2018-12-08

Understanding regulatory factors in the skin during vitiligo

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A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 8, 2018

PhD Program in Biomedical Sciences

Interdisciplinary Graduate Program

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This work was undertaken in the Graduate School of Biomedical Sciences

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February 11th 2019

ACKNOWLEDGMENTS

I must thank Dr. John Harris, my thesis mentor for his guidance and support throughout my thesis. Dr. Harris has taught me how to design experiments and to appreciate the larger implications of my data. I could not have succeeded without Dr. Harris' ability to creatively approach obstacles and I am grateful for his invaluable direction while I navigated the many twists and turns that I encountered during my graduate career. I am also grateful to my committee members for their encouragement and direction.

I cannot thank my family enough for their immeasurable support. Mom, thank you for supporting me all this time. There have been so many, exams, papers and late nights and you have encouraged me through all of them. Dad, thank you for teaching me the virtue of hard work. Your example has inspired me to persevere through the difficult times. To my siblings Charles, Clara, Gloria and Richard thank you for the joy you have brought me all these years. I have truly been blessed to have you all as role models and friends.

Finally, I want to thank my wife Nadeschka. You have been my rock for the past 6 years of our marriage and your constant encouragement is the driving force behind all my success. I thank God for putting you in my life, we made it.

ABSTRACT

Vitiligo is an autoimmune disease of the skin characterized by epidermal depigmentation that results from CD8+ T cell-mediated destruction of pigment producing melanocytes. Vitiligo affects up to 1% of the population and current treatments are moderately effective at facilitating repigmentation by suppressing cutaneous autoimmune inflammation to promote melanocyte regeneration. In order to cause disease, CD8+ T cells must overwhelm the mechanisms of peripheral tolerance in the skin and if we understand the suppressive mechanisms that are compromised during vitiligo, we can potentially use this information to improve existing treatments or engineer novel interventions. Therefore, my goal is to characterize the regulatory factors in the skin that suppress depigmentation during vitiligo. Our lab has developed a mouse model of vitiligo that accurately reflects human disease and I used this model to demonstrate that regulatory T cells suppress CD8+ T cell-mediated depigmentation and interact with CD8+ T cells in the skin during vitiligo. In this model of disease, I investigated the molecules involved in regulatory T cell function and observed that the chemokine receptors CCR5 and CCR6 play different roles in regulatory T cell suppression. While CCR6 facilitates regulatory T cell migration to the skin, CCR5 is dispensable for migration but required for optimal regulatory T cell function. Additionally, I used our mouse model to demonstrate that Langerhans cells suppress the incidence of disease during vitiligo. Taken together the results from these studies provide novel insights into the mechanisms of suppression during vitiligo.

LIST OF FIGURES

LIST OF ABBREVIATIONS USED COMMONLY IN THIS WORK

4-TBP: Tertiary butyl phenol

AA: Alopecia areata

AICD: Activation-induced cell death

APCs: Antigen presenting cells

B6: C57BL/6 mice

BCL-2: B-cell lymphoma 2

BrdU: Bromodeoxyuridine

C. Albicans: Candida albicans

cAMP Cyclic adenosine monophosphate ()

CCL17: Chemokine (C-C motif) ligand 17 (TARC)

CCL19: Chemokine (C-C motif) ligand 19

CCL20: Chemokine (C-C motif) ligand 20 (MIP-3α – Macrophage inflammatory protein 3 alpha; LARC - Liver and activation-regulated chemokine)

CCL21: Chemokine (C-C motif) ligand 21

CCL22: Chemokine (C-C motif) ligand 22 (Macrophage-derived chemokine)

CCL3: Chemokine (C-C motif) ligand 3

CCL4: Chemokine (C-C motif) ligand 4 (MIP-1α – Macrophage inflammatory protein 1 alpha)

CCL5: Chemokine (C-C motif) ligand 5 (RANTES)

CCR10: C-C chemokine receptor type 9 (GPR-2)

CCR4: C-C chemokine receptor type 4 (CD194)

CCR5: C-C chemokine receptor type 5 (CD195)

CCR6: C-C chemokine receptor type 6 (CD196)

CCR7: C-C chemokine receptor type 7 (CD197)

CCR8: C-C chemokine receptor type 8 (CD198)

CCR9: C-C chemokine receptor type 9 (CD199)

CD103: Cluster of Differentiation 103 (ITGAE – Integrin, alpha E)

CD207: Cluster of Differentiation 207 (Langerin)

CD25: Cluster of Differentiation 25 (IL2RA – Interleukin-2 receptor alpha chain)

CD28: Cluster of Differentiation 28

CD3: Cluster of Differentiation 3

CD4: Cluster of Differentiation 4

CD40: Cluster of Differentiation 40

CD45: Cluster of Differentiation 45 (PTPRC – Protein tyrosine phosphatase receptor type C)

CD62L: Cluster of Differentiation 62L (L-selectin)

CD8: Cluster of Differentiation 8

CD80: Clus**t**er of Differentiation 80 (B7-1)

CD86: Cluster of Differentiation 86 (B7-2)

CHS: Contact hypersensitivity

c-Kit: Stem cell growth factor

CLA: Cutaneous lymphocyte antigen

Cre: Cre recombinase

CTLA4: Cytotoxic T-lymphocyte-associated protein 4 (CD152)

CXCL10: C-X-C motif chemokine 10 (IP-10 – Interferon gamma-induced protein)

CXCL11: C-X-C motif chemokine 11

CXCL9: C-X-C motif chemokine 9 (MIG – Monokine induced by gamma interferon)

CXCR3: C-X-C motif chemokine receptor 3

DC: Dendritic cell

dDC: Dermal dendritic cell

DTR: Diphtheria toxin receptor

DTX: Diphtheria toxin

EAE: Experimental autoimmune encephalomyelitis

E-Selectin: CD62E (ELAM-1- endothelial-leukocyte adhesion molecule-1)

FACS: Fluorescence-activated cell sorting (Flow cytometry),

FLOX: Flanked by LoxP

FoxP3: Forkhead box P3

FucT7: α-1,3-fucosyltransferase VII ()

GAGs: Glycosaminoglycans

GFP: Green fluorescent protein

gp100: Glycoprotein 100 (PMEL – Melanocyte protein)

GVHD: Graft-versus-host-disease

GWAS: Genome wide association studies

Het: Heterozygous

HLA: Human Leukocyte Antigen

Homo: Homozygous

HSV-1: Herpes Simplex Virus 1

huLang-DTA: Human Langerin diphtheria toxin A

ICOS: Inducible T-cell costimulator (CD278)

IFNγ: Interferon-gamma

IFNγR: Interferon-gamma receptor

IL10: Interleukin 10

IL15: Interleukin 15

IL2: Interleukin 2

IL35: Interleukin 35

K14: Keratin-14

Ki67: antigen Ki-67

Krt14-Kitl: Keratin-14-Kit ligand

LHC: Langerhans cells

LHC-KO: huLangerin-DTA

LN: Lymph node

mAb: Monoclonal antibody

MART1: Melanoma antigen recognized by T cells-1

MBEH: Monobenzyl ether of hydroquinone

MelanA: Melanoma antigen

MFI: Median fluorescence intensity

MHC: Major histocompatibility complex

MITF: Microphthalmia-associated transcription factor

NLR: NOD-like receptors

OVA: Ovalbumin

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PD-L1: Programmed death-ligand 1

PFU: Plaque forming units

PPD: Para-phenylenediamine

preTCR: Pre T Cell Receptor

Rag$^{-/-}$: Recombination activating gene KO

REX3: Reporting **E**xpression of C**X**CR**3** ligands

RFP: Red fluorescent protein

RNA-seq: RNA sequencing

S1P: Sphingosine-1-phosphate

SDLN: Skin draining lymphnode

SLE: Systemic lupus erythematosus

SLO: Secondary lymphoid organ

SNP: Single nucleotide polymorphism

TCR: T cell receptor

Teffs: effector T cells

Tem: T effector memory cell

TGF-β1: Transforming growth factor beta 1

Thy1.1: CD90.1 – Cluster of Differentiation 90.1

Thy1.2: CD90.2 – Cluster of Differentiation 90.2

TLR: Toll-like receptors

TNFα: Tumor necrosis factor-α

Tregs: Regulatory T cells

TRP1: Tyrosinase-related protein 1

TRP2: Tyrosinase-related protein 2

Tyr369: human Tyrosine epitope

UVB: Narrow band ultraviolet B

WT: Wildtype mice

α4β7: integrin $\alpha_4\beta_7$

αCD4: GK1.5 CD4 depleting antibody

αDEC-205: OVA chemically linked to an antibody against CD205

CHAPTER I: INTRODUCTION

1.1 Vitiligo is an autoimmune disease characterized by epidermal depigmentation.

Vitiligo is an autoimmune disease that affects up to 1% of the population and is characterized by patchy epidermal depigmentation resulting from the loss of pigment-producing melanocytes in the skin [1, 2]. Two forms of vitiligo exist with distinct clinical features: non-segmental, or generalized, vitiligo and segmental vitiligo [1, 2]. Generalized vitiligo, characterized by widespread depigmentation, affects the majority of patients, while segmental vitiligo is less common (~5%) and occurs in a unilateral distribution that does not cross the midline [1-3]. Vitiligo equally affects men and women; 50% of patients exhibit depigmentation before the age of 20 and 80% before 30. Depigmentation can affect any part of the skin, but depigmentation most frequently presents on the face, hands, feet, and genitals [1, 2]. The risk for developing other autoimmune disease like thyroiditis, type 1 diabetes, pernicious anemia, Addison's disease, or alopecia areata is increased for vitiligo patients and their family members [1, 2]. Interestingly, while lesional skin lacks epidermal melanocytes, melanocytes in hair follicles located within depigmented skin are often spared [4]. This is likely a result of the immune privilege of the hair follicle [5] and successful treatments induce epidermal repigmentation that spreads from melanocyte stem cell reservoirs that persist protected from autoimmunity in hair follicles [4].

1.2 Treatments for vitiligo are effective but can be improved

No cure for vitiligo exists but there are a number of treatment options. Narrow band ultraviolet B (UVB) radiation is one of the preferred treatments for patients. Both adults and children with vitiligo positively respond to this treatment strategy and studies report that narrow

band UVB therapy resulted in more than 75% repigmentation in a majority of adults and children who received treatment [6, 7]. This treatment requires two treatments per week for at least 9 months to achieve maximal repigmentation and this may present a challenge, even for patients who are fortunate enough to live near a treatment center [1]. Hair-bearing skin responds well to treatment while other locations do not, and relapse frequently occurs within a year of discontinuing treatment [8].

Topicals like corticosteroids and calcineurin inhibitors can also be used to treat depigmentation. A majority of patients treated with potent corticosteroids exhibited more than 75% repigmentation in the treated areas, and these drugs are most useful for treating small, localized areas of depigmentaion [9]. This treatment does have adverse side effects, including epidermal atrophy, steroid-induced acne, and rosacea, so prolonged use should be avoided [10]. Calcineurin inhibitors can be used for sensitive areas of skin like the face, and have been shown to be effective in multiple double blind trials [11-14] but may need to be combined with UVB therapy to be most effective [15].

Vitiligo patients can also pursue surgical options if other treatment methods fail. Minigrafting [16], which encompasses transplantation of autologous epidermal cell suspensions [17], or application of ultrathin epidermal grafts [18] can be used therapeutically and, in one study, patients with stable generalized vitiligo exhibited extensive repigmentation of an area treated with a transplanted epidermal cell suspensions as well as UVB light [19]. Surgery is expensive, invasive, and only effective in a small subset of patients with highly stable disease rendering this option unreasonable for many patients. While the treatments available for vitiligo patients can promote repigmentation, new durable treatments that are minimally invasive will

improve the quality of life of afflicted patients. In order to design novel interventions or improve existing ones, it is crucial to understand the factors driving pathogenesis.

1.3: Evidence that genetics contribute to vitiligo pathogenesis

The pathogenesis of vitiligo has been extensively studied and a growing body of evidence suggests that a myriad of factors synergize to initiate and maintain depigmentation. Vitiligo patients are likely genetically predisposed to disease. While the general population has a 1% chance of developing vitiligo, first degree relatives of vitiligo patients have a 6% chance of developing disease and identical twins have a 23% chance of developing disease [20]. This observation strongly supports a role for genetics in disease pathogenesis. Further supporting this, linkage and association studies of vitiligo and HLA suggest that the HLA region is involved in the genetic susceptibility to vitiligo [21] and Liu et al. conducted an analysis that uncovered a strong association of vitiligo with HLA-A2 [22]. These initial observations hinted that genetics contribute to disease and future genome wide association studies (GWAS) reaffirmed these findings. Jin et al. in particular performed GWASs of vitiligo and found up to 60 distinct genetic loci associated with disease, with many of these regions encoding factors involved in melanocyte function, immunoregulation, adaptive or innate immunity [23-26]. These results demonstrate that vitiligo patients may be genetically predisposed to disease and implicate pathways involved in pathogenesis.

1.4: Evidence that intrinsic defects in melanocytes contribute to vitiligo pathogenesis

Intrinsic defects in melanocytes may contribute to vitiligo pathogenesis. Separate studies comparing the ability of melanocytes from vitiligo patients or healthy patients to grow in in vitro

studies reported somewhat conflicting results. Puri et al. cultured melanocytes isolated from the skin of adult patients and healthy controls and while melanocytes from healthy patients readily grew in culture, melanocytes from non-lesional vitiligo skin did not begin to grow until 8 to 11 days post culture [27]. After this initial lag, melanocytes from vitiligo and healthy skin grew at comparable rates but only melanocytes from healthy skin could be passaged multiple times [27]. These observations suggest that melanocytes in vitiligo skin, even those from uninvolved skin, may have some inherent growth defect. However, in contrast to these findings, Medrano and Nordlund et al. were able to establish long lasting cultures with melanocytes from vitiligo skin [28]. In this study melanocytes obtained from adult non-lesional vitiligo skin exhibited no lag before growth and were passaged multiple times suggesting melanocytes in vitiligo skin have no defects that inhibit their ability to grow in culture.

Several studies note structural abnormalities in vitiliginous melanocytes compared to normal melanocytes. When Boissy et al. used electron microscopy to examine cultured melanocytes from non-lesional skin, they observed that the rough endoplasmic reticulum is morphologically different in melanocytes during vitiligo [29]. In light of these observations they suggested that melanocytes in normally pigmented skin have morphological aberrations that render them susceptible to disease [29]. Immunohistochemical analysis comparing healthy skin to vitiligo skin revealed that melanocytes express less c-kit in the skin during vitiligo [30]. Since c-kit signaling plays a key role in melanocyte proliferation, migration, and survival [31, 32], this apparent reduction in c-kit expression may result in reduced melanocyte longevity during disease.

Melanocytes also exhibit functional defects during vitiligo and these may result from the structural defects that have been reported. Using electron microscopy, Tobin et al. observed

extracellular melanin granules in the interstitial spaces of non-lesional basal epidermis and concluded that melanocytes inefficiently transfer melanosomes in vitiligo skin [33]. Together these studies demonstrate that melanocytes in the skin during vitiligo exhibit various structural and functional defects that could lead to increased susceptibility to immune destruction. It is important to note that many of the previously detailed observations were made using more subjective techniques, like immunohistochemistry or electron microscopy, that are susceptible to sampling error and more work must be done to elucidate the full contribution of melanocyte defects to disease pathogenesis.

1.5: Evidence that exposure to environmental factors contributes to disease pathogenesis

Skin exposed to specific chemicals can exhibit depigmentation characteristic of vitiligo. The first example of this occurred when factory workers who wore gloves that contained monobenzyl ether of hydroquinone (MBEH) developed what was described as “occupational leukoderma” [34]. A significant proportion of workers developed patchy depigmentation on the skin that was exposed to the gloves and a number of them developed depigmented lesions on distal skin that did not contact gloves as well [34]. Oliver et al. performed patch tests on affected workers with ingredients used in the gloves and observed that patches containing MBEH ultimately induced depigmentation [34]. Later studies, thoroughly reviewed by Harris [35], confirmed that MBEH and other chemicals like 4-TBP and para-phenylenediamine (PPD) found in some commonly used products like hair dye, can indeed induce depigmentation [36, 37]. To explain how chemicals induce vitiligo a number of groups presented evidence linking increased melanocyte susceptibility to cellular stress to pathogenesis during vitiligo.

1.6 Evidence that Cellular stress contributes to disease pathogenesis

To maintain viability cells have to deal with a number of environmental or intracellular stressors that perturb homeostasis. These stressors can include DNA-damaging UV light, the accumulation of misfolded proteins during protein synthesis, heat shock, or chemical toxins that activate cellular sensors to initiate a cellular stress response [38]. During this response, multiple intracellular signaling pathways converge to ultimately manage stress and repair damage or mediate regulated cell death [38]. Epidermal melanocytes are particularly vulnerable to cellular stress due to their constant melanin production and exposure to UV light but a number of published studies demonstrated that melanocytes from vitiligo patients manage stress less efficiently than those from healthy controls. Maresca et al. observed that melanocytes cultured from non-lesional vitiligo skin were more susceptible to the toxic effects of an oxidizing agent than melanocytes from healthy donors [39] and another study similarly demonstrated that melanocytes from vitiligo patients exhibit increased susceptibility to UVB induced oxidative stress [40]. This increased susceptibility to stress could lead to melanocyte death and subsequent depigmentation when melanocytes undergo protein synthesis or encounter exogenous stressors like chemicals or UV light. In addition, a number of groups report high concentrations of H_2O_2 in the epidermis of vitiligo skin along with reduced levels of catalase an enzyme that protect cells from oxidative damage and both of these observations indicate increased cellular stress in the skin during vitiligo [41-46]. Taken together, these findings implicate a contribution of stress to disease pathogenesis.

In some cases MBEH induced depigmentation at sites distant from that point of initial contact [34, 47, 48]. This suggests that exogenous stressors can instigate a whole body response against melanocytes to promote depigmentation and their toxicity is not limited to melanocytes

at the site of contact. To explain this phenomenon, Harris suggests that during vitiligo melanocytes that poorly manage stress respond to environmental factors by releasing self-antigen. Antigen presenting cells (APCs) phagocytize melanocyte antigens while simultaneously receiving a danger signal that primes the APC to initiate an adaptive immune response against melanocytes [49]. Melanocytes cultured with chemicals previously shown to promote depigmentation, like 4tertiary butyl phenol (4-TBP) or MBEH, activated dendritic cells (DCs) to either kill melanocytes or induce cytotoxic melanocyte-targeting T cells [50, 51]. These observations support the hypothesis that environment factors can promote depigmentation by initiating an immune response against melanocytes. While intrinsic melanocyte defects and environmental factors likely contribute to the initiation of vitiligo, a growing body of evidence supports a clear role for autoimmunity in driving depigmentation during disease.

1.7: Evidence that humoral immunity contributes to disease pathogenesis

Early studies detected autoantibodies in the serum of vitiligo patients and these results were among the first to suggest a role for autoimmunity in disease pathogenesis. Harning et al. compared serum from patients with active vitiligo, inactive vitiligo, and no disease, and only observed antibodies to pigmented melanoma cells in the serum from patients with active disease [52]. Other studies reaffirmed this finding as they also identified melanocyte-specific autoantibodies using varying strategies [53, 54] but few of these studies identified antibodies targeted against surface expressed proteins [55, 56]. These findings suggest autoantibodies play a role in vitiligo pathogenesis but while CD4 and CD8 T cells infiltrate the skin during vitiligo, B cells are absent in lesional skin and thus it is possible other immune mechanisms contribute to melanocyte loss [57].

1.8: Melanocyte specific CD8+ T cells mediate epidermal depigmentation

Convincing evidence exists that CD8+ T cells drive depigmentation during vitiligo. CD8+ T cells are lymphocytes that express the CD8 co-receptor along with a TCR that recognizes pathogen peptides presented on MHC I molecules on infected cells or on cross-presenting APCs. During a normal immune response, activated CD8+ T cells are dispatched to eliminate infected cells that display pathogenic antigens on surface expressed MHC I molecules [58]. Ogg et al. used HLA-A2 restricted tetramers to demonstrate that high frequencies of CD8 T cells in PBMCs from vitiligo patients exhibit specificity against MelanA peptides and express CLA, a receptor that facilitates skin homing [59]. Ogg et al. also observed that MelanA specific CD8+ T cells from vitiligo patient PBMCs were capable of lysing peptide-pulsed target cells and melanoma cells in vitro suggesting that the circulating T cells that exist in vitiligo patients are capable of destroying melanocytes [59]. Lang et al. used an ELISPOT assay to demonstrate that CD8+ T cells in the PBMCs of vitiligo patients recognize melanocyte antigens in actively progressing vitiligo [60]. PBMCs from patients reacted strongly against MelanA/MART1 peptides as well as peptides derived from gp100 and tyrosinase proteins [60]. Additionally CD8+ T cells in PBMCs isolated from vitiligo patients produced IFNγ in response to stimulation with peptides from MelanA/MART1 and tyrosinase [60]. This data suggests that MelanA/MART1, gp100, and tyrosinase represent important antigenic targets for an autoimmune T cell response in vitiligo patients [60]. The studies performed by Ogg et al. and Lang et al. reported less melanocyte reactivity in PBMCs from controls without disease [59, 60]. In the few instances where control PBMCs exhibited reactivity against melanocyte antigens, T cells did not express the skin homing receptor CLA [59, 60].

The aforementioned studies established an increased presence of melanocyte specific CD8 T cells in the peripheral blood and subsequent studies discovered the presence of CD8+ T cells in lesional skin. Activated T cells were among the lymphocytes infiltrating perilesional skin when histology was performed on biopsies from vitiligo patients [61]. A later study reaffirmed that most of the skin-infiltrating lymphocytes during vitiligo are CD8+ T cells and these T cells were skin-homing, predominantly expressed the type 1 cytokines IFNγ and TNFα, and clustered near disappearing melanocytes [57, 62]. Van den Boorne et al. isolated T cells from perilesional skin or healthy controls and used HLA-A2 restricted tetramers to screen the ability of T cells to recognize peptides generated from melanocyte proteins like gp100, MART-1 and tyrosinase [63]. There were significantly increased levels of T cells recognizing melanocyte antigens in populations from perilesional skin compared to those from healthy skin and these melanocyte targeting T cells exhibited the capability to destroy melanocytes [63]. CD8+ T cells or CD8-depleted lymphocytes isolated from perilesional skin were co-cultured with pigmented skin explants from non-lesional skin [63]. After analyzing skin explants with immunohistochemistry or immunofluorescence they observed that lymphocytes infiltrate the skin and localize near melanocytes in both experimental groups but only CD8+ T cells isolated from patient skin induced melanocyte apoptosis [63]. Taken together these data firmly establish that CD8+ T cells drive vitiligo pathogenesis by targeting and destroying melanocytes in lesional skin during disease.

1.9: Mouse models developed to study Vitiligo

To investigate the mechanisms of pathogenesis during vitiligo a number of mouse models have been developed and are extensively reviewed in Essien et al. [64]. While none of these

models perfectly encapsulate all the factors involved in pathogenesis, each can address specific components of disease if used appropriately. The mi^{vit}/mi^{vit} mouse spontaneously develops hair depigmentation without immune involvement due the loss of melanocyte function caused by a point mutation in the MITF gene [65, 66]. MITF regulates melanocyte development and genes important for melanin production like tyrosinase [67] but since the identical mutation results in partial albinism and deafness instead of vitiligo, this model does not appear to accurately mimic human vitiligo [68, 69]. Despite these differences this model could be useful for studying how melanocyte defects contribute to vitiligo.

Zhu *et al.* developed a mouse model of vitiligo in which the application of MBEH to the abdomen of young mice resulted in local hair depigmentation that spread to distant sites. In $Rag^{-/-}$ mice depigmentation is restricted to the site of local depigmentation suggesting that while direct toxicity of MBEH may be responsible for localized depigmentation at the site of application, an adaptive immune response is required for spread to other sites. $CD8^+$ T cells infiltrate MBEH treated skin and this model may be useful for investigating how environmental factors and stress promote depigmentation [70].

Mice immunized with various vectors carrying human melanocyte antigens like TRP1 [71] and TRP2 [72, 73] exhibit hair depigmentation. In these models, melanocyte-specific autoantibodies are induced and either CD4+ T cells [71] or CD8+ T cells [72, 73] mediate depigmentation. TCR transgenic hosts bred to express human HLA-A2 and T cells with TCRs recognizing human melanocyte antigens develop depigmentation [74, 75]. In one of these models depigmentation is driven by CD8+ T cells and depended on IFNγ similar to human disease [74], but in the other model CD3+CD4-CD8- T cells drive depigmentation, a scenario very different

from human disease [75]. Nonetheless, these models are suitable for studying the role of T cell responses during vitiligo.

In several inducible models of vitiligo, TCR transgenic T cells are adoptively transferred into hosts to induce depigmentation. In these models CD4+ T cells with TCRs recognizing human TRP1 [76] or CD8+ T cells with TCRs recognizing human gp100 [77-79] drive hair or epidermal depigmentation in host mice. The Harris mouse model of vitiligo is well-suited to study disease progression since this model develops prominent epidermal depigmentation while sparing the hair [79]. This scenario mimics the clinical presentation of human vitiligo, providing an opportunity to study mechanisms that direct T cell migration to the epidermis and topical agents that modify disease severity. Since this model also maintains immune privilege of the hair follicle [5], it allows the investigation of factors promoting epidermal repigmentation that has been shown to spread from melanocyte stem cell reservoirs that persist protected from autoimmunity in hair follicles [4]. The Harris model of vitiligo provides the opportunity to study vitiligo in context that closely matches human disease and I used this model to investigate the regulatory factors in the skin during vitiligo.

1.10 A myriad of factors contribute to vitiligo pathogenesis

The etiology of vitiligo is complex and a number of factors likely combine to cause skin depigmentation. Intrinsic melanocyte defects and exposure to environmental factors may initiate processes that activate CD8+ T cells to target melanocytes and drive disease. Since patients seek treatment for vitiligo once lesions have already appeared, focusing on the autoimmunity that drives depigmentation, as opposed to factors that initiate disease, will best serve patients looking for an effective treatment or cure. If we can learn how to dampen the CD8+ T cell response

against melanocytes in the skin we can halt melanocyte destruction and establish favorable conditions for repigmentation. To accomplish this one must appreciate that the skin hosts an extensive network of immune players that all interact to effectively protect the body from infections. Skin resident APCs, along with T cells, coordinate to initiate immune responses and in healthy people can also support peripheral tolerance to protect the skin from autoimmunity. Our group focuses on understanding the factors that drive CD8+ T cell-mediated autoimmunity and this work focuses on regulatory factors in the skin that suppress CD8+ T cell-mediated depigmentation during vitiligo. The following work will review the literature on factors that regulate effector T cell function during an autoimmune response in the skin.

1.11: T cell development in the thymus deletes auto-reactive T cells

Like all T cells, CD8+ T cells develop in the thymus. T cell progenitors that express neither CD4 nor CD8 leave the bone marrow and enter the thymus where they receive signals that initiate processes that lead to the expression of a functional surface pre T Cell Receptor or preTCR. The affinity of the preTCR for MHC-self-peptide complexes expressed in the thymus determines whether or not a cell survives positive and negative selection. Cells bearing TCRs with low affinity for MHC-self-peptide complexes presented on thymic epithelium are positively selected and receive signals that lead to survival. Any positively selected cells expressing TCRs with strong affinity for MHC-self-peptide complexes presented on thymic APCs are negatively selected and receive signals that initiate apoptosis. Cells that survive positive and negative selection receive signals that induce the expression of a mature CD8 or CD4 TCR and egress from thymus to the circulation. This process of thymic selection serves as the first check to

protect the body from autoimmunity by preventing the escape of auto-reactive T cells to peripheral circulation.

1.12: Naïve T cell activation and migration to skin

A CD8+ T cell that leaves the thymus will circulate between the blood stream and secondary lymphoid organs (SLOs) until it encounters the antigen recognized by its TCR. In the SLO organs like lymph nodes (LNs), DCs that have migrated from the site of infection present MHCI-bound antigen to T cells while simultaneously providing co-stimulation and cytokine signals to activate naïve CD8+ T cells [80-83]. Activated naïve T cells differentiate into effector T cells (Teffs) and exponentially proliferate until the amount of antigen-specific Teffs reaches more than 10,000 fold their original number [84].

During this clonal expansion Teffs migrate to sites of inflammation in peripheral tissues [85]. There they interact with pathogen-infected cells that express surface bound antigen-MHCI complexes and clear the infection by inducing apoptosis in infected cells with cytotoxic granules or by releasing inflammatory cytokines [86]. The lymphoid environment in which a T cell is activated has been shown to influence Teff migration after leaving SLOs. Naïve CD8+ T cells cultured with antigen-pulsed DCs isolated from skin draining lymph nodes (SDLNs) expressed higher levels of molecules required for skin entry and migration [87]. These T cells migrated efficiently to inflamed skin while T cells activated by DCs from gut-derived lymphoid tissues migrate to the gut [87]. Teffs licensed to migrate to the skin upregulate the adhesion molecule CLA. CLA binds E-selectin molecules that are constitutively expressed by the endothelial cells lining blood vessels in the skin and this interaction allows T cells to roll against the wall of the vessel, the initial step of T cell entry into the skin [88, 89].

1.13: Mechanisms that prevent Teff-mediated host tissue damage

Immune-mediated tissue damage can result from an improperly regulated CD8+ Teff response. During hemophagocytic lymphohistiocytosis a failure to clear viral infections results in excessive CD8+ Teff activation that leads to the over expression of inflammatory cytokines and subsequent immune-mediated tissue damage [90]. Proinflammatory and anti-inflammatory signals must be balanced to ensure effective pathogen clearance with minimal host tissue damage and the immune system has evolved strategies to properly regulate Teff-mediated inflammation. TCR signaling, co-stimulation, and cytokine signals that Teffs receive during an infection induce the expression of anti-apoptotic BCL-2 molecules that promote Teff survival [91]. After Teffs clear an infection, the signals that drive Teff activation are gone and as a result Teffs stop expressing anti-apoptotic BCL-2 molecules and express pro-apoptotic molecules [92-94]. Thus the clearance of infection establishes conditions that lead to Teffs apoptosis to prevent potential Teff-mediated tissue damage. Activation-induced cell death (AICD) results from repeated TCR stimulation of previously activated Teffs and culminates in apoptosis mediated by FAS “death receptor” signaling to prevent excessive Teff responses [95, 96]. Teff responses during infection are regulated to prevent damage to host tissues and the immune system has also evolved mechanisms to suppress Teff-mediated autoimmunity. In some instances self-targeting T cell clones escape deletion in thymus and the mechanisms that suppress their autoimmune activity in peripheral tissue are collectively termed peripheral tolerance. During vitiligo these mechanisms must be overwhelmed to allow Teff activity to mediate depigmentation, and studying vitiligo provides a unique opportunity to understand how impaired peripheral tolerance contributes to

pathogenesis during vitiligo and other forms of autoimmunity. A number of immune cells can participate in the peripheral tolerance, and regulatory T cells (Tregs) play a crucial role.

1.14: Discovery of Tregs

Tregs are a subset of CD4+ T cells that potently suppress pathogenic immune responses mediated by autoreactive cells, and maintain tissue immune homeostasis. Researchers first discovered a thymus-derived population capable of suppressing autoimmunity in the late 60s. Experiments performed by Gershon and Kondo in 1970, revealed that thymus-derived lymphocytes prevent the inflammatory activity induced by bone marrow-derived cells transferred into recipient mice [97]. Nishizuka and Sakakura reported that removing the thymus of 2 to 4 day old mice, led to autoimmune ovary damage, inflammation in various tissues and the production of tissues specific autoantibodies [98]. In line with this discovery, another group found that removal of the thymus followed by sublethal irradiation in adult rats induces multiple forms of autoimmunity, including diabetes [99, 100]. Subsequent studies investigated the mechanism of the thymic-derived suppressor T cells. These efforts identified a network of soluble factors whose suppressive capabilities were attributed to a molecule termed I-J that mapped to the MHC locus [101]. Controversy concerning the existence of suppressor T cells arose after analysis of the MHC gene revealed that the region I-J mapped to could not encode an I-J polypeptide [102]. In addition to this finding, the inability to identify a reliable distinguishing marker for suppressor T cells combined with difficulty preparing antigen-specific suppressor clones, resulted in widespread skepticism regarding the existence of a suppressive T cell population. Finally, studies perform in the early 80’s verified suppressor T cells exist.

Work performed by Sakaguci et al. reaffirmed the suppressive capabilities of T cell populations. When Sakaguchi et al. removed the thymus from 2-4 day old mice to induce autoimmunity, they found that splenocytes from these mice could induce similar autoimmune inflammation when transferred to healthy T cell deficient neonatal mice [103]. Additionally, they found that thymocytes from healthy adult mice injected 2 weeks after thymus removal could prevent autoimmunity that normally results from thymectomy [104]. The splenocytes that could adoptively transfer autoimmunity and the suppressive cells that could prevent it were both identified as CD4+ cells. This suggested that two populations of CD4s exist; one capable of effector function and another capable of suppressor function. Subsequent experiments identified that $CD5^{high}CD45RB^{low}CD4+$ T cells possess suppressive capabilities as isolated $CD5^{low}CD4+$ T cells and $CD45RB^{high}CD4+$ T cells mediated multi-organ autoimmunity when transferred into T cell deficient mice [105, 106]. In both these cases co-transfer of whole CD4+ T cells prevented autoimmunity [105, 106]. Sakaguchi et al. later identified that CD25, the high affinity IL2 receptor α-chain, is preferentially expressed on the $CD5^{high}CD45RB^{low}$ fraction of peripheral CD4+ T cells in naïve mice compared to peripheral CD8+ T cells and other CD4+ T cells [107]. CD25-CD4+ splenocytes induced autoimmunity when transferred into athymic mice and transferring CD25+CD4+ T cells inhibited autoimmunity confirming CD25 as a reliable marker for T cells with regulatory capabilities, Tregs [107].

1.15 Foxp3 identifies Tregs and regulates Treg function.

As argued by Schmidt et al., the discovery of CD25 as a reliable marker for Tregs opened the door for researchers to explore their presence and function [108]. Tregs identified by CD25 can be found in peripheral tissues of normal mice 3 days after birth and make up 5-10% of CD4 T cells in the periphery of adult naïve mice [109]. However, CD25 is not simply a marker for

Tregs, but the receptor is also crucial for Treg growth, survival and function. This is evidenced by the fact that IL-2 deficient mice have reduced numbers of Tregs, spontaneously develop autoimmunity, and disease in $CD25^{-/-}$ mice can be prevented by the adoptive transfer of Tregs [110, 111].

CD25 identifies Tregs in humans as well, but while Tregs express very high levels of CD25, activated CD4+ T cells express CD25, albeit at lower levels [112]. Forkhead box P3 (FoxP3), a transcription factor that regulates Treg identity and function, was later identified as a marker for Tregs in humans and in mice. Scurfy mice that have a mutation in the gene encoding FoxP3 have no Tregs and exhibit widespread autoimmunity [113] similar to autoimmunity in patients with Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), also caused by mutations in the *FOXP3* gene [114]. Fontenot et al. observed that Tregs express Foxp3 and the $Foxp3^{-/-}$ mice that they generated had fewer CD4+CD25+ Tregs. They also developed a lymphoproliferative disorder that resolved when CD4+CD25+ Tregs were transferred [115]. Fontenot et al. used retrovirus to mediate the ectopic expression of Foxp3 in naïve CD4+CD25- T cells and found that these cells gained regulatory capabilities in vivo, suggesting Foxp3 is a crucial regulator of Treg function and is not only necessary but sufficient for this function [115]. Further supporting this claim, Hori et al. also reported retroviral transduction of Foxp3 is sufficient to give peripheral CD4+CD25- T cells suppressive capabilities and in humans ectopic overexpression Foxp3 allowed naïve T cells to mimic the suppressive function of Tregs in vitro [116, 117]. In mice Tregs express Foxp3 while resting or activated T cells do not express significant levels [115], but in humans Tregs express Foxp3 and activated T cells can transiently express the transcription factor [118].

1.16: Natural Tregs develop in the thymus.

Two classes of Tregs have been identified based on their development, induced Tregs and natural Tregs. Induced Tregs develop in the periphery when naïve CD4+ T cells acquire suppressive capabilities after exposure to cytokines like TGF-β1 [119]. Natural Tregs develop in the thymus and the paucity of specific markers to differentiate between the two complicate attempts to study their differences. The following will focus on natural Treg development. In humans, Tregs develop in the thymus and can be found in the thymus at 12 to 13 gestational weeks. Fetal Tregs have the ability to suppress CD4+CD8- T cell proliferation in vitro and express canonical markers of Treg fate like CD25, FOXP3 and CTLA4 [120]. The mechanisms that commit progenitors to Treg fate in the thymus are still being studied. Observations that fetal thymic Tregs express markers associated with TCR stimulation like CD69 suggest positive selection based on affinity to MHC-self-peptide complexes contributes to Treg differentiation [120]. Caramalho et al. identified IL2 and IL15, ligands for CD25, as key cytokines involved in Treg development that preferentially promote Treg proliferation and survival in the thymus [121]. In addition to IL2 and IL15, various other signaling molecules contribute to Treg differentiation in thymic progenitors, suggesting that a combination of TCR stimulation and molecular signals likely control Treg development in the thymus [121-123].

In mice, induced and natural Tregs have been described and natural Tregs develop in the thymus. Similar to Treg development in humans, progenitor commitment to Treg differentiation in the thymus likely depends on a combination of different signals. In the thymus, Treg development depends on the expression of a TCR with strong affinity to MHC-self-peptide complexes, as Treg differentiation was enhanced in mice where superantigens that bind TCRs with high affinity were presented by MHC molecules on thymic epithelium [124]. Studies detailed that TCR-driven selection is accompanied by additional signaling from CD28, IL2/IL15

and TGFβ that drive Treg development in the thymus [125-127]. TCR affinity for MHC-self-peptide complexes in combination with specific molecular signals drives Treg development in the thymus.

1.17: Tregs can use a variety of mechanisms to mediate suppression.

Autoreactive T cells can escape negative selection in the thymus and when this occurs Tregs play an important role in maintaining peripheral tolerance. Tregs can suppress naïve Teffs through a variety of mechanisms that ultimately prevent Teff activation and proliferation [128]. Tregs can also suppress activated T cells as Tregs inhibit the proliferative response of memory CD4+ Teffs to alloantigen [129] and Tregs suppress memory CD8+ T cell responses to HSV-1 infection [130]. Tregs can suppress Teffs indirectly through mechanisms that reduce co-stimulation signals antigen presenting cells provide to Teffs or directly through secreted anti-inflammatory cytokines or cell contact-mediated mechanisms. I will briefly describe these mechanisms below.

Suppression by modulation of APC co-stimulatory signals

A DC can initiate immune responses by activating Teffs through multiple signals. First, DCs provide TCR signaling that initiates Teff activation when DCs present antigen on MHC molecules to Teff TCRs. DCs also provide CD28 co-stimulatory signaling that increases Teff survival and proliferation when CD80/CD86 expressed by DCs, ligates CD28 expressed by Teffs. When CD4+CD25+ T cells are co-cultured with bone marrow-derived DCs in the presence of anti-CD3 antibodies, Tregs reduced DC surface expression of CD80 and CD86 [131]. Tregs are still able to reduce CD80 and CD86 when DCs are activated with anti-CD40 antibodies and reduce mRNA levels of CD80 [131]. Another in vitro study echoed these results

but reported that this process requires CTLA4. They found that CTLA4$^{-/-}$ Tregs co-cultured with DCs could not reduce DC CD80 or CD86 expression [132]. DCs with reduced levels of co-stimulatory molecules primed Teff responses less efficiently. Oderup et al. reported that DCs pre-cultured with Tregs exhibited reduced CD80 and CD86 expression and induced poor CD4+ T cell proliferation in secondary cultures [133]. In these cultures, the addition of anti-CD28 antibodies rescued CD4+ T cell proliferation [133]. These observations suggest that Tregs can suppress Teffs indirectly by reducing the co-stimulatory signals that DCs provide.

Suppression by anti-inflammatory cytokines

IL-10. Tregs secrete cytokines that mediate Treg induced suppression. IL10 is an immunosuppressive cytokine that contributes to Treg suppression in several in vivo studies. Asseman et al. demonstrated that Tregs from IL10$^{-/-}$ mice fail to prevent disease in a mouse model of colitis while WT Tregs can abrogate inflammation [134]. Similarly, IL-10$^{-/-}$ Tregs fail to protect mice from inflammation in a mouse model of EAE while WT Tregs could suppress active disease [135]. These observations suggest IL-10 contributes to Treg suppression but the fact that IL-10$^{-/-}$ mice only exhibit colitis and not widespread autoimmunity, suggests Tregs may not always require IL-10 to suppress Teffs [136]. IL-10-mediated Treg suppression seems to depend on where Tregs encounter Teffs. IL-10$^{-/-}$ Tregs and WT Tregs can suppress CD4+ T Cell proliferation and IFN-γ production in the lymph node but IL-10$^{-/-}$ Tregs fail to control CD4+ T Cell IFNγ production in ear skin [137]. Tregs may not use IL-10 to suppress in peripheral tissue like skin and these findings highlight the importance of studying Treg mechanisms of suppression in assays that consider the context of Treg suppression.

