and in endosomes and have complex expression patterns in different cell types and tissues\(^\text{90,91}\). The subcellular localization of TLRs, and the cell-type specific and temporal patterns of TLR expression may result in the activation of different downstream signaling pathways and distinct cytokine induced gene expression. TLRs are activated by different ligands. TLR3, detects double stranded-RNA, which is a common feature of both DNA and RNA viruses\(^\text{92}\). Whereas TLR7 and TLR8 recognize viruses with single-stranded RNA genomes\(^\text{93-95}\), and TLR9 recognizes unmethylated CpG motifs which are present in DNA viruses\(^\text{96-98}\). Not only can TLRs directly sense viral nucleic acids, but evidence suggests that endosomal TLR3 can detect viral dsRNA coming from apoptotic cells that are engulfed\(^\text{99}\). Since viral infection often leads to apoptosis, indirect detection of viral
products from dead cells facilitates cross presentation of viral antigens to CD8 T cells and viral control\textsuperscript{81}. This data also suggests that presentation of viral antigens by antigen presenting cells (APCs) can occur even from viruses unable to infect APCs, such as vaccinia virus. Also, since viruses inhibit host anti-viral responses, presentation of viral antigens by uninfected APCs are protected from viral defense mechanisms\textsuperscript{81}.

Although PRRs use different downstream signaling components, they converge on the activation of two main transcription factors; NF-κB and interferon regulatory factor (IRF) proteins. The NF-κB pathway is responsible for the induction of pro-inflammatory cytokines, chemokines, and other inflammatory mediators\textsuperscript{100}. Activation of NF-κB occurs through transforming growth factor β-activated kinase 1 (TAK1), which then leads to the activation of IKK kinase and IκBα phosphorylation\textsuperscript{100}. Downstream activation of NF-κB following PRR signaling induces the expression of hundreds of genes that regulate cell proliferation, apoptosis, and differentiation. NF-κB activation impacts CD4 T cell differentiation through induction of cytokines but also through T cell intrinsic mechanisms\textsuperscript{100-102}. Studies show that following TCR signaling, recruitment of the NF-κB family member RelA by T-bet, the master transcriptional regulator of Th1 cells, is important for optimal IFNγ production\textsuperscript{103}. T cells deficient in c-Rel show defective Th1 immune responses and IFNγ production\textsuperscript{104}. NF-κB activation likely impacts CD8 T cell differentiation as well\textsuperscript{100,102}.

PRR signaling also leads to the activation of TANK-binding kinase 1 (TBK-1), which phosphorylates the transcription factors interferon regulatory factor (IRF) 3/7 to induce
transcription of target genes including type I IFN alpha and beta\textsuperscript{105,106}. Endosomal TLRs 7, 8 and 9 use the adaptor protein myeloid differentiation primary response gene (MyD88) to initiate signaling, which can also associate with IRF3/7 independently of TBK-1\textsuperscript{107,108}. But endosomal TLR3 and surface TLR4 use the adaptor molecule TIR domain containing adaptor inducing IFN-β (TRIF) to activate TBK-1 and phosphorylate IRF3/7. The cytosolic RLRs also activate TBK-1 using the mitochondria-associated protein mitochondrial anti-viral signaling protein (MAVS)\textsuperscript{109}. Finally, cytosolic DNA receptors like cGAS use the adaptor stimulator of IFN genes (STING) to activate IRF3/7 and type I IFN production\textsuperscript{110}.

The majority of cells constitutively express IRF3 but only specialized cells express IRF7. One of these specialized cell types are known as plasmacytoid DCs (pDCs), which constitutively express IRF7 and can produce up to 95% of type I IFNs during systemic infections\textsuperscript{111}. Although the role of pDCs in vitiligo is not clear, pDCs have been shown to drive autoimmune disease in SLE as well as other auto-inflammatory diseases\textsuperscript{112-114}.

1.13: Type I IFN signaling pathway

Canonical type I IFN signaling involves the activation of Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways\textsuperscript{115,116}. Type I IFNs are a family of cytokines including IFN-α, β, ε, κ, ω, which all bind to the same heterodimeric receptor IFNAR1 and IFNAR2\textsuperscript{117}. Upon receptor ligand binding, conformational changes leads to auto-phosphorylation and activation of the associated JAK proteins which phosphorylate downstream STATs. STAT proteins associate to form hetero- or homo-pairs and translocate to the nucleus to induce transcription of genes. JAK-STAT signaling
is tightly regulated to prevent immunopathology and the suppressor of cytokine signaling (SOCS) family of proteins is one mechanism by which type I IFN signaling is regulated\textsuperscript{118}. SOCS proteins modulate JAK-STAT at early stages by direct binding to the receptor or to associated kinase proteins\textsuperscript{119}. SOCS proteins can also regulate signaling by targeting receptors or JAK proteins for proteosomal degradation.

IFNAR1/IFNAR2 associate with the Janus kinases JAK1 and TYK2 which phosphorylate specific tyrosine residues of signal transducer and activator of transcription (STAT) proteins\textsuperscript{117,120}. TYK2 associates with IFNAR1 and JAK1 with IFNAR2. Canonical signaling transduction leads to receptor dimerization, and cross-phosphorylation of associated kinases. The phosphorylation and activation of Janus kinases allows for STAT proteins to dock followed by kinase mediated phosphorylation of STAT1/STAT2 heterodimers. STAT1/2 form a complex with the transcription factor IRF9 known as the IFN-stimulated gene factor 3 (ISGF3) transcription factor complex. ISGF3 translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) in the promoters of IFN-stimulated genes (ISGs).

Type I IFNs can induce activation of a variety of STAT proteins including STAT1, STAT3, STAT4, STAT5, and STAT6 homodimers. They also stimulate formation of STAT1-2, STAT1-3, STAT1-4, STAT1-5, STAT2-3, and STAT5-6 heterodimers and activation of different STAT complexes is cell type and context dependent\textsuperscript{115,121,122}. Many of these STAT complexes do not bind to ISRE elements in the genome to activate transcription but instead bind to IFNγ-activated site (GAS) elements. Some ISGs have both ISRE and GAS elements in their promoters but others contain only ISRE or GAS
elements\textsuperscript{123,124}. Therefore, the transcription of specific genes and the immune responses generated by type I IFN receptor signaling is dependent on the downstream activation of certain STAT proteins. Also, IFN\textalpha{} and IFN\textbeta{} have different binding affinities and therefore can induce different antiviral, and immunomodulatory responses\textsuperscript{125-127}. For instance, interferon beta has a 50 fold higher receptor binding affinity to IFNAR1 than interferon alpha\textsuperscript{128}.

Type I interferon signaling leads to induction of hundreds of interferon stimulated genes (ISGs) that work to coordinate and complexly regulate the anti-viral immune response\textsuperscript{81,86}. ISGs inhibit all stages of the viral life cycle but also modulate innate and adaptive immune response. In addition to ISG induction, the mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) pathway, and mammalian target of rapamycin (mTOR) pathway are activated downstream of type I IFN signaling\textsuperscript{83}. These pathways regulate cell survival, apoptosis, pro-inflammatory cytokine production, and metabolism.

\textbf{1.14: Function of ISGs in viral host defense}

Viral detection by PRRs and downstream type I IFN and ISG induction leads to the transcription of many genes that function to inhibit all stages of the viral life cycle including viral attachment, fusion, replication, transcription, translation, and release\textsuperscript{81}. For example, the interferon stimulated gene 2’-5’ oligoadenylate synthases (OAS) function to activate the nuclease RNase L, resulting in degradation of viral RNA transcripts\textsuperscript{121}. Degradation of viral RNA inhibits viral replication and can promote sensing of viral products by PRRs to enhance the anti-viral state\textsuperscript{129,130}. Another induced
ISG is protein kinase R (PKR). PKR is part of the eukaryotic initiation factor 2α (eIF2α) kinase family. PKR activation leads to phosphorylation of eIF2α, which blocks translation of cellular and viral mRNAs. Because many viruses require host machinery to complete their life cycle, blocking host mRNAs is equally important. Some ISGs function more specifically. For example, APOBEC3 targets retroviruses such as HIV by inhibiting reverse transcription through induction of mutations. Another class of induced ISGs are the Myxoma GTPases known as MX family proteins. These GTPases differ in their ability to restrict virus infection and have specific protective effects against VSV and influenza.

1.15: Type I Interferon Signaling on Effector Cells

Type I IFNs can modulate the immune system in a cell type, time, and context dependent manner. Type I IFNs mediate their effects either directly, through IFNAR signaling or indirectly through the induction of other pro-inflammatory cytokines or chemokines. Two effector cell populations that are responsible for killing of infected cells, NK cells and CD8 T cells, are directly and indirectly affected by type I IFNs. Type I IFNs have direct effects on NK cell survival and cytotoxicity. Direct type I IFN signaling increases NK cell cytotoxic function by inducing cytotoxic effector molecules like perforin and granzymes. Type I IFNs also promote NK cell survival indirectly through STAT1-dependent induction of IL-15.

Type I IFNs contribute indirectly to the activation of effector cell populations like CD8 T cells through maturation of DCs, which can aid in cross presentation of naïve T cells. Similarly, type I IFN signaling has a direct co-stimulatory effect on CD8 T cell
proliferation, differentiation and expansion\textsuperscript{139-141}. One study showed that type I IFN provides a third signal to CD8 T cells during antigen recognition via STAT4-activation that stimulates survival, development and cytolytic function including IFN$\gamma$\textsuperscript{139}. However, type I IFN was shown to limit non-specific T cell expansion in a STAT1 dependent manner\textsuperscript{142}, highlighting the differential effects of type I IFNs. Timing of type I IFN signaling can also affect the magnitude of the CD8 T cell response. CD8 T cells exposed to type I IFNs before or during the time of antigen exposure induced significant effector function capabilities including production of IFN$\gamma$\textsuperscript{143,144}. However, sustained type I IFN can have immunosuppressive effects on CD8 T cells by inducing T cell exhaustion\textsuperscript{145,146}.

Direct cellular type I IFN signaling is critical for the anti-viral CD8 T cell response to lymphocyte choriomeningitis virus (LCMV). Direct type I IFN was not required for proliferation but was required for the survival of CD8 T cells during the priming phase of LCMV infection and for memory formation\textsuperscript{140}. In contrast to LCMV, CD8 T cell responses to vaccinia virus infection do not require direct type I IFN signaling, but instead require IL-12, which can also provide co-stimulatory signals to T cells during activation\textsuperscript{147,148}. Furthermore, low dose vaccination with modified vaccinia virus did not require type I IFN signaling for cellular immunity as CD8 T cell responses to MVA vaccination were similar between wild type and IFNAR deficient mice\textsuperscript{149}. These results highlight the context-specific role of type I IFN on effector CD8 T cells, and these studies provide evidence of two types of CD8 T cells; those that require type I IFNs for expansion and function and those that do not. Type I IFNs also indirectly affect CD8 T cells. For example, studies show that IL-15 enhances CD8 T cell proliferation and
effector memory formation *in vivo*\(^{150}\), and we have shown its importance in vitiligo for resident memory T cell survival\(^{67}\).

Type I IFNs also modulate T cell responses through their actions on dendritic cells (DCs). Type I IFNs are important for the activation of immature DCs. Type I IFN signaling directly induces expression of MHC class I and II and the costimulatory molecules CD80 and CD86\(^{151,152}\), which supports naïve T cell activation. Presentation of class I peptides are derived from antigens in the host cells cytosol, while class II peptides are derived from extracellular proteins or from self-proteins that are degraded in the endosomal pathway\(^{153}\). Certain APCs, including CD8\(\alpha^+\) DCs, can take up and process extracellular antigens and present peptides on MHC Class I to CD8 T cells in a process known as cross-presentation\(^{154}\). Type I IFNs signaling on DCs has been shown to enhance cross-presentation to CD8 T cells\(^{155,156}\). Because activation of CD8 T cells licenses them to kill infected cells or tumor cells, cross-presentation is critical for immunity against tumors and viruses. Type I IFNs were also shown to induce IL-12 production by DCs and IL-12 has potent co-stimulatory effects on CD8 T cells during priming\(^{157}\). Type I IFNs can also enhance T cell activation by increasing migration of DCs to the lymph nodes through the upregulation of chemokines receptors like CCR7\(^{158,159}\). Thus the direct and indirect effects of type I IFNs on effector cells act to enhance anti-viral immunity.

### 1.16: Type I interferons in autoimmunity

Type I IFNs can have both beneficial and deleterious effects on human autoimmune diseases. For example, type I IFNs drive pathogenesis in systemic lupus erythematosus
(SLE)\textsuperscript{160}, but have protective effects for the autoimmune disease multiple sclerosis (MS)\textsuperscript{161}. SLE is a complex and multifactorial disease but about 87\% of patients have a type I IFN gene signature and type I IFNs are able to predict disease outcomes\textsuperscript{162} and are used as biomarkers. One of the primary sources of IFN production in SLE is by pDCs. Increased type I IFNs may result from the accumulation of extracellular and intracellular self-nucleic acids through increased cell damage/apoptosis or defects in cell clearance\textsuperscript{163,164}. Dysregulated PRR activation driving type I IFN production in SLE has also been implicated in disease. Using a mouse model of SLE, TLR9 signaling was shown to suppress immune activation in the skin because TLR9 deficient mice developed more clinical manifestations of SLE, and this was dependent on the presence of TLR7\textsuperscript{165}. Another disease mediated by dysregulated type I FIN production is Sjogrens syndrome\textsuperscript{166}. Sjogrens syndrome is an autoimmune disease that affects the exocrine glands and patients with an interferon gene signature have increased B cell activating factor (BAFF) expression and increased autoantibodies\textsuperscript{167,168}. Interestingly, a phase 2 study on the use of an anti-BAFF antibody for patients with Sjogrens syndrome found that two subpopulations of patients may exist; one with a type I IFN signature and one with a type II IFN signature\textsuperscript{169}. Type I IFN receptor signaling was also shown to induce pathogenic responses to self in a rat model of virus induced diabetes\textsuperscript{170}. In contrast, IFN\textbeta-1a has clinical efficacy for the treatment of relapsing remitting multiple sclerosis (RRMS)\textsuperscript{171}. The mechanism of action is not fully understood, but studies show that the induction of IL-10, an anti-inflammatory cytokine, blockade of T cell migration through reduced metalloprotease and adhesion molecule expression, and induction of Tregs may
play a role in its efficacy\textsuperscript{172,173}. Thus, type I IFNs can promote pathogenic or protective responses in autoimmunity and its mechanism of action is complex, owing to the heterogeneous immune responses involved in autoimmune development and the pleiotropic effects of type I IFNs.

1.17: Type I and type II interferon signaling overlap and antagonism

Type I and type II interferon, IFN\textsubscript{\textgamma}, bind to different cell surface receptors, but can induce transcription of multiple overlapping gene targets and thus promote similar immune programs. This is because the activation of STAT1 homodimers occurs downstream of both type I and type II IFN signaling\textsuperscript{117,174}. Type I IFNs can induce CXCL9 and CXCL10 to attract and stimulate effector CD8 T cells\textsuperscript{175}. Type I IFN induction of pro-inflammatory cytokines including CXCL9 and CXCL10 through STAT1 homodimer activation is regulated in part by STAT3\textsuperscript{176}. STAT3 antagonizes STAT1 by competing for STAT1 association and through DNA binding. Thus the contribution of type I IFN in inducing CXCL9 and CXCL10 may be part due to the expression of cellular STAT3. Studies reveal that both type I and type II interferon can act synergistically\textsuperscript{177}. Type I IFN signaling induces STAT1 expression, which can prime cells to respond to cytokines that use STAT1 for downstream signaling including IFN\textsubscript{\textgamma}\textsuperscript{178}. As discussed earlier, because some ISGs contain both ISRE and GAS elements in their promoters, they can be induced by both type I and type II interferon. IFITM1, an anti-viral protein that prevents viral entry into host cells, is one example of a gene regulated by both type I and type II IFN.\textsuperscript{179}
Type I and type II interferon can also induce opposing effects and a good example of this is following microbial infections\textsuperscript{180,181}. Mycobacterium leprae induces two different clinical presentations; one in which few skin lesions develop resulting from low bacterial count and the second in which bacteria become disseminated and the host immune response fails. Gene expression data revealed an inverse correlation between IFN-β and IFN\textsubscript{γ}. The authors reveal that IFN-β induced IL-10 led to disseminated disease through inhibition of the antimicrobial pathways induced by IFN\textsubscript{γ}\textsuperscript{181}. Thus defining the biological function of type I IFN in vitiligo and its potential regulation of IFN\textsubscript{γ} responses is critical for our understanding of disease pathogenesis.

1.18: Vaccinia Virus

Vaccinia virus (VACV) is a member of the highly conserved Poxviridae family and is one of the most extensively studied viruses because VACV served as the vaccine that eradicated smallpox, the human disease produced by the Poxviridae variola virus\textsuperscript{182}. In vitro studies have revealed that VACV has broad cellular tropism\textsuperscript{183}. Despite this, a specific host receptor for VACV entry has not been identified. Vaccinia virus is a large (130,000 – 375,000 nts) linear double stranded DNA virus that replicates in the cytoplasm. Specifically, studies report that VACV DNA synthesis occurs within the endoplasmic reticulum in enclosed mini-nuclei\textsuperscript{184,185}. Because VACV does not require the host cell nucleus, VACV is capable of replicating in differentiated keratinocytes, cells that have shed their nuclei. However, infection of human skin with vaccinia virus revealed productive infection in dermal fibroblasts and dermal endothelial cells but reduced infection in keratinocytes\textsuperscript{186}. Only early VACV gene products were detected in
epidermal Langerhans cells, dermal dendritic cells, and skin T cells, suggesting that these cell types either have mechanisms to suppress viral infection or have reduced permissibility of vaccinia virus entry. Interestingly, infection of keratinocytes with VACV led to production of immune suppressive cytokines including IL-10 and TGF-beta. The authors propose this may be an invasion strategy of VACV that also impacts CD8 T cell responses. Differences in cellular or tissue permissiveness to VACV may result from the disparate expression of host factors required for VACV life cycle or differences in expression of restriction factors such as viral RNA and DNA sensors and their signaling pathways that limit viral replication.

