Innate Immune Mechanisms That Regulate Disease Flare in Systemic Lupus Erythematosus

by

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Dedication

To my parents, for always believing in me, even when I didn’t believe in myself. Thank you will never be enough.

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Abstract

Systemic lupus erythematosus (SLE) is a heterogenous autoimmune disease characterized by dysfunction within the innate and adaptive immune systems. The breakdown in immune tolerance that accompanies this promotes aberrant B cell activation, production of autoantibodies targeting self-nucleic acids, overproduction of pro-inflammatory cytokines, and widespread tissue damage. While some patients experience persistently active disease, most SLE patients follow a relapsing-remitting course with periods of clinical quiescence interspersed with unpredictable disease flares. Delineating the underlying mechanisms that contribute to pathogenesis of SLE as well as those that trigger flares will aid in development of novel therapeutics to improve patient outcomes.

Several cytokines have been implicated in SLE including type I interferons (IFNs) and the IL (interleukin)-1 superfamily. Type I IFNs are overexpressed in both the circulation and non-lesional skin of SLE patients compared to controls. Levels of type I IFNs correlate with disease activity and severity and promote increased death of SLE keratinocytes after exposure to ultraviolet (UV) light. This likely contributes to photosensitivity, or enhanced sensitivity to UV light, which is experienced by up to 90% of patients. The IL-1 family of cytokines includes IL-1beta (IL-1β) which is activated by the inflammasome, a component of the innate immune system that detects infectious or endogenous danger signals. Inflammasome inhibition in lupus-prone mice improves several disease manifestations including nephritis. The specific mechanisms by which cutaneous type I IFNs contribute to photosensitivity and IL-1β influences lupus

pathogenesis are currently unknown. The primary focus of this dissertation is to examine the role of type I IFNs in the skin and IL-1β in the kidney.

The first objective of this study was to explore the activation and regulation of cell death pathways in keratinocytes exposed to type I IFNs and UV irradiation. Immortalized human keratinocytes were treated with IFN-α and UVB light in combination with apoptosis, necroptosis, and pyroptosis inhibitors or neutralizing antibodies. Cell death was measured by Annexin V and propidium iodide or cleaved caspase-3 staining. We identified enhanced activation of caspase-8-dependent apoptosis in IFN-primed keratinocytes after UVB exposure. This apoptosis occurred independently of known caspase-8-activating death ligands but was dependent on interferon regulatory factor 1 (IRF1). Lastly, we observed increased UVB-induced keratinocyte apoptosis in mice that overexpressed epidermal IFN-κ compared to wild-type mice. Together, these data suggest that photosensitivity exhibited by SLE patients may result from IFN priming of keratinocytes that drives IRF1 expression and sensitizes cells to undergo increased apoptosis after minimal exposures to UVB. Continued investigation into mechanisms by which this occurs will provide prophylactic options to prevent SLE flares.

The second part of our study involved investigating how loss of IL-1β affects disease severity in the lupus-prone NZM2328 mouse model. We generated a knockout strain (NZM-*Il1b*$^{-/-}$) and examined common manifestations of disease. Surprisingly, loss of IL-1β did not affect overall survival, autoantibody generation, or renal immune cell infiltration; however, it did worsen renal immune complex deposition and proteinuria, specifically in female mice. IL-17 and TNF signaling pathways were enriched in the kidneys of female NZM-*Il1b*$^{-/-}$ mice, suggesting a potential mechanism by which nephritis is aggravated. Female patients with lupus nephritis also demonstrate upregulation of these signaling pathways, suggesting they may be of clinical

relevance. Together, these data suggest that blocking IL-1β may need to be approached with caution in SLE patients, especially those with nephritis, to prevent potential exacerbation of disease.

Chapter 1 – Introduction

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1.1 Systemic Lupus Erythematosus

1.1.1 Epidemiology

Systemic lupus erythematosus (SLE) is a chronic, multisystem autoimmune disease associated with significant morbidity and mortality (1). It predominantly affects women between puberty and menopause, with a striking 9:1 female to male ratio of disease occurrence (2). In fact, SLE has recently been identified as a leading cause of death among young females (3). Incidence rates of SLE vary based on the study, but range from approximately 1 to 10 per 100,000 person-years, with rates of prevalence ranging from 20 to 70 per 100,000 (2). Epidemiological studies have identified that people from racial and ethnic minorities are disproportionality affected by SLE and experience more severe disease and increased mortality (4).

1.1.2 Clinical Manifestations

SLE is a highly heterogenous disease that can present, both clinically and biologically, in vastly different ways. For most patients, SLE follows a relapsing-remitting course in which periods

of quiescence are followed by disease flares at varying intervals (5). Patients commonly experience generalized symptoms such as fatigue, unexplained fevers, and arthralgia. Nearly any organ system can be involved, including the skin, kidneys, brain, and lungs, and even the specific complications and outcomes within each organ can vary greatly between affected individuals (1). The extraordinary complexity of this disease makes research efforts to understand the mechanisms underlying disease pathology vital for effective patient care. The more we understand about this disease, the more personalized, targeted therapies we can develop to treat this exceptionally heterogenous patient population and improve patient outcomes.

1.1.3 Cutaneous Lupus Erythematosus

Up to 70% of patients with SLE experience cutaneous manifestations, termed cutaneous lupus erythematosus (CLE) (6). CLE can also occur in the absence of systemic autoimmunity, although a subset of patients will go on to develop SLE later in their disease course (7). Three distinct types of CLE exist: acute CLE (ACLE), subacute CLE (SCLE), and chronic CLE (CCLE). The hallmark feature of ACLE is the malar (or "butterfly") rash that typically presents following sun-exposure but does not scar (8). SCLE often appears in sun-exposed areas as erythema that later progresses to psoriasiform lesions that can heal with long-lasting hyperpigmentation (8). CCLE is further subdivided into discoid LE (DLE), LE profundus (LEP), chilblain LE (CHLE), and LE tumidus (LET) (9). DLE is the most common form, making up approximately 80% of CLE diagnoses (10). DLE lesions most frequently affect the scalp, ears, and cheeks and heal with hyperpigmentation and atrophic scarring (10).

1.1.4 Lupus Nephritis

Renal involvement, or lupus nephritis (LN), occurs in more than half of all SLE patients and contributes substantially to morbidity and mortality associated with SLE. The majority of

cohort studies that have been conducted report a higher prevalence of LN in male patients than in female patients, with the male-to-female ratio of LN in patients with SLE ranging from 1.1:1 to 1.7:1 (11-15). LN is also more prevalent in patients with juvenile-onset SLE compared to those with adult-onset SLE (16-18) and in African American, Hispanic, and Asian individuals than in White individuals with SLE (19-24).

LN develops when excessive kidney inflammation damages glomeruli, preventing the kidneys from properly filtering out waste. This ultimately results in protein leakage into the urine, or proteinuria, which serves as an important biomarker for assessing kidney damage. Approximately 10-30% of patients with severe LN will progress to end-stage renal disease, or kidney failure, within 15 years of diagnosis (25).

Kidney biopsies are considered the gold standard for confirming the initial LN diagnosis as well as for determining the subtype of disease. LN subtypes are classified based on glomerular findings and this provides important framework for prognosis and treatment of disease (26). Mesangial immune complex deposition is diagnosed as minimal mesangial (Class I) or mesangial (Class II), the presence of active or chronic glomerular lesions are diagnosed as focal (Class III) or diffuse (Class IV), combined membranous and mesangial LN is diagnosed as Class V, and Class VI is considered end-stage LN which involves the presence of >90% global glomerulosclerosis (27). Flares of LN are associated with poor long-term kidney outcomes for patients with SLE, thus, an important component of disease management is prediction of flares for prompt treatment to improve outcomes (28). Therefore, patients should be periodically screened even during periods of remission or low disease activity.

A variety of factors drive disease pathogenesis in LN, however, the exact etiology is still unclear. Immune cell infiltration into the kidneys is considered a hallmark of LN and this

contributes to renal inflammation and tissue damage (27). An increased neutrophil signature in the blood has been strongly associated with active LN in SLE patients, however, neutrophilia is not typically observed histologically in the kidney itself (27, 29). Microarray analyses show that monocytes are the most prominent differentially expressed cells in LN making up approximately 30-50% of the immune cells in glomeruli of LN patients (30). T cells play a central role in the pathogenesis of LN through providing help for B cells, resulting in autoantibody production, injuring renal parenchymal cells via cytotoxic effects, and modulating T helper and effector cell functions (31). B cells are also pathogenic in LN and perform a variety of roles, including production of autoantibodies, processing and presenting antigen to T cells, and regulating dendritic cell function (31). Age associated B cells, which have been implicated in autoimmunity (32), have been identified in LN kidneys as well (33). Activated NK cells are also increased in LN glomeruli where they serve as major sources of interferon gamma (IFN-γ) and cytolytic molecules (30, 33). Pathway analyses have indicated that similar signaling pathways are activated in LN as are activated during viral infections (30). In fact, high interferon response scores are found in nearly all immune cell populations in the kidneys of a majority of LN patients (33).

1.1.5 Treatment

Management of SLE presents challenges for clinicians, largely due to extreme disease heterogeneity and relatively limited treatment options. Available therapies include corticosteroids, antimalarials, and immunosuppressive drugs. A number of new biological therapies have recently been developed and tested in SLE patients. Unfortunately, most of these clinical trials have ended following a failure to show significant benefit. To date, only two biologics have been FDA-approved for treatment of SLE. Belimumab, a human monoclonal antibody that targets B-cell activating factor (BAFF), was the first to receive approval in 2011 for SLE and active LN (34, 35).

BAFF is a key survival cytokine for B cells that is overexpressed in SLE. Levels of BAFF correlate with disease activity and anti-double stranded DNA (dsDNA) antibody concentrations (36-38). The newest advancement in SLE treatment came in 2021 with the FDA-approval of anifrolumab, a human monoclonal antibody to type I interferon receptor subunit 1 (IFNAR1) (39, 40). It has been approved for use in adults with moderate to severe SLE, and clinical trial results suggest improvements in skin disease as well (40). Recently, the FDA also approved voclosporin to be used in combination with standard-of-care therapy in adults with active LN (41, 42). Voclosporin is an immunosuppressive medication that works by inhibiting a protein called calcineurin, which is important in T cell activation and can help limit renal damage. Better understanding of the pathogenesis of SLE and the ongoing shift toward personalized medicine will aid in the development of novel therapeutics that better address the varied patterns of disease.

1.1.6 Risk Factors

The etiology of SLE is multi-factorial and incompletely understood, however, growing evidence suggests it involves a complex interplay between a variety of factors including genetics, epigenetics, hormones, and environmental triggers.

Genetics

Studies of twins, which are frequently used to quantify the role of gene inheritance in disease susceptibility, demonstrate a concordance rate for SLE of approximately 24% in monozygotic twins, compared to only 2% in dizygotic twins, suggesting a robust, but partial, role for genetics in disease susceptibility (43). As such, genetics alone are typically not enough to drive SLE pathogenesis and other factors are needed to trigger disease onset in predisposed individuals. While SLE is a biologically complex and clinically heterogenous disease, the genetic variants that

confer susceptibility are primarily involved in common pathways, including complement activation, nucleic acid sensing and degradation, and type I IFN signaling and regulation.

Monogenic lupus is a form of the disease that is caused by a single gene defect. It typically presents with severe disease manifestations in pediatric patients. Because of the early onset of monogenic lupus, it is thought that genetic factors may play a more important role than environmental factors (44). More than 30 genes have been identified as causing the monogenic form of SLE. In contrast, approximately 100 susceptibility loci have been described for polygenic, multifactorial SLE (44). In this more common form of the disease, individuals carrying particular gene variants are predisposed to SLE, but an environmental trigger is likely needed to precipitate the onset of disease.

There is a strong association between SLE and genes involved in type I IFN signaling, production, and response. Three members of the interferon regulatory factor (IRF) family (*IRF5, IRF7, IRF8*) have been genetically associated with SLE (45). Several studies have identified an association between single-nucleotide polymorphisms (SNPs) and functional variants in *IRF5* with SLE risk (46-48). *IRF5* can both be activated by IFN-α as well as induce transcription of *IFNA* genes, suggesting its ability to produce a positive feedback loop of IFN-induced responses (49). *IRF5* can also enhance transcription of other pro-inflammatory cytokines, several of which have known roles in SLE, including *IL6*, *TNF*, and *IL17* (45). Additionally, in the absence of *IRF5*, murine models of lupus have decreased levels of autoantibodies that may result from IRF5 regulating transcription of *Prdm1* (encodes Blimp-1, a regulator of plasma cell differentiation), thereby reducing autoantibody production (50). Studies also support a role for IRF7 in SLE risk. SNPs in the IRF7 region correlate with IFN levels and autoantibody profiles in patients with SLE from multiple ethnic backgrounds and murine studies suggest that IRF7 may be important for

autoantibody production (51). Lastly, *IRF8* gene variants have been associated with SLE susceptibility, likely through modulation of type I IFN responses (52).

Signal transducer and activator of transcription 4 protein (STAT4) is a transcription factor required for inflammation in response to stimulation by interleukin (IL)-12, IL-23 and IFN-α. Multiple genome-wide association studies (GWAS) and gene association studies provide evidence for a strong association between STAT4 and SLE (52). *STAT4* polymorphisms in patients with SLE have been associated with increased production of autoantibodies, augmented responses to IL-12 and IFN-α, and more severe nephritis (53-56). Of particular interest, *STAT4* polymorphisms have also been associated with development of DLE in a Polish population (57).

The human leukocyte antigen (HLA) complex encodes more than 200 genes and is the most gene-dense region of the genome. The HLA region has been consistently identified as the strongest predictor of genetic risk in SLE GWAS among various ethnic populations (52). In particular, genetic variants in the class II and III regions predispose an individual to SLE. The class II region contains genes encoding glycoproteins that are expressed on antigen presenting cells for peptide processing and presentation to T cells. The class III region contains genes with important immunological roles including those encoding for proteins of the complement system (58).

The complement system is made up of more than 30 proteins that are involved in innate immune processes including pathogen defense and clearance of cellular debris (59). Defects in early complement proteins (C1q, C1r/C1s, C2, and C4) can result in insufficient removal of apoptotic bodies, accumulation of autoantigens and immune complexes, and systemic inflammation. This can trigger autoimmunity and has been linked to SLE. In fact, although these mutations are relatively rare, frequency of SLE is nearly 90% in patients with C1q deficiency and roughly 65% in C1r/C1s deficiency, 75% in C4 deficiency, and 10% in C2 deficiency (44).

TREX1 (3’ repair exonuclease 1) is an exonuclease that degrades cytoplasmic DNA fragments (60). Specifically, it plays an important role in degrading self-DNA in order to safeguard from autoimmune responses. Loss of TREX1 function results in accumulation of cytoplasmic DNA and aberrant activation of the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway. This stimulates type I IFN responses and systemic inflammation. Mutations in the *TREX1* gene are associated with a spectrum of autoimmune disorders including type I interferonopathies and forms of lupus (60). A heterozygous missense mutation (D18N) in *TREX1* has been identified in individuals with familial chilblain lupus (61). Multiple heterozygous missense and frameshift changes in *TREX1* have been identified in SLE patients (61).

Epigenetics

Epigenetic mechanisms involve genetic modifications that occur without changing the DNA sequence (62). These changes are reversible, but heritable, and control the accessibility of the DNA in order to regulate gene activity. DNA methylation and histone modifications are two epigenetic events that have been identified as being relevant in the pathophysiology of lupus.

DNA methylation refers to the addition of a methyl group to a cytosine residue (most often CpG dinucleotides) by DNA methyltransferases (DNMTs) (63). This generally results in silencing of gene expression. DNA demethylation is the process by which these methyl groups are removed from cytosines. This can be either a passive (during cell replication newly incorporated cytosines remain unmethylated) or active (enzymatic reactions process the methylated cytosine back to a naked cytosine) process (63). Demethylation generally promotes gene expression.

Monozygotic twins discordant for SLE have DNA methylation and expression changes in genes with biological functions relevant to SLE, highlighting the importance of epigenetic alterations in disease pathophysiology (64). In particular, global DNA hypomethylation that

inversely correlates with the SLE Disease Activity Index (SLEDAI) has been identified in $CD4^+$ T cells in SLE compared to healthy controls (65-67). Further, expression levels of DNMT1 are significantly reduced in SLE (65-67). However, whether levels of DNMT1 correlate with either SLEDAI or global DNA methylation varies between studies, suggesting that there may be variability between patient populations (65-68). Of particular interest, significant hypomethylation has been identified in IFN-regulated genes (69, 70) in SLE and this has been associated with autoantibody production (71) and nephritis (72). Differentially methylated regions in genes important for mediating proliferation and apoptosis have been reported in naïve $CD4^+$ T cells from SLE patients with malar and discoid rashes, suggesting the involvement of epigenetic predisposition in development of cutaneous manifestations of SLE (73). Additionally, recent work indicates that Hippo signaling is the top differentially methylated pathway in keratinocytes from SLE patients compared to controls (74). As a result, enhanced Hippo signaling contributes to enhanced UV-mediated apoptosis in SLE keratinocytes via cytoplasmic retention of the anti-apoptotic transcriptional coactivator YAP (74).

Histone modifications are another common form of epigenetic changes. Histones are the proteins around which DNA is arranged to form the nucleosome. Post-translational modifications, including acetylation, methylation, and phosphorylation, among others, can be made in histone tails and this plays an important role in regulation of gene expression through controlling accessibility to transcription factors. Chromatin can be divided into either actively transcribed euchromatin that is lightly packed or transcriptionally inactive heterochromatin that is in a condensed form (62). Both euchromatin and heterochromatin are generally characterized by their levels of specific histone modifications.

Dysregulated histone marks have been identified as important in a number of autoimmune and inflammatory disorders, including SLE. This has been best studied in genes encoding a variety of cytokines. For example, increased expression of the pro-inflammatory cytokine IL-17 in SLE patients is, in part, the result of permissive modifications to histone proteins at the *IL17* gene cluster in T cells (75, 76). The *IL2* gene has also been reported to undergo genetic silencing in T cells from SLE patients due to repressive histone modifications. As IL-2 is important for generation of T regulatory cells (Tregs), this promotes an effector phenotype for SLE T cells (77, 78). Other cytokines differentially regulated by histone modifications, specifically increased acetylation, in lupus include tumor necrosis factor alpha (TNF-α) and IL-10 in monocytes and T cells, respectively (79, 80).

Sex-bias

The striking female predominance in SLE suggests a potential hormonal component to disease development. While the role of sex hormones in modulating pathogenesis of lupus is not fully understood, some links have been made (81). Estrogens are known to influence immune system regulation. Of importance for SLE, estrogen plays a role in B cell maturation, selection, and activation which can support autoantibody production (82). Supplementation of lupus-prone mice with estrogen accelerates the onset of disease and mortality (83). In addition, oophorectomized female lupus-prone mice display an increased lifespan, while castrated male lupus-prone mice exhibit disease more similar to intact female lupus-prone mice (84). Studies have made differing conclusions on the influence of exogenous hormones (e.g. taking oral contraceptives containing estrogen or postmenopausal hormone therapy) on developing SLE or increasing the risk of lupus flares (85-88). These inconsistencies may be partially explained by the characteristics of the study groups, which often make human studies of SLE challenging, and may

suggest that hormone levels are frequently altered in SLE patients, but whether this has any actual casual effect has not been established.

The increased prevalence of SLE in females is unlikely to be explained entirely by sex hormones. Indeed, sex-hormone-independent mechanisms have been identified that predispose females to autoimmunity. In one study, sex-biased genes were enriched even in healthy female skin and found to overlap with genetic risk variants previously associated with SLE risk (89). One such female-biased gene is vestigial-like family member 3 (*VGLL3*), a putative transcription factor that promotes expression of several known SLE-related genes including *BAFF*, *MMP9*, and *ICAM1* (89). In addition to being more highly expressed in female skin, VGLL3 also shows female-specific nuclear localization (89). Intriguingly, although disease is less common in males, those who do develop CLE are reported to have increased expression and nuclear localization of VGLL3, comparable to females (89). Epidermal overexpression of murine VGLL3 drives cutaneous and systemic autoimmune disease that is remarkably similar to SLE, with development of severe rash, B cell expansion, anti-dsDNA autoantibody production, renal immune complex deposition, and end-organ damage (90). Ongoing research efforts are continuing to elucidate the role of these non-sex-hormone-related factors in modulating female-bias in SLE.

Environment

Substantial research, including epidemiological evidence, indicates that environmental triggers are pivotal in the pathophysiology of SLE. Several factors have been associated with risk of SLE including cigarette smoke, use of certain medications, Epstein-Barr virus (EBV) infection, and exposure to ultraviolet light (UV).

Many studies have reported an increased risk of SLE among cigarette smokers (91-95), with current smoking generally being a stronger risk factor than previous smoking (92-94).

Whether there is a dose-response relationship between level of exposure to cigarette smoke and a higher risk of SLE is unclear, with varied results in most studies (91-93, 96, 97). Actively smoking also seems to increase the severity of disease in SLE patients. Current smokers have been shown to have higher SLEDAI scores, more serious cutaneous manifestations, greater organ system involvement, and accelerated development of kidney failure compared to former or never smokers (91, 97-100). The mechanistic role of cigarette smoke in autoimmunity is not fully understood. Cigarette smoke contains a very high level of free radicals that can induce oxidative stress and directly damage DNA, both of which have been implicated in SLE (101-103). Further, cigarette smoke can induce epigenetic changes and increase production of several pro-inflammatory cytokines (104-106).

Chronic use of certain drugs can result in drug-induced LE (DILE). DILE usually occurs after months to years of usage and the disorder resolves once the medication is stopped. The first reported case of DILE occurred with sulfadiazine treatments in the 1940s (107). More than 100 drugs from a multitude of categories have been implicated in this disorder since then (108, 109). Two of these drugs, procainamide and hydralazine, are considered high-risk drugs for DILE with around 10-20% risk, however, use of these drugs in clinical settings is on the decline (110, 111). As more drugs and biologics are being developed, the number of new DILE cases continues to rise (112). The current proposed pathogenic mechanisms of DILE involve genetic predisposition, epigenetic alterations, and photosensitization, among others (113). The role of genetic susceptibility in development of DILE is widely accepted, however, the specific mechanisms involved vary based on the drug type. For example, drugs like procainamide, hydralazine, and isoniazid are metabolized by acetylation via N-acetyltransferase enzymes and individuals that are slow acetylators are more prone to accumulation of autoantibodies while taking these drugs (114-

116). These drugs and their metabolic products can also act as inhibitors of the complement pathway limiting effective clearance of immune complexes (117, 118). Drugs known to induce DILE including procainamide and hydralazine have been shown to inhibit T cell DNA methylation by blocking the actions of DNMTs (119, 120). Intriguingly, evidence suggests that photosensitizing drugs may further cutaneous inflammation and IFN responses that promote development of a cutaneous form of DILE (121).

Many studies support an association between EBV infection and SLE, although the precise mechanisms that underly this are not well understood (122). EBV infection is nearly ubiquitous among adults as it remains in a latent state and can periodically reactivate following initial infection. It is possible that reactivation of a previously latent infection could serve as a triggering event for individuals already genetically predisposed to SLE, although this would likely be difficult to definitively confirm. Higher EBV seroconversion rates have been observed in SLE patients compared to healthy controls (123-125). Further, patients have higher viral loads of EBV which may be indicative of reactivation of virus (126, 127). Lastly, autoimmune responses may be induced due to molecular mimicry between EBV antigens and SLE autoantigens. Antibodies against the EBV nuclear antigen EBNA-1 cross-react with a variety of autoantigens including Sm and Ro (128). This suggests that EBV infection may promote loss of tolerance to self-antigens leading to potential pathogenic responses and onset of SLE.

It is well established that exposure to even low levels of UV light can drive disease flares in patients with established SLE, termed photosensitivity. However, it is less clear whether UV exposure can also increase the risk of developing SLE in the first place. A recently published prospective study was the first to assess the association of UV exposure with the risk of incident SLE (129). The Nurses' Health Study, which enrolled nearly 240,000 US nurses, collected lifestyle

and medical data through biennial questionnaires and medical record reviews. Ambient UV exposure was estimated based on residential addresses and a high spatiotemporal resolution erythemal UV exposure model. Perhaps surprisingly, cumulative UV exposure was not found to be significantly associated with overall SLE risk in this study. However, it was demonstrated that UV exposure was associated with increased risk of malar rash at SLE onset. This study did have several limitations that must be considered, however, including lack of diversity among participants (mostly white females working as nurses in the US) and not accounting for time spent outdoors. Further studies should be conducted to confirm whether UV exposure is a risk factor for specific subtypes of lupus or specific manifestations of the disease and whether this risk varies in other parts of the world or in other ethnic groups or skin types.