IL-35. Collinson et al. identified IL-35 as a molecule capable of mediating Treg suppression. IL-35 is predominantly expressed by Tregs and $IL35^{-/-}$ Tregs are less suppressive in vitro and fail to control autoimmunity in an in vivo model of inflammatory bowel disease [138]. IL-35 suppresses T cell proliferation and T cells retrovirally transduced with vectors encoding IL-35, acquired regulatory capabilities [138].

TGFβ. TGFβ is an immunomodulatory cytokine and a number of separate studies report contradicting evidence regarding the necessity of TGFβ for Treg suppression. In vitro assays performed by Piccirillo et al. demonstrated that suppressive Treg function does not require TGFβ, as Tregs were still able to suppress Teff proliferation in response to TCR stimulation in the presence of anti-TGF-β1 antibody [139]. Tregs also suppressed Teff proliferation in Teffs incapable of responding to TGFβ signaling and Tregs from $TGF\beta^{-/-}$ mice suppressed Teffs in vitro, further demonstrating TGFβ is dispensable for Treg suppression [139]. In contrast to this, Levings et al. demonstrated that human Tregs require TGFβ for optimal suppression, as Tregs exhibited a reduced capacity to suppress TCR-mediated CD4+ T Cell proliferation in the presence of anti-TGF-β1 antibody [140]. Read et al. also reported that Treg suppression is mediated by TGFβ signaling in a mouse model of colitis as adoptively transferred Tregs lost the ability to prevent colitis when mice were treated with anti-TGFβ antibody [141].

Contact-dependent Mechanisms of Treg suppression

While direct suppression of Teffs by Tregs can be mediated by secretion of the immunomodulatory cytokines described above, mechanisms of suppression that require Tregs to directly contact Teffs have also been described. Cyclic adenosine monophosphate (cAMP) is a molecule that suppresses T cell growth, differentiation and proliferation [142]. Bopp et al. observed that Tregs express high levels of cAMP while CD4+ Teffs did not express appreciable

amounts [143]. CD4+ Teffs exhibited an increase in cAMP after they were co-cultured with Tregs, and Tregs suppressing CD4+ Teffs transfer cAMP to Teffs via gap junctions in co-cultures [143]. This mechanism required contact, as separating Tregs from CD4+ T cells with a membrane abrogated Treg suppression [143].

Several studies demonstrate that Tregs use surface bound TGF-β1 to suppress in a contact-dependent manner. Nakamura et al. reported that activated Tregs express surface bound TGF-β1 and antibodies that block TGF-β1 ablate the ability of Tregs to suppress CD4+CD25- T cell proliferation in vitro [144]. Andersson et al. also reported that in the presence of an antibody blocking surface bound TGF-β1, Tregs cannot suppress CD4+CD25- Teffs in in-vitro co-cultures [145]. In these studies, Treg-mediated suppression required contact as Tregs could not suppress CD4+CD25- T cells when separated by a membrane [144, 145]. In a mouse model of diabetes, Green et al. reported that Tregs cannot control disease mediated by CD8+ T cells without TGFβ signaling and suggest that Treg suppression requires contact since disease is least severe in mice with Tregs expressing high levels of surface TGF-β1 [146].

Tregs can also directly kill Teffs through contact-dependent mechanisms that require granzyme. Grossman et al. observed that human Tregs isolated from peripheral blood upregulated granzyme A after activation with anti CD3/CD28 antibodies in the presence of IL2. These activated Tregs killed CD8+ and CD4+ target cells in vitro using perforin-dependent mechanisms [147]. Another study reported that Tregs may also use granzyme B for suppression as murine granzyme B deficient Tregs failed to efficiently suppress CD8+ T cells in co-cultures [148].

While many mechanisms of Treg suppression have been described, questions remain about which mechanisms Tregs use in different contexts. As argued by Schmidt et al., Tregs may

not use only one distinct mechanism to suppress Teffs [108]. Tregs may have a variety of different options at their disposal and the mechanism that they use could depend on numerous factors like the activation status of the Teffs they encounter or the location where they encounter Teffs. Conflicting results where a specific molecule is crucial for suppressive function in one instance and dispensable for suppression in another suggest that Tregs can use redundant compensatory mechanisms to mediate suppression. The context of Treg-Teff interactions could determine which mechanisms Tregs use and it is important to consider this when designing assays to test Treg function. For example, Tregs can suppress Teffs by decreasing the co-stimulatory signals that APCs deliver. This strategy will successfully suppress the activation of naïve T cells in SLOs but will be ineffective when Tregs encounter already activated Teffs in peripheral tissue. A lot of work has been done to elucidate Treg function in vitro but these systems may not be able to fully appreciate the context of suppression and thus may not be able to inform how Tregs suppress in the periphery. In our model of vitiligo presents a model where Tregs suppress Teffs in the periphery and could help clarify how Tregs suppress Teffs in peripheral tissue.

1.18: Tregs in the skin

A growing body of evidence supports the fact that Tregs exist in peripheral tissue and Tregs have been reported in human and mouse skin. Using flow cytometry, Scharschmidt et al. found a wave of Tregs enter mouse skin as early as postnatal day 6 and Tregs account for 20-60% of the CD4+ T cells in the skin of adult mice [149]. Tregs also exist in human skin as Sanchez Rodriguez et al. found that about 20% of CD4+ T cells in surgically obtained skin from healthy patients are Foxp3 expressing Tregs [150]. This group categorized the Tregs they found

in healthy skin as activated memory Tregs, since more than 90% of Tregs in healthy skin express markers characteristic of effector memory T cells like CD45RO and higher levels of activation markers like CTLA-4, CD25 and ICOS [150]. Rosenblum et al. used a mouse model to demonstrate that memory Tregs reside in mouse skin and provide expedited protection against skin inflammation [151]. In this model initial antigen expression activates and expands antigen-specific Tregs in SDLNs that subsequently accumulate in the skin during antigen-induced disease [151]. After disease resolves, activated Tregs remain in skin even in the absence of antigen and facilitate a potentiated inflammatory response when antigen is re-expressed [151].

Immunofluorescence staining on healthy human skin revealed that Tregs reside in the dermis clustered near hair follicles [150]. Similar observations were made in mice, as immunohistochemical staining of 13 day-old mice revealed that Tregs primarily localized to hair follicles [152]. Hair follicle development plays a crucial role in the migration of Tregs to skin in developing mice and Scharschmidt et al. demonstrated this when they reported that the inducible ablation of murine hair follicle resulted in reduced Tregs accumulation in the skin of 13 day old mice [152]. Hair follicle-dependent Treg skin migration is augmented by commensal microorganisms and mediated by the chemokine CCL20 made by hair follicle epithelial cells [152]. Treg homeostasis in the skin most likely remains tied to hair follicle biology as murine Tregs are more abundant and activated in telogenic than anagenic skin [153] and areas of human skin with more hair follicles (face and scalp) have more Tregs than areas with low hair follicle density (trunk and upper proximal extremities) [150].

Tregs circulating in mice and humans can traffic to the skin and the signals that Tregs can use to enter the skin have been studied. Murine Tregs require CCR6 to follow hair follicle-derived CCL20 to migrate to the skin during neonatal development [152]. CCR4 may also

facilitate Treg migration to the skin. Murine Tregs, activated by antigen in lymph nodes, express CCR4 and CD103 and $CCR4^{-/-}$ Tregs exhibit a reduced ability to accumulate in the skin and control cutaneous inflammation [154]. Eby et al. demonstrated that the over expression of the CCR4 ligand CCL22 in murine skin, drove increased cutaneous Treg accumulation in mice further supporting a role for CCR4 in skin migration [155]. The enzyme α-1,3-fucosyltransferase VII (FucT7) was also demonstrated to be important for Treg skin migration as $FuvT7^{-/-}$ Tregs transferred into scurfy mice could migrate well to most tissues except the skin [156].

1.19: Tregs in autoimmune skin diseases.

Autoimmune diseases affecting the skin are mediated by various forms of cutaneous auto-inflammatory activity. In a number of these diseases, many have implicated decreased Treg number in the skin or impaired Treg function as a causative factor for the break in tolerance that allows autoimmunity to proceed.

In alopecia areata (AA) CD8+ T cells mediate hair loss by attacking the bulb of the hair follicle [157]. Han et al. observed that there are fewer Tregs in the scalp lesions of AA patients [158] and increasing Treg number in lesions may resolve AA. Castela et al. subcutaneously injected the lesional scalp of patients with low dose recombinant IL2 and saw partial hair regrowth in injected lesions and increased Treg number when comparing biopsies obtained before treatment and at the end of the study [159].

The role of Tregs in systemic lupus erythematosus (SLE) remains unclear and conflicting reports on the numbers and function of Tregs in SLE have been published. Using flow cytometry, Bonelli et al. observed fewer Tregs in the peripheral blood of patients with active and inactive SLE compared to healthy controls [160] and Valencia et al. reported that Tregs isolated

from patients with active SLE failed to suppress the proliferation and cytokine production of CD4+ T cells [161]. These results suggest that reduced Treg number and impaired Treg function could contribute to SLE pathogenesis but a few studies contradict this hypothesis. Studies done by Alvarado-Sanchez et al. report no difference in Treg number in the PBMCs of SLE patients [162] and studies done by Vargas-Rojas et al. found that Tregs from SLE patient exhibit normal suppressive ability but CD4+ Teffs from SLE patients are resistant to Treg suppression [163].

Psoriasis patients suffer from widespread, scaly plaques that persist for weeks to months. Immunohistochemical analysis of punch biopsies from the lesional skin of psoriasis patients revealed lower Treg/CD4 ratios in perilesional skin compared to distal non-lesional skin and skin from healthy patients, suggesting decreased Treg number in the skin may contribute to pathogenesis [164]. Increased numbers of CD4+ T cells likely also contribute to disease but a favorable ratio of Tregs to Teffs is probably necessary to control inflammation. Tregs in PBMCs from psoriasis patients exhibit a reduced ability to inhibit Teffs in vitro suggesting impaired Tregs function may contribute to disease as well [165]. In an imiquimod induced mouse model of psoriasis, Tregs accumulate in lesional skin and Treg depletion results in increased CD8 Teff accumulation in the skin and exacerbated disease [166]. These observations suggest that the number of Tregs in the skin affects psoriasis pathogenesis.

In the diseases previously detailed, the ability of effectors to mediate inflammation during skin autoimmunity may result from impaired Treg number or ability to suppress inflammation. If Teffs are mounting a massive immune response against skin tissue, the number of Tregs must be important to ensure effective concentrations of anti-inflammatory cytokines or enough opportunity for contact-dependent mechanisms of suppression to work. Too few Tregs or reduced Treg function could conceivably perpetuate a cycle where Teffs proceed unchecked.

Defects in Treg function or decreased Treg number may contribute to the reduced peripheral tolerance that allows disease pathogenesis during vitiligo and in this work I will investigate the contributions of Tregs to vitiligo. DCs are another cutaneous immune population that participate in peripheral tolerance.

1.20: DCs are APCs capable of initiating innate and adaptive immune responses.

When an infection occurs in peripheral tissue innate immune cells are quickly activated to non-specifically address infection but adaptive immune cells, like CD8+ and CD4+ T cells, are often required to ensure a sterilizing immune response. Antigen presenting cells (APCs) are unique in their ability to encounter pathogens and initiate both innate and adaptive responses. Dendritic cells (DCs) are APCs that express both classes of human leukocyte antigen (HLA) molecules, allowing them to present antigen to CD8+ and CD4+ T cells and subsequently activate the T cells to mediate adaptive responses. In mice DCs express MHC I and MHC II and can activate murine CD8+ T cells and CD4+ T cells. DCs frequently phagocytize both self and foreign proteins in the skin and rely on molecular sensors called Toll-like receptors (TLRs) or NOD-like receptors (NLRs) to signal whether DCs require to initiate an immune response. Surface bound or internal TLRs and NLRs recognize pathogen products and activate DCs. Upon activation DCs produce inflammatory cytokines that initiate innate immunity, process and present pathogenic antigen and migrate to lymph nodes where they present antigen to and activate CD4+ and CD8+ T cells bearing TCRs with affinity to the pathogenic antigen [167-169].

1.21: Dendritic cells exist in skin and some can initiate T cell activation.

In 1868, Paul Langerhans used gold chloride and a light microscope to discovered non-pigmented cells in human skin and while he initially thought they were nerve cells, they were later identified as skin resident DCs [170, 171]. Today we know that a few populations of DCs populate the skin and they can be distinguished based on the surface expression of specific proteins. Henri et al. used flow cytometry to look at $MHCII^{high}$ DC populations in murine epidermis and dermis and found epidermal Langerhans cells (LHCs) that are CD207+CD103- and dermal dendritic cells (dDCs) that are CD207+CD103+ or CD207-CD103- [172, 173]. Skin dendritic cells can activate T cells to initiate adaptive immunity, as Henri et al. demonstrated that CD207+CD103+ dDCs isolated from the skin of transgenic mice with epidermal keratinocytes overexpressing ovalbumin (OVA) induced the proliferation of OT-1 CD8 T cells in in-vitro co-cultures [172]. In these assays, only CD207+CD103+ dDCs were able to induce T cell proliferation, suggesting that not all skin DCs can activate T cells to initiate adaptive responses [172]. In fact a number of published studies established that DCs can suppress inflammatory immune responses and these DCs have been called tolerogenic DC.

1.22: Tolerogenic dendritic cells can suppress inflammation.

Some of the first evidence that demonstrated DCs can inhibit inflammation was presented by Jonuleit et al. They generated immature DCs and mature DCs from human PBMCs and observed that while mature DCs could induce the proliferation of naïve CD4+ T cells in in-vitro co-cultures, immature DCs induced irreversible impaired proliferation in naïve CD4+ T cells [174]. T cells co-cultured with immature DCs upregulated CTLA4 (which is also constitutively expressed by Tregs), produced anti-inflammatory IL10, and acquired the ability to suppress the antigen driven proliferation of Th1 T cells [174]. This data suggest DCs are able to induce a

regulatory-like state in naïve T cells promoting immunosuppression instead of inflammation. One in vivo study demonstrated that DCs internalize and present OVA antigens on MHC I and remain immature when mice are subcutaneously injected with OVA chemically linked to an antibody against CD205 (αDEC-205:OVA) [175]. After 12-14 days OT-1 cells injected into these mice are absent from LNs, spleen and blood and in a separate experiment, mice injected with αDEC-205:OVA mount no immune response after being re-challenged with OVA [175]. This data suggests that antigen presentation by immature DCs can induce peripheral tolerance and additional evidence reports that the maturation status of a DC when it presents antigen determines whether a DC promotes inflammation or tolerance.

DCs exposed to TNF-α adopt a semi-mature phenotype and upregulate maturation markers like CD80, CD86, and CD40 but express reduced levels of IL-1β and IL-6 compared to DCs matured with LPS and anti-CD40 antibody [176]. TNFα-exposed semi-mature DCs protected mice from disease in a mouse model of EAE, but DCs matured with LPS plus anti-CD40 could not [176]. Semi-mature DCs were also able to induce IL-10 producing Tregs and together these observations suggest specific cytokine signals induce tolerogenic function in DCs [176].

Mature DCs may also promote tolerance in the right conditions by inducing Tregs. Varhasselt et al. reported that DCs generated from human PBMCs and matured with LPS induced the proliferation of CD4+ T cells better than immature DCs, when CD4+ T cells were cultured with either immature or mature DCs for 6 days [177]. CD4+CD25- T cells cultured with mature DCs acquired FoxP3 mRNA expression and were able to suppress the proliferation and cytokine production of allogeneic T lymphocytes activated by DCs in secondary cultures [177].

DCs express the appropriate molecules to interact with both CD8+ and CD4+ T cells and tolerogenic DCs may induce Tregs to establish an anti-inflammatory environment.

CD4+Foxp3+ inducible Tregs that develop from CD4+Foxp3- progenitors in peripheral lymphoid tissue instead of the thymus have been described by a number of groups. Yamagiwa et al. isolate naïve human CD4+ T cells that were CD45RA+ RO−. These CD4 cells incubated with TGF-β1 for 5 days acquired the ability to suppress T cells in secondary in vitro cultures [119]. Similarly, murine CD4+CD25- cells incubated with anti-CD3 antibody, anti-CD28 antibody and TGF-β1 for 5 days in vitro, acquired Foxp3 expression [178]. These cells were able to suppress the proliferation of CD4+ T cells in response to anti-CD3 and CD28 antibodies in secondary in vitro cultures [178]. Together these data show that TGFβ can drive the induction of Tregs from CD4+Foxp3- progenitors and others have reported that the context of antigen presentation can induce Tregs as well. Kretschmer et al. transferred CD4+25- T cells into a $Rag^{-/-}$ mouse and then intraperitoneally injected mice with influenza antigen coupled to anti-DEC-205 antibody (αDEC-HA) to target antigen to DCs without inducing DC maturation [179]. Adoptively transferred CD4+ cells proliferated, acquired CD25 and Foxp3 expression and also acquired suppressive capabilities in vivo and in vitro [179]. Kretschmer et al. examined optimal conditions for Treg induction and observed that low doses of αDEC-HA most efficiently induced Tregs [179]. Additionally, injecting anti-CD40 with αDEC-HA increased DC activation and reduced Treg induction [179]. Kretschmer's observations suggest that low amounts of antigen presented by unactivated DCs can induce Tregs from naïve CD4 progenitors. Naïve CD4 cells can acquire regulatory function and tolerogenic DCs may coordinate with Tregs to maintain peripheral tolerance through a variety of mechanisms that lead to Treg induction.

1.23: Langerhans cells are dendritic cells that reside in the epidermis and express specific markers.

LHCs are the primary DC in the epidermis. Murine LHCs express MHCII and human LHCs express HLA-DR and whether or not LHCs prime adaptive responses or act as tolerogenic DCs is still debated. LHCs reside in the suprabasal layer of the epidermis in close proximity to melanocytes and attached to keratinocytes [180] and since they are the main APC in the outer most layer of skin, LHCs are potentially the immune system's first point of contact with pathogens infecting the skin. In vitiligo melanocytes are targeted by the immune system and since LHCs are present in the skin adjacent to melanocytes, they may play some role in the immune processes mediating pathogenesis during disease.

LHCs are distributed throughout the epidermis and in humans LHCs represent approximately 2% of all cells in the epidermis [181]. The density of LHCs in the skin varies as the palms and soles have the fewest LHCs, 200 per mm^2, and the face and neck have the most LHCs, 970 per mm^2 [182]. In epidermal sheets from adult mice, there are approximately 1000 LHCs per mm2 [183, 184]. LHCs can be identified by the expression of a few particular markers. Birbeck granules are organelles found exclusively in LHCs and served as early markers to identify them [185] until intracellular and extracellular CD207 or Langerin, a type II Ca2+-dependent lectin displaying mannose-binding specificity, was discovered as an LHC identifier [186, 187]. Other DCs in the dermis express Langerin as well but these DCs can be differentiated from LHCs based on the expression of CD103, EpCAM and CD8; with LHCs defined as CD207+CD103-EpCAM+CD8- [172, 173].

1.24: Langerhans cell development.

The ontogeny of LHCs has been well-studied in mice. LHC precursors seed the epidermis early in development and undergo a proliferative burst before LHC density in the skin settles to what we see in adult animals. Chorro et al. used flow cytometry to find LHC precursors in the epidermis of fetal mice at embryonic day 18. They found the number of epidermal precursors in the epidermis jumps from 50-100 cells/mm2 at embryonic day 18 to 1,000 cells/mm2 at postnatal day 2 [188]. This increased precursor number resulted from a proliferative burst in the first week of life as they reported 60-70% of precursors in the skin expressed the nuclear proliferation factor Ki67 at postnatal day 2 [188]. Tripp et al. prepared epidermal suspensions from epidermal sheets of mice and used flow cytometry to quantify MHCII and CD207 expression. At postnatal day 3, MHCII+CD207^{dim} populations could be identified and after 3 weeks, MHCII+ LHCs in the epidermis expressed levels of CD207 comparable to adults [184].

Once epidermal LHC numbers stabilize, they are extremely resilient. LHCs are radioresistant and progenitors can divide in the skin to replace LHCs that migrate to peripheral lymphoid tissue during the steady state or infection. Krueger et al. grafted human skin onto immunocompromised mice and found that human LHCs survived in the skin for up to 9 weeks, demonstrating that LHCs maintain their numbers for long periods of time without recruiting new bone marrow-derived progenitors [189]. Merad et al. lethally irradiated mice to generate bone marrow chimeras and found that while more than 90% of DCs, peritoneal macrophages, and blood monocytes came from donor bone marrow, 97% of LHCs in the skin were of host origin up to 18 months after transplantation [190]. These results imply that LHCs are resistant to lethal irradiation and further support the idea that LHCs can maintain epidermal numbers without recruiting cells from the bone marrow. In order to maintain epidermal numbers, LHCs precursors that reside in the skin undergo homeostatic proliferation. Merad et al. dosed their chimeras with

BrdU and found that after 30 days approximately 30% of the host-derived LHCs proliferated [190]. Kamath et al. also dosed mice with BrdU and found that 27% of LHCs in ear skin incorporated BrdU in a span of 14 days [191]. Together these results show that during adult life a significant portion of LHCs proliferate or proliferating precursors present in the skin give rise to new LHCs. LHCs also leave the skin during acute inflammation and under these extreme circumstances blood-derived LHCs precursors enter the skin and permanently replace LHCs [190, 192].

1.25: Langerhans cells can migrate from the epidermis and enter skin draining lymph nodes.

LHCs migrate from the epidermis to skin draining lymph nodes during the steady state and require CCR7 to do so [193]. LHCs also migrate to skin draining lymph nodes after exposure to inflammatory stimuli like UV light, tape stripping or contact sensitizers [190, 192, 194]. One group further demonstrated this when they saw more LHCs migrating to skin draining lymph nodes in mice intradermally injected with West Nile virus or Semliki Forest virus than in mice dosed with vehicle [195]. Whether LHCs present antigen in an immunogenic or tolerogenic manner once they get to skin draining lymph nodes, is still not completely understood and a number of groups have presented conflicting evidence when attempting to answer that question.

A number of studies used contact hypersensitivity (CHS) assays in mice to answer whether LHCs can prime T cell responses. To initiate a CHS response mice are first exposed to an antigen that is usually applied to shaved abdominal skin. This initial antigen exposure or sensitization stage will activate skin DCs that subsequently migrate to SDLNs and present the applied antigen to T cells with antigen-specific TCRs in an MHC-dependent manner. Antigen presentation primes the T cells and secondary exposure of murine skin to the same antigen some

days later, the elicitation stage, will provoke an immune response as skin DCs present the antigen to SDLN T cells and recruit them to antigen exposed skin [196, 197]. CHS experiments and other assays performed in the absence of LHCs in some instances revealed that LHCs prime T cell responses and sometimes suggested LHCs were dispensable for T cell priming and even suppressed priming.

1.26: Evidence suggesting Langerhans cells prime T cell-mediated adaptive responses.

To see what role LHCs play in initiating adaptive responses Bennett et al. used the Langerin-DTR mouse. Dosing this mouse with diphtheria toxin will deplete LHCs after 24 hours for 4 weeks allowing Bennett et al. to conditionally deplete LHC during CHS to test the requirement for LHC in priming T cell responses [198]. Bennett et al. induced a CHS response in LHC-depleted mice 3 days after i.p. injection of diphtheria toxin (DTX), and observed a reduced allergic response in mice without LHCs compared to controls with LHCs [198]. This result suggests that LHCs contribute to T cell priming, but this study was performed before Langerin+ dDCs were discovered and thus fails to consider that the reduced inflammation could result from reduced Langerin+ dDC numbers. Sullivan et al. used FACS sorting to isolate LCs from epidermal cell suspensions based on MHC II expression, cultured them with antigen in vitro and adoptively transferred these cells into naïve mice. These haptenized LCs were able to mediate CHS in naive animals suggesting LHCs can prime T cells [199].

Data obtained in models aside from CHS also support an immunogenic role for LHCs. IL-1β injected intradermally into mouse ears upregulated MHC II expression on LHCs and when these LHCs were isolated from epidermal ear suspensions, they potently induced T cell proliferation when they were cultured with T cells in the presence of antiCD3 mAb [200].

Human LHCs isolated from epidermal sheets have also been shown to stimulate allogeneic T cells in vitro, and in another study LHCs isolated from the epidermis induce the proliferation of naïve CD4+ T cells dDCs in vitro [201, 202]. Flacher et al. reported that mouse LHCs could prime antigen specific T cells. They targeted OVA antigen to LHCs by intradermally injecting mice with OVA coupled to an anti-DEC-205 antibody. They obtained epidermal sheets and culture sheets in vitro to isolate migratory LHCs and then cultured LHCs with CFSE labeled OVA specific OT-I CD8+ T cells or OT-II CD4+ T cells. LHCs were able to prime both OT-I and OT-II proliferation further supporting an immunogenic role for LHCs [203]. Mayerova et al. generated bone marrow chimeras using K14-OVAp mice, with keratinocytes that express OVA, as hosts. They lethally irradiated hosts and reconstituted mice with bone marrow from WT mice or bm8 mice that have a mutation in MHCI and can't present OVA [204]. They found that OT-1 cells could still proliferate in chimeras where only LHCs can present antigen suggesting LHCs can prime T cell responses [204]. Together this data suggests LHCs can prime T cell responses but a growing amount of evidence suggests LHCs play a tolerogenic role.

1.27: Evidence suggesting Langerhans cells are dispensable for T cell priming or mediate tolerance.

Some CHS studies have also demonstrated that LHCs are dispensable for T cell priming. Kissenpfennig et al. generated Langerin-DTR-EGFP mice with LHCs that express GFP and the DTR receptor [205]. Dosing mice with DTX effectively depleted LHCs and immune responses are comparable after CHS is induced in the absence or presence of LHCs [205]. This evidence suggests that LHCs do not contribute to T cell priming and Kaplan et al. made similar observations in mice that constitutively lacked LHCs. Unlike previous DTR models, in the

huLang-DTA mouse expression the active subunit of DTX is driven by the human Langerin promoter [206]. As a result, LHCs are constitutively depleted and Langerin+ dDC are unaffected [206]. Kaplan et al. induced CHS in these mice and observed that immune responses are increased by 2-fold in the absence of LHCs suggesting that LHCs are not only dispensable for priming immune responses, they may suppress immune responses as well [206]. Igyarto et al. also report exaggerated CHS responses in huLang-DTA mice and they saw increased numbers of antigen specific T cells in the skin and SDLNs LHC deficient mice [207]. Tregs were unaffected by the absence of LHCs as Igyarto et al. saw no difference in Treg number between WT and huLang-DTA mice and Tregs in SDLNs expressed similar levels of the skin homing receptor CCR4 in WT and LHC deficient mice [207]. LHC-derived IL-10 suppresses T cell priming during CHS as Langerin-Cre × IL-$10^{fl/fl}$ mice exhibited increased CHS responses compared to littermate controls [207].

Shibaki et al. developed K14-mOVA mice that express membrane-bound OVA, under the control of a keratin 14 (K14) promoter to model self-antigen presentation and argue LHCs mediate peripheral tolerance [208]. They immunized K14-mOVA mice and littermate control mice with OVA and while control exhibited significant footpad swelling, K14-mOVA mice developed no significant immune reaction suggesting that transgenic mice are tolerant to OVA [208]. LCs from K14-mOVA mice but not WT mice could activate OT-I cells in in vitro co-cultures suggesting that LHCs may mediate peripheral tolerance by presenting antigen to T cells in SDLNs [208]. Together these observations show that LHCs don’t participate in T cell priming during adaptive response and can in some cases induce tolerance.

Since LHCs express both HLA molecules in humans and MHC molecules in mice they can present antigen to CD8+ T cells and CD4+ T cells. Skin resident memory Tregs that are

capable of attenuating immune responses upon activation have been described [151] and LHCs could conceivably mediate peripheral tolerance in the skin by activating these Treg populations. In support of this hypothesis, Seneschal et al. found that human LHCs can induce the proliferation of skin resident memory Tregs. LHCs or dDCs isolated from the skin of multiple individual donors were cultured with skin resident memory T cells in vitro. After 6 days LHCs, but not dDCs, induced T cell proliferation and the proliferating T cells in this culture were CD4+CD25+FoxP3+ skin resident memory Tregs [209]. Tregs obtained from this culture were able to suppress the proliferation of skin resident T effector memory (Tem) cells in secondary co-cultures and since LHC-mediated Treg proliferation is significantly reduced in the presence of HLA-DR and HLA-DP blocking antibodies, the proliferation of skin resident memory Tregs is antigen-dependent [209].

Using immunoflorescent microscopy Seneschal found Tregs contacting LHCs in freshly obtained human skin and after performing Ki67 staining, found Tregs proliferate in the epidermis [209]. These in vivo observations were consistent with their findings that LHCs can induce skin resident Treg proliferation. LHCs cultured with CFSE labeled skin resident T cells in the presence of low doses of heat-inactivated *C. albicans* induces increased proliferation of Tregs [209]. Interestingly, high doses of *C. albicans* induced Tem proliferation and these studies suggest LHCs can be immunogenic or tolerogenic depending on the context of antigen presentation [209]. This result seems to reflect the conflicting findings of those studying the contributions of LHCs to immunity. In the data previously reviewed, LHCs can prime immunity in some cases but under the right circumstances LHCs can induce tolerance. Additional studies performed in in vivo models of inflammation may contribute valuable information to this

discussion and our model of vitiligo presents a valuable opportunity to study how LHCs behave in a model of autoimmunity.

While some cells reside in tissue like LHCs, others must be recruited to the periphery to participate in inflammation or peripheral tolerance. To get to peripheral tissues, cells like Teffs or Tregs that are activated and expanded in the SLO organs follow chemokines that provide migratory signals. Chemokines and their receptors coordinate a balanced immune response by carefully orchestrating the recruitment of inflammatory and tolerogenic mediators. If we understand the anti-inflammatory chemokine signals that facilitate tolerance and the pro-inflammatory signals that facilitate Teff migration during vitiligo, we can design interventions that target the appropriate pathways to curtail cutaneous inflammation and promote repigmentation. As a part of this study I investigated the chemokine signals that promote Treg migration during vitiligo and the following reviews the role of chemokine in immunity.

1.28: Chemokines and their receptors direct cell migration.

Chemokines are small positively charged cytokines that can direct the migration of cells expressing the appropriate receptors. The immune system protects the entire body, and to respond to inflammation at any particular locale immune cells rely on the expression of chemokines to traffic to the appropriate places. Chemokines can be segregated into categories based on their structure. The two major groups are “CXC chemokines” that have two cysteine residues near the N-terminus that are separated by another amino acid, and “CC chemokines”, with two adjacent cysteine residues near the N-terminus [210]. “C chemokines” with one cysteine residue and “CX_3C chemokines” with two cysteine residues separated by 3 amino acids exist as well, but few chemokines with these structural arrangements exist [210]. Cells can

secrete chemokines to direct cell migration or tether chemokines on their surface via interactions with molecules known as glycosaminoglycans (GAGs). Surface chemokine immobilization mediated by GAGs was demonstrated in vivo [211] and Proudfoot et al. used chemokines with reduced ability to oligomerize and bind GAGs to demonstrate that GAG-binding and chemokine oligomerization are essential for the ability of chemokines to promote trafficking [212]. Chemokine tethering ensures the stable expression of chemokine at the local production site to mediate the directed migration along an immobilized gradient for cells with the appropriate receptor [212, 213]. Chemokine receptors are differentially expressed on the surface of all leukocytes and cells that express the cognate receptor for a specific chemokine can migrate in response to chemokine gradients. Structurally, chemokine receptors have seven transmembrane domains that mediate signaling via G proteins after binding chemokine [214]. An effective immune response depends on the proper localization of immune cells and chemokines, and their receptors position immune cells during inflammation.

1.29: Chemokine signaling facilitates T cell priming in secondary lymphoid organs.

The repertoire of chemokine receptors that T cells express changes when T cells are primed in the SLOs. Ferguson et al. labeled OT-1 cells with CFSE, adoptively transferred them into mice with OVA-pulsed BMDCs, and used flow cytometry to reveal that the receptors CCR5 and CXCR3 are upregulated on activated OT-1s [215]. CCR5 is the receptor for CCL3, CCL4 and CCL5 and CXCR3 is the receptor for CXCL9, CXCL10 and CXCL11 [216]. Both of the receptors can be expressed by various immune cells, including CD8+ T cells, Th1 CD4+ T cells, and Tregs [216]. Ferguson’s observations demonstrated that initial priming events induce the

expression of CCR5 and CXCR3 on activated CD8+ T cells and CCR5 expression by CD8 T cells can facilitate CD8+ T cells interactions with antigen presenting DCs in lymphoid tissue.

Castellino et al. used two-photon microscopy to image interactions between OT-II CD4+ T cells, OT-I CD8+ T cells, and DCs in the LNs of live mice after OVA immunization and they observed that interactions between T cells and DCs were facilitated by DC- and CD4+ T cell-expressed CCL3 and CCL4 [217]. Blocking antibodies against CCL3 or CCL4 consistently reduced these interactions and $CCR5^{-/-}$ CD8 T cells contacted DCs with less frequency than WT CD8s, supporting the importance of CCR5 in mediating contact between CD8+ T cells and DCs during priming [217]. Furthermore, treating mice with CCL3 and CCL4 blocking antibodies interferes with the ability of mice to form a functional memory CD8+ T cell population in response to immunization with OVA, suggesting that CCR5 plays a crucial role in the initiation of immune responses [217]. Hughes et al. also used imaging to demonstrate that CCR5 mediates contact between DCs and CD8+ T cells in lymphoid tissue [218].

Other chemokines may direct T cells to antigen presenting DCs during priming. DCs express CCL17 to attract CCR4-expressing CD8+ T cells and DC-derived CCL17 increased contact time between DCs and CD8+ T cells [219]. Furthermore, DCs in the lymph node tether T cells with CXCL10 [220], and in vitro studies demonstrated that the chemokines CCL19, CCL21, CXCL10, and CCL5 were bound to the surface of LN-derived DCs and tethered T cells in an antigen-independent manner [221]. These observations demonstrate that DCs can make chemokines to attract and tether T cells in lymphoid tissue and increase the efficiency of T cell priming in the initial phases of an immune response.

In peripheral tissue DCs have also been shown to use chemokines to facilitate interactions with disease-causing effectors. Immunofluorescence staining on psoriatic skin lesions revealed that CCL20 and CCR6 were highly expressed among dermal clusters between HLA-DR+ DCs and skin-infiltrating CD3+ T cells that routinely form in lesional skin [222]. DCs and T cells analyzed by flow cytometry both expressed CCR6 in psoriatic skin a CCL20/CCR6 signaling likely mediates the interactions observed between the two cells in lesional skin [222].

1.30: Chemokine signaling drives activated T cell migration to sites of peripheral inflammation.

The chemokine signals that facilitate the migration of activated T cells out of the lymphoid organs have been extensively studied. After activation, effector T cells downregulate receptors like CCR7 that facilitate entry into the LN and after multiple divisions, activated T cells upregulate the S1P1 receptor that mediates LN exit [223]. After LN exit, T cells follow chemokine gradients that guide them to sites of inflammation and the chemokine receptors that an T cell expresses upon activation depends on the lymphoid environment that the cell is primed in. T cells activated by DCs from SDLNs expressed skin homing receptors like CCR4 and CLA and migrated more efficiently to inflamed skin than those activated by DCs from gut-derived lymphoid tissues [87]. DCs from gut-associated lymphoid tissue induce the expression of the chemokine receptor CCR9 and the integrin $\alpha_4\beta_7$, both crucial for gut specific homing, on activated CD8+ T cells [224, 225]. Activated CD4+ T cells can differentiate into a number of subclasses and the chemokine receptors that they express are tied to their differentiation status and the cytokines that they encounter during activation [216].

1.31: Chemokine signaling promotes Treg localization during homeostasis.

Chemokine signaling also plays a role in Treg function and localization during homeostasis. Two groups used flow cytometry to analyze the expression of chemokine receptors on circulating Tregs isolated from peripheral blood and found that the repertoire of chemokines expressed by Tregs changes from birth to adulthood. Tregs from neonatal cord blood express chemokine receptors that mediate lymphoid tissue homing like CD62L, CCR7, and CXCR4, and chemokine receptors that mediate gut homing such as CCR9 and α4β7 [226, 227]. By 3 years of age Tregs in peripheral blood upregulate inflammatory chemokine receptors and chemokine receptors that mediate entry into the skin and lung, such as CXCR3, CCR2, CCR4, CCR5, CCR6, CCR8; a few Tregs express the gut homing receptors CCR9 and α4β7 [226, 227]. Chemotaxis assays performed on Tregs isolated from infants and adults demonstrated that Tregs exhibit different migratory properties depending on the age of the donor [226]. Tregs from cord blood migrated in response to CCL19 and CCL25, the ligands for CCR7 and CCR9, respectively, while adult Tregs migrated in response to CCL22, the ligand for CCR4 [226]. These findings demonstrate that the shift in homing receptor expression observed on Treg cells seems to correspond to their actual migration properties.

Murine models allow us to study early chemokine signals that govern Treg migration, and chemokine signaling positions murine Tregs in the skin during early development. Using flow cytometry, Scharschmidt et al. found a wave of Tregs enter mouse skin after postnatal day 6 [149] and require CCR6 to follow hair follicle-derived CCL20 to migrate to the skin during neonatal development [152]. RT-PCR revealed that CCL20 RNA was upregulated in the skin of neonatal mice during the height of Treg migration to the skin and Tregs isolated from the skin of

mice during this period expressed CCR6 RNA [152]. In vitro, the exposure of human fetal skin explants to commensal skin bacteria or bacterial peptides augmented CCL20 expression, highlighting a role for commensal bacteria in promoting cutaneous Treg populations. Flow cytometry revealed that Tregs in neonatal skin expressed significantly higher levels of CCR6 than Tregs in SDLNs and skin-resident CD4+ and CD8+ Teffs [152]. $CCR6^{-/-}$ Tregs co-transferred into $RAG2^{-/-}$ mice with WT Tregs exhibited a decreased ability to migrate to the skin of 2 week old mice compared to WT Tregs, and these results demonstrated that CCL20/CCR6 interactions play an important role in establishing skin resident Treg populations during neonatal development [152].

1.32: Chemokine signaling promotes Treg migration and suppression in peripheral tissues.

Various chemokine signals promote Treg migration to peripheral tissues during inflammation. Tregs upregulate specific chemokine receptors in response to activating stimuli and this facilitates lymph node exit and migration to non-lymphoid tissue entry. Lee et al. reported that murine Tregs initially express secondary lymphoid homing receptors after they develop in the thymus, and these $CD62L^{+}CCR7^{+}CXCR4^{low}$ Tregs preferentially migrate to the spleen and LNs [228]. Tregs in LNs up-regulated inflammatory receptors like CCR2, CCR4, CCR5, CCR6, CCR8, CCR9, CXCR3, CXCR5, and CXCR6, while simultaneously down-regulating LN homing receptors in response to antigen exposure [228]. This shift in chemokine receptor expression upon antigen exposure facilitated efficient Treg migration to non-lymphoid tissues such as bone marrow and the small intestine [228]. Another in vitro study analyzed the ability of Tregs from peripheral human blood to migrate to various chemokines and revealed that Tregs readily migrated to CCL1, the ligand for CCR8, as well as CCL22 and CCL17, the ligands

for CCR4 [229]. Immunofluorescence confirmed that Tregs in human peripheral blood express skin homing receptors CCR8 and CCR4, suggesting circulating Tregs are primed to follow chemotactic signals to peripheral tissue [229].

In a mouse model of GVHD Tregs required CCR8 signaling to prevent lethal GVHD. WT and $CCR4^{-/-}$ Tregs adoptively transferred into hosts were able to suppress lethal GVHD but $CCR8^{-/-}$ Tregs were unable to mediate a similar protective effect [230]. $CCR8^{-/-}$ Tregs were able to efficiently migrate to all lymphoid and non-lymphoid tissues analyzed but exhibited an increased susceptibility to death due to their inability to interact with DCs [230]. In this instance CCR8 signaling does not facilitate Treg-mediated suppression by promoting migration to peripheral tissues. Instead, CCR8 signaling facilitates Treg function by mediating interactions with DCs that promote Treg survival. In another transplantation model, Treg chemokine signaling facilitates the suppressive capabilities of Tregs. Zhang et al. observed that Tregs migrated from blood to a transplanted allograft where they promoted graft survival [231]. They observed that Tregs required CCR2, CCR4, and CCR5, as well as P- and E-selectin ligands to ultimately suppress T effector cell migration and proliferation in LNs and allografts [231].

CCR4 signaling mediates Treg suppression in various models of inflammation, including a cardiac transplantation and IBD model of disease [232, 233]. Notably, Sather et al. demonstrated that the CCR4 signal facilitates Treg migration to the skin during inflammation. They performed flow cytometry on tissue from $Foxp3^{GFP}$ mice and observed Tregs distributed among all the tissues they analyzed, including the skin [154]. While Tregs in all tissues express some level of CCR4, nearly all the Tregs in the skin expressed CCR4 and CD103 and Tregs in SDLNs upregulated CCR4 and CD103 after exposure to antigen [154]. Sather et al. generated mixed bone marrow chimeras with congenically marked WT and $CCR4^{-/-}$ Tregs to compare their

ability to migrate to various tissues and found that CCR4$^{-/-}$ Tregs exhibited significantly impaired migration to the skin and lungs compared to WT Tregs [154]. CCR4 signaling in Tregs was important for protecting these tissues from inflammation, as only WT Tregs and not CCR4$^{-/-}$ Tregs adoptively transferred into neonatal scurfy mice could prevent systemic autoimmunity and skin inflammation, to which scurfy mice eventually succumb [154]. WT Tregs prevented inflammation in the skin, lungs, and liver of scurfy mice, but CCR4$^{-/-}$ Tregs only prevented inflammation in the liver and not the skin or lungs [154]. These results highlight a role for CCR4 in promoting Treg migration and suppression in the skin and lung.