1.19: Cellular Immunity to Vaccinia Virus

Studies investigating the protective immune responses against VACV indicate a role for both the innate and adaptive immune system. Infection of mice with Western Reserve strain of VACV showed that both antibody production, and CD4 and CD8 T cell responses play a role in protection. Mice deficient in either B or CD4 T cells had significantly higher VACV titers compared to wild type mice, and the authors further reveal the importance of CD4 T cells in mounting a humoral response to VACV. Mice lacking CD8 T cells did not show any differences in VACV titers. However, infection induced robust IFNγ production by CD8 T cells and these cells are required for protection against VACV rechallenge. Other studies reveal an important role of Th1 cytokines, including TNFα, IFNγ, and type I IFNs in controlling vaccinia virus infection. Natural killer (NK) cells also play an important role in both the detection of vaccinia virus infected cells and in controlling vaccinia virus replication. Studies reveal that
type I IFN production following VACV infection is required for NK cell activation and for early control of vaccinia virus since type I IFN receptor deficient NK cells were unable to control VACV replication or induce lysis of target cells\textsuperscript{191}.

1.20: Detection of vaccinia virus by the host

VACV is a double stranded DNA virus, thus VACV is likely to trigger cellular PRRs that sense DNA\textsuperscript{192}. Cyclic GMP-AMP synthase (cGAS) is a nucleotidyltransferase that generates cyclic GMP-AMP (cGAMP) upon binding to dsDNA in the cytosol\textsuperscript{193}. cGAMP acts as a secondary messenger that binds to stimulator of interferon genes (STING) to induce activation of IRFs and NF-κB\textsuperscript{89,194,195}. STING is an endoplasmic reticulum-associated protein that induces type I IFN production in response to viral, tumor, or self DNA. Early studies reported the importance of STING for inducing CD8 T cell responses against vaccinia virus infection\textsuperscript{194}. Further studies revealed that cGAS-STING was required for the detection of vaccinia virus in mouse cDCs and for downstream activation of IRF3 and type I IFN production\textsuperscript{196}. Interestingly, infection of vaccinia virus in murine cDCs but not pDCs led to type I IFN production\textsuperscript{196}. Type I IFN production was dependent on IRF3/7 and type I IFN receptor signaling. The authors also report a minor role for TLR9, which detects DNA containing CpG motifs, and MyD88 in host vaccinia virus sensing. Absent in melanoma 2 (Aim2), a cytosolic sensor that directly binds DNA, may also detect VACV\textsuperscript{197}. Studies revealed that vaccinia virus infection in Aim2 deficient dendritic cells showed a complete abrogation of IFNβ compared to infection in WT DCs, suggesting that Aim2 may also be involved in the sensing of vaccinia virus\textsuperscript{197}. 
In addition to type I IFNs, studies reveal that vaccinia virus infection led to induction of the pro-inflammatory cytokines TNFα, IL-6, IL-1β, and IL-12 and these cytokines and chemokines were also induced following infection of human macrophages with vaccinia virus 198. Expression of the chemokine MIP-2 by vaccinia virus infection was dependent on TLR2, TLR6, and MyD88. Using shRNA targeted knock down of cytosolic PRRs, the authors suggest that IFIH1 (MDA5) is important for inducing IFNβ in response to vaccinia virus infection in macrophages198. Collectively, these studies suggest that vaccinia virus detection and downstream innate immune signaling activation may vary depending on the host cell type or tissue and expression of certain PRRs or viral intermediates.

1.2.1: Vaccinia virus defense mechanisms

Many viruses have adapted ways to inhibit host cell detection and downstream type I IFN production. In fact, viruses can inhibit almost every part of the type I IFN pathway including production of secreted decoy receptors, chemokines or cytokines, directly inhibiting signaling components, as well as inhibition of specific ISGs199. Vaccinia virus is no exception and about 20 genes have been identified that interfere with intracellular interferon signaling, chemokine production, and cytokine production200. Vaccinia virus encodes a few proteins that inhibit the broad effects of host anti-viral defense. E3/E3L can inhibit PKR activity by directly binding to PKR and preventing PKR-induced apoptosis201. E3 can also bind to dsRNA and prevent activation of 2’-5’ OAS-RNaseL202,203. Vaccinia virus can also block the functions of eIF2α through 2 additional proteins K3 and C7204. K3 is a homolog of eIF2α and inhibits PKR and prevents eIF2α
phosphorylation\textsuperscript{205}. In addition, VACV can evade detection by the immune system by targeting PRRs and components of downstream signaling. The vaccinia virus protein A46 can directly bind to all four TLR4 adaptors MyD88, MAL, TRIF, and TRAM and inhibit the downstream activation of NF-κB, IRF and MAPK pathways induced by TLR signaling\textsuperscript{206,207}, suggesting the importance of these signaling pathways in VACV host defense\textsuperscript{206,208}. For instance, studies reveal that TRIF mediated activation of IRF3 and induction of IFN by TLR3 or TLR4 suppressed VACV replication in macrophages\textsuperscript{206}. Likewise, another early expressed VACV protein A52 can interfere with downstream TLR molecules TRAF6 and IRAK-2, and can enhance IL-10 production through TRAF6 to induce immunosuppression\textsuperscript{209}. Protein K7/K7R inhibits IFNβ through inhibition of TBK1/IKK-ε mediated IRF3 activation, because K7 can bind to DDX3\textsuperscript{210}. NL1 has a similar function to K7 and can block NF-κB and IRF3 activation by direct binding of the inhibitor IKK complex and TBK1. In addition to N1/N1L, VACV also encodes several NF-κB inhibitors which may be more potent than N1 in suppressing NF-κB signaling\textsuperscript{211}. Vaccinia virus can also and interfere directly with type I IFN signaling with encoded protein B19/B19R, which acts as a decoy and is a secreted type I IFN receptor that binds to type I IFNs thus preventing binding to the host type I IFN receptor\textsuperscript{212}. VACV also encodes a soluble IFNγ receptor, B8/B8R, to inhibit host IFNγ signaling\textsuperscript{213,214} and can inhibit the activity of CC chemokines through the protein C23/C23L which binds to CC chemokines\textsuperscript{215}. Because vaccinia virus encodes specialized proteins to inhibit type I IFN signaling, infection with wild type strains does not induce strong type I IFN production\textsuperscript{216}.
1.22: Attenuated vaccinia virus strain vs wild type strains

Recombinant vaccinia virus expressing gp100 was generated from an attenuated non-replicating poxvirus variant. Modified vaccinia Ankara (MVA) is a replication-defective virus strain derived by passaging more than 570 times in chicken embryo fibroblasts. It has been used to vaccinate in Germany and presented with no adverse side effects but its effectiveness against smallpox remains unknown. Sequencing studies revealed that MVA has several large deletions, about 30kb, in its terminal regions and contains numerous point mutations relative to the replication-competent VACV strains. Many of the deletions/mutations are in viral defense genes, preventing the virus from overcoming the host anti-viral state. MVA lacks a few immunomodulatory genes encoding soluble receptors for type I IFNs, TNF and CC chemokines, and only has one copy of the E3L gene responsible for PKR inhibition. MVA makes early, intermediate, and late proteins but only immature virions. One major difference between parental strains and MVA is that unlike wild type vaccinia strains, MVA can infect and causes enhanced activation of human dendritic cells. It is speculated that this is one of the reasons for its high immunogenicity. Since MVA lacks multiple genes required for inhibition of type I IFNs, infection with this attenuated strain induces type I IFN production while infection with the wild-type vaccinia strain fails to induce type I IFNs, and results in attenuated innate immune responses to TLR ligands LSP and poly IC.
DNA viruses require the conversion of ribonucleotides to deoxynucleotides for DNA replication. Ribonucleotide reductase (RR) is the key enzyme responsible for this process and catalyzes the reduction of rNDPs to dNDPs\textsuperscript{227}. RR enzymes are composed of large and small protein subunits\textsuperscript{227}. Mammalian cells encode a single large subunit gene and 2 small subunit genes. Orthopoxviruses including VACV encode both a large and small ribonucleotide reductase subunit protein\textsuperscript{228} identified to be \textit{I4L} and \textit{F4L}\textsuperscript{229-231}. Studies have shown that inactivating the VACV large subunit \textit{I4L} did not affect DNA replication or viral particle production when compared to the wild type strain and was only mildly attenuated\textsuperscript{232,233}. In contrast, inactivation of the small subunit \textit{F4L} led to significant attenuation of VACV\textsuperscript{234}. However, these studies were performed independently and used different VACV strains. A comparison study was conducted that generated both large and small RR subunit VACV mutant strains\textsuperscript{235}. In this study the authors report that the small RR subunit is more critical for VACV replication. Small subunit VACV mutant strains exhibited plaque forming defects, impaired replication kinetics \textit{in vitro}, and impaired DNA synthesis compared with \textit{I4L} mutants\textsuperscript{235}. The authors also reveal that the small VACV RR subunit \textit{F4L} forms an active complex with host large RR subunit proteins to ensure viral replication\textsuperscript{235}. Infection of mice with VACV mutants revealed that the small subunit \textit{F4L} mutant was highly attenuated as shown by significantly reduced viral titters on day 5 post infection\textsuperscript{235}.

Recombinant vaccinia virus expressing gp100 was generated by insertion of the human gp100 minigene through homologous recombination into the VACV locus encoding the
small subunit of viral ribonucleotide reductase (RR)\textsuperscript{64}. Minigenes were constructed using a recombination plasmid pKT1401\textsuperscript{64}. pKT1401 was then recombined into the VACV RR locus\textsuperscript{64}. Since, human gp100 was inserted into the small RR subunit, this VACV strain was expected to be highly attenuated based on previous studies\textsuperscript{235}.

We tested whether the recombinant VACV expressing gp100 induced type I IFN production following infection in mice. To test this, we used an IFNβ reporter mouse that expresses enhanced yellow fluorescent protein (EYFP). The reporter mouse was generated using an EYFP targeting vector in which a knockin of EYFP was inserted into the endogenous \textit{ifnb} locus immediately following the STOP codon of the \textit{ifnb} open reading frame. IFNβ reporter expression was validated using different IFN inducing agents and visualization of EYFP by flow cytometry and immunofluorescence. We induced vitiligo in mice using the protocol described in 1.8 and then examined IFNβ reporter expression in the spleen of mice following VACV infection. Our results show that the percentage of IFNβ-EYFP expressing cells increases over the course of infection in WT hosts. At day 3 post infection about 10% of CD45 positive splenocytes express EYFP, while at days 7 and 10 post infection an average of 40% of CD45 positive splenocytes express EYFP. This data is preliminary, but suggests that infection with recombinant VACV expressing gp100 in WT mice induces robust type I IFN production.
Type I IFNs are critical anti-viral proteins and VACV host immunity requires both type I IFN and IFNγ for effective clearance. Our IFNβ data suggests that type I IFN production induced following infection with recombinant gp100 expressing VACV is important for host anti-viral immunity. Interestingly, IFNβ reporter expression peaked at 7 days post infection and was sustained at 10 days post infection. Thus, the attenuation of VACV through insertion of gp100 into the small subunit RR gene may delay and attenuate its replication, but also enhances the susceptibility of this virus strain to type I IFN mediated anti-viral immunity.

**Figure 1.3: IFNβ-EYFP reporter expression increases following infection with VACV in mice.**

A) EYFP reporter expression following infection with VACV or uninfected controls at days 3, 5, 7, and 10 post infection. B) Quantification of the percentage of CD45 positive EYFP-IFNβ expressing cells.
1.23: Melanoma and vitiligo: an overview

Melanoma is an aggressive skin cancer that arises from mutations in melanocytes and has a high mortality rate and mean survival of only 6-11 months\textsuperscript{236,237}. It is now well established that the innate and adaptive immune system play an integral part in the surveillance, recognition, and persistence of tumors including melanoma\textsuperscript{238,239}. CD8 T cells recognizing tumor-specific antigens are critical for the anti-tumor response, and have been the target of immunotherapies for melanoma\textsuperscript{240,241}. The association between vitiligo and melanoma has been long recognized after many case studies reported the development of vitiligo following treatment of melanoma\textsuperscript{242,243}. Research in tumor immunology has revealed a strong correlation between tumor immunity and autoimmunity and this is because of the overlap of antigens expressed by both normal and malignant cells\textsuperscript{63,244}. Early in vitro studies of melanoma cell lines and melanocytes from normal skin led to the identification of a class of antigens expressed by both.

Antigens expressed by both melanoma cells and normal human melanocytes are classified as melanocyte differentiation antigens (MDAs)\textsuperscript{63,245}. Some of these identified antigens include the melanocyte proteins tyrosinase and tyrosinase related proteins (TRP-1 and TRP-2), as well as premelanosome protein (Pmel-1/gp100) and MART1/Melan-A, all which play roles in melanin synthesis. The association between vitiligo and melanoma is well established following early case reports in which patients developed vitiligo after melanoma immunotherapy\textsuperscript{242,243,246}. Many studies have followed and it is accepted that vitiligo is an independent positive prognostic factor for melanoma patients\textsuperscript{247}. Patients
receiving melanoma immunotherapy have increased incidence of vitiligo\textsuperscript{248,249,250}. Similarly, melanoma patients that developed vitiligo following treatment with anti-PD-1 (pembrolizumab) or anti-CTLA4 (ipilimumab) immunotherapies have increased overall survival compared to those who did not develop vitiligo\textsuperscript{251}. It is not completely understood why the development of vitiligo is a positive prognostic factor but many hypothesize it is due to enhanced CD8 T cell responses against malignant melanoma that leads to enhanced CD8 T cell responses against epidermal melanocytes. Genetic studies also indicate an inverse relationship between vitiligo and cancer risk, suggesting that enhanced immune activation in vitiligo protects against melanoma\textsuperscript{252}.

In addition to these clinical findings, studies in mice have also modeled this relationship to better understand the mechanisms behind the association. Studies by the Turk lab reveal that following dermal B16 inoculation, depletion of Tregs, and resection of the tumor can lead to vitiligo development evident by hair depigmentation\textsuperscript{253}. In this model the authors show increased resident memory CD8 T cell formation in the skin of mice that developed vitiligo compared to those that did not. They further reveal that resident memory CD8 T cells were required for protection against melanoma re-challenge\textsuperscript{253}. Another report also describes the role of resident memory CD8 T cells in mediating immunosurveillance of nascent tumors and protection against melanoma\textsuperscript{254}. Collectively, these studies suggest that melanocyte/melanoma specific CD8 T cell memory formation that occurs following vitiligo development provides protection against tumor development through continued skin immunosurveillance and suggests that CD8 T cells are a major contributor to the link between vitiligo and melanoma.
1.24: Type I IFNs and melanoma

It is well established that type I IFNs have significant effects in promoting tumor immunogenicity through dendritic cell maturation, survival and cross-presentation of tumor antigens to T cells\textsuperscript{138,255}. Studies report that the activation of cGAS-STING by tumor derived DNA in DCs is required for induction of type I IFNs and activation of the anti-tumor response leading to tumor regression\textsuperscript{256}. A recent study revealed that infection of cDCs with heat or UV inactivated MVA led to significantly higher type I IFN levels than MVA alone and this was mediated through the STING pathway\textsuperscript{257}. Intra-tumoral injection of inactivated MVA led to induction of antitumor immunity against melanoma in mice\textsuperscript{257}. The authors also show that the use of local MVA vaccination along with systemic immune checkpoint blockade generated synergistic antitumor effects\textsuperscript{257}. Other studies have revealed that IFNaR deficient mice have defective tumor induced T cell priming, and that type I IFN signaling on DCs is critical for tumor rejection, and for accumulation in the tumor\textsuperscript{258,259}.

The efficacy of type I IFNs in the treatment of melanoma is controversial. Early studies supported a role for type I IFN in stimulating the anti-tumor response to melanoma. These studies showed that induction of cell death following radiotherapy led to release of tumor associated nucleic acids, activation of innate immune PRRs and production of type I IFNs. Production of type I IFNs by myeloid cells following radiotherapy were shown to be critical for tumor regression and type I IFNs induced CXCR3 mediated migration of CD8 T cells into the tumor\textsuperscript{260,261}. Because of these initial findings, IFN therapy was
hypothesized to have great success in the clinic. High dose IFN-α-2b therapy was approved for treatment of stage IIB/III melanoma\textsuperscript{262}. IFN-α induced MHC Class I on immune cells and induced apoptosis of melanoma cells\textsuperscript{263}. Although initial studies found that adjuvant IFN-α improved the overall survival of patients, it became clear that only a subset of patients respond to IFN therapy\textsuperscript{264,265}. Pegylated IFN, IFN-α-2b combined with polyethylene glycol, was designed to increase the half-life of IFN-α and improve adjuvant therapy\textsuperscript{266}. PEG-IFN was approved for adjuvant therapy for stage III melanoma in 2011\textsuperscript{267,268}. The immunomodulatory effects of IFN adjuvant therapies were shown to enhance innate immune recognition of tumor derived nucleic acids, migration of CD8α\textsuperscript{+} DCs into the tumor, and thus had potent effects on T cell-mediated cytotoxicity\textsuperscript{269-271}. However, one clinical study reported significant improvements in relapse-free survival, but no difference in overall survival. The other study reported no significant improvement following IFN-α adjuvant therapy and treatment was associated with high toxicity.

Despite the robust immunostimulatory properties of type I IFNs on DCs and CD8 T cells on the anti-tumor response, its use in the clinic did not prove to be as successful. This may be a result of type I IFN mediated resistance. One mechanism of resistance is downregulation of the type I IFN receptor, IFNaR. Studies in a mouse model of melanoma revealed that IFNaR signaling is critical for the suppression of melanoma progression\textsuperscript{272}. Using a IFNAR1 mutant that is resistant to downregulation, the authors reveal delayed melanoma development, reduced metastatic disease and improved
responses to immunotherapies like anti-PD-1. Another mechanism of IFN resistance is modulation of signaling downstream of IFNaR. One study revealed an upregulation of STAT5 in IFN resistant melanoma cells that led to the suppression of STAT1 activation through the upregulation of the cytokine inhibitor CIS\(^{273}\). Melanoma tumors have also become resistant to immune-checkpoint inhibitors such as anti-PD-1 by modulation of JAKs\(^{274}\). Sequencing studies of two patients’ resistant to pembrolizumab revealed a loss of function mutations in JAK1 and JAK2. The presence of JAK1 mutations led to resistance to IFN\(\gamma\), IFN-\(\alpha\), and IFN-\(\beta\), whereas JAK2 mutations only abrogated IFN\(\gamma\) responsiveness\(^{274}\).

Another explanation for the failure of type I IFN adjuvant therapy in the clinic is that type I IFNs can contribute to an immunosuppressive tumor microenvironment. Since type I IFNs can induce PD-1 on T cells, PD-L1 on tumor cells\(^{275}\), IL-10, and the expression of indoleamine 2,3-dioxygenase (IDO) it remains likely that sustained or chronic IFN in the tumor microenvironment may lead to suppression of the anti-tumor response and resistance to immune checkpoint immunotherapies\(^{145,276-278}\). However, a better understanding of the role of type I IFNs on the tumor microenvironment and the development of combination therapies to overcome resistance mechanisms to IFN will improve the treatment of melanoma.