1.2 Photosensitivity in SLE

Up to 93% of lupus patients experience heightened sensitivity to sunlight and other sources of UV radiation, a phenomenon known as photosensitivity (130-132). Exposure to even minimal amounts of UV radiation can trigger cutaneous and systemic disease flares in patients that include development of skin lesions, joint pain, fever, and fatigue. Further, repetitive incidences of this can lead to long-term organ damage. Patients with photosensitive skin disease report having severely decreased quality of life with high levels of depression and anxiety (133). With limited treatment options, patients must take precautions to limit their sun exposure, such as wearing protective clothing, using sunscreen, and avoiding the outdoors during peak UV hours (134). An increased understanding of the mechanisms driving photosensitivity in lupus will open new treatment avenues for controlling these responses. Our current understanding of the mechanisms contributing to photosensitivity in lupus is summarized in Figure 1-1.

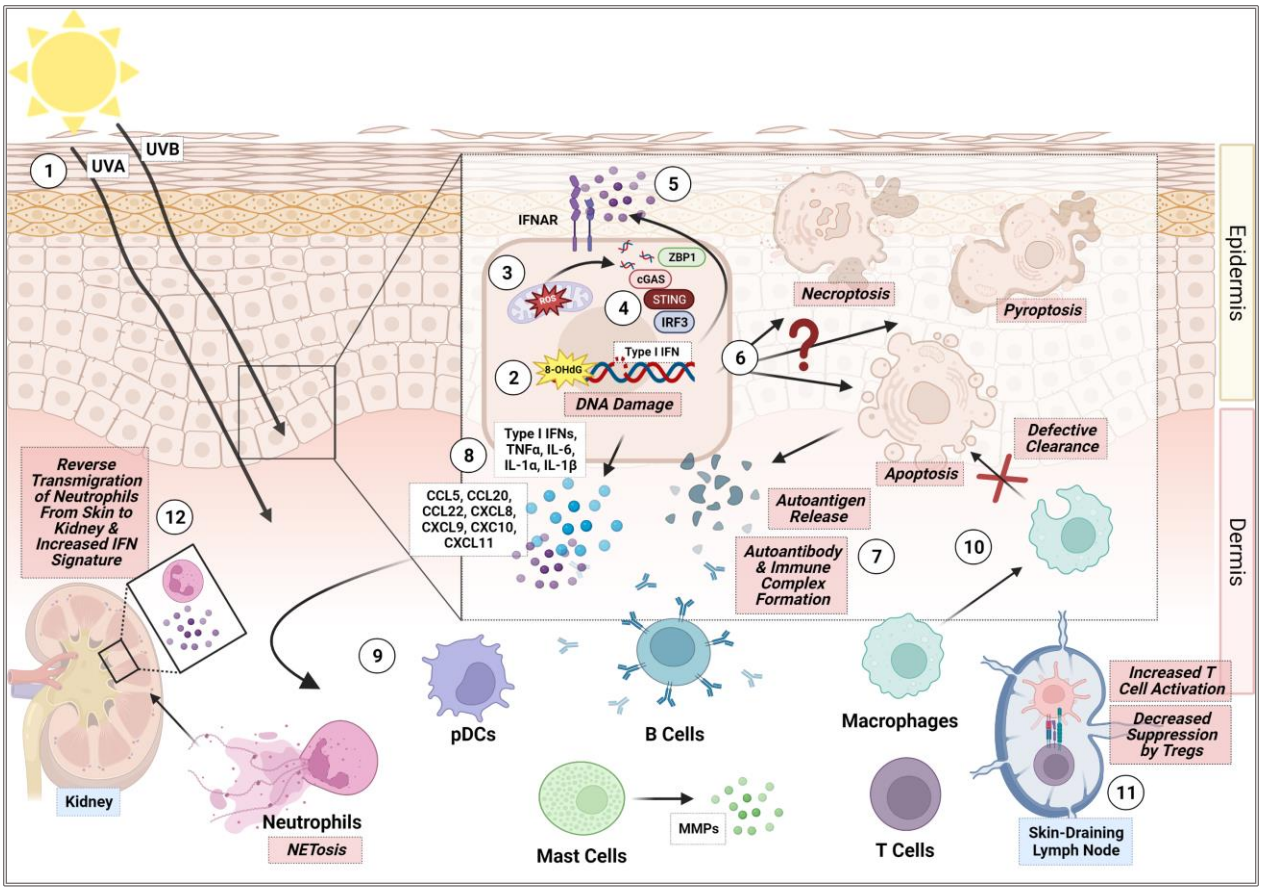

Figure 1-1 – Summary of mechanisms of photosensitivity in lupus.
(1) UVA and UVB light from the sun penetrate the dermis and epidermis, respectively. UVB light is absorbed by keratinocytes. (2) UVB induces DNA and (3) mitochondrial damage. (4) Mutated mitochondrial DNA can activate cytosolic DNA sensors including ZBP1 and cGAS-STING and (5) potentiate type I IFN production and signaling through the type I IFN receptor (IFNAR). (6) A subset of damaged keratinocytes will die, likely through apoptosis, necroptosis, or pyroptosis; however, the specific pathway activated in lupus keratinocytes is unclear. (7) Dying cells release a variety of autoantigens that can be bound by autoantibodies to form immune complexes. (8) Inflammatory cytokines and chemokines are produced in response to these insults, and this (9) promotes recruitment and activation of immune cells. (10) Phagocytic clearance of apoptotic cells may be defective in lupus resulting in increased release of autoantigens, furthering cutaneous inflammation. (11) In skin-draining lymph nodes, T cells are highly activated resulting from type I IFN-mediated suppression of T regulatory (Treg) cells. (12) Neutrophils recruited to the skin can undergo reverse transmigration to the kidney where an increased IFN signature is observed after UV exposure, suggesting a potential mechanism by which UV can induce a systemic inflammatory response. Created with BioRender.com.

1.2.1 Ultraviolet Light

UV light emitted by the sun is classified based on wavelength into UVA (400-320nm), UVB (320-280nm), and UVC (280-100nm), with shorter wavelengths possessing higher energy. UVA accounts for 95% of the solar UV radiation that reaches the Earth's surface, and the longer wavelengths of UVA rays can penetrate the dermis. Most UVB and all UVC radiation is absorbed by the ozone layer and, therefore, does not naturally reach our skin. The portion of UVB that

permeates the earth's atmosphere enters the epidermis where it is largely absorbed by keratinocytes (135).

1.2.2 DNA Damage and Sensing

DNA damage

UVA and UVB distinctly influence skin physiology with outcomes dependent on duration and intensity of exposure. The damage that results following UV exposure can be mediated either directly through absorption of incident light by cellular components, including DNA and proteins, or indirectly via photosensitization reactions (136, 137). UVB radiation is primarily responsible for damage resulting from direct absorption. When DNA absorbs UVB photons, intra-strand linkages can form between neighboring pyrimidines. The resulting photodimers include cyclobutane pyrimidine dimers (CPDs), pyrimidine-6,4-pyrimidone photoproducts (6,4PPs), and their Dewar isomers (138). 6,4PPs are more highly mutagenic than CPDs, however, they make up only 20-30% of total photoproducts while CPDs comprise 70-80% (139). These bulky, helix-distorting DNA lesions are recognized, removed, and repaired by the highly conserved nucleotide excision repair (NER) pathway in order to ensure genomic integrity (140). Mutations in genes associated with the NER cause a number of human genetic disorders that are associated with photosensitivity including xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (141).

UVA exerts the majority of its cellular effects indirectly through generation of reactive oxygen species (ROS) that induce oxidation of macromolecular structures (136). These oxidants can damage proteins and lipids, resulting in modulation of cellular signaling pathways and membrane structures, which can have detrimental effects on the cell (142, 143). Oxidative modification of DNA can also occur promoting formation of oxidized bases such as 8-hydroxy-

2’-deoxyguanosine (8-OHdG), which can mispair with A leading to transversions. To limit this mutagenicity, the oxidized guanine must be repaired via the oxyguanine glycosylase 1 (OGG1)-initiated base excision repair (BER) pathway (144). Oxidative DNA damage has been shown to potentiate inflammation and, therefore, has important implications for autoimmunity. In fact, 8-OHdG is highly immunogenic and abundant in UV-induced lupus skin lesions (145). Furthermore, loss of OGG1 in the pristane-induced mouse model of SLE results in dysregulated IFN responses and aggravated skin pathology. Reduced expression of *OGG1* is also observed in lesional skin of patients with discoid lupus, supporting a role for OGG1 in UV-induced skin pathology in autoimmunity (146).

Cytosolic DNA sensing

Genomic DNA from UV-irradiated epithelial cells can induce primary human monocytes to secrete more IFN-α than those exposed to DNA from non-irradiated epithelial cells (145). This suggests that a UV-induced modification of DNA is at least partially responsible for upregulation of type I IFNs in lupus skin. Lending more support to this idea, colocalization of 8-OHdG and IFN-inducible MxA is observed in the epidermis of UV-induced lupus skin lesions (145).

Integration of the 8-OHdG and IFN response may occur via STING signaling. STING coordinates signals from the cytoplasmic DNA sensor cGAS, and is negatively regulated by the pro-autophagic protein unc-51-like kinase 1 (ULK1) (147). Upon UV-induced DNA damage, ULK1 stability is disrupted by the loss of the activating molecule autophagy and beclin 1 regulator 1 (AMBRA1) (148). The resulting increase in STING activity causes activation of IRF3, potentiating type I IFN secretion and exacerbating autoimmunity in response to UV exposure (148). Further, TREX1 generally degrades cytosolic self-DNA in order to prevent activation of cytosolic DNA sensors. DNA that has undergone oxidative modification is less susceptible to

TREX1-mediated degradation and, therefore, is a potent activator of the cGAS-STING DNA sensing pathway (145).

Z-DNA

The familiar right-handed DNA, also known as B-DNA, can be "flipped" to a left-handed conformation by DNA sequences called flipons without breaking or damaging the DNA (149). This altered left-handed DNA is known as Z-DNA (150). Z-DNA is often formed in regions that contain endogenous retroelements, including short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs). Alu SINE sequences make up approximately 10% of the genome and commonly contain inverted repeats separated by only a few thousand bases (151, 152). The transcripts produced from these regions fold back and pair on each other creating long dsRNA that can activate cytosolic RNA sensors and type I IFN production (153).

With elevated levels of type I IFN production playing a crucial role in SLE, it is perhaps unsurprising that Z-DNA has been associated with its pathogenesis. Anti-dsDNA antibodies are highly specific for SLE and serum from SLE patients has been shown to preferentially bind to Z-DNA over B-DNA (154-157). Indeed, anti-Z-DNA antibodies are found in the serum of lupus patients and correlate with clinical disease manifestations, suggesting a pathogenic role for Z-DNA in SLE (158). Further supporting this notion, drugs associated with DILE have been shown to facilitate the B-to-Z transition of DNA (159, 160). For example, hydralazine can induce the Z-DNA conformation and one study found that 82% of individuals taking this drug had anti-Z-DNA antibodies, while age-matched healthy controls had none (160).

Adenosine deaminase acting on RNA 1 (ADAR1) is an RNA-editing enzyme that can bind to Z-DNA through its Zα binding domain (161). ADAR1 edits RNA through deamination of adenosines to inosines (A-to-I editing) which can destabilize dsRNA structures and prevent

recognition of self-dsRNA (162). The constitutively expressed p110 isoform of ADAR1 is protective against induction of apoptosis in cells under stress (163). UV irradiation can induce the translocation of ADAR1p110 from the nucleus to the cytosol following its phosphorylation by MKK6-p38-MSK MAP kinases and Exportin-5-mediated nuclear export. Once localized to the cytosol, ADAR1p110 binds to mRNAs containing a dsRNA structure in their 3'untranslated regions (3'UTRs). This binding process results in competitive inhibition of the binding of another dsRNA-binding protein called Staufen-1 to the target mRNAs. Staufen-1 participates in Staufen1-mediated mRNA decay (SMD), an mRNA degradation process (164, 165). Importantly, many anti-apoptotic genes are known to have these dsRNA-containing 3'UTRs, therefore, ADAR1p110 protects these transcripts from undergoing SMD (163).

The p150 isoform of ADAR1 is induced in response to IFN signaling and has an essential role in the negative regulation of immune responses to dsRNA (150). Indeed, ADAR1 mutations can confer autoimmunity in both humans and mouse models (166-168). Disorders such as Aicardi-Goutières syndrome (AGS) have been attributed to loss-of-function mutations in the Zα domain of ADAR1. This leads to the hallmark type I interferonopathy seen in AGS, supporting a role for Z-DNA binding in preventing dysregulated IFN responses (169).

In contrast to AGS, excessive ADAR1 activity may be implicated in SLE. In fact, ADAR1 is more highly expressed in SLE lymphocytes (170-172) and SLE patients have been shown to have increased levels of global RNA-editing (173). Research indicates that elevated levels of ADAR1 are associated with higher rates of RNA-editing as well as dysregulated editing of "non-typical" editing sites (174, 175). This can lead to generation of neo-autoantigens that induce immune responses and exacerbate autoimmunity (173).

In addition to ADAR1, Z-DNA binding protein 1 (ZBP1) is the only other mammalian Zα-containing protein that has been identified (176). ZBP1 is a key innate immune sensor of foreign and endogenous DNA and RNA that can activate both programmed cell death and inflammatory responses (177). It recognizes the Z-form of DNA and RNA in a sequence-independent manner. One of only four human proteins known to do so, ZBP1 contains receptor-interacting protein homotypic interaction motif (RHIM) domains (178). This allows it to interact with RHIM-domain-containing receptor-interacting protein kinase 3 (RIPK3) and induce parallel activation of apoptosis, necroptosis, and pyroptosis pathways, termed PANoptosis (179). In mice, ZBP1 activity is restrained under normal conditions by RIPK1 via RHIM-dependent interactions (180, 181). Mice with epidermal-specific loss of RIPK1 exhibit keratinocyte necroptosis and skin inflammation that is prevented by ZBP1-deficiency (180). How ZBP1-sensing of nucleic acids may contribute to photosensitive responses in SLE has yet to be addressed, but may be of interest as *ZBP1* is more highly expressed in both females vs. males and SLE patients vs. healthy controls (182).

1.2.3 Cell Death

Apoptosis

When these various repair mechanisms are unsuccessful, cells may continue to proliferate resulting in errors in DNA synthesis that allow genomic mutations to accumulate and promote carcinogenesis. Alternatively, cells may undergo cellular senescence, or permanent cell cycle arrest, to suppress malignant transformation, however, this process contributes to photoaging (183, 184). Cells can also initiate apoptosis as a protective mechanism when there is irreparable DNA damage. Apoptosis is a form of programmed cell death that is generally mediated by caspases and occurs through either the intrinsic pathway (mediated by mitochondrial dysfunction) or the

extrinsic pathway (mediated by activation of external death receptors). During apoptosis, cells undergo morphological changes that involve plasma membrane blebbing and separation of cellular fragments into apoptotic bodies. The plasma membrane remains intact to prevent release of inflammatory cellular contents, making this an immunologically silent process (185).

Death receptors found on keratinocytes are members of the TNF receptor (TNFR) family that can initiate apoptosis upon activation by ligand binding. These include Fas/CD95 and its ligand FasL/CD95L, TNF-related apoptosis-inducing ligand (TRAIL)-Rs and their ligand TRAIL (see below), and TNFR and its ligand TNF-α (186). Each ligand-receptor pair follows a general pathway. When a ligand binds to its corresponding receptor, the receptor undergoes oligomerization and recruits Fas-associating protein with death domain (FADD) (187). Procaspase-8 is recruited and binds to FADD forming the death-inducing signaling complex (DISC) (188). Procaspase-8 undergoes auto-proteolytic cleavage and cleaves BH3 interacting-domain death agonist (BID) and/or procaspase-3 either at the plasma membrane as part of the DISC or in the cytosol following its release (189). The truncated form of BID (tBID) translocates to the mitochondria where it induces cytochrome c release. Procaspase-9 and cytochrome c together with APAF-1 form the apoptosome. Caspase-9 is activated by the apoptosome and subsequently activates downstream effector caspases-3 and -7 which cleave target molecules. This induces apoptosis of the cell (190). Depending on the cell type, apoptotic stimuli, and particular circumstances, the pathways following BID or procaspase-3 cleavage can act independently or synergistically to amplify the initial death signal (191). The necessity of this amplification largely depends on whether the amount of active caspase-8 at the DISC is sufficient to directly activate caspase-3 (191).

Exogenous stimuli, such as UV irradiation, or endogenous stress, such as ROS-induced DNA damage and endoplasmic reticulum stress, can induce the intrinsic apoptosis pathways (192). Pro-apoptotic proteins BAK and BAX are activated and induce mitochondrial outer membrane permeabilization (MOMP), allowing proteins normally sequestered in the mitochondria to diffuse into the cytosol (193, 194). Similarly to the mitochondrial pathway induced by the extrinsic pathway, cytochrome c is released from the mitochondria, the apoptosome is formed, and caspases-9, -3, and-7 are activated resulting in apoptosis (195). Intrinsic apoptosis can also be caspase-independent following release of apoptosis inducing factor (AIF) and endonuclease G from the mitochondria. These proteins can translocate to the nucleus, where they induce chromatin condensation, DNA fragmentation, and, ultimately, apoptosis (194, 196).

Accumulation of apoptotic cells has been noted in the epidermis of SLE patients after UV exposure, which may be secondary to increased cell death and/or decreased clearance (197). Reduced clearance of apoptotic cells is associated with decreased levels of serum complement proteins C1q, C4, and C3 (198, 199). Importantly, photosensitivity is more common among patients with deficiencies of C4A (200) and C2 (201). Other forms of cell death have been reported in CLE (see below).

Necroptosis

Necroptosis is a form of regulated cell death that is initiated by ligation of death receptors, similar to extrinsic apoptosis, but ultimately results in cell death via swelling and rupture of the plasma membrane, similar to necrosis (202). This pathway occurs in the absence of functional caspase-8 or FADD and generally involves RIPK1-induced activation and oligomerization of RIPK3 followed by engagement of the effector protein mixed lineage kinase domain like pseudokinase (MLKL) (203). Once activated, MLKL translocates to the plasma membrane where

it mediates plasma membrane rupture. Cellular contents, including danger associated molecular patterns (DAMPs), pro-inflammatory cytokines, and other alarmins, are released into the microenvironment eliciting an inflammatory response (202).

Constitutive IFN signaling is needed to maintain expression of MLKL above a critical threshold to allow for MLKL oligomerization and necroptotic cell death (204). Mitochondrial stress, such as that induced by UV radiation, can lead to DNA-damage that triggers activation of the cGAS-STING pathway and promotes type I IFN production (205). Individuals with SLE have increased levels of type I IFNs that are further increased following UV exposure, suggesting necroptosis as a potential mechanism by which patients develop inflammatory photosensitive responses. Further supporting a role for necroptosis in SLE, enhanced epidermal expression of RIPK3 is detected in interface dermatitis which is frequently observed in CLE skin lesions and can be triggered by UV irradiation (206).

Pyroptosis

Pyroptosis is a form of regulated cell death canonically mediated by activation of inflammasomes (202). Inflammasomes are cytoplasmic, multiprotein complexes of the innate immune system that are assembled in response to sterile or infectious stimuli (207). Activation of these complexes results in proteolytic cleavage of pro-caspase-1. In turn, activated caspase-1 cleaves pro-IL-1beta (IL-1β) and pro-IL-18, both pro-inflammatory cytokines, to their mature forms. Gasdermin D (GSDMD), the executioner of pyroptosis, is also cleaved and activated (208). The N-terminal fragment of GSDMD (GSDMD-N) translocates to the inner leaflet of the plasma membrane where it binds to phosphatidylinositol phosphates and phosphatidylserine (209). GSDMD-N oligomerizes generating a pore that induces cell swelling and rapid plasma membrane

permeabilization in addition to release of IL-1β and IL-18. Similar to necroptosis, this is a lytic form of cell death that elicits an inflammatory response.

Keratinocytes are reported to undergo caspase-1-induced pyroptosis in response to UV radiation (210). The NLRP3 and NLRP1 inflammasomes are reported to be activated in response to UVB exposure in keratinocytes (210-213). NLRP1 has been identified as the most prominent inflammasome sensor in human skin (214), while expression of NLRP3 is significantly increased in a dose-dependent manner following UV irradiation (215). Moreover, NLRP3 polymorphisms have been associated with SLE risk and severity of disease activity, including in the skin, supporting a potential role for its involvement in mediating photosensitivity (216).

1.2.4 Clearance Mechanisms

For apoptosis to remain an immunologically silent process, dying cells must be quickly located, recognized, engulfed, and digested by phagocytes (217). Clearance of apoptotic cells, known as efferocytosis, can be divided into four key steps (218). First, apoptotic cells release soluble mediators that recruit phagocytes to the site of cell death. These are referred to as "find-me" signals and can include low levels of nucleotides ATP and UTP, lysophosphatidylcholine, fractalkine, and sphingosine 1-phosphate (219-222). Second, apoptotic cells express "eat-me" signals on their cell surface so that phagocytes will recognize and engulf them. The most common and best characterized of these signals is the externalization of phosphatidylserine (PtdSer) to the outer plasma membrane, which occurs early in the apoptotic process (223). Phagocytes express a variety of receptors that recognize these signals either directly or via bridging molecules (224). Third, once ingested, the phagocyte must process the cellar corpse. This involves a series of steps referred to as phagosome maturation that leads to its eventual degradation (225). Lastly, in

response to this process, the phagocyte releases anti-inflammatory cytokines and mediators that prevent an inflammatory response in the tissue (226).

If efferocytosis does not occur efficiently, cells undergo secondary necrosis releasing intracellular materials that act as danger signals to neighboring cells and can lead to severe tissue injury. Reduced clearance of apoptotic cells by phagocytes from SLE patients compared to healthy controls likely contributes to induction of inflammatory lesions after UV exposure (198, 199, 227). This reduced uptake is suggested to be serum dependent, rather than resulting from an intrinsic defect, and is associated with decreased levels of serum complement proteins C1q, C4, and C3 (198, 199). Complement plays an important role in regulating apoptotic clearance via opsonization and deficiencies in early complement components predispose individuals to the development of SLE (228). As many as 75-90% of patients with homozygous deficiency of C1 or C4 are reported to have SLE or lupus-like disease (229). Additionally, photosensitivity is more common among patients with deficiencies of C4A (200) and C2 (201).

While it appears that many of the same mechanisms used for apoptotic cell clearance are used for cells undergoing other forms of cell death, this has not yet been fully elucidated (230). Lytic forms of cell death, including both necroptosis and pyroptosis, involve plasma membrane rupture and subsequent release of intracellular contents. Many of the same “find-me” signals that are intentionally released by apoptotic cells are also released as a consequence of lysis during inflammatory cell death (231). This promotes chemotaxis of phagocytes to the site of the dying cells. Further, while externalization of PtdSer does not occur as an active process as it does during apoptosis, it does still become exposed on the cell surface due to loss of enzymatic activity required to maintain PtdSer inside the cells (232). However, while similar mechanisms seem to be involved

in phagocytosis of apoptotic and non-apoptotic cells, clearance of the latter has been reported to occur less quickly and less efficiently (233).

1.2.5 Autoantigens

UVB exposure induces translocation of intracellular antigens including Ro/SSa and La/SSB to the surface of apoptotic keratinocytes rendering cells susceptible to binding by circulating autoantibodies (234-237). These autoantigens tend to cluster in close proximity to sites of increased ROS generation leaving them vulnerable to oxidative modifications that further enhance their immunogenicity (238). The presence of anti-Ro and anti-La autoantibodies as well as increased expression of Ro/SSA and La/SSB on keratinocytes correlate with patient photosensitivity (239-242). UVB can also increase autoantibody binding to other autoantigens including Sm, RNP, Ku, and ribosomal-P (243-245) and this is associated with photosensitivity in lupus patients (245-247).