CXCR3 is normally expressed by activated CD8+ T cells and Th1 CD4+ T cells but Tregs can also express CXCR3, and in some instances require CXCR3 signaling to mediate suppression. Koch et al. used flow cytometry to observe that Tregs in the spleens of WT mice express CXCR3 and found that Tregs upregulate CXCR3 expression in response to Th1 inflammation [234]. T-bet is a transcription factor that controls the expression of Th1-associated genes like CXCR3, and Tregs seemed to require Th1 identity to suppress inflammation as WT Tregs but not T-bet$^{-/-}$ Tregs could suppress lymphadenopathy and splenomegaly upon adoptive transfer to scurfy mice [234]. In a model of EAE, Tregs require CXCR3 to migrate to the site of inflammation. When EAE is induced in WT and CXCR3$^{-/-}$ mice, CXCR3$^{-/-}$ mice exhibit more severe chronic disease and increased loss of myelin and axonal injury compared to WT mice [235]. Inflammation is more severe in the absence of CXCR3 and this correlates with a reduction of Tregs among CNS-infiltrating cells in CXCR3$^{-/-}$ mice [235]. CXCR3 signaling can also mediate Treg suppression in the skin. Suga et al. induced contact hypersensitivity (CHS) in WT and CXCR3$^{-/-}$ mice and observed that recovery from Th1-type CHS was delayed in CXCR3$^{-/-}$ mice [236]. After using microscopy to quantify fluorescently-labeled Tregs in inflamed ears,

Suga et al. counted less Tregs in the skin of $CXCR3^{-/-}$ mice compared to WT mice during CHS, suggesting that Tregs require CXCR3 to migrate to the skin and suppress Th1-mediated inflammation [236]. $CXCR3^{+}$ Tregs injected i.v. into $CXCR3^{-/-}$ mice before CHS induction were capable of normalizing recovery from Th1 type inflammation, further supporting a role for CXCR3 in mediating Treg suppression in the skin [236].

$CCR6^{-/-}$ Tregs migrate to the site of inflammation less efficiently than WT Tregs in an inducible mouse model of EAE [237]. Chaudhry et al. observed that $Stat3^{flox}$×$Foxp3^{CRE}$ Tregs and not WT Tregs fail to prevent colitis when adoptively transferred into $Rag^{-/-}$ mice with disease-causing CD4+ T effectors [238]. While $Stat3^{flox}$×$Foxp3^{CRE}$ Tregs can suppress Teffs in vitro, they express significantly lower levels of CCR6 and exhibit a diminished capacity to migrate to the gut when compared to Tregs from littermate controls [238]. Similarly, $CCR6^{-/-}$ Tregs exhibited diminished suppressive capabilities compared to WT Tregs in another mouse model of colitis and the number of $CCR6^{-/-}$ Tregs relative to effectors was reduced compared to WT Tregs [239]. These studies demonstrate that CCR6 can mediate Treg suppression in the gut and central nervous system during inflammation by facilitating their migration to these peripheral tissues. CCL20/CCR6 signaling has also been reported to mediate Treg suppression of inflammation in the skin. Barth et al. subcutaneously injected the footpads of WT and $CCR6^{-/-}$ mice with *Leishmania major* and found that footpad swelling was enhanced in the absence of CCR6 [240]. Disease-causing T cells were more abundant in the skin of $CCR6^{-/-}$ mice and the number of Tregs was significantly reduced in the skin draining lymph nodes of $CCR6^{-/-}$ mice compared to WT mice [240]. Interestingly the number of Tregs in the skin was comparable between WT and $CCR6^{-/-}$ mice but nonetheless the importance of CCR6 for recruiting Tregs to

the draining lymph node to suppress excessive effector cell activation and subsequent skin damage is clear.

CCR5 signaling can also mediate Treg migration to peripheral tissue and subsequent suppression during inflammation. In a mouse model of GVHD, $CCR5^{-/-}$ Tregs were less effective than WT Tregs in suppressing GVHD and prolonging median survival time of engrafted host mice [241]. While $CCR5^{-/-}$ Tregs isolated from host GVHD target tissues like the spleen and liver were capable of suppressing T cell proliferation in vitro, significantly fewer $CCR5^{-/-}$ Tregs infiltrated GVHD affected tissues [241]. This suggests that the reduced ability of $CCR5^{-/-}$ Tregs to suppress GVHD stems from a migratory defect and not a functional defect. Similarly, CCR5 signaling promotes the accumulation of Tregs in the heart in a model of myocardial infarction that subsequently prevents uncontrolled post-infarction inflammation [242]. CCR5 ligands were highly expressed in the infarcted myocardium of WT mice and after the induction of myocardial infarction in WT and $CCR5^{-/-}$ mice there were significantly fewer Tregs in the mononuclear infiltrate from the infarcted hearts of $CCR5^{-/-}$ mice [242]. This reduced Treg migration accompanied increased inflammation indicated by the marked upregulation of proinflammatory cytokines in the hearts of $CCR5^{-/-}$ mice [242].

In paracoccidioidomycosis, a fungal infection, Tregs accumulate in skin lesions and contribute to persistent infection by suppressing local antifungal immune responses [243]. Tregs in the skin during infection express CCR5 and CCR5 ligands are highly expressed in lesional skin [243]. In a mouse model of this disease, Tregs in $CCR5^{-/-}$ mice exhibit impaired migration to pulmonary lesions [244] and together these studies suggest CCR5 may promote Treg localization and suppression in peripheral tissues. CCR5 signaling has also been reported to promote the cutaneous localization of Tregs during inflammation. Yurchenko et al. used RT-

PCR and flow cytometry to demonstrate that Tregs isolated from the LNs of mice express more CCR5 at the transcriptional and protein level than CD4 T cells [245]. Consequently, Tregs migrated more readily than CD4+ T cells in response to the CCR5 ligands CCL3, CCL4, and CCL5 during in vitro transwell assays [245]. Tregs isolated from $CCR5^{-/-}$ mice suppress the in vitro proliferation of T cells in response TCR stimulation as well as WT Tregs, but when $Rag^{-/-}$ mice are infected with *Leishmania major* and reconstituted with effectors, $CCR5^{-/-}$ Tregs fail to control effector T cell expansion and IFN-γ production in inflamed dermis, while WT Tregs are effective [245]. $CCR5^{-/-}$ Tregs migrated to the inflamed dermis less efficiently than WT Tregs [245] and these results suggest that CCR5 promotes the migration of Tregs to the skin during inflammation to mediate effector suppression.

1.33: Working toward better understanding of the regulatory factors involved during vitiligo.

In order to mediate depigmentation, CD8+ T cells must overwhelm the mechanisms of peripheral tolerance that the body has evolved to prevent autoimmunity. While the factors that contribute to depigmentation have been well studied, few have addressed the suppressive factors at play during disease. Vitiligo skin is characterized by patchy depigmentation with white spots surrounded by unaffected skin, and cases of complete depigmentation are rare. Why don't disease causing Teffs depigment the entire epidermis? What causes Teffs to eventually stop where they do, at the lesional border? To answer these questions I hypothesize that regulatory factors function in the skin during vitiligo to keep Teffs from mediating their maximum destructive potential. If we understand the mechanisms of suppression used during vitiligo, we can use this information to improve existing treatment strategies or discover new ones. Tregs and LHCs reside in the skin during vitiligo and have been shown to potentiate T cell responses

during inflammation, as well as autoimmunity. In light of this they are properly positioned to protect melanocytes in the skin during disease and I investigated whether these populations suppress depigmentation during vitiligo.

In the following chapters I use a number of strategies within our mouse model of vitiligo to investigate the contributions of Tregs and LHCs to vitiligo. I investigated the severity of disease in the absence of Tregs in **Chapter I** and explore their interactions with Teffs in the skin during human and mouse disease. In **Chapter II** I investigated whether LHCs play in immunogenic or tolerogenic role during vitiligo and the mechanisms of their function during disease. Finally in **Chapter III** I investigated the chemotactic signals that facilitate Treg function during vitiligo.

CHAPTER II: REGULATORY T CELLS SUPPRESS DEPIGMENTATION IN A MOUSE MODEL OF VITILIGO

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Contribution Summary:

K.I.E helped to design the experiments, performed the experiments, and helped to analyze the data and write the manuscript. J.P.S performed suction blistering to obtain skin infiltrating cells from human skin, D.B. performed IHC on biopsies from vitiligo patients, M.R helped design BrdU incorporation experiments. J.E.H. helped to design the experiments, analyze the data, and write the manuscript.

2.1: Abstract

In this study I explored the role of T regulatory cells (Tregs) in suppressing effector T cell responses during vitiligo. In a mouse model of vitiligo we observed that depletion of Tregs with both CD4-depleting antibody and in diphtheria toxin-treated Krt14-Kitl×Foxp3-DTR hosts resulted in worsened disease as well as an increase in Teff skin infiltration, suggesting that Tregs also play a suppressive role in vitiligo. While characterizing the distribution of Tregs in the skin, I observed that regions of skin with more Tregs depigment less readily in our mouse model of disease. This suggests that Tregs function in the skin under homeostatic conditions to suppress depigmentation and that Treg number in the skin is important for suppressing disease. In vitiligo patients the Treg/Teff ratio is decreased in lesional skin compared to non-lesional skin, further reflecting what I observed in our mouse model and further supporting the importance of Treg number in the skin for suppression. Using in situ confocal microscopy we observed that Tregs directly contact Teffs in lesional mouse skin during vitiligo, and we observed similar interactions in human lesional skin, suggesting that Tregs interact with Teffs in the skin to potentially suppress through contact-dependent mechanisms.

2.2: Introduction

Tregs in human vitiligo

Treg suppressive function may be compromised during vitiligo. Tregs isolated from the PBMCs of vitiligo patients were reported to have a reduced ability to suppress the proliferation and cytokine production of Teffs isolated from the vitiligo PBMCs in vitro [246]. In contrast, another study reported that Tregs in PBMCs from vitiligo patients and healthy controls equally suppress CD4+ T Cell proliferation but Tregs from vitiligo patients have a reduced capacity to migrate to skin [247]. Whether the presence of Tregs in the skin of vitiligo patients affects disease severity has not been definitively proven. Ahmed et al. compared Treg numbers from vitiligo patients and healthy controls and saw no significant differences in the skin or in PBMCs [248]. Terras et al. also saw no differences in skin Treg number after performing immunohistochemistry on skin biopsies from vitiligo patients and healthy controls [249]. In contrast, another study where immunohistochemistry was performed on skin biopsies from vitiligo patients found the CD4/CD8 ratio is decreased among the skin infiltrating cells of vitiligo patients [62]. Abdallah et al. also performed immunohistochemical analysis of skin biopsies and saw a significant reduction in the number of Foxp3+ Tregs [250]. In another study Klarquist et al. stained frozen sections from lesional vitiligo and healthy skin with CD3 and FoxP3 to count Tregs and observed that the percentage of Tregs among skin infiltrating T cells is reduced in vitiligo patients compared to healthy controls [247]. In this study Klarquist found circulating Treg numbers were unchanged in vitiligo patients but CCL22 expression – which facilitates Treg skin migration – was reduced in vitiligo, potentially explaining the reduced Treg number in vitiligo skin [247]. These studies suggest that reduced Treg number in the skin during vitiligo may contribute to depigmentation.

Treg suppression in mouse models of vitiligo

A few groups performed studies in mouse models of vitiligo to demonstrate that increased Treg recruitment to the skin correlates with reduced disease severity. In two different murine models of spontaneous vitiligo, gene gun treatment to induce CCL22 expression in the skin led to increased cutaneous Treg migration and decreased depigmentation [155]. Another group found that adoptively transferring 2×10^5 Tregs to 3 week old vitiligo prone mice increased numbers of Tregs in the skin and almost completely abrogated development of disease [251]. Teffs in SDLNs are not suppressed and in light of this, the authors correlated disease prevention with increased Treg number and suppression specifically in the skin [251]. Miao et al. injected vitiligo prone mice with PD-L1-Fc and this enhanced Treg accumulation in the skin and markedly reversed depigmentation [252]. These results support the hypothesis that Treg number in skin is important for reducing Teff-driven depigmentation and that mouse models of vitiligo will prove invaluable for understanding exactly how Tregs work during vitiligo.

A number of groups have developed mouse models of vitiligo to investigate the role of Tregs by CD4 or CD25 depletion. Gregg et al. developed a spontaneous mouse model of vitiligo based on the human tyrosine epitope, Tyr_{369}, recognized by CD8+ T cells. They bred AAD mice that express a recombinant MHC I molecule containing the binding region of human HLA-A*0201 specific for Tyr_{369} to albino mice that do not express tyrosine in the thymus and thus do not establish immunological tolerance to tyrosine [74]. As result, autoreactive T cells with TCRs targeting the melanocyte protein tyrosinase escape thymic deletion and mediate depigmentation as early as postnatal day 3 [74]. Chatterjee et al. used the h3AT2 mouse, which spontaneously develops vitiligo and expresses an HLA-A2 restricted human TCR that recognizes the tyrosinase epitope $tyrosinase_{368\text{-}376}$ [75, 251]. Both of these groups observed an increase in the severity of

vitiligo after treating mice with CD4 depleting antibody or CD25 depleting antibody that preferentially targets Tregs [74, 251] and these results suggest that CD4+CD25+ Tregs suppress depigmentation during vitiligo.

Despite the increasing evidence that Tregs affect disease severity, many questions remain regarding Treg function. We have developed a mouse model of vitiligo in which hosts develop depigmentation characteristic of human disease. In our model, IFNγ drives CD8+ T cell destruction of melanocytes that are retained in the epidermis and the hair retains pigment, even in depigmented skin. The similarities between our mouse model of vitiligo and human disease allow us to investigate how Tregs affect vitiligo in the proper context and potentially address some of the inconsistencies about how Tregs function in vitiligo and other cases of peripheral inflammation. In light of this, I used our mouse model of vitiligo to investigate the role of Tregs during vitiligo.

2.3 Results

2.3.1: CD4 Depletion Exacerbates Vitiligo.

I tested previously established methods of Treg depletion in our mouse model of vitiligo to assess the severity of disease in the absence of Tregs. I hypothesized that mice without Tregs will exhibit increased disease since two other groups have observed that Tregs suppress vitiligo in their mouse models of disease using CD4 or CD25 depleting antibodies [74, 251]. I induced vitiligo as previously described in Krt14-Kitl×FoxP3-GFP mice, with Tregs expressing GFP under control of the FoxP3 reporter. After 2 weeks, I treated one group of mice with 0.2mg of GK1.5 CD4 depleting antibody once weekly to deplete Tregs. To serve as controls, I treated another group of mice with PBS. After 5 weeks of treatment, a separate observer blinded to the

experimental groups quantified the severity of depigmentation on ears, nose, hind footpad and tail skin. Each site was given a vitiligo score that corresponds to the percent of skin depigmented and the score at each site can be combined to represent a total vitiligo score. When I compared the severity of disease, I observed that CD4-depleted mice exhibited almost 3 times more depigmentation than PBS treated mice, and CD4-depleted mice had an average vitiligo score of 13.6 ± 1.408 compared to an average score of 4.3 ± 1.375 in PBS controls (Fig 1A). This mirrors observations from the other previously described mouse models of vitiligo. Previous studies in our mouse model demonstrated that disease severity correlates with the number of disease causing Teffs in the skin [79, 253]. Seven weeks after disease induction, I harvested skin from mice, mechanically separated the dermis from the epidermis and used flow cytometry to count the number Teffs in digested tissues. I found significantly more Teffs in the skin of mice treated with CD4-depleting antibody compared to mice treated with PBS (Fig 1B). I also observed more Teffs in the SDLNs of mice treated with CD4-depleting antibody but this difference was not significant. The number of Tregs relative to Teffs was significantly decreased in the SDLNs, tail and ear skin of CD4-depleted mice (Fig 1C), confirming that CD4 depletion affects Treg number. This data suggests that CD4 T cells are dispensable for disease progression and a subset of CD4 expressing cells suppresses depigmentation by limiting the number of Teffs in the skin during disease.

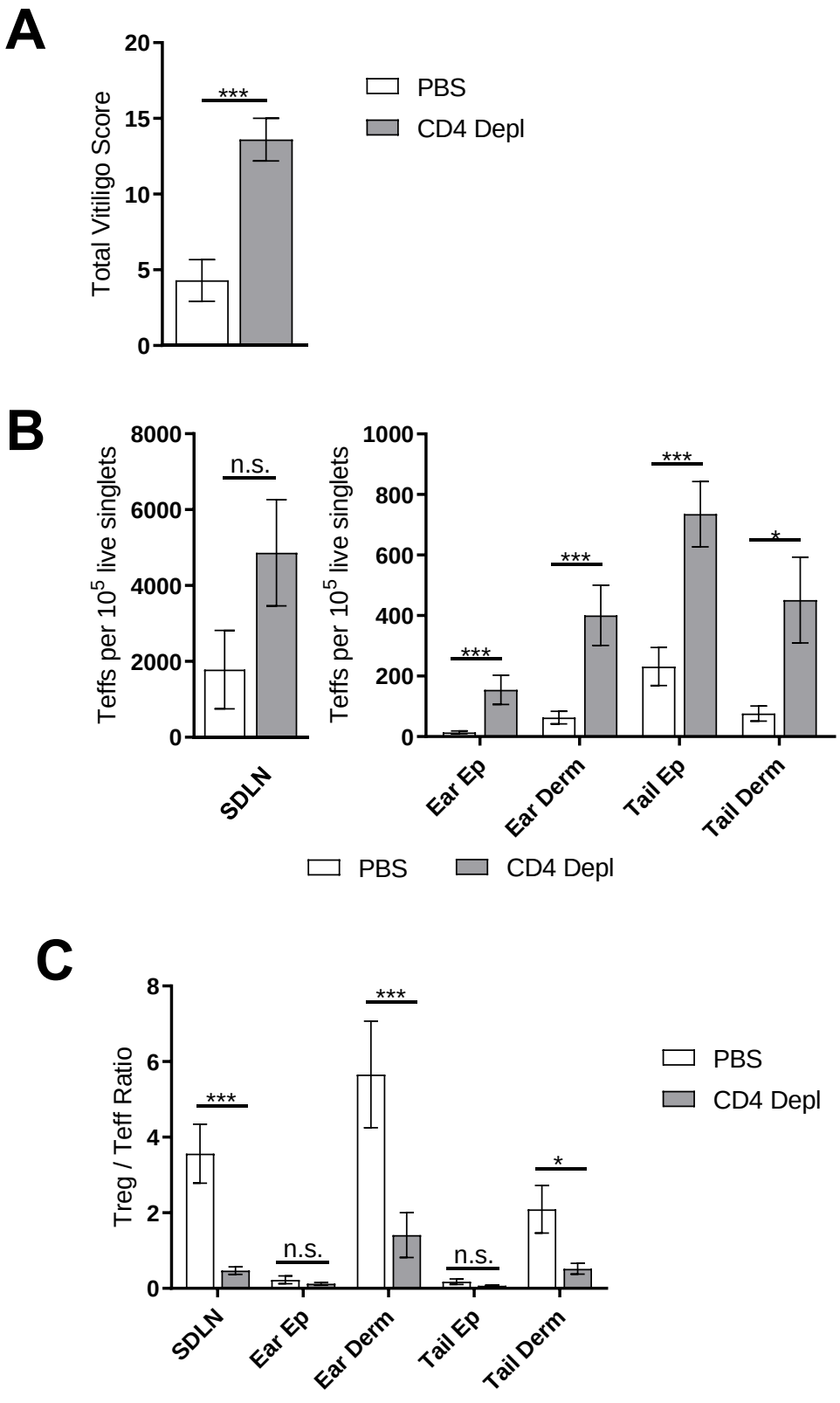


Figure 1: CD4 depletion exacerbates vitiligo.

Figure 1: CD4 depletion exacerbates vitiligo. Tissues harvested from Krt14-Kitl×FoxP3-GFP mice with vitiligo and treated with either PBS or GK1.5 CD4 depleting antibody (CD4 Depl) were analyzed by flow cytometry 5 weeks after disease induction. Graphs depict; **(A)** quantification of depigmentation; **(B)** the average number of Thy1.1+ Teffs normalized to the number of live single cells in the indicated tissues; or **(C)** The number of GFP+ Tregs divided by the number of Thy1.1+ Teffs in the indicated tissues represented as a Treg/Teff ratio; ***N=10, student T-test, bars represent s.e.m.***

2.3.2: CD4 depletion does not increase Teff proliferation in the skin.

I hypothesized that increased Teff proliferation in the skin was responsible for the significant increase in Teff number in the absence of Tregs. To test this, I induced vitiligo and injected Bromodeoxyuridine (BrdU) i.v. 6 hours before takedown into mice that received CD4-depleting antibody or PBS. BrdU is a thymidine analog that cells incorporate into newly synthesized DNA during replication and proliferating cells can be identified with antibodies against BrdU. I sacrificed mice 3 or 5 weeks post disease induction, processed skin and SDLN tissue into single cell suspensions, permeablized and stained cells with fluorescent anti-BrdU antibodies and identified proliferating cells with flow cytometry. I found few Teffs in mice treated with PBS or CD4-depleting antibody incorporated BrdU in the skin. In the SDLNs I observed a significant increase in Teff proliferation in mice treated with CD4 depleting antibody at week 3 and week 5 (Fig 2A). Since Teffs in the skin do not incorporate more BrdU after treatment with CD4 depleting antibody, it is unlikely that Teff proliferation in the skin was responsible for the increase in Teff number in the absence of Tregs. In contrast to Teffs, I observed that CD45- cells incorporated BrdU in the skin at week 3 and week 5 and amount of BrdU incorporating cells does not significantly change after treatment with CD4 depleting antibody.

2.3.3: Tregs suppress depigmentation in a mouse model of vitiligo.

CD4 depletion will affect Tregs and all other CD4+ cells. To address the role of only Tregs in vitiligo, I generated Krt14-Kitl×FoxP3-DTR mice. In these mice Tregs express the diphtheria toxin receptor under control of the FoxP3 promoter and treatment with diphtheria toxin (DTX) will specifically and conditionally deplete Tregs [254]. I chose to conditionally

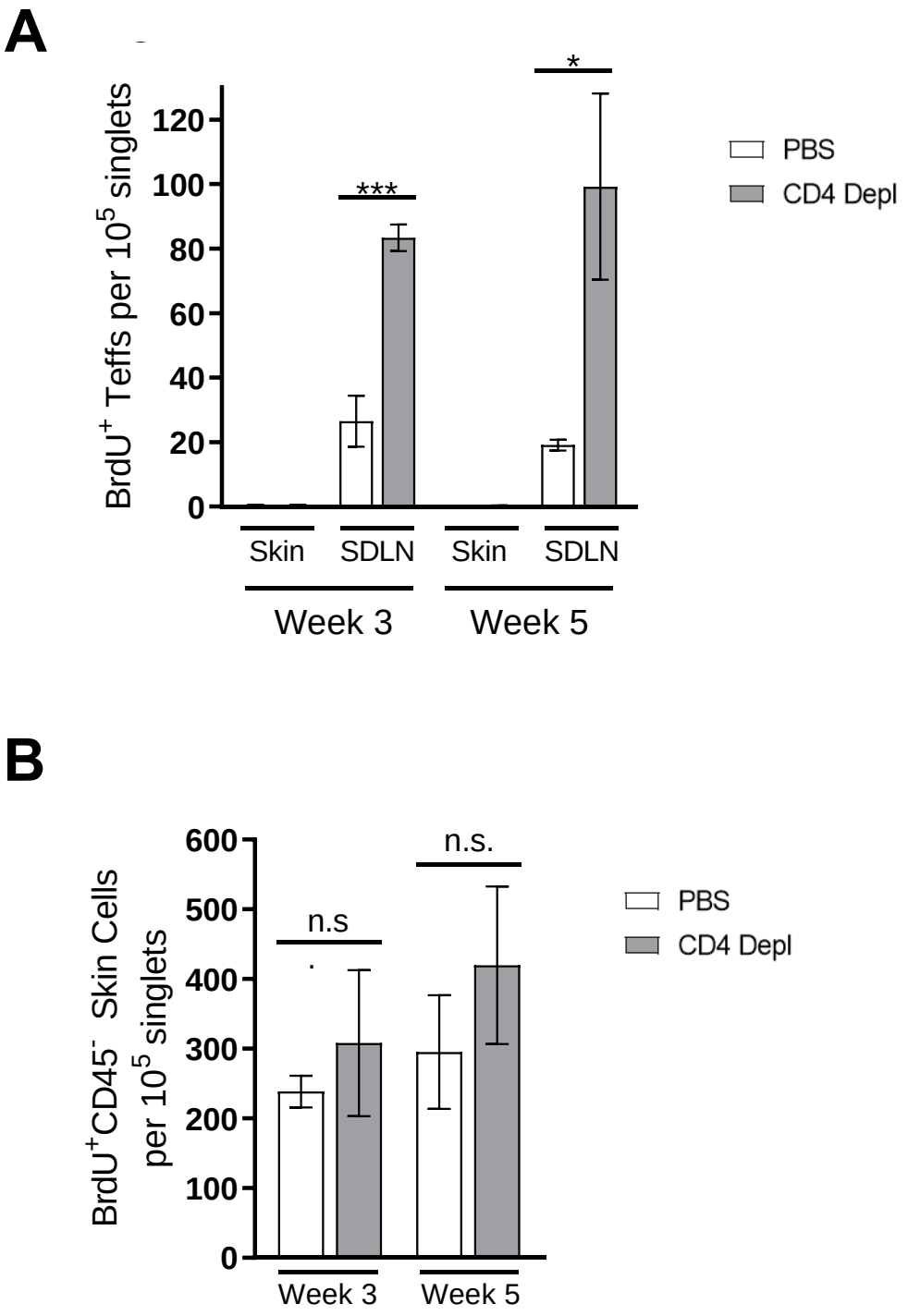


Figure 2: CD4 depletion does not increase Teff proliferation in the skin during murine vitiligo.

Figure 2: CD4 depletion does not increase Teff proliferation in the skin. Vitiligo mice treated with either PBS or GK1.5 CD4 depleting antibody (CD4 Depl) were dosed with Bromodeoxyuridine (BrdU) i.v. 6 hours before harvested tissues were analyzed using flow cytometry. **(A)** Graph depicts the average number of BrdU incorporating Thy1.1+ Teffs normalized to the number of single cells in the indicated tissues; **(B)** Graph depicts the average number of BrdU incorporating CD45- cells normalized to the number of single cells in the skin is shown; ***N=3, student T-test, bars represent s.e.m.***

deplete Tregs since they are crucial for development and survival, as scurfy mice with mutations in the Foxp3 gene die within 3 to 4 weeks of age [113]. Depletion of Tregs only during vitiligo will allow mice to develop normally without other lethal autoimmunity. I induced vitiligo in Krt14-Kitl×FoxP3-DTR mice and, 2 weeks post disease induction, I specifically depleted Tregs by treating mice with 50µg/kg of DTX or PBS every other day for 2 weeks. I chose to treat mice for 2 week windows since repeated DTX administration results in toxicity after continuous dosing for 2 weeks. At 7 weeks post disease induction, a separate observer blinded to the experimental groups quantified the severity of depigmentation on ears, nose, hind footpad and tail skin to generate a vitiligo score. I observed that depigmentation was increased in DTX treated mice compared to PBS treated mice (Fig 3A). I processed the SDLNs and skin of these mice into single cell suspensions and used flow cytometry to compare Teff numbers in mice treated with DTX or PBS. I observed the number of Teffs in the SDLN of DTX treated mice is increased but not significantly (Fig 3B). I also observed an increase in the number of Teffs in the ear skin but interestingly not in the tail skin of DTX treated mice (Fig 3B). When I measured disease severity I observed that the difference in depigmentation between DTX treated and PBS treated mice was more pronounced in ear skin than in tail skin (Fig 3C). This may be explained by the fact the Teff number does not increase in the tails of mice treated with DTX (Fig 3B). Overall Krt14-Kitl×FoxP3-DTR mice exhibit increased depigmentation when treated with DTX. These results mirror findings from the CD4 depletion studies and suggest that Tregs are the CD4+ population that suppresses depigmentation during vitiligo.

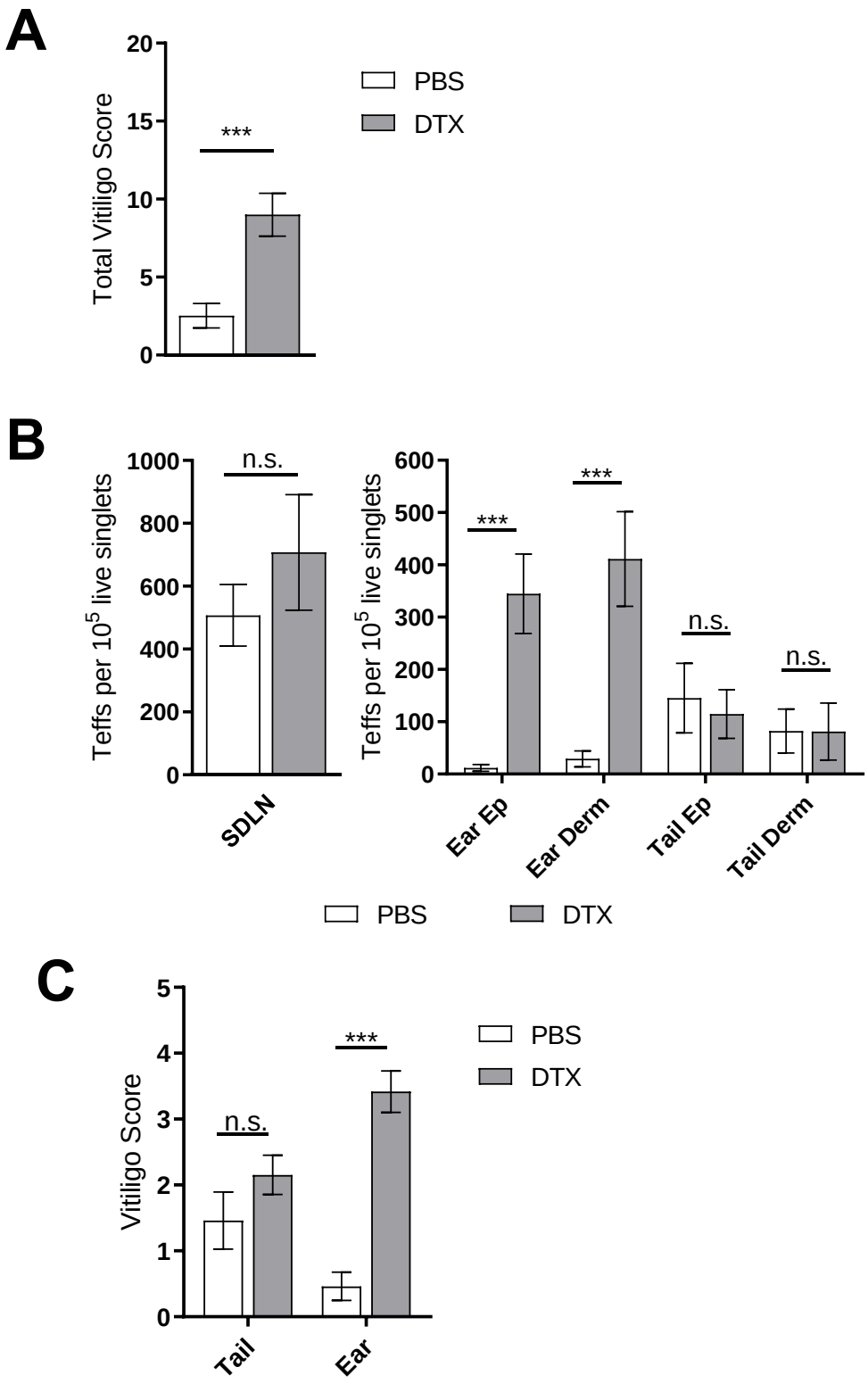


Figure 3: Tregs suppress depigmentation in a mouse model of vitiligo.

Figure 3: Tregs suppress depigmentation in a mouse model of vitiligo. Krt14-Kitl×FoxP3-DTR vitiligo mice were treated with PBS or diphtheria toxin (DTX) and tissues harvested 7 weeks post disease induction were analyzed by flow cytometry. **(A)** Graph depicts depigmentation quantified by a blinded observer 7 weeks post disease induction; ***N=19 PBS, N=13 DTX; students T-test; bars represent s.e.m.*****; (B)** Graph depicts the average number of Thy1.1+ Teffs normalized to the number of live single cells in the indicated tissues; ***N=8; students T-test; bars represent s.e.m.*****; (C)** Graph depicts depigmentation on tail and ear skin of mice quantified by a blinded observer; ***N=12, student T-test, bars represent s.e.m.***

2.3.4: Treg number in the skin correlates with disease severity.

Vitiligo is more severe in mice treated with DTX but Treg depletion seems to affect ears more than tails (Fig 3C). To explore why different regions of skin seem more susceptible to disease after Treg depletion I used flow cytometry to count Tregs in the skin of 8-week-old Krt14-Kitl×FoxP3-GFP mice without vitiligo. After mechanically separating ear and tail dermis from epidermis and processing skin and SDLN tissue into single cell suspensions, I counted GFP+ Tregs in each location. I counted few Tregs in the ear or tail epidermis and I observed that the majority of the Tregs reside in the dermis (Fig 4A). I compared the number of Tregs between the ear and tail dermis and found that there are almost four-fold more Tregs in the ear dermis (Fig 4A). I compared the extent of depigmentation between the ears and tails of 177 mice with a total vitiligo score of at least 5 and I observed that tail skin generally exhibits more depigmentation than ear skin 5 to 7 weeks after disease induction (Fig 4B). In light of this data I reasoned that the increased number of Tregs in ear skin may promote a more immunosupressive environment in ear skin than in tail skin. This could be why tails are more susceptible to depigmentation than ears and why Treg depletion affects ears more than tails.

After observing that the number of Tregs in mouse skin may affect disease severity I sought to use flow cytometry to compare Treg numbers between lesional and non-lesional skin of human vitiligo patients. In collaboration with others in the lab, we used suction blistering to isolate skin infiltrating cell populations from the lesional and non-lesional skin of vitiligo patients and I used flow cytometry to count CD3+CD8+ T cells and CD3+CD4+CD25+FOXP3+ Tregs. I observed that the Treg/CD8 ratio is decreased in lesional skin compared to non-lesional skin (Fig 4C) suggesting Treg number in the skin may correlate with disease severity.

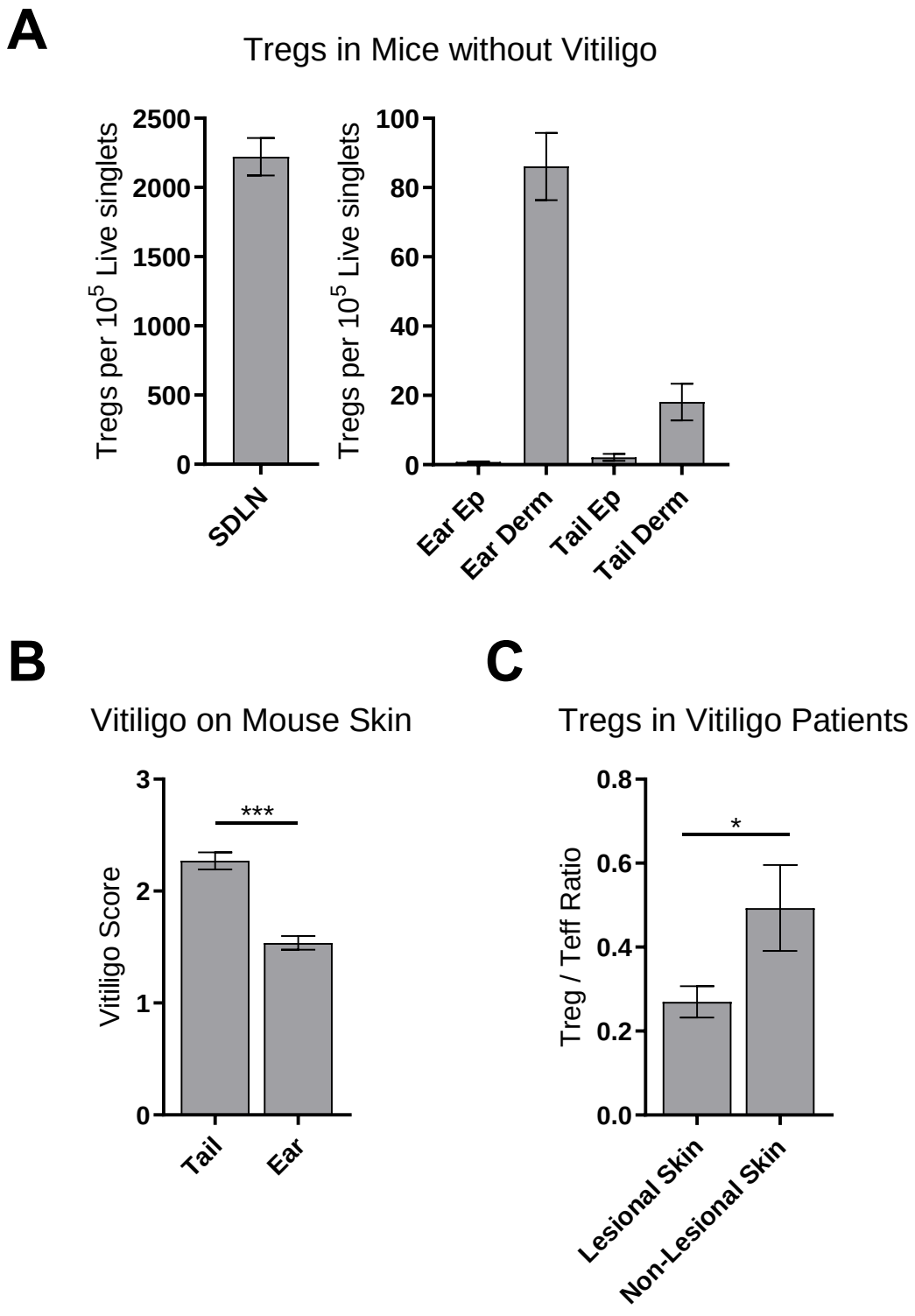


Figure 4: Treg number in the skin inversely correlates with disease severity.

Figure 4: Treg number in the skin correlates with disease severity. (A) Tissues harvested from 8-week-old Krt14-Kitl×FoxP3-GFP mice without vitiligo were analyzed by flow cytometry and graphs depict the average number of GFP+ Tregs normalized to the number of live single cells in the indicated tissues; ***N=12, students T-test, bars represent s.e.m.*; (B)** Comparison of depigmentation between the ears and tails of mice with a total vitiligo score of at least 5; ***N=177, students T-test, bars represent s.e.m***; **(C)** Cells isolated from the skin of vitiligo patients by suction blistering were analyzed by flow cytometry and graph depicts the number of Tregs divided by the number of Teffs in a sample is represented as a Treg/Teff ratio; ***N=18 lesional, N=10 non-lesional, bars represent s.e.m*.**

2.3.5: Tregs and Teffs accumulate in the skin with similar kinetics.