1.25: **Vaccinia virus as a cancer vaccine**

Vaccinia virus (VACV) is part of the family of viruses known as *Poxviridae*, genus *Orthopoxvirus*. Because of its highly conserved nature, immunization with VACV provides cross-protection against all other orthopoxviruses such as variola and
monkeypox\textsuperscript{279}. VACV is a double stranded DNA virus with a large genome (~180Kb) and 200 genes, which allows for the insertion and high level expression of large foreign genes, making them ideal for recombinant vaccine vectors\textsuperscript{279}. VACV can efficiently express large transgenes or express multiple transgenes up to 40 Kb\textsuperscript{280,281}. Another advantage of VACV as a vaccine is that it replicates completely in the cytoplasm and poses no risk of integration of inserted DNA into the genome. Because VACV has proven to be efficacious and safe, it is an attractive vector for cancer immunotherapies\textsuperscript{282}. VACV has been explored for the treatment of multiple cancer types but I will focus on VACV vaccines for melanoma immunotherapy. One of the first uses of vaccinia virus in cancer was for metastatic melanoma in which the Wyeth strain of vaccinia virus was used by direct injection into the tumor\textsuperscript{283}. Results showed an objective response rate of 50\% with only mild side effects resulting from the live virus infection. An early clinical study revealed that injection of a replication restrictive VACV strain expressing IL-2 in patients with mesothelioma was well tolerated and persisted even in the presence of VACV antibodies\textsuperscript{284}. VACV has been extensively used to express tumor-associated antigens in hopes of activating specific cytotoxic T lymphocyte (CTL) responses in preclinical studies. Modified vaccinia virus Anakara engineered to express the melanocyte differentiation antigen tyrosinase was shown to induce robust CTL responses\textsuperscript{285}. Other studies investigated whether recombinant VACV expression of an ER targeted MART1/Melan-A mini gene or expression of the complete MART1/Melan-A protein induced better CTL responses\textsuperscript{286}. The authors reveal that the expression of the mini gene induced 10-1000 fold stronger responses than expression of the full length protein. In
another study, the expression of three melanocyte differentiation antigens, MelanA, gp100, and tyrosinase were expressed by recombinant vaccinia virus vaccine and used to immunize metastatic melanoma patients\textsuperscript{287}. Vaccination led to increased frequencies of cytotoxic T cells specific for these antigens and was associated with improved clinical responses.

Another vaccination strategy has been to infect DCs to activate CTL responses against certain tumor associated antigens. Not only can vaccinia vector expressing DCs present melanocyte differentiation antigens to CTLs directly but apoptotic DCs can be phagocytosed by nearby DCs resulting in amplification of the CTL response. A few studies used this approach by infecting mature human dendritic cells with recombinant VACV expressing melanocyte differentiation antigens such as gp100 to induce CD8 T cell responses against tumor cells\textsuperscript{288,289}. These studies showed induction of strong T cell responses against melanocyte differentiation antigens. A clinical trial also used autologous CD34 positive DCs transduced with recombinant vaccinia virus expressing tyrosinase\textsuperscript{290}.

Two early clinical trials used recombinant fowlpox, also a \textit{Poxviridae} family member, and recombinant vaccinia virus for the treatment of metastatic melanoma and revealed increased frequencies of CTL responses in some patients and the treatment was well tolerated\textsuperscript{291,292}. Another clinical trial used a non-replicative vaccinia virus expressing multiple melanocyte differentiation antigens (MART-1, gp100 and tyrosinase) as well as the co-stimulatory molecules CD80 and CD86 for use in metastatic melanoma\textsuperscript{293}. Their
study showed that 15 out of 18 patients had significant CTL responses against these antigens with 3 patients showing tumor regression.

More recently, the use of a Herpes Simplex Virus (HSV)-1 based talimogene laherparepvec (TVEC) has been approved for the treatment of metastatic melanoma. TVEC is a genetically modified HSV-1 that preferentially replicates in tumor cells. Lysis of tumor cells following administration of TVEC can enhance cross presentation of tumor antigens and lead to anti-tumor immunity at distant uninjected sites. Cancer cells usually downregulate type I IFNs to promote unrestrained proliferation, and this is advantageous for oncolytic viruses such as HSV-1 and VACV, which allows for their unrestrained replication in cancer cells. A recent report engineered HSV-1 and VACV to express tumor associated antigens on the viral envelope and revealed that this could induce strong CTL responses against B16F10 melanomas in mice. In these studies, VACV performed as well or better than HSV-1 at stimulating robust anti-tumor immunity. Thus, the use of VACV for immunotherapy for melanoma is safe and the development of improved VACV therapies is ongoing. But a better understanding of the signals driving VACV induced CTL responses will aid in generation of new improved VACV therapies.
The data presented in this chapter contains original text from Rebecca L. Riding. Rebecca L. Riding designed and executed experiments, analyzed the data, and wrote the manuscript that is currently under review in Pigment Cell and Melanoma Research. Dr. Jillian Richmond helped perform experiments (Figure 1D and Figure 2C), analyzed data (Figure 1D and Figure 2C), and edited the manuscript presented in this chapter. Dr. John E. Harris designed experiments and critically reviewed the work. Keitaro Fukuda provided experimental and intellectual advice on the data presented in this chapter. Ann Rothstein critically reviewed the work presented in this chapter.
CHAPTER 2
THE ROLE OF TYPE I INTERFERON IN VITILIGO PATHOGENESIS

2.1: Introduction

Vitiligo is a disfiguring skin disease in which autoreactive CD8+ T cells target and destroy melanocytes, the pigment-producing cells of the epidermis, leading to patchy depigmentation. Vitiligo affects roughly 1% of the population worldwide and currently there are no FDA approved medical treatments to reverse the disease. Our lab and others have shown that signaling through type II interferon, IFN-gamma (IFNγ), is critical for disease pathogenesis in human patients and in a mouse model of vitiligo. Previous work has identified epidermal IFNγ signaling and the induction of the chemokine ligands CXCL9 and CXCL10 in promoting migration of anti-melanocyte CXCR3-expressing CD8+ T cells to the epidermal-dermal junction where they mediate elimination of melanocytes.

The role of type I interferon signaling in vitiligo pathogenesis is unclear. Vitiligo patients have an increased incidence of other type I interferon-driven autoimmune diseases, such as systemic lupus erythematosus (SLE) and Sjogrens syndrome, and their association with vitiligo suggests an overlap in disease-associated pathways. In addition, genome wide association studies identified variants in interferon-induced with helicase C domain 1 (IFIH1) and toll like receptor adaptor molecule 1 (TICAM1) as vitiligo susceptibility loci. Activation of IFIH1 or TICAM1 leads to induction of type I IFNs and expression of interferon-stimulated genes. Finally, both IFNγ and type I IFN receptor signaling can lead to activation of STAT1 homodimers to induce transcription of multiple
overlapping gene targets, including CXCL10, due to the sharing of promoter binding elements\textsuperscript{116,301}.

A few reports show a correlation between the presence of type I IFNs and vitiligo development. One study showed a modest increase in IFNB1 mRNA in vitiligo uninvolved skin compared to healthy control skin but there was no difference in gene expression compared to lesional skin \textsuperscript{302}. This suggests that the expression of type I IFN may be more closely associated with immune tolerance in the skin rather than progressive disease. Another study reported increased numbers of CD123 positive cells in the skin of vitiligo patients and moderately increased levels of CXCL9 and MxA, and concluded that type I IFN was responsible for this induction\textsuperscript{77,303}, although these markers can also be associated with IFN\textgamma. Furthermore, multiple case studies have reported the development of vitiligo in Hepatitis C Virus patients following therapy with PEG-IFN\alpha and ribavirin\textsuperscript{48-50}. However, this rare phenomenon is not only linked to vitiligo but also to the development of other autoimmune diseases such as type I diabetes and SLE\textsuperscript{304-306}.

Here, we sought to determine whether type I IFN plays a functional role in vitiligo. In this study, we used two different mouse models of human disease. The first model is dependent on activation of autoimmune T cells using a vaccinia virus vaccine. The second model is a novel mouse model that is dependent on adoptive transfer of bone marrow-derived dendritic cells to prime autoimmune T cells. We find that induction of vitiligo using vaccinia virus vaccine resulted in severe disease in IFNaR-deficient hosts because of vaccinia virus persistence and the subsequent hyperactivation of autoimmune
effector CD8⁺ T cells. Consistent with this observation, we determined that vaccination with vaccinia virus improved anti-tumor immunity against melanoma in IFNaR-deficient hosts compared to treatment in WT hosts. We show functional evidence that type I IFN is not required for vitiligo, since induction of vitiligo with antigen-pulsed dendritic cells resulted in similar disease development in both WT and IFNaR-deficient hosts. Our results further define the pathways responsible for vitiligo pathogenesis and will inform new therapies. These studies highlight the role of type I IFN in vaccine-induced adaptive immune responses and suggest targeting IFNaR to benefit vaccine efficacy during melanoma therapy.

2.2: IFNaR-deficient hosts develop accelerated and severe virally-induced vitiligo compared to WT hosts

To directly compare the relative contribution of type I and type II interferon signaling in vitiligo pathogenesis, we induced vitiligo in WT, IFNaR-deficient, and IFNGR-deficient hosts as previously described⁵⁷. In brief, vitiligo was induced by adoptive cell transfer of naïve CD8⁺ T cells specific for the melanocyte protein premelanosome (PMEL) and these cells were activated by same day infection with recombinant vaccinia virus (VV) expressing their cognate antigen. Effector PMEL CD8⁺ T cells traffic to the skin to target melanocytes and hosts develop epidermal depigmentation on the ears, nose, footpads, and tail between 5-7 weeks after PMEL transfer⁵⁷,30⁷. Surprisingly, induction of vitiligo in IFNaR-deficient hosts led to enhanced disease compared to wildtype hosts and resulted in complete depigmentation at some skin sites (Figure 2.1A-D). This was in stark contrast to IFNGR-deficient hosts, which were protected from disease (Figure 2.1C-D) and had significantly reduced PMEL numbers in
the epidermis and dermis (Figure 2.2A-C), consistent with our previous studies that targeted IFNγ using an antibody\textsuperscript{57}. In IFNaR-deficient hosts, depigmentation was accelerated, with severe disease visible by 5 weeks post vitiligo induction (Figure 2.1E). We next asked whether IFNaR signaling affects PMEL trafficking into the skin. PMEL number was slightly increased in the skin at week 3, but significantly increased at week 5 post vitiligo induction in IFNaR-deficient mice (Figure 2.1F-G). The altered kinetics of PMEL infiltration into the skin of IFNaR-deficient mice correlate with the acceleration of vitiligo. These results suggest that in contrast to IFNGR signaling, IFNaR signaling is not required during the effector phase of vitiligo development.
Figure 4.1: Type I interferon receptor-deficient mice develop accelerated and severe vitiligo. Representative images of mouse ears, nose, footpads, and tail 7 weeks post vitiligo induction for A) WT, B) IFNaR KO, and C) IFNGR KO hosts. D) Vitiligo score at 7 weeks post vitiligo induction. E) Vitiligo scores of WT (black) and IFNaR KO (grey) mice at weeks 3, 5 and 7 pooled from 2 to 3 separate experiments; mean ± standard deviation of the mean. Normalized number of PMEL in the footpad epidermis (F) and dermis (G) of WT and IFNaR KO mice at weeks 3 and 5 post vitiligo induction.
2.3: IFNaR signaling on host radioresistant cells is important for controlling disease development

In wild type and IFNaR-deficient vitiligo hosts, transferred wild type PMELs can respond to type I IFNs. Since type I IFN signaling can directly enhance the proliferation and effector capabilities of CD8$^+$ T cells\textsuperscript{140,308}, we asked whether type I IFNs acted directly on PMELs to mediate severe disease. To test this, we transferred wild-type or IFNaR-deficient PMELs into wild type and IFNaR-deficient hosts. Whether the T cells were IFNaR-deficient or IFNaR-sufficient had no impact on the course of the disease (Figure 2.3A-B). However, IFNaR expression on host cells played a critical role as both WT and IFNaR-deficient T cells into IFNaR-deficient hosts developed significantly more severe vitiligo than the corresponding wild type hosts (Figure 2.3A-B). These results suggest

\textit{Figure 2.2: Significantly reduced PMEL numbers in the tail epidermis of IFNGR KO mice at 7 weeks post vitiligo induction.} A) Epidermal (B) Dermal, and (C) inguinal lymph node PMEL numbers normalized to 10,000 live single cells.
that type I IFN signaling directly on autoreactive PMEL does not modulate disease severity.

To determine whether IFNaR deficiency on host hematopoietic or radioresistant cells was required for enhanced vitiligo, we generated bone marrow chimeric mice by reconstituting lethally irradiated WT or IFNaR-deficient mice with WT or IFNaR-deficient BM stem cells. Chimeric mice that lacked IFNaR on radioresistant cells (IFNaR\textsuperscript{RC}) developed significantly more severe disease compared to wild type recipients (Figure 2.3C). IFNaR-deficiency in the hematopoietic cells had more modest effects (Figure 2.3C). There was increased PMEL trafficking to the epidermis and dermis of IFNaR\textsuperscript{RC} mice at 3 weeks post vitiligo induction (Figure 2.3D-F). Thus, IFNaR deficiency on radioresistant cells is responsible for the accelerated migration of autoimmune CD8\textsuperscript{+} T cells in hosts with vitiligo.
Figure 2.3: IFNaR signaling on host radioresistant cells is important for controlling disease development. A) Representative mouse images at 7 weeks post vitiligo induction. B) Vitiligo scores at week 7 post vitiligo induction. C) Vitiligo scores of chimeric mice 7 weeks post vitiligo induction. Normalized PMEL numbers in the epidermis (D), dermis (E), and draining lymph node (F) of chimeric mice at 5 weeks post vitiligo induction.
2.4: IFNaR-deficient mice have increased circulating CD44$^+$ effector memory PMEL with enhanced effector function

In addition to increased migration of PMEL into the skin of IFNaR-deficient mice, we observed significantly increased numbers of circulating PMEL in the skin draining lymph nodes and spleen of IFNaR-deficient hosts as early as 3 weeks after vitiligo induction (Figure 2.4A-B), and the number of T cells remained significantly increased relative to wild type mice for up to 7 weeks. Phenotypic analyses of circulating PMEL revealed a significant increase in effector memory, CD44$^+$CD62L$^-$ PMEL in IFNaR-deficient host mice compared to WT (Figure 2.4C-D). Since recirculating memory PMEL are important in mediating melanocyte death in vitiligo mice$^{309}$, these results suggest that increased numbers of recirculating memory PMEL are likely to contribute to disease severity.
**Figure 2.4: IFNaR-deficient mice have increased circulating CD44^+ effector memory PMEL.** Normalized PMEL numbers in the skin draining lymph nodes (A) and spleen (B) at weeks 3, 5, and 7 post vitiligo induction. C) Representative flow cytometry plots pre-gated on PMEL showing CD44 and CD62L marker expression. D) Percent of CD44^+CD62L^- PMEL in the spleen of WT and IFNaR KO mice at weeks 3, 5, and 7 post vitiligo induction.
2.5: Increased PMEL function in IFNaR-deficient hosts leads to enhanced CXCL9 and CXCL10 chemokine ligand expression.

We next asked whether PMEL in IFNaR-deficient hosts exhibited increased effector potential. Vitiligo was induced in mice and then tissue cell suspensions were stimulated with anti-CD3/CD28 overnight. Epidermal PMEL in IFNaR-deficient hosts produced significantly more TNFα and IFNγ compared to WT controls (Figure 2.5A-C). Importantly, poly functional TNFα⁺ IFNγ⁺ PMEL were significantly increased in the epidermis of IFNaR-deficient host mice (Figure 2.5B). This data demonstrates that enhanced severity of vitiligo in IFNaR-deficient mice results from increased PMEL function.

The IFN-induced chemokine ligands CXCL9 and CXCL10 promote migration of CXCR3⁺ PMEL T cells into the skin to destroy melanocytes during vitiligo⁷⁰,⁷⁵. Because we observed increased IFNγ production by PMEL in IFNaR-deficient mice, we monitored CXCL9 and CXCL10 expression levels in the skin over the course of disease using reporter REX3 mice ⁷⁹. We previously described that chemokine production by epidermal keratinocytes is important for disease progression⁷⁴, and we found that epidermal keratinocyte expression of CXCL9 and CXCL10 was significantly higher in IFNaR-deficient mice compared to WT mice (Figure 2.5D-F), as well as production by dermal phagocytes. These results suggest that increased IFNγ production by PMEL in
IFNaR-deficient mice amplifies expression of the chemokines CXCL9 and CXCL10, thereby enhancing the migration of effector PMEL into the skin.

**Figure 2.5: Increased PMEL function in IFNaR-deficient hosts leads to enhanced CXCL9 and CXCL10 chemokine ligand expression.** A) Representative flow plots of IFNγ and TNFα production by PMEL following ex vivo stimulation with 5 µg/mL anti-CD3 and 2 µg/mL anti-CD28 in the epidermis and dermis of WT and IFNaR KO mice. Percentage of IFNγ+ and IFNγ+ TNFα+ PMEL in the epidermis (B) and dermis (C). D) CXCL9 and CXCL10 reporter expression by epidermal keratinocytes and dermal MHC II+ phagocytes in the ear skin of WT and IFNaR KO mice at week 3 post vitiligo induction. Percent CXCL10 single positive and CXCL9+CXCL10+ double positive keratinocytes (E) and dermal phagocytes (F) in WT and IFNaR KO mice.
2.6: PMEL do not require IFNGR signaling to mediate disease.