1.2.6 Immune Complexes

Immune complex deposition along the dermal-epidermal junction, known as the lupus band, is a common feature of both CLE and SLE skin (248). Formation of the lupus band can be induced by UV stimulation (249) and its presence, as well as the number of immunoreactants found within the deposits, positively correlates with disease activity (250). These immune complexes can amplify the inflammatory response through a variety of mechanisms. Importantly, autoantibodies in complex with RNA or DNA fragments can be internalized by FcγRII on plasmacytoid dendritic cells (pDCs) resulting in activation of endosomal toll like receptor (TLR)-7/9 and production of IFN-α that can potentiate cutaneous lesion development (251-253). Immune complexes can also stimulate inflammasome activation (254, 255) and B cell expansion (256), which may further perpetuate the cycle of inflammation in the skin following UV exposure.

1.2.7 Cytokines

Type I IFNs

UV exposure influences cytokine production in a highly context-dependent manner. In wild-type (WT) mouse skin, repeated exposure to UVB radiation enhances STING-dependent production of type I IFNs in a bimodal fashion, with early production likely by keratinocytes (257) and later production by infiltrating immune cells, including inflammatory monocytes (258). IFNAR-knockout mice display more severe skin inflammation following UVB exposure, suggesting that type I IFNs play a protective role in healthy skin (258). This suppressive effect may occur via IFN-induced upregulation of the RNA-binding protein tristetrapolin, which limits expression of pro-inflammatory genes such as TNF-α and IL-6 (259). Intriguingly, UVB exposure not only triggers a type I IFN signature in the skin of WT mice, but also systemically in the blood and kidneys (260) and this may be one mechanism by which UV triggers systemic lupus flares.

Type I IFN response genes are more highly expressed in both lesional (261) and non-lesional (262) lupus keratinocytes compared to healthy controls and this may trigger a more inflammatory response to UV radiation. Increased expression of type I IFNs, including *IFNK*, is also observed in CLE lesions (263, 264). Following UVB stimulation, SLE keratinocytes secrete more IFN-κ compared to healthy controls (257) and this increases keratinocyte sensitivity to UV radiation (263). Conditioned media from UVB irradiated SLE keratinocytes stimulates dendritic cell activation in an IFN-dependent manner, suggesting epithelial-derived IFN-κ primes SLE skin for heightened UVB-induced inflammation (263). Furthermore, in the lupus-prone NZM2328 mouse model, UVB exposure induces a type I IFN response that subsequently represses regulatory T cells (Tregs) and promotes T cell activation and expansion (265). Together, these data support a more pathogenic role for UV-induced type I IFNs in lupus skin.

Other cytokines and chemokines

UV light also triggers production of several pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1α/β (266-272), that may contribute to cutaneous inflammation provoked during sun-induced lupus flares. These cytokines can, in turn, promote production of inflammatory chemokines such as CCL5, CCL20, CCL22, and CXCL8 by epidermal keratinocytes and enhance leukocyte recruitment into the skin (253). Supporting a role for UV-induced injury in the inflammatory phenotype of cutaneous lupus, CCL5 and CXCL8 are among the most differentially regulated chemokines in CLE (253). Another relevant chemokine in CLE is CCL27, a skin-specific chemokine that is produced in response to TNF-α and IL-1β stimulation (273) by epidermal basal keratinocytes and then released into the dermis following UV light provocation (253). CCL27 then increases recruitment of memory T cells into the skin that can release large amounts of IFN-γ and further perpetuate inflammation (253, 273, 274).

TNF-like weak inducer of apoptosis (TWEAK) and its receptor fibroblast growth factor-inducible 14 (Fn14) play a role in modulation of inflammatory responses in the skin by activating nuclear factor kappa B (NFκB) in keratinocytes (73). Activation of the TWEAK/Fn14 signaling pathway is significantly increased in lesional skin of SLE patients. Additionally, mRNA expression of TWEAK, Fn14, and several genes turned on by this pathway including CCL5, monocyte chemoattractant protein-1 (MCP-1) and CXCL10 is higher in these lesions compared to healthy controls (74). Overlap of Fn14 and Ro52 is observed in the upper epidermis of lesional skin suggesting a possible role for TWEAK/Fn14 activation in Ro-52 mediated photosensitivity of CLE patients, similar to what has been observed in mouse models (74).

1.2.8 Immune Cells

Cell adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and E-selectin, are important for facilitating leukocyte migration into the skin. UV radiation influences expression of ICAM-1 on keratinocytes in a biphasic manner with early inhibition followed by late induction (275). This late induction by UV may be an important component in initiation of photosensitive diseases. In fact, ICAM-1 and E-selectin are more highly upregulated in patients with SLE compared to healthy controls, and more so in active vs. inactive SLE (276). Expression of IFN-inducible MxA correlates with expression of these adhesion molecules as well as with T cell and monocyte/macrophage infiltration, suggesting a role for UV-induced IFN in promoting immune cell recruitment into the skin (277).

Mast cells

Mast cells are recruited into UVB-exposed skin in response to keratinocyte-derived IL-15 and CCL5 (278). Moreover, the number of mast cells in CLE skin is higher compared to healthy controls and is further elevated in sun-exposed vs. sun-protected CLE skin (278). Mast cells may contribute to cutaneous inflammation via production of matrix metalloproteinases (MMPs). MMPs belong to a family of proteinases that have broad functions ranging from tissue degradation and repair to immunomodulation (279). Altered expression of MMPs is suggested to play a role in the pathogenesis of several autoimmune diseases (280), including lupus, but the specific mechanisms by which they do so is still being investigated. In support of this hypothesis, CLE lesions exhibit elevated expression of activated MMPs, including MMP-1 and MMP-9, with levels of active MMP-9 correlating with cutaneous disease severity (278, 281). MMP-9 is involved in degradation of extracellular matrix proteins and activation of cytokines and chemokines, and this can promote immune cell recruitment, autoantigen generation, and perpetuation of inflammation (280, 282-285). On the contrary, some studies suggest a protective role for MMP-9 in the development of

systemic autoimmunity. In $B6^{lpr/lpr}$ mice, which develop mild SLE-like disease, MMP-9 deficiency worsened lymphadenopathy and splenomegaly, increased autoantibody production and tissue injury, and significantly reduced survival (286). This may result from the ability of MMP-9 to destroy autoantigens and, therefore, limit formation of immune complexes and facilitate their clearance (286). Further investigation into the role of UVB-recruited mast cells and their secreted MMPs is needed to clarify their roles in autoimmunity and determine whether their effects may be organ-specific.

Plasmacytoid dendritic cells

UVB exposure triggers plasmacytoid dendritic cell (pDC) recruitment into the skin that is associated with dermal fibroblast production of the chemoattractant chemerin (287). Lupus-prone MRL/lpr mice exhibit increased skin pDC accumulation as well as increased production of chemerin in response to UVB irradiation compared to wild type mice (287). Skin lesions of lupus patients have also been shown to express elevated levels of chemerin (288). Furthermore, UV exposure promotes pDC accumulation at the dermal-epidermal junction to a greater extent in SLE skin vs. healthy control skin (289). However, recent work has identified that pDCs become functionally impaired in settings of preclinical autoimmunity and established SLE (264). These compromised pDCs undergo senescence and have a decreased ability to produce cytokines, including type I IFNs, suggesting that these cells may not actually have a role in driving cutaneous autoimmune disease (264). Rather, non-hematopoietic sources of type I IFNs, particularly keratinocytes, are more likely to be responsible for enhancing photosensitive responses in lupus skin.

Langerhans cells

UV exposure results in the activation of epidermal growth factor receptor (EGFR), a transmembrane protein involved in regulating proliferation, differentiation, and survival in various cell types, including keratinocytes. This enhances keratinocyte replication leading to epidermal hyperplasia that protects against subsequent UV-induced skin injury (290). Langerhans cells (LCs), a population of antigen-presenting cells that can reside in the epidermis, activate EGFR via the actions of LC-expressed metalloprotease a disintegrin and metalloprotease 17 (ADAM17) (291). Activation of these signaling pathways limits UV-induced keratinocyte apoptosis. LCs play a further role in limiting skin injury following UV radiation through their phagocytosis of apoptotic keratinocytes (292). Importantly, reduced numbers of LCs are found in SLE skin compared to healthy control skin (291). Additionally, reduced epidermal EGFR phosphorylation is seen in SLE skin as well as in photosensitive SLE mouse models (291). Together, these data suggest that LC dysfunction may be an important component of photosensitivity in lupus.

T cells

Human skin has a large population of resident T cells that provides surveillance and repair functions following exposure to UV light. Specifically, UVB radiation induces release of ATP from keratinocytes (293) that can activate these skin-resident T cells and increase their production of IL-17 (294). This upregulates keratinocyte expression of two DNA damage associated genes, TWEAK and Growth arrest and DNA damage associated gene 45 (GADD45) (295, 296), and, thus, limits DNA damage in the keratinocytes (294). Local type I IFN production triggered by UV light enhances production of Th1-associated chemokines CXCL9, CXCL10, and CXCL11 which supports T cell recruitment into the skin (253, 297). This influx of $CD4^+$ T cells is followed by induction of Tregs with immunosuppressive functions (298). As discussed above, in a mouse model of SLE, T cell activation and expansion following UVB exposure is increased compared to

wild type controls as a result of type I IFN-dependent Treg inhibition (265). In further support of a role for T cell-mediated inflammation in photosensitivity, UV exposure promotes accumulation of T cells at the dermal-epidermal junction and these cells persist in lesions over time (299, 300).

B cells

B cells also play a role in UV-induced immunosuppression in healthy skin. Specifically, UVB irradiation activates regulatory B cells in skin-draining lymph nodes, potentially via IL-10, that can inhibit dendritic cell-mediated activation of T cell immunity (301). Intriguingly, new roles for skin-associated B cells in both driving and suppressing cutaneous inflammation have recently been identified (302). Elevated numbers of B cells have been observed in lesional DLE skin relative to controls (303-306), however, the disease specific functions of these B cells have yet to be determined. As such, it is not currently known if differential activation of regulatory or inflammatory B cells after UV may be involved in development of photosensitive responses in autoimmunity.

Neutrophils

Neutrophils are considered first responders of the immune system and, as such, are among the first cells to be recruited into the skin following UVB irradiation (258, 307-311). In healthy skin, these responding neutrophils express high levels of IL-10 that contributes to an immunosuppressive environment (312). Intriguingly, neutrophil infiltration after UVB exposure is significantly reduced in the skin of patients with photosensitive disorders such as polymorphous light eruption and this likely limits immunosuppression (310).

An important feature of neutrophils is their ability to extrude neutrophil extracellular traps (NETs), through a process termed NETosis, that are primary composed of chromatin fibers, antimicrobial peptides, and various enzymes (313, 314). Low density granulocytes (LDGs) are an

abnormal subset of neutrophils identified in lupus patients that have an increased capacity to release NETs resulting in enhanced externalization of autoantigens and other immunostimulatory molecules relevant in autoimmunity (315). SLE patients with high levels of circulating LDGs have higher prevalence of cutaneous disease, suggesting LDGs may be pathogenic to cutaneous tissue (316). Moreover, CLE lesions have been shown to contain increased numbers of netting neutrophils, however, no cell-specific markers have been identified for LDGs so it is not yet known if these neutrophils correspond to LDGs or normal-density neutrophils (315). UV can stimulate NETosis (317), and therefore, may reflect a mechanism by which NETs are released and promote inflammation in the skin of CLE patients. Fascinatingly, skin exposure to UVB light also stimulates neutrophil migration into the kidneys where they contribute to renal inflammation, injury, and type I IFN signatures (318), suggesting an important mechanistic link between photosensitivity and systemic disease flares.

1.3 IL-1β in SLE

In addition to type I IFNs, many other cytokines play a critical role in modulation of disease flares in SLE. IL-1β and IL-18 are members of the IL-1 family of cytokines, a superfamily comprised of eleven proteins. Members of this family range from highly inflammatory cytokines to antagonistic and anti-inflammatory cytokines (319). IL-1β is the most widely studied among the IL-1 family for both its role in host defense and autoimmunity. IL-1β signals through IL-1R, which is ubiquitously expressed, therefore, tight regulation of IL-1β expression and activation is necessary to avoid excessive inflammation (320). To help regulate this, IL-1β is not constitutively expressed and requires a two-step process to reach its functional form (319). Expression of the precursor form occurs in response to microbial molecules that stimulate pattern recognition receptors (PRRs), then additional processing is required as detailed in Section 1.3.1.

1.3.1 Activation of IL-1β

Inflammasome-mediated activation

Inflammasomes are multimeric protein complexes of the innate immune system that are assembled in the cytosol following recognition of a variety of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (321). PAMPs are a diverse class of conserved molecular patterns that are derived from microbes and recognized as foreign by the immune system, while DAMPs are endogenous molecules that are released by damaged or dying host cells and act as danger signals. Inflammasome complexes are comprised of a sensor protein, inflammatory caspases, and, generally, an adapter protein (322). Several cytoplasmic pattern recognition receptors (PRRs) are able to serve as the sensor protein including those in the NOD-like receptor (NLR) family and those in the PYHIN protein family. Many of these sensors interact with an adaptor molecule called apoptosis-associated speck-like protein containing a CARD (ASC). ASC connects the sensor to monomers of pro-caspase-1. Pro-caspase-1 is activated via proximity-induced autoproteolysis, then cleaves the immature forms of IL-1β and IL-18 to their active signaling forms (323). Active caspase-1 also cleaves GSDMD to induce pyroptotic cell death (209), as described in section 1.2.3. Inappropriate regulation of inflammasome activation is associated with a variety of diseases, including neurodegenerative, metabolic, and autoimmune diseases.

Alternative mechanisms of activation

While processing by caspase-1 is the most recognized mechanism by which IL-1β is activated, it is not the only pathway through which this occurs. Pro-IL-1β can also be processed by other serine proteases including proteinase-3 in neutrophils (324). In addition, proteases such

as elastase, MMP9, mast cell chymase, and granzyme A can cleave pro-IL-1β extracellularly (319, 324, 325).

1.3.2 Secretion of IL-1β

Upon activation, IL-1β is secreted from cells in an unconventional manner that is not fully understood, despite extensive research efforts. IL-1β lacks a signal sequence preventing it from following the classical ER-Golgi secretion route (326). Several mechanisms have been proposed and it is likely that the specific pathway by which IL-1β leaves the cell is context- and cell type-dependent. These proposed mechanisms can be generally divided into four classifications: passive release from dying cells, translocation across the plasma membrane, translocation in intracellular vesicles followed by exocytosis, and release in exosomes or microvesicles (327).

1.3.3 Function of IL-1β

IL-1β is produced by a variety of cell types (especially macrophages, monocytes, and epithelial cells) and is widely distributed among both lymphoid and non-lymphoid tissues (328, 329). This cytokine is a potent endogenous pyrogen, meaning it is responsible for induction of fever (330, 331). Increased body temperature creates an unfavorable environment for invading pathogens and augments the performance of immune cells so they can better fight infection (332). IL-1β also aids in host defenses through amplifying proliferation and differentiation of T and B cells (333-335); inducing neutrophilia (336); stimulating production of acute phase proteins, prostaglandins, chemokines, and inflammatory cytokines (337, 338); regulating corticosteroids and glucose homeostasis (339, 340); and serving as an adjuvant of antigen-specific antibody responses (341-343).

1.3.4 Dysregulation of IL-1β

Autoinflammatory diseases

Dysregulation of IL-1β can lead to a range of inflammation-related diseases (344). Many autoinflammatory diseases are associated with defects in IL-1β processing and release. These diseases are often hereditary, monogenic syndromes that are associated with defects in the innate immune system that lead to unrestricted activation of IL-1β (345-348). Cryopyrin-associated periodic syndromes (CAPS) are diseases caused by mutations in *NLRP3*. *NLRP3* encodes the sensor protein of the NLRP3 inflammasome which is the dominant route for IL-1β processing and secretion. These mutations are gain-of-function, therefore, patients with CAPS have chronic inflammation resulting from overproduction of active IL-1β (349). Another example of an IL-1β -mediated autoinflammatory disease is familial Mediterranean fever (FMF) which results from a mutation in the pyrin-encoding gene *MEFV* of NLRP3 (350). Gout, systemic-onset juvenile idiopathic arthritis, and adult-onset Still's disease are additional diseases characterized by overproduction of IL-1β (351-353). These diseases have been shown to generally respond well to IL-1β neutralization with canakinumab (354-359) or IL-1R1 blockade with anakinra (351, 353, 355, 360-362).

Autoimmune diseases

IL-1 secreted by both healthy control and SLE monocytes has been shown to stimulate IgG synthesis, an important component in driving disease pathology in lupus, suggesting it could be a relevant target for treatment (363). *IL1B* and *IL-1RN* (encodes IL-1Ra) polymorphisms have been associated with SLE risk in a number of patient populations (364-366). Contradictory findings, however, have been reported in the literature regarding levels of IL-1β in SLE patients. Some studies have identified increased expression of IL-1β in the serum of SLE patients (367, 368) and in lupus-prone mouse models (369), while other studies observed no differences (370, 371). These

discrepancies may result from varied patient populations, disease heterogeneity, or timing of measurements. It is also possible that IL-1β serum levels may be comparable to controls while being elevated in the tissue. In fact, *Il1b* mRNA levels are increased in MRL/lpr mice with LN and can be detected in the renal cortices of these animals (372). Administration of recombinant IL-1β further accelerated renal disease and mortality, suggesting its involvement in nephritis (372). Further, levels of IL-1Ra are lower in the serum of patients with LN and this coincides with kidney involvement and disease flares (373).

Polymorphisms in genes encoding components of the inflammasome itself, including *NLRP1* and *NLRP3*, have been associated with SLE risk (216, 374). These polymorphisms have been linked to specific disease manifestations such as skin and renal involvement as well as overall severity of disease. Several inflammasome scaffolds are reportedly increased in SLE patients including IFI16 and AIM2 (375). NLRP3 as well as caspase-1 are overexpressed in lupus nephritis biopsies, suggesting the kidneys may be primed for enhanced inflammasome activation (376). Further, immune complexes and NETs, both known inflammasome activators, are increased in SLE patients (255, 377). Common lupus treatments, such as antimalarials, have inhibitory effects on inflammasome activation suggesting a potential mechanism of action for these therapies (254, 255, 378). Lastly, direct or indirect inhibition of NLRP3 or loss of caspase-1 in murine models of lupus have been shown to be protective from autoantibody generation and lupus nephritis as well as reduce type I IFN signatures (379-383). Together, these data suggest involvement of inflammasomes and inflammasome-derived IL-1β in SLE pathogenesis, however, continued investigation into the specific mechanisms involved is warranted.

1.4 Rationale and Specific Aims

The goal of this dissertation was to further our understanding of the innate immune mechanisms involved in driving disease in SLE. Specifically, we wanted to examine the ways in which pro-inflammatory cytokines implicated in SLE pathogenesis influence disease progression in two of the most commonly affected organs – the skin and the kidney. We focus on the role of cutaneous type I IFNs in influencing responses to sunlight in the skin and the role of inflammasome-activated IL-1β in lupus nephritis. Our results will aid in the development of novel therapies for lupus patients with cutaneous and renal involvement to prevent painful lesions, repetitive disease flares, and devastating long-term systemic organ damage.

Aim 1: Chapter 2 – Interferon Alpha Promotes Caspase-8 Dependent Ultraviolet-Light Mediated Keratinocyte Apoptosis via Interferon Regulatory Factor 1.

The objective of this aim was to elucidate the specific pathways regulated by type I IFNs to enhance UVB-driven death of keratinocytes. The rationale for this work was that by determining the role type I IFNs play in promoting keratinocyte death in lupus skin we will better understand the mechanisms regulating photosensitivity and thus prevent its ability to exacerbate disease. Our hypothesis was that type I IFNs promote enhanced activation of lytic cell death pathways in keratinocytes after exposure to UV light that drives inflammatory responses and contributes to lesion development and disease flares. We tested this hypothesis using a variety of inhibitors that target components of different cell death pathways and measuring the effect on IFN and UV-mediated cell death. Our results will have an important positive impact on lupus research by contributing to the knowledge of pathways mediating photosensitivity downstream of type I IFN hyper-production.

Aim 2: Chapter 3 – Loss of interleukin-1 beta is not protective in the lupus-prone NZM2328 mouse model.

The objective of this aim was to examine the specific contributions of the inflammasome-activated cytokine IL-1β in SLE pathogenesis. The rationale for this work was that inflammasome blockade has shown promising results for improving disease manifestations in lupus, therefore, inhibition of IL-1β could be a more targeted approach for treatment. We chose to use the NZM2328 (NZM) mouse model for this aim as these mice spontaneously develop lupus-like disease that recapitulates many components of human SLE including female predominance, autoantibody production, and glomerulonephritis. Our hypothesis was that loss of IL-1β would reduce morbidity and mortality in NZM mice. We generated NZM-*Il1b*$^{-/-}$ mice and monitored disease progression through measuring serum levels of anti-dsDNA IgG, proteinuria, renal immune complex deposition, and overall survival. Inhibitors of IL-1β and IL-1β signaling are already being used in the clinic, therefore, our results will have important implications regarding the use of these drugs to treat SLE patients.

Chapter 2 – Interferon Alpha Promotes Caspase-8 Dependent Ultraviolet-Light Mediated Keratinocyte Apoptosis via Interferon Regulatory Factor 1

Portions of this chapter have been submitted for publication:

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2.1 Abstract

Ultraviolet (UV) light is a known trigger of both cutaneous and systemic disease manifestations in lupus patients. Lupus skin has elevated expression of type I interferons (IFNs) that promote increased keratinocyte death after UV exposure. The mechanisms by which cell death is increased by type I IFNs are unknown. Here, we examine the specific cell death pathways that are activated in keratinocytes by type I IFN priming and UVB exposure. We identify enhanced activation of caspase-8 dependent apoptosis, but not pyroptosis or necroptosis, in type I IFN and UVB exposed keratinocytes. Further, UVB treatment of mice that overexpress IFN-κ in the epidermis resulted in increased apoptosis in the skin compared to levels observed in wild-type mice. This increase in keratinocyte apoptosis was not dependent on known death ligands but rather dependent on type I IFN-upregulation of interferon regulatory factor 1 (IRF1). Together, these data suggest that enhanced sensitivity to UV light exhibited by lupus patients results from type I IFN priming of keratinocytes that drives IRF1 expression, resulting in caspase-8 activation and increased apoptosis after minimal exposures to UVB.

2.2 Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with heterogeneous clinical manifestations that can affect nearly all major organ systems, including the skin. Skin involvement can occur on its own, as cutaneous lupus erythematosus (CLE), or in combination with systemic disease. A characteristic feature of SLE and CLE is photosensitivity, affecting up to 93% of patients (132). For photosensitive patients, exposure to minimal amounts of sunlight can trigger cutaneous or systemic disease flares; however, mechanisms driving these responses are not well characterized.

Exposure to ultraviolet (UV) light emitted by the sun distinctly influences skin physiology dependent on wavelength as well as duration and intensity of exposure. In healthy skin, exposure to UV radiation generates an immunosuppressive environment that limits severe tissue injury (384). However, in SLE skin, exposure to UVB radiation results in overactivation of the immune system that induces autoimmune responses and lesion development(385, 386). While the mechanisms underlying these differences are likely multifactorial, several studies suggest an important role for type I interferons (IFNs) in driving these differential responses.

Type I IFNs are implicated in SLE and CLE pathogenesis and progression as well as keratinocyte cell death. Recent trials blocking type I IFN signaling using anifrolumab show efficacy for improving severity of skin disease (40). Expression of type I IFN response genes is increased in lesional and non-lesional keratinocytes from lupus patients compared to those from healthy controls (261, 262). Moreover, cutaneous type I IFN production from both keratinocytes and infiltrating immune cells is increased after UVB exposure (257, 258, 287). Importantly, keratinocyte secretion of type I IFNs is more significantly increased in lupus keratinocytes compared to controls after UVB treatment (257, 264). While short bursts of type I IFNs are

protective against UVB-induced inflammation in healthy skin (258), they seem to be more pathogenic in SLE skin where they are chronically present and promote pro-inflammatory cytokine secretion, immune cell recruitment, and T cell activation (253, 257, 265). Further, we have previously demonstrated that keratinocytes from SLE patients exhibit increased UVB-induced cell death compared to healthy controls that is dependent on type I IFN signaling (263). The manner in which type I IFNs regulate this enhanced cell death phenotype is unknown.