Previous studies in our mouse model demonstrated that Teffs migrate to the skin to facilitate disease but the kinetics of Treg migration were not explored. To investigate Treg migration in our mouse model I induced vitiligo in Krt14-Kitl×FoxP3-GFP mice and used flow cytometry to quantify the number of Teffs and Tregs in the SDLNs and skin of mice 3, 5, 7 and 12 weeks post disease induction. I identified very few Tregs in the ear epidermis and the number of Tregs remained minimal at all weeks as disease progressed (Fig 5a). The majority of Tregs in the ear were in the dermis and I observed the number of Tregs reached its peak at 5 weeks post disease induction, what we have previously observed as the peak of disease. By week 12 the number of Tregs contracts to levels comparable to week 3 early in disease progression (Fig 5a). I observed Tregs in the tail epidermis but the number of Tregs peaked at week 3, early in disease, and remained almost the same for the duration of disease (Fig 5b). I also found that similar to the ear, most of the Tregs in the tail were in the dermis and Treg numbers in the tail dermis begin to increase between week 0 and week 3 and peak at week 5 (Fig 5b). After week 5, Treg numbers begin to wane and reached levels comparable to week 3 early in disease progression (Fig 5b). During disease, at all weeks, I observed the most Tregs in the ear dermis. In fact, the number of Tregs in the ear dermis at the beginning of disease induction is comparable to the number of Tregs in the tail dermis at week 5, when I observed the most Tregs in the tail. The peak number of Tregs in ear dermis is on average more than two fold greater than the peak number of Tregs in the tail (Fig 5a,b). While the magnitude of Treg number is different in the ear and tail dermis, overall the trend is the same: the number of Tregs begins to increase between 0 and 3 weeks post disease induction and peaks at 5 to 7 weeks before contracting to levels comparable to the early in disease progression. In SDLNs, I observed the opposite of what I saw in the skin. Treg

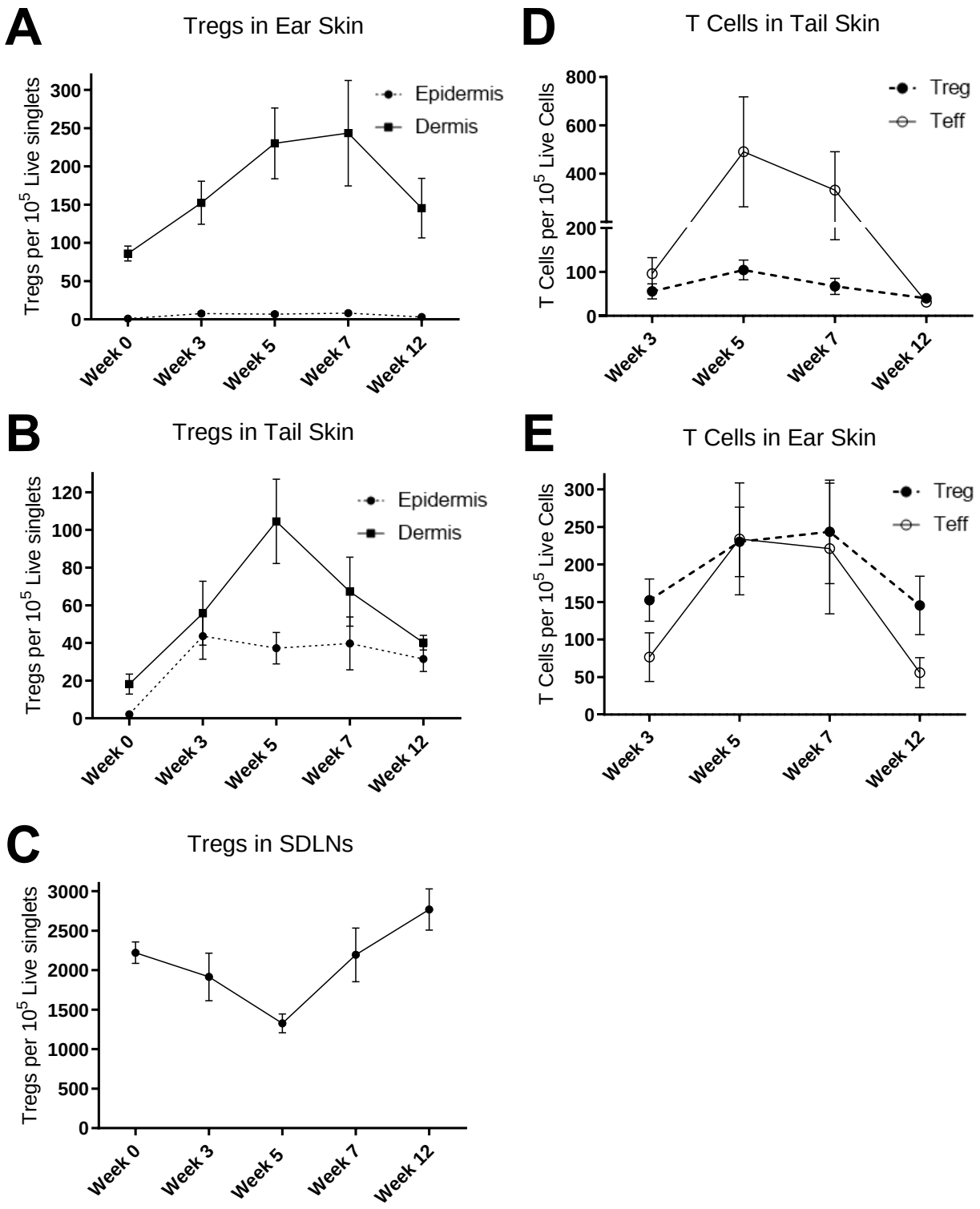


Figure 5: Tregs and Teffs accumulate in the skin with similar kinetics.

Figure 5: Tregs and Teffs accumulate in the skin with similar kinetics. Tissues harvested from Krt14-Kitl×FoxP3-GFP vitiligo mice were analyzed by flow cytometry at the indicated times post disease induction. **(A-C)** Graphs depict the average number of GFP+ Tregs normalized to the number of live single cells in the indicated tissues; or **(D,E)** The average number of GFP+ Tregs and Thy1.1+ Teffs normalized to the number of live single cells in the indicated tissues. ***N=10, bars represent s.e.m.***

numbers start higher and dip at week 5, when Treg recruitment is at its peak in the skin (Fig 5c). By week 7 the number of Tregs begins to increase and at week 12 as disease stabilizes, the number of Tregs in SDLNs reaches levels comparable to those at disease induction (Fig 5c).

When I looked at Teff numbers in skin I observed more Teffs in tail skin than in ear skin (Fig 5d,e), potentially due the decreased number of Tregs in the tail compared to the ear (Fig 5a,b). When I compared Treg migration to Teff migration during disease, I observed that while there are generally more Teffs than Tregs in skin, Treg and Teff numbers increase and decrease simultaneously suggesting that Teffs and Tregs migrate to the skin with similar kinetics (Fig 5d, e). Both populations peak between 5 and 7 weeks post disease induction, during the height of disease. Interestingly, when I compared Treg migration and Teff migration in ear skin I observed that at the peak of disease, numbers of Tregs and Teffs are almost equal (Fig 5e). This may explain why ear skin is more protected supporting the previous observations I made in human skin and those reported in the literature that suggest the Tregs/Teff ratio in skin affects disease severity.

2.3.6: Tregs directly contact Teffs in the skin during vitiligo.

After observing that Tregs and Teffs migrate to the skin with similar kinetics, I investigated whether Tregs and Teffs interact in the skin during vitiligo. To do this I induced vitiligo in Krt14-Kitl×FoxP3-GFP mice with GFP+ Tregs as previously described. I crossed B6-RFP mice with PMEL mice to generate Teffs that express RFP and induced vitiligo with RFP+ Teffs (Fig 6a). After 5 weeks I harvested lesional ears, split the dorsal and ventral sides, mounted the ears on slides and used fluorescent and confocal microscopy to examine them en face. Using florescent microscopy I observed that GFP+ Tregs and RFP+ Teffs cluster together in the ear

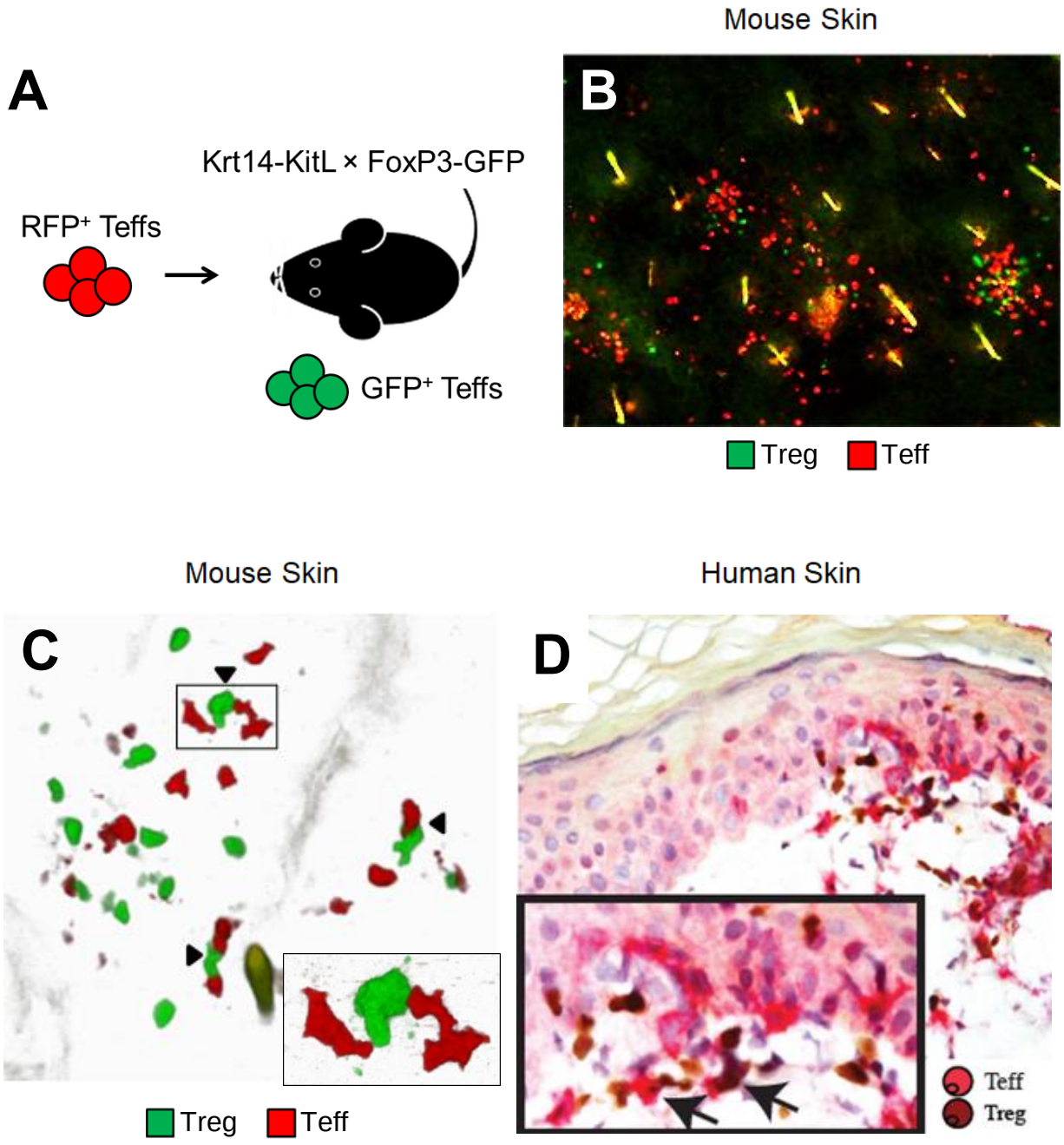


Figure 6: Tregs directly contact Teffs in the skin during vitiligo.

Figure 6: Tregs directly contact Teffs in the skin during vitiligo. (A) Vitiligo was induced in Krt14-Kitl×FoxP3-GFP mice as previously described using RFP+ Teffs; **(B)** Representative florescent microscopy image (20x) of lesional ears from Krt14-Kitl×FoxP3-GFP mice 5 weeks post vitiligo induction; **(C)** Representative confocal microscopy image (40x) of lesional ears in Fig. 6b. Black arrows identify Tregs extending projections to directly contact Teffs in Treg-Teff clusters; **(D)** Representative immunohistochemistry image (20x) of human perilesional skin stained for CD8 to identify Teffs and Foxp3 to identify Tregs.

skin during vitiligo (Fig 6b). I used confocal microscopy to examine these Treg-Teff clusters with greater resolution and I observed a number of Tregs and Teffs in direct contact in ear skin (Fig 6c). Teffs appear to extend projections to contact Tregs suggesting that this contact is intentional and not merely coincidental (Fig 6c). In collaboration with other members in our lab, we performed immunohistochemistry on lesional skin from vitiligo patients and stained for CD8 to identify Teffs and Foxp3 to identify Tregs. I found that human Tregs closely associated with Teffs mirroring the findings in our mouse model (Fig 6d). Together, this data suggests that Tregs migrate to the skin with kinetics similar to Teffs and contact Teffs in lesional skin. I have observed that Tregs suppress Teffs to control depigmentation and the seemingly intentional contact between the two cells that I observed in ear skin suggests that Tregs may use contact-dependent mechanisms of suppression to control disease.

2.3.7: Tregs do not induce CCR7 to control Teff number in the skin.

After observing contact between Tregs and Teffs in the skin, I investigated the mechanisms that Tregs could be using to suppress Teffs. In previous studies I found minimal BrdU incorporation in the skin with or without Tregs, suggesting that Tregs suppress Teffs without inhibiting their proliferation in the skin. In *in vitro* suppression assays conducted by Maeda et al., Tregs induced the expression of the chemokine receptor CCR7 on human melanocyte-specific Teffs that they suppressed [255]. The ligands for CCR7 are the chemokines CCL19 and CCL21, and CCR7 expression mediates T cell exit from the skin via CCL19 and CCL21 rich afferent lymphatics [256, 257]. Therefore, I hypothesize that Tregs control depigmentation during vitiligo by up-regulating CCR7 on autoreactive Teffs that they encounter in the skin to promote Teff egress from the skin (Fig 7a).

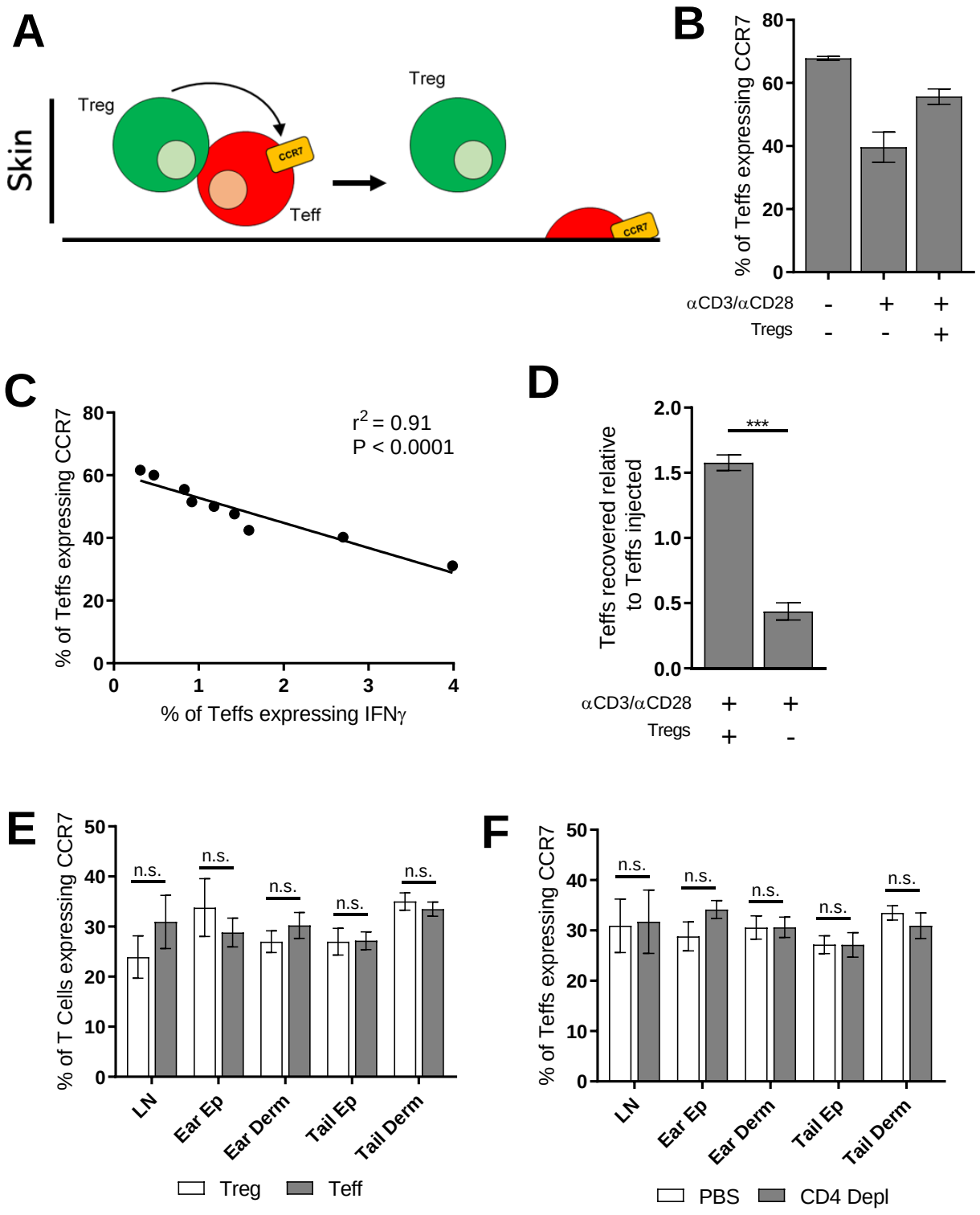


Figure 7: Tregs do not induce CCR7 to control Teff number in the skin.

Figure 7: Tregs do not induce CCR7 to control Teff number in the skin. (A) Once Tregs contact Teffs in the skin, Tregs induce surface CCR7 expression on Teffs to promote Teff egress from the skin**;** Naïve splenic Teffs cultured with or without Tregs for 2 days in uncoated wells or wells coated with αCD3/αCD28 antibody were analyzed using flow cytometry. Graphs depict **(B)** the percentage of Teffs expressing CCR7; ***N=3***, ***students T-test, bars represent s.e.m.*****;** or **(C)** the percentage of Teffs expressing CCR7 correlated with the percentage of Teffs expressing IFNγ**; (D)** Teffs cultured alone or with Tregs for 2 days in wells coated with αCD3/αCD28 antibody were isolated from in vitro cultures, combined at a 1:1 ratio and injected into mouse ears. After 18 hours SDLNs were harvested and flow cytometry was used to quantify the proportion of Teffs recovered relative to their proportion at input; ***N=16, students T-test, bars represent s.e.m.*****;** Tissues harvested from Krt14-Kitl×FoxP3-GFP vitiligo mice treated with PBS or GK1.5 CD4 depleting antibody (CD4 Depl) were analyzed by flow cytometry. Graphs show **(E)** The percentage of Thy1.1+ Teffs and GFP+ Tregs expressing CCR7 in PBS treated mice**;** or **(F)** the percentage of Thy1.1+ Teffs expressing CCR7 in PBS or CD4 Depl treated mice; ***N=10, students T-test, bars represent s.e.m.***

I performed *in vitro* suppression assays to investigate whether Tregs drive CCR7 expression on the Teffs used to induce vitiligo in our mouse model. I isolated Tregs from Krt14-Kitl×FoxP3-GFP mice and melanocyte-specific Teffs from TCR transgenic donors. I co-cultured Tregs and naïve Teffs for 48 hours in the presence of αCD3/αCD28 antibodies and used flow cytometry to quantify CCR7 expression on Teffs. I observed that significantly more Teffs expressed CCR7 in co-cultures with Tregs than those in cultures without Tregs and Teffs cultured alone without αCD3/αCD28 stimulation expressed the most CCR7 (Fig. 7b). I observed an inverse correlation between CCR7 staining and IFNγ expression when I quantified levels in Teffs co-cultured with Tregs at a 0:1, 0.5:1 and 1:1 Treg:Teff ratio. This supports the hypothesis that increased Treg-mediated suppression induces CCR7 expression (Fig. 7c).

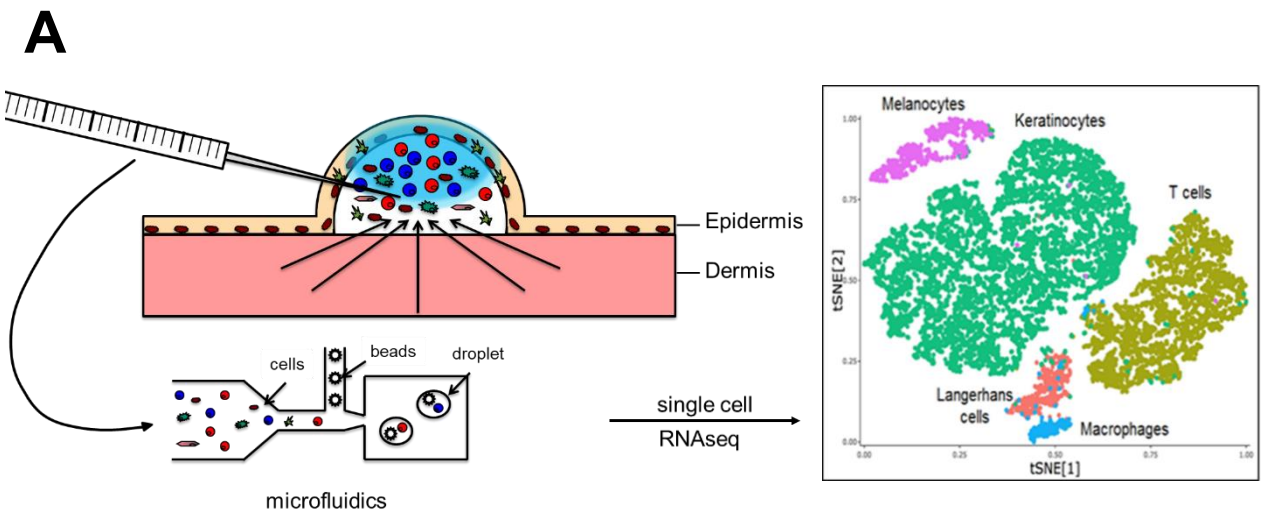
After observing increased CCR7 expression by Teffs cultured with Tregs, I investigated whether Tregs can promote increased Teff egress from skin. To do this I stimulated Teffs *in vitro* with αCD3/αCD28 antibodies in the presence or absence of Tregs. After 48 hours I harvested the cells and used flow cytometry to quantify Teffs in cultures before I combined Teffs cultured alone with Teffs from Treg co-cultures at a 1:1 ratio. I intradermally injected the Teff mixture into the ears of C57BL/6 mice and after 18 hours I harvested the ear draining lymph nodes then used flow cytometry to compare the numbers of Teffs that migrated out of the ear. I used congenic markers to differentiate Teffs cultured alone from Teffs cultured with Tregs. In draining lymph nodes, I recovered significantly more Teffs that were stimulated in the presence of Tregs than Teffs that were stimulated alone (Fig. 7d). This observation supports our hypothesis that Tregs can promote Teff egress from the skin but subsequent experiments targeting CCR7 are needed to confirm whether Treg-induced CCR7 expression governs Teff exit.

To investigate whether Teffs express CCR7 in the skin during vitiligo, I induced disease in Krt14-Kitl×FoxP3-GFP mice and used flow cytometry to compare CCR7 expression between Tregs and Teffs in the SDLNs and skin of mice 7 weeks post disease induction. Comparable numbers of Tregs and Teffs express CCR7 in the SDLNs and skin during vitiligo (Fig. 7e). This was surprising since I hypothesized that Tregs drive CCR7 on Teffs to promote their exit and if Tregs in the skin express comparable levels of CCR7 as Teffs, it is unlikely that CCR7 is functioning as an "exit" signal that exclusively affects Teffs during vitiligo.

To investigate whether Tregs induce CCR7 on Teffs as a mechanism of suppression during vitiligo, I induced vitiligo in Krt14-Kitl×FoxP3-GFP hosts and depleted Tregs as previously described with GK1.5 CD4 depleting antibody. When I used flow cytometry to quantify CCR7 expression on Teffs in the SDLN and skin after seven weeks, I observed that comparable numbers of Teffs express CCR7 during vitiligo with or without Tregs (Fig. 7f). This suggests that Tregs do not control Teff number in the skin by promoting their egress through CCR7 upregulation.

2.3.8: Tregs in lesional human skin express less TGF-β1 than Tregs in healthy skin.

Tregs can use surface bound TGF-β1 to suppress in a contact-dependent manner [144-146], and Tregs in our mouse model could be suppressing Teffs in the skin through a mechanism involving surface bound TGF-β1. In collaboration with other lab members, we isolated Tregs from healthy skin and lesional vitiligo skin using suction blistering and profiled genes expressed by Tregs in human skin with single cell RNA sequencing (Fig. 8a). Tregs in lesional vitiligo skin express less TGF-β1 than Tregs in healthy skin and this could explain why Tregs fail to control Teffs in the skin during vitiligo (Fig. 8b). Future experiments in our mouse model targeting surface bound TGF-β1 on Tregs will elucidate whether the molecule plays a role in suppression.



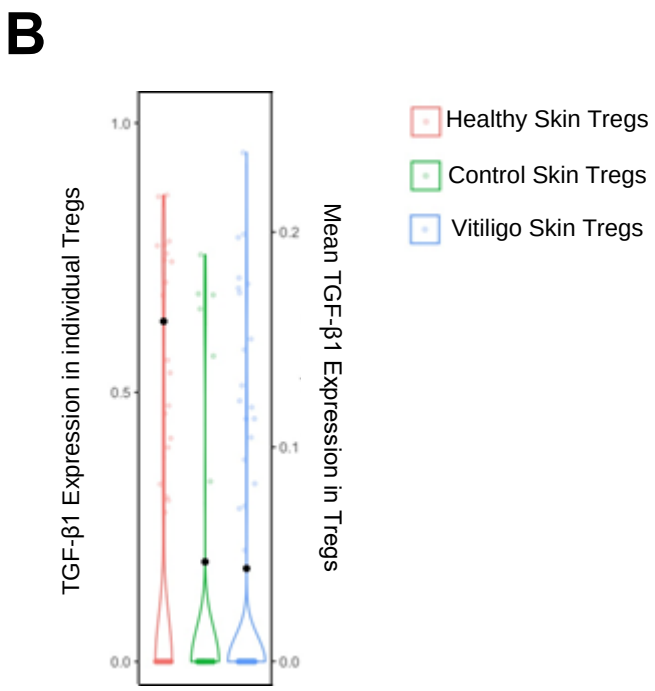


Figure 8: Tregs in lesional human skin express less TGF-β1 than Tregs in healthy skin.

Figure 8: Tregs in lesional human skin express less TGF-β1 than Tregs in healthy skin. (A) Skin infiltrating cells were aspirated from a blister induced by heat and negative pressure on the skin of healthy patients or vitiligo patients and subjected to single cell RNA-sequencing. The tSNE plot generated from single cell RNA-seq data demonstrates that different cell types within the infiltrate are separated based on the expression of cell specific genes; **(B)** the violin plot generated from RNA-seq data shows quantification of TGF-β1 expression by Tregs in healthy human skin and vitiligo skin; the left y axis measures the magnitude of individual events (colored dots) and the right y axis measures the magnitude of the group average (black dot); ***N=74 healthy skin; N=79 control skin; N=248 vitiligo skin*.**

2.4 Discussion

Many studies have addressed the role of Tregs in vitiligo. Defects in Treg function [246, 258] and reduction in Treg number have been reported [246, 259] but the field remains conflicted on exactly how Tregs affect depigmentation during disease. In our mouse model of vitiligo melanocytes are retained in mouse skin and CD8+ T cell-mediated depigmentation affects the skin instead of the hair, providing a unique opportunity to study vitiligo in a context similar to human disease. In light of this I used our model to address the role that Tregs play in the development of vitiligo.

After inducing vitiligo, I treated mice with CD4 depleting antibody and observed that CD4 depletion exacerbates depigmentation. As measured by a blinded observer, CD4-depleted mice have nearly 3-fold more total body depigmentation than mice treated with PBS. Other groups have used their mouse models of vitiligo to investigate the role of Tregs by CD4 or CD25 depletion. Gregg et al. and Chatterjee et al. also observed an increase in the severity of vitiligo after treating mice in their models of disease, with CD4 depleting antibody or CD25 depleting antibody that preferentially targets Tregs [74, 251]. These results suggest that a CD4s are dispensable for disease progression and that CD4+CD25+ Tregs suppress depigmentation during vitiligo.

There are a few key differences between the mouse models used to test Treg involvement that could influence how results are interpreted. In the Gregg model CD8 T cells drive epidermal depigmentation and extensive hair depigmentation while in our model of disease, the hair is spared even in depigmented skin. While both models are suitable for examining the ability of Teffs to mediate melanocyte destruction, the epidermis and the hair follicle have distinct characteristics. The hair follicle is an immune privileged site with its own complex biology and

these differences could confound conclusions [5]. In our model the pattern of depigmentation reflects human disease and gives us a unique opportunity to study vitiligo in a context closely resembling what we see in patients. Compelling evidence exists that identifies CD8+ T cells as the cell type that mediates human vitiligo [63, 260]. In the h3TA2 mouse depigmentation is mediated by a CD3+CD4-CD8- T Cell and this difference could complicate attempts to make conclusions about human disease. Our model relies on CD8+ T cells to mediate depigmentation and again gives us an opportunity to study vitiligo in a context closely resembling what we see in patients. One advantage the other models hold is that they provide the opportunity to study disease in a spontaneous context. To induce vitiligo in our model we infect mice with recombinant vaccinia virus to break tolerance. One caveat of our approach is while we deplete Tregs two weeks after virus infection, when a CD8+ T Cell virus response should already be resolved, Treg depletion could impair viral immune responses [261] and the increased viral load could confound conclusions. In future experiments, tissue titering should be performed on tissues to confirm that viral titers are comparable between PBS treated and Treg-depleted mice.

I used flow cytometry to count T cell populations is PBS treated and CD4-depleted mice. I saw that GFP+ Tregs were not completely depleted in mice treated with CD4 depleting antibody. CD4 depletion did significantly reduce the number of Tregs relative to Teffs, represented as a Treg/Teff ratio in the skin and SDLNs. In future experiments, a higher dose of CD4 depleting antibody could prove more effective but the reduced Treg/Teff ratio in skin suggests that the number of Tregs in lesional skin affects disease severity. This observation aligns with studies in which immunohistochemistry on skin biopsies from vitiligo patients revealed that the CD4/CD8 ratio is decreased among the skin infiltrating cells of vitiligo patients [62], and the percentage of Tregs among skin infiltrating T cells is reduced in vitiligo patients

compared to healthy controls [247]. Treg/Teff ratios are also decreased in the SDLN and my study does not rule out Treg suppression in SDLNs. Future studies that restrict Tregs to the lymph node would definitively answer whether Treg suppression in the skin or LN is important but it is likely that suppression occurs in both locations. Some studies report differences in Treg number between vitiligo skin and healthy skin and other studies report no difference so the field is conflicted on whether Treg number in the skin inversely correlates with disease severity. This study suggests Treg number in the skin is important for suppressing depigmentation.

I observed that CD4 depletion leads to an increased accumulation of Teffs in the skin. Similarly, Chatterjee observed an increase in the amount of skin infiltrating CD3+ effector cells resulting from treatment with CD25 depleting antibody [251]. Gregg et al. also depleted Tregs in their model of vitiligo but they did not report the impact of Treg depletion on Teff skin infiltration [74]. Increased Teff number in the absence of Tregs suggests that Tregs control depigmentation during vitiligo by limiting the number of disease-causing Teffs in the skin. Other models of autoimmunity demonstrate that Tregs may facilitate suppression by limiting Teff number in peripheral tissue as Treg depletion resulted in increased epidermal CD8+ T cells infiltration and exacerbated skin inflammation in a mouse model of psoriasis [166].

I hypothesized that Tregs could limit Teff number by limiting Teff proliferation in the skin and this was why there were more Teffs after Treg depletion. I saw little to no Teff proliferation in the skin, with or without Tregs suggesting Tregs do not inhibit Teff proliferation in the skin during vitiligo. Tregs use many mechanisms to suppress that require cell-cell contact, secretion of inhibitory cytokines or even downregulation of co-stimulatory signals that antigen presenting cells provide Teffs [108]. These mechanisms have been extensively studied and result in the suppression of naïve Teff activation or proliferation [108]. The Teffs that Tregs encounter

in the skin have presumably already been activated and proliferate in SDLNs before receiving signals to go to skin. In fact, by flow cytometry we have seen Teffs in our model express activation markers like CD69. Thus, it is unlikely that Teffs would undergo subsequent rounds of proliferation in the skin or that Tregs would use mechanisms that aim to prevent activation or proliferation in the skin. This could explain why we see little BrdU incorporation in the skin but in contrast see appreciable amounts of BrdU incorporation in SDLN. Teffs proliferate more in SDLNs of mice treated with CD4 depleting antibody. This increase in Teff proliferation in SDLNs of CD4-depleted mice could explain the increased Teff skin infiltration, as more Teffs are available to migrate from SDLNs to the skin of mice without Tregs during vitiligo. This would suggest that Treg suppression of Teffs in the SDLNs is important but does not rule out the possibility that Tregs suppress Teffs in skin. Regardless, this result suggests the mechanism Tregs use to suppress Teffs in the skin do not suppress Teff proliferation. Again, experiments where Tregs are restricted from skin and present only to SDLNs will help answer if Tregs need to be in skin to with Teffs to suppress depigmentation.

CD4 depletion will affect Tregs but also all other CD4 bearing cells. CD25 depletion will preferentially affect Tregs since they express elevated levels of CD25 [108] but activated cells can also express CD25. To specifically deplete Tregs I treated FoxP3-DTR mice with DTX and saw increased levels of depigmentation similar to CD4 depletion. This suggests that Tregs are the CD4+ cell that suppresses depigmentation during vitiligo. It is worth noting that Teffs can still drive vitiligo even though Tregs are present, but vitiligo is much worse when Tregs are depleted. This suggests that even though spots are forming, Tregs are still actively suppressing Teffs and keeping depigmentation from being as extensive as it could be in their absence.

I found that most of the Tregs in the skin were in the dermis and this mirrors observations in human skin where Tregs are found clustered around hair follicles [150]. Interestingly, I noticed that ear skin was affected by Treg depletion more than tail skin. Additionally, when we induce vitiligo in mice tails readily depigment but ears seem protected. Since more Tregs exist in the ear dermis than in the tail dermis in mice without vitiligo and during disease, I hypothesize that the increased number of Tregs in ear skin promotes a more immunosuppressive environment in ear skin. This would explain why ears do not depigment as readily as tails.

Whether the presence of Tregs in the skin of vitiligo patients affects disease severity has not been definitively proven and studies quantifying Tregs in lesional skin reported conflicting results [62, 247-249]. In our studies analyzing skin infiltrating cells in vitiligo patients I observed that the Treg/Teff ratio is reduced in lesional skin compared to non-lesional skin. The absolute Treg number was comparable between lesional and non-lesional skin but the number of Teffs increased in lesional skin. Nonetheless, these findings suggest that the number of Tregs relative to the number of Teffs in the skin may be important for controlling pathogenesis. Rosenblum et al. used a mouse model to demonstrate that resident memory Tregs reside in skin and provide expedited protection against skin inflammation [151]. This finding supports the notion that Tregs function in skin and the Tregs we see in skin could be important for suppressing Teff function. Studies in mouse models of vitiligo reported that increased Treg recruitment to the skin resulted in reduced disease, further supporting the hypothesis that Treg number in skin can reduce Teff driven depigmentation [155, 251, 252]. In light of this, it is reasonable to hypothesize that the increased number of Tregs in the ear compared to the tail before and during vitiligo, promotes a more immunosuppressive environment in ear skin that renders the ear less susceptible to depigmentation than the tail. This strongly suggests that Treg activity in the skin is important for

Treg suppression during vitiligo but does not preclude the contribution of Treg suppression in SDLNs.

One group used flow cytometry to examine differences in Treg number between different areas of human skin. Treg number seemed to correlate with hair follicle density as areas of skin with more hair follicles (face and scalp) had more Tregs than areas with low hair follicle density (trunk and upper proximal extremities) [150]. Vitiligo more commonly presents on the face which contradicts the idea that increased Tregs in certain areas of skin confers more protection but other factors like antigen density may contribute to depigmentation in people [262].

In lesional mouse ear skin I observed Tregs extending projections to contact Teffs suggesting contact between the T cells is intentional not merely coincidental. Tregs directly contact Teffs in lesional human skin from vitiligo patients mirroring what we see in mice. This contact is significant because it suggests that Tregs interact with Teffs in the skin during vitiligo and may be using contact-dependent mechanisms to suppress Teffs in the skin. The mechanisms Tregs use to suppress Teffs have been extensively studied but a lot of these studies were performed in vitro. Tregs can suppress Teffs using a variety of mechanisms and Vignali et al. argue that the mechanisms Tregs use could depend on where they encounter inflammatory activity [263]. Therefore, experiments performed in vitro could lead to incomplete conclusions since they cannot examine Treg function in the proper context. Our mouse model of vitiligo gives a unique opportunity to study mechanisms of Treg suppression in an in vivo model of peripheral tolerance and our observation that Tregs interact with Teffs in lesional skin suggests Tregs use contact-dependent mechanisms to suppress in peripheral tissue. A few mechanisms of contact-dependent suppression have been described. Bopp et al. reported that Tregs express high levels of cAMP and Tregs suppress CD4+ T cells by transferring cAMP to CD4+ T cells via gap

junctions in co-cultures [143]. This suppression was contact-dependent [143] and Tregs could be using this mechanism to suppress Teffs in the skin during vitiligo. A number of studies demonstrate that Tregs use surface bound TGF-β1 to suppress in a contact-dependent manner [144-146] and Tregs in lesional vitiligo skin express less TGF-β1 than Tregs in healthy skin. If surface bound TGF-β1 contributes to Treg suppression, reduced TGF-β1 expression could lead to the uncontrolled Teffs activity evident in the skin during vitiligo. Tregs could use membrane bound TGF-β1or cAMP to suppress Teffs during vitiligo and these possibilities should be explored in future studies.

I investigated whether Tregs suppress depigmentation by inducing CCR7 on Teffs to drive their egress from the skin during vitiligo. While Teffs suppressed by Tregs expressed more CCR7 than Teffs activated alone in vitro, the number of Teffs expressing CCR7 in the skin did not significantly change when mice were treated with CD4 depleting antibody. The latter observation suggests that Tregs do not target CCR7 to suppress Teffs during disease and using CCR7 deficient Teffs to induce vitiligo in our mouse model would further address this question. If Treg-driven induction of CCR7 on Teffs is necessary to control depigmentation by limiting Teff number in the skin, hosts with CCR7 deficient Tregs will exhibit increased disease and increased cutaneous Teff accumulation. If disease is comparable between hosts with WT and CCR7 deficient Teffs, than Tregs likely do not induce CCR7 on Teffs to suppress depigmentation.

Defining the mechanisms that Tregs use to suppress autoimmunity during vitiligo would help us further understand how Tregs function in the periphery and potentially help discover new therapies to treat patients or improve existing ones.

2.5: Materials and Methods

Mice

KRT14-Kitl*4XTG2Bjl (Krt14-Kitl) mice were a generous gift from B. J. Longley (University of Wisconsin). I isolated Teffs from PMEL TCR transgenic mice that are Thy1.1+ and were obtained from The Jackson Laboratory, stock no. 005023, B6.Cg Thy1a/CyTg(TcraTcrb)8Rest/J. RFP mice were obtained from The Jackson Laboratory, stock no. 005884, B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J. RFP mice were bred to PMEL TCR transgenic mice to generate Teffs that express RFP. The following strains were bred to Krt14-Kitl mice to use as hosts in a mouse model of vitiligo: FoxP3-GFP mice generated by VJ Kuchroo, FoxP3-DTR mice (The Jackson Laboratory, stock no. 016958, B6.129(Cg)-Foxp3$^{tm3(DTR/GFP)Ayr}$/J). The Krt14-Kitl allele was bred heterozygous on all mice used in experiments. All mice were bred on a C57BL/6J background and mice were maintained in pathogen-free facilities at the University of Massachusetts Medical School (UMMS), and procedures were approved by the UMMS Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Vitiligo induction

Vitiligo was induced through adoptive transfer of CD8+ T cells isolated from PMEL mice (Teffs) as described previously [79]. Teffs were isolated from the spleens of PMEL TCR transgenic mice through negative selection using a Miltenyi Biotec (Bergisch Gladbach, Germany) CD8 isolation kit according to the manufacturer's instructions. 10^6 purified Teffs were injected intravenously into 8 to 12 week old hosts sublethally irradiated with 500 rads 1 day before transfer. Hosts were also infected with 10^6 plaque-forming units (PFU) of rVV-hPMEL

through intraperitoneal injection (N. Restifo, National Cancer Institute, NIH) on the same day of adoptive transfer. Depigmentation was reported using a vitiligo score. The vitiligo score was determined by an observer blinded to the experimental groups, using a point scale based on the extent of depigmentation at four locations, the ears, tail, nose and hind footpads as previously described [79]. Each location was examined, and the extent of depigmentation was assigned a number based on the estimated percentage of depigmentation at the anatomic site. Both left and right ears and left and right rear footpads were estimated together and therefore evaluated as single sites. The scoring system used is as follows: no depigmentation = 0, >0 to 10% = 1 point, >10 to 25% = 2 points, >25 to 75% = 3 points, >75 to <100% = 4 points and 100% = 5 points. The "vitiligo score" was the sum of the scores at all four sites, with a maximum score of 20 points.

CD4 depletion and Treg depletion by DTX treatment

For CD4 depletion experiments vitiligo was induced in Krt14-Kitl×FoxP3-GFP mice as previously described. After 2 weeks, I treated mice once a week with 0.2mg of GK1.5 CD4 depleting antibody (CD4-Depl) (BioXcell; West Lebanon, NH; Cat: BE0003-1) or PBS by intraperitoneal (i.p.) injection. After 5 weeks of treatment, a blinded observer quantified depigmentation on ears, nose, hind footpad and tail skin to generate a vitiligo score and tissues were harvested to be processed for flow cytometry. For DTX depletion experiments vitiligo was induced in Krt14-Kitl×FoxP3-DTR mice as previously described. After 2 weeks, I treated mice with 50µg/kg of diphtheria toxin (DTX) (Sigma-Aldrich; Darmstadt, Germany; Cat: D0564) or PBS every other day for 2 weeks. At 7 weeks post disease induction, a blinded observer

quantified depigmentation on ears, nose, hind footpad and tail skin to generate a vitiligo score and tissues were harvested to be processed for flow cytometry.