Previous work has established that IFNγ and downstream chemokine production is required for vitiligo pathogenesis, but the role of autocrine IFNγR signaling on effector CD8 T cells is unknown. Autocrine IFNγR signaling has been reported to enhance the differentiation and effector capabilities of CD8 T cells as is type I IFN310. Since IFNγ production in IFNaR-deficient hosts was increased compared to WT, we asked whether signaling through the IFNγR on PMEL was required for this phenotype. To test this, we induced vitiligo in WT or IFNaR-deficient hosts with WT or IFNγR-deficient PMELs. Results reveal that autocrine IFNγR signaling is not required for vitiligo induction in WT mice (Figure 2.6A). These results also reveal that enhanced IFNγ production by PMEL in IFNaR-deficient hosts is not contributing to PMEL function through autocrine IFNγR signaling (Figure 2.6A). There were not significant differences in PMEL numbers in the skin between groups (Figure 2.6B-C). Although, as we previously described, IFNaR-deficient hosts have increased PMELs in the lymph nodes and spleen (Figure 2.6D-E). There was a significant difference in WT vs IFNγR-deficient PMEL number in the lymph node in IFNaR-deficient hosts, and this may suggest that autocrine IFNγR signaling regulates the CD8 T cell memory pool at this site, although more experiments need to be performed311 (Figure 2.6D).
Reduced expression of PD-1 and PD-L1 in IFNaR-deficient hosts

Since type I IFNs induce expression of negative immune regulators such as programmed cell death-1 and programmed cell death ligand 1 (PD-L1), which act to suppress T cell immune responses, we sought to determine whether inhibitory molecule expression was altered in IFNaR-deficient mice. Both the percentage of PD-1 expressing PMEL and the mean fluorescent intensity (MFI) of PD-1 was significantly reduced on PMELs in the epidermis and dermis of IFNaR-deficient hosts compared to wild type hosts (Figure 2.7A-D). However, there was no difference in PD-1 expression on PMEL in the skin draining lymph nodes (Figure 2.7E). In addition, IFNaR<sup>RC</sup> chimeras revealed significantly reduced PD-1 expression on PMEL (Figure 2.8A-C). Examination of PD-L1 on cross priming CD8<sup>α</sup> dendritic cells revealed a significant reduction in IFNaR-deficient hosts compared to wild type (Figure 2.7F-G). These results suggest that enhanced vitiligo

Figure 2.6: Autocrine IFNGR signaling on PMEL does not mediate severe disease in IFNaR-deficient hosts. A) Vitiligo scores week 7 after vitiligo induction. Effector CD8<sup>+</sup> PMEL numbers normalized to live single cells in the epidermis (B) dermis (C) skin draining lymph node (D) and spleen (E).
in IFNaR-deficient mice may result from dysregulated inhibitory molecule expression on both T cells and antigen presenting cells.
Figure 2.7: **Reduced expression of negative immune regulators in IFNaR-deficient hosts.** A) Representative flow plots of epidermal and dermal PD-1 expression on PMEL in WT and IFNaR KO host mice. B) PD-1 MFI on PMEL in the skin epidermis and dermis. Each data point represents an individual animal; n = 5 with 2-3 replicate experiments performed. Percentage of PD-1 on epidermal (C) dermal (D) and draining lymph node (E) PMEL at weeks 3, 5, and 7 post vitiligo induction pooled from 2 to 3 separate experiments; mean ± standard deviation of the mean. F) PD-L1 expression by CD8α dendritic cells in the spleen of WT and IFNaR KO mice at week 3 post vitiligo induction. G) Representative histogram and quantification of PD-L1 expression as determined by flow cytometry on WT and IFNaR KO CD8α DCs at week 3 post vitiligo induction. Each data point represents an individual animal with 2-3 replicate experiments performed.
2.8: IFNaR-deficient hosts display persistent vaccinia virus infection

Early studies show that both type I IFN and IFNγ are required for defense against vaccinia virus as mice deficient for either IFNaR or IFNGR showed increased susceptibility to infection compared to wild type mice. Although our mouse model of vitiligo is induced with a highly attenuated vaccinia virus strain and we inject virus-specific CD8+ T cells (PMELs) into mice that have the capability to clear infected cells, we asked whether IFNaR-deficient mice had reduced viral clearance. IFNaR-deficient host mice had significantly increased viral loads in the ovary at 9 days and 3 weeks post-infection compared to wild type hosts (Figure 2.9A), however, no virus was detected in
the skin of wild type or IFNaR-deficient mice (Figure 2.9A). These results suggest that increased viral replication in IFNaR-deficient hosts may be responsible for increasing the activation of PMELs and thus the development of severe autoimmune vitiligo.

**Figure 2.9: IFNaR-deficient hosts display vaccinia virus persistence.** A) Vaccinia virus PFU in WT and IFNaR KO mouse ovaries and ear skin determined by plaque assay at day 9 and week 3 post infection. Each data point represents an individual animal, pooled from 2 separate experiments.
2.9: Development of vitiligo in mice using PMEL pulsed-dendritic cells to prime T cells in vivo instead of virus

To determine whether IFNaR-mediated restraint of the autoimmune T cell response was a result of increased vaccinia virus replication, we developed a novel mouse model of vitiligo that is independent of induction with a vaccinia virus vaccine. In this model, vitiligo is induced by sublethal irradiation of Krt14-Kitl* host mice the day before adoptive T cell transfer. The following day, bone marrow derived dendritic cells (BMDCs) pulsed with PMEL peptide are co-transferred with PMEL CD8+ T cells into hosts (Figure 2.10A). Hosts are injected with rmIL-2 for the following 3 days to support PMEL CD8+ T cell engraftment (Figure 2.10A). Following induction of disease, hosts develop epidermal depigmentation on the nose, ears, footpads and tail, similar to vitiligo induced with a vaccinia viral vector (Figure 2.10B).

To determine whether IFNγ signaling is also required for disease pathogenesis in BMDC-induced vitiligo, we used IFNGR-deficient mice as hosts. Vitiligo induced in IFNGR-deficient hosts with BMDC-PMEL showed protection from disease and a significant reduction of PMEL in the epidermis and dermis (Figure 2.10C-F). However, engraftment of PMEL was unaffected, as PMEL numbers were similar in the lymph node (Figure 2.10F). These results reveal IFNγ as the shared pathogenic pathway in both BMDC-induced and vaccinia virus-induced vitiligo and confirm its importance in driving vitiligo progression.
2.10: Depletion of resident memory CD8 T cells using anti-CD122 antibody treatment in BMDC-PMEL induced vitiligo hosts stimulates repigmentation.
Previous studies revealed that vitiligo mice and human vitiligo patients contain resident memory CD8 T cells (TRM) in the skin and that TRM play a role maintaining disease. Furthermore, mechanistic studies in the vaccinia virus induced mouse model revealed that treatment of mice targeting the survival factor IL-15 using anti-CD122 antibody resulted in significant repigmentation of the skin and correlated with the depletion of TRM from the skin. We sought to determine whether BMDC-PMEL induced vitiligo also generated a population of resident memory PMEL CD8 T cells. Studies reveal that at 7 weeks post vitiligo induction the majority of PMEL in the epidermis of WT hosts express the TRM markers CD69 and CD103 (Figure 2.11A-B). Preliminary data in BMDC-PMEL induced vitiligo mice suggests that treatment of mice with anti-CD122 antibody led to significant repigmentation of the skin compared to mice treated with PBS (Figure 2.11C). Treatment with anti-CD122 led to reduced numbers of PMEL in the epidermis and dermis as well as skin draining lymph nodes (Figure 2.11D-F). More experiments need to be performed to determine whether PMEL numbers are significantly changed with treatment as in vaccinia virus induced vitiligo. However, collectively these studies validate the BMDC-PMEL model as a new model of vitiligo that is dependent on IFNγ signaling and that undergoes repigmentation following TRM depletion.
Figure 2.11: Vitiligo induced with BMDC-PMEL leads to development of epidermal TRM, which upon depletion using anti-CD122 antibody reverses vitiligo. A) Representative flow cytometry plot of epidermal CD69 and CD103 expressing PMEL. B) Quantification of the percent CD69^+CD103^+ PMEL in the epidermis of WT mice. C) Percent tail pigment before and after PBS or anti-CD122 treatment using ImageJ analysis to quantify pigmentation. Normalized PMEL CD8 T cell numbers in the epidermis (D) dermis (E) and skin draining lymph nodes (F) of BMDC-PMEL vitiligo mice 7 weeks post vitiligo induction.
2.11: IFNaR-deficient hosts do not develop severe vitiligo when disease is induced by dendritic cells in the absence of vaccinia virus

To determine the role of type I IFN in vitiligo using a mouse model independent of the vaccinia virus vaccine, we induced vitiligo using BMDC-PMEL in IFNaR-deficient and wild type hosts. Disease severity was comparable between the two groups, revealing that type I IFN is not required during the effector phase of vitiligo development or for disease progression (Figure 2.12A-D). These results demonstrate that the extent of disease activity exhibited by CD8⁺ PMEL effector cells in the vaccinia virus-induced model was directly linked to the viral induction phase of the disease. Delayed clearance of vaccinia virus in IFNaR-deficient mice extends the priming phase of the disease, possibly resulting in greater activation of the effector T cells through a variety of mechanisms. These studies further show that timing and balance of type I and type II interferons during anti-viral immune responses is critical to prevent pathogenic immune responses to self.
2.12: Vaccinia virus vaccine therapy in IFNaR-deficient mice significantly enhances the anti-tumor response to melanoma

Our results reveal that type I IFN limits viral persistence and enhances the activation of anti-melanocyte T cells. Since vaccinia virus vectors have also been used to prime T cells for tumor vaccination immunotherapies\(^ {287,293,315,316} \), we decided to ask whether a similar loss of type I IFN would improve tumor control in a model of adoptive T cell therapy enhanced by vaccinia virus vaccine (Figure 2.13A). B16-F10 melanomas were injected into WT or IFNaR-deficient mice. Six to eight days after melanoma injection, mice were infected with recombinant vaccinia virus expressing gp100 in combination with adoptive transfer of PMEL CD8\(^+\) T cells (Figure 2.13A). IFNaR-deficient mice showed improved tumor control compared to wild type mice (Figure 2.13B-C). Significant reduction in tumor weight in IFNaR-deficient host mice correlated with an increase in tumor
infiltrating PMEL CD8$^+$ T cells (Figure 2.13D) and a significant reduction in the expression of the immune checkpoint inhibitor PD-1 on PMEL T cells (Figure 2.13E-F). These results reveal that the absence of type I IFN during viral vaccination leads to a more robust anti-melanocyte CD8$^+$ T cell response in both autoimmunity and melanoma immunotherapy.
2.13: Discussion

Here we describe the role of type I IFN in vitiligo using two different mouse models of disease. We report that in contrast to IFNγ, type I IFN signaling is not required during the
effector phase of vitiligo development or progression in mice. These results are important to inform future therapies for vitiligo patients.

Our results further reveal that administration of a vaccinia virus vaccine in IFNaR-deficient mice led to enhanced priming of CD8+ T cells and development of more severe vitiligo than in IFNaR-sufficient hosts. Type I IFNs are critical for controlling virus infections, and we report that type I IFN signaling is necessary to control the replication of even an attenuated vaccinia virus strain.

Type I IFNs can contribute to impaired host adaptive immune responses and viral persistence during chronic infections \[^{145,317}\]. Blockade of type I IFN during chronic LCMV and HIV reactivates the anti-viral CD8+ T cell response leading to clearance of the virus through reduction of PD-1 on T cells and PD-L1 on dendritic cells \[^{145,317}\]. Similar to these observations, we show that type I IFN signaling on radioresistant cells during vaccinia virus persistence limits T cell expansion and activation. Increased activation of T cells in IFNaR-deficient mice following vaccinia virus vaccine may result from prolonged antigen exposure, because of persistent antigen presentation. Studies reveal that the amount of antigen presented by DCs can increase the CD8 T cell response\[^{318}\]. Therefore, increased antigen density may increase PMEL CD8 T cell responses in IFNaR deficient hosts. An alternative hypothesis may be that infection of DCs with VACV in WT and IFNaR deficient hosts led to differences in T cell activation. One report showed that infection of DCs with VACV induced DC apoptosis. As a result, VACV led to impaired T cell responses compared to DC infection with influenza\[^{319}\].
VACV infection of DCs and subsequent apoptosis may result from type I IFN signaling. Thus, enhanced vitiligo in IFNaR deficient hosts may result from reduced apoptosis of infected DCs compared to WT hosts. Persistent infection may also impact the regulatory activity of dendritic cells, as we observed reduced PD-L1 on the CD8α dendritic cells responsible for T cell priming.

Previous clinical studies report that injection of type I IFN into the skin has induced local, and sometimes distant depigmentation consistent with vitiligo. These reports demonstrate that overexposure of the skin to increased doses of type I IFN are capable of promoting depigmentation, although this does not necessarily indicate that type I IFN plays a role in normal vitiligo pathogenesis and we show functional evidence that type I IFN is not required for disease. Thus, the reported development of autoimmunity following IFN treatment may be dependent on chronic infection or an individuals’ predisposition to autoimmunity. Administration of type I IFNs in the setting of chronic infection may lead to the activation of cross-reactive T cells that cause autoimmune pathology, or alternatively that type I IFN-induced inflammation in the right context can recruit autoreactive T cells into the skin, similar to Koebner phenomenon.  

The generation of a robust cytotoxic T lymphocyte (CTL) response against melanoma is critical for the success of immunotherapy. Similar to the immune-suppressive effects of type I IFN during chronic viral infections, negative effects of type I IFNs occur in cancer inflammation and can lead to resistance to treatment. After observing more severe
vitiligo in IFNaR-deficient hosts following administration of a vaccinia virus vaccine, we sought to determine whether stimulation of the anti-tumor response to melanoma could be enhanced in IFNaR-deficient mice. Our results demonstrate that in the absence of type I IFN signaling, adoptive CD8+ T cell therapy in combination with a vaccinia virus vaccine enhances the anti-melanoma response.

A major limitation to the use of viral vaccines in cancer immunotherapy has been their lack of immunogenicity. Our results suggest that blockade of type I IFNs during vaccinia virus administration can improve the immunogenicity of the vaccine, likely resulting from increased CD8+ T cell priming during vector persistence. Studies using oncolytic viruses for cancer immunotherapy have also reported benefits of blocking type I IFNs to increase infection and improve responses to therapy. A recent study reported increased tumor regression after IFNaR blockade during oncolytic virus therapy in combination with adoptive T cell therapy in mice. Our findings suggest that blockade of IFNaR during vaccinia virus vaccine for cancer immunotherapy may enhance both immunogenicity and persistence of the vector, thus improving vaccine efficacy, and may induce long-term benefits for tumor eradication.
PREFACE TO CHAPTER 3

Chapter 3 contains original text which represents the unpublished work of Rebecca L. Riding. Rebecca L. Riding executed and designed experiments presented in this chapter and analyzed the data. Keitaro Fukuda contributed immensely to this project by providing experimental and intellectual advice.
CHAPTER 3

REGULATION OF T CELL PRIMING BY DENDRITIC CELL ACTIVATION

3.1: Introduction

Dendritic cells bridge the gap between the innate and adaptive immune system because they continuously sample antigen within their environment and are responsible for maintaining immune homeostasis\textsuperscript{326}. Infection with a pathogen or sterile inflammation can lead to PRR activation and induces DC maturation. DC maturation involves the upregulation of MHC molecules, increased formation of MHC-peptide complexes as well as the upregulation of the co-stimulatory molecules CD80, CD86, and CD40\textsuperscript{327}. PRR activation also triggers production of pro-inflammatory cytokines and type I IFNs. Mature DCs then traffic to the lymphoid organs where they can prime naïve T cells to become activated. Therefore, DCs play an integral part in fine tuning the adaptive immune responses through modulating T cell differentiation and function. Naïve T cells require three signals to become activated; cognate peptide MHC-TCR interaction, co-stimulation, and cytokines\textsuperscript{328}. Differential cytokine production downstream of PRR activation in DCs can induce different T cell programs. For example, in humans Gram negative bacteria induces DC production of IL-12 but not gram positive bacteria\textsuperscript{329,330}. Mycobacterium tuberculosis can induce DC production of IL-10\textsuperscript{331}. IL-12 typically promotes Th1 responses whereas IL-10 inhibits Th1 responses, therefore differential PRR activation and the cytokine milieu is important for driving T cell immunity\textsuperscript{332}. 
The aberrant activation of PRRs has been implicated in driving autoimmune disease. One example of this is the recognition of immune complexes that contain self-nucleic acids in the endosome, which drives SLE. Specifically, self-nucleic acids released in the endosome can trigger endosomal TLRs such as TLR7 or TLR9 and induce pro-inflammatory cytokines or type I IFN production. STING activation has also been implicated in autoimmunity. TREX1 is an exonuclease that is responsible for digesting DNA in the cytosol. Without TREX1, cytosolic DNA can activate innate immune signaling pathways such as cGAS-STING. Genetic mutations in TREX1 cause the type I IFN autoimmune disease Aicardi-Goutières syndrome (AGS). Because DCs are critical in driving T cell responses and because a few PRRs and signaling adaptors (NLRP1, MDA5, TRIF) are associated with vitiligo susceptibility, we sought to investigate the roles of PRRs in activating T cells in vitiligo mice.

To investigate the role of PRR activation in DCs, we used the BMDC-PMEL vitiligo model (Chapter 2) in which bone marrow derived dendritic cells (BMDCs) are pulsed with PMEL peptide and then co-adoptively transferred into SCF recipient mice with PMEL CD8 T cells. This model allows for the manipulation of BMDCs by using genetically altered mice as BMDC donors to investigate the role of different PRRs on generating PMEL T cell responses in vitiligo.

3.2: BMDC accumulation in the skin tissue following transfer into host mice

We first asked where transferred BMDCs accumulate following injection into WT SCF hosts. To test this, we used BMDCs from a congenic mouse strain expressing CD45.1.
CD45 is a protein tyrosine phosphatase expressed by all hematopoietic cells and wild type C57BL/6 mice normally carry the CD45.2 allele. Since our host SCF mice are on the C57BL/6 background strain, they also carry the CD45.2 allele. Therefore, we acquired CD45.1 expressing C57BL/6 mice from the Jackson Laboratory and isolated BMDCs from these recipients for transfer into SCF CD45.2 hosts. This allows for the tracking of CD45.1 positive cells following injection into CD45.2 hosts. We induced vitiligo as previously described (Ch.2). Briefly, CD45.2 WT SCF hosts were subletally irradiated and the following day CD45.1 BMDC-PMEL and PMEL CD8 T cells were co-transferred into hosts. Tissues were harvested and flow cytometry was used to detect CD45.1 BMDCs over time.