In this paper, we investigate the role of type I IFNs in activation and regulation of cell death pathways in UVB-irradiated keratinocytes. We show that type I IFNs promote enhanced UVB-driven apoptosis of keratinocytes rather than inflammatory forms of cell death including necroptosis or pyroptosis. Further, we demonstrate that this is a caspase-8-dependent process that occurs in the absence of death-ligand signaling. Lastly, we show evidence that these increased rates of apoptosis require IFN-induced upregulation of interferon regulatory factor 1 (IRF1) in keratinocytes, a known modulator of caspase-8 (387). Thus, our work places IFN-enhanced apoptosis, and not inflammatory forms of cell death, as a critical step in driving photosensitivity in SLE patients.

2.3 Materials and Methods

2.3.1 Cell Culture

N/TERTs (388), an immortalized human keratinocyte line, were used for in vitro assays. Cells were grown in serum-free keratinocyte growth medium (supplemented with 30 µg/mL bovine pituitary extract (BPE), 0.2 ng/ mL epidermal growth factor (EGF) and 0.3 mM calcium chloride) as previously described (Gibco, Grand Island, NY) (263). N/TERTs were maintained at 37°C with 5% CO_2.

2.3.2 Mice

10–14-week-old female wild-type C57BL/6 mice were purchased from Charles River Laboratories for use in this study. Mice overexpressing *Ifnk* under the keratin 14 promoter (to promote epidermal overexpression) on the C57BL/6 background were generated as previously described (389). All mice were housed in specific pathogen-free facilities in the Unit for Animal Laboratory Medicine facility at the University of Michigan. All animal procedures were performed in accordance with our University of Michigan Institutional Animal Care and Use Committee on Use and Care of Animals -approved protocols.

2.3.3 UVB Irradiation

Dorsal fur was removed from mice via shaving and depilation with Veet (Reckitt Benckiser, UK). Mice were placed in restrainers with facial protection and treated once with ±250 mJ/cm^2 UVB light using the UV-2 ultraviolet irradiation system (Tyler Research). UVB light was provided by cascade-phosphor ultraviolet generators that emit 310nm UVB radiation.

2.3.4 Flow Cytometry

N/TERTs were treated ±1000 U/ml IFN-α for 16 hours, then treated ±50 mJ/cm^2 UVB light. Four hours later, cells were trypsinized and pelleted together with supernatant to ensure no loss of detached dying cells.

For Annexin V and propidium iodide staining: Cells were washed twice in flow block (1% bovine serum albumin and 1% horse serum in PBS). 100,000 cells were stained with FITC-Annexin V and propidium iodide (Biolegend, San Diego, CA) for 15 minutes. Flow cytometry data was collected via a BD LSR II flow cytometer and analyzed using FlowJo V10.

For cleaved caspase-3 staining: Cells were washed twice in PBS. Approximately 500,000

cells were stained with Fixable Viability Dye eFluor™ 780 (Invitrogen, Waltham, MA) for 30 minutes then fixed with 4% paraformaldehyde for 20 minutes. Following fixation, cells were washed in IFA-Tx buffer (4% FBS, 0.1% Triton X-100, 100mM HEPES pH 7.4, in 0.9% sodium chloride), then stained with anti-cleaved caspase-3 (1:500; #9661 or 9664, Cell Signaling Technology, Danver, MA) for 1 hour at 4°C and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:200; Invitrogen, Waltham, MA) for 1 hour at 4°C. Flow cytometry data was collected via a BD LSR II flow cytometer and analyzed using FlowJo V10.

For neutralizing antibody experiments, N/TERTs were treated with isotype control (Mouse IgG1 Isotype R&D MAB002; Mouse IgG2B Isotype R&D MAB0041) or neutralizing antibodies targeting TRAIL (100ng/ml; Human TRAIL/TNFSF10 Antibody; R&D MAB375), TNF-α (5ug/ml; Human TNF-alpha Antibody; R&D MAB210) or FasL (1ug/ml; Human Fas Ligand/TNFSF6 Antibody; R&D MAB126) immediately following UVB exposure.

2.3.5 RNA isolation and quantitative reverse-transcription PCR

Total RNA was isolated from cultured cells using Direct-Zol RNA MiniPrep kit (Zymo Research, Irvine, CA) or RNeasy Plus Mini Kit (Qiagen, Germantown, MD) following manufacturers' instructions. 200ng of RNA was converted to cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California). qRT-PCR was performed in technical triplicates using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Waltham, Massachusetts,) on either QuantStudio or ABI Prism 7900HT Real-Time qPCR Systems (Applied Biosystems) with the assistance of the University of Michigan Advanced Genomics Core. The primers used were as follows (all listed 5' → 3'): *CASP8* (forward, AGAAGAGGGTCATCCTGGGAGA; reverse, TCAGGACTTCCTTCAAGGCTGC), *IRF1* (forward, CTGTGCGAGTGTACCGGATG; reverse, ATCCCCACATGACTTCCTCTT), *TNFSF10*

(forward, TGCGTGCTGATCGTGATCTTC; reverse, GCTCGTTGGTAAAGTACACGTA), *XAF1* (forward, GAGCGCCCTGTTGAGTGTAA; reverse, CACAGTAGGACTCGTGGAGC), *GAPDH* (forward, CTGGGCTACACTGAGCACC; reverse, AAGTGGTCGTTGAGGGCAATG), *BACTIN* (forward, CCTCGCCTTTGCCGATCC; reverse, GCGCGGCGATATCATCATCC). Gene expression was normalized to β-actin or GAPDH and relative fold change compared to control was calculated using the comparative $2^{-\Delta\Delta CT}$ method.

2.3.6 Western Blot

Total protein was isolated from cultured keratinocytes and cellular supernatants using RIPA buffer supplemented with protease and phosphatase inhibitors and protein concentration was measured by Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) per manufacturer's directions. 10ug of protein was run on 4 to 12% precast polyacrylamide gels (Invitrogen, Waltham, MA) and transferred to 0.45µm polyvinylidene difluoride (PVDF) membranes (Thermo Scientific, Waltham, MA). Membranes were blocked with 5% nonfat dry milk and incubated overnight at 4°C with primary antibodies (1:000 dilution; XAF1 (E1E4O) Rabbit mAb #13805; TRAIL (C92B9) Rabbit mAb #3219; IRF-1 (D5E4) XP® Rabbit mAb #8478; Cell Signaling Technology, Danvers, MA) followed by HRP-conjugated goat anti-rabbit IgG (1:10,000 dilution, sc-2301; Santa Cruz Biotechnology, Dallas, TX). Protein bands were detected by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA) and imaged by Omega Lum C (Gel Company, San Francisco, CA). Quantification was completed with ImageJ software relative to β-actin loading control (1:1000 dilution; #4967; Cell Signaling Technology, Danvers, MA).

2.3.7 ELISA

The supernatants from cultured N/TERTs were assaying for TNF-α using the TNF alpha Human ELISA Kit (Invitrogen, Waltham, MA) following the manufacturer's instructions.

2.3.8 Generation of stable knockdown lines using viral shRNAs

Knockdown of XAF1 in the N/TERT keratinocyte cell line was performed by transduction with a lentivirus expressing two different shRNAs targeting human XAF1 or an shRNA control (shCTL). Two different lentivirus-based plasmids of Mission shRNA (clone numbers TRCN0000426403 and TRCN0000134449) against human XAF1 and the shCTL vector TRC2 pLKO.5-puro non-mammalian shRNA (sHC202) were obtained from Sigma-Aldrich (Burlington, MA). 293T cells were co-transfected with the shRNA of interest and packaging plasmids psPAX2 and pMD2 by the Lipofectamine 2000 (Invitrogen, Waltham, MA) method in OptiMEM (Gibco). Six hours following transfection, media was replaced with fresh keratinocyte growth medium. Twenty-four hours post-transfection, media containing lentivirus was passed through 0.45µm filters, supplemented with 8 µg/ml polybrene (Sigma), and used to transduce sub-confluent N/TERTs. Fresh keratinocyte growth medium was added to the 293T cells and this transduction process was repeated 8 and 24 hours later. The shRNA- and shCTL-transduced N/TERTs were selected for using 10-12ug/ml puromycin and cells were maintained in 8ug/ml puromycin until day of experiment.

2.3.9 siRNA KD and CellEvent Caspase-3/7 Assay

N/TERTs were plated in 96 well plates and incubated overnight. 100uM human IRF1 and CASP8 Accell siRNA SMARTPool and Accell Non-targeting Control Pool stocks were prepared in 1x siRNA buffer (Horizon Discovery, Cambridge, UK) per manufacturer's instructions. Accell siRNA stocks were diluted 1:100 in Accell siRNA Delivery Media (Dharmacon B-005000-500)

and added to each well. 32 hours later, cells were treated ±1000 U/ml IFN-α (without removing the siRNA) for 16 hours. Cells were treated ± 50mJ/cm^2 UVB in PBS. Supernatants containing siRNA in delivery media were mixed with Cell Event Caspase-3/7 Green Detection Reagent (final concentration = 5uM; Invitrogen, Waltham, MA) and added back to wells. 1uM staurosporine (Sigma-Aldrich, Burlington, MA) treatment was used as a positive control. Cells were incubated for 6 hours and counterstained with Hoechst (final concentration 0.5%). Images were acquired using a Zeiss microscope (Zeiss, Oberkochen, Germany) at indicated magnifications. Three images were taken per well. The fold change of active caspase-3/7 cells was quantified by manual counting (# cells positive for active caspase-3/7 (green) / # cells positive for Hoechst (blue) x100) relative to UV treated and the average for the three images was plotted.

2.3.10 Immunohistochemistry

For detection of cleaved caspase-3 in mouse skin, formalin-fixed, paraffin-embedded sections were heated at 60°C for 1 hour, deparaffinized, rehydrated, and heated at 100°C for 20 minutes in Tris-EDTA buffer (pH 9.0) for antigen retrieval. Slides were washed, treated with 3% hydrogen peroxide in PBS for 5 minutes, blocked in goat serum for 1 hour, and incubated with Human/Mouse Cleaved Caspase-3 (Asp175) antibody (1:100, MAB835 R&D Systems, Minneapolis, MN) overnight at 4°C. Isotype controls (#3900; Cell Signaling Technology, Danvers, MA) were stained in parallel with each set of slides. All slides were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories, Newark, California), followed by incubation with VECTASTAIN Elite ABC reagent (Vector Laboratories, Newark, California) and detection with 3,3'-diaminobenzidine (BD, Franklin Lakes, NJ) under a light microscope. Slides were counterstained with hematoxylin, dehydrated, and mounted. Images were acquired using a Zeiss microscope (Zeiss, Oberkochen, Germany) at indicated

magnifications. Epidermal caspase-3^+ cells were quantified by manually counting the # of positive cells (brown) / total # of nuclei in the epidermis averaged for three 20x fields of view.

2.3.11 TUNEL staining

For TUNEL staining in mouse skin, formalin-fixed, paraffin-embedded sections were utilized, and staining was performed according to the manufacturer's protocol (Sigma-Aldrich, Burlington, MA). Briefly, slides were dewaxed and rehydrated and incubated with Proteinase K solution. Slides were then treated with TUNEL reaction mixture in a humidified chamber, washed in PBS, and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Newark, California). Images were acquired using a Zeiss microscope (Zeiss, Oberkochen, Germany) at indicated magnifications. Epidermal $TUNEL^+$ cells were quantified by manually counting the # cells positive for TUNEL (red) / # of cells positive for DAPI (blue) in the epidermis.

2.3.12 Statistical analysis

All data was graphed and statistics were performed using GraphPad Prism 9. Data are presented as the mean ± SEM. For comparisons between two groups, paired or unpaired two-tailed t tests were used for normally distributed data and Mann-Whitney or Wilcoxon matched-pairs signed rank tests were used for non-normally distributed data. P-values <0.05 were considered as statistically significant.

2.4 Results

2.4.1 Type I IFNs promote keratinocyte apoptosis after UVB exposure

Type I IFNs are implicated in increasing keratinocyte cell death after exposure to UVB light (263). We first sought to confirm this by treating N/TERTs, an immortalized human

keratinocyte line, with IFN-alpha (IFN-α) prior to exposure to UVB light. To examine cell death, we used flow cytometry and defined cells likely to be apoptotic as the Annexin V^+ Propidium $Iodide^-$ (AV^+PI^-) population and cells likely undergoing other forms of cell death in which membrane permeability is disrupted as the AV^+PI^+ population (Fig. 2-1A). Four hours after UVB exposure, the percentage of AV^+PI^- and not AV^+PI^+ cells increased significantly with UVB treatment alone and this was further increased by IFN-α priming (Fig. 2-1B). These results confirmed that type I IFNs promote enhanced keratinocyte cell death after UVB exposure and suggests that this may occur through activation of apoptotic pathways.

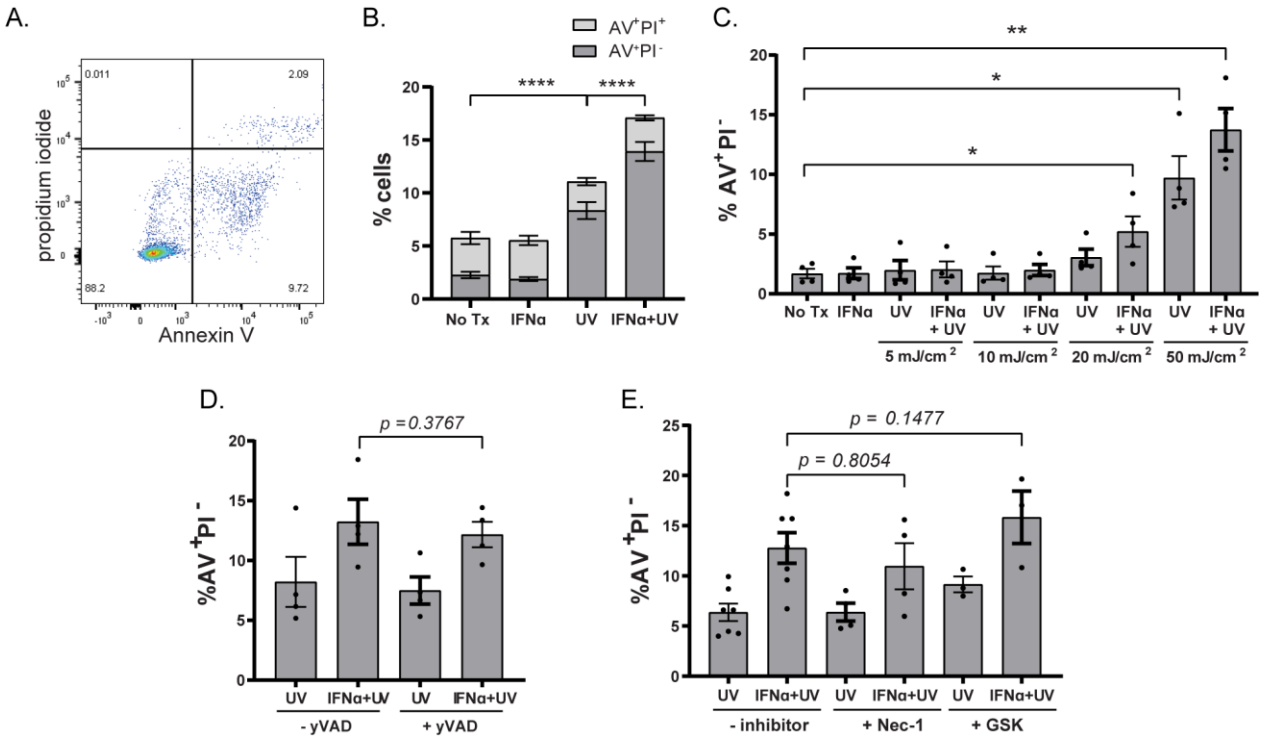

Figure 2-1 – Type I IFNs promote keratinocyte apoptosis after UVB exposure.
(A) Representative flow cytometry gating strategy. Debris was gated out using FSC and SSC and single cells were assessed for Annexin V (AV) and propidium iodide (PI). **(B-C)** N/TERTs were treated with 1000 U/ml IFN-α for 16 hours prior to exposure to **(B)** 50 mJ/cm² UVB (n=17) or **(C)** 0-50 mJ/cm² (n=4). Four hours later, cells were stained with AV/PI and assessed by flow cytometry. **(D-E)** N/TERTs were pretreated with **(D)** caspase-1 inhibitor Ac-YVAD-cmk (yVAD, 10uM), **(E)** RIPK1 inhibitor Necrostatin-1 (Nec-1, 10uM) or RIPK3 inhibitor GSK'872 (GSK, 10uM) prior to IFN-α and UVB stimulation and AV/PI staining as before (n=3-8). Data analyzed by paired t tests for normally distributed data and Wilcoxon tests for non-normally distributed data. *<0.05, ***<0.001, ****<0.0001.

An important component of photosensitivity in lupus is that patients not only experience more severe reactions to sunlight, but they are also more sensitive to lesser amounts of UV light (390). The minimal erythema dose (MED), or the lowest dose of UV light required to induce reddening of the skin, of SLE patients is lower compared to that of healthy controls (390), but whether this phenomenon is driven by cutaneous type I IFNs is not known. Based on this, we wanted to determine if type I IFN priming of N/TERTs could lower the dose of UV light needed to increase cell death. As expected, priming with IFN-α lowered the dose of UVB required to significantly increase the percentage of AV^+PI^- cells (Fig. 2-1C). This suggests that the ability of IFN-α to increase UVB-driven cell death in vitro mimics what is observed clinically in SLE patients with lower MEDs.

Several cell death pathways can be activated by UVB irradiation including apoptosis, pyroptosis, and necroptosis (210). While externalization of phosphatidylserine and subsequent detection by Annexin V has long been considered specific for apoptosis, recent studies have reported this occurring in non-apoptotic forms of inflammatory cell death (232). Because pyroptosis and necroptosis are inflammatory forms of cell death we hypothesized that increased activation of these pathways by IFN priming could be a factor in driving UVB-induced inflammation in lupus skin. To test this, we used small-molecule inhibitors of caspase-1 (Ac-YVAD-cmk) to block pyroptosis and of receptor-interacting serine/threonine-protein kinase-1 (RIPK1; Necrostatin-1) and RIPK3 (GSK'872) to block necroptosis. Cells were pretreated with the individual inhibitors then stimulated with IFN-α and UVB. Inhibition of pyroptosis or necroptosis had no effect on the percentage of AV^+PI^- cells (Fig.2-1D-E), suggesting activation of these pathways did not contribute to the observed cell death phenotype. Together, these results suggest that type I IFN priming sensitizes keratinocytes to increased UVB-induced apoptosis.

2.4.2 Type I IFN-priming enhances caspase-8 driven extrinsic apoptosis in UVB-irradiated keratinocytes

Apoptosis is generally mediated by activation of proteolytic enzymes called caspases; however, caspase-independent apoptotic pathways also exist (391). To determine the requirement for caspases in type I IFN and UVB-regulated apoptosis, we pretreated cells with Z-VAD-FMK, a pan-caspase-inhibitor, prior to IFN-α and UVB stimulation. IFN-α-induced upregulation of UVB-mediated apoptosis was entirely abrogated by pan-caspase inhibition (Fig. 2-2A). Apoptosis most often occurs through either the caspase-9 driven intrinsic pathway or the caspase-8 driven extrinsic pathway (185). UVB radiation is known to induce both intrinsic and extrinsic apoptosis (192, 392), therefore, we next examined dependence on these pathways in type I IFN primed keratinocytes following UVB exposure. To do so, we pretreated cells with either the caspase-8 inhibitor Z-IETD-FMK, or the caspase-9 inhibitor Z-LEHD-FMK, prior to IFN-α and UVB stimulation. Intriguingly, only caspase-8 inhibition significantly reduced the percentage of AV^+PI^- cells following combination treatment with IFN-α and UVB (Fig. 2-2B), while caspase-9 inhibition had no effect on this population (Fig. 2-2C).

As a secondary means for confirming the effect of caspase-8 inhibition on IFN-α-enhanced, UVB-induced apoptosis we examined caspase-3 cleavage, which occurs downstream of caspase-8 activation. To do this, we stained cells with antibody specific for cleaved caspase-3 (CC3) and a fixable viability dye (FVD) to differentiate between early ($CC3^+FVD^-$) and late apoptosis ($CC3^+FVD^+$) (Fig. 2-2D). We found that, as expected, UVB exposure alone significantly increased the percentage of $CC3^+FVD^-$ cells, and IFN-α priming prior to UVB further increased this population (Fig. 2-2E). In agreement with the Annexin V staining, inhibition of caspase-8 almost completely abrogated UVB-mediated and IFN-enhanced apoptosis as measured by $CC3^+FVD^-$

(Fig. 2-2F). Inhibition of caspase-9 did significantly decrease the percentage of $CC3^{+}FVD^{-}$ cells after UVB treatment, but the effect on IFN-mediated enhancement was modest (Fig. 2-2G). We further showed that IFN-α significantly increased expression of caspase-8 in N/TERTs (Fig. 2-2H).

To complement our pharmacological studies, we also used a genetic approach to confirm caspase-8 dependence. Accell siRNA was used to knockdown expression of caspase-8 in N/TERTs (siCASP8) relative to a non-targeting control line (Fig. 2-2I). siCASP8 and non-targeting control cells were treated with IFN-α and UVB and caspase-3 cleavage was assessed. Here, we used fluorescent microscopy to detect cells with activated caspase-3 as insufficient cells are obtained with Accell siRNA knockdown for flow cytometry. While use of non-targeting control siRNA resulted in a slight blunting of the effects of IFN priming compared to levels observed without siRNA, IFN priming still resulted in enhanced caspase-3 activation compared to UV treatment alone in the presence of non-targeting control siRNA (Fig. 2-2J). Importantly, in agreement with the inhibitor data, loss of caspase-8 significantly reduced the percentage of $CC3^{+}$ cells following UVB exposure and eliminated the IFN-enhancement of apoptosis (Fig. 2-2K,L).

Together, these data suggest that type I IFN priming drives an enhanced apoptotic phenotype in keratinocytes following exposure to UVB that depends on activation of caspase-8.