BrdU pulsing experiments

Vitiligo was induced in Krt14-Kitl×FoxP3-GFP mice as previously described and after 2 weeks I treated mice with CD4 depleting antibody once a week with 0.2mg of GK1.5 CD4 depleting antibody (αCD4) or PBS. After 3 or 5 weeks mice were injected with Bromodeoxyuridine (BrdU) (BD Pharmingen; San Jose, CA; Cat: 552598) i.v. 6 hours before takedown and tissues were harvested to be processed for flow cytometry. BrdU incorporation was detected by intracellular staining with the APC BrdU Flow Kit (Cat: 552598) performed according to the manufacturer's instructions.

Flow Cytometry

Ears, tails and skin-draining lymph nodes were harvested. Lymph nodes were mechanically disrupted. To separate the dermis and epidermis, harvested ear and tail skin samples were incubated with dispase (50 U/ml and 5 U/ml in PBS, respectively) (Roche) for 1 hour at 37°C. Epidermis was removed and mechanically disrupted with 70-mm cell strainers. Dermis samples were incubated with 1 mg/ml collagenase IV and 2 mg/ml DNase I (Sigma-Aldrich) in RPMI for 45 minutes at 37°C on a shaker. After incubation dermis was dissociated by gentle agitation. Cells from lesional human skin were obtained by suction blistering. The following antibodies were obtained from BioLegend (San Diego, CA): mouse (CD3, CD8b, CD4, CD45 and CD90.1) and human (CD3, CD4, CD8, CD45, CD25 and FOXP3). For extracellular staining cells were washed in FACS buffer (1% FBS in PBS) and then incubated with antibodies at a 1:200 dilution

at 4° C in the dark for 20 minutes. For intracellular FoxP3 staining, surface staining was performed and cells were fixed and permeablized using the FoxP3/Transcription factor staining buffer set from eBioscience (Santa Clara, CA) according to the manufacturer's recommendations. The data were collected and analyzed with a BD LSR II flow cytometer (BD Biosciences) and FlowJo (Tree Star Inc.).

Quantifying Treg and Teffs during vitiligo

To quantify Tregs in mice without vitiligo tissues from 8 week old Krt14-Kitl×FoxP3-GFP mice were harvested to be processed for flow cytometry. Tissue processing and surface staining were performed as previously described and CD45+CD3+CD4+GFP+ Tregs were quantified using a BD LSR II flow cytometer (BD Biosciences). To quantify Tregs and Teffs in mouse tissues during vitiligo I induced vitiligo in Krt14-Kitl×FoxP3-GFP mice as previously described and after 3, 5, 7 and 12 weeks post disease induction tissues were harvested to be processed for flow cytometry. Tissue processing and surfaces staining were performed as previously described and CD45+CD3+CD8+CD90.1+ Teffs and CD45+CD3+CD4+GFP+ Tregs were quantified using a BD LSR II flow cytometer (BD Biosciences). To quantify Tregs and Teffs in human skin during vitiligo skin infiltrating T cells were obtained from lesional and non-lesional skin by suction blistering. Suction blistering was performed as previously described [264]. Blisters that were approximately 1 cm in diameter were induced on the skin by using the Negative Pressure Instrument Model NP-4 (Electronic Diversities, Finksburg, MD). Suction chambers were placed on the skin with 10-15 mm Hg of negative pressure and a constant temperature of 40° C. After 30 to 60 minutes blisters formed and the blister fluid was aspirated through blister roofs using a 1 mL insulin syringe. Single cell RNA sequencing was performed on blister fluid obtained. Skin

infiltrating cells within the blister fluid were pelleted at 330×g for 10 minutes and then stained to be analyzed with flow cytometry. CD45+CD3+CD4+CD25+FOXP3+ Tregs and CD45+CD3+CD8+ Teffs were quantified using a BD LSR II flow cytometer (BD Biosciences). All flow cytometry data was analyzed with FlowJo (Tree Star Inc.).

Imaging Treg-Teff interactions

To visualize interactions between murine Tregs and Teffs, vitiligo was induced in Krt14-Kitl×FoxP3-GFP mice as previously described using RFP expressing Teffs from PMEL×RFP donors. After 5 weeks, mice were euthanized and lesional ears were excised. Following light treatment of ears with Nair to remove hair, the dorsal and ventral sides were separated and mounted on slides for imaging. All confocal imaging was performed on a Leica SP8 confocal microscope. To visualize interactions between Tregs and Teffs in lesional human skin during vitiligo immunohistochemistry was performed on biopsies from peri-lesional vitiligo skin. Teffs were identified by CD8 staining and Tregs were identified by FoxP3 staining.

Statistical analyses

All statistical analyses were performed with GraphPad Prism software. Dual comparisons were made with unpaired Student's t test.

CHAPTER III: LANGERHANS CELLS SUPPRESS THE INCIDENCE OF DISEASE IN A MOUSE MODEL OF VITILIGO

Kingsley I. Essien, Jillian M. Richmond and John E. Harris

Contribution Summary:

K.I.E helped to design the experiments, performed the experiments, and helped to analyze the data and write the manuscript. J.M.R helped perform LHC-KO experiments, mixed bone marrow chimera experiments and helped to analyze the data from these experiments. J.E.H. helped to design the experiments, analyze the data and write the manuscript.

3.1: Abstract

Langerhans cells (LHCs) are a dendritic cell (DC) population that resides in the epidermis where they survey the skin for the presence of foreign pathogen. Since LHCs are tightly packed adjacent to melanocytes in the epidermis, they are ideally positioned to interact with Teffs infiltrating the skin during vitiligo and in this study, I explored the contributions of LHCs to disease. Numerous studies examining the role that LHCs play in models of inflammation, report conflicting results. In some of these studies LHCs behaved in an immunogenic manner while in others LHCs played a tolerogenic role. Our mouse model of vitiligo provides a unique opportunity to bring clarity to this in the context of vitiligo. In our studies we observed that the incidence of disease increased in the absence of LHCs, suggesting that LHCs suppress depigmentation by reducing disease incidence. Epidermal Teff number also increases in the absence of LHCs, and the epidermal/dermal Treg ratio decreases so I hypothesize that LHCs may suppress disease by promoting the proper positioning of Tregs. LHCs have been reported to interact with Tregs in the skin. After observing that LHC-derived CXCL10 is necessary for suppression, I hypothesized that LHCs attract both Tregs and Teffs to the epidermis with CXCL10 and then tether both cells with MHC-TCR interactions in order to increase the efficiency of Treg-mediated Teff suppression. After observing LHC-Treg-Teff interaction in lesional mouse skin, I induced vitiligo in hosts with MHCII-deficient LHCs and observed that MHCII-TCR interactions are dispensable for LHC-mediated suppression.

3.2: Introduction

What role do LHCs play during vitiligo?

LHCs are of special interest in vitiligo. They exist in the epidermis in close proximity to melanocytes so during vitiligo they can potentially present melanocyte antigens to mediate disease pathogenesis, or contribute to the peripheral suppression of disease causing Teffs. A few groups have studied LHCs during vitiligo and when Brown et al. compared LHC numbers between lesional and uninvolved skin from vitiligo patients they found no significant differences [265]. Another group similarly found that the number of epidermal LHCs in vitiligo skin was similar to the number of LHCs observed in the healthy skin [266]. In contrast Itoi et al. performed immunohistochemistry on skin biopsies from vitiligo patients and observed an increased number of LHCs in lesional skin compared to non-lesional skin [267]. Itoi et al. also concluded LHCs were more activated in lesional skin based on observations that LHCs in lesional skin were larger and had more Birbeck granules [267]. These results led them to conclude that LHCs stimulate CD8+ T cell responses during vitiligo.

Wang et al. also performed immunohistochemistry on non-lesional and peri-lesional skin from vitiligo patients and counted more CD207+ LHCs in peri-lesional skin than in non-lesional skin [268]. Dermal dendritic cells in peri-lesional skin localized near the dermal-epidermal junction and there were more dDCs in peri-lesional skin than in non-lesional skin [268]. Wang et al. observed LHCs in peri-lesional skin expressed higher levels of HLA-DR suggesting LHCs are more activated in lesional skin during vitiligo, and similar to Itoi et al., they hypothesize that skin DCs drive T cells responses during vitiligo [268]. More work needs to be done to elucidate the role LHCs play during vitiligo and by using our mouse model of disease we used the many

genetic tools available in mice to study the contributions of LHCs to immunity in a model of autoimmune antigen presentation.

3.3: Results

3.3.1: The absence of Langerhans cells increases the incidence of disease in a mouse model of vitiligo

In collaboration with others in the lab, I induced vitiligo in mice without langerhans cells (LHCs) to investigate the role of LHCs during disease. In huLangerin-DTA mice, the expression of the active subunit of diphtheria toxin is driven by huLangerin an LHC-specific promoter [206]. In these mice, LHCs are constitutively absent but langerin expressing dermal dendritic cells are unaffected [206]. I bred Krt14-Kitl×huLangerin-DTA (LHC-KO) mice to generate hosts that retain melanocytes in the epidermis while constitutively lacking LHCs and I induced vitiligo in these mice as previously described. I induced vitiligo in Krt14-Kitl mice to serve as wild-type (WT) controls. At 7 weeks post disease induction, a blind observer quantified the severity of depigmentation on ears, nose, hind footpad and tail skin to generate a vitiligo score. I compared depigmentation between WT and LHC-KO mice and observed that the extent of depigmentation is not significantly affected by the absence of LHCs; WT mice had an average vitiligo score of 4.8 ± 0.6 and LHC-KO mice had an average score of 5.4 ± 0.6 (Fig. 9a). While the absence of LHCs does not affect the extent of depigmentation, it does increase the incidence of disease. I observed that 14 of the 48 WT mice induced with vitiligo exhibit no disease (Fig. 9a) and this characteristic of disease incidence in our mouse model – 1/3 of mice do not depigment (not shown). However only 1 of the 34 LHC-KO mice induced with vitiligo exhibited no depigmentation (Fig. 9a) and considering this we hypothesized that LHC-KO mice suppress

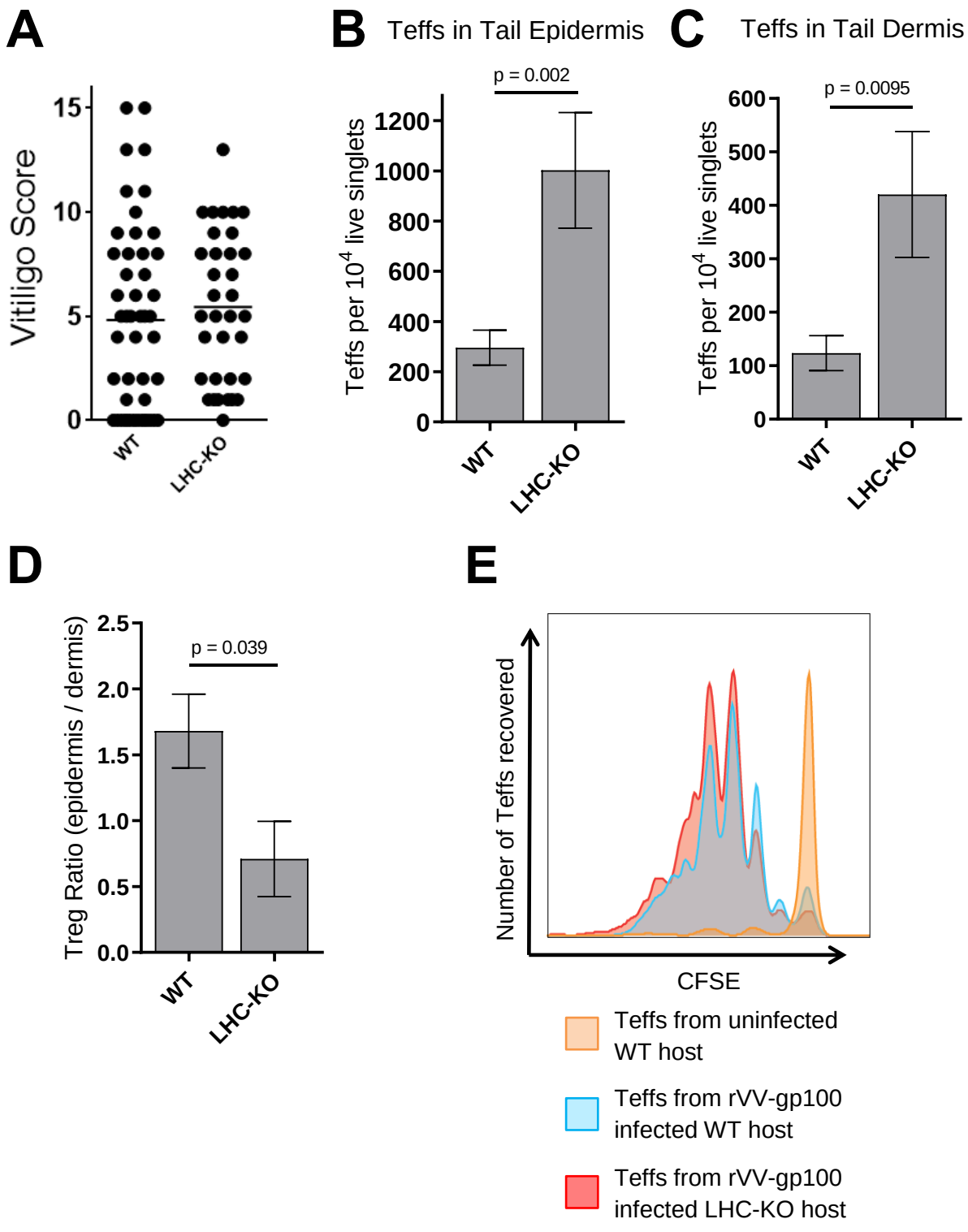


Figure 9: The absence of Langerhans cell increases the incidence of disease.

Figure 9: The absence of Langerhans cells increases the incidence of disease. Tissues harvested from LHC-KO vitiligo mice were analyzed by flow cytometry 7 weeks post disease induction. **(A)** Graph depicts depigmentation on mice with vitiligo; ***WT N=48, LHC-KO N=34, students T-test, bars represent s.e.m.;*** **(B, C)** Graphs show the average number of Thy1.1+ Teffs normalized to the number of live single cells in the tail epidermis and dermis; or **(D)** the number of epidermal FoxP3+ Tregs divided by the number of dermal Tregs represented as a Treg ratio; ***WT N=32, LHC-KO N=25, students T-test, bars represent s.e.m.;*** **(E)** CFSE labeled Teffs harvested from the spleens of uninfected or rVV-gp100 infected mice were analyzed by flow cytometry. The representative flow plot shows CFSE dilution in Teffs isolated from the spleens of mice infected for 3 days; ***N=3***.

depigmentation during vitiligo with mechanisms that ultimately reduce the frequency of depigmentation.

Seven weeks after disease induction, I harvested tail skin from both groups of mice, mechanically separated the dermis from the epidermis and used flow cytometry to count the number Teffs in digested tissues. I counted significantly more Teffs in the epidermis of LHC-KO mice than WT mice, 1002 ± 230 cells compared to 295.6 ± 69.7 cells, respectively (Fig. 9b). More Teffs accumulated in the dermis of LHC-KO mice than in the dermis of WT mice as well, 420.5 ± 117.8 cells compared to 123.7 ± 32.8 cells, respectively (Fig. 9c). This further suggests that LHCs control disease and may do so by controlling Teff number in the skin. To ensure that increased depigmentation was not an artifact of altered priming of Teffs LHC-KO mice, I adoptively transferred CFSE-labeled Teffs into LHC-KO or WT mice and infected both groups with 1×10^6 PFU rVV-gp100. After three days I harvested spleens from both groups of mice and used flow cytometry to compare Teff CFSE dilution as a measure of Teff proliferation. I found that the absence of LHCs does not affect Teff priming as Teffs in WT and LHC-KO mice exhibited comparable levels of CFSE dilution (Fig. 9e).

Seneschal et al. reported that human LHCs can induce the activation and proliferation of skin resident Tregs [209]. Since LHCs suppress vitiligo in our mouse model of disease I reasoned that the absence of LHCs could affect Treg number during vitiligo. To test this, I induced vitiligo in WT and LHC-KO mice and after 7 weeks I harvested tail skin from both groups of mice, mechanically separated the dermis from the epidermis and used flow cytometry to count the number Tregs in digested tissues. I compared numbers of Tregs between LHC-KO and WT mice and saw an increase in the number of dermal Tregs and a decrease in the number of epidermal Tregs in the absence of LHCs. I represented this as an epidermal/dermal ratio and

this ratio is decreased in LHC-KO mice compared to WT mice, 0.71 ± 0.28 compared to 1.68 ± 0.28, respectively (Fig. 9d). Considering this I hypothesized that LHCs suppress disease by properly positioning Tregs in the epidermis during vitiligo to promote their suppressive interactions with Teffs.

3.3.2: Tregs express CXCR3 and localize with CXCL10 expression in the skin during vitiligo

I began to question how LHCs could promote Treg positioning and reasoned that they could make chemokines that Tregs follow. I previously observed that Teffs and Treg migrate to the skin with similar kinetics (chapter 1 Fig. 5D, E). This suggests that Tregs could be following the same signal as Teffs to get to the skin and previous studies from our group established that Teffs require CXCR3 to follow CXCL10 to migrate to the epidermis and mediate depigmentation [253]. The receptor for CXCL10 is CXCR3 and if Tregs follow the same signal as Teffs they must express CXCR3. Published studies have reported that Tregs express CXCR3 [234] so I investigated whether Tregs express the chemokine receptor in the skin during vitiligo. I induced vitiligo in Krt14-Kitl×FoxP3-GFP and 5 weeks later I harvested tail and ear skin and used flow cytometry to measure levels of CXCR3 on Tregs in the skin during vitiligo. I observed that the same number of Tregs and Teffs express CXCR3 in the ear and tail epidermis and dermis during vitiligo (Fig. 10a). In light of this, I hypothesized that Tregs can follow the same CXCL10 gradients that Teffs do to get to the skin and cluster with Teffs (ch.1, Fig 6). To investigate the possibility that CXCR3 expressing Tregs follow CXCL10 in the skin during vitiligo I crossed a CXCL10/CXCL9 reporter mouse (REX3, **R**eporting **E**xpression of C**X**CR**3** Ligands) to Krt14-Kitl×FoxP3-GFP to generate mice with GFP expressing Tregs and the CXCL10 promoter driving BFP expression (Fig. 10b). I induced vitiligo in these mice and after 5

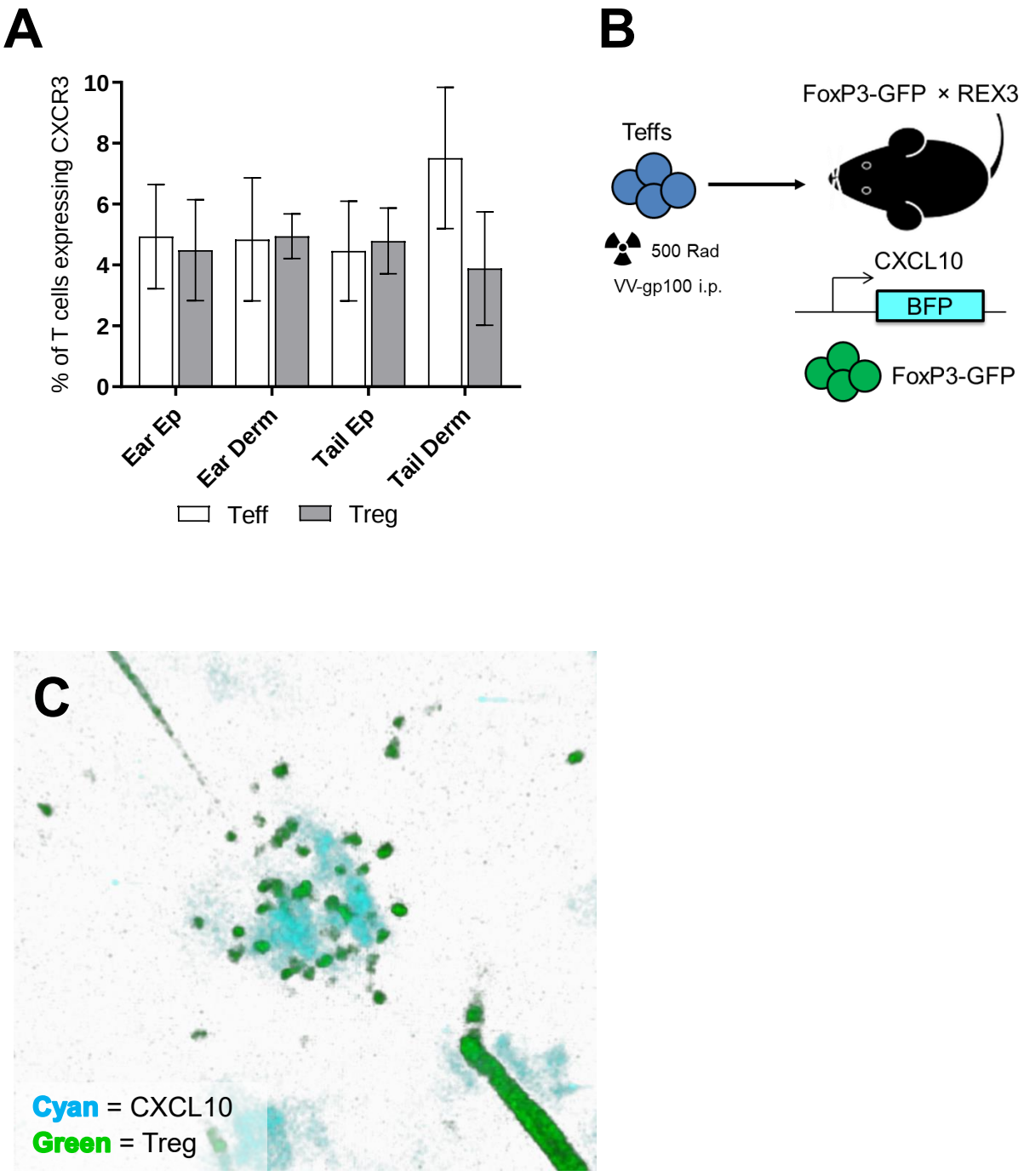


Figure 10: Tregs express CXCR3 and localize with CXCL10 in the skin during vitiligo

Figure 10: Tregs express CXCR3 and localize with CXCL10 in the skin during vitiligo. (A) Skin harvested from Krt14-Kitl×FoxP3-GFP vitiligo mice was analyzed by flow cytometry 5 weeks post disease induction and the graph depicts the percentage of Thy1.1+ Teffs and GFP+ Tregs; ***N=7, students T-test, bars represent s.e.m.*; (B)** Vitiligo was induced as previously described in FoxP3-GFP×REX3 mice with GFP+ Tregs and BFP expression driven by the CXCL10 promoter; **(C)** Representative confocal microscopy image (40x) of lesional ears from FoxP3-GFP×REX3 mice with vitiligo 5 weeks post disease induction; ***representative image of 2 experiments with 3 mice***.

weeks I harvested lesional ears, split the dorsal and ventral sides, mounted the ears on slides and used confocal microscopy to image ears en face. I observed that Tregs co-localized with areas of CXCL10 expression in the skin during vitiligo (Fig. 10c) and this observation supported the hypothesis that Tregs localize with Teffs by following the same chemokine signals.

3.3.3: LHC-derived CXCL10 promotes optimal suppression in a mouse model of vitiligo

Our group previously reported CXCL10 expression is upregulated in mouse and human skin during vitiligo [253]. We induced vitiligo in Krt14-Kitl×REX3 mice to investigate the source of CXCL10 and found that the majority of CXCL10 is expressed in the epidermis during vitiligo and LHCs are one of the major producers of CXCL10 [269]. After observing that LHCs suppress vitiligo, make CXCL10, affect the epidermal positioning of Tregs that express CXCR3, and localize with CXCL10 in the skin during vitiligo, I hypothesized that LHCs use CXCL10 to attract Tregs and Teffs in the skin and facilitate the interactions that we see between the two cell types (ch.1, Fig 6). To test this I investigated whether LHCs suppression requires CXCL10. Because CXCL10 conditional knockout mice have not been developed, I generated mixed chimeras to determine the role of LHC-derived CXCL10 during vitiligo in collaboration with others in the lab. I lethally irradiated LHC-KO host and reconstituted them with 50% bone marrow from LHC-KO donors (contribute 50% of BM-derived cells except LHCs) and 50% marrow from WT or $CXCL10^{-/-}$ mice (contribute to the entire LHC compartment and 50% to all other compartments) (Fig 11). After 8 weeks I verified that LHCs reconstituted the epidermal niche by harvesting ears, fixing ears to slides, removing the dermis and staining the remaining epidermal sheets for langerin to identify LHCs. There are no LHCs in LHC-KO hosts and in chimeras receiving $CXCL10^{-/-}$ or WT bone marrow, BM-derived LHCs fill the empty LHC niche and distribute in similar patterns (Fig. 12a).

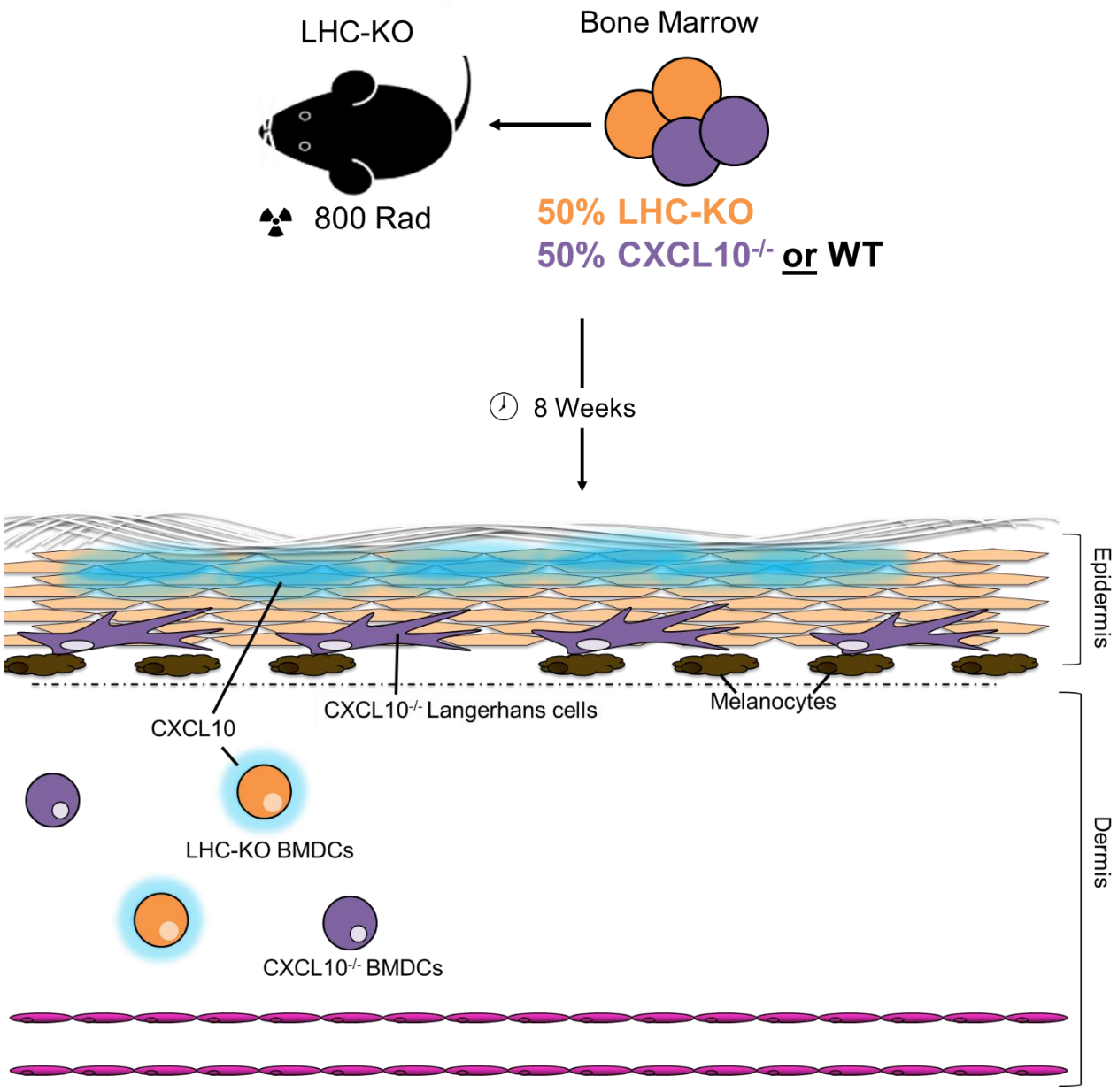


Figure 11: Generating mixed bone marrow chimeras with CXCL10$^{-/-}$ langerhans cells.

Figure 11: Generating mixed bone marrow chimeras with CXCL10$^{-/-}$ langerhans cells.

Lethally irradiated LHC-KO hosts were reconstituted with 50% bone marrow from LHC-KO donors and 50% marrow from WT or CXCL10$^{-/-}$ mice. After 8 weeks LHC-KO donor bone marrow will reconstitute 50% of BM-derived cells except LHCs and CXCL10$^{-/-}$ or WT bone marrow will reconstitute 100% of the empty LHC compartment and 50% of all other BM-derived cells.

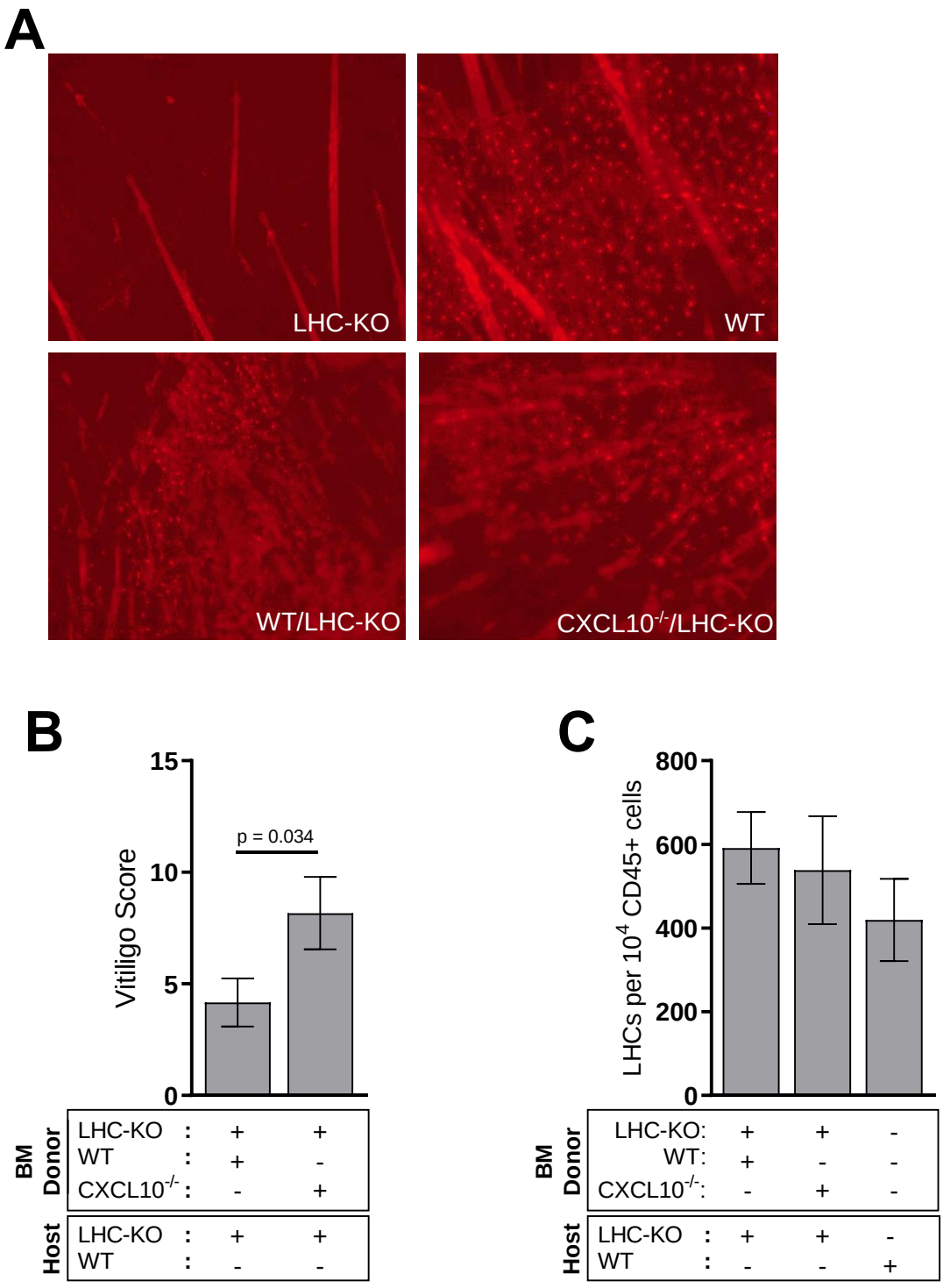


Figure 12: Langerhans cells require CXCL10 for optimal suppression.

Figure 12: Langerhans cells require CXCL10 for optimal suppression. (A) Representative immunofluorescent microscopy images of ear epidermal sheets harvested from LHC-KO mice, WT mice, and mixed bone marrow chimeras were stained with anti-CD207 antibody to identify LHCs; **WT/LHC-KO are mixed chimeras with 50% WT and 50% LHC-KO bone marrow, CXCL10$^{-/-}$/LHC-KO are mixed chimeras with 50% CXCL10$^{-/-}$ and 50% LHC-KO bone marrow*, representative images from 2 experiments***; Tail skin harvested from chimeric vitiligo mice was analyzed by flow cytometry 7 weeks post disease induction. Charts show **(B)** quantification of depigmentation on mixed bone marrow chimeras; or **(C**) quantification of the average number of MHCII+ LHCs normalized to the number CD45+ cells in the tail epidermis; ***N=6, students T-test, bars represent s.e.m.***

In collaboration with others in the lab I induced vitiligo in chimeric mice 8 weeks after bone marrow reconstitution and 7 weeks post disease induction, a separate observer blinded to the experimental groups quantified the severity of depigmentation on ears, nose, hind footpad and tail skin to generate a vitiligo score. I observed that chimeras with CXCL10$^{-/-}$ LHCs exhibited increased depigmentation compared to chimeras with WT LHCs, average total scores of 8.2 ± 1.6 and 4.2 ± 1.1, respectively (Fig. 12b). I also harvested tail epidermis, processed the tissue into a single cell suspension and use flow cytometry to find comparable numbers of LHCs between WT mice and chimeras reconstituted with CXCL10$^{-/-}$ LHCs or WT LHCs (Fig. 12c). The findings suggest that LHCs require CXCL10 to suppress disease and support the hypothesis that LHCs use CXCL10 to facilitate interactions between Tregs and Teffs.

3.3.4: Visualizing interactions between LHCs, Tregs and Teffs in murine skin during vitiligo

CXCL10 promotes depigmentation in our mouse model of vitiligo and keratinocyte-derived CXCL10 orchestrates epidermal Teff positioning to drive disease [253, 269]. Since LHC-derived CXCL10 promoted suppression I questioned how LHCs could suppress disease while keratinocytes promote disease using the same molecule. I reason that LHCs, unlike KCs, constitutively express MHC II and can interact with Tregs via MHCII-TCR interactions. In light of this I hypothesized that LHCs attract epidermal Tregs and Teffs with CXCL10 and then tether both cells through MHC-TCR interactions to promote direct cell-cell contact, and thus increase the efficiency of Treg-mediated suppression of Teffs (Fig. 13a). This interaction is likely as Seneschal et al. found that human LHCs activate skin resident Tregs in an MHC-TCR-dependent manner and observed Tregs in contact with LHCs in the epidermis [209]. To visualize whether Teffs, Tregs, and LHCs interact during vitiligo I adapted a method previously described [270] to

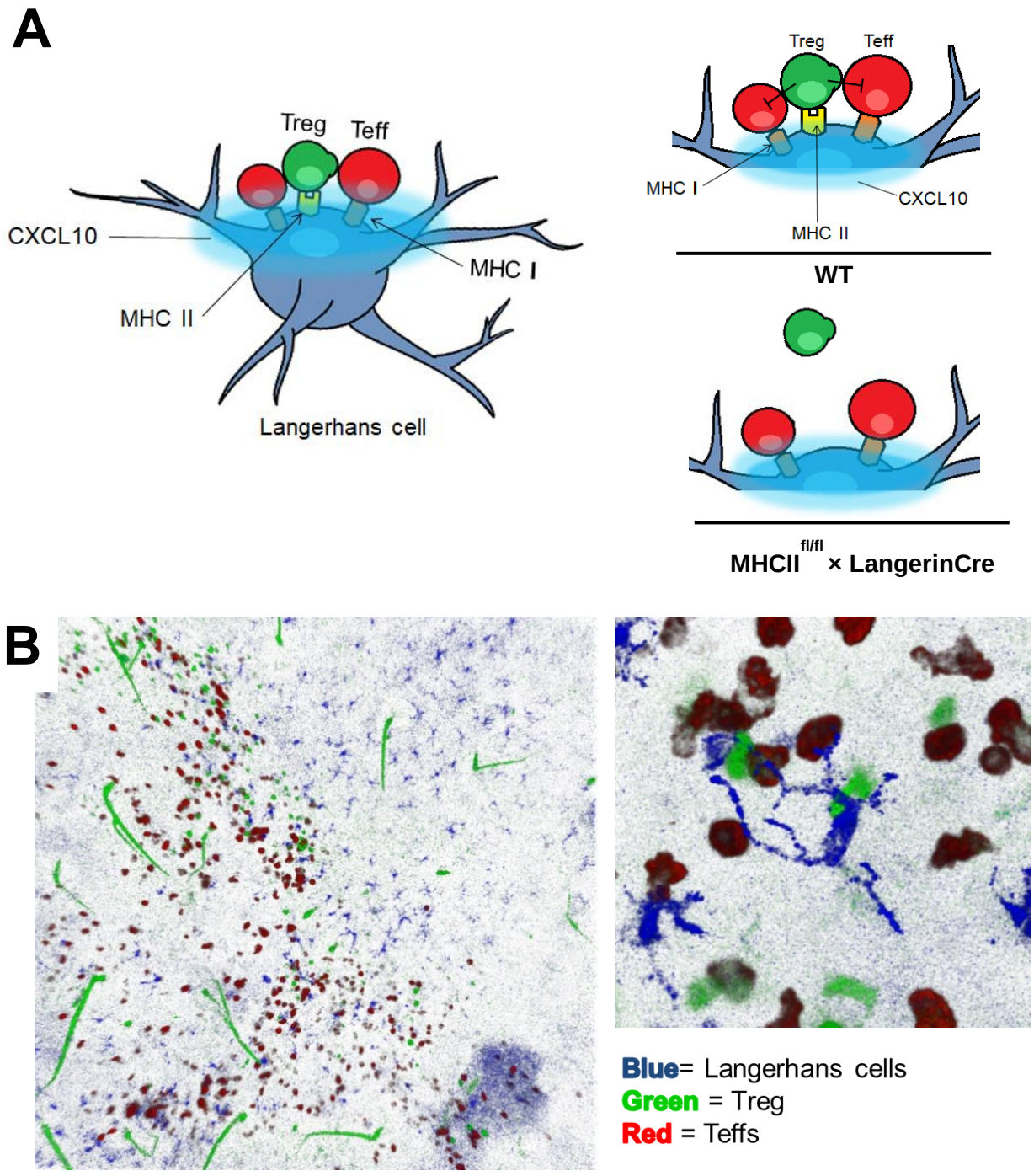


Figure 13: Langerhans cells as a scaffold to facilitate interactions between Tregs and Teffs

Figure 13: Langerhans cells as a scaffold to facilitate interactions between Tregs and Teffs. **(A)** LHCs attract both Tregs and Teffs that are in the epidermis with CXCL10 and then tether both cells with MHC-TCR interactions to increase the efficiency of Treg-mediated Teff suppression **(left panel)**. $MHCII^{flox/flox}$×LangerinCre mice with LHCs lacking MHCII, may still have Tregs in the skin capable of suppressing Teffs but the efficiency of their suppressive contact with Teffs may be reduced **(right panels)**; Lesional ears harvested from Krt14-KitL×FoxP3-GFP mice were stained with αLangerin antibody 5 weeks post RFP+ Teff transfer and analyzed using confocal microscopy. **(B)** A representative confocal microscopy image shows that LHCs (blue) frequently associated with both Teffs (red) and Tregs (green) in the skin during vitiligo; ***The left panel is a 10x image and the right panel is a 40x image, Representative images from 1 experiment with 3 mice*.**

label LHCs while preserving the integrity of the ears 3D architecture. I induced vitiligo in Krt14-KitL×FoxP3-GFP hosts with RFP expressing Teffs and 5 to 7 weeks later, ears developed depigmentation and I intradermally injected lesional ears with an antibody cocktail containing αLangerin-APC. I used confocal microscopy to image ears two hours after intradermal injection and found that LHCs frequently associated with both Teffs and Tregs (Fig. 13b). In fact, dendritic projections from LHCs appeared to hold interacting Tregs and Teffs close together. These observations support the hypothesis that LHCs facilitate interactions between Tregs and Teffs in the skin during vitiligo.