CD45.1 BMDCs were not found in any tissue tested at early time points (Day 1 and 3) post injection into SCF hosts (data not shown). But interestingly, CD45.1 BMDCs can be detected in the epidermis and dermis of SCF hosts at day 7 post injection (Figure 3.1A-B). CD45.2 BMDCs are also detected in the lymph nodes and spleen at this time point (Figure 3.1B). Additional studies in the lab reveal that BMDCs can be detected in the spleen and tumor microenvironment of C57BL/6 hosts up until day 20 post transfer (Keitaro Fukuda, manuscript submitted). In vitiligo, CD45.1 BMDCs are present in the skin even before the appearance of PMEL CD8 T cells (data not shown). This is of interest to our studies because it may suggest that transferred BMDCs can activate CD8 T cell responses at the skin site or may drive migration of PMEL CD8 T cell into the skin.
Figure 3.1: Transferred WT-BMDCs traffic to the epidermis and dermis in SCF hosts. A) Representative flow cytometry plots of CD45^+ BMDCs in the epidermis, dermis, and spleen of SCF host mice day 7 post transfer. B) Normalized BMDC numbers in host tissues at day 7 post transfer. Each dot represents an individual host mouse.
3.3: Autocrine type I IFN signaling on BMDCs is not required for vitiligo development

Previous studies described in chapter 2 revealed that following BMDC-PMEL transfer, host IFNaR signaling is not required for vitiligo induction. However, type I IFNs are critical for the maturation of DCs through induction of antigen presentation, induction of co-stimulatory molecules, and mediating migration of DCs to the lymph node for priming of T cell immunity. Thus, we sought to determine whether autocrine IFNaR signaling was required on BMDCs to initiate PMEL CD8 T cell activation using this model. To test this, we induced vitiligo in WT hosts using either WT or IFNaR-deficient BMDCs. Induction of vitiligo with IFNaR-deficient BMDCs revealed comparable disease scores to mice induced with WT BMDCs (Figure 3.2A). Therefore, we can conclude that IFNaR signaling on BMDCs is not required for induction of PMEL CD8 T cells using this model. Interestingly, PMEL numbers in the epidermis and dermis where significantly increased with IFNaR-deficient BMDCs compared to WT-BMDCs (Figure 3.2B-C). There was no difference in PMEL numbers between groups in the lymph nodes or spleen (Figure 3.2D-E).

Although these results suggest that BMDCs do not require IFNaR signaling to mediate stimulation of T cells, both the VACV and BMDC-induced vitiligo models use expression of their cognate antigen (gp100) to intentionally induce CD8 T cell activation. Thus, the effects of type I IFNs on the priming and activation of endogenous autoreactive T cells may not be captured in these models.
Figure 3.2: IFNaR signaling on BMDCs is not required for induction of vitiligo in mice. A) Vitiligo score at week 7. Normalized PMEL CD8 T cell number in the epidermis (B) dermis (C) skin draining lymph node (D) and spleen (E) 7 weeks post vitiligo induction. Results are pooled from two separate experiments.
3.4: Transfer of mature BMDCs induces significantly enhanced vitiligo in mice

As previously described in chapter 2 and in the previous figure, IFNaR signaling was not required on host cells or on BMDCs for vitiligo induction using BMDC-PMEL. However, this model may bypass the effects of innate immune activation and effects of type I IFN in enhancing T cell immunity. Because maturation of DCs following PRR activation is important for inducing T cell immunity and because type I IFNs play a critical role during this process, we sought to determine whether stimulation of BMDCs with a TLR ligand would affect vitiligo development in mice. To test this, we used a synthetic oligonucleotide that contains unmethylated CpG dinucleotides called CpG ODN1826. DNA containing CpG motifs are found at a significantly higher frequency in bacterial DNA compared to mammalian DNA and are the ligand that activates TLR9. Following bone marrow isolation and 8 days of culture with granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4, BMDCs were peptide pulsed and either left untreated or treated with CpG ODN1826 (TLR9 ligand) for 1 hr. WT or TLR9 ligand stimulated BMDCs were used to induce vitiligo in WT or IFNaR KO hosts. Results reveal that stimulation of BMDCs with TLR9 agonist led to significantly increased vitiligo score (Figure 3.3A). This suggests that maturation of BMDCs following PRR activation can enhance the T cell response. The effects of mature BMDC transfer observed in WT hosts was ameliorated following transfer into IFNaR KO hosts (Figure 3.3B), suggesting that IFNaR signaling on host cells is important for the potentiating
effects of TLR ligand stimulated BMDCs. Previous studies have shown that murine BMDCs express TLR9 and that stimulation by CpG induced expression of the costimulatory molecules CD86 and CD40 and induces high levels of the pro-inflammatory cytokines IL-12, IL-6, TNFα and the chemokine ligand CXCL10\textsuperscript{336}. TLR9 stimulation also leads to type I IFN production by DCs. Therefore, TLR ligand stimulation of BMDCs may increase vitiligo through maturation of DCs including induction of co-stimulatory molecules, and production of cytokines that can enhance T cell activation.
3.5: Aim2-deficient BMDCs induce significantly enhanced vitiligo in mice.

The activation of PRRs by tumor derived or self-DNA is a potent activator of adaptive immune responses. One example of this is the activation of cGAS-STING pathway by tumor derived DNA\textsuperscript{256}. Studies show that the activation of STING in tumor DCs leads to a robust cytotoxic T lymphocyte response against tumor cells. However, less is known about the activation of other DNA sensors during this process. Absent in melanoma 2 (Aim2) is one of many innate immune sensors that detect cytosolic DNA\textsuperscript{197}. It can detect both foreign and self-DNA that becomes mislocalized in the cytosol. Aim2 also induces...
inflammasome assembly that activates caspases\textsuperscript{337}. Activation of the Aim2 inflammasome leads to the maturation and secretion of the cytokines IL-1β and IL-18. Evidence suggests that dysregulation of the Aim2 inflammasome can lead to the production of autoantibodies in a mouse model of lupus\textsuperscript{338}.

In a mouse model of melanoma in which adoptive T cell therapy using PMEL CD8 T cells and BMDC-PMEL vaccine is used to induce tumor regression, Dr. Fukuda revealed that Aim2 KO-BMDCs induced robust PMEL infiltration into the tumor, and showed improved tumor regression (\textit{manuscript submitted}). We sought to test the role of DNA sensing by Aim2 on the CD8 T cell response to vitiligo. Induction of vitiligo in WT hosts using Aim2 KO BMDCs led to significantly enhanced vitiligo severity compared to induction with WT BMDCs (Figure 3.4A). Enhanced disease in hosts induced with Aim2 KO-BMDCs correlated with significantly increased PMEL numbers in the epidermis and dermis compared to hosts induced with WT BMDCs (Figure 3.4B-C). There were only modest increases in PMEL numbers in the lymph node following induction with Aim2 KO BMDCs (Figure 3.4D-E).
We became interested in better understanding how the activation of Aim2 regulates melanocyte specific T cell responses during vitiligo in mice. Previous work reported that following stimulation with DNA, Aim2 KO BMDCs have increased IFNβ and CXCL10

**Figure 3.4: Aim2 KO BMDCs induce more severe vitiligo compared to WT BMDCs. A) Vitiligo disease score at week 7. Normalized PMEL CD8 T cell numbers in the epidermis (B) dermis (C) skin draining lymph node (D) and spleen (E) 7 weeks post vitiligo induction.**
production compared to WT BMDCs. Corrales et al reveal that activation of the STING signaling components TBK1 and IRF3 were increased in Aim2 KO BMDCs compared to WT, suggesting that Aim2 regulates STING activation. The authors suggest a mechanism by which activation of the Aim2 inflammasome and caspase-1 following DNA stimulation inhibits the STING pathway. Based on this data, we hypothesized that Aim2 KO BMDCs enhance vitiligo through the upregulation of IFNβ and enhancement of signaling through the STING pathway as is described by previous work. Although autocrine IFNaR signaling was not required on BMDCs in our previous experiments, enhanced IFNβ produced by Aim2 KO BMDCs through STING activation may act directly on Aim2 KO BMDCs to further their maturation and this may be one explanation for the enhanced disease severity seen using Aim2 KO BMDCs.

To test this hypothesis, we induced vitiligo in SCF hosts using WT, Aim2 KO, Aim2/IFNaR DKO, STING KO, and Aim2/STING DKO BMDCs. Our results reveal significantly increased disease scores when Aim2 KO-BMDCs are used to induce disease compared to WT-BMDCs as was observed in Figure 3.4 (Figure 3.5A). Interestingly, ablation of IFNaR or STING on Aim2 KO-BMDCs did not result in significantly reduced scores (Figure 3.5A). These results suggest that the potentiating effects of Aim2 KO-BMDCs on vitiligo development are not a result of enhanced STING signaling and that STING signaling by BMDC-PMEL is not required for effector T cell stimulation in this model. Although disease scores in Aim2/IFNaR DKO-BMDCs were reduced compared to Aim2-KO, STING-KO, and Aim2/STING DKO-BMDCs, it did not reach statistical significance (Figure 3.5A). These results suggest that the enhanced vitiligo following
induction with Aim2 KO-BMDCs is not a result of autocrine IFNaR signaling, or that autocrine IFNaR signaling plays only a small role in potentiating disease by Aim2 KO-BMDCs. There were no significant differences in PMEL CD8 T cell numbers in the skin or secondary lymphoid organs between the groups (Figure 3.5B-E).
Figure 3.5: Enhanced disease mediated by Aim2 KO-BMDCs is not a result of enhanced STING or IFNaR signaling. A) Vitiligo disease score at 7 weeks post induction. Normalized PMEL CD8 T cell numbers in the epidermis (B), dermis (C), skin draining lymph nodes (D), and spleen (E) 7 weeks post vitiligo induction. Each dot represents one individual animal and the data is representative of two separate experiments pooled.
3.6: CXCL10 production by BMDC-PMEL is not required for induction of T cell responses.

Our lab has previously shown that the chemokines CXCL9 and CXCL10 are critical for vitiligo progression in mice and are enhanced in the skin of patients with vitiligo\textsuperscript{53,70}. We have shown that chemokine production by keratinocytes is critical for initiating disease, as loss of STAT1 signaling in keratinocytes led to significantly reduced disease in mice\textsuperscript{74}. Since Aim2 KO-BMDCs increase expression of CXCL10\textsuperscript{339}, and because DCs can recruit CXCR3 expressing T cells into tumors\textsuperscript{260,261}, we sought to determine whether CXCL10 production by Aim2 KO-BMDCs contributed to the phenotype we observed. To test this, we induced vitiligo in WT SCF hosts using WT, CXCL10 KO or Aim2/CXCL10 DKO BMDCs. Our results reveal that CXCL10 production by BMDC-PMEL is not required for vitiligo induction and that enhanced vitiligo following transfer of Aim2 KO-BMDCs is not a result of increased CXCL10 expression (Figure 3.6A). Vitiligo disease scores were not significantly different between WT-BMDCs and CXCL10 KO-BMDCs (Figure 3.6A). Aim2/CXCL10 DKO BMDCs showed increased disease scores but it did not reach statistical significance as a result of low power and because of high vitiligo scores in WT-BMDC induced hosts (Figure 3.6A). There were no significant differences in PMEL CD8 T cell numbers in the skin or secondary lymphoid organs between WT-BMDC and experimental groups (Figure 3.6B-E). Because CXCL10 production by keratinocytes is critical for inducing migration of PMEL CD8 T cells into the skin to promote disease, these results suggest that CXCL10 by the presenting DCs may not be as critical for stimulating migration of PMEL into the skin.
Figure 3.6: CXCL10 by Aim2 KO BMDC-PMEL does not mediate enhanced vitiligo development. A) Vitiligo disease score at week 7. Normalized PMEL CD8 T cell numbers in the epidermis (B) dermis (C) skin draining lymph node (D) and spleen (E) 7 weeks post vitiligo induction.
3.7: The role of TLR adapters MyD88 and TRIF in mediating T cell responses against melanocytes in vitiligo.

TLR adaptors are TIR domain-containing proteins that associate with TLRs and mediate downstream signaling\textsuperscript{340}. TRIF is one of six known TLR adaptor molecules; MyD88, MAL, TRIF, TRAM, SARM, and BCAP. Specific adaptor molecule recruitment to TLRs can alter the downstream signaling pathways and immune responses generated following TLR activation. I will focus on the signaling pathways mediated by MyD88 and TRIF. Both MyD88 and TRIF activate MAPKs and IKKs to induce transcription factors such as NF-κB, and interferon regulatory factor (IRF) family members. All TLRs except for TLR3 use MyD88 for signaling but TRIF is required for gene induction downstream of TLR3\textsuperscript{341}. TLR4 is a unique receptor in that it utilizes both MyD88 and TRIF for signaling\textsuperscript{342}. While TRIF is recruited directly to TLR3 for signaling, TRIF is recruited indirectly to TLR4 by the adaptor TRAM\textsuperscript{343,344}. The N-terminal domain of TRIF interacts with TBK1 and IKKi/IKKe leading to activation of IRF3\textsuperscript{106,345}. The C-terminal domain of TRIF activates NF-κB and apoptosis pathways\textsuperscript{346,347}. TLR3 signaling through TRIF leads to the activation of IRF3 and induces expression of IFNβ. TLR4 can activate multiple downstream pathways, but signaling through TRIF leads to IFNβ production. Studies show the importance of TRIF in anti-viral defense in humans. Although TRIF mediated TLR3 is associated with defense against dsRNA viruses, it is also important for resistance against DNA viruses and ssRNA viruses\textsuperscript{348}. TRIF has also recently been shown to interact directly with STING to mediate downstream signaling\textsuperscript{349}. 
Few studies have looked at the role of TRIF in autoimmunity. In a mouse model for experimental autoimmune encephalomyelitis (EAE), TRIF activation was reported to suppress disease\textsuperscript{350}. Suppression of EAE was a result of the induction of endogenous IFNβ and CCL2\textsuperscript{350}. Another report provides evidence for TRIF mediated suppression of autoimmunity in a model of STZ induced diabetes\textsuperscript{351}. The authors reveal that TRIF deficiency in DCs leads to acceleration of disease. TRIF signaling was shown to protect against IL-17-dependent arthritis model\textsuperscript{352}, possibly through the induction of type I IFNs known to suppress arthritis. TRIF has also been shown to play a role in tissue repair because it was required for clearance of axonal debris microglia\textsuperscript{353}, and TRIF KO mice show delayed wound healing\textsuperscript{354,355}.

Interestingly, GWAS studies have identified TICAM1, the gene that encodes TRIF, as a risk allele for vitiligo. The functional role of TRIF in vitiligo pathogenesis remains unclear, but new insights from single cell RNA sequencing analysis of vitiligo patient skin revealed a significant enrichment of TICAM1 in lesional macrophages compared to healthy skin macrophages (Figure 3.7A). The violin plot in Figure 3.7 is graphing both the normalized expression of TICAM1 on a per cell basis (left y-axis) and the mean TICAM1 expression of all macrophages per skin type (right y-axis). The black dot represents the mean expression of TICAM1 in macrophages from blister fluid in healthy, non-lesional, and lesional skin (right y-axis), where the individual points are plotted using the left-y-axis and show individual cell TICAM1 expression. The majority of cells express very little TICAM1, as shown by points at the bottom of the graph. However,
there are cells that express high levels. Thus, for us to see the average mean expression per skin type, we plotted this using the scale on the right y-axis. You can interpret this graph to show you both the cell specific TICAM1 expression (left y-axis) and the mean expression of all macrophages per skin type shown by the black dot (right y-axis). The right y-axis and mean expression is much lower because there are many macrophages that did not express TICAM1 or were below the detection limit of the scRNA seq data. Differential gene expression analysis was run using findDEgenes function which calls EdgeR. All genes with false discovery rate of $< 0.1$ and abs (logFC) $> 0.5$ were used. Using these statistical tests, macrophages in vitiligo lesional skin express significantly more TICAM1 compared to healthy individual macrophages.
Figure 3.7: TICAM1 transcripts are significantly enriched in macrophages in lesional skin compared to healthy individuals. A) Violin plot of healthy (pink), non-lesional (green) and lesional (blue) of TICAM1 transcripts.
Because of the potential implications of TRIF signaling in mediating vitiligo development in human patients, we sought to determine the effects of TRIF signaling in dendritic cells on the development of vitiligo using the BMDC vitiligo mouse model. To test this, we induced vitiligo in WT SCF hosts using WT BMDCs or TRIF KO-BMDCs. Results reveal that induction of vitiligo with TRIF KO-BMDCs led to the development of increased (although not significant) vitiligo disease scores (Figure 3.8A). Although PMEL CD8 T cells numbers were not significantly increased in the skin tissues, PMEL numbers were trending towards increased when TRIF-KO BMDCs were used to induce disease compared to WT BMDCs (Figure 3.8B-C). There were no significant differences in PMEL numbers in the skin draining lymph node or spleen (Figure 3.8D-E).
Figure 3.8: TRIF KO-BMDCs induce slightly worse disease in WT SCF hosts compared to WT-BMDCs. A) Vitiligo scores at week 7. Normalized PMEL CD8 T cells numbers in the epidermis (B) dermis (C) skin draining lymph nodes (D) and spleen (E). Each data point represents one mouse, pooled from 2 separate experiments.
Because MyD88 mediates signaling downstream of all TLRs, except TLR3, and because TLR4 uses both MyD88 and TRIF signaling pathways, we sought to determine the effect of MyD88 signaling in BMDCs on vitiligo pathogenesis. We induced vitiligo in WT SCF hosts using either WT-BMDCs or MyD88-KO BMDCs. Results revealed no significant differences in disease score or PMEL CD8 T cell infiltration in the skin (Figure 3.9A-E). Interestingly, there was a trend towards reduced disease and reduced PMEL numbers in WT SCF hosts induced with MyD88-KO-BMDCs compared to WT-BMDCs. This observation led to the inverse phenotype mediated by TRIF-KO BMDCs, which resulted in increased disease score and PMEL numbers (Figure 3.8).
Figure 3.9: MyD88 KO-BMDCs induce similar disease scores compared to WT-BMDCs in WT SCF hosts. A) Vitiligo scores at week 7. Normalized PMEL CD8 T cells numbers in the epidermis (B) dermis (C) skin draining lymph nodes (D) and spleen (E). Each data point represents one mouse, pooled from 2 separate experiments.
Our results are similar to observations reported in another recent study. The authors reported delayed onset of STZ induced diabetes in mice with MyD88 deficient myeloid cells\textsuperscript{351}. Although diabetes development was similar between mice with TRIF deficient myeloid cells and WT mice, the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO) was reduced in mice with TRIF deficient myeloid cells, and they observed a significant reduction in skin draining lymph node Tregs\textsuperscript{351}. This is in contrast to mice with MyD88 deficient myeloid cells in which IDO expression was induced\textsuperscript{351}. These results suggest that signaling through TRIF in DCs may induce pathways that suppress autoimmune development, whereas signaling through MyD88 does not. The role of MyD88 and TRIF in vitiligo development requires further investigation, but our preliminary results suggest that TRIF signaling may also act to induce negative regulators to suppress the autoimmune response.