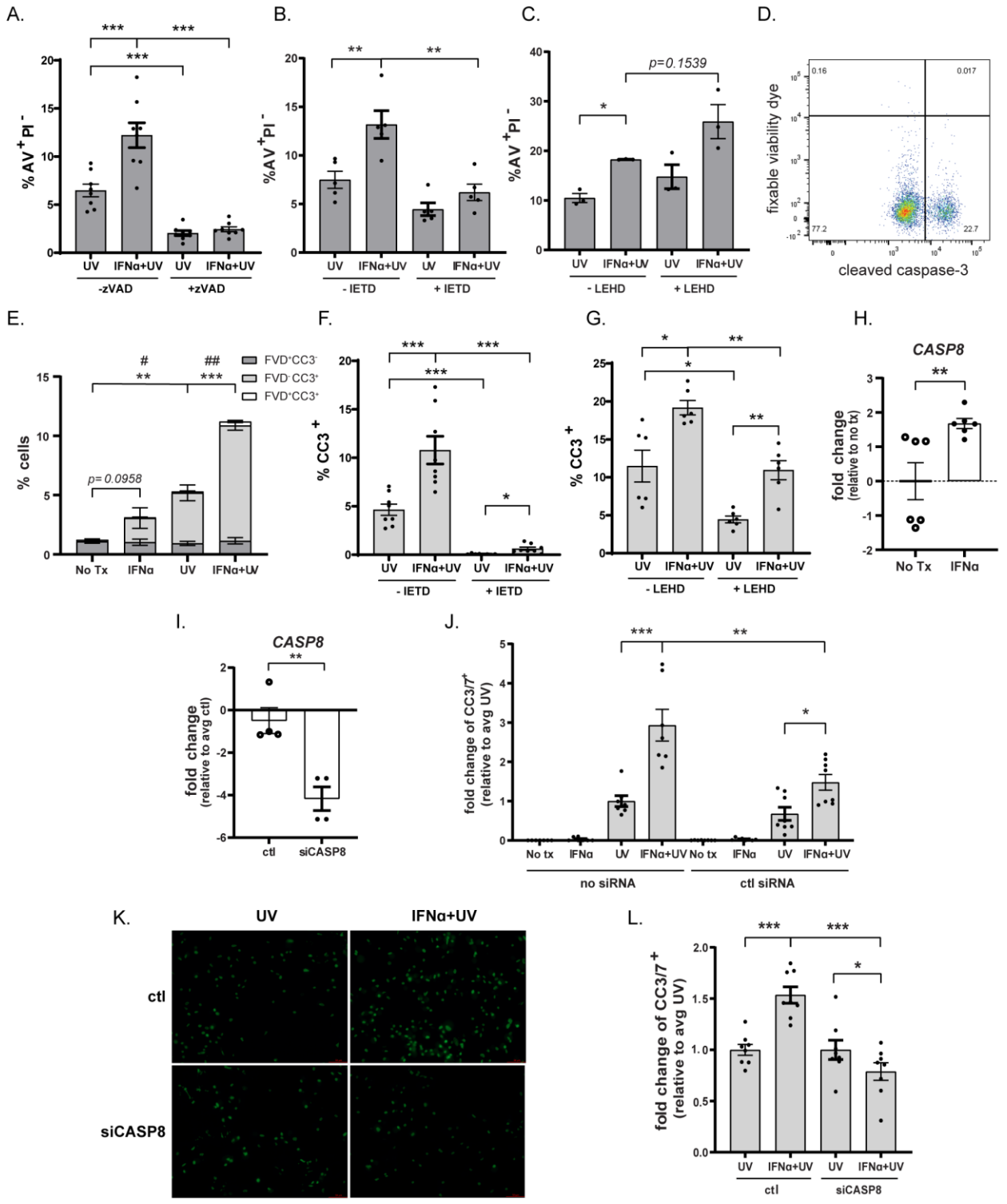

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Figure 2-2 – Type I IFNs promote caspase-8 mediated keratinocyte apoptosis after UVB exposure.
N/TERTs were pretreated with **(A)** pan-caspase inhibitor Z-VAD-FMK (zVAD, 10uM), **(B,E)** caspase-8 inhibitor Z-IETD-FMK (IETD, 10uM), or **(C,F)** caspase-9 inhibitor Z-LEHD-FMK (LEHD, 10uM) prior to treatment with 1000 U/ml IFN-α for 16 hours then exposed to 50 mJ/cm² UVB. Four hours later, cells were stained with **(a-c)** Annexin V (AV) and propidium iodide (PI) or **(E-G)** cleaved caspase-3 antibody (CC3) and fixable viability dye (FVD) and assessed by flow cytometry (n=3-8). Representative gating strategy shown in **(D)**. Debris was gated out using FSC and SSC and single cells were assessed for CC3 and FVD. **(H)** N/TERTs were treated with 1000 U/ml IFNα for 16 hours and *CASP8* gene expression was measured by qRT-PCR (n=6). **(I)** N/TERTs were incubated with 1uM Accell non-targeting siRNA (ctl) or Accell CASP8 siRNA (siCASP8) for 48 hours and mRNA expression of *CASP8* was measured by qRT-PCR (n=4). **(J)** N/TERTs were treated with or without 1uM Accell non-targeting (ctl) siRNA for 48 hours, treated with 1000 U/ml IFN-α for 16 hours, then exposed to 50mJ/cm² UVB. Cells were stained with CellEvent Caspase-3/7 Green Detection Reagent and imaged 6 hours later (n=7-8). **(K-L)** N/TERTs were incubated with 1uM ctl siRNA or siCASP8 for 48 hours, treated with 1000 U/ml IFN-α for 16 hours, then exposed to 50mJ/cm² UVB. Cells were stained with CellEvent Caspase-3/7 Green Detection Reagent and imaged 6 hours later (n=8). Data analyzed by paired or unpaired t tests for normally distributed data or Mann-Whitney for non-normally distributed data. (d) p-values designated by asterisks (*) correspond to $CC3^+FVD^-$ populations and p-values designated by number signs ($^{\#}$) correspond to $CC3^+FVD^+$ populations. * or $^{\#}$<0.05, ** or $^{\#\#}$<0.01, ***<0.001, ****<0.0001.

2.4.3 Epidermal overexpression of IFN-κ increases UVB-induced apoptosis in vivo

To investigate how modulation of type I IFNs affects UVB-induced apoptosis *in vivo*, we used mice that overexpress IFN-kappa (IFN-κ) 2-4 fold above normal levels in the epidermis under the control of the keratin 14 promoter ($IFN\kappa^{EPI}$ mice) (389, 393). C57BL/6 (wild-type) and $IFN\kappa^{EPI}$ mice were treated with or without UVB on their dorsum. Levels of apoptosis in the epidermis were examined 24 hours following stimulation with 250 mJ/cm^2 UVB via terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays and immunostaining for cleaved caspase-3. Consistent with our cell culture data, we detected no difference in baseline levels of apoptosis between wild-type and $IFN\kappa^{EPI}$ mice (Fig. 2-3A-D). However, following acute UVB exposure, $IFN\kappa^{EPI}$ mice had significantly elevated levels of TUNEL staining (Fig. 2-3A,B) and cleaved caspase-3 staining (Fig. 2-3C,D). These data support a role for type I IFNs in increasing UVB-induced apoptosis *in vivo*.

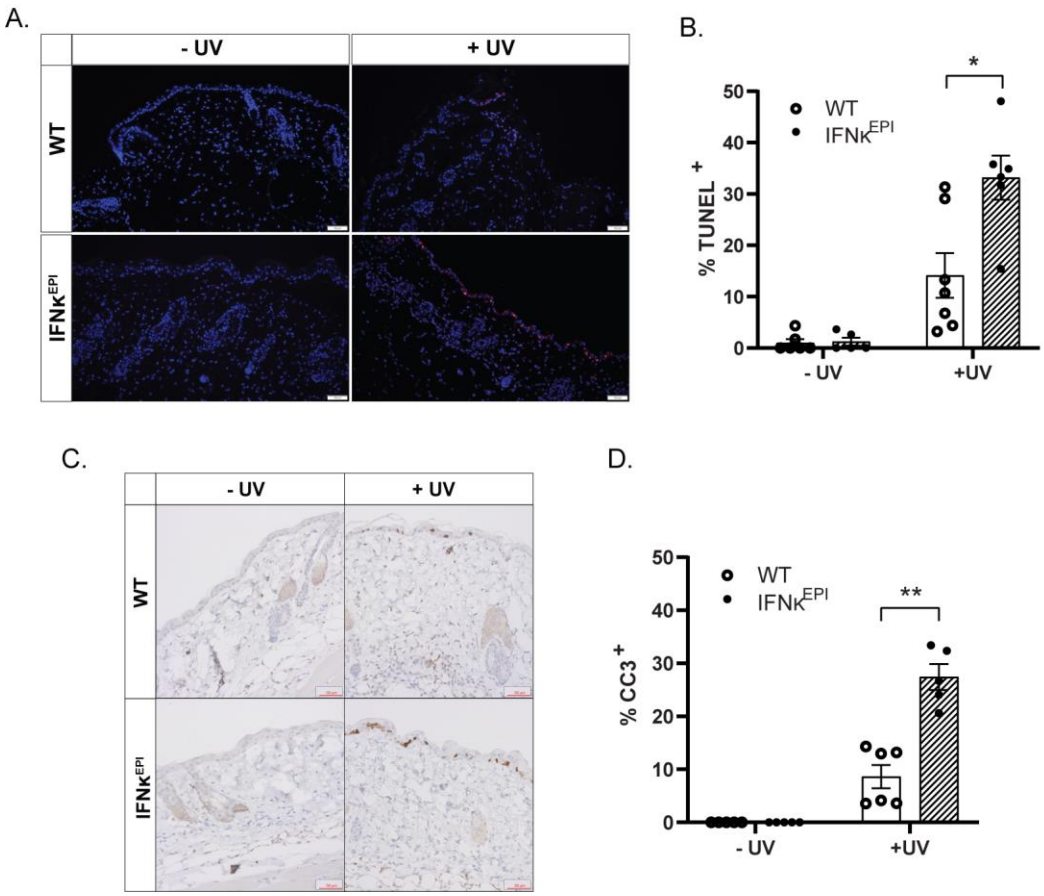


Figure 2-3 – Epidermal overexpression of IFN-κ increases UVB-induced apoptosis in vivo.
Mice that overexpress IFN-κ in the epidermis under the control of the keratin-14 promoter ($IFN\kappa^{EPI}$) and C57BL/6 wild-type (WT) mice were treated with or without 250 mJ/cm² UVB on their dorsum (n=5-7). Levels of apoptosis were examined 24 hours later via **(A-B)** terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays or **(C-D)** cleaved caspase-3 (CC3) immunostaining. Data analyzed by Mann-Whitney test. *<0.05, **<0.01.

2.4.4 Type I IFN-enhanced apoptosis after UVB exposure is XAF1-independent

To better understand the mechanism by which type I IFNs influence keratinocyte responses to UVB exposure, we next explored whether apoptosis-related genes were differentially expressed in IFN-α treated primary keratinocytes. To this end, keratinocytes from non-lesional SLE skin and healthy control (HC) skin were treated with IFN-α and subjected to RNA-sequencing (RNA-seq) analysis as previously reported (394). The pro-apoptotic gene X-linked inhibitor of apoptosis (XIAP)-associated factor 1 (*XAF1*) was highly upregulated by IFN-α treatment in HC keratinocytes, and even more so in SLE keratinocytes (Fig. 2-4A). XAF1 can enhance apoptosis

through a variety of mechanisms including through sensitization to extrinsic apoptosis (395). Thus, we hypothesized that XAF1 upregulation by type I IFNs renders keratinocytes more sensitive to UVB-induced apoptosis.

A.

Gene	Healthy Control + IFNα		SLE + IFNα	
	log2 FC	P-value	log2 FC	P-value
IRF1	1.66	5.26E-06	2.09	3.95E-07
CASP8	1.08	1.37E-13	0.83	1.20E-26
XAF1	4.03	5.12E-02	4.79	2.01E-06
TNFSF10	4.08	2.93E-11	4.37	7.79E-27
TNF	0.37	7.54E-02	0.37	4.61E-02
TNFRSF1B	1.36	6.76E-03	1.80	2.04E-06
TNFRSF12A	-0.10	3.75E-01	0.18	1.03E-03
TNFSF12	0.04	8.71E-01	0.21	2.99E-01
FAS	0.18	1.67E-01	0.27	4.49E-04
FASL	ND	---	ND	---

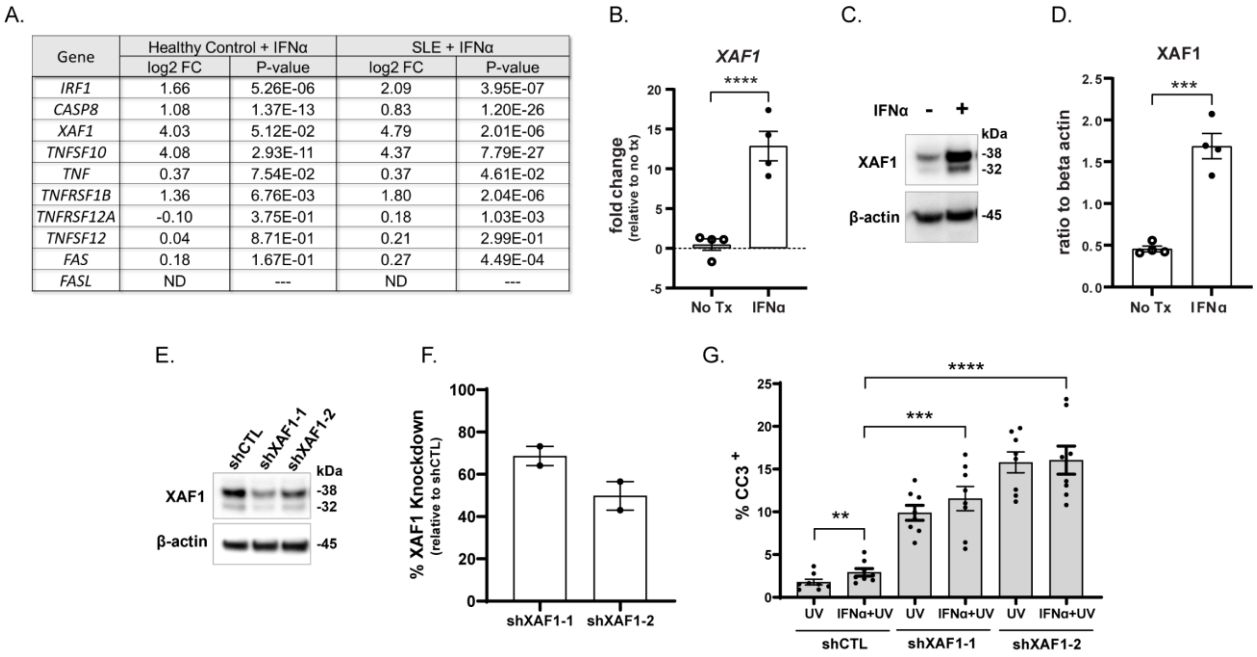


Figure 2-4 – Type I IFN-enhanced apoptosis after UVB exposure is XAF1-indpendent.
(A) Keratinocytes were isolated from non-lesional SLE skin and healthy control (HC) skin, treated with 1000 U/ml IFNα for 6 hours, and gene expression was analyzed by RNA-sequencing as previously reported[394]. Gene expression values are represented as log2 fold change (FC) and p-values <0.05 are considered significant. ND = not detected. **(B-D)** N/TERTs were treated with 1000 U/ml IFNα for 16 hours and XAF1 **(B)** mRNA expression was measured by qRT-PCR (n=4) and **(C-D)** protein expression was measured by Western blot (n=4). **(E-G)** Stable XAF1-knockdown N/TERT lines were generated by transducing N/TERTs with lentivirus expressing either of two shRNAs targeting XAF1 (shXAF1-1, -2) or a control shRNA (shCTL). **(E-F)** Protein knockdown was confirmed by Western blot (n=2). **(G)** shCTL, shXAF1-1, and shXAF1-2 N/TERTs were treated with 1000 U/ml IFNα for 16 hours then exposed to 50 mJ/cm² UVB. Four hours post UVB, apoptosis was measured by cleaved caspase-3 (CC3) and fixable viability dye (FVD) staining (n=8). Data analyzed by paired or unpaired t tests. **<0.01, ***<0.001, ****<0.0001.

To test this, we first confirmed that IFN-α treatment of N/TERTs results in increased expression of XAF1 mRNA (Fig. 2-4B) and protein (Fig. 2-4C,D). We next generated two stable XAF1-knockdown N/TERT lines by transducing N/TERTs with lentivirus expressing shRNA targeting *XAF1* (shXAF1-1 and -2) or a control shRNA (shCTL). Knockdown efficacy of XAF1 was confirmed by Western blot (Fig. 2-4E,F). shXAF1 N/TERTs were stimulated with IFN-α and UVB as before and caspase-3 cleavage was assessed. Surprisingly, loss of XAF1 resulted in a

robust increase in UVB-induced keratinocyte apoptosis compared to control (Fig. 2-4G). Therefore, IFN-upregulated XAF1 is not responsible for increasing keratinocyte apoptosis after UVB exposure and in fact, XAF1 may serve as a negative regulator of cell death after UVB exposure.

2.4.5 Type I IFN-enhanced apoptosis after UVB exposure is death ligand-independent

Previous studies have suggested that cutaneous disease in lupus models is, in part, driven by members of the tumor necrosis factor (TNF) and TNF-receptor (TNFR) superfamilies (396, 397). We, therefore, examined our RNA-seq data to determine if differential regulation of these pro-apoptotic genes by IFN-α could be promoting UVB-driven apoptosis in our system. TNF receptor superfamily member 12A (*TNFRSF12A*; encodes Fn14) was slightly upregulated by IFN-α treatment in the SLE keratinocytes only, however, there was no change in TNF superfamily member 12 (*TNFSF12*; encodes TWEAK) expression in HC or SLE keratinocytes (Fig. 2-4A). Further, while *FAS* was moderately increased by IFN-α treatment in the SLE keratinocytes only, *FASLG* (encodes FasL) expression was not detected in these samples (Fig. 2-4A). Thus, these pathways did not exhibit differences that would explain IFN-enhanced apoptotic changes. However, our RNA-seq analysis did identify differential expression of other TNF/TNFR superfamily members that can induce caspase-8-dependent apoptosis, as well as increased expression of *CASP8* (encodes caspase-8) itself. Specifically, *TNFSF10* (encodes TNF-related apoptosis-inducing ligand (TRAIL)) and *TNFRSF1B* (encodes TNFR2) were upregulated in IFN-α treated keratinocytes and more so in SLE compared to HCs (Fig. 2-4A). Thus, we hypothesized that IFN-induced alterations in TRAIL and/or TNF signaling mediate increased extrinsic apoptosis of keratinocytes after UVB exposure.

In agreement with the RNA-seq data, N/TERTs stimulated with IFN-α had increased expression of TRAIL mRNA (Fig. 2-5A) and protein (Fig. 2-5B,C). As our analysis only identified differences in receptor expression and not TNF-α itself, we confirmed that N/TERTs secrete TNF-α following UVB treatment (Fig. 2-5D). There was a small, but significant increase in TNF-α secretion with IFN priming prior to UVB exposure, suggesting that IFN-induced changes in TNF-receptor expression paired with increased cytokine secretion after UVB stimulation may drive increased signaling. To determine whether increased TRAIL or TNF signaling was responsible for the enhanced apoptotic phenotype we observed, we used neutralizing antibodies against TRAIL and TNF-α. We first confirmed that treatment with these blocking antibodies prevented apoptosis in cells treated with recombinant TRAIL or TNF-α (Fig. 2-5E,G). N/TERTs were treated with the neutralizing TRAIL or TNF antibodies or isotype control following stimulation with IFN-α and UVB. Surprisingly, inhibition of neither TRAIL nor TNF-α influenced IFN-enhanced apoptosis after UV exposure (Fig. 2-5F,H). To account for any compensatory effect that may occur when inhibiting an individual ligand, we treated N/TERTs with both anti-TRAIL and anti-TNF-α. Again, we saw no change in levels of apoptosis suggesting no involvement of these ligands in driving IFN-enhancement of apoptosis (Fig. 2-5I). We also confirmed that inhibition of FasL had no effect on IFN-α and UVB induced apoptosis (Fig. 2-5J). Together, these data suggest that type I IFN-enhanced apoptosis after UVB exposure occurs independently of several known death ligands.

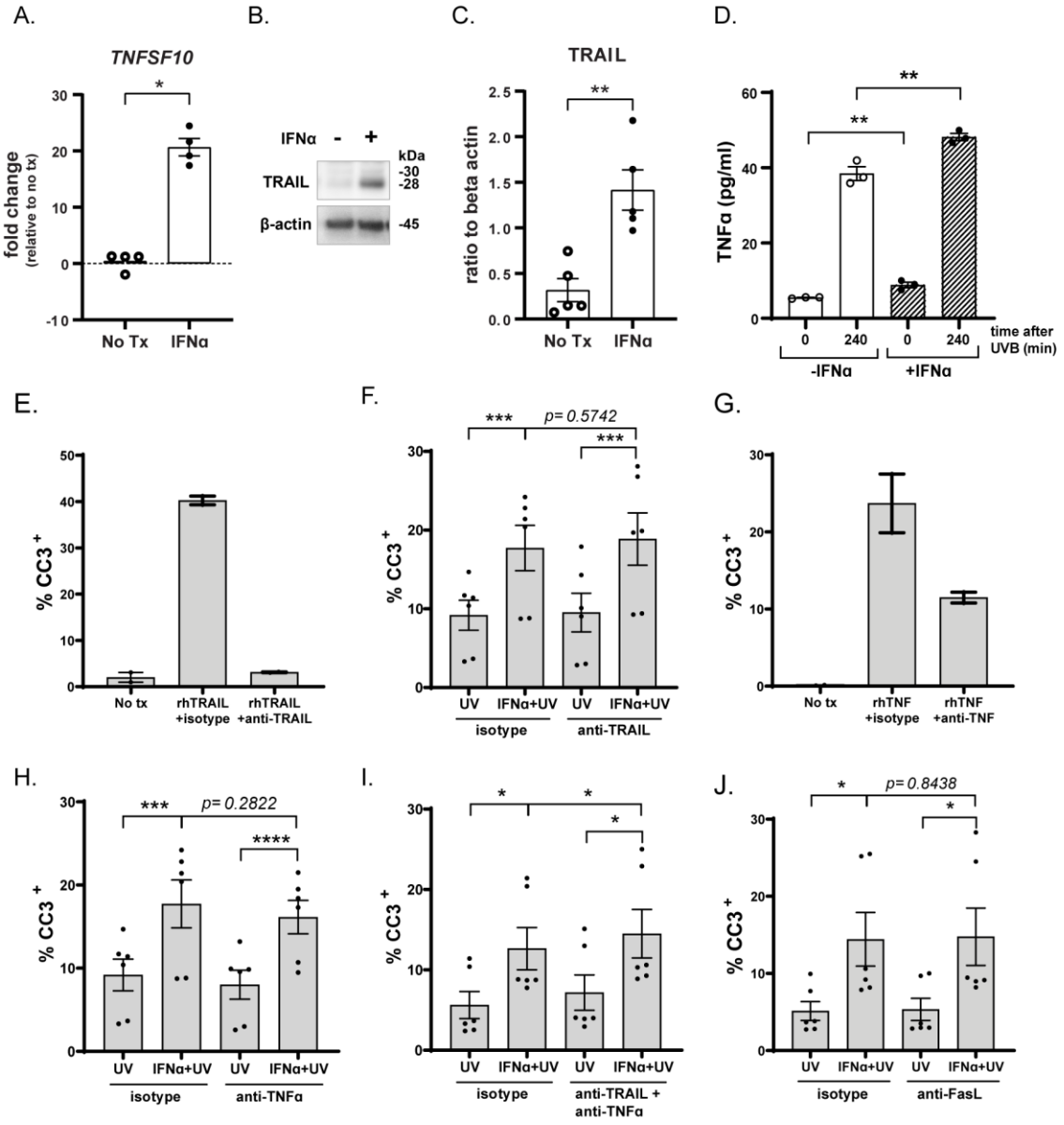


Figure 2-5 – Type I IFN-enhanced apoptosis after UVB exposure is death ligand-independent.
N/TERTs were treated with 1000 U/ml IFN-α for 16 hours and **(A)** TNFSF10 gene expression was measured by qRT-PCR (n=4) and **(B-C)** TRAIL protein expression was measured by Western blot (n=5). **(D)** N/TERTs were treated with 1000 U/ml IFN-α for 16 hours prior to exposure to 50 mJ/cm² UVB. TNF-α was measured in the supernatant at the indicated times after UVB exposure by ELISA (n=2-3). **(E)** N/TERTs were treated with 100ng/ml anti-TRAIL or isotype antibody and 50 ng/ml recombinant human TRAIL for 24 hours. Cells were stained with cleaved caspase-3 (CC3) antibody and fixable viability dye (FVD) and analyzed by flow cytometry (n=2). **(G)** Primary human fibroblasts were treated with 5ug/ml anti-TNF-α, 1ug/ml actinomycin D, and 0.75ng/ml recombinant human TNFα for 24 hours. Cells were stained with CC3 antibody and FVD to measure apoptosis (n=2). **(F,H,I)** N/TERTs were treated with 1000 U/ml IFN-α for 16 hours prior to exposure to 50 mJ/cm² UVB. Immediately following UVB exposure, cells were treated with neutralizing antibodies targeting **(F)** TRAIL (100ng/ml), **(H)** TNF-α (5ug/ml), or **(I)** TRAIL+TNF-α. Four hours post UVB exposure, apoptosis was measured with CC3 and FVD staining (n=6). **(J)** N/TERTs were treated with 1000 U/ml IFN-α for 16 hours prior to exposure to 50 mJ/cm² UVB. Immediately following UVB exposure, cells were treated with neutralizing antibody targeting FasL (1ug/ml). Four hours post UVB exposure, apoptosis was measured with CC3 and FVD staining (n=6). Data analyzed by paired or unpaired t test for normally distributed data and Wilcoxon matched-pairs signed rank test or Mann-Whitney test for non-normally distributed data.* <0.05, **<0.01, ***<0.001.