3.3.5: MHC-TCR interactions are dispensable for LHC-mediated suppression during murine vitiligo

To test whether MHC-TCR interactions between Tregs and LHCs are functionally required for LHC-mediated suppression, I crossed Krt14-KitL×Langerin-cre and $MHCII^{flox}$ mice to generate LHC-specific MHCII-deficient mice. I induced vitiligo in $MHCII^{flox/flox}$×LangerinCre+ using $MHCII^{flox/flox}$×LangerinCre- and $MHCII^{+/flox}$×LangerinCre+ littermates as controls. After 7 weeks mice developed depigmentation and a separate observer blinded to the experimental groups quantified the severity of depigmentation on ears, nose, hind footpad and tail skin to generate a vitiligo score. I observed no significant difference in vitiligo between mice with MHC deficient LHCs and littermate controls (Fig. 14a). I harvested tail skin, processed the tissue into single cell suspensions and used flow cytometry to analyze cell types in the skin. I first confirmed that LHCs lacked MHC II in $MHCII^{flox/flox}$×LangerinCre+ animals (Fig. 14b). I also noticed no difference in epidermal Teff number between $MHCII^{flox/flox}$×LangerinCre+ hosts and littermate controls (Fig. 14c). I also observed no

differences in the epidermal/dermal Treg ratio between MHCII$^{flox/flox}$×LangerinCre+ hosts and littermate controls (Fig. 14d). These results suggest MHC-TCR-mediated interactions between LHCs and Tregs may be dispensable for LHC suppression during vitiligo.

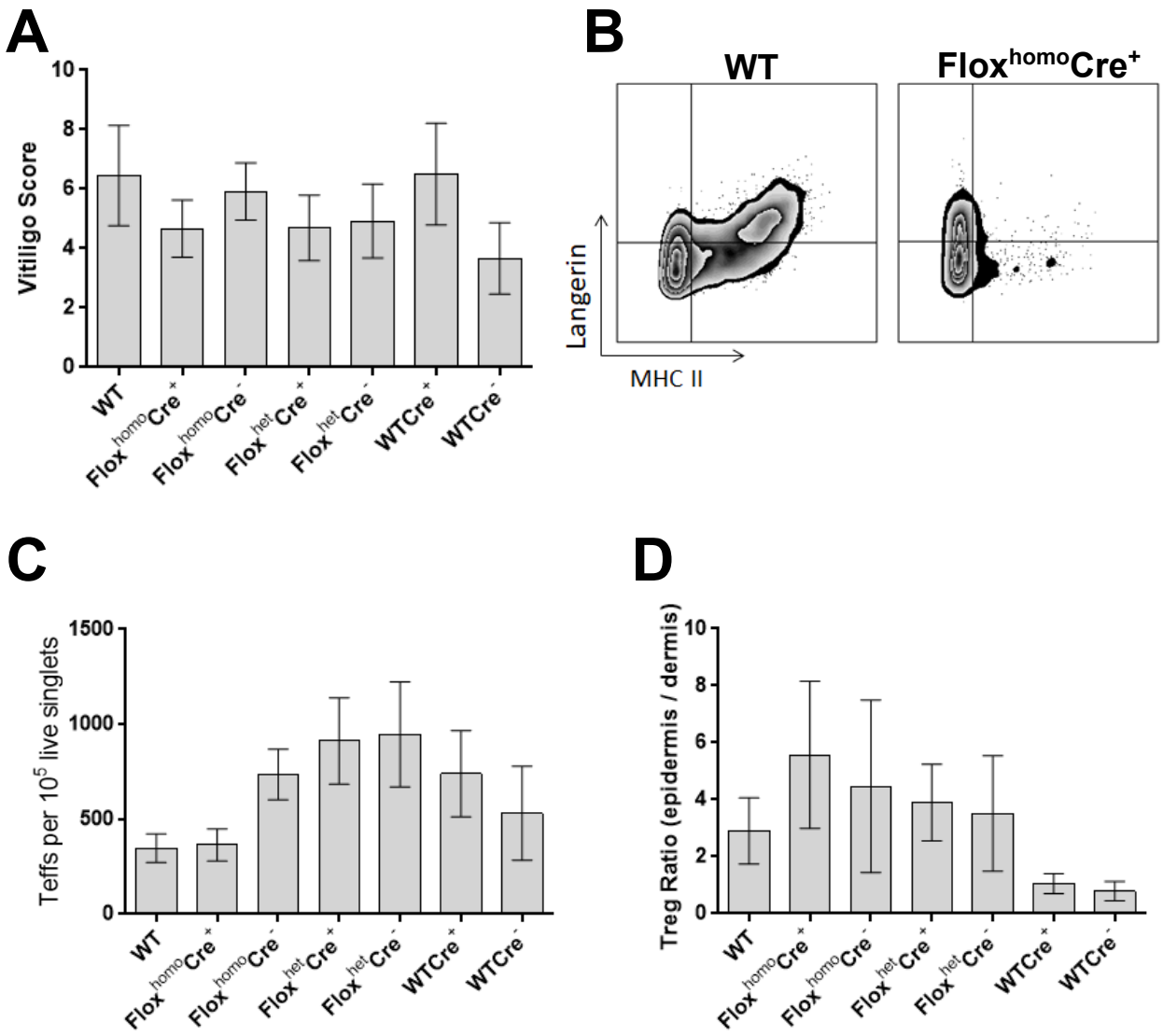


Figure 14: MHC-TCR interactions are dispensable for Langerhans cell suppression.

Figure 14: MHC-TCR interactions are dispensable for Langerhans cell suppression. Tail skin harvested from MHCII$^{flox/flox}$×LangerinCre+ mice and littermate controls with vitiligo was analyzed by flow cytometry 7 weeks post disease induction. **(A)** Graph shows quantification of depigmentation on mice with vitiligo; **(B)** Representative flow plots showing MHCII expression on LHCs in WT and Flox^{homo}Cre^{+} tails; **(C)** Graphs depict the average number of Thy1.1+ Teffs normalized to the number of live single cells in tail skin; or **(D)** the number of epidermal FoxP3+ Tregs divided by the number of dermal Tregs represented as a Treg ratio; ***WT N=11; Flox^{homo}Cre^{+} N=18; Flox^{homo}Cre^{-} N=12; Flox^{het}Cre^{+} N=13; Flox^{het}Cre^{-} N=13; WTCre^{+} N=10; WTCre^{-} N=6, bars represent s.e.m.***

3.4: Discussion

Whether LHCs play an immunogenic or tolerogenic role during inflammation is still debated and the role LHCs play during vitiligo is not completely understood. I observed that while the extent of depigmentation was comparable between LHC-KO and WT mice, the incidence of disease is increased in the absence of LHCs (Fig 9a). This suggests that LHCs suppress depigmentation during vitiligo by suppressing incidence of disease and I hypothesized that LHCs might act as an immunological checkpoint that autoimmunity must overwhelm for disease to proceed. Studies investigating the role that LHCs play during vitiligo reported conflicting results regarding the number and activation status of LHCs during disease [265-268]. No functional studies depleting LHCs were performed since the aforementioned works were done in human patients and this study functionally demonstrates that LHCs play a suppressive role during vitiligo.

Several studies report LHCs can play either an immunogenic or tolerogenic role during immune responses and CHS experiments using the same LHC-KO mouse used in this study, demonstrated that LHCs are dispensable for priming and suppress adaptive immunity [206]. I observed that the incidence of vitiligo is increased in the absence of LHCs (Fig 9a) mirroring previous findings in the context of T cell-mediated autoimmunity instead of delayed-type hypersensitivity response. A DCs maturation status influences whether a DC is immunogenic or tolerogenic [174-177] and the initial application of a hapten during CHS induction, could artificially activate DCs and influence that decision. In our model of vitiligo, we do not directly activate skin DCs to initiate disease providing a physiological context distinct from CHS to examine whether LHCs prime T cell responses. Models where OVA expression is driven by a skin specific promoter provide a physiological context to study disease as well but OT-I and OT-

II responses are abnormally sensitive and this must be taken into account when interpreting results [271]. Our model of disease also has certain caveats as we examine LHC suppression in the context of a monoclonal T cell response and break tolerance by infecting mice I.P. with recombinant vaccinia virus but my observations that LHCs suppress the incidence of disease suggest LHCs are dispensable for T cell priming and suppress adaptive responses.

I observed that the number of Teffs was increased in the skin of animals lacking LHCs (Fig 9b,c) and in light of this, I hypothesized that LHCs suppress depigmentation by controlling Teff number in the skin. Igyarto et al. observed an increase in disease and numbers of antigen specific T cells in the skin after inducing CHS in huLang-DTA mice [207] reflecting the observations I made in our model of vitiligo. Since I observed that Tregs suppress depigmentation during vitiligo by controlling Teff number (Fig. 1,3) and the number of Tregs in the skin is important for suppressing depigmentation (Fig 4), I hypothesized that Tregs may be affected in LHC-KO mice. I observed an increase in the number of dermal Tregs in LHC-KO mice and the epidermal/dermal ratio is decreased in LHC-KO mice compared to WT mice (Fig. 9e). Considering this I hypothesized that LHCs suppress disease by properly positioning Tregs in the epidermis during vitiligo to promote their suppressive interactions with Teffs. Igyarto et al. observed no difference in Treg number between LHC deficient and WT mice after inducing immunity and the observations I made conflict this finding. Other studies demonstrate that tolerogenic DCs interact with Tregs to promote an immunosuppressive environment [176, 177] and LHCs have been shown to induce the proliferation of skin resident Tregs [209], so it is possible that LHCs coordinate with Tregs during vitiligo to suppress autoimmunity and it seemed reasonable to test my hypothesis that LHCs mediate suppression by properly positioning Tregs.

Previous studies in our mouse model revealed that CXCL10 expression is upregulated in mouse and human skin during vitiligo [253]. The majority of CXCL10 is expressed in the epidermis during vitiligo and keratinocytes and LHCs are the major epidermal producers of CXCL10 [269]. I hypothesized that LHC-derived CXCL10 properly positions Tregs in the skin for optimal suppression. Few Tregs in the skin expressed CXCR3 on their surface when I analyzed skin infiltrating Tregs by flow cytometry but it is possible that Tregs internalize the receptor after exposure to chemokine. We know Teffs require CXCR3 and comparable numbers of Teffs in the skin expressed the receptor. When I analyzed lesional skin using confocal microscopy Tregs clustered around areas of CXCL10 expression but I cannot rule out the possibility that other cells or chemokines are mediating Treg clustering. These caveats must be considered but based on the observation that Tregs express CXCR3 and localize with CXCL10 expression in the skin, I felt it was reasonable to hypothesize LHC-derived CXCL10 was important for positioning Tregs.

In mixed bone marrow chimeras LHCs required CXCL10 for optimal suppression and this contrasted previously obtained data that demonstrated keratinocytes use CXCL10 to drive depigmentation [269]. To reconcile how the same chemokine could be used by two different cells to achieve opposite outcomes, I hypothesized that LHCs attract epidermal Tregs and Teffs with CXCL10 and then tether both cells through MHC-TCR interactions to promote direct cell-cell contact, and thus increase the efficiency of Treg-mediated suppression of Teffs. Seneschal et al. reported that LHCs in human skin induced skin resident Treg proliferation in an MHCII-dependent manner and LHCs interact with skin resident Tregs in the epidermis or near the dermal-epidermal junction [209], supporting the notion that functional interactions between LHCs and Tregs occur in the skin.

Interactions I observed between LHCs, Tregs and Teffs in the skin support my hypothesis that LHCs facilitate interactions between Tregs and Teffs in the skin during vitiligo (Fig. 11b). The possibility that contact between Tregs, Teffs and LHCs occurs merely by coincidence cannot be ignored. LHCs are tightly packed in the epidermis adjacent to melanocytes and KCs and Teffs and Tregs that must squeeze into extracellular spaces as they enter the skin during vitiligo are likely to encounter LHCs. I tested whether MHC-TCR interactions mediate this scaffold and I observed no significant difference in depigmentation between mice with MHC deficient LHCs and littermate controls (Fig. 12a). There was also no difference in epidermal Teff number between $MHCII^{flox/flox}$×LangerinCre+ hosts and littermate controls (Fig. 12c) and no differences in the epidermal/dermal Treg ratio (Fig. 12d). These results suggest MHC-TCR-mediated interactions between LHCs and Tregs may be dispensable for LHC suppression during vitiligo.

Taken together the observations made in this study suggest LHCs suppress the incidence of disease in a mouse model of vitiligo. In the absence of LHCs the number of Teffs infiltrating the skin increases and the epidermal/dermal Treg ratio decreases. How LHCs use CXCL10 to suppress depigmentation while keratinocytes use CXCL10 to promote depigmentation remains unanswered as LHCs do not require MHCII-TCR interactions to suppress vitiligo in our mouse model. It is possible that LHC-derived CXCL10 is enough to tether Teff and Tregs without MHC-TCR interactions. Groom et al. report that DCs in the lymph node tether T cells with CXCL10 [220] and if CXCL10 can tether Tregs and Teff to LHCs than it is still possible that LHCs can increase the efficiency of Treg-Teff interacts to facilitate suppression during vitiligo. Inducing vitiligo in $IFN\gamma R^{fl/fl}$ × LangerinCre mice would further test whether LHCs require CXCL10 for suppression as LHCs in this mouse would be unable to make CXCL10, expressed as a result of IFNγR signaling. Also LHCs may not alter Tregs and act directly on Teffs to

control their numbers. Igyarto et al. reported LHCs make the suppressive cytokine IL-10 and require IL-10 express for optimal suppress of CHS induced immune responses [207]. Autoimmunity progresses and is even exacerbated in the absence of LHCs in a mouse model of vitiligo suggesting that LHCs suppress adaptive immunity. This observation contributes to the ongoing discussion concerning whether LHCs function in an immunogenic or tolerogenic manner during immune responses.

3.5: Materials and Methods

Mice

KRT14-Kitl*4XTG2Bjl (Krt14-Kitl) mice were a generous gift from B. J. Longley (University of Wisconsin). I isolated Teffs from PMEL TCR transgenic mice that are Thy1.1+ and were obtained from The Jackson Laboratory, stock no. 005023, B6.Cg Thy1a/CyTg(TcraTcrb)8Rest/J. RFP mice were obtained from The Jackson Laboratory, stock no. 005884, B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J. RFP mice were bred to PMEL TCR transgenic mice to generate Teffs that express RFP. The following strains were bred to Krt14-Kitl mice to use as hosts in a mouse model of vitiligo: FoxP3-GFP mice generated by VJ Kuchroo, huLangerin-DTA (LHC-KO) mice (provided by D.H. Kaplan, University of Minnesota), REX3 mice (provided by A. Luster, Massachusetts General Hospital), $MHCII^{flox/flox}$ mice (The Jackson Laboratory, stock no. 013181, B6.129X1-H2-Ab1tm1Koni/J), LangerinCre mice (NCI Mouse Repository, strain no. 01X66, B6.Cg-Tg(CD207-cre)1Dhka/Nci) and $CXCL10^{-/-}$ mice (The Jackson Laboratory, stock no. 006087, B6.129S4-Cxcl10tm1Adl/J). REX3×Krt14-Kitl×FoxP3-GFP mice used as hosts for vitiligo were generated by crossing Krt14-Kitl×FoxP3-GFP mice and Krt14-Kitl×REX3 mice. Krt14-Kitl×$MHCII^{flox/flox}$×LangerinCre mice used as hosts for vitiligo were generated by crossing Krt14-Kitl×$MHCII^{flox/flox}$ mice and Krt14-Kitl ×LangerinCre mice. The Krt14-Kitl allele was bred heterozygous on all mice used in experiments. All mice were bred on a C57BL/6J background and mice were maintained in pathogen-free facilities at the University of Massachusetts Medical School (UMMS), and procedures were approved by the UMMS Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Vitiligo induction

Vitiligo was induced through adoptive transfer of CD8+ T cells isolated from PMEL mice (Teffs) as described previously [79]. Teffs were isolated from the spleens of PMEL TCR transgenic mice through negative selection using a Miltenyi Biotec (Bergisch Gladbach, Germany) CD8 isolation kit according to the manufacturer's instructions. 10^6 purified Teffs were injected intravenously into 8 to 12 week old hosts sublethally irradiated with 500 rads 1 day before transfer. Hosts were also infected with 10^6 plaque-forming units (PFU) of rVV-hPMEL through intraperitoneal injection (N. Restifo, National Cancer Institute, NIH) on the same day of adoptive transfer. Depigmentation was reported using a vitiligo score. The vitiligo score was determined by an observer blinded to the experimental groups, using a point scale based on the extent of depigmentation at four locations, the ears, tail, nose and hind footpads as previously described [79]. Each location was examined, and the extent of depigmentation was assigned a number based on the estimated percentage of depigmentation at the anatomic site. Both left and right ears and left and right rear footpads were estimated together and therefore evaluated as single sites. The scoring system used is as follows: no depigmentation = 0, >0 to 10% = 1 point, >10 to 25% = 2 points, >25 to 75% = 3 points, >75 to <100% = 4 points and 100% = 5 points. The "vitiligo score" was the sum of the scores at all four sites, with a maximum score of 20 points.

Flow Cytometry

Ears, tails and skin-draining lymph nodes were harvested. Lymph nodes were mechanically disrupted. To separate the dermis and epidermis, harvested ear and tail skin samples were incubated with dispase (50 U/ml and 5 U/ml in PBS, respectively) (Roche) for 1 hour at 37°C.

Epidermis was removed and mechanically disrupted with 70-mm cell strainers. Dermis samples were incubated with 1 mg/ml collagenase IV and 2 mg/ml DNase I (Sigma-Aldrich) in RPMI for 45 minutes at 37°C on a shaker. After incubation dermis was dissociated by gentle agitation. The following antibodies were obtained from BioLegend (San Diego, CA): mouse Thy1.1, CD8, CD45, MHCII, CD3, CD207, FOXP3, For extracellular staining cells were washed in FACS buffer (1% FBS in PBS) and then incubated with antibodies at a 1:200 dilution at 4°C in the dark for 20 minutes. For intracellular FoxP3 staining, surface staining was performed and cells were fixed and permeablized using the FoxP3/Transcription factor staining buffer set from eBioscience (Santa Clara, CA) according to the manufacturer's recommendations. The data were collected and analyzed with a BD LSR II flow cytometer (BD Biosciences) and FlowJo (Tree Star Inc.).

Priming Experiment

Teffs were isolated from the spleens of PMEL TCR transgenic mice through negative selection using a Miltenyi Biotec (Bergisch Gladbach, Germany) CD8 isolation kit according to the manufacturer's instructions. Teffs were labeled with CellTrace CFSE from Thermo Fisher Scientific (Waltham, MA) before 10^6 purified Teffs were injected intravenously into 8 to 12 week old hosts that were also infected with 10^6 plaque-forming units (PFU) of rVV-hPMEL through intraperitoneal injection (N. Restifo, National Cancer Institute, NIH). After 3 days spleens were isolated from mice, mechanically disrupted and CFSE dilution in splenic Teffs was analyzed with a BD LSR II flow cytometer (BD Biosciences) and FlowJo (Tree Star Inc.).

Mixed bone marrow chimera generation

To generate mixed bone marrow chimeras, LHC-KO hosts were lethally irradiated with 800 rads one day prior to reconstituting hosts with bone marrow. Donor bone marrow was isolated from C57BL/6J mice, CXCL10-/- mice and LHC-KO mice. In one group of mice 5×10^6 total bone marrow cells consisting of equal numbers of cells from CXCL10-/- and LHC-KO mice were transferred i.v. into previously irradiated hosts. In the other group of mice 5×10^6 total bone marrow cells consisting of equal numbers of cells from C57BL/6J and LHC-KO mice were transferred i.v. into previously irradiated hosts. As controls previously irradiated hosts were reconstituted with bone marrow from C57BL/6J mice. After 8 weeks I induced vitiligo in mixed bone marrow chimeras as previously described.

Confocal Microscopy

For imaging experiments to visualize Tregs co-localizing with CXCL10 expression, vitiligo was induced in REX3×Krt14-Kitl×FoxP3-GFP mice as previously a described. After 5 weeks mice were euthanized, ears excised and following light treatment of ears with Nair to remove hair, the dorsal and ventral sides were separated before being mounted on slides for imaging. For imaging experiments to visualize interactions between LHCs, Tregs and Teffs, vitiligo was induced in Krt14-Kitl×FoxP3-GFP mice as previously described using RFP expressing Teffs from PMEL×RFP donors. After 5 weeks lesional ears were intradermally injected with 40ul of an antibody cocktail containing 0.05mg/ml Langerin-APC clone 4C7 from Biolegend (Dedham, MA) and 0.35mg/ml FC block 2.4G2. Two hours after intradermal injection, mice were euthanized and ears excised. Following light treatment of ears with Nair to remove hair, the dorsal and ventral sides were separated and mounted on slides for imaging. All confocal imaging was performed on a Leica SP8 confocal microscope.

Epidermal sheet staining

The dorsal and ventral sides of ears were mechanically separated, and each side was fixed to a glass slide, epidermis side down, with clear nail polish. 50 U/ml of dispase (Roche) was dropped into a hydrophobic barrier drawn around ears on the slides and slides were incubated at 37° C for an hour. After incubation the dermis was gently removed and following the remaining epidermal sheet was fixed in 4% paraformaldehye for 30 minutes at room temperature. After being washed with PBS epidermal sheets were incubated for 1 hour at 25° C with 2% (wt/vol) BSA in PBS and stained overnight at 4° C with a 1:1000 dilution of primary antibody, CD207 clone 4C7 from Biolegend (Dedham, MA), in 2% BSA. Sheets were washed thoroughly in PBS and were incubated with a 1:2000 dilution of secondary antibody, donkey anti-mouse AF647 or AF555 from Life Technologies (Carlsbad, CA) in PBS 1% BSA (wt/vol) for 90 minutes at 37° C. Epidermal sheets were washed thoroughly with PBS and mounted on slides with Prolong Gold from Life Technologies (Carlsbad, CA).

Statistical analyses

All statistical analyses were performed with GraphPad Prism software. Dual comparisons were made with unpaired Student's t test.

CHAPTER IV: TREGS REQUIRE CCR5 AND CCR6 FOR OPTIMAL SUPPRESSION IN A MOUSE MODEL OF VITILIGO

Kingsley I. Essien, James P. Strassner, Kyle J Gellatly, Patrick E. McDonel, Manuel Garber and John E. Harris

Contribution Summary:

K.I.E helped to design the experiments, performed the experiments, and helped to analyze the data and write the manuscript. J.P.S performed suction blistering to obtain skin infiltrating cells from human skin and performed single cell RNA-sequencing on the infiltrate from blisters. K.J.G and P.E.M helped to analyze single cell RNA-sequencing data. M.G. helped to design the RNA-seq experiments and analyze the RNA-seq data. J.E.H. helped to design the experiments, analyze the data, and write the manuscript.

4.1: Abstract

Previous studies in our mouse model of vitiligo revealed that Teffs require CXCR3 to migrate to the skin in response to CXCL9 and CXCL10 chemokine signals. I also found that T regulatory cells (Tregs) suppress depigmentation in vitiligo and directly contact Teffs in human and murine lesional skin, suggesting that Tregs must colocalize with Teffs in the skin to efficiently suppress their function. In light of these observations, I investigated the mechanisms of Treg recruitment to the skin during vitiligo. To investigate the chemokine signals that promote Treg migration, we induced vitiligo in $RAG^{-/-}$ hosts that received wild-type (WT) or $CXCR3^{-/-}$ Tregs. WT and $CXCR3^{-/-}$ Treg recipients had comparable levels of depigmentation and comparable numbers of Teffs and Tregs in the skin, suggesting that CXCR3 is dispensable for the ability of Tregs to migrate to the skin and suppress vitiligo. In previous studies we observed that CCL5 and its receptor CCR5 are highly induced in the skin of vitiligo patients and mice, and published GWAS results identified CCR6 as a vitiligo susceptibility gene. We found that Tregs express CCR5 and CCR6 in mouse and human skin during vitiligo, and that eliminating either chemokine receptor on Tregs inhibited their ability to suppress depigmentation during vitiligo, however they affected Tregs differently. While the number of $CCR6^{-/-}$ Tregs was decreased in the skin of mice with vitiligo compared to WT Tregs, $CCR5^{-/-}$ Tregs were present in normal numbers, supporting a role for CCR5 in their function. Our results reveal that both CCR5 and CCR6 are required for optimal suppression of Teff-mediated depigmentation.

4.2: Introduction

Chemokine signaling regulates immune responses during vitiligo.

One of the fundamental features of the immune system is its ability to carefully balance effector responses with mechanisms of immunosuppression and the chemokine signals that mediate T cell infiltration during vitiligo are well known. Rashighi et al. compared the gene expression profiles between lesional skin biopsies from vitiligo patients as well as healthy controls and found that chemokines associated with a Th1 immune response were upregulated in vitiligo skin. IFNγ-dependent chemokines like CCL5 and the CXCR3 ligands CXCL10, CXCL9, CXCL11 were highly induced during vitiligo and the most upregulated chemokines among these were CXCL10 and CCL5 [253]. Rashighi et al. found that the CCR4 ligands CCL17 and CCL22 were induced as well, but to a much lower extent than the aforementioned IFNγ-dependent chemokines [253]. Rashighi et al. analyzed the gene expression profile in lesional ear skin in their mouse model of vitiligo and observed that CCL5, CXCL9 and CXCL10 were all markedly induced [253]. This expression profile was skewed towards an IFNγ-dependent signature, mirroring gene expression in patients. In patient serum levels of CXCL10 protein was significantly increased, and CXCL10 drove depigmentation in a mouse model of vitiligo [253]. Disease-causing Teffs required CXCR3 expression to enter the skin and mediate depigmentation, and treating mice with CXCL10 neutralizing antibody during disease induction almost completely abrogated disease [253]. Furthermore, treating already depigmented mice with CXCL10 neutralizing antibody induced repigmentation, establishing CXCL10 as a crucial signal for mediating Teff migration to the skin [253].

In the same mouse model of disease Richmond et al. demonstrated that treating mice with CXCR3 blocking and depleting antibodies prevents disease and induces the repigmentation of

already depigmented skin [272]. This result further supports a role for CXCR3 signaling in facilitating Teff migration to the skin during disease and since nearly all CD8s isolated from lesional skin express CXCR3 [264], drugs targeting this pathway may promote repigmentation in patients.

CXCR3 promoted Treg recruitment in other inflammatory models with a Th1 signature [234] and whether or not CXCR3 mediates Treg recruitment to the skin during vitiligo needs to be explored when considering interventions that disrupt CXCR3 signaling. Another study observed that protein levels of CCL5, CXCL8 and CXCL10 were increased in serum from patients with active vitiligo compared to patients with stable vitiligo or healthy controls [273]. Blister fluid from lesional skin revealed a significant increase in CXCL10 and CCL5 protein in the skin of patients with active disease compared to the skin of patients with stable disease or healthy controls [273]. Using flow cytometry they found that significantly more CD8+ T cells express CCR5 and CXCR3 in the peripheral blood of patient with active vitiligo compared to patients with stable vitiligo or healthy controls [273]. These results mirror previous findings that suggest IFNγ-dependent chemokines play a role in mediating vitiligo.

Few have explored the chemotactic signals that drive Treg migration to the skin during vitiligo. Jin et al. identified an association of generalized vitiligo with SNPs in the CCR6 gene in a vitiligo genome wide association study [24]. Since CCR6 has been shown to mediate Th17 and Treg migration during inflammation [237-239] and establish Tregs in the skin during neonatal development [152], it is likely that a SNP affecting CCR6 would affect Tregs and not Teffs during vitiligo. Thus a loss of function or impaired function mutation could tip the balance in favor of proinflammatory migration during vitiligo. In one study Klarquist et al. used flow cytometry to compare chemokine receptor expression between Tregs isolated from the PBMCs

of vitiligo patients or healthy controls. They observed that levels of CCR4, CCR5 and CCR8 were comparable between circulating Tregs in vitiligo patients and controls [247]. Klarquist et al. performed immunostaining on skin biopsies to compare chemokine expression between healthy and lesional skin. While levels of CCL1 and CCL17 were similar between healthy and lesional skin, CCL22 expression was significantly reduced in vitiligo skin and the authors suggest that this decrease leads to reduced Treg migration to lesional skin and consequently, diminished suppression in lesional skin [247].

In two different murine models of spontaneous vitiligo, Eby et al. used gene gun treatment to express CCL22 expression in the skin and saw increased cutaneous Treg migration and decreased depigmentation [155]. These results support a role for CCR4/CCL22 signal as sufficient to promote Treg skin migration, but one caveat of this treatment method was its limited durability, as treated mice depigmented two weeks after the end of treatment [155]. It is possible that other chemokines along with CCL22 mediate Treg migration and that targeting these chemokines as well could provide longer lasting protection. The chemokine signals that mediate Treg migration to the skin during vitiligo are not completely understood. Fully elucidating the chemokine signals that draw Tregs to the skin could lead to the discovery of new treatments or refine existing treatments for patients with vitiligo. In this study I use a mouse model of vitiligo to understand the chemokine signals that Tregs follow during vitiligo.

In our mouse model I observed exacerbated disease in the absence of Tregs and a greater increase in depigmentation in ear skin than in tail skin after Treg depletion (Fig. 1 & 3). In our model of vitiligo, ear skin depigments to a lesser extent compared to tail skin (Fig. 4), and I found that there are more Tregs in ear skin than tail skin in mice both before vitiligo induction and during disease progression (Fig. 4 & 5). Together this data suggests that the number of Tregs

in the skin affects disease severity. In support of this hypothesis, I found a lower Treg/Teff ratio in lesional skin than in non-lesional skin (Fig. 4). I found Tregs in direct contact with Teffs in mouse and human skin, suggesting that Treg suppression occurs in the skin (Fig. 6) and I questioned what signals help Tregs find Teffs in the skin to mediate suppression. Understanding the signals that mediate Treg migration to the skin during could improve treatments that aim to increase Treg number in the skin.

4.3: RESULTS

4.3.1: Tregs do not require CXCR3 to suppress disease in a mouse model of vitiligo.

We previously published that CXCL10, the ligand for CXCR3, is upregulated in the skin of vitiligo patients and mice, and Teffs require CXCR3 to migrate to the epidermis in a CXCL10-dependent manner and mediate depigmentation [253, 272]. Since I observed that Tregs and Teffs migrate to the skin with similar kinetics (Fig. 5), that Tregs express CXCR3, and that Tregs localize with CXCL10 expression in mouse skin during vitiligo (Fig. 9), I hypothesized that Tregs follow the same signal as Teffs, CXCL10, to get to the skin. In this scenario Tregs can interact with and suppress Teffs since both T cells will inevitably end up in the same areas if they follow the same signal.

To test whether Tregs require CXCR3 to migrate to the skin and mediate suppression during vitiligo, I modified a $Rag^{-/-}$ mouse model of melanoma [274]. Since $Rag^{-/-}$ mice lack T cells, I can induce vitiligo in $Rag^{-/-}$ hosts by adoptively transferring Teffs and Treg-depleted CD4 T cells. I can then use $Rag^{-/-}$ hosts to screen whether Tregs require CXCR3 to suppress vitiligo by comparing the extent of depigmentation between hosts reconstituted with $CXCR3^{-/-}$ or WT Tregs. I can use this strategy to test the necessity of any molecule for Treg suppression during

vitiligo instead of breeding KO strains to use as hosts in our mouse model. I generated Krt14-Kitl×Rag$^{-/-}$ hosts that lack T cells and retain melanocytes in the epidermis. I adoptively transferred 10^6 Treg-depleted CD4 cells and 10^5 Tregs that I isolated from spleens WT B6 mice of donors using miltenyi columns. After 4 weeks I used flow cytometry to quantify CD45+CD3+Thy1.2+GFP+ Tregs in SDLNs, ear skin and tail skin. I observed that in most areas, Treg numbers in Rag$^{-/-}$ mice were comparable to Treg numbers in our Krt14-Kitl×FoxP3-GFP mice (Fig. 13a). The only exception was the ear dermis where we observed more Tregs in Rag-/- hosts than in Krt14-Kitl×FoxP3-GFP mice, an average of 263.02 ± 55.37 cells compared to 86.10 ± 9.73 cells, respectively (Fig. 15a). To ensure that the addition of Tregs suppresses depigmentation I induced vitiligo in sublethally irradiated Krt14-Kitl×Rag$^{-/-}$ hosts with 10^6 Teffs, 10^6 Treg-depleted CD4s and 10^5 WT Tregs or no Tregs (Fig. 15b). I infected hosts with rVV-gp100 on the day of adoptive transfer. After 5 weeks, I compared ear skin depigmentation between groups, since I previously observed that ear skin exhibited the most pronounced difference in depigmentation between untreated and Treg-depleted hosts (Fig. 3). I observed that hosts that received no Tregs had extensive ear depigmentation but hosts that received WT Tregs had minimal ear depigmentation, 4.17± 0.17 compared to 1.33 ± 0.61, respectively (Fig. 15c). This result mirrors my previous findings that demonstrate Tregs suppress depigmentation and establishes a model assay to screen Treg function.

To test whether Tregs require CXCR3 to control depigmentation, I induced vitiligo in our Krt14-KitL×Rag$^{-/-}$ hosts with 10^6 Teffs, 10^6 Treg-depleted CD4s and 10^5 WT Tregs, CXCR3$^{-/-}$ Tregs, or no Tregs (Fig 16a). After 5 weeks, a blinded observer quantified the extent of depigmentation on ear skin to generate a vitiligo score and I compared ear skin depigmentation between groups. I again observed that hosts that received no Tregs had extensive ear

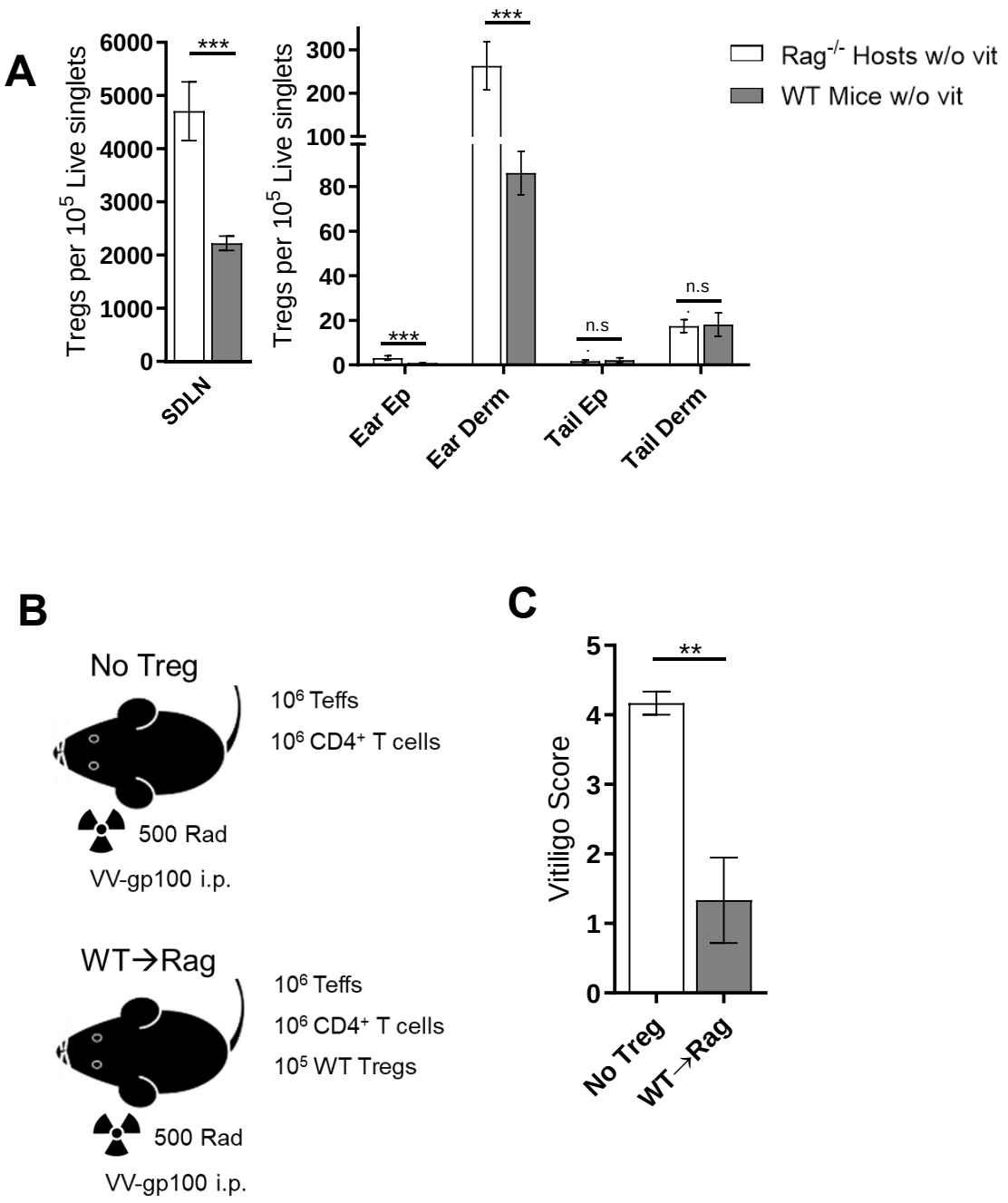


Figure 15: Tregs suppress depigmentation in a Rag$^{-/-}$ model of vitiligo.

Figure 15: Tregs suppress depigmentation in a Rag$^{-/-}$ model of vitiligo. Tissues harvested from Krt14-Kitl×FoxP3-GFP mice (WT) and Rag$^{-/-}$ hosts 4 weeks after adoptive Treg transfer were analyzed by flow cytometry. **(A)** Graph shows the average number of CD45+CD3+Thy1.2+GFP+ Tregs normalized to the number of live single cells in the indicated tissues; ***Rag$^{-/-}$ mice: N=4, students T-test, bars represent s.e.m.***; ***FoxP3-GFP mice: N=12, students T-test, bars represent s.e.m***; **(B)** Sublethally irradiated, rVV-gp100 infected Krt14-Kitl×Rag$^{-/-}$ hosts received 10^6 Teffs, 10^6 Treg-depleted CD4s and 10^5 WT Tregs or no Tregs; and in **(C)** graph depicts quantification of ear depigmentation in Rag$^{-/-}$ hosts from both groups represented as a vitiligo score; ***N=6, students T-test, bars represent s.e.m.***

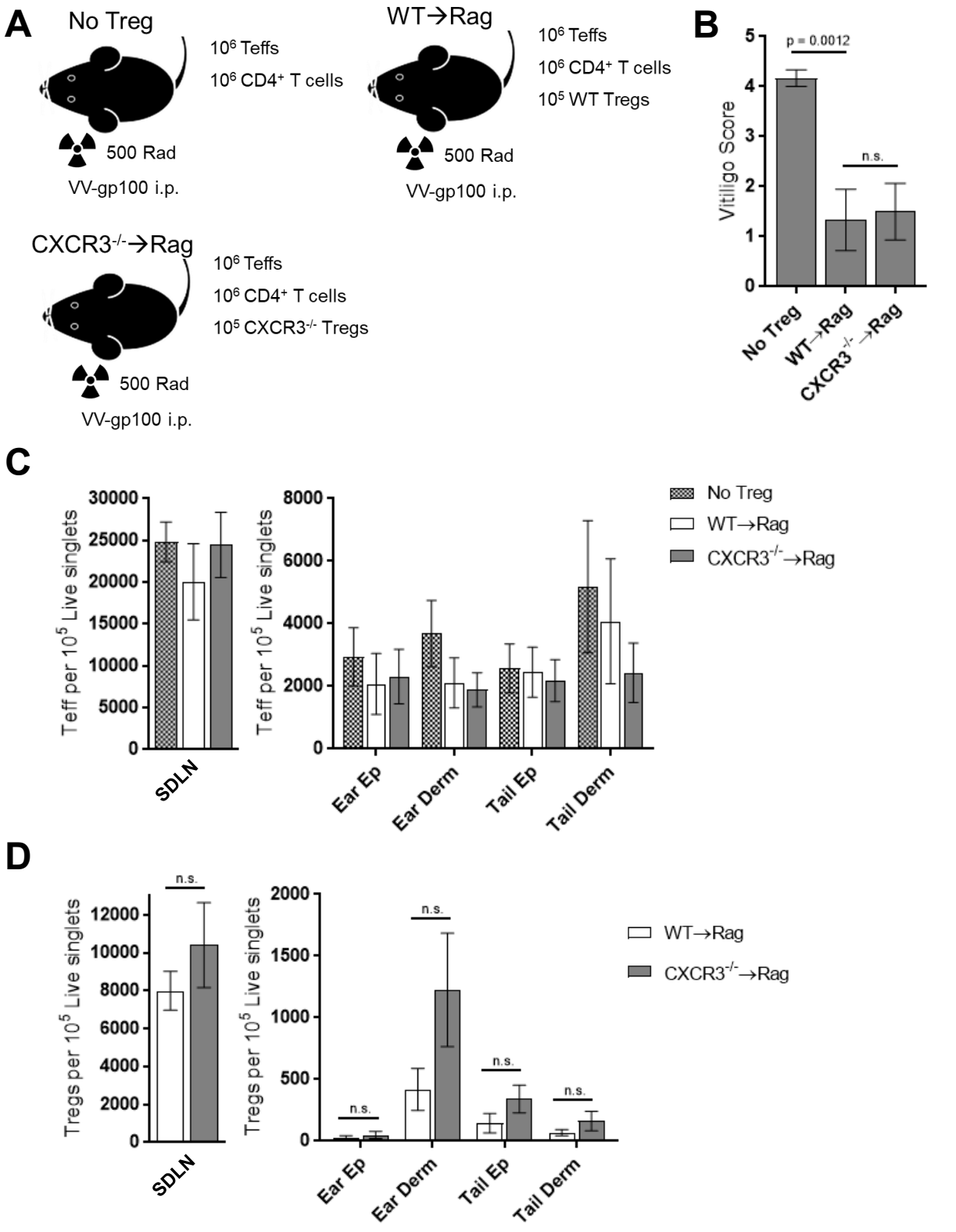


Figure 16: Tregs do not require CXCR3 to suppress depigmentation in a $Rag^{-/-}$ model of vitiligo.