A possible explanation for why disease severity is increased when TRIF KO-BMDCs are used to induce vitiligo compared to WT-BMDCs could be attributed to cell death. TRIF can induce apoptosis through RIP, FADD, and caspase-8\textsuperscript{347}. Following TLR ligand binding, TRIF interacts with RIP1 and through RIP3 can trigger cell death. Also, when caspase-8 is inhibited, TRIF can drive necroptosis\textsuperscript{356}. Therefore, determining whether the survival of TRIF deficient BMDCs is altered compared to WT BMDCs may help explain its effects on vitiligo development.
3.8: Discussion

Our studies discussed in this chapter focused on identifying where BMDCs accumulate following transfer into SCF hosts, and investigating the roles of PRR activation and downstream signaling in BMDCs on the development of vitiligo in mice. Our findings reveal that BMDCs are present in the skin epidermis and dermis at least 7 days post transfer in WT SCF hosts. Unpublished results from Dr. Fukuda also reveals that BMDC-PMEL remain in the tumor and spleen in a model of B16F10 melanoma through day 20 post transfer. Our results showing BMDC-PMEL in the epidermis and dermis following transfer into WT hosts suggests that transferred BMDC-PMEL may be able to sample antigen from the skin microenvironment. The activation of BMDC PRRs by DAMPs or PAMPs may lead to the upregulation of costimulatory molecules and proinflammatory cytokines, which may alter the T cell responses against melanocytes in vitiligo following PMEL antigen recognition\textsuperscript{327}. Interestingly, BMDCs are present in the skin tissue of mice before any PMEL CD8 T cells arrive in the skin. This suggests that pro-inflammatory cytokines, or chemokines produced by transferred BMDCs may not only affect PMEL CD8 T cell function, but could also affect the migration of PMEL CD8 T cells into the host skin tissue. We hypothesize that BMDCs are acting in the skin tissue to modulate T cell responses because of our observation that PMEL CD8 T cell numbers are significantly different at the skin site, and remain unchanged or have more modest effects in the secondary lymphoid organs (Figure 3.2 and 3.4).
We investigated whether PRR activation of BMDCs before transfer unto hosts could affect the development of vitiligo compared to immature BMDCs. We used a TLR9 ligand, a synthetic CpG oligonucleotide (ODN), to stimulate BMDCs before transfer into host mice. Transfer of CpG ODN stimulated BMDCs significantly increased vitiligo disease score compared to unstimulated BMDCs. These results suggest that activation of PRRs in BMDCs can affect the PMEL CD8 T cell response and alter disease progression in vivo. All BMDCs are pulsed with PMEL peptide, so differences observed in vitiligo score may result from differences in surface MHC expression, but are not the result of differences in the ability of BMDCs to process antigen. IL-12 produced following CpG stimulation promotes Th1 responses through increasing IFNγ production in both CD4 and CD8 T cells. Thus, this may be one explanation for enhanced disease development following stimulation by TLR9 ligand. However, it is still unclear where and when naïve PMEL CD8 T cells are activated following transfer into host mice. Thus, increased vitiligo disease score seen following induction with CpG stimulated BMDCs may result from activation in the secondary lymphoid organs or in the skin. The kinetics and duration of changes induced following CpG activation of BMDCs, such as co-stimulatory molecule expression or pro-inflammatory cytokine production, may impact the PMEL CD8 T cell response. For instance, it is not clear whether CpG stimulated BMDCs remain phenotypically stable following transfer or whether their phenotype is plastic and changes after transfer into host mice.
Since stimulation of TLR signaling in BMDCs led to clinical differences in vitiligo score, we investigated the role of the DNA sensors STING and Aim2 in vitiligo development. Dr. Fukuda’s studies in a mouse model of adoptive T cell therapy and DC vaccine for melanoma immunotherapy revealed improved tumor regression when Aim2- KO BMDCs were used compared to WT BMDCs as a result of enhanced PMEL migration into the tumor. In parallel to these studies, we revealed that induction of vitiligo using Aim2 KO-BMDCs led to significantly enhanced vitiligo compared to induction with WT BMDCs. Aim2 KO BMDCs express increased IFNβ, and CXCL10 as a result of increased STING signaling\textsuperscript{339,359}. We hypothesized that increased IFNβ or CXCL10 production by Aim2 KO BMDCs may increase disease in mice, as CXCL10 is critical for driving disease progression in mice\textsuperscript{70} and IFNβ, although not required for disease could impact the T cell response. However, induction of vitiligo using Aim2/STING DKO, Aim2/IFNaR DKO, and Aim2/CXCL10 DKO BMDCs did not reverse the phenotype observed using Aim2 KO BMDCs. Therefore, it is clear that the effects of Aim2 KO BMDCs are not mediated through the STING pathway. Ligand binding to Aim2 also promotes the formation of the Aim2 inflammasome protein complex, which is responsible for the processing and release of IL-1β and IL-18 through caspase-1 activation\textsuperscript{337}. We hypothesized that the processing and secretion of IL-1β and IL-18 by BMDCs may play a role in suppressing disease. Both IL-18 and IL-1β have been implicated in promoting regulatory T cell (Treg) responses, which are important for suppressing effector CD8 T cells in autoimmunity and in vitiligo\textsuperscript{360-362}. In a model of asthma, IL-18 production by DCs acted directly on T cells to drive conversion into
Tregs\textsuperscript{363}. In another study, IL-1 acted as a signal 3 cytokine to induce Treg expansion and differentiation\textsuperscript{364}. Thus, one explanation for increased disease induced with Aim2 KO BMDCs is reduced Treg expansion and recruitment as a result of reduced IL-1\(\beta\) and IL-18 (\textit{Keitaro Fukuda, unpublished}) in Aim2 KO BMDCs. To determine whether recruitment or expansion of Tregs is reduced following induction of vitiligo with Aim2 deficient BMDCs compared to WT BMDCs, we could perform Foxp3 staining or use Foxp3-GFP mice as hosts.

Another explanation Aim2 KO BMDCs may be leading to increased disease score is through the chemokine ligand, CCL22. CCL22 production by DCs can recruit Tregs into the tumor microenvironment and type I IFN was reported to inhibit CCL22 production\textsuperscript{365,366}. Since Aim2 KO BMDCs produce increased levels of type I IFN, this may indirectly lead to enhanced disease by suppressing CCL22, reducing Treg migration into the skin of mice. Thus, although induction of vitiligo with Aim2/IFNaR DKO BMDC did not completely reverse the effects of Aim2 KO BMDCs, it did lead to reduced disease scores. Aim2 could also be suppressing disease through suppression of an unknown pathway, or antagonism of another cytosolic DNA sensor.

Other questions that remain unanswered are whether BMDCs are being matured \textit{in vivo} through PRR activation, what are the PRR ligands triggering transferred BMDCs \textit{in vivo}, and where are transferred BMDCs being triggered by PRR ligands \textit{in vivo}. One hypothesis is that BMDCs located in the skin microenvironment are sensing PAMPs, or
DAMPs at the skin site leading to differential priming or activation of effector PMEL CD8 T cells. Another possibility is that sublethal irradiation, which induces cell death and DAMPs, may lead to the activation of PRRs in transferred BMDCs after they pick up debris in host mice. More experiments are required to answer these questions and to better understand how BMDC KO cells can mediate clinical differences in vitiligo development following transfer into WT hosts.
CHAPTER 4
DISCUSSION

4.1: Uncovering signals involved in vitiligo pathogenesis

Susceptibility of vitiligo is multifactorial and includes genetic, environmental, and stochastic factors that drive epidermal depigmentation (reviewed in ch.1)\(^2\). Previous studies reveal that IFN\(\gamma\) signaling and induction of the Th1 chemokine ligands CXCL9 and CXCL0 drive vitiligo progression\(^{70,367}\). However, the role of type I IFNs in driving vitiligo progression is unclear. Current therapies for vitiligo have variable success for patients and are not durable. Although JAK inhibitors, which target the IFN\(\gamma\) pathway have had success for vitiligo patients\(^{368}\)(ongoing clinical trials), these treatments are not FDA approved yet and are also not durable. A better understanding of additional signals driving disease as well as signals driving different stages of disease will lead to the development of better targeted and durable therapies.

4.2: Innate immunity in vitiligo

One theory that suggests a role for innate immunity in the initiation of disease is based on evidence that melanocytes from vitiligo patients have increased cellular stress and have increased sensitivity to exogenous stressors\(^{28-30}\). Cell stress can cause the release of damage associated molecular patterns (DAMPs) that are sensed by PRRs\(^{369}\). Activation of PRRs leads to induction of type I IFNs and other pro-inflammatory cytokines through activation of NF-\(\kappa\)B. Inflammation induced through type I IFNs and other cytokines may drive CD8 T cells into the skin tissue to initiate depigmentation\(^{370}\). Type I IFNs also
induce maturation of dendritic cells\textsuperscript{371}, which are responsible for the cross priming of CD8 T cells, and thus can induce adaptive autoimmune responses.

Another reason to investigate the role of type I IFNs in vitiligo pathogenesis is that type I IFNs induce the transcription of hundreds of ISGs including the chemokine ligands CXCL9 and CXCL10\textsuperscript{121,301}, which we previously identified as driving vitiligo progression and maintenance\textsuperscript{70}. Therefore, IFN\textgreek{g} and type I IFNs may act synergistically to drive disease and we sought to determine whether type I IFNs were involved in vitiligo progression.

### 4.3: Controversy over the role of type I IFNs in vitiligo

Vitiligo is associated with multiple autoimmune diseases and studies reveal that overlap in genetic susceptibility loci such as HLA may contribute to this risk\textsuperscript{17,18}. A few autoimmune diseases associated with vitiligo are type I IFN driven such as Sjogrens syndrome and SLE\textsuperscript{12,16}. One may hypothesize that their association links type I IFN signaling to vitiligo pathogenesis. Although this is a possibility, vitiligo is also highly associated with autoimmune diseases that are not primarily type I IFN driven\textsuperscript{13,299}. Thus, the association with other autoimmune diseases may result from other shared genetic susceptibility loci encoding immune proteins or from environmental triggers.

Only one report has provided evidence of type I IFNs in the skin of vitiligo patients\textsuperscript{77}. The authors performed immunohistochemistry staining of skin tissue from healthy and vitiligo patients using the markers MX1, a type I IFN stimulated gene, and CD123\textsuperscript{+}, often a marker for pDCs\textsuperscript{77}. The study reveals an increase in MX1 and CD123 staining in
progressive vitiligo skin compared to healthy skin. CD123 staining was primarily observed in the dermis, which is not the location of disease, while MX1 staining was observed in the epidermis. Although an increase in staining was observed, this method is not very quantitative, and the differences between healthy and vitiligo patient skin were small. Therefore, further investigation is required to determine whether type I IFNs are involved in disease progression.

Other evidence suggesting a pathogenic role of type I IFN in vitiligo development are case studies that report the development of vitiligo following PEGylated IFNα injection and ribavirin therapy in HCV patients. Some patients developed vitiligo at the injection site, while others showed development of lesions at distant sites. However, this rare phenomenon is not only linked to vitiligo but also to the development of other autoimmune diseases such as type I diabetes and SLE. Linkage of PEGylated IFN therapy with vitiligo, SLE, and Sjogrens also links chronic virus infection and type I IFN to the development of autoimmune disease. Thus, in a context dependent manner type I IFNs may initiate vitiligo during chronic infection, or may stimulate the ISGs CXCL9 and CXCL10 in the skin to recruit potentially autoreactive CD8 T cells to initiate disease.

4.4: Type I IFN is not required for vitiligo progression in mice

Our results reveal that vitiligo development occurs independent of type I IFN signaling in mice using two different mechanisms to activate autoreactive cells. In the first model, VACV infection is used to activate transferred PMEL CD8 T cells to drive disease. In this model, IFNGR KO hosts were protected from disease but IFNaR KO hosts
developed severe disease. Further studies revealed that severe disease was a consequence of persistent VACV infection in IFNaR KO hosts. The role of persistent infection in triggering robust CD8 T cell responses against melanocytes will be discussed in section 5.5. In the second model, bone marrow derived dendritic cells pulsed with PMEL peptide were used to activate transferred PMEL CD8 T cells. Our studies revealed no difference in vitiligo disease score in IFNaR KO hosts compared to WT hosts. Collectively this data suggests that type I IFN signaling does not act as the effector cytokine driving vitiligo progression following activation of autoreactive cells by their cognate antigen. These results confirm that IFNγ signaling and downstream transcription of the chemokine ligands CXCL9 and CXCL10 are necessary and sufficient to drive disease forward. These results support the development of therapies that target the IFNγ signaling pathway, and not type I IFN signaling.

Since we intentionally activate transferred PMEL CD8 T cells using VACV or BMDCs expressing their cognate antigen, these mouse models test the role of type I IFNs during the effector phase of vitiligo development. Therefore, the role of type I IFNs during disease initiation requires further investigation.

Monobenzone is a phenol known to stress melanocytes, that has led to the development of vitiligo in human patients. One study reported that monobenzone induced melanocyte stress led to the release of exosomes and stimulated shedding of melanocyte antigens by inducing autophagy. Release of exosomes and autophagy led to DC activation and induction of a potent T cell response toward melanoma cells in vitro.
This study is the first to identify melanocyte DAMPs in the activation of T cells. We were also interested in this phenomenon and performed studies on mice. Topical monobenzone treatment in mice did not exacerbate vitiligo in SCF hosts (unpublished) and we did not see any aberrant T cell responses in treated mice. Thus, mouse melanocytes may not be susceptible to monobenzone induced stress. To ask questions about disease initiation through melanocyte stress in mice we could try other known cell stressors such as hydrogen peroxide.

Whether melanocyte stress and exosome release occurs in vitiligo patient skin remains unknown. Our lab uses suction blistering to analyze cells and proteins involved in the disease process in the skin. We can blister patients with “confetti vitiligo”, which is clinically active vitiligo skin. Confetti lesions are the closest we can get to the initiation of a depigmented lesion since patients do not come into the clinic until after vitiligo appears on their skin. Thus, capturing the initiation events in vitiligo patients is challenging. We are now using scRNA sequencing and proteomics analyses to better understand these processes. A deeper and closer look at innate immune proteins and gene expression induced during active disease may capture innate immune cells and proteins involved in the initiation of vitiligo. Our scRNA sequencing data has already revealed that macrophages in vitiligo patient skin express higher levels of innate immune signaling components, such as MX1 and TICAM1. Thus, we are hopeful that this data will pinpoint cell types or signaling pathways that are involved in disease initiation.
I believe the best way to determine whether innate immunity or type I IFNs are involved in the initiation of the autoimmune response in vitiligo is to perform in vitro co-culture experiments. Our lab is working hard to develop in vitro 3D skin cultures to model T cell infiltration into the skin and the killing of melanocytes. Using 3D skin, we can apply different stressors, such as Monobenzone or hair dyes, which also contain phenols, to stress human melanocytes. With the addition of autologous PBMCs, purified CD8 T cells or human DCs, we can model initiation events in vitiligo. Comparing melanocytes, T cells and DCs derived from healthy individuals and vitiligo patients will also provide insight into whether melanocytes are intrinsically different and stimulate different T cell responses. We can then measure innate immune activation through gene expression, ELISA, or proteomic studies. 3D skin modeling experiments are technically challenging and require much optimization but will provide incredible insight into the initiation of disease, as well as other aspects of vitiligo progression.

4.5: Type I IFN signaling can lead to enhanced T cell responses

Although vitiligo progressed in mice independent of type I IFN signaling, our results using BMDCs to activate PMEL CD8 T cells provides evidence that innate immune signals including type I IFN production may enhance disease progression and autoreactive T cell priming. The first piece of evidence comes from our studies in which maturation of immature BMDCs following stimulation with a TLR9 ligand led to a significant increase in vitiligo disease score. We did not test the effects of TLR9 ligand stimulation on co-stimulatory molecule expression or cytokine production by BMDCs prior to transfer into WT hosts. However, previous studies have revealed that TLR9
ligand stimulation increases expression of the co-stimulatory molecules CD80 and CD86 and induces expression of the pro-inflammatory cytokines IL-12, which drives Th1 responses, as well as type I IFNs\textsuperscript{336}. We tested whether type I IFN signaling by host cells was important for enhancing disease following TLR9 activation of BMDCs and reveal that type I IFNs do in fact play a role in increasing disease severity. Where transfer of TLR9 stimulated mature BMDCs induced enhanced disease in WT hosts, TLR9 stimulated mature BMDCs transferred into IFNaR KO hosts did not result in significantly enhanced disease. Therefore, type I IFN signaling is partially responsible for enhanced T cell activation and vitiligo development following TLR9 stimulation. The other piece of evidence that suggests type I IFNs play a role in potentiating disease is from our studies revealing that Aim2 KO BMDCs induce significantly increased vitiligo compared to WT BMDCs. We show that this phenotypic effect is not a result of signaling through STING, or through CXCL10, but we do observe reduced scores when Aim2/IFNaR DKO BMDCs are used to induce disease. Although this effect was not statistically significant, it suggests that enhanced disease in hosts induced with Aim2 KO BMDCs is partly a result of autocrine IFNaR signaling.

Our studies answered two important questions. First, we revealed functional evidence that vitiligo progression occurs independently of type I IFN signaling. Second, we provide evidence for the role of type I IFN signaling in enhancing vitiligo development and autoreactive T cell activation by DCs during T cell priming.

\textit{4.6: Persistent or chronic infection may trigger activation of melanocyte specific T cells leading to vitiligo}
Genetic susceptibility to vitiligo is only a piece of the overall risk for vitiligo development. This suggests that environmental factors including infection can be a trigger for disease. Viral triggers of vitiligo remain unclear, but multiple viruses are associated with the development of other autoimmune diseases\(^{374}\). Interestingly, vitiligo development has been associated with two chronic infections, HCV and HIV (discussed in Ch. 1)\(^{44,49}\). Molecular mimicry, bystander activation of autoreactive cells, and epitope spreading are a few mechanisms by which viral infection may trigger autoreactive immune responses\(^{375}\). Molecular mimicry is the idea that T cells specific for foreign antigens can cross react against self-antigens. It is widely accepted that a single T cell can respond to various distinct peptides and that the MHC-peptide complexes can trigger cross-reactivity by the same TCR. This bystander activation of autoreactive cells by DCs can occur following infection with a virus\(^{375}\). Epitope spreading is the idea that priming of the immune response to a specific antigen spreads to initiate activation of cells specific for a different portion of the same protein or to a different protein\(^{375}\). This phenomenon could be beneficial for viral infections and for stimulating immune responses against tumors, however, it is detrimental when it results in activation of self-reactive T cells. It remains plausible that chronic infection with HCV or HIV can lead to the activation of melanocyte-reactive CD8 T cells through mechanisms described above and result in vitiligo development, although this has not been studied. In HCV, cases describing vitiligo development occur following injection with type I IFN. Type I IFNs act to mature DCs and enhance cross-presentation, so this may result in the activation of melanocyte-reactive cells in these patients. In HIV, the presence of the chronic virus is associated
with vitiligo, and treatment reverses vitiligo. Thus, in these cases epitope spreading may result in vitiligo.