2.4.6 IRF1 is upregulated in keratinocytes by type I IFN treatment and drives enhanced UVB-driven apoptosis

Interferon regulatory factor-1 (IRF1) is a transcription factor and tumor suppressor (398) that mediates ligand-independent apoptosis via caspase-8 activation in breast cancer cells (387). As expected, IRF1 expression was increased by IFN-α treatment in both primary keratinocytes (Fig. 2-4A) and N/TERTs (Fig. 2-6A-C). We used Accell siRNA to knockdown expression of IRF1 (siIRF1) in N/TERTs (Fig. 2-6D). siIRF1 and non-targeting control N/TERTs were treated with IFN-α and UVB and caspase-3 cleavage was assessed using fluorescent staining of activated

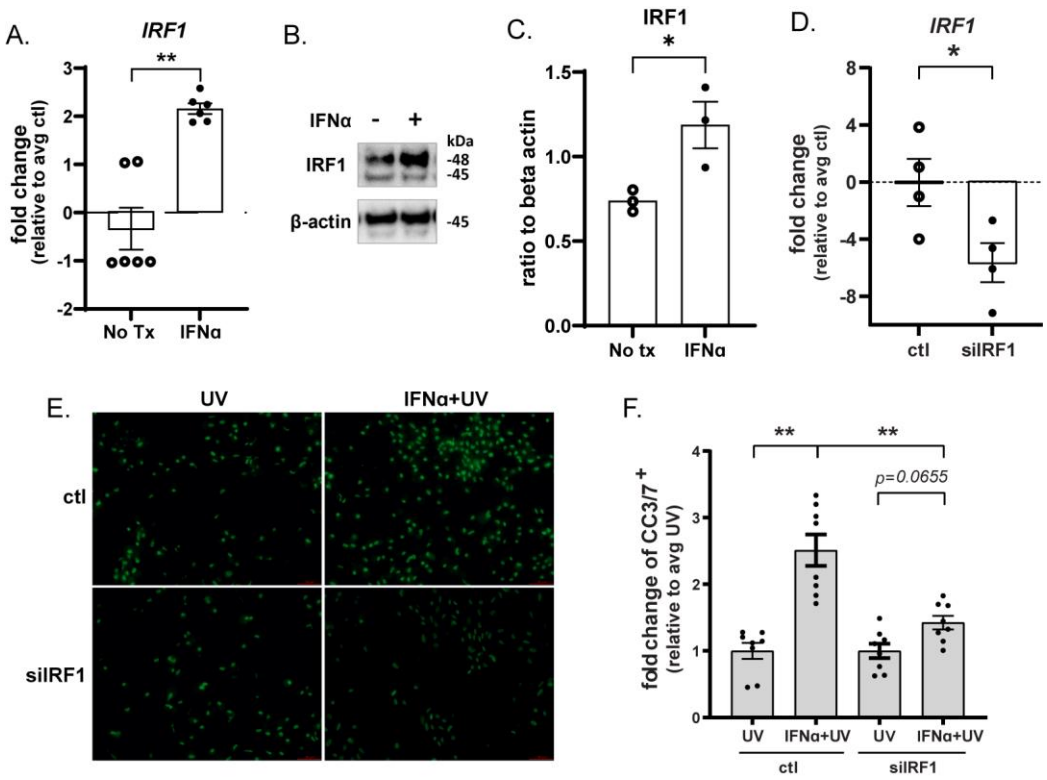


Figure 2-6 – Type I IFN-enhanced apoptosis after UVB exposure is IRF1-dependent.
N/TERTs were treated with 1000 U/ml IFN-α for 16 hours and **(A)** *IRF1* gene expression was measured by qRT-PCR (n=6) and **(B-C)** IRF1 protein expression was measured by Western blot (n=3). **(D)** N/TERTs were incubated with 1uM Accell non-targeting siRNA (ctl) or Accell IRF1 siRNA (siIRF1) for 48 hours and mRNA expression of IRF1 was measured by qRT-PCR (n=4). **(E-F)** N/TERTs were incubated with 1uM ctl siRNA or siIRF1 for 48 hours, treated with 1000 U/ml IFN-α for 16 hours, then exposed to 50mJ/cm² UVB. Cells were stained with CellEvent Caspase-3/7 Green Detection Reagent and imaged 6 hours later (n=8). Data analyzed by paired or unpaired t test for normally distributed data or Wilcoxon matched-pairs signed rank test for non-normally distributed data. *<0.05, **<0.01.

caspase-3 given the small number of cells used for this assay. There was no difference in baseline levels of UV-mediated caspase-3 activation with IRF1 knockdown; however, suppression of IRF1 significantly reduced the effect of IFN-α priming to induce enhanced apoptosis (Fig. 2-6E,F). This suggests that IFN induction of IRF1 is required for enhanced apoptosis following UVB exposure.

2.5 Discussion

In this paper, we identified that type I IFN priming of keratinocytes prior to UVB exposure resulted in increased caspase-8 driven apoptosis that was independent of XAF1 and death ligands, but dependent on IRF1. Further, we observed a similar phenomenon *in vivo* in which epidermal overexpression of IFN-κ increased UVB-induced apoptosis. Dissecting these pathways provides important insights into how skin rich in type I IFN production may drive predisposition to triggering of cutaneous manifestations of lupus by sunlight exposure.

Exposure to UV light is traditionally associated with keratinocyte apoptosis, while stimulation with type I IFNs has been implicated in activation of several cell death pathways. For example, type I IFN stimulation of apoptosis-resistant fibroblasts and colon adenocarcinoma cell lines induces necroptosis (399, 400). Conversely, type I IFN signaling during influenza infection of respiratory epithelial cells induces a switch from apoptosis to pyroptosis to promote pro-inflammatory responses (401). Our lab has previously shown by TUNEL staining that keratinocytes from SLE patients die more after UV exposure compared to those from healthy controls and that this is dependent on IFN signaling (263). Here, we expand on these findings by clarifying that IFN-priming of keratinocytes does not alter the apoptotic pathways activated by UVB exposure, just amplifies them. Further, while UVB exposure is associated with activation of both extrinsic and intrinsic pathways of apoptosis (192, 392) we demonstrate specific enhancement of caspase-8-driven apoptosis by type I IFNs. These findings contribute to our mechanistic

understanding of how the inflammatory environment present even in non-lesional SLE skin can promote lesion induction and disease flares upon UV exposure (262).

Our data demonstrate a role for IRF1 in regulating the IFN-mediated enhancement of UVB-induced apoptosis. It is well established that IRF1 regulates a large number of genes associated with apoptosis, including caspase-8 (402, 403). We have shown that IFN-treated keratinocytes upregulate expression of caspase-8 and IRF1 and that both are required for IFN-enhancement of UVB-driven apoptosis; however, the full mechanism by which this occurs needs further investigation. It may be that increased expression of caspase-8 following type I IFN treatment sensitizes cells for activation of apoptotic signaling pathways upon a subsequent stimulus. Since we have shown this process to be independent of known death ligands, we hypothesize that UVB stimulation induces clustering of death receptors that allows for activation of caspase-8 in the absence of ligand binding. Previous work has shown that UV induces aggregation of Fas through reorganization of membrane lipid rafts that results in apoptosis of melanoma cells (404). Whether this occurs in IFN-α primed, UVB exposed keratinocytes to initiate apoptosis should be examined. Of note, loss of IRF1 did not entirely abrogate apoptosis (Fig 6e-f), but rather reduced levels to those seen with UV exposure alone. This suggests that IRF1 specifically drives the IFN enhancement of apoptosis rather than UV-induced apoptosis as a whole. In healthy skin, apoptosis that proceeds UV exposure generally occurs as a protective response to remove irreversibly damaged precancerous cells. Thus, the ability to modulate the rate of apoptosis in IFN-exposed cells without entirely turning it off has attractive therapeutic potential.

Intriguingly, we identified a strong upregulation of XAF1 in IFN-α-stimulated keratinocytes, however, loss of XAF1 further enhanced the rates of keratinocyte apoptosis after UVB exposure (Fig. 4d). Although XAF1-knockdown does have some effect on the IFN-mediated

enhancement of apoptosis, the overall increase in UVB-mediated apoptosis that occurs likely negates any potential benefit that limiting this IFN-mediated phenotype could have. While XAF1 has conventionally been regarded as a pro-apoptotic protein, our study is the first to investigate its role in UVB-mediated apoptosis. XAF1 can act in a variety of ways to promote apoptosis including through stabilization of p53 (405, 406). Interestingly, reduction of p53 levels in keratinocytes has been shown to enhance the apoptotic susceptibility of cells to UV irradiation (407) as well as to IFN treatment (408), suggesting pro-survival functions for p53. Further, studies in HT29 colon carcinoma cell lines showed that expression of p53 after UV irradiation stimulates apoptosis, while expression of p53 prior to irradiation can protect against apoptosis (409). In our study, keratinocytes were pretreated with type I IFNs prior to exposure to UV light, therefore, IFN-induction of XAF1 may result in p53 stabilization that turns on its pro-survival functions. This IFN-mediated stabilization of p53 through XAF1 induction, although seemingly counterintuitive, may partially explain the increase in apoptosis observed following shRNA silencing of XAF1. Further studies are needed to confirm this.

We have previously confirmed that the dose of Ac-YVAD-cmk we used inhibits caspase-1 activation. Dosing for Necrostatin-1, GSK'872, and Z-LEHD-FMK was determined based on the literature, however, we were not able to validate this dosing in our model which presents a limitation of our work and should be further tested. Further, it is important to note that in our model, we used IFN-α stimulation to mimic the type I IFN rich environment of lupus skin and to allow us to dissect the specific contributions of IFNs to cell death. We showed that this lowers the dose of UV required for apoptosis induction (Fig 1b), similar to the lowered MED of lupus patients. However, this system does not fully recapitulate the altered immune landscape of the diseased state that results in abnormal UV responses in lupus skin. Therefore, this phenomenon should be

further examined in lupus-prone mouse models and lupus patients. This would account for additional immunopathological abnormalities present including circulating autoantibodies, immune complexes, and increased immune cell infiltration and activation after UV exposure.

Altogether, our study dissects the specific cell death pathways activated in keratinocytes by type I IFNs and UVB. We demonstrate an important role for IFN-mediated increases in IRF1 in driving enhanced apoptosis of keratinocytes after UVB exposure. As mechanisms for phagocytic clearance of apoptotic cells are suggested to be defective in lupus (198, 199, 227, 410), this likely leads to elevated levels of secondary necrosis and release of intracellular autoantigens. Circulating autoantibodies can bind to their respective antigens and activate a multitude of inflammatory responses that perpetuate the cycle of inflammation in the skin and promote lesion induction. In conclusion, our data contributes to a more thorough understanding of the mechanisms by which the type I IFN rich environment of lupus skin contributes to aberrant responses to UVB. This opens new avenues for potential therapeutic options for patients whose disease is flared by the sun.

Chapter 3 – Loss of Interleukin-1 Beta Is Not Protective in the Lupus-Prone NZM2328 Mouse Model

Portions of this chapter have been accepted for publication in Frontiers in Immunology:

Loftus SN, Liu J, Berthier CC, Gudjonsson JE, Gharaee-Kermani M, Tsoi LC, Kahlenberg JM. Loss of Interleukin-1 beta is not protective in the lupus-prone NZM2328 mouse model.

3.1 Abstract

Aberrant activation of the innate immune system is a known driver of lupus pathogenesis. Inhibition of the inflammasome and its downstream signaling components in murine models of lupus has been shown to reduce severity of disease. Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine released from cells following inflammasome activation. Here, we examine how loss of IL-1β affects disease severity in the lupus-prone NZM2328 mouse model. We observed a sex-biased increase in immune complex deposition in the kidneys of female mice in the absence of IL-1β that corresponds to worsened proteinuria. Loss of IL-1β did not result in changes in overall survival, anti-dsDNA autoantibody production, or renal immune cell infiltration. RNA-sequencing analysis identified upregulation of TNF and IL-17 signaling pathways specifically in females lacking IL-1β. Increases in these signaling pathways were also found in female patients with lupus nephritis, suggesting clinical relevance for upregulation of these pathways. Together, these data suggest that inhibition of the inflammasome or its downstream elements that block IL-1β signaling may need to be approached with caution in SLE, especially in patients with renal involvement to prevent potential disease exacerbation.

3.2 Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease involving aberrant immune responses, production of autoantibodies, and multi-organ system involvement. The etiology of SLE is multifactorial, likely involving a complex interplay between genetic risk factors and environmental triggers (411). Dysregulation of several innate and adaptive immune signaling pathways contributes to the inflammation characteristic of the disease. Multiple cytokines have been implicated in the pathogenesis and progression of SLE including B-cell activating factor (BAFF), type I interferons (IFN), and members of the interleukin-1 (IL-1) superfamily (412-414). While drugs targeting BAFF (belimumab) and the type I IFN receptor (anifrolumab) have been approved for treatment of SLE, the specific roles of IL-1 cytokines in SLE have remained more elusive (34, 40).

IL-1 beta (IL-1β) and IL-18, members of the IL-1 superfamily, are synthesized as inactive precursor molecules that are processed to their mature, biologically active form following activation of the inflammasome. Enhanced inflammasome activation is observed in lupus macrophages and monocytes and studies suggest that inhibition of inflammasome signaling attenuates disease severity in murine models of lupus (377, 382, 383, 415). Specifically, suppression of inflammasome activation through blockade of caspase-1 or the NLRP3 inflammasome has been shown to reduce severity of lupus nephritis (LN) and decrease autoantibody production (382, 383).

Most studies to date have focused on the role of the inflammasome complex in the modulation of disease activity, and many of them have only highlighted correlative associations between IL-1β/IL-18 and disease severity (371, 383). As a result, the specific roles and mechanisms of these individual cytokines in SLE are incompletely understood. Given that IL-1β

can trigger a broad range of responses that drive systemic inflammation and exacerbate damage during chronic disease, we hypothesized that inhibition of this cytokine would limit disease severity in a lupus-prone mouse model.

In this study, we examined how loss of IL-1β modulates disease in the NZM2328 lupus-prone mouse model. We observed no differences in overall survival or autoantibody production between NZM and NZM-*Il1b*$^{-/-}$ mice. Surprisingly, we identified a female-specific increase in immune complex deposition in the kidneys in the absence of IL-1β. Consistent with these results, female NZM-*Il1b*$^{-/-}$ mice also had increased proteinuria compared to NZM controls. RNA-sequencing analysis of the kidneys identified sex-specific differences in TNF and IL-17 signaling pathways that are also observed in female LN patients. These results demonstrate an unexpected, potentially protective role for IL-1β in LN in a sex-biased manner. This suggests that IL-1β blockade could have unintended consequences for disease progression in LN patients.

3.3 Materials and Methods

3.3.1 Mice

New Zealand Mixed (NZM) 2328 lupus-prone mice were a gift from Dr. Chaim Jacob, University of Southern California. NZM-*Il1b*$^{-/-}$ mice (NZM2328 mice lacking functional IL-1β) were generated through the University of Michigan Transgenic Animal Model Core as previously described (416). All mice were bred and housed in specific pathogen-free facilities at the University of Michigan and treated in accordance with our University of Michigan IACUC-approved protocol. Survival studies were conducted for 72 weeks in male and female mice or until mice became moribund following development of proteinuria. Mice were monitored for development of lupus via weekly urine collection starting at 20 weeks of age. Blood was sampled

via saphenous vein bleed every other week. At euthanasia, terminal bleeding was performed via cardiac puncture and tissues were harvested.

3.3.2 Quantification of autoantibodies

Anti-dsDNA IgG levels were quantified in the serum using the Mouse Anti-dsDNA IgG ELISA Kit (Alpha Diagnostics International), according to manufacturer's protocols.

3.3.3 Proteinuria analysis

Urine samples were assessed for microalbumin using the mouse Albuwell M Kit (Exocell, Philadelphia, PA) and creatinine using the mouse QuantiChrom™ Creatinine assay kit (BioAssay Systems; Hayward, CA), both according to the manufacturer's protocols. Mircoalbumin-to-creatinine ratios were calculated to estimate 24-hour urinary protein excretion.

3.3.4 Immune complex deposition scoring

Glomerular immune complex deposition was quantified on frozen kidney sections via staining for C3 and IgG deposition as previously described (417). Briefly, sections were stained with FITC-conjugated anti-C3 (1:250; Immunology Consultants Laboratory, Portland, OR) and Texas-Red-conjugated anti-IgG (1:250; Invitrogen, Waltham, MA) for 1 hour at 4°C. DAPI was used to visualize DNA. Glomerular immune complex staining was quantified using FIJI ImageJ and a semi-automated looping program to capture fluorescence within a user defined area designed by the BRCF Microscopy Core at the University of Michigan.

3.3.5 Immunohistochemistry

For detection of immune cells in mouse kidney, formalin-fixed, paraffin-embedded sections were heated at 60°C for 1 hour, deparaffinized, rehydrated, and heated at 100°C for 20

minutes in Retrievagen A (pH 6.0) (for CD11c; BD Biosciences, Franklin Lakes, NJ) or Tris-EDTA buffer (pH 9.0) (for CD11b, CD4, and CD8) for antigen retrieval. Slides were washed, treated with 3% hydrogen peroxide in PBS for 5 minutes, blocked in goat serum for 1 hour, and incubated with Recombinant Anti-CD11c antibody [EPR21826] (1:50; ab219799), Recombinant Anti-CD4 antibody [EPR19514] (1:500; ab183685), Recombinant Anti-CD8 alpha antibody [EPR21769] (1:2000; ab217344), or Recombinant Anti-CD11b antibody [EPR1344] (1:4000; ab133357) (Abcam, Boston, MA) overnight at 4°C. Isotype controls (#3900; Cell Signaling Technology, Danvers, MA) were stained in parallel with each set of slides. All slides were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories, Newark, California), followed by incubation with VECTASTAIN Elite ABC reagent (Vector Laboratories, Newark, California) and detection with 3,3'-diaminobenzidine (BD, Franklin Lakes, NJ) under a light microscope. Slides were counterstained with hematoxylin, dehydrated, and mounted. Images were acquired using a Zeiss microscope (Zeiss, Oberkochen, Germany) at indicated magnifications. Positive cells were quantified by manually counting the # of positive cells (brown) averaged for five 20x fields of view.

3.3.6 RNA-sequencing

Kidney RNA was isolated via Zymo Direct-zol RNA isolation kit and libraries were generated using standard poly-A prep kits (New England Biolabs) with the assistance of the U-M Advanced Genomics Core. An average of 61 million read pairs were obtained per sample via Nextseq 6000. Paired-end reads (151bp for each end) were generated for the RNA-seq experiments. After quality control and adapter trimming, we conducted read alignment (418) and gene quantification (419) using mm10 and gencode vM18, respectively. DESeq2 was used for read

normalization and modeling (420). Only genes with on average greater than one read per sample were used in subsequent analysis.

3.3.7 Human renal biopsy samples and calculation of IL-17 and TNF signaling pathway signature scores

Gene expression analysis of glomeruli and tubulointerstitial compartments from the European cDNA Bank (ERCB) human renal biopsies were used, as previously described (421, 422). In brief, the discovery cohort included pre-transplant healthy living-donors (LD; 5 females, 9 males for glomeruli; 6 females, 3 males for tubules) and patients demographic characteristics representative of lupus nephritis disease (LN; 19 females, 3 males for glomeruli; 6 females, 3 males for tubules) and WHO class III and/or IV. The validation cohort included 5 LD (4 females, 1 male for glomeruli and tubules) and LN patients (10 female, 3 male for glomeruli; 18 female, 6 male for tubules), also WHO class III and/or IV (ref: www.Nephroseq.org).

IL-17 and TNF pathway scores were calculated using the algorithm described by Feng et al (423), and as previously published (261), using sex-matched LD controls. The IL-17 and TNF signaling pathway genes were extracted from KEGG (https://www.genome.jp/entry/pathway+hsa04657 and https://www.genome.jp/entry/hsa04668). The IL-17 pathway score was calculated for glomeruli using 77 and 80 of the 94 genes that were expressed in the discovery and validation datasets, respectively, and for tubules using 74 and 82 of the 94 genes that were expressed in the discovery and validation datasets, respectively (Table A-1). The TNF pathway score was calculated for glomeruli using 99 and 108 of the 112 genes that were expressed in the discovery and validation datasets, respectively, and for tubules using 95 and 109 of the 112 genes that were expressed in the discovery and validation datasets, respectively (Table A-2).

3.3.8 Upstream regulators analyses

Ingenuity Pathway Analysis (IPA) software was used to identify potential upstream transcriptional regulators which may be involved in the regulation of the genes differentially expressed in NZM-*Il1b*$^{-/-}$ compared to NZM mice.

3.3.9 Statistical Analysis

Data (Fig. 3-1,2, Fig. 3-4) were graphed and statistics were performed using GraphPad Prism 9. Data are presented as the mean ± SEM (Fig. 3-1,2) or with median line (Fig.3-4). For comparisons between two groups, unpaired two-tailed t tests or Mann-Whitney tests were used. For survival studies, log-rank testing was used. P-values <0.05 were considered as statistically significant.

3.4 Results

3.4.1 Deletion of IL-1β does not improve survival of lupus-prone mice

Lupus-prone NZM2328 (NZM) mice spontaneously develop lupus-like characteristics including autoantibody production and glomerulonephritis with females developing more severe manifestations at an earlier age of onset compared to males (424). To investigate the role of IL-1β in driving disease pathology in this model, we used NZM mice with a homozygous deletion of *Il1b* (NZM-*Il1b*$^{-/-}$) (416).

We first examined how loss of IL-1β impacted the overall survival of both male and female mice. While disease progression was much slower in male mice, as expected, there were no differences in survival outcomes within the sexes between NZM and NZM-*Il1b*$^{-/-}$ mice (Fig. 3-1A). Next, we measured production of anti-dsDNA antibodies, a hallmark of disease that generally begins by five months of age in female NZM mice and is delayed by approximately four weeks in

males (424). Serum levels of anti-dsDNA IgG increased over time in both male and female mice but were not significantly different between NZM and NZM-*Il1b*$^{-/-}$ (Fig. 3-1B).