Figure 16: Tregs do not require CXCR3 to suppress depigmentation in a Rag$^{-/-}$ model of vitiligo. (A) Sublethally irradiated, rVV-gp100 infected Krt14-Kitl×Rag$^{-/-}$ hosts received 10^6 Teffs, 10^6 Treg-depleted CD4s and no Tregs, 10^5 WT Tregs or 10^5 CXCR3$^{-/-}$ Tregs; and in **(B)** graph depicts quantification of ear depigmentation in Rag$^{-/-}$ hosts represented as a vitiligo score; Tissues harvested from Rag$^{-/-}$ hosts were analyzed by flow cytometry 5 weeks post vitiligo induction and graphs show the average number of **(C)** Thy1.1+ Teffs; or **(D)** CD3+Thy1.2+ Tregs normalized to the number of live single cells in the indicated tissues**; *N=6, students T-test, bars represent s.e.m.***

depigmentation and hosts that received WT Tregs had minimal ear depigmentation (Fig. 16b). Hosts that received CXCR3$^{-/-}$ Tregs had comparable levels of ear depigmentation as hosts receiving WT Tregs, with vitiligo scores of 1.50 ± 0.56 compared to 1.33 ± 0.61, respectively (Fig. 16b). This observation suggests that Tregs do not require CXCR3 to prevent Teff-mediated depigmentation. I harvested SDLNs, ear and tail skin, separated the dermis from the epidermis and used flow cytometry to quantify CD45+CD3+Thy1.1+ Teffs in the skin and SDLNs. I found comparable numbers of Teffs in the SDLNs and ear skin of mice receiving CXCR3$^{-/-}$ and WT Tregs (Fig. 16c). Hosts that received no Tregs had the most Teffs in ear skin (Fig. 16c). I also used flow cytometry to quantify Tregs in SDLNs, ear and tail skin and I observed that WT and CXCR3$^{-/-}$ Tregs migrate to the ear skin of Rag$^{-/-}$ mice during vitiligo (Fig. 16d). I observed more CXCR3$^{-/-}$ Tregs in the ear dermis and the fact that this difference is not statistically significant may be explained by the power of the study. While Teffs require CXCR3 to get to the skin and mediate disease, these results suggest that CXCR3 is dispensable for Treg suppression and migration to the skin. These findings also suggest that treatments targeting the CXCL10/CXCR3 axis to counteract Teff-mediated depigmentation will not affect Treg suppression, which appears to operate independent of CXCR3.

4.3.2: Tregs in human and murine skin express CCR6 during vitiligo.

After I determined that Tregs do not require CXCR3 to suppress depigmentation, I investigated which chemokines and receptors drive Treg migration to the skin during vitiligo. GWAS studies have identified CCR6 as a vitiligo susceptibility gene [275], CCR6 has been reported to facilitate Treg migration to mouse skin [152] and areas of inflammation in other models of disease [237], and based on these observations I hypothesized that CCR6/CCL20

signaling contributes to Treg migration during vitiligo.

In collaboration with other members of the lab, I used suction blistering to isolate cells from the skin of vitiligo patients and using single cell RNA-seq, I identified Teffs based on CD8 expression and Tregs based on CD4 and FOXP3 expression. Single cell RNA-seq revealed that Teffs and Tregs express CCR6 at the RNA level (Fig. 17a). I used flow cytometry to quantify CCR6 expression on CD45+CD8+ Teffs and CD45+CD4+CD25+FOXP3+ Tregs isolated from the lesional skin of vitiligo patients by suction blistering. I observed that approximately 90% of Tregs and 60% of Teffs isolated from lesional skin express CCR6 (Fig. 17b). Tregs also express significantly higher levels of CCR6 than Teffs, with an average median florescence intensity (MFI) of 2719 ± 479.1 compared to 750 ± 70.4, respectively (Fig. 17c).

I harvested SDLNs, ear and tail skin from Krt14-KitL×Foxp3-GFP mice and used flow cytometry to investigate receptor levels on GFP+ Tregs without vitiligo. I didn't find appreciable numbers of Tregs in the epidermis in mice without vitiligo so I focused Tregs in the SDLNs and dermis for analysis. I found that few Tregs in SDLNs express CCR6, 9.11 ± 1.02%, but that 52.26 ± 5.082% of Tregs in ear skin and 40.14 ± 6.87% of Tregs in tail skin express CCR6 (Fig. 17d). Tregs in the skin also express significantly higher levels of CCR6 than Tregs in the SDLNs with an average MFI of 146.2 ± 35.5 in SDLNs compared to an average MFI of 406.5 ± 45 in ear skin and 384.8 ± 42.9 in tail skin (Fig. 17e). These observations suggest that CCR6-expressing Tregs are enriched in the skin due to homing and this supports findings that Tregs require CCR6 to get to the skin.

I induced vitiligo in Krt14-KitL×Foxp3-GFP mice and 5 weeks later I harvested SDLNs, ear and tail skin, processed the tissue into single cell suspensions and used flow cytometry to measure CCR6 expression on Teffs and Tregs during vitiligo. I found that few Tregs and Teffs

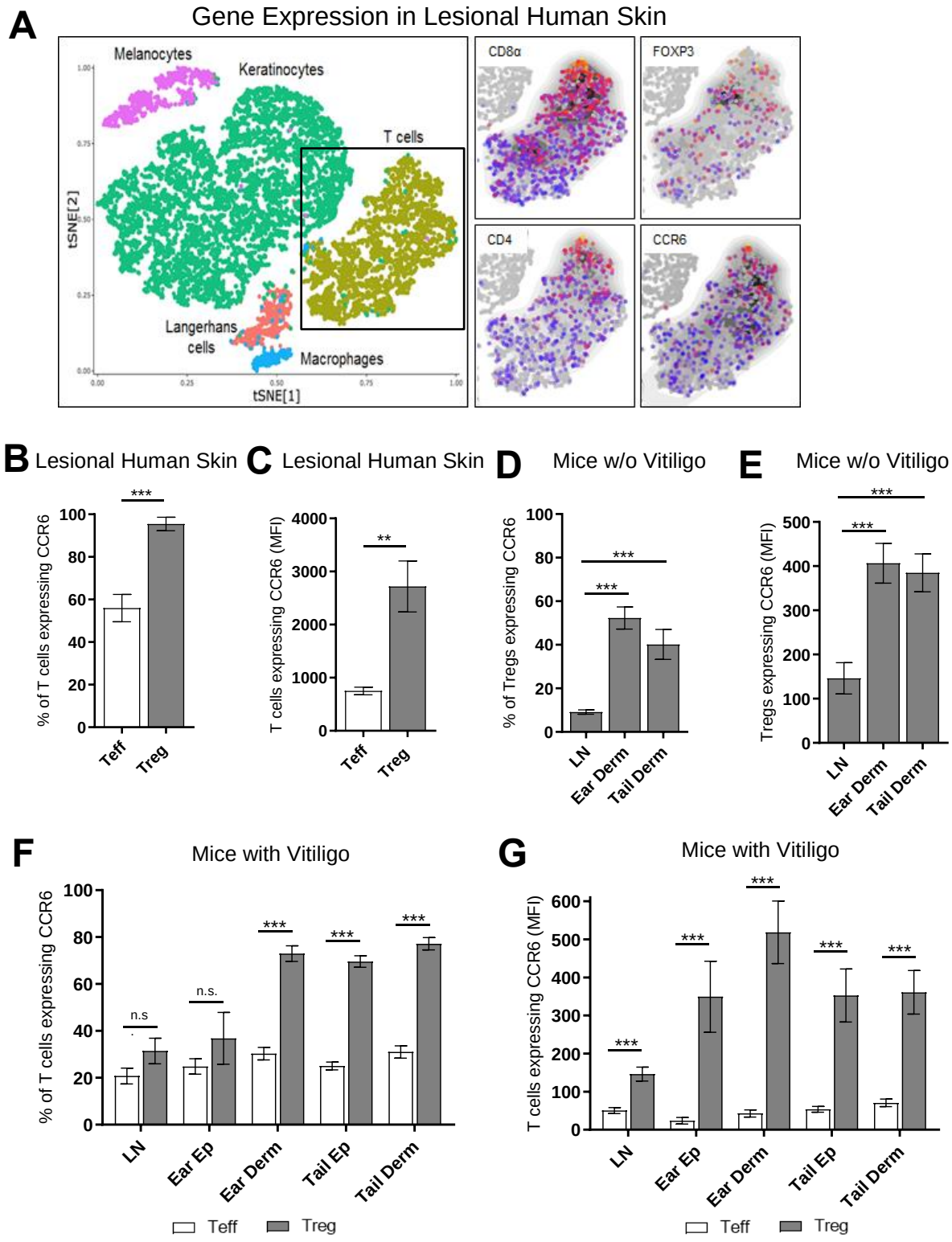


Figure 17: Tregs in human and murine skin express CCR6 during vitiligo.

Figure 17: Tregs in human and murine skin express CCR6 during vitiligo. Skin infiltrating cells isolated from the lesional skin of patients by suction blistering were analyzed by single cell RNA-sequencing and flow cytometry. **(A)** The tSNE plot generated from the RNA-seq data shows different cell types within lesional skin and skin Tregs express CD4, FoxP3 and CCR6; graphs depict flow cytometry data quantifying **(B)** the average percentage of T cells expressing CCR6; or **(C)** the average MFI of CCR6 expression on T cells in lesional skin; ***N=5, students T-test, bars represent s.e.m.;*** GFP+ Tregs in tissues harvested from 8-week-old Krt14-Kitl×FoxP3-GFP were analyzed by flow cytometry and **(D)** graphs depict the average percentage of Tregs expressing CCR6; or **(E)** the average MFI of CCR6 expression on Tregs in the indicated tissues; ***N=8, students T-test, bars represent s.e.m.;*** GFP+ Tregs and Thy1.1+ Teffs in tissues harvested from Krt14-Kitl×FoxP3-GFP vitiligo mice were analyzed by flow cytometry and graphs depict **(F)** the average percentage of T cells expressing CCR6; or **(G)** the average MFI of CCR6 expression on T cells in the indicated tissues ***N=10, students T-test, bars represent s.e.m.***

express CCR6 in the SDLNs but in the skin more Tregs make CCR6 than Teffs and this difference is statistically significant everywhere except the ear epidermis (Fig. 17f). I observed a similar trend when I compared the MFI between Tregs and Teffs during vitiligo as Tregs express significantly higher levels of CCR6 than Teffs in SDLNs and ear and tail skin (Fig. 17g). I observed that Tregs express CCR6 in the skin during vitiligo and express more CCR6 than Teffs, suggesting that treatments aimed at recruiting more Tregs into the skin by targeting the CCR6/CCL20 axis will preferentially affect Tregs.

4.3.3: Tregs in human and murine skin express CCR5 during vitiligo.

In previous gene expression studies CCL5, the ligand for CCR5, was upregulated in the lesional skin of vitiligo patients [253]. CCR5 has also be has been reported to facilitate Treg function and migration in other inflammatory disease models [241, 242] and based on these observations I hypothesized that CCR5 signaling may contribute to Treg migration during vitiligo.

In collaboration with other members of the lab, I used suction blistering to isolate cells from the lesional skin of vitiligo patients and single cell RNA-seq to analyze gene expression in skin infiltrating cells. I identified Teffs based on CD8 expression and Tregs based on CD4 and FOXP3 expression and I observed that Teffs and Tregs express CCR5 at the RNA level (Fig. 18a). I used flow cytometry to quantify CCR5 expression on T cells isolated by suction blistering. I identified Teffs as CD45+CD8+ and Tregs as CD45+CD4+CD25+FOXP3+ and observed that an average of 76.66 ± 10.47% of Teffs express CCR5 on their surface and an average of 98.5 ± 0.89% of Tregs express CCR5 (Fig. 18b). Tregs express more CCR5 than Teffs by MFI as well with averages of 5465 ± 175 and 2455 ± 551.7, respectively. This

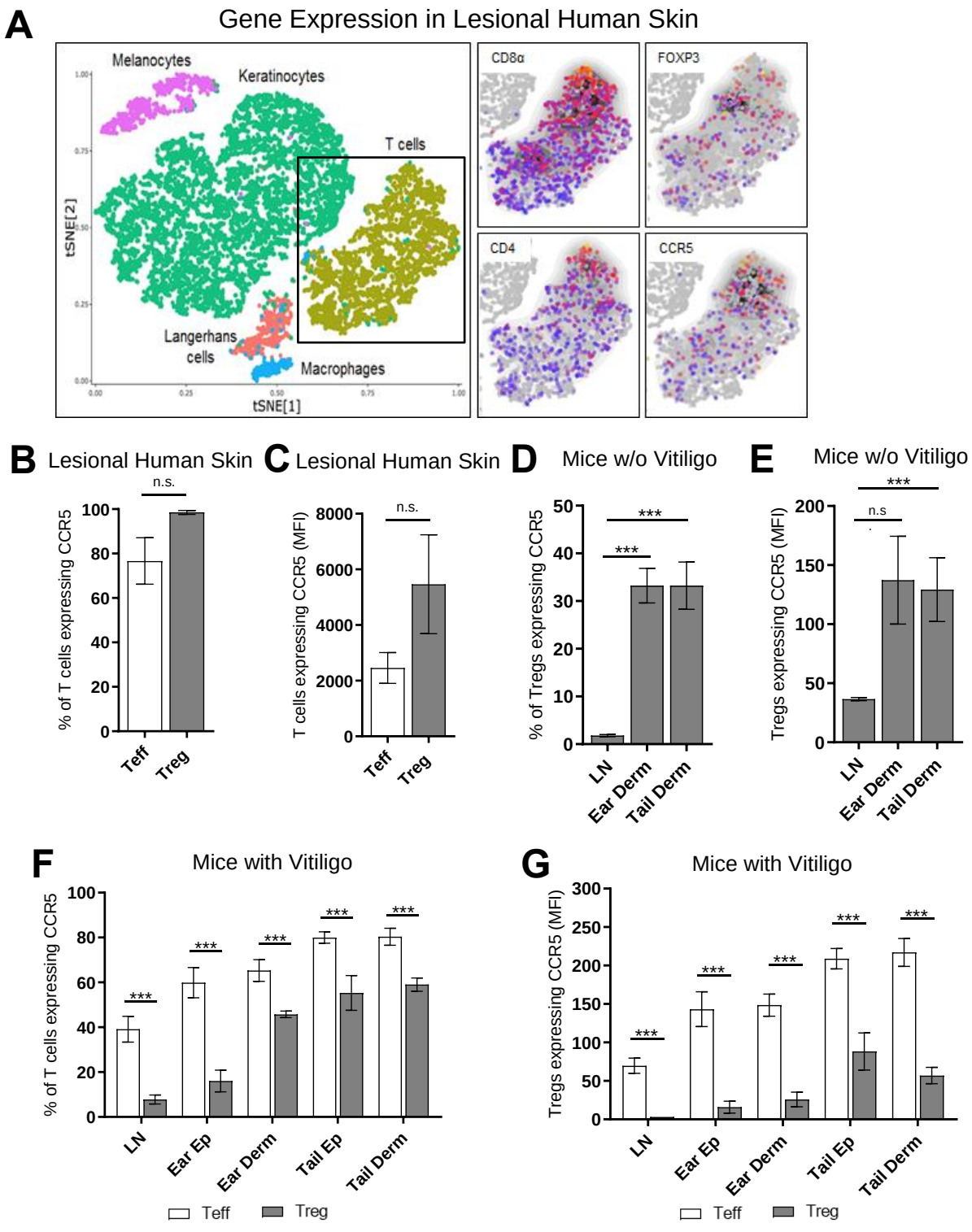


Figure 18: Tregs in human and murine skin express CCR5 during vitiligo.

Figure 18: Tregs in human and murine skin express CCR5 during vitiligo. Skin infiltrating cells isolated from the lesional skin of patients by suction blistering were analyzed by single cell RNA-sequencing and flow cytometry. **(A)** The tSNE plot generated from the RNA-seq data shows different cell types within lesional skin and skin Tregs express CD4, FoxP3 and CCR5; graphs depict flow cytometry data quantifying **(B)** the average percentage of T cells expressing CCR5; or **(C)** the average MFI of CCR5 expression on T cells in lesional skin; ***N=5, students T-test, bars represent s.e.m.;*** GFP+ Tregs in tissues harvested from 8-week-old Krt14-Kitl×FoxP3-GFP were analyzed by flow cytometry and **(D)** graphs depict the average percentage of Tregs expressing CCR5; or **(E)** the average MFI of CCR5 expression on Tregs in the indicated tissues; ***N=12, students T-test, bars represent s.e.m.;*** GFP+ Tregs and Thy1.1+ Teffs in tissues harvested from Krt14-Kitl×FoxP3-GFP vitiligo mice were analyzed by flow cytometry and graphs depict **(F)** the average percentage of T cells expressing CCR5; or **(G)** the average MFI of CCR5 expression on T cells in the indicated tissues ***N=10, students T-test, bars represent s.e.m.***

difference in MFI was not statistically significant (Fig. 18c).

To investigate CCR5 levels on Tregs without vitiligo, I harvested SDLNs, ear and tail skin from Krt14-KitL×Foxp3-GFP mice and used flow cytometry to analyze receptor expression on GFP+ Tregs. I didn't find appreciable numbers of Tregs in the epidermis in mice without vitiligo so I focused on Tregs in the SDLNs and dermis for analysis. I found that 1.81 ± 0.23% of Tregs in SDLNs express CCR5 and significantly more Tregs express CCR5 in ear and tail skin, 33.23 ± 3.61% of Tregs in ear skin and 33.23 ± 4.97% of Tregs in tail skin (Fig. 18d). I observed the same trend when I compared CCR5 expression by MFI between Tregs in the SDLNs and skin as the average MFI of Tregs in the SDLN is 36.58 ± 1.33 compared to 137.3 ± 37.2 and 129.2 ± 27.0 in ear and tail skin, respectively (Fig. 18e). These finding suggest Tregs upregulate CCR5 in the skin and may require CCR5 for optimal migration to the skin during vitiligo.

To investigate CCR5 expression during vitiligo I used our mouse model of disease. I induced vitiligo in Krt14-KitL×Foxp3-GFP mice and 5 weeks later I harvested SDLNs, ear and tail skin, processed the tissue into single cell suspensions and used flow cytometry to measure CCR5 expression on Teffs and Tregs during vitiligo. I observed that Tregs express CCR5 during vitiligo but fewer Tregs than Teffs express CCR5 in all tissues (Fig. 18f). I also observed that Teffs express more CCR5 when I compared MFI between Tregs and Teffs in all tissues (Fig. 18g). This difference is statistically significant in all tissues. While Teffs and Tregs both express CCR5, Tregs seem to express high levels of CCR5 in the skin and not the SDLNs during vitiligo. Based on these findings I hypothesize that CCR5/CCL5 signaling contributes to Treg migration to the skin during vitiligo.

4.3.4: Tregs require CCR6 but not CCR5 for optimal migration in a mouse model of vitiligo.

After I observed that Tregs express CCR6 and CCR5 in the skin during vitiligo I hypothesized that these chemokine receptors could promote Treg migration and suppression in the skin. To test this I used the $Rag^{-/-}$ model of vitiligo that I modified to investigate the ability of $CCR6^{-/-}$ and $CCR5^{-/-}$ Tregs to suppress Teff-mediated depigmentation. I induced vitiligo in Krt14-KitL×$Rag^{-/-}$ hosts with 10^6 Teffs, 10^6 Treg-depleted CD4 T cells and no Tregs or 10^5 Tregs isolated from the spleens of WT B6 mice, $CCR6^{-/-}$ mice or $CCR5^{-/-}$ mice (Fig. 19a). After 5 weeks, a blinded observer quantified the extent of ear skin depigmentation to generate a vitiligo score and I compared depigmentation between groups. Consistent with previous results, I observed that hosts that did not receive Tregs exhibited significantly more depigmentation than hosts that received WT Tregs, average vitiligo score of 3.69 ± 0.21 compared to 0.7 ± 0.29, respectively (Fig. 19b). Interestingly, hosts that received $CCR6^{-/-}$ Tregs had significantly more depigmentation than hosts with WT Tregs, average vitiligo score of 3.43 ± 0.37 compared to 0.69 ± 0.29 (Fig. 19b). In fact, disease is comparable in hosts without Tregs and hosts with $CCR6^{-/-}$ (Fig. 19b).

Hosts receiving $CCR5^{-/-}$ Tregs exhibited significantly more depigmentation than hosts receiving WT Tregs, average vitiligo score of 1.77 ± 0.38 compared to 0.69 ± 0.29, respectively. (Fig. 19b). This difference in depigmentation was statistically significant but the magnitude of depigmentation was much less in hosts receiving $CCR5^{-/-}$ Tregs than in hosts receiving $CCR6^{-/-}$ Tregs, with average vitiligo scores of 1.77 ± 0.38 and 3.43 ± 0.37, respectively (Fig. 19b). This suggests that Tregs require both receptors for optimal suppression but may require CCR6 to a greater extent than CCR5.

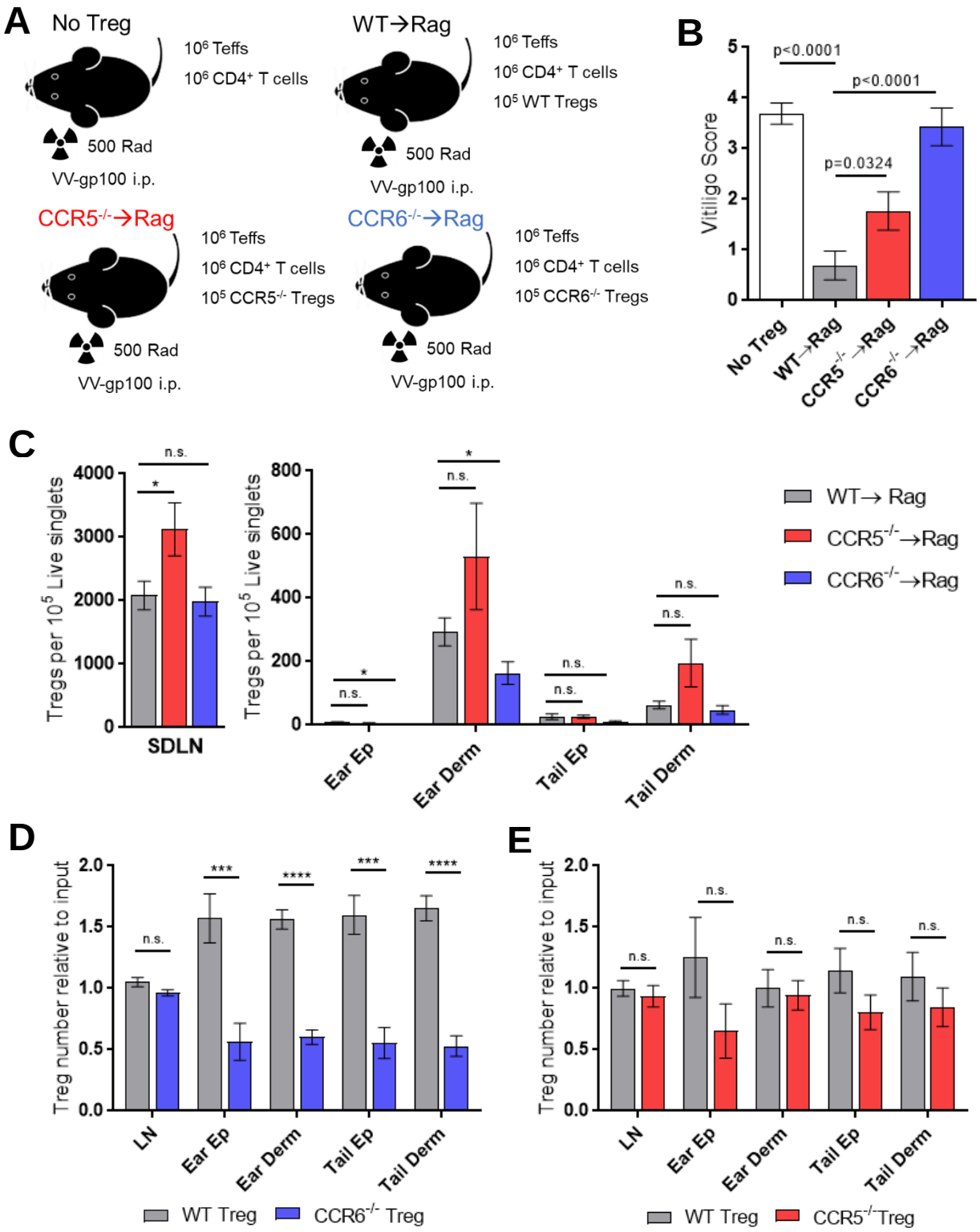


Figure 19: Tregs require CCR6 but not CCR5 for optimal migration in a mouse model of vitiligo.

Figure 19: Tregs require CCR6 but not CCR5 for optimal migration in a mouse model of vitiligo. (A) Sublethally irradiated, rVV-gp100 infected Krt14-Kitl×Rag$^{-/-}$ hosts received 10^6 Teffs, 10^6 Treg-depleted CD4s and no Tregs, 10^5 WT Tregs, 10^5 CCR5$^{-/-}$ or 10^5 CCR6$^{-/-}$ Tregs; and in **(B)** graph depicts quantification of ear depigmentation in Rag$^{-/-}$ hosts 5 weeks post vitiligo induction, represented as a vitiligo score; ***N≥13, students T-test, bars represent s.e.m.*; (C)** Tissues harvested from Rag$^{-/-}$ hosts were analyzed by flow cytometry and graph show the average number of CD3+Thy1.2+ Tregs normalized to the number of live single cells in the indicated tissues***; N=9, students T-test, bars represent s.e.m.;*** Tissues harvested from in Rag$^{-/-}$ vitiligo mice that received equal amounts of Thy1.1+1.2+ WT Tregs and Thy1.2+ CCR6$^{-/-}$ Tregs or Thy1.1+1.2+ WT Tregs and Thy1.2+ CCR5$^{-/-}$ Tregs were analyzed by flow cytometry. Graphs depict **(D)** the proportion of WT or CCR6$^{-/-}$ Tregs in the indicated tissue relative to input, ***N=8, students T-test, bars represent s.e.m.;*** or **(E)** the proportion of WT or CCR5$^{-/-}$ Tregs in the indicated tissue relative to input, ***N=7, students T-test, bars represent s.e.m.***

I harvested SDLNs, ear and tail skin, processed the tissue into single cell suspensions and used flow cytometry to quantify CD45+CD3+Thy1.2+ Tregs. I observed that the number of Tregs in the SDLNs was comparable between hosts that received WT Tregs and hosts that received $CCR6^{-/-}$ Tregs (Fig. 19c). In the ear skin however, there were significantly fewer $CCR6^{-/-}$ Tregs than WT Tregs (Fig. 19c) and while there were fewer $CCR6^{-/-}$ Treg than WT Tregs in the tail skin as well, this difference was not statistically significant (Fig. 19c). These observations suggest that Tregs require CCR6 for optimal migration to the skin during vitiligo.

In SDLNs, I observed significantly more Tregs in hosts receiving $CCR5^{-/-}$ Tregs than in hosts receiving WT Tregs (Fig. 19c). The absence of CCR5 did not impair the ability of Tregs to migrate to skin as I observed more Tregs in the ear and tail dermis of mice receiving $CCR5^{-/-}$ Tregs than in mice receiving WT Tregs (Fig. 19c). This increase was not statistically significant but suggests that CCR5 is dispensable for Treg migration to the skin.

I compared the ability of $CCR6^{-/-}$ Tregs and WT Tregs to migrate to the skin during vitiligo in a competitive adoptive transfer model. I isolated WT Tregs from Thy1.1+1.2+ mice and $CCR6^{-/-}$ Tregs from $CCR6^{-/-}$Thy1.2+ mice and induced vitiligo in Krt14-KitL×$Rag^{-/-}$ mice with 10^6 Teffs, 10^6 Treg-depleted CD4 T cells, 5.0×10^4 WT Tregs and 5.0×10^4 CCR6-/- Tregs. After 5 weeks, I harvested SDLNs, ear and tail skin and used flow cytometry to assess the relative capacity of Thy1.1+Thy1.2+ WT and Thy1.2+ $CCR6^{-/-}$ Tregs to migrate into the tissues collected. I adoptively transferred $CCR6^{-/-}$ and WT Tregs at approximately a 1:1 ratio and the proportion of WT Tregs to $CCR6^{-/-}$ Tregs I quantified in SDLNs was comparable to the proportion at input (Fig. 19d). In the skin the proportion of $CCR6^{-/-}$ Tregs to WT Tregs is reduced compared to the proportion at input, suggesting that $CCR6^{-/-}$ Tregs have a reduced

capacity to migrate to the skin during vitiligo compared to WT Tregs (Fig. 19d). This migratory defect translates to an impaired ability of CCR6$^{-/-}$ Tregs to control depigmentation.

Similarly, I compared the ability of CCR5$^{-/-}$ Tregs and WT Tregs to migrate to the skin during vitiligo in a competitive adoptive transfer model. I isolated WT Tregs from Thy1.1+1.2+ mice and CCR5$^{-/-}$ Tregs from CCR5$^{-/-}$Thy1.2+ mice and induced vitiligo in Krt14-KitL×Rag$^{-/-}$ mice with 10^6 Teffs, 10^6 Treg-depleted CD4 T cells, 5.0×10^4 WT Tregs and 5.0×10^4 CCR5$^{-/-}$ Tregs. After 5 weeks, I harvested SDLNs, ear and tail skin and used flow cytometry to assess the relative capacity of Thy1.1+Thy1.2+ WT and Thy1.2+ CCR5$^{-/-}$ Tregs to migrate into the tissues collected. I adoptively transferred CCR5$^{-/-}$ and WT Tregs at approximately a 1:1 ratio and the proportion of WT Tregs to CCR5$^{-/-}$ Tregs I quantified in SDLNs was comparable to the proportion at input (Fig. 19e). In the skin the proportion of CCR5$^{-/-}$ Tregs to WT Tregs is slightly reduced but not significantly different, suggesting that CCR5$^{-/-}$ Tregs have a comparable capacity to migrate to the skin during vitiligo as WT Tregs (Fig. 19e).

I observed that Tregs require CCR6 and CCR5 to optimally suppress depigmentation during vitiligo. However, while CCR6$^{-/-}$ Tregs are reduced in the skin compared to WT Tregs, comparable numbers of CCR5$^{-/-}$ Tregs and WT Tregs are present in the skin. This suggests that unlike CCR6, CCR5 contributes to Treg suppression through a mechanism distinct from promoting Treg migration to the skin.

4.3.5: Tregs do not require CCR5 to cluster with Teffs in the skin.

In collaboration with other members of the lab, I used suction blistering to isolate cells from the lesional skin of vitiligo patients and single cell RNA-seq to analyze gene expression in skin infiltrating cells. I observed that T cells are the main producers of CCL5, a CCR5 ligand, in

lesional human skin (Fig. 20a). Melanocytes and macrophages also expressed CCL5 but CCL5 expression was concentrated in the T cell cluster (Fig. 20a). When I compared CCL5 expression between skin infiltrating CD4+ T cells and CD8+ T cells and I observed that CD8+ T cells make more CCL5 than CD4+ T cells (Fig. 20b). Teff-derived CCL5 may promote the clustering I observed between Tregs and Teffs in the skin during disease (Fig. 6) and thus facilitate Treg suppression by helping cutaneous Tregs co-localize with Teffs (Fig 20c-d). In this scenario, a $CCR5^{-/-}$ Treg would still be able to migrate to the skin but would exhibit a reduced ability to co-localize with and suppress Teffs (Fig. 20e). To investigate this possibility, I induced vitiligo in Krt14-KitL×$Rag^{-/-}$ hosts with 10^6 RFP+ Teffs, 10^6 Treg-depleted CD4 T cells and 10^5 GFP+ $CCR5^{-/-}$ Tregs or 10^5 GFP+ WT Tregs. After 5 weeks, I harvested lesional ears and used confocal microscopy to examine them en face. I observed $CCR5^{-/-}$ Tregs clustering with Teffs suggesting that CCR5/CCL5 signaling does not facilitate Treg suppression by driving Treg co-localization with Teffs (Fig. 20f).

4.3.6: Tregs require CCR5 but not CCR6 for optimal effector function.

After observing $CCR5^{-/-}$ Tregs clustering with Teffs in lesional skin, I used an in vitro suppression assay to compare the ability of WT, $CCR5^{-/-}$ and $CCR6^{-/-}$ Tregs to suppress cytokine expression by Teffs upon activation. I used αCD3/αCD28 antibody to stimulate Teffs cultured alone or in the presence of no Tregs, WT Tregs, $CCR5^{-/-}$ Tregs or $CCR6^{-/-}$ Tregs and after 72 hours I used flow cytometry to quantify TNFα and IFNγ expression by Teffs. WT and $CCR6^{-/-}$ Tregs exhibited a comparable ability to suppress Teffs suggesting that the increased depigmentation in hosts receiving $CCR6^{-/-}$ Tregs is not due to a migratory and not functional defect (Fig. 21a, b). $CCR5^{-/-}$ Tregs marginally suppress Teff activation as significantly fewer

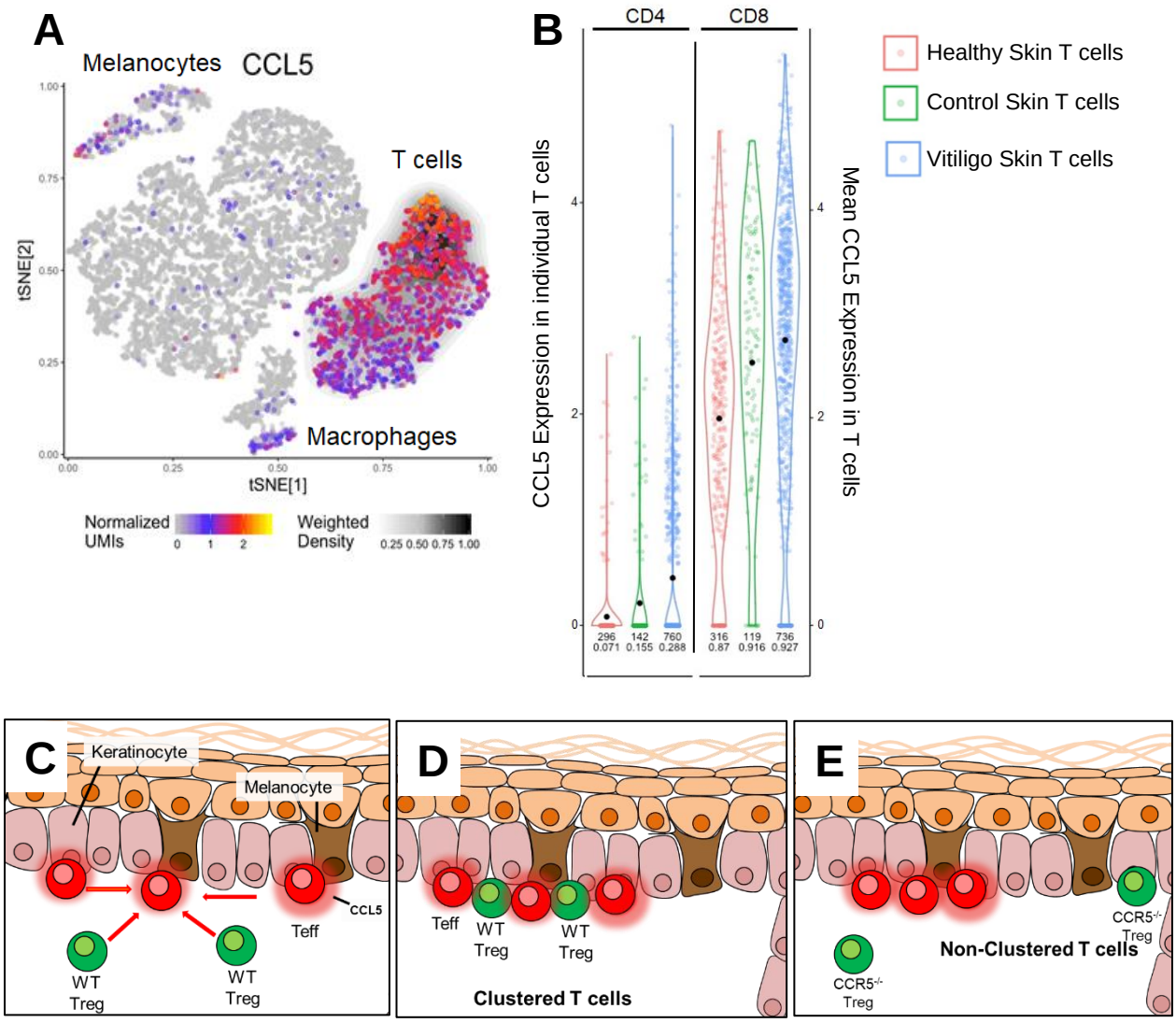


Figure 20: Tregs do not require CCR5 to cluster with Teffs in the skin.

Figure 20: Tregs do not require CCR5 to cluster with Teffs in the skin. Skin infiltrating cells isolated from the lesional skin of patients were analyzed by single cell RNA-sequencing. **(A)** The tSNE plot generated from single cell RNA-seq data shows CCL5 expression in human skin; and **(B)** the violin plot generated from single cell RNA-seq data shows the quantification of CCL5 RNA expression by CD4 and CD8 T cells in healthy human skin and vitiligo skin. The left y axis measures the magnitude of individual events (colored dots) and the right y axis measures the magnitude of the group average (black dot); ***N=296 healthy skin CD4 T cells; N=142 control skin CD4 T cells; N=760 vitiligo skin CD4 T cells; N=316 healthy skin CD8 T cells; N=119 control skin CD8 T cells; N=736 vitiligo skin CD8 T cells*****; (C-E)** Teff-derived CCL5 may promote the clustering between CCR5 expressing Tregs and Teffs in the skin during vitiligo, facilitating Treg suppression of Teffs and $CCR5^{-/-}$ may exhibit a reduced ability to co-localize with Teffs within the skin; **(F)** Ears harvested from sublethally irradiated, rVV-gp100 infected Krt14-Kitl×$Rag^{-/-}$ hosts that received RFP+ Teffs, 10^6 Treg-depleted CD4s and GFP+ $CCR5^{-/-}$ Tregs were analyzed using confocal microscopy. Representative confocal images show GFP+ $CCR5^{-/-}$ Tregs clustering with RFP+ Teffs.

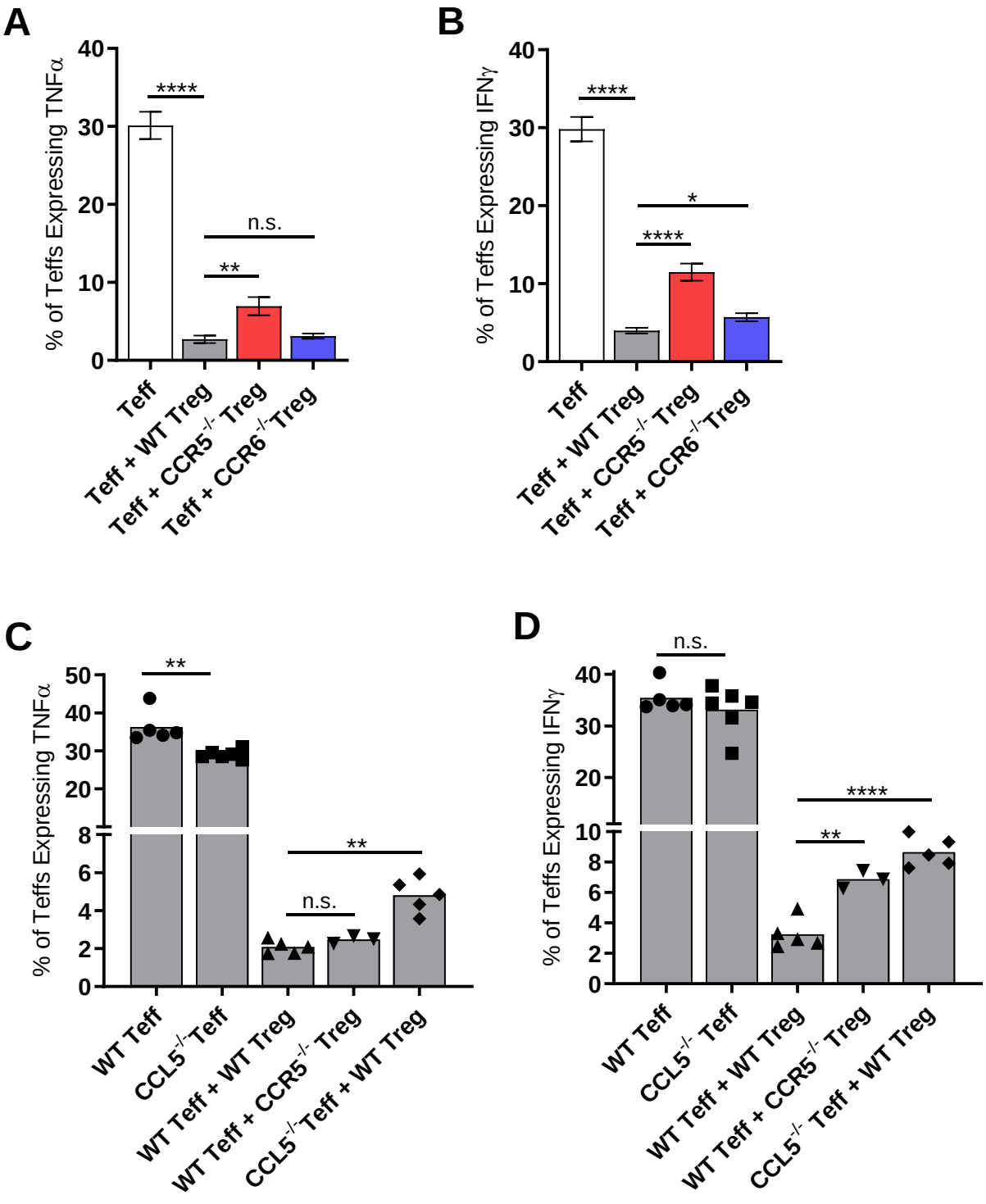


Figure 21: Tregs require CCR5 but not CCR6 for optimal effector function.