Previous studies report that the precursor frequency of T cells specific for a certain pathogen are high and studies estimate that a naïve mouse has around 14,000 T cells specific for VACV$^{376,377}$. We did not investigate the role of bystander CD8 T cell activation in the development of severe vitiligo in IFNaR KO hosts. However, activation of bystander VACV specific CD8 T cells during persistent VACV infection in IFNaR KO hosts may have contributed to disease development.

Interestingly, most viruses associated with autoimmunity are viruses that establish chronic infection. This suggests that immune dysregulation associated with chronic infection leads to autoimmunity. Chronic infection can lead to aberrant activation of PRRs and production of pro-inflammatory cytokines. Pro-inflammatory cytokines not only lead to maturation of DCs, which may present unwanted antigens to T cells, but also can lead to recruitment of cross-reactive T cells into a peripheral tissue, leading to initiation of autoimmunity.

**4.7: Effects of type I IFN signaling on vitiligo is complex**

Collectively our results suggest that vitiligo progresses independent of type I IFN signaling (Chapter 2), but that production of type I IFNs and type I IFN signaling during T cell activation can enhance disease development (Chapter 3). We also provide evidence
that type I IFNs may have significant direct effects on the residence of CD8 T cells and thus impact the maintenance of vitiligo (Appendix A). Following intradermal injection of IFNβ, we show that CD69, an early T cell activation marker and marker of resident memory CD8 T cells was significantly upregulated (3 fold). This may explain why treatment with IFN in HCV patients could trigger autoimmunity. For instance, type I IFNs can induce the recruitment of potentially autoreactive cells into the skin of patients through induction of the chemokine ligands CXCL9 and CXCL10, while also upregulating markers of tissue residence leading to vitiligo development. Our studies in mice reveal that type I IFN is not required for the effector phase of vitiligo, but the role of type I IFN in human vitiligo is still unclear. Interestingly, multiple immune genes identified by GWAS as vitiligo susceptibility genes include innate immune signaling proteins. The identified innate immune TLR adaptor, TICAM1 (TRIF), is associated with vitiligo and is also significantly upregulated in vitiligo lesional skin by scRNA sequencing. We investigated the functional role of TRIF on vitiligo development in mice (Ch. 3). It is unclear whether the association of these innate immune risk alleles such as TICAM1 are linked to type I IFN production, apoptosis, or other signals that are induced downstream. Therefore, examination of human patient samples for the activation of innate immune molecules will be important to determine their role in disease and we have the tools to perform these studies using blister fluid or in vitro 3D skin models. Macrophages and dendritic cells, which are critical innate immune cells triggered by DAMPs and PAMPs are located in the skin dermis. Optimizing blister fluid to capture more of these cell types in our analyses may help identify innate immune signals driving
human disease. Blistering patients earlier during the course of their disease, such as a newly developed lesion, may help catch the role of innate immunity in the initiation of disease.

4.8: Increasing viral vaccine therapy with interferon blockade

As reviewed in Chapter 1, VACV has been used in pre-clinical and clinical studies for the use in the treatment of metastatic melanoma and has increased CTL responses but overall did not have significant effects on overall survival of patients, or only some patients responded to treatment\textsuperscript{279}. VACV vaccines for melanoma are continuing to be developed. Interferon adjuvant therapy for melanoma was also not successful in clinical trials, despite its robust stimulatory role in generating CTL responses in pre-clinical studies, and it was shown to have high toxicity\textsuperscript{262,270,271}. To test the effects of type I IFN signaling during VACV vaccine therapy, we infected WT or IFNaR KO hosts with recombinant VACV in combination with adoptive PMEL CD8 T cell therapy. Treatment led to significant increases in tumor infiltrating PMEL CD8 T cells in IFNaR KO hosts compared to WT hosts. Expression of PD-1 on PMEL CD8 T cells was reduced in IFNaR KO hosts suggesting enhanced cytotoxic ability, and we observed improved tumor regression in IFNaR KO hosts compared with WT hosts. One explanation for this effect is prolonged antigen exposure as a result of persistent VACV in IFNaR KO mice. Live VACV vaccines have been used in the clinic for treatment of melanoma with relatively minimal side effects and were well tolerated\textsuperscript{283}. Thus, blocking type I IFN following administration of VACV vaccine may enhance the persistence of the vector, prolonging the CTL response, and leading to increased or complete responses in melanoma patients.
Another possibility for why blocking type I IFN may be beneficial is that VACV, which has a broad cellular tropism, can also infect tumor cells. Tumor cells often downregulate IFNaR signaling, which allows for their unrestrained growth. However, this is advantageous for VACV, and increases its ability to replicate in tumor cells. Therefore, blockade of type I IFN during VACV therapy may in fact increase its replication in cancer cells, lead to activation of DCs by tumor derived DNA, and increase recruitment of CD8 T cells into the tumor. Finally, type I IFNs can induce immunosuppressive cytokine production such as IL-10, which has been shown to limit VACV spread. This may dampen the CTL response, along with induction of PD-1 and PD-L1, which limit T cell responses. It is of interest to determine whether VACV vaccine and adoptive T cell therapy in combination with PD-1 blockade will have synergistic effects.

Some believe that a major limitation to current recombinant viral vaccines is that it induces strong T cell responses to the viral epitopes and not the recombinant tumor derived antigen epitopes. Dissection of a clinical trial in which an attenuated strain of vaccinia virus expressing melanoma associated antigens revealed that the vaccine generated a cytotoxic T lymphocyte (CTL) response that was dominated by VACV viral specific responses instead of responses specific for the recombinant protein MelanA. Thus the effectiveness of the vaccine to induce T cell responses against the tumor associated antigen MelanA is blunted as a result of robust T cell responses against the viral antigens. An alternative thought it that the generation of T cell responses to the virus may lead to antigen spreading or activation of cross-reactive T cells that actually increase the anti-tumor response. Generating anti-viral CD8 T cells following VACV
vaccine administration would especially be beneficial when an oncolytic or tumor targeting VACV vaccine is used because these anti-viral cells would destroy tumor cells. Because blockade of type I IFN resulted in persistent infection following rVACV vaccine, generation of endogenous anti-viral CD8 T cells may have contributed to the anti-tumor response seen in IFNaR deficient host mice. Multiple studies have shown that immunotherapies are often combined with cyclophosphamide, a lymphodepletion regime that induces type I IFN production. This can lead to the upregulation of MHC class I, which can help aid in the anti-tumor response but also be detrimental if the activation of CTLs leads to activation of cross-reactive cells and subsequent autoimmunity or other off-target toxicities. Therefore, blocking type I IFN following viral vaccine therapy combined with adoptive T cell therapy may minimize off target consequences of the vaccine.

Type I IFN production by tumor derived DCs has been shown to be critical in the priming of anti-tumor CD8 T cells. However, we believe that in the context of administration of a viral vaccine expressing tumor derived antigens, blockade of type I IFN is beneficial. Blockade of type I IFN overcomes the suppressive mechanisms of the tumor microenvironment through reducing PD-1 and PD-L1 upregulation that would normally occur following viral infection through type I IFN. We observed that type I IFN signaling was not required for the priming and the anti-tumor response against melanoma following vaccinia virus vaccine. This may be virus specific, as CD8 T cells do not require type I IFN signaling to mediate the anti-viral response and clearance of VACV. Whether this
would hold true for other viruses in which CD8 T cells do require type I IFN is unclear. More pre-clinical studies need to be performed to determine whether blockade of type I IFN may be beneficial. We are now performing experiments using an anti-IFNaR antibody in combination with VACV vaccine and adoptive T cell therapy. The timing of type I IFN blockade is an important question that needs to be addressed. A major concern in implementing treatment with IFN blockade with a vaccinia virus vaccine is whether blockade of type I IFN would lead to dissemination of VACV and how this may effect immunocompromised individuals.

4.9: Final thoughts and conclusions

Our results provide mechanistic insight into the role of type I IFNs in vitiligo and we reveal that their role in disease is complex. Type I IFNs are not required for the effector phase of disease in mice, however, their production during T cell activation led to significantly enhanced disease severity in mice. Type I IFNs produced during T cell activation may result from the activation of PRRs from DAMPs or pathogen infections and our studies provide evidence for environmental insults or innate immune activation in driving effector T cell responses in vitiligo.

Our results also offer evidence that combined VACV vaccine and adoptive T cell therapy induces robust CTL responses in hosts lacking IFNaR and suggest that IFNaR blockade during treatment has synergistic effects that overcome regulatory mechanisms of tumor suppression. IFNaR blockade may not only increase viral vector persistence but increase
immunogenicity. Because vitiligo and melanoma are strongly linked, our results also reveal that IFNaR blockade, which leads to improved tumor regression may induce vitiligo due to the robust CD8 T cell response. Although this is an adverse effect of treatment, vitiligo development has been shown to have significant benefits for melanoma patients. Melanoma patients have improved survival outcomes. Based on studies in mice, generation of melanocyte/melanoma resident memory T cells following vitiligo development after melanoma immunotherapy may protect against future disease relapse. Therefore, these studies provide critical insights into the use of IFNaR blockade in viral vaccine therapies for melanoma.
CHAPTER 5
MATERIALS AND METHODS

Mice

All mice were maintained in pathogen-free facilities at UMMS, and procedures were
approved by the UMMS Institutional Animal Care and Use Committee and in accordance
with the NIH Guide for the Care and Use of Laboratory Animals. The following mouse
strains were used for these studies and are available from The Jackson Laboratory: PMEL
TCR transgenic mice (stock no. 005023), KRT14-Kitl*4XTG2Bjl (Krt14-Kitl*) mice
(stock no. 009687), IFNgR1-deficient mice (stock no. 003288), CXCL10-deficient mice
(stock no. 006087) and IFNaR1-deficient mice (provided by A. Rothstein, now available
as stock no. 032045, MMRRC). Aim2-deficient mice of C57BL/6 background were
obtained from Genentech. Sting-deficient mice were kindly provided by Dr. D. Stetson
(University of Washington) and backcrossed for more than 10 generations at the UMMS.
Aim2-deficient mice were intercrossed with Sting-deficient, IFNaR-deficient, or Cxcl10-
deficient mice to produce Aim2/Sting DKO, Aim2/IFNaR DKO, and Aim2/Cxcl10 DKO
mice. All mice used for vitiligo studies were on a C57BL/6J background, or a mixed 129-
B6 background that had been backcrossed for at least 10 generations. Age and sex-
matched mice were used, and both male and female mice of all strains were tested to
avoid gender bias.
**Vitiligo induction using vaccinia virus**

Vitiligo was induced as previously described\(^{307,367}\). Briefly, PMEL CD8+ T cells were isolated from the spleens of PMEL TCR transgenic mice through negative selection on microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Purified CD8+ T cells (1×10^6) were injected intravenously into sublethally irradiated (500 rads 1 day before transfer) Krt14-Kitl* hosts. Recipient mice also received i.p. injection of 1×10^6 pfu rVV-hPMEL (N Restifo, NCI, NIH) on the same day of transfer. Scoring of vitiligo progression in mice was done by a blinded investigator, using a point scale based on the estimated depigmentation on the ears, nose, tail, and footpads. Replicate experiments were performed at least twice.

**Generation of BMDCs for peptide-pulsed DC vaccine**

Bone marrow-derived dendritic cells (BMDCs) were generated according to a modified version of a previously described method\(^ {383,384}\). Briefly, bone marrow cells isolated from the femurs and tibia of 7–14-week-old mice were filtered through a 70-µm nylon strainer, red blood cells lysed by ACK lysis buffer (Sigma Aldrich), and cultured in BMDC medium (RPMI-1640 containing 10% FBS, 100 U/mL PS, 2 mM L-glutamine (Gibco), 50 µM 2-mercaptopethanol (Sigma Aldrich), 20 ng/mL GM-CSF (PeproTech), and 10 ng/mL IL-4 (PeproTech)). The BMDC medium was replaced on days 3 and 6. On day 8, non-adherent cells were harvested and BMDC purity was assessed by flow cytometry to ensure staining for markers CD11c, MHC II, CD11b, and CD86. For BMDC vaccination, non-adherent cells were pulsed for 3 h at 37°C with 10 µM of the human gp100\(_{25-33}\) (hgp100) peptide (GenScript) in Opti-MEM media (Gibco) and washed three times with
PBS before their use. For induction of vitiligo in mice, $1.0 \times 10^6$ CD11c$^+$MHC II$^+$ hgp100 pulsed BMDCs were co-injected intravenously into sublethally irradiated (500 rads, 1 day before transfer) WT, IFNaR-deficient or IFNGR-deficient hosts.

**Bone marrow chimeras**

Bone marrow was isolated from femurs and tibias of donor mice. Recipient mice were lethally irradiated (800 rads at least 5h before transplant) and administered $5 \times 10^6$ bone marrow cells intravenously. Mice were left untreated for 8 weeks for reconstitution of bone marrow cells prior to use in the vitiligo model.

**B16F10 Tumor Model**

The murine melanoma B16F10 cell line was obtained from ATCC. B16F10 cells were cultured in DMEM (Corning) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin (PS). B16F10 cells were profiled at passage 4-9 to prevent heterogeneity introduced by long-term culture. B16F10 cells were confirmed negative for Mycoplasma species by RAPIDMAP-21 (Taconic Biosciences) and were maintained at $37^\circ C$ in a humidified atmosphere of 5% CO$_2$.

For use in melanoma immunotherapy experiments, B16F10 melanoma cells ($1.0 \times 10^5$) were resuspended in 100 µL of PBS and implanted subcutaneously into the right flank of 6–12-week-old WT and IFNaR deficient mice. Tumor size was measured in two dimensions by caliper and is expressed as the product of two perpendicular diameters. PMEL CD8 T cells were isolated from the spleens of PMEL TCR transgenic mice through negative selection on microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. After 6-8 days of tumor injection, purified PMELs ($1.0 \times 10^6$)
were transferred by intravenous injection into sublethally irradiated (500 rad, day -1) hosts. On the same day mice were infected with 10^6 pfu of rVV-hPMEL to activate PMEL in vivo.

**Plaque Assay**

The day before infection, CV-1 cells were seeded in a 6 well plate at 1 million cells/well in 2 mLs DMEM + 10% fetal bovine serum (FBS) and incubated at 37°C overnight. The following day, serial dilutions of virus infected tissue supernatants or viral stock control was performed. Culture medium was removed from CV-1 cells and 600 ul-1mL of virus infected tissue supernatant was added to each well and incubated at 37°C for 1 hour.

After incubation, 2 mLs of a heated agarose solution containing 0.6% agarose and 2X MEM+20% FBS mixed at 1:1 was added to each well. Plates were incubated at 37°C for 72 hours. Cells were fixed by adding 1.5 mLs of 10% formaldehyde. Following fixation, excess fixative and agar was removed carefully to avoid scraping the plate underneath. Cells were then stained with 200 ul of 2% Crystal violet diluted in water.

**Flow Cytometry**

Tissues were harvested at the indicated times and processed as previously described. Skin was incubated for 1 hour at 37°C in 5U/mL of Dispase II (Roche, Basel, Switzerland) for tail or 50U/mL Dispase II for ears or footpad skin. Epidermal skin was removed and mechanically dissociated into a single cell suspension for staining. Dermis was incubated in 1mg/mL collagenase IV (1 mg/ml; Roche) and 2 mg/mL DNAse I (Sigma Aldrich, St. Louis, MO) for 1 hour at 37°C before mechanical dissociation. All murine flow cytometry cell suspensions were blocked with 2.4G2 (Bio X Cell) following
Tissue staining with Live Dead Blue (1:1000 Invitrogen) and relevant cell surface antibodies was performed at 4°C for 30 minutes. Subsequently, cells were washed one and then fixed with either Cytofix/Cytoperm solution (BD Biosciences). Fixed cells were either washed twice with Perm/Wash Buffer (BD Biosciences) or incubated for 20 minutes in Perm/Wash before intracellular staining. For intracellular staining, relevant antibodies diluted in Perm/Wash Buffer (BD Biosciences) were applied to fixed cells and incubated for 30 minutes at 4°C. Intracellular staining of Foxp3 was performed using the Foxp3/Transcription Factor Staining kit (eBioscience) after surface staining. For intracellular cytokine staining, tissue supernatants were incubated overnight with plate bound anti-CD3ε (5 µg/ml) and soluble anti-CD28 (2 µg/ml) in the presence of Brefeldin A (Biolegend) for 3-4 hrs before staining with antibodies against cell surface and intracellular markers. Data was collected with the LSR II or Cytek Aurora and analyzed with FlowJo software.

**Blister Induction and Processing**

Suction blisters (1 cm in diameter) were induced on the skin using the Negative Pressure Instrument Model NP-4 (Electronic Diversities, Finksburg MD). Suction chambers were applied to the skin with 10-15 mm Hg of negative pressure and a constant temperature of 40°C. Usually blisters form between 30-60 minutes after initiation of the procedure but this varies depending on the skin site and disease state. After blister formation the interstitial fluid is aspirated through the roof using a 1 ml insulin syringe (28 gauge). The interstitial fluid is pelleted at 300 x g for 10 minutes. The supernatant can be collected and frozen for future analysis by ELISA. The cell pellet can be analyzed by staining with
antibodies for flow cytometry.

**ELISA**

Levels of CCL20 from blister interstitial fluid was quantified per the manufacturer’s instructions (R&D- DY360-05). Optical densities were measured using a Perkins Elmer Envision 2102 multilabel reader and used to calculate concentrations using a 4-parameter logarithmic standard curve using GraphPad Prism.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism software (GraphPad, La Jolla, CA). Dual comparisons were made with unpaired Student’s t test. Groups of three or more were analyzed by analysis of variance with Tukey or Dunnett posttests. P values less than 0.05 were considered significant.
APPENDIX A

ADDITIONAL EFFECTS OF TYPE I INTERFERON ON VITILIGO DEVELOPMENT

A.1: Do neutrophils play a role in vitiligo development?

Neutrophils are granulocytes that are found in the blood and are early responders to infection. Neutrophils have also been shown to play a role in mediating inflammation in many diseases, cancer and autoimmunity. Neutrophils traffic to different parts of the body through chemokine gradients and specifically express the chemokine receptor CXCR2 allowing for migration into tissues secreting the ligands CXCL1 and CXCL2. Type I interferons suppress the expression of these chemokine ligands. One study showed that influenza induced type I IFN production suppressed CXCL1 and CXCL2, leading to improved survival from secondary challenge with bacterial pneumonia. Another study found that endogenous type I IFNs actively suppress the production of CXCL1 and CXCL2, and IFNβ deficient mice had increased neutrophil recruitment into tumors and significantly enhanced CXCL1 and CXCL2 levels. Type I interferon production by epithelial cells can suppress the recruitment of neutrophils through CXCL1 and CXCL2. CXCL2 was significantly upregulated in the skin of mice following HSV-1 infection and type I IFNs ameliorated expression in the ganglia and to a lesser extent in
the skin\textsuperscript{388}. These studies suggest that type I IFN mediated regulation of the chemokines CXCL1 and CXCL2 may be important to suppress skin inflammation.