3.4.2 Absence of IL-1β results in increased immune complex deposition in the kidneys

LN is characterized by the deposition of immune complexes within the kidney that can promote renal inflammation and damage contributing to development of proteinuria. We thus quantified immune complex deposition in the kidneys. Surprisingly, we observed a significantly

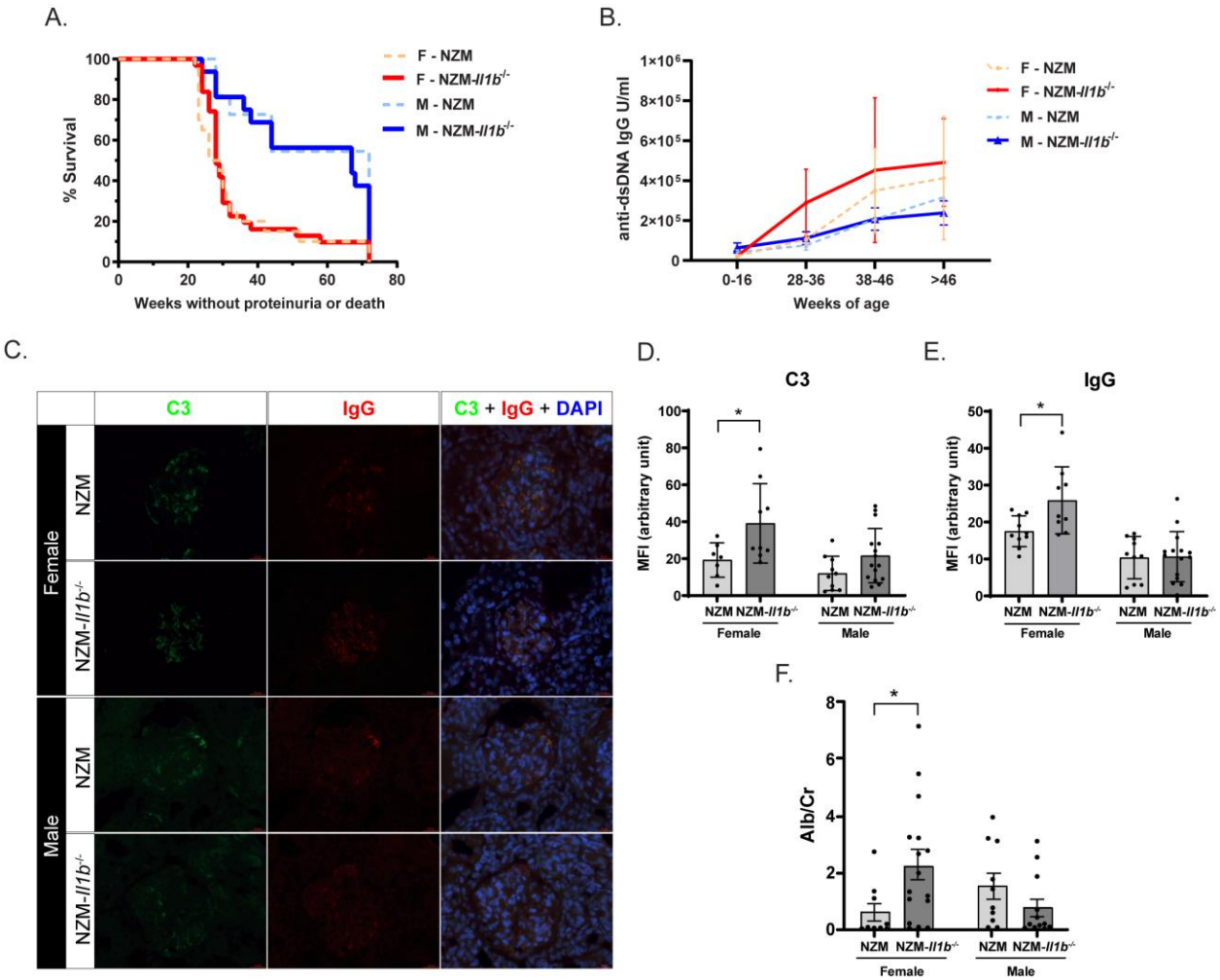


Figure 3-1 – Absence of IL-1β has no effect on survival but increases renal immune complex deposition.
(A) Survival of female (F) and male (M) NZM and NZM-*Il1b*$^{-/-}$ mice was monitored for 72 weeks or until mice became moribund following development of proteinuria (n=11-31). **(B)** Levels of anti-dsDNA IgG were measured in the serum at the indicated age ranges by ELISA (n=3-20 per age range). **(C)** Representative images of immunofluorescent microscopy of renal cortex containing glomeruli. Green = FITC-C3, Red = Texas Red-IgG, Blue = DAPI. Levels of **(D)** C3 and **(E)** IgG were quantified using a semi-automated analysis program (n=7-15). **(F)** The ratio of microalbumin (Alb) to creatinine (Cr) was assessed in the urine at time of death as a measure of proteinuria (n=10-16). Data analyzed by log-rank test, unpaired t test or Mann-Whitney test. *<0.05.

increased staining of both C3 (*p=0.0397*) and IgG (*p=0.0176*) in the kidneys of female NZM-*Il1b*$^{-/-}$ mice compared to NZM controls, indicating increased immune complex deposition. No significant difference was observed between male NZM and NZM-*Il1b*$^{-/-}$ mice (C3: *p=0.0798*; IgG: *p=0.9238*) (Fig. 3-1C-E). We then examined the urine to see if this increase in immune complexes translated into any meaningful change in proteinuria. In line with the immune complex staining, female NZM-*Il1b*$^{-/-}$ mice had increased albumin to creatinine ratios (*p=0.0196*), indicative of damage to the filtration barrier, compared to their NZM counterparts, while no significant difference was observed for males at this timepoint (*p=0.1593*) (Fig. 3-1F).

Together, these data suggest that while IL-1β does not affect overall survival or autoantibody production in lupus-prone mice, it may play an important sex-specific protective role in the kidney by limiting immune complex deposition and resultant proteinuria.

3.4.3 Loss of IL-1β does not change immune cell infiltration into kidneys

LN is characterized by an inflammatory cascade involving immune cell infiltration into the kidneys followed by production of pro-inflammatory cytokines and chemokines that contribute to kidney damage (425). To determine if gross differences in immune cell infiltration were observed in the female NZM-*Il1b*$^{-/-}$ kidneys, we examined the presence of CD4^{+}, CD8^{+}, CD11b^{+}, and CD11c^{+} cells by immunostaining. While CD4^{+} (female: *p=0.3513*; male: *p=0.2725*) and CD8^{+} (female: *p=0.3306*; male: *p=0.2966*) T cells, as well as CD11b^{+} myeloid cells (female: *p=0.7987*), were detected in the kidneys, there were no significant differences in the numbers of these cells between female NZM and NZM-*Il1b*$^{-/-}$ mice (Fig. 3-2A-F). Male NZM-*Il1b*$^{-/-}$ mice did have significantly more CD11b^{+} cells compared to NZM (*p=0.0195*) (Fig. 3-2E,F). CD11c^{+} dendritic cells were not identified in the kidneys (Fig. 3-2G). Based on these data, we see similar populations of immune cells in the kidneys of these mice.

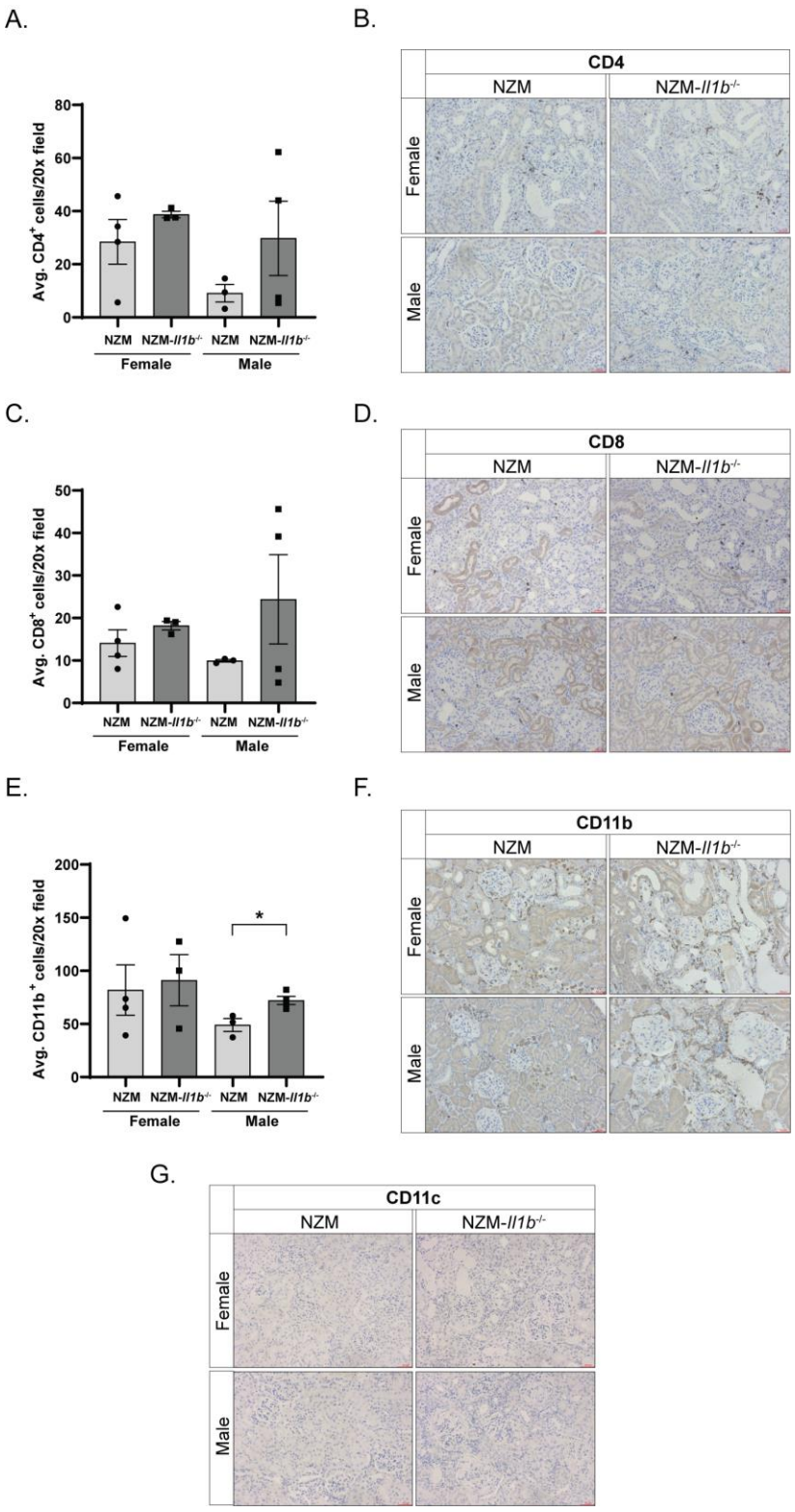


Figure 3-2 – Loss of IL-1β does not change immune cell infiltration into kidneys.
Quantification and representative images of **(A-B)** $CD4^+$, **(C-D)** $CD8^+$, **(E-F)** $CD11b^+$, and **(G)** $CD11c^+$ cells in the kidney per x20 field averaged across three images each (n=3-4). Data analyzed by unpaired t tests. *<0.05.

3.4.4 IL-17 and TNF signaling pathways are activated in females in absence of IL-1β

To better understand the impact of IL-1β deletion on lupus kidneys, genome-wide transcriptome analysis using RNA-sequencing (RNA-seq) was performed and transcriptional differences in the kidneys of male and female NZM and NZM-*Il1b*$^{-/-}$ mice were examined. We performed uniform manifold approximation and projection (UMAP) for dimension reduction on the transcriptome data, and we highlighted that male and female mice separated from each other only in NZM-*Il1b*$^{-/-}$ but not NZM kidney samples, emphasizing the sex bias seen in the mouse model (Fig. 3-3A). Indeed, when comparing differentially expressed genes (DEGs) between NZM and NZM-*Il1b*$^{-/-}$ kidneys (using FDR<0.01 and fold change of 2), we identified 1,046 and 1,992 genes that were up-and down-regulated respectively for female mice (Fig. 3-3B), while for male there were only 112 up and 382 down genes.

Pathway analysis from those DEGs highlighted the TNF ($p=7.59x10^{-5}$) and IL-17 signaling ($p=1.21x10^{-6}$) pathways among the top uniquely regulated pathways (Fig. 3-3C) in the NZM-*Il1b*$^{-/-}$ mice. Heatmaps of the genes involved in the modulated TNF and IL-17 pathways confirmed a robust upregulation of TNF and IL-17-related genes in kidneys of female NZM-*Il1b*$^{-/-}$ mice compared to the kidneys of male NZM-*Il1b*$^{-/-}$ mice (Fig. 3-3D,E). Ingenuity Pathway Analysis was used to predict upstream regulators for the DEGs identified in the kidneys of female and male NZM-*IL1b*$^{-/-}$ vs NZM mice (Table A-3). TNF was identified as the top upstream regulator in female mice. These data suggest that enhancement of the IL-17 and TNF signaling pathways occurs in a sex-biased manner in the absence of IL-1β signaling and that TNF may be a driving differentiator of this signal.

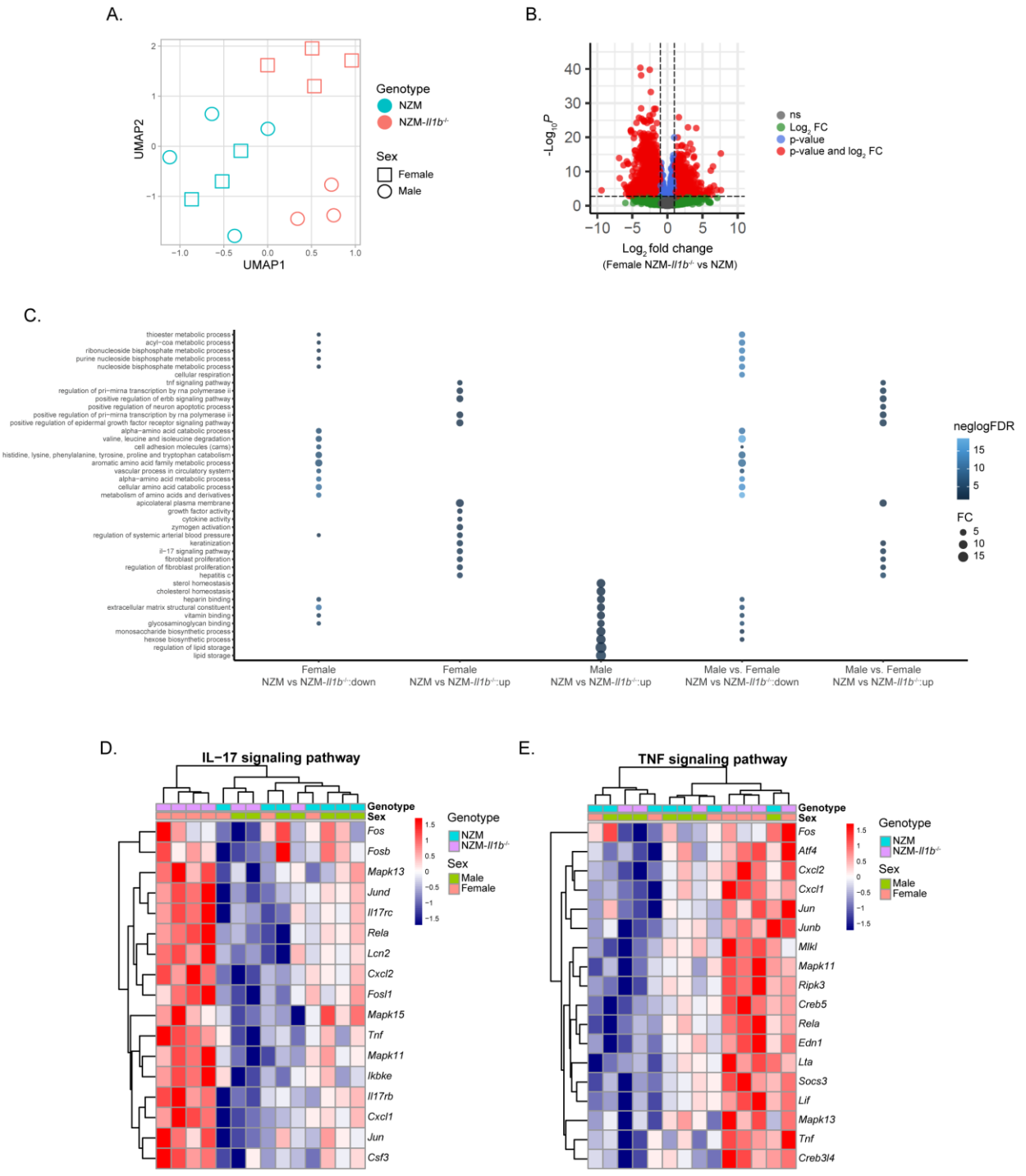


Figure 3-3 – Sex-biased increase in IL-17 and TNF signaling pathway gene expression in absence of IL-1β.
(A) Dimension reduction (UMAP) plot for RNA-seq kidney samples; **(B)** Volcano plot to show summary statistics of the differential expression analysis comparing NZM-*Il1b*$^{-/-}$ vs NZM in female mice. **(C)** Pathway analysis of differentially expressed genes between NZM and NZM-*Il1b*$^{-/-}$ kidneys highlighted the TNF (p=7.59x10-5) and IL-17 signaling (p=1.21x10-6) pathways among the top regulated pathways. **(D-E)** Heatmaps illustrating standardized expressions of genes participating in the **(D)** IL-17 and **(E)** TNF signaling pathways.

To determine whether sex-biased regulation of IL-17 and TNF pathways has clinical relevance in LN patients, we studied available gene expression data of microdissected renal biopsies from healthy living-donors and patients with class III and/or IV LN (Table A-4,5). IL-17 and TNF signaling pathway signature scores (see methods) were significantly elevated in the glomeruli of female LN patients compared to living donors (*p=0.0186* and *0.0091*, respectively), but not in males (*p=0.7921* and *0.2188*, respectively) (Fig. 3-4A-B).

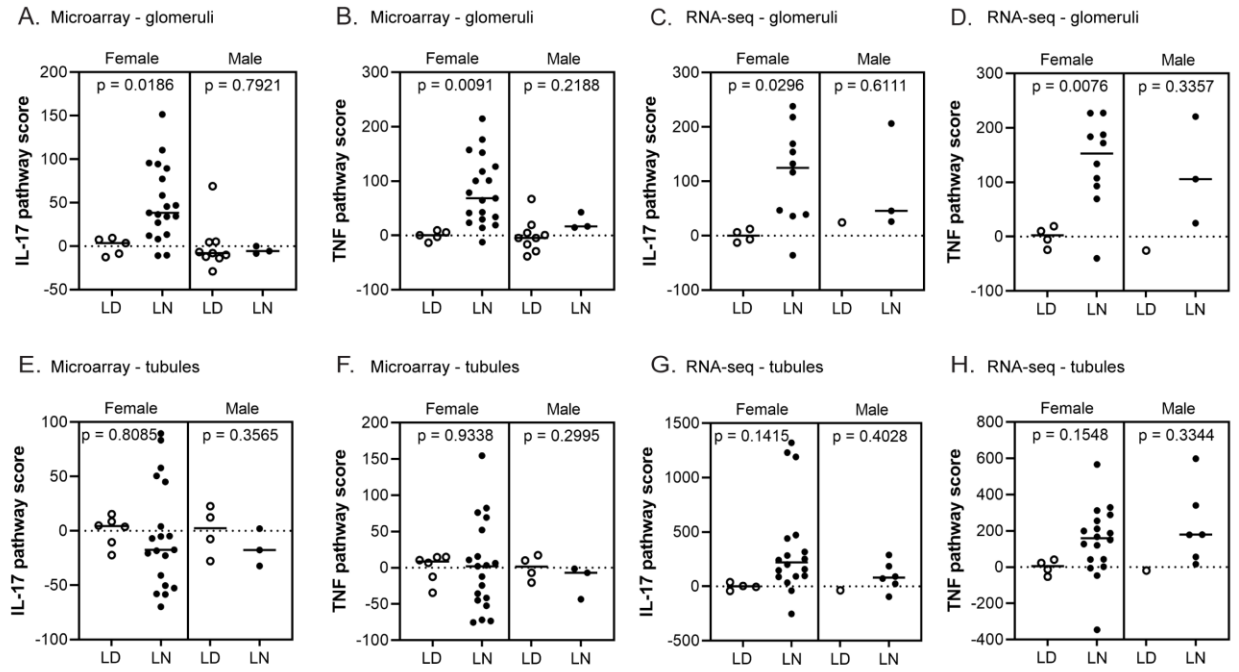


Figure 3-4 – IL-17 and TNF pathway scores increased in female lupus nephritis patients.
Microarray gene expression analysis of **(A)** IL-17 and **(B)** TNF pathway scores in the glomerular compartment of human lupus nephritis (LN) and healthy living donor (LD) biopsies: IL-17 and TNF signaling pathway scores were significantly higher in female LN (n=19) compared to LD controls (n=5) (p=0.0186 and 0.091 respectively), but not in males (n=9 LD and 3 LN) (p=0.7921 and 0.2188 respectively). RNA-sequencing gene expression analysis of **(C)** IL-17 and **(D)** TNF pathway scores in the glomerular compartment of human lupus nephritis (LN) and healthy living donor (LD) biopsies: IL-17 and TNF signaling pathway scores were significantly higher in female LN (n=10) compared to LD controls (n=4) (p=0.0296 and 0.0076 respectively), but not in males (n=1 LD and 3 LN) (p=0.6111 and 0.3357 respectively). Microarray gene expression analysis of **(E)** IL-17 and **(F)** TNF pathway scores in the tubulointerstitial compartment of human LN and LD biopsies: IL-17 and TNF signaling pathway scores were not significantly different in female LN (n=19) compared to LD controls (n=6) (0.8085 and 0.9338, respectively) or in males (n=4 LD and n=3 LN) (p=0.3565 and 0.2995, respectively). RNA-sequencing gene expression analysis of **(G)** IL-17 and **(H)** TNF pathway scores in the tubulointerstitial compartment of human LN and LD biopsies: IL-17 and TNF signaling pathway scores were not significantly different in female LN (n=18) compared to LD controls (n=4) (p=0.1415 and 0.1548, respectively) or in males (n=1 LD and 6 LN) (p=0.4028 and 0.3344, respectively).

Those results were validated in a second independent LN patient cohort (Fig. 3-4C,D). Interestingly, these signatures are not seen in the tubule compartments from the same patients

highlighting that this may be a glomerular-specific pathology (Fig. 3-4E-H). This further supports our murine data suggesting involvement of these pathways in promoting sex-biased differences in kidney disease in the absence of IL-1β.

3.5 Discussion

In this study, we uncovered a novel sex-biased protective role for IL-1β in limiting renal pathology in the lupus-prone NZM2328 mouse model. While loss of IL-1β did not significantly alter survival or autoantibody production, it did increase glomerular immune complex deposition and worsen proteinuria in a female-specific manner. We observed no differences in immune cell infiltration into the kidneys to explain these differences. RNA-seq analysis identified upregulation of genes involved in IL-17 and TNF signaling pathways in female, but not male, NZM-*Il1b*$^{-/-}$ compared to NZM mice. Importantly, upregulation of these signaling pathways was also observed in female LN patients compared to healthy controls, suggesting that activation of IL-17 and TNF may have relevance in the sex-bias in human disease and should be further investigated.

Our data support an unexpected role for IL-1β in limiting renal damage in LN. Previous studies have offered differing interpretations of the role of IL-1β in SLE. Indeed, inhibition of the activating caspase and inflammasome complexes have protective effects on LN in many murine models. Both IL-1β and IL-18 are secreted following inflammasome activation, however, the individual contributions of these cytokines to SLE are incompletely understood. Serum levels of IL-18 are elevated in SLE patients and correlate with disease activity and organ damage (371, 426, 427). Further, in murine lupus models, IL-18 serum levels correlate with LN severity (383, 428). Our data suggest that IL-1β is not critical for nephritis progression but may in fact have a protective effect that is revealed when its function is inhibited in isolation (as opposed to full inflammasome complex targeting). Indeed, recent studies support a protective effect for signaling through the IL-

1 receptor in podocytes during glomerular diseases (429, 430). Our data exhibit more proteinuria, but not inflammation, in the absence of *Il1b*, which may suggest a role for protective IL-1 podocyte signaling in our model as well. Further, in our human data, the IL-17 and TNF signatures are only increased in the glomeruli, not the tubules, of female LN patients suggesting that this might be a podocyte-associated effect of IL-1β. Lastly, IL-1β has been shown to induce expression of CD16 (FcγRIII), a receptor involved in immune complex clearance, suggesting that the worsened renal pathology in our model may, in part, result from reduced ability to clear immune complexes (431, 432).

Enthusiasm for IL-1 inhibition in SLE has remained low until late. A small clinical trial of anakinra, a recombinant version of IL-1Ra, was conducted with four SLE patients with severe, treatment-refractory polyarthritis (433). While subjective improvements were observed in patients, one patient had an arthritic flare, and studies in larger cohorts of patients were not conducted. No signs of SLE flares in the kidneys were identified but enrolled patients did not have a history of renal disease and specific clinical markers of lupus nephritis were not measured. Recently, other studies of IL-1 blockade using anakinra have shown promise in improving SLE-associated recurrent fevers, pericarditis, and macrophage activation syndrome, highlighting potential benefits for specific acute manifestations of lupus (434-437). Our data, however, suggests that chronic inhibition of IL-1 signaling, especially in patients with renal involvement, should be approached with caution to potentially prevent aggravation of disease.

Elevated levels of type I IFNs are a prominent feature of SLE that plays an important role in driving development and progression of lupus nephritis (438). Previous work suggests that inflammasome activation negatively regulates the expression of type I IFNs through IL-1β-mediated signaling in the context of malaria infection (439). In our study, we did not identify

differential expression of type I IFN signaling pathways between the NZM-*Il1b*$^{-/-}$ and NZM kidneys. This lack of difference could suggest that regulatory mechanisms in an infection setting may differ from those in an autoimmune setting in which inflammatory pathways, including type I IFN pathways, are already highly activated and dysregulated. Prior work indicates that females mount higher type I IFN responses that make them more prone to autoimmune diseases (440). This suggests that, perhaps, loss of the repressive effects of IL-1β nudges the type I IFN-dependent nephritis even further. This could partially explain the sexual dimorphism that is seen in these mice.

In summary, we have described an unexpected inflammatory regulatory role for IL-1β in renal injury in female NZM2328 mice. In its absence, mice develop worsened proteinuria and immune complex deposition in a sex-specific manner. We hypothesize that the more highly dysregulated inflammatory environment present in female NZM mice is needed for this sex-biased response. Further research into the specific mechanisms at play in our model is needed; until then, caution regarding the use of inflammasome and IL-1 inhibitors in human lupus (especially in patients with LN) should be considered.