Figure 21: Tregs require CCR5 but not CCR6 for optimal effector function. Naïve Teffs cultured alone or with WT, $CCR5^{-/-}$ or $CCR6^{-/-}$ Tregs in wells coated with αCD3/αCD28 for 3 days were analyzed by flow cytometry. Graph shows the average percentage of Teffs expressing **(A)** TNFα; or **(B)** IFNγ; ***N≥10, students T-test, bars represent s.e.m.;*** Naïve WT or $CCL5^{-/-}$ Teffs cultured alone or with WT or $CCR5^{-/-}$ Tregs in wells coated with αCD3/αCD28 antibody for 3 days were analyzed by flow cytometry. Graphs show the average percentage of Teffs expressing (C) TNFα or**; (D)** IFNγ; ***N≥10, students T-test, bars represent s.e.m.***

Teffs express cytokine when cultured with $CCR5^{-/-}$ Tregs than when cultured alone (Fig. 21a, b) but $CCR5^{-/-}$ Tregs exhibit a reduced ability to suppress Teffs compared to WT Tregs. Significantly more Teffs expressed cytokines when cultured with $CCR5^{-/-}$ Tregs than with WT Tregs (Fig. 21a, b), suggesting that CCR5/CCL5 signaling may be required for optimal Treg function.

To further investigate the impact of CCR5/CCL5 signaling, I compared the ability of Tregs to suppress WT Teffs and $CCL5^{-/-}$ Teffs. I observed that comparable numbers of WT and $CCL5^{-/-}$ Teffs expressed TNFα and IFNγ when stimulated by αCD3/αCD28 antibodies in the absence of Tregs (Fig. 21c, d). As I previously observed, $CCR5^{-/-}$ Tregs suppress WT Teffs less efficiently than WT Tregs do but WT Tregs did not suppress $CCL5^{-/-}$ Teffs as well as they suppressed WT Teffs (Fig. 21c, d). This suggests that Teff-derived CCL5 may be required for optimal Treg function and during vitiligo $CCR5^{-/-}$ Tregs exhibit a functional defect and not a migratory defect.

4.4: DISCUSSION

In this study I investigated the chemokine signals that drive Treg migration to the skin during vitiligo and I found that Tregs require CCR5 and CCR6 for optimal suppression in a mouse model of disease. The chemokine driven migration of Tregs to peripheral tissues facilitated Treg- mediated suppression in numerous models of inflammation [154, 230, 231, 235-238, 240, 241, 243, 244]. While the role Tregs play during vitiligo is still not completely understood, a number of studies suggest that Treg number in skin is important for reducing Teff-driven depigmentation during disease. Two separate groups that analyzed Treg number in lesional skin reported a reduction of Tregs in the skin of vitiligo patients [247, 250]. Studies performed in mouse models of vitiligo revealed that depigmentation is exacerbated in the absence of CD4+ or CD25+ T cells [74, 251] and strategies that increased Treg number in murine skin during vitiligo led to reduced disease [155, 251, 252]. While these studies demonstrate that the presence of Tregs in the skin can suppress depigmentation, little is known about the chemokine signals that promote Treg migration during vitiligo and if we understand these signals we can design treatments that target these pathways. Eby et al. demonstrated this when they suppressed disease in a mouse model of vitiligo by overexpressing CCL22 to draw murine Tregs to the skin [155].

I found that Tregs did not require CXCR3 to suppress depigmentation in our mouse model of disease. CXCR3/CXCL10 signaling is critical for the progression of disease by promoting Teff migration to the skin [253, 272]. In previous studies I observed that murine Treg and Teff migration patterns during vitiligo are similar. In addition, murine Tregs express CXCR3 and localize with CXCL10 expression in lesional skin during vitiligo. In light of these findings I hypothesized that CXCR3 could promote Treg suppression and migration to the skin. However

in a $Rag^{-/-}$ model of vitiligo CXCR3 signaling was dispensable for Treg migration and suppression.

This finding is in contrast with results from previously published studies that found Tregs required CXCR3 to migrate to peripheral sites of inflammation and mediate suppression [234-236]. In two of these studies Tregs required CXCR3 to suppress inflammation with a Th1 signature [234, 236], the same signature of disease in our model of vitiligo [253], but only one study investigated whether Tregs require CXCR3 to suppress inflammation in the skin [236]. This study found increased inflammation and fewer Tregs in the inflamed skin of $CXCR3^{-/-}$ mice during a CHS response, so I was surprised to find Tregs do not require CXCR3 to suppress depigmentation in our model of vitiligo or efficiently migrate to lesional skin. Inflammation in CHS models an allergic response induced by re-exposure to hapten, and disease in our model of vitiligo models an autoimmune response to antigens expressed by melanocytes in murine skin [79]. CD4+ T cells drive CHS responses while CD8+ Teffs drive vitiligo in our model and this difference could explain why Tregs do not require CXCR3 to mediate suppression in our model of vitiligo but do require CXCR3 to suppress CHS responses. Nonetheless, the fact that CXCR3 signaling is dispensable for Treg suppression during vitiligo suggests that treatments targeting CXCR3/CXCL10 signaling will only affect Teffs and not Tregs. This could reduce disease-causing cells in the skin and promote an anti-inflammatory environment in the skin that could lead to repigmentation.

After observing that CXCR3 is dispensable for Treg-mediated suppression during vitiligo, I investigated whether CCR6 promoted Treg suppression during disease. Since CCR6 signaling mediated the migration of Tregs to murine skin during neonatal development [152], Tregs required CCR6 to suppress inflammation in the skin [240], and CCR6 represents a

susceptibility gene for vitiligo identified in genome wide association studies [24], I hypothesized that CCR6 could promote Treg suppression during vitiligo. I observed that Tregs required CCR6 to efficiently migrate to the skin and suppress depigmentation in a $Rag^{-/-}$ model of vitiligo. The number of $CCR6^{-/-}$ Tregs was comparable to the number of WT Tregs in SDLNs and the only migratory defect $CCR6^{-/-}$ Tregs exhibited was skin homing. This suggests that CCR6 promotes Treg suppression during vitiligo by facilitating Treg migration to the skin. These findings mirror previously published findings that established Tregs require CCR6 to migrate to the skin to suppress inflammation [240]. One study in particular reported that $CCR6^{-/-}$ Tregs transferred into $Rag^{-/-}$ mice did not migrate to neonatal skin as well as WT Tregs [152]. This finding presents a caveat to our study, as CCR6 may facilitate homeostatic skin migration to fill the empty Treg niche in mouse skin and may not mediate Treg migration during vitiligo. This caveat is an inevitable consequence of the $Rag^{-/-}$ vitiligo model but to further confirm a role for CCR6 in facilitating Treg migration to the skin to suppress depigmentation, I could induce vitiligo in WT mice and treat mice with CCR6 blocking or neutralizing antibodies to see if this exacerbates depigmentation by reducing Treg number in the skin. Since Tregs express significantly more CCR6 than Teffs, CCR6 neutralization will likely spare Teff migration.

Whether decreased Treg number in the skin contributes to pathogenesis during vitiligo still needs to be studied and reduced Tregs numbers have been reported in the lesional skin of vitiligo patients [247, 250]. A number of studies report increasing Treg number in the skin can suppress depigmentation in animal models of disease, but my studies provide functional evidence that suggests reducing Treg number only in the skin, and not in the lymph nodes, leads to increased disease. These observations add to the increasing amount of evidence that suggests Tregs suppress Teff-mediated depigmentation directly in the skin. Fewer $CCR6^{-/-}$ Tregs than WT

Tregs were in the skin of $Rag^{-/-}$ mice during vitiligo but $CCR6^{-/-}$ Treg were not completely absent from the skin. This suggests that there are redundant chemokine signals that mediate Treg migration during vitiligo and more work needs to be done to completely elucidate these signals.

I observed significantly increased depigmentation in hosts with $CCR5^{-/-}$ Tregs than in host with WT Tregs in our $Rag^{-/-}$ vitiligo model and, based on these observations, I concluded that Tregs require CCR5 for optimal suppression during vitiligo. Interestingly the increase in depigmentation is less severe in hosts reconstituted with $CCR5^{-/-}$ Tregs than in hosts with $CCR6^{-/-}$ Tregs, suggesting that Tregs require CCR6 more than CCR5 for suppression. CCR5 has been implicated in Treg-mediated suppression of inflammation in peripheral tissues [241-245] and one of these studies demonstrated that CCR5 signaling promoted efficient suppression and Treg migration to inflamed skin in a $Rag^{-/-}$ model of *Leishmania major* infection [245]. My observations partially reflect these findings as Tregs required CCR5 to optimally suppress skin depigmentation but I was surprised to find that $CCR5^{-/-}$ Tregs were capable of migrating to the skin during vitiligo. Despite the presence of $CCR5^{-/-}$ Tregs in the skin, depigmentation was still significantly worse than in mice with WT Tregs. This suggests that CCR5 signaling contributes to Treg suppression by promoting Treg function and not Treg migration to the skin in a mouse model of vitiligo.

In support of this, I found that CCR5/CCL5 signaling is important for Treg suppression in in vitro suppression assays. Several studies demonstrate a role for CCR5 in promoting Tregs suppression [241-245]. However, these studies limit the role of CCR5/CCL5 signaling to driving the chemotaxis that positions Tregs in sites of inflammation. A couple of studies also used in vitro suppression assays to investigate the suppressive capabilities of $CCR5^{-/-}$ Tregs and reported no differences between WT and $CCR5^{-/-}$ Tregs [241, 245]. These studies assessed the ability of

Tregs to suppress proliferation in CD4+CD25- T cells instead of CD8+ Teff but in RNA-seq studies performed in collaboration with other members of the lab, CD8+ T cells in vitiliginous skin expressed much more CCL5 than skin infiltrating CD4+ T cells. If CD8+ T cells make more CCL5 than CD4+ T cells upon activation, suppression assays that use CD4+ Teffs as targets may not recognize subtle differences between WT and $CCR5^{-/-}$ Tregs.

A study performed in a model of colorectal cancer reports that CCR5/CCL5 signaling enhances the migration of Tregs to tumor sites and enhances the ability of Tregs to suppress CD8+ T cells in vitro and in vivo [276]. These studies partially support my observations that CCR5/CCL5 signaling promotes optimal Treg function and not migration during vitiligo, but in the colorectal models of disease the source of the CCL5 is the tumor. Based on the data that we have gathered, Teffs are the primary producers of CCL5 in the skin during vitiligo and thus participate in their own suppression by expressing the CCL5 that promotes optimal Treg function.

I have not ruled out the possibility that CCR5 facilitates Treg suppression by positioning of Tregs in clusters with Teffs in the skin during vitiligo. It is possible that the clusters of Teffs and Tregs that we see in lesional mouse skin are formed as an activated Teff gets to the skin and draws both CCR5-expressing Tregs and Teff in close proximity through the expression of CCL5. Tregs can still get to the skin and encounter Teffs to enact suppression, but CCR5 may help Tregs colocalize with and suppress Teffs more efficiently. Similar effects have been reported in the literature [217, 220] and one study demonstrated that DCs and T cells in the LNs expressed CCR5 ligands to facilitate clusters that form when an immune response is initiated [217]. In light of this, it is possible that T cell-derived CCL5 could facilitate Treg suppression by drawing CCR5 expressing Teffs and Tregs to clusters where Tregs enact suppression.

While I did observe CCR5$^{-/-}$ Tregs clustering with Teffs in lesional murine skin, in future imaging experiments I would need to compare the frequency of contact between Teffs and WT or CCR5$^{-/-}$ Tregs. If the frequency of interactions between Tregs and Teffs is reduced in the absence of CCR5, this would support a role for CCR5 in promoting clustering between Tregs and Teffs in the skin.

In this study I investigated the chemokine signals that promote Treg suppression during vitiligo. I observed that CCR5 and CCR6 signaling promotes Treg suppression but only CCR6 signaling does this by facilitating the migration of Tregs to the skin. CCR5 signaling appears to promote optimal Treg function and the different impacts of these chemokine receptors on Tregs is uniquely highlighted by our mouse model of vitiligo. Understanding the signals that govern Treg migration to the skin to suppress autoimmunity during vitiligo would potentially help discover new therapies to treat patients or improve existing ones.

4.5: Materials and Methods

Mice

KRT14-Kitl*4XTG2Bjl (Krt14-Kitl) mice were a generous gift from B. J. Longley (University of Wisconsin). I isolated Teffs from PMEL TCR transgenic mice that are Thy1.1+ and were obtained from The Jackson Laboratory, stock no. 005023, B6.Cg Thy1a/CyTg(TcraTcrb)8Rest/J. Krt14-Kitl×Rag$^{-/-}$ (Rag$^{-/-}$) mice were generated by crossing Krt14-Kitl mice with the B6.129S7-Rag1^{tm1Mom}/J strain (The Jackson Laboratory, stock no. 002216). Krt14-Kitl×FoxP3-GFP mice were generated by crossing Krt14-Kitl mice with FOXP3^{tm1Kuch} generated by VJ Kuchroo. Thy1.1+Thy1.2+ mice were generated by crossing C57BL/6J mice with B6.PL-Thy1^{a}/CyJ mice (The Jackson Laboratory, stock no 000406). B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (CD45.1) mice were used as CD4 donors and were obtained from The Jackson Laboratory, stock no 002014. CCR6$^{-/-}$ mice were obtained from The Jackson Laboratory, stock no. 005793, B6.129P2-Ccr6^{tm1Dgen}/J. CCR5$^{-/-}$ mice were obtained from The Jackson Laboratory, stock no. 005427, B6.129P2-Ccr5^{tm1Kuz}/J. CXCR3$^{-/-}$ mice were obtained from The Jackson Laboratory, stock no. 005796, B6.129P2-Cxcr3^{tm1Dgen}/J. The Krt14-Kitl allele was bred heterozygous on all mice used in experiments. All mice were bred on a C57BL/6J background and mice were maintained in pathogen-free facilities at the University of Massachusetts Medical School (UMMS), and procedures were approved by the UMMS Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Vitiligo induction

Vitiligo was induced through adoptive transfer of CD8+ T cells isolated from PMEL mice (Teffs) as described previously [79]. Teffs were isolated from the spleens of PMEL TCR transgenic mice through negative selection using a Miltenyi Biotec (Bergisch Gladbach, Germany) CD8 isolation kit according to the manufacturer's instructions. 10^6 purified Teffs were injected intravenously into 8 to 12 week old hosts sublethally irradiated with 500 rads 1 day before transfer. Hosts were also infected with 10^6 plaque-forming units (PFU) of rVV-hPMEL through intraperitoneal injection (N. Restifo, National Cancer Institute, NIH) on the same day of adoptive transfer. Depigmentation was reported using a vitiligo score. The vitiligo score was determined by an observer blinded to the experimental groups, using a point scale based on the extent of depigmentation at four locations, the ears, tail, nose and hind footpads as previously described [79]. Each location was examined, and the extent of depigmentation was assigned a number based on the estimated percentage of depigmentation at the anatomic site. Both left and right ears and left and right rear footpads were estimated together and therefore evaluated as single sites. The scoring system used is as follows: no depigmentation = 0, >0 to 10% = 1 point, >10 to 25% = 2 points, >25 to 75% = 3 points, >75 to <100% = 4 points and 100% = 5 points. The "vitiligo score" was the sum of the scores at all four sites, with a maximum score of 20 points.

$Rag^{-/-}$ vitiligo induction

Vitiligo was induced in $Rag^{-/-}$ mice through adoptive transfer of purified Teffs isolated as previously described. Treg-depleted CD4+ T cells were isolated from the spleens of C57BL/6J or CD45.1 mice through negative selection using Miltenyi Biotec (Bergisch Gladbach, Germany) CD4+CD25+ isolation kits. Tregs were isolated from the spleens of C57BL/6J mice, $CXCR3^{-/-}$

mice, $CCR5^{-/-}$ mice or $CCR6^{-/-}$ mice through positive selection using Miltenyi Biotec (Bergisch Gladbach, Germany) CD4+CD25+ isolation kits. To induce vitiligo in $Rag^{-/-}$ mice 10^6 Teffs, 10^6 CD4s and no Tregs, 10^5 WT Tregs, $CXCR3^{-/-}$ Tregs, $CCR5^{-/-}$ Tregs or $CCR6^{-/-}$ Tregs were adoptively transferred into 8 to 12 week old $Rag^{-/-}$ hosts. Hosts were sublethally irradiated with 500 rads 1 day before transfer and hosts were infected with 10^6 PFU of rVV-hPMEL through intraperitoneal injection (N. Restifo, National Cancer Institute, NIH) on the same day of adoptive transfer. In competitive $Rag^{-/-}$ vitiligo models vitiligo was induced by adoptive transfer of 10^6 Teffs, 10^6 CD4+ T cells and equal numbers of WT and $CCR5^{-/-}$ or $CCR6^{-/-}$ Tregs. WT Tregs were isolated from the spleens of Thy1.1+Thy1.2 C57BL/6J mice as previously described. Hosts were sublethally irradiated with 500 rads 1 day before transfer and hosts were infected with 10^6 PFU of rVV-hPMEL through intraperitoneal injection (N. Restifo, National Cancer Institute, NIH) on the same day of adoptive transfer.

Flow Cytometry

Ears, tails and skin-draining lymph nodes were harvested. Lymph nodes were mechanically disrupted. To separate the dermis and epidermis, harvested ear and tail skin samples were incubated with dispase (50 U/ml and 5 U/ml in PBS, respectively) (Roche) for 1 hour at 37°C. Epidermis was removed and mechanically disrupted with 70-mm cell strainers. Dermis samples were incubated with 1 mg/ml collagenase IV and 2 mg/ml DNase I (Sigma-Aldrich) in RPMI for 45 minutes at 37°C on a shaker. After incubation dermis was dissociated by gentle agitation. Cells from lesional human skin were obtained by suction blistering. The following antibodies were obtained from BioLegend (San Diego, CA): mouse (CD3, CD8b, CD45, CD45.1, CD45.2, CD90.1, CD90.2, CCR5, CCR6) and human (CD3, CD4, CD8, CD45, CD25, FOXP3, CCR5

and CCR6). For extracellular staining cells were washed in FACS buffer (1% FBS in PBS) and then incubated with antibodies at a 1:200 dilution at 4° C in the dark for 20 minutes. CCR5 and CCR6 staining was done at 37° C in the dark for 30 minutes. For intracellular FoxP3 staining, surface staining was performed and cells were fixed and permeablized using the FoxP3/Transcription factor staining buffer set from eBioscience (Santa Clara, CA) according to the manufacturer's recommendations. The data were collected and analyzed with a BD LSR II flow cytometer (BD Biosciences) and FlowJo (Tree Star Inc.).

Suction blistering

Suction blistering was performed as previously described [264]. Blisters that were approximately 1 cm in diameter were induced on the skin by using the Negative Pressure Instrument Model NP-4 (Electronic Diversities, Finksburg, MD). Suction chambers were placed on the skin with 10-15 mm Hg of negative pressure and a constant temperature of 40° C. After 30 to 60 minutes blisters formed and the blister fluid was aspirated through blister roofs using a 1 mL insulin syringe. Single cell RNA sequencing was performed on blister fluid obtained. Skin infiltrating cells within the blister fluid were pelleted at 330×g for 10 minutes and then stained to be analyzed with flow cytometry.

Statistical analyses

All statistical analyses were performed with GraphPad Prism software. Dual comparisons were made with unpaired Student's t test.

CHAPTER V: DISCUSSION

5.1: Regulatory factors in the skin during vitiligo need further investigation

A myriad of factors drive the epidermal depigmentation that characterizes vitiligo (reviewed in ch. 1.3 – 1.13). One theory suggests that intrinsic defects render melanocytes less capable of handling stress and exposure to external stressors can initiate innate responses that subsequently activate melanocyte targeting CD8+ Teffs. Teffs can then migrate to the skin where they destroy pigment producing melanocytes. Lesions are disfiguring and patients tend to have self-esteem issues due to the visible nature of vitiligo. Few treatments for vitiligo currently exist (reviewed in ch. 1.2). These therapies can induce repigmentation but success is variable and in some cases not durable.

Depigmentation frequently occurs in patches with lesional skin surrounded by unaffected skin. This suggests that there are regulatory factors at play that keep Teffs from exerting their full destructive potential. Melanocytes in the hair follicle are often protected during vitiligo, even in depigmented skin due the immune privilege that protects the follicle. In light of this, it is possible that suppressive immune factors prevent Teffs from depigmenting the entire epidermis and I hypothesize that regulatory factors in the skin suppress depigmentation during vitiligo. Several mechanisms that end Teff responses during inflammation have been described (reviewed in ch. 1.13) and cells like tolerogenic DCs or Tregs can suppress Teff activity in peripheral tissue to prevent autoimmunity. In this work I investigated the roles that Tregs and LHCs, two populations in the skin during vitiligo, play during disease.

5.2: A case for Treg suppression in the skin during vitiligo.

Several groups report the presence of Tregs in the skin during vitiligo and drew conflicting conclusions in attempts to correlate Treg number with disease severity. These human studies did not functionally test whether Tregs suppression in the skin plays a role in disease. Additionally, studies in mouse models of vitiligo that investigated Treg function tested the ability of Tregs to suppress hair depigmentation instead of epidermal depigmentation and used depletion strategies that could potentially affect cells aside from Tregs. Furthermore, these depletion strategies affected Tregs in the entire body thus more work needs to be done to assess whether Tregs suppress Teffs in the skin.

In chapter II I observed that vitiligo is exacerbated in a mouse model of disease when Tregs are depleted. The depletion strategy I used exclusively targeted Tregs in a mouse model of vitiligo while previous studies used CD4 or CD25 depletion that target Tregs as well as all other CD4 or CD25 bearing cells. The depletion experiments that I conducted globally depleted Tregs and thus do not completely address the contribution of Treg suppression in the skin since Treg numbers could also be diminished in the SDLNs. Whether Tregs suppress Teffs in the skin or SDLNs is an important question to address since answering this question would direct how we treat disease. Strategies that exclusively deplete Tregs in the skin or sequester Tregs to SDLNs are needed to definitively answer this question but if Tregs directly suppress Teffs that they encounter in the skin, interventions aimed at increasing cutaneous Treg number in lesional skin could promote repigmentation.

Data obtained from vitiligo patients supports this idea of an inverse correlation between cutaneous Treg number and disease severity as I observed a reduced Treg/Teff ratio in lesional skin compared to non-lesional skin. While the absolute number of Tregs was comparable between lesional and non-lesional skin, the fact that Treg number did not increase proportionally

with Teff number in lesional skin supports the notion that maintaining a favorable ratio of Tregs to Teffs in the skin may control depigmentation. In our mouse model of vitiligo Tregs and Teffs accumulate at almost equal number in ear skin which is less prone to disease than tail skin, where Teffs outnumber Tregs. The increased number of Tregs in ear skin could establish a more immunosuppressive environment and explain why depigmentation is decreased. This observation reflects the inverse correlation we see between cutaneous Treg number and depigmentation in vitiligo patients. Considering this, treating depigmentation by increasing Treg number in the skin may be a viable treatment option for patient.

Observations reported in this work strongly suggests that Tregs suppress Teffs directly in the skin during vitiligo but do not preclude Treg suppression in SLOs. Suppression in both locales is likely important during disease and I can functionally address this question in future experiments with the tools we have developed to use in our mouse model of disease. I believe the observations that I have reported highlight an underappreciated role for Treg-mediated Teff suppression in the skin during vitiligo. In our mouse model of vitiligo Tregs directly contact Teffs in lesional skin (Fig. 6) and in our $Rag^{-/-}$ model of disease, I observed increased ear depigmentation in hosts with $CCR6^{-/-}$ Tregs that can migrate to SDLNs but exhibit a reduced ability to migrate to skin (Fig. 19). In the latter experiment, the absence of CCR6 on Tregs reduces Treg number in the skin without significantly affecting Treg number in SDLNs and thus highlights the importance of cutaneous Treg number for controlling Teff-mediated depigmentation. These results do not preclude the possibility of Treg-mediated Teff suppression in SDLNs but do present compelling evidence that Treg suppression in the skin contributes to the regulation of Teff-mediated depigmentation during vitiligo.

I did not definitively define the mechanisms of suppression that Tregs use during vitiligo. After seeing contact between Tregs and Teffs in the skin I hypothesized that Tregs may use contact-dependent mechanisms of suppression to regulate Teff activity in the skin and explored mechanisms that Tregs could be using. I pursued the hypothesis that Tregs suppress depigmentation by inducing CCR7 on Teffs to drive their egress from the skin during vitiligo. In in vitro suppression assays more naïve Teffs expressed CCR7 in co-cultures with Tregs than those in cultures without Tregs and this could be because Tregs induce CCR7 on Teffs or Tregs prevent naïve Teffs from maturing and down-regulating CCR7. To address this caveat, I would use previously activated Teffs or Teffs isolated from lesional tissue in co-cultures with Tregs. Nonetheless, if Tregs induce CCR7 expression on Teffs to regulate cutaneous Teff number during disease, the number of CCR7 expressing Teffs would decrease in absence of Tregs. Treg depletion did not affect the number Teffs expressing CCR7 in our mouse model of disease and thus it is unlikely that Tregs induce CCR7 on Teffs as an "exit" signal during vitiligo. Since CCR7 regulates lymphocyte exit it may not be possible to catch Teffs in the skin once they have been suppressed and this may explain why receptor expression is unaffected by Treg depletion. To address this, I can induce vitiligo with CCR7 deficient Teffs and compare disease severity and Teff accumulation in the skin.

In future experiments I can combine the tools that we have I developed to use in our mouse model to further investigate the mechanisms Tregs use to suppress Teffs. Data obtained from single cell RNA-sequencing performed on skin infiltrating cells from lesional skin provides insight in the genes that are differentially expressed between Tregs in lesional skin and healthy or non-lesional skin. Single cell RNA-seq identified that Tregs in lesional vitiligo skin express less TGF-β1 than Tregs in healthy skin (Fig. 8) and I can begin to test if TGF-β1 plays a role in

Treg suppression during vitiligo by inducing vitiligo in $Rag^{-/-}$ hosts reconstituted with TGF-$\beta1^{-/-}$ Tregs. Using this method of discovery in RNA-seq experiments followed by functional screening experiments in $Rag^{-/-}$ mice, I can quickly screen for molecules required for Treg suppression during vitiligo. If we discover the mechanisms that Tregs use to suppress Teffs during vitiligo, we can use that information to improve how we treat patients.

5.3: Defining the distinct signals that facilitate Treg migration and function during vitiligo.

Our group characterized the signals that drive Teff migration to the skin during vitiligo but few have investigated the chemokine signals Tregs follow to the skin during disease (reviewed in ch 4.2). Comparisons of chemokine receptor expression on circulating Tregs in patients and healthy controls revealed no significant differences and more work needs to be done to fully elucidate the signals Teffs follow to get to the skin during vitiligo.

I developed a $Rag^{-/-}$ model of vitiligo to study the signals that Tregs use to migrate to the skin. Since $Rag^{-/-}$ mice lack all T cells, I can reconstitute $Rag^{-/-}$ hosts with Tregs from any C57BL/6J donor when I induce vitiligo. This strategy gives us the advantage of being able to screen molecules that Tregs need for suppression during vitiligo without having to breed Krt14-KitL hosts with Treg specific deletions. In chapter IV, I used this $Rag^{-/-}$ model to test the chemokine signals that Tregs follow. I found that Tregs required CCR6 for optimal suppression in a mouse model of vitiligo and Treg numbers in the skin are reduced when CCR6 is absent (Fig. 19). This result functionally demonstrated that reducing Treg number in the skin while not affecting Treg number in SDLNs, results in increased depigmentation. Previous studies investigating Tregs affected Treg number in the whole body and the one study that exclusively affected Treg number in the skin reported increased Treg number suppresses depigmentation.

My results suggest the decreased Treg number relative to Teff number that we see in the skin in lesional patient skin could be one of the factors that allow Teffs to target melanocytes during vitiligo.

Scharschmidt et al reported that CCR6$^{-/-}$ Tregs transferred into Rag$^{-/-}$ mice did not migrate to neonatal skin as well as WT Tregs [152]. In our Rag$^{-/-}$ studies CCR6 may actually facilitate homeostatic skin migration to fill the empty Treg niche in mouse skin instead of mediating Treg migration during vitiligo. This caveat is an inevitable consequence of the Rag$^{-/-}$ vitiligo model and a disadvantage compared to our traditional mouse model of disease. In future experiments I can specifically address this caveat by comparing the ability of WT and CCR6$^{-/-}$ Tregs to migrate to the skin in the absence of vitiligo in a competitive transfer model. This will address whether inflammation during vitiligo draws Tregs into the skin during disease and to further confirm a role for CCR6 in Treg migration during vitiligo I will generate Krt14-Kitl mice with CCR6$^{-/-}$ Tregs to use as hosts during vitiligo.

I also observed that Tregs required CCR5 for optimal suppression in a Rag$^{-/-}$ model of vitiligo but Tregs did not require CCR5 to get to the skin (Fig. 19). Instead CCR5/CCL5 signaling promoted optimal Treg function in vitro and these observations highlight an underappreciated role for CCR5 in enhancing Treg function instead of inducing Treg migration (Fig. 20). A few other groups have reported similar unconventional chemokine receptor signaling. CCR8 signaling potentiated the suppressive function of Tregs in vitro and in a model of EAE [277] and CCR5/CCL5 enhances the ability of Tregs to suppress CD8+ T cells in vitro and in vivo [276]. Furthermore, another study demonstrated that CCR5 signaling, which normally activates G proteins to mediate migration, can also activate the JAK/STAT pathway and regulate cell growth via alterations in JAK activity [278]. These observations combined with

those presented in this work challenges the conventional theories that hold chemokine receptor signaling merely directs cell migration. In some instances, chemokine receptor signaling may potentiate function and this exciting hypothesis can be explored in future experiments.

5.4: Defining the role of LHCs during vitiligo.

LHCs are the only DC population in the epidermis and ideally positioned to interact with Teffs in the skin during vitiligo. Few have studied the role LHCs play during vitiligo and these studies report conflicting results regarding LHCs number and function during disease (reviewed in ch. 3.2). More work needs to be done to elucidate the role LHCs play during vitiligo. LHCs can behave in and immunogenic or tolerogenic manner in other models of inflammation (reviewed in ch. 1.26 – 1.27) and our results obtained in our mouse model of disease can contribute to the debate regarding the role LHCs play during other forms of autoimmunity.

In our mouse model, I observed that while the extent of depigmentation was comparable between LHC-KO and WT mice, the incidence of disease is increased in the absence of LHCs (Fig 9). How LHCs affect the incidence of disease without affecting disease severity of is an important question that this work did not conclusively answer. It is possible that LHCs behave as an immunological check point Teffs must overcome in order to mediate depigmentation during the initiation phase of vitiligo. Our mouse model of disease is well-suited to study disease progression but not intended to model disease initiation since we infect with virus to induce autoimmunity. As a result, we may not be able to fully appreciate the role LHCs play using our mouse model. Spontaneous mouse models of vitiligo (review in chapter 1.9) accurately replicate processes involved in disease initiation and should be adapted to further investigate how LHCs impact disease. The Teff donor mice that we use in our model of vitiligo spontaneously develop

depigmentation over time and in future experiments, I will cross Teff donor mice with LHC-KOs to generate mice that spontaneously develop depigmentation while lacking epidermal LHCs. This will allow me to investigate whether LHCs impact disease initiation and address some of the remaining questions surrounding the role of LHCs during vitiligo.

The observation that Tregs and Teffs directly interact in the skin during disease further suggested that Tregs suppress Teffs in the skin during vitiligo and led us to question how Tregs find Teffs in the skin. LHCs express the appropriate molecules to interact with both cell types so it is possible that LHCs participate in Teff suppression by facilitating interactions between Teffs and Tregs in the skin in an MHC-TCR-dependent manner. In this scenario LHCs would increase the efficiency of interactions between Tregs and Teffs but Tregs could still migrate to the skin to suppress Teffs. In chapter III I observed that LHCs suppression was not MHCII-TCR-dependent but LHC-derived CXCL10 was necessary for optimal suppression. LHCs could participate in suppression by tethering Tregs and Teffs directly with CXCL10 to facilitate interactions between the two cell types and I can test this hypothesis in future experiments. In chapter II I observed more Tregs in ear skin than tail skin and I focused on ear skin as the main area of Treg suppression in chapter II and IV. In chapter III I investigated LHCs function in tail skin and if LHCs do synergize with Tregs to suppress Teffs, I should have focused on ear skin in LHC-KO experiments. This caveat must be considered when interpreting the data present and ear tissue should be analyze in future experiments investigating LHC function during vitiligo.

Another interesting possibility is that LHCs are directly suppressing Teffs in the skin. LHCs have been shown to suppress Teffs responses using immunosuppressive cytokines like IL-10 (reviewed in chapter 1.27) and considering this, it is possible that LHCs use IL-10 to suppress Teffs that they encounter in the skin. I could explore this possibility by inducing vitiligo in hosts

with IL-10$^{-/-}$ LHCs to see if this replicates the phenotype that I observed in LHC-KO mice with vitiligo.

Whether LHCs play an immunogenic or tolerogenic role during inflammation is still debated. Autoimmunity progresses and is even exacerbated in the absence of LHCs in our model of vitiligo suggesting that LHCs suppress Teff function. While I have not completely characterized the mechanism of LHC suppression, the observations reported in this work contribute to the ongoing discussion concerning LHCs function in vitiligo and other immune responses.

5.5: Combining observations: a model for Treg-mediated Teff suppression in the skin during vitiligo.

In vitiligo patients, cases of complete depigmentation are rare and I hypothesize that regulatory factors function in the skin during vitiligo to keep Teffs from mediating their maximum destructive potential. My observations are consistent with the notion that that Teff suppression in the skin contributes to the overall suppression of depigmentation during vitiligo. I observed an inverse correlation between cutaneous Treg number and disease severity in murine and human skin and a reduction in cutaneous Treg number in Rag$^{-/-}$ experiments with CCR6$^{-/-}$ Tregs, resulted in increased depigmentation. One question that remains is how do Tregs localize with Teffs in the skin to mediate suppression? I hypothesize that LHCs provide chemotactic signals to direct Tregs to Teffs and that this interaction happens at the epidermal dermal interface. The majority of Tregs are in the dermis during disease (Fig. 5) but I still observed epidermal LHCs interacting with Treg and Teffs. If these interactions occur at the epidermal/dermal junction, basal LHCs will be properly positioned to interact with epidermal

and dermal populations. I observed that Tregs require CCR5 signaling for optimal suppressive function and I hypothesize that once Tregs contact Teffs in the skin, Teff-derived CCL5 potentiates Treg function (Fig. 22). More work needs to be done to completely comprehend how skin resident cell types interact during inflammation, but this model begins to fill some gaps in our understanding of the complex network of cells and signals at play during skin immune responses including vitiligo.

5.6: Conclusions

During vitiligo, Tregs suppress Teff-mediated depigmentation and require CCR6 and CCR5 for optimal suppression. LHCs also suppress incidence of disease and may participate in suppression by promoting interactions between Tregs and Teffs (Fig. 22). New effective treatments for vitiligo that will induce long lasting repigmentation will help the 1% of the population afflicted by disease. Understanding the regulatory factors that suppress Teff-mediated depigmentation in the skin could inform the creation of treatments designed to augment the suppressive mechanisms occurring in the skin during vitiligo.

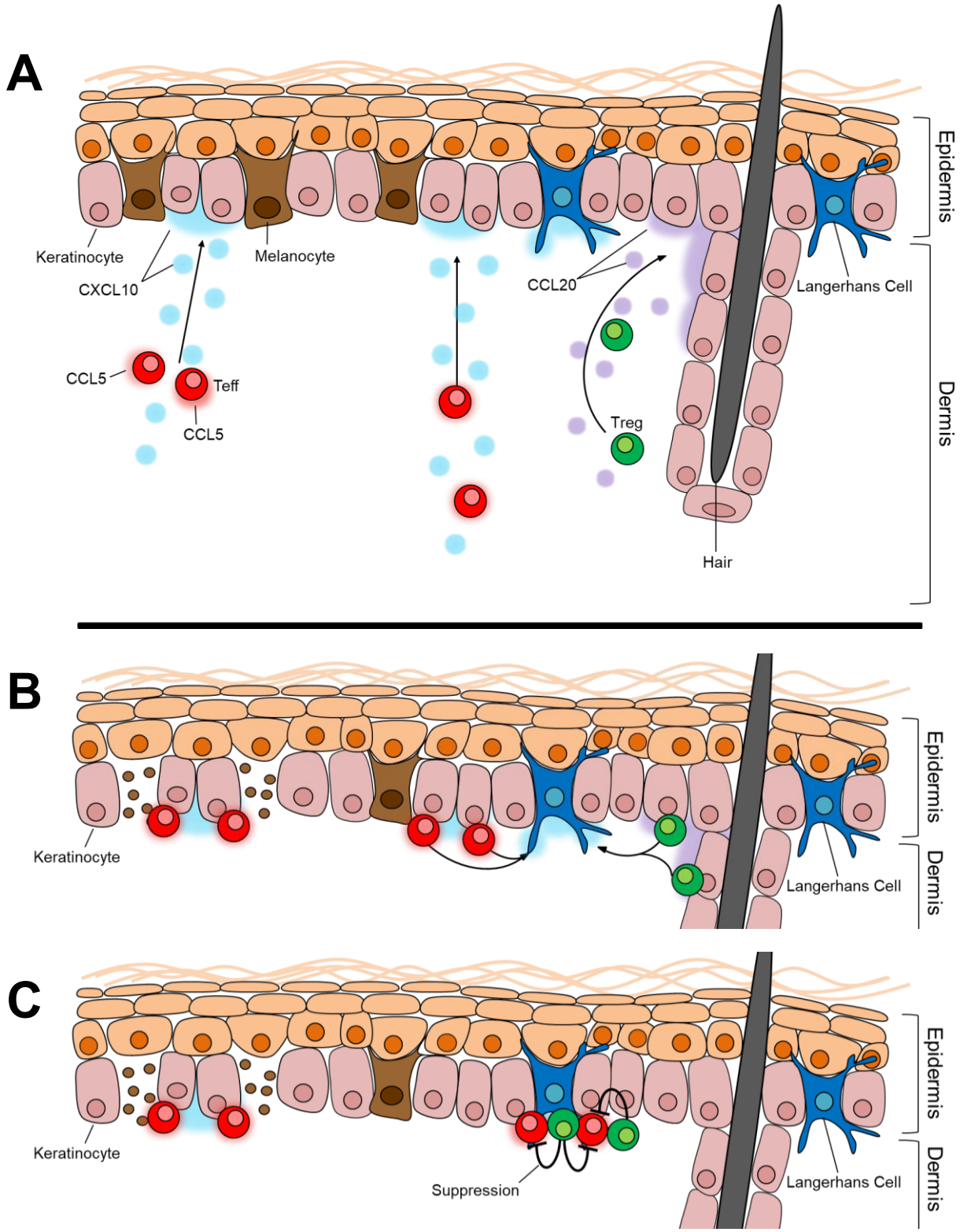


Figure 22: A model for Treg-mediated Teff suppression in the skin during vitiligo.

Figure 22: A model for Treg-mediated Teff suppression in the skin during vitiligo. (A) During vitiligo CXCL10/CXCR3 signaling promotes Teff migration to the epidermis while CCL20/CCR6 signaling promotes Treg migration to the epidermal/dermal interface. **(B,C)** At the basal layer of the epidermis LHC-derived CXCL10 attracts both cutaneous Tregs and Teffs to facilitate Treg-mediated Teff suppression. Once Tregs contact Teffs, Teff-derived CCL5 potentiates Treg suppressive function.

CHAPTER VI: REFERENCES

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