Expression of these chemokine ligands not only drives migration of neutrophils into tissues, but any CXCR2 expressing cell. It was reported that expression of the human homologue of CXCL1/2 known as IL-8 can induce trans-endothelial migration of human skin homing T cells\textsuperscript{389}. Other papers suggest that IL-8 responsive CD8 T cells have an effector phenotype and enhanced cytotoxic ability\textsuperscript{390,391}. Therefore, expression of these chemokine ligands in skin could also enhance CD8 T cell migration and function in vitiligo.

Nanostring, a technology that involves the direct detection of RNA transcripts was performed on WT and IFNaR KO vitiligo mouse ears to determine differential gene expression at week 7 post vitiligo induction. Preliminary results showed that the chemokine ligands CXCL1 and CXCL2 were significantly upregulated in IFNaR KO vitiligo hosts compared to WT (Figure A.1A-B). This data corroborates previous reports that type I IFN suppresses these chemokine ligands. CXCL1 and CXCL2 were also upregulated in the skin of bone marrow chimera hosts that lacked IFNaR on radioreistant cells (Figure A.1A-B). The upregulation of CXCL1 was confirmed by qPCR at week 7 post vitiligo induction (Figure A.1C) but the upregulation of CXCL2 was not significantly upregulated (Figure A.1D). These results suggest that severe disease
development in IFNaR KO hosts compared to WT may be a result of increased expression of these chemokine ligands in skin.

**Figure A.1:** Chemokine ligands CXCL1 and CXCL2 are upregulated in IFNaR KO hosts compared to WT hosts. A) CXCL1 and (B) CXCL2 transcripts in the ear skin of naïve, WT and IFNaR vitiligo mice performed using Nanostring Technologies. C) CXCL1 and (D) CXCL2 transcripts by qPCR of ear skin of WT and IFNaR KO mice at weeks 3, 5, and 7 post vitiligo induction.

To determine whether neutrophils were recruited to the skin during vitiligo in mice and whether upregulation of these chemokine ligands in IFNaR KO mice led to increased
neutrophil recruitment we performed flow cytometry. Detection of neutrophils in skin was determined by CD11b and Ly6G co-expression. Results revealed that neutrophils are recruited to the skin of vitiligo mice but in small numbers (Figure A.2A-C). Numbers of neutrophils in the epidermis increase from week 3 to week 5 in WT and IFNaR KO hosts (Figure A.2A-C). However, we found no differences in neutrophil numbers in the skin between WT and IFNaR KO hosts (Figure A.2A-C). These results conclude that increased skin CXCL1 and CXCL2 in IFNaR KO hosts did not alter neutrophil migration in vitiligo and are not responsible for enhancing severity of disease.
A.2: Do CXCR2 ligands CXCL1 and CXCL2 affect PMEL CD8 T cells during vitiligo in IFNaR KO hosts?

Because previous studies report the role of CXCL1 and CXCL2 chemokine ligands in the migration and function of effector CD8 T cells\(^{389-391}\), we sought to determine their effect on PMEL CD8 T cells in vitiligo. We hypothesized that increased numbers and function...
of PMEL in IFNaR KO hosts may result from the upregulation of skin CXCL1 and CXCL2 in IFNaR KO hosts. We tested this by inducing vitiligo in WT and IFNaR KO hosts with WT PMEL or CXCR2-deficient PMEL, the receptor for CXCL1 and CXCL2. Results reveal that CXCR2 signaling on PMEL is not required for disease in mice and is not responsible for the enhanced disease seen in IFNaR KO hosts (Figure A.3A). Results did reveal a significant reduction in CXCR2-deficient PMEL numbers in the epidermis and dermis compared to WT PMEL in WT hosts (Figure A.2B-C). These results suggest that CXCL1 and CXCL2 may also mediate recruitment of PMEL into the skin during vitiligo. Competition experiments need to be performed in order to determine whether CXCR2-deficient PMEL are disadvantaged in their recruitment to the skin compared to WT PMEL. However, CXCR2 signaling on PMEL is dispensable for disease development since the PMEL that did navigate into the skin tissue were able to cause disease comparable to WT PMEL (Figure A.3A). These results also confirm that CXCL1 and CXCL2 are not mediating severe disease in IFNaR KO hosts through direct action on PMEL.
**Figure A.3: PMEL do not require CXCR2 signaling to mediate vitiligo in mice.**

A) Vitiligo score at week 7. Normalized PMEL numbers in the epidermis (B) dermis (C) skin draining lymph node (D) and spleen (E).
A.3: Does treatment of mice with exogenous interferon affect vitiligo progression?

Multiple case studies have reported that PEGylated IFNα treatment for HCV patients correlated with development of vitiligo\textsuperscript{48,49}. Since type I IFNs stimulate many adaptive immune responses, it suggests that excess type I IFNs may drive vitiligo pathogenesis. To model this in mice, we injected mice subcutaneously (s.c.) with 10,000 IU of IFNα or IFNβ. To avoid affecting the anti-viral response to VACV, we waited to treat WT hosts until VACV is cleared, which is about 10 days. Systemic delivery of IFNα or IFNβ resulted in comparable vitiligo disease score to untreated hosts (Figure A.4A). PMEL numbers were also comparable between IFN treated and untreated WT hosts (A.4B-E). These results suggest that the presence of excess IFN does not lead to enhanced vitiligo in this model.
Figure A.4: Systemic type I IFN therapy did not induce more severe disease in WT SCF hosts. A) Vitiligo disease score 7 weeks post vitiligo induction. Normalized PMEL CD8 T cell numbers in the epidermis (B), dermis (C), skin draining lymph nodes (D), and spleen (E).
To model PEGylated IFN injection for HCV patients, and to determine whether type I IFNs injected directly into the skin tissue could induce severe disease in hosts predisposed to vitiligo through PMEL transfer, we injected PBS, or IFNβ intradermally into WT hosts. Preliminary results revealed no significant difference in vitiligo score or PMEL number in the skin (Figure A.5A-D). However, epidermal and dermal PMEL numbers were slightly increased following IFNβ treatment and additional studies are required to determine whether local IFN injection enhances PMEL retention or survival in the skin. An interesting observation was that IFNβ significantly induced (3 fold) the expression of CD69 on PMEL CD8 T cells in the epidermis and dermis (Figure A.5E-G). A study reported by Shiow et al. identified the mechanism of CD69 upregulation following type I IFN. They revealed that CD69 was upregulated following type I IFNs and that CD69 negatively regulates S1P1 to promote lymphoid retention. Our previous work shows that resident memory CD8 T cells, that co-express CD69 and CD103 are critical in promoting vitiligo maintenance. Therefore, although preliminary results reveal that IFNβ treatment didn’t enhance development of vitiligo during the effector phase of disease, type I IFN treatment may drive vitiligo maintenance through the upregulation of CD69 and retention of melanocyte-reactive CD8 T cells in the skin. Increased expression of CD69 may also explain the increased PMEL numbers in the skin tissue in IFNβ treated hosts. Treatment of vitiligo hosts exhibiting stable disease with intradermal IFNβ would help determine the role of IFN during the maintenance of vitiligo.
**Figure A.5: Local IFNβ injection into skin leads to robust induction of CD69 on CD8<sup>+</sup> PMEL.** A) Vitiligo score of WT and IFNβ treated mice at 7 weeks. Normalized PMEL CD8 T cell number in the epidermis (B) dermis (C) and skin draining lymph node (D). E) Representative flow cytometry plots of CD69 and CD103 expression on skin PMEL from WT and IFNβ treated mice. Representative histograms (F) and mean fluorescent intensity (G) of CD69 on skin PMEL from WT and IFNβ treated mice.
A.4: Is regulatory T cell recruitment different in WT and IFNaR KO hosts?

Regulatory T cells are a subset of T cells that express the transcription factor Foxp3 and are responsible for maintaining peripheral tolerance to self. Studies suggest that Tregs play a role in suppressing melanocyte-specific CD8 T cells responses in vitiligo\textsuperscript{360,361}. The ratio of CD4 to CD8 T cells in the blood are reduced in vitiligo patients compared to healthy controls\textsuperscript{361}. Evidence also supports that Treg suppression of CD8 T cell proliferation and cytolytic function is significantly reduced in vitiligo patients compared to healthy individuals\textsuperscript{360}. In mouse models of vitiligo, the overexpression of CCL22 in the skin, a chemokine attractant for Tregs, led to enhanced Treg number in the skin and significantly reduced depigmentation\textsuperscript{365}. Another study, which adoptively transferred Tregs into host mice and treated hosts with rapamycin, led to lasting remission of vitiligo\textsuperscript{393}. Unpublished studies in our lab showed that Tregs are critical for the suppression of vitiligo in our mouse model (Kingsley Essien Thesis). These studies suggest that an imbalance of Treg number or Treg dysfunction may lead to vitiligo development and that Tregs play a critical role in suppressing melanocyte-specific CD8 T cells. Because IFNaR- deficient mice developed such severe disease and Tregs suppress disease in our mouse model, we asked whether there was a difference in Treg numbers compared to WT hosts. We analyzed Treg numbers by flow cytometry using either a Foxp3-GFP reporter mice or by intracellular staining of Foxp3. Our results reveal that Tregs are not significantly reduced in the skin of IFNaR KO hosts compared to WT hosts following induction of vitiligo using VACV at either week 3 or 5 (Figure A.6A,C,E,G). Numbers of PMEL CD8 T cells are significantly increased in IFNaR KO hosts as
previously described in chapter 2 (Figure A.6B and F). At 3 weeks post vitiligo induction Treg numbers are slightly increased in IFNaR KO hosts compared to WT (Figure A.6C). Thus, Treg recruitment to the skin in IFNaR deficient hosts is similar to WT hosts. However, as a result of the increased PMEL numbers in IFNaR deficient hosts compared to WT hosts, the Treg/PMEL ratio is significantly lower in IFNaR deficient hosts at weeks 3 and 5 post induction (Figure A.6D and H).

Figure A.6: Tregs are recruited into the epidermis at equal numbers in IFNaR deficient hosts compared to WT hosts. A) Representative flow cytometry plots of epidermal Tregs by intracellular staining with Foxp3 at week 3 post vitiligo induction. B) Normalized PMEL numbers in the skin epidermis at week 3. C) Normalized Treg numbers in the epidermis at week 3. D) Treg/PMEL ratio in the skin epidermis at week 3. E) Representative flow cytometry plots of epidermal Tregs using Foxp3-GFP mice at week 5 post vitiligo induction. F) Normalized PMEL numbers in the skin epidermis at week 5. G) Normalized Treg numbers in the epidermis at week 5. H) Treg/PMEL ratio in the skin epidermis at week 5.
PREFACE TO APPENDIX B

Appendix B contains original text, which represents the unpublished work of Rebecca L. Riding and Mitchell Thorn (Pfizer Inc.) and is currently prepared for submission. Rebecca L. Riding performed the experiments, analyzed data, and co-wrote and critically reviewed the manuscript with Mitchell Thorn. Dr. James Strassner, Dr. Zainab Abbas, Dr. Maggi Ahmed, and Evangeline Kim performed blistering experiments. Wei Li designed experiments. Dr. John E. Harris designed experiments and critically reviewed the work.
APPENDIX B

STABILIZATION AND IMMUNOPHENOTYPING OF T CELLS ISOLATED FROM BLISTER FLUID OF PSORIATIC LESIONS

B.1: Stabilization of blister fluid cells using TransFix preserves immunophenotyping markers.

Psoriasis is a chronic auto-inflammatory disease that primarily affects the skin and joints and is mediated by T cells\textsuperscript{394,395}. Pathogenesis is multifactorial but is marked by infiltration of Th17 cells that drive inflammation at the skin site and studies show that IFN\textsubscript{γ} may promote this recruitment\textsuperscript{396,397}. Although advancements in targeted psoriasis therapies are available, the tools to assess treatment efficacy in a clinical trial setting are limited. Clinical trials of psoriasis therapies require information-rich assays that can assess multiple parameters relevant to drug activity, including PK/PD, target engagement, efficacy, inflammation status of the affected tissue and cellular composition of healthy and lesional areas of skin. Flow cytometry is a powerful tool that can simultaneously assess the sizes of T cell subsets and the expression of biomarkers associated with psoriasis. In this study we sought to assess the feasibility of evaluating T cells isolated from blister fluid of psoriatic skin lesions by flow cytometry. Blistering is performed by inducing negative pressure and heat to the skin for a brief period of time. The interstitial fluid that collects within each blister contains immune cells and proteins that may be important during the local inflammatory response\textsuperscript{53}. Blister induction and immune-phenotyping of cells present in blister fluid is not only a valuable basic science tool, but also has potential to be an important tool for investigating immune biomarkers for
dermatological diseases because blistering is rapid, painless, and non-scaring. The link between elevated Th17 cells present in the skin and development of psoriasis further suggests that blister fluid cell analysis may be an important component of clinical trials that evaluate psoriasis therapies\textsuperscript{397}.

Sample stability including cell viability and biomarker stability are important considerations for clinical assays when shipping from the clinical to analytical site is required. This is a particular challenge for flow cytometry assays because, typically, samples collected at the clinical site are shipped to the analytical laboratory, necessitating sample stability for 24 hours or longer. In this study we observed that the frequency of viable blister fluid cells decreased significantly from 64% at 2hrs post collection to 43% at 6 hrs post collection (Figure B.1A). Therefore, we tested a commercial cell stabilizer, TransFix, to preserve the sample while retaining the cellular markers used in our T cell immunophenotyping panel. The panel includes markers for CD4 and CD8 cells as well as markers for the chemokine receptors expressed by Th1 and Th17 cells. We performed flow cytometry analyses of fresh PBMC and blister fluid cells compared to cells preserved in TransFix for 24 hrs. The C-C motif chemokine receptor 6 (CCR6) is highly expressed on Th17 cells that are abundant in psoriasis lesion infiltrates and play an important role in psoriasis pathogenesis\textsuperscript{398}. Therefore, detection of CCR6, CCR4 and the chemokine receptor CXCR3, expressed by Th1 cells were analyzed. Comparison between CD4 and CD8 T cells from fresh and TransFix stabilized blister fluid is shown in Figure B.1. Both CD4 and CD8 T cell subsets are abundant in psoriatic lesions. While some
patients had equal ratios of CD4 and CD8 T cells in the blister fluid, others had predominantly CD4 T cells (Figure B.1B-D). CD4 and CD8 T cells within psoriasis patient blister fluid were predominantly Th17 by expression of CCR6, but we also detected CD4 and CD8 T cells expressing phenotypic markers for Th1-17 cells by co-expression of CCR6 and CXCR3 (Figure B.1C-D). Importantly, the frequency and cell number of these populations was preserved using TransFix stabilizer (Figure B.1B-D).
Figure B.1: TransFix stabilization of blister fluid cells in psoriasis patient lesions. A) Cell viability of blister fluid cells fresh or 6 hrs post collection. B) Representative flow cytometry plots of fresh and TransFix blister fluid samples showing CD4 and CD8 T cells and chemokine expression. C) Frequency of the cell population from the parent gate in blister fluid samples. D) Absolute cell number of cell population within blister fluid samples
**B.2: Analysis of blister fluid from psoriasis patients may be used for evaluation of clinical responses to treatment**

It is now clear that patient blood is a poor indicator of disease state in peripheral tissues such as the skin. We performed paired immunophenotyping analyses of psoriasis patient PBMCs and blister fluid cells. We find significant differences in the immunophenotype of CD4\(^+\) and CD8\(^+\) T cells in psoriasis patient blood compared to blister fluid (Figure B.2A-D). The majority of CD4\(^+\) and CD8\(^+\) T cells in psoriasis patient skin are CD45RA- and represent mature memory T cells. In the blood, the majority of CD4\(^+\) T are neither Th17, Th1-17 or Th1, whereas in the skin, CD4\(^+\) T cells are predominantly Th17 marked by expression of CCR6 (Figure B.2A-B). We also detect a population of CD4\(^+\) T cells in blister fluid that express both CCR6 and CXCR3, a chemokine receptor expressed by Th1 cells and refer to these cells as Th1-17. These cells like Th17 are highly enriched in psoriasis skin compared to the blood (Figure B.2A-B). Another finding shared by the majority of psoriasis patients tested is that while CD4\(^+\) CCR6/CCR4 co-expression was modest in peripheral blood, the majority of CD4\(^+\) T cells in the skin co-express CCR6 and CCR4 (Figure B.2A-B). CD8\(^+\) T cells in the skin of psoriatic lesions also adapt a Th17 phenotype including a subset of Th1-Th17 cells present, which are enriched in psoriasis skin compared to blood (Figure B.2C-D). Similar to CD4\(^+\) T cells, CD8\(^+\) T cells in the blood are not CCR6/CCR4 double positive but the majority of CD8\(^+\) T cells in lesional skin do co-express CCR6 and CCR4 (Figure B.2C-D).
To determine whether CCR6 ligand, CCL20, is increased in psoriasis patient skin compared to the skin of healthy individuals, we performed ELISA on the blister fluid supernatant. Results show an increase in CCL20 in psoriasis lesional skin compared to healthy control skin, revealing active inflammation at the skin site (Figure 2E).
Our results reveal that blister fluid sample stabilization using TransFix preserves T cell infiltrates for clinical analyses. Our results also reveal a significant enrichment of Th17 and Th1-17 cells in psoriasis lesions, which are not detected in the blood of patients.

**Figure B.2: Elevated Th17 and CCR4-CCR6 co-expressing CD4 and CD8 T cells in psoriatic lesions compared to blood.** A) Representative flow cytometry plots comparing the phenotype of CD4 and CD8 T cells in PBMCs and lesional blister fluid from psoriasis patients. B) Quantification of the frequencies of CD4 T cell subsets in the blood and blister fluid. C) Quantification of the frequencies of CD8 T cell subsets in PBMCs and lesional blister fluid. D) ELISA of CCL20 levels in the blister fluid of healthy individuals compared to lesional psoriasis skin.
results further suggest that the infiltration of Th17 and Th1-17 cells in psoriasis lesional skin can be used to predict successful treatment responses during clinical trials and that analysis of blister fluid of psoriatic skin may provide important clues into psoriasis pathogenesis.

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