Chapter 4 – Discussion and Future Directions

These chapters have explored the role of innate cytokines involved in lupus pathogenesis and progression. We showed that type I IFNs enhance keratinocyte apoptosis, but not necroptosis or pyroptosis, after UVB exposure. This occurs through a caspase-8- and IRF1-dependent mechanism, but surprisingly, occurs independently of IFN-inducible XAF1, TRAIL, and TNF. Additionally, we show a similar phenomenon *in vivo* where epidermal overexpression of IFN-κ in a transgenic mouse model also increases keratinocyte apoptosis following stimulation with a single dose of UVB light. We further showed that loss of IL-1β, a highly inflammatory cytokine activated through the inflammasome, is not protective in the lupus-prone NZM2328 mouse model. Intriguingly, our data suggests a potential sex-biased protective role for IL-1β in the kidneys of nephritic mice. In fact, in its absence, female, but not male, mice developed more severe proteinuria and increased renal immune complex deposition. RNA-sequencing analysis of the kidneys identified upregulation of TNF and IL-17 signaling pathways specifically in female NZM-*Il1b*$^{-/-}$ mice that may account for these differences. We also identify upregulation of these pathways in kidney biopsies from lupus nephritis patients, suggesting involvement of these pathways in disease. Further research is needed to clarify how IFN-inducible IRF1 drives caspase-8 dependent apoptosis after UVB exposure in the skin and to determine how loss of IL-1β enhances TNF and IL-17 signaling in nephritic kidneys to worsen disease in a female-biased manner.

4.1 Are there other mechanisms by which apoptosis is enhanced in SLE?

While we have identified IRF1 and caspase-8 as important regulators of type I IFN-enhanced keratinocyte apoptosis induced by UVB exposure, there are likely other factors contributing to epidermal apoptosis and inflammation in SLE. Of particular interest is the potential involvement of altered nucleic acid sensing in enhancing type I IFN expression and UVB-mediated apoptosis.

Alu elements are a class of short interspersed elements (SINEs), a type of retrotransposon, that contain many alternating purine-pyrimidine base sequences that are known to form Z-DNA (441). ADAR1 binds to these Z-DNA-forming regions. The repetitive structure of Alu RNA forms dsRNA that ADAR1 can also bind to in order to perform its RNA-editing functions. This results in destabilization of the dsRNA and prevents immune system activation. Interestingly, type I IFNs can induce transcription of Alu elements and Alu transcripts are upregulated in whole blood samples from SLE patients compared to healthy controls (442). It may be that IFN excessively stimulates transcription of these Alu elements to such an extent that there is not enough ADAR1 available to destabilize the dsRNA that forms. This would account for the increased levels of Alu transcripts found in SLE patients. These dsRNA-forming Alu transcripts could go on to activate cytosolic RNA sensors, induce more IFN production, and form a feed-forward loop of inflammation.

Differential regulation of ADAR1 isoforms may also contribute to pathological responses such as those seen in SLE. ADAR1-p150 is induced by IFN, thus, levels of this isoform would be expected to change in response to changes in IFN signaling. Because SLE patients typically have a prominent type I IFN signature, it is plausible that this would promote increased expression of the p150 isoform. Studies have shown that levels of ADAR1 usually correlate with increased

editing activity both in terms of editing rate and editing of non-typical sites (175). Elevated global RNA-editing levels are found in the blood of SLE patients compared to healthy controls (173) and expression of ADAR1 is increased in SLE T cells (170-172). This may drive the generation of neoantigens that are recognized by the immune system to activate inflammatory responses in SLE.

Dysregulated activity of the ADAR1-p110 isoform may influence cellular responses to UV stimulation. This isoform has negative regulatory functions in modulating UV-induced apoptosis by inhibiting the degradation of anti-apoptotic transcripts (163). While, in the healthy state, both the p110 and p150 isoforms of ADAR1 contribute to RNA-editing activities, p150 is the dominant editing isoform as it has an additional binding domain that allows it to bind to a broader range of RNA molecules (443). With SLE patients having more type I IFNs that induce more Alu transcripts that need to be edited in order to prevent immune recognition, it may be that the editing activity of p110 becomes more critical. Increased editing from this isoform could also help explain the increased rates of RNA-editing in SLE. This, in turn, would mean that less ADAR1-p110 is readily available to translocate to the cytosol to protect anti-apoptotic transcripts upon UV irradiation. This would allow SMD to take place, pushing the cell towards apoptotic cell death. Together, this could provide an additional mechanism by which IFN-exposed keratinocytes undergo increased rates of UVB-induced apoptosis.

Alternatively, it may be that there is an increased demand for RNA-editing activity in SLE that, particularly in the context of UV exposure, cannot be met because the p110 isoform is carrying out other functions. Rather than the role of p110 being subverted to RNA-editing duties, it continues with its role in inhibiting degradation of anti-apoptotic transcripts. This, however, could leave some of dsRNA in a stable form able to activate cytosolic RNA sensors and induce

production of additional type I IFNs that could enhance UVB-induced apoptosis as described in Chapter 2.

While a role for necroptosis in driving inflammatory responses to UV light in IFN-rich skin was ruled out in our study, ZBP1 may still be an important mediator of photosensitivity in SLE. Recent work identified a cell death-independent function of ZBP1 during times of mitochondrial dysfunction (444). Upon mitochondrial DNA (mtDNA) stress, Z-DNA accumulates in the mitochondria and the cytosol. ZBP1 expression is strongly induced and this promotes cGAS localization to the cytosol where these sensors interact via the RHIM domains of ZBP1 and the N-terminus of cGAS. The DNA binding domains of both ZBP1 and cGAS are bridged by DNA. This DNA binding activates the cGAS-STING pathway and subsequent type I IFN production. ZBP1 enhances the cytosolic retention of cGAS in the cytosol potentiating a sustained IFN response. While this study was done in fibroblasts and cardiomyocytes, ZBP1 is expressed in keratinocytes and is known to contribute to skin inflammation (445).

It is well known that UVB irradiation induces mitochondrial dysfunction, therefore, future research should examine the involvement of these pathways in SLE. Keratinocytes treated with or without IFN-α and SLE and healthy control keratinocytes could be treated with UVB light and stained for Z-DNA. The percentage of cGAS in nuclear vs. cytosolic fractions could be determined by Western blot and co-immunoprecipitation experiments would confirm ZBP1-cGAS interactions. In the context of our data showing that type I IFN enhances UVB-induced apoptosis, it would be intriguing to knockout ZBP1 in keratinocytes and observe how this affects the apoptotic phenotype. This is of particular relevance as we have identified this process to be IRF1-dependent and IRF1 is a known transcriptional regulator of ZBP1 (446).

4.2 Is dysregulated plasma membrane rupture a driver of inflammation in SLE?

Lytic cell death pathways such as pyroptosis and necroptosis are inflammatory secondary to plasma membrane rupture (PMR) and release of PAMPs and DAMPs into the extracellular space. Until recently, PMR was thought to be a passive event that occurred following pore formation and cellular swelling mediated by osmotic pressure. A new study has challenged this long-standing theory, finding that PMR is actively regulated by a cell surface protein called ninjurin-1 (NINJ1) (447). Prior to this study, NINJ1 was already recognized for a role in regulating cellular adhesion (448), and previous work suggested its involvement in programmed cell death through modulation of cell-cell interactions (449). However, no direct ties between NINJ1 and cell death had previously been made.

While our initial hypothesis was that the type I IFN rich environment in lupus skin would promote a switch to primarily lytic keratinocyte cell death following UVB stimulation, we determined that IFN-primed keratinocytes undergo increased rates of apoptosis. This, however, leaves us with the question of why this "immunologically silent" form of cell death leads to so much inflammation. One possibility, which has been explored in previous studies and will be further discussed in Section 4.3, is that defective clearance mechanisms in SLE result in inefficient removal of apoptotic debris. Apoptotic cells that are not properly removed undergo secondary necrosis and intracellular contents are released into the extracellular space as they would have following lytic cell death.

NINJ1 has yet to be studied in the context of lupus but has been implicated in other autoimmune and autoinflammatory diseases. A role for NINJ1 in modulating disease processes has been identified in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis (MS). Expression of NINJ1 is increased on the endothelial cells of the blood-

brain barrier as well as on brain infiltrating myeloid cells in both EAE and in active MS lesions (450, 451). Blocking NINJ1 in EAE models reduced clinical disease activity as a result of reduced adhesion and infiltration of leukocytes across the blood-brain barrier (450-452). This work suggests that NINJ1 is a potential therapeutic target for modulating disease, however, how inhibition of NINJ1 affected PMR was not evaluated in these studies. Further, a recent study observed upregulation of NINJ1 in patient PBMCs during flares of gout (453). Loss of NINJ1 in both a monosodium urate (MSU)-induced rat and mouse model resulted in reduced joint swelling and reduced gout flare, respectively, that correlated with PMR induction (453).

NINJ1 is a target of p53, a tumor suppressor protein, and its expression can be induced by DNA damage in a p53-dependent manner. p53 can be activated by UV irradiation (454) and lesional CLE keratinocytes express higher levels of p53 (455). It is, therefore, reasonable to expect that these increased levels of p53 in CLE keratinocytes would translate to increased levels of NINJ1. Expression of NINJ1 should be measured in CLE lesions and healthy control skin by IHC or via qRT-PCR and Western blot analyses of keratinocytes isolated from skin biopsies. It would also be interesting to determine whether NINJ1 expression in keratinocytes is influenced by IFN treatment with or without UVB stimulation. Given that NINJ1 is induced in keratinocytes by ionizing radiation in a p53-dependent manner (456), it is reasonable to assume that a similar response may occur following UVB exposure. Whether levels of NINJ1 correlate with rates of PMR is unknown but, if they do, this could provide rationale for the secondary necrosis that is likely occurring following the increased rates of UVB-induced apoptosis we identified in our study.

Based on the studies carried out in EAE and gout models, it would be intriguing to examine how blocking NINJ1, either via neutralizing antibody or genetic knockout, may affect disease in a

lupus-prone mouse model as well as in IFNκ^{EPI} mice. This could be tested both at baseline and in the context of UVB exposure. Levels of epidermal apoptosis could be measured as well as different parameters of disease such as proteinuria, anti-dsDNA antibody concentrations, and immune complex deposition. If there were improvements in these disease-mediated factors in the absence of NINJ1, it would likely be the result of modulating PMR and limiting release of autoantigens. It is also possible that the cell adhesion and recruitment roles of NINJ1 could be involved in lupus as well. Levels of lactate dehydrogenase (LDH), which is known to be released from secondary necrotic cells (457), could be measured to determine whether inhibition of NINJ1 reduces PMR. Additionally, leukocyte numbers in affected organs (e.g. kidney, skin, lymph nodes) could be measured to examine differences in migration that could result from defective cell adhesion in the absence of NINJ1. These experiments could help differentiate between the two demonstrated roles of NINJ1 in modulating disease.

4.3 Do defective clearance mechanisms contribute to inflammatory responses to UVB in SLE?

Mechanisms regulating the clearance of apoptotic debris by phagocytic cells is thought to be defective in SLE, however, whether this is the result of intrinsic defects or altered serum factors is still up for debate. UV irradiation of keratinocytes activates a disintegrin and metalloproteases (ADAMs) (458, 459). ADAMs are a family of transmembrane and secreted proteins with roles in cell adhesion, migration, proteolysis, and signaling (460). Many ADAMs, including ADAM10 and ADAM17, contain functional metalloproteinase domains that can proteolytically process the ectodomains of various signaling molecules and cell surface receptors. Among these surface receptors cleaved by ADAM10/17 are Tyro3, Axl, and MerTK (TAM) receptors, a family of IFN-inducible receptor tyrosine kinases (461). TAM receptors share the ligands growth arrest specific-

6 (Gas6) and Protein S (ProS), which act as bridging molecules between TAM receptors and PtdSer on apoptotic cell membranes (462). This binding results in the PtdSer signal being transduced by the TAM receptor-ligand pair, triggering phagocytic engulfment of the apoptotic cell. However, ADAM10/17 activity can cleave off the ectodomain of the TAM receptors causing them to be shed from the cell surface. Once in the soluble form, these TAM receptors have an inhibitory effect, as they can act as a decoy receptor by binding the bridging ligands and masking PtdSer on apoptotic cells, preventing efficient recognition and clearance (463, 464).

SLE patient plasma is reported to have increased levels of soluble TAM receptors that correlate with disease activity and severity (465-467), suggesting a pathogenic role for these receptors in disease that could help clarify how apoptosis, a non-inflammatory mechanism of cell death, occurs in parallel with excessive inflammation in lupus skin. We were surprised to find that type I IFNs enhanced apoptosis in our study, as activation of inflammatory pathways of cell death would have provided a rational explanation to this. However, defective clearance of apoptotic cells that ultimately ends in PMR would serve as an alternate justification for this inflammation that should be further explored.

Levels of ProS and Gas6 in SLE patients varies between studies and subtypes of disease. Free ProS levels are reportedly lower in most studies (468-472), while levels of Gas6 are increased in some SLE patients (470, 472, 473) and decreased in others (465, 474). It is important to note that ProS and Gas6 are also important regulators of thrombosis. Gas6 contributes to platelet aggregation and inhibiting Gas6 has been shown to prevent thrombosis in mice (475). Therefore, levels of ProS and Gas6 may be different in SLE patients at high risk for thrombosis. Interestingly, autoantibodies to ProS have been identified in SLE patients and are associated with risk of thrombosis (476). The presence of anti-ProS antibodies might help explain the decreased levels of

free ProS in many SLE patients Additionally, it may be possible that differential expression of these TAM ligands could influence efficiency of phagocytic clearance, but additional research would be needed to determine if this contributes to photosensitivity in SLE.

TAM signaling plays a fundamental role in mediating innate immune responses. Activation of TAM receptors promotes an anti-inflammatory response involving negative regulation of TLRs, induction of suppressor of cytokine signaling (SOCS) proteins, and suppression of type I IFNs (477). Remarkably, induction of SOCS proteins, specifically SOCS1 and SOCS3, is dependent on TAM receptors hijacking the IFNAR/STAT1 signaling cassette (477). As a result, TAM-induced SOCS proteins inhibit type I IFN signaling and, thus, limit inflammation. SOCS proteins are reportedly dysregulated in SLE, and loss of these suppressive mechanisms may contribute to the inflammation characteristic of the disease. Expression of *SOCS1* is decreased in SLE PBMCs and patients with active SLE have lower expression compared to those with inactive disease (478). Furthermore, in a Chinese population, photosensitivity was associated with *SOCS1* levels in SLE patients (478).

These studies bring about two hypotheses regarding mechanisms of defective clearance and loss of inhibitory signaling that follows efficient induction of phagocytosis. Firstly, excessive amounts of IFN signaling present in lupus skin may overwhelm this regulatory system and prevent activation of SOCS proteins via the TAM-mediated hijacking of the IFNAR/STAT1 pathway. There is a limited amount of STAT1 protein available per cell, thus, it may be possible that activation of the TAM pathway could be dysregulated and contribute to unchecked levels of inflammation after UV exposure. Secondly, as discussed above, increased expression of soluble TAM receptors in SLE may prevent activation of TAM signaling by preventing ligation to apoptotic cell-expressed PtdSer. In turn, this would prevent TAM-induced activation of SOCS1/3

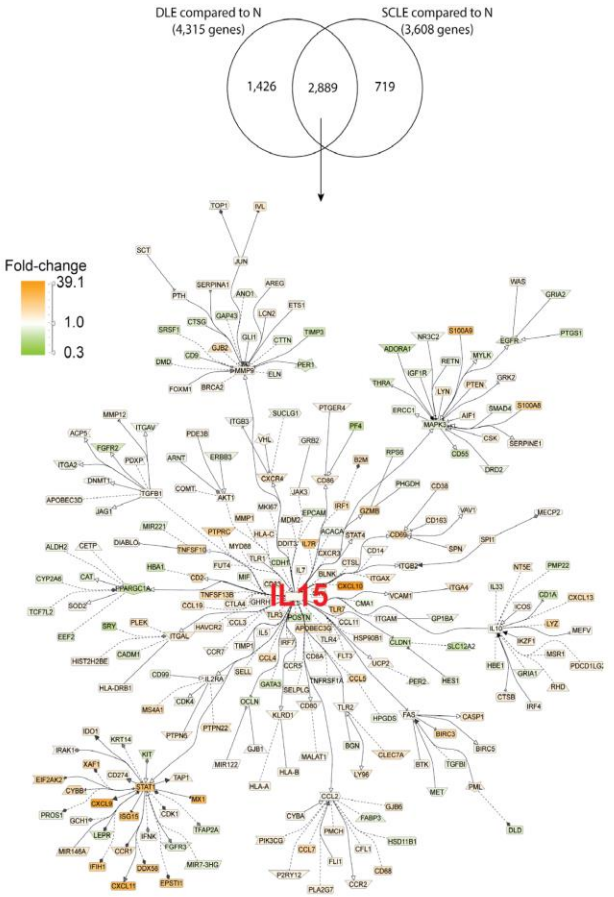
and limit the anti-inflammatory effects typically provided by these proteins. This would fit with the reports that *SOCS1* expression is decreased while type I IFN expression is increased in SLE patients.

4.4 How do skin-infiltrating immune cells further inflammatory responses in SLE?

Our work supports keratinocytes as being important players in the inflammatory responses that drive photosensitivity in lupus. However, *in vivo* we know that keratinocytes do not exist in isolation and other cell types contribute to the inflammation characteristic of sunlight-induced lesions and disease flares. A role for natural killer (NK) cells has been identified in cutaneous diseases such as psoriasis (479) and atopic dermatitis (480), however, NK cells are understudied in lupus. NK cells are reported to be decreased in number in circulation of SLE patients (481), however, few studies have investigated whether this is secondary to these cells moving to the tissue. Due to the cytotoxic nature of NK cells, it may be that they are involved in lesion development in UV-exposed lupus skin. Therefore, future research should determine the role of NK cells in the skin of cutaneous lupus patients.

Regulation of NK cell development, homeostasis, and proliferation (482) depends on IL-15, a cytokine that can be produced by dendritic cells after stimulation with type I IFNs (483). Serum levels of IL-15 are significantly increased in SLE patients (484) and expression of the proliferation marker Ki67 on SLE NK cells correlates with these higher levels of IL-15 as well as with disease severity (485). Using the Genomatix Pathway System, we evaluated CLE lesions and identified upregulation of IL-15 as a prominent node (Fig 4-1A). This supports IL-15 as an important component of gene expression changes in CLE. Additionally, our RNA-seq data indicate that IFN-α treatment of keratinocytes results in increased expression of several genes involved in NK cell regulation. These include IL-15, chemokines that promote NK cell recruitment such as

A.



B.

	HC vs IFNa log2FC	HC vs IFNa p value	SLE vs IFNa log2FC	SLE vs IFNa p value
NK CELL CHEMOKINES				
CXCL10	9.66	2.84E-02	10.50	1.47E-98
CXCL11	9.34	1.76E-02	10.38	6.74E-301
CCL5	1.08	8.40E-04	1.34	1.22E-06
NK CELL CYTOTOXICITY				
IL15	2.24	8.51E-16	2.22	1.67E-21
CADM1	0.83	1.22E-06	0.58	1.21E-02

C.

	BALB/c: no UV vs. UV log2FC	BALB/c Adj. q value	NZM: no UV vs. UV log2FC	NZM Adj. q value
Ccl2	**0.69**	**6.16E-01**	**2.35**	**4.80E-03**
Cxcl3	**2.91**	**5.19E-01**	**7.87**	**3.00E-03**
Rarres2	**1.20**	**3.20E-01**	**1.81**	**5.36E-03**
Ccl3	3.67	1.93E-02	5.61	1.42E-04
Ccl4	4.62	2.55E-03	7.76	1.17E-03

Figure 5 – Upregulation of NK cell-related cytokines and chemokines by IFNs and in lupus patients and mouse models. (**A**) The Genomatix Pathway System was used to evaluate shared gene expression changes in CLE lesions. Genes in orange are upregulated and genes in green are downregulated in CLE lesions. (**B**) Primary healthy control (HC) or SLE keratinocytes were primed +/- 1000U/ml IFN-α for 6 hours. Results expressed as mean fold change (log_2) in comparison to control values. (**C**) Dorsal skin of NZM2328 and BALB/c mice (n=5 each) was treated with 100mJ/cm^2 UVB for 5 days. 24 hours after the 5th UV treatment, gene expression in the skin was analyzed by RNA-seq. Results expressed as mean fold change (log_2) in comparison to control values (no UV). Bolded genes are increased in NZM>BALB/c.

CXCL10, CXCL11, and CCL5 (486) and factors that promote NK cell activation such as CADM1 (487) (Fig. 4-1B). We also observe heightened expression of several NK cell chemokines in the skin of lupus-prone NZM2328 mice vs. wild-type mice after exposure to UVB light (Fig. 4-1C).

Based on these preliminary data, it is possible that chronic type I IFN overexpression primes for secretion of chemokines that recruit NK cells to the skin and cytokines that enhance their cytotoxicity toward keratinocytes. This could be tested by measuring healthy control vs. SLE NK cell migration and killing of IFN-primed keratinocytes *in vitro* and examining the role of IFN-κ in NK cell recruitment and cytotoxicity after UVB exposure *in vivo*. This work would support the establishment of a new role for IFNs in lupus skin through their impact on NK cell recruitment and activation and highlight a new mechanism for NK cell-mediated keratinocyte death.

It is also possible that cytotoxic $CD8^+$ T cells (CTLs) contribute to induction of apoptotic mechanisms in lupus skin. Similar to NK cells, CTLs carry out their killing function directly through release of cytotoxic granules containing apoptosis-inducing granzymes and pore-forming perforin. Granzyme B is one of the serine protease granzymes that is released by CTLs (488). It is delivered into the cytoplasm of targeted cells where it can target cellular substrates and initiate apoptosis. Granzyme B has been identified in dermal and epidermal lymphocytic infiltrate in CLE skin as well as in the intercellular spaces surrounding apoptotic basal keratinocytes (489). Moreover, in scarring CLE subtypes, increased expression of granzyme B is found in lesional lymphocytes and this was strongly correlated with expression of the MxA (490).

IFN-inducible cytokines including CXCL10 and CXCL11 are involved in T cell trafficking with CXCR3 highly expressed on CTLs (491) and this may drive CTL recruitment to the site of CLE lesion formation. Furthermore, type I IFNs were shown to have the capacity to augment

granzyme B-mediated cytotoxicity of CTLs within the islets of patients with type I diabetes (492). Together, this supports a potential role for type I IFN-driven recruitment and activation of CTLs to the skin following UVB-exposure where they contribute to keratinocyte apoptosis and tissue destruction. This could be addressed in future studies by examining the presence of CTLs within the epidermis of UV-exposed IFNκ^{EPI} and wild-type mice and determining how depletion of these cells influences keratinocyte apoptosis, as well as by staining for granzyme B in the skin.

4.5 Why is inflammasome blockade protective in SLE, but loss of IL-1β is not?

Our initial hypothesis was that loss of IL-1β in the lupus-prone NZM2328 model would limit disease severity and improve overall survival. This was largely based on previous work that shows that inflammasome blockade is protective in mouse models of lupus. Because IL-1β is a quintessential pro-inflammatory cytokine that has been implicated in a variety of inflammatory and autoimmune diseases, it was logical to speculate that it would be involved in driving inflammation in SLE. Our work clearly suggests, however, that this may not be the case. How then is it that inhibiting the inflammasome is protective, but inhibiting IL-1β, which is activated by the inflammasome, is not?

The most straightforward explanation to this question is that another component of the inflammasome is responsible for driving the pathogenic effects of the inflammasome in SLE. Processing of pro-IL-18 occurs simultaneously with processing of pro-IL-1β by caspase-1. The mature forms of these proteins are both released from the cell and go on to induce inflammatory responses in a paracrine manner. Based on our data and the significant body of literature supporting a role of for IL-18 in SLE, it is likely that this is the more relevant cytokine that is activated by the inflammasome. To briefly summarize the available data regarding IL-18 in lupus, levels of IL-18 are increased in the serum and skin of SLE patients compared to healthy controls (371, 493),

treatment with exogenous IL-18 worsened disease severity in the MRL/lpr mouse model (494), and levels of IL-18 correlated with severity of disease, including LN (495).

IL-18, together with IL-12, activates NK cells and induces production of IFN-γ (496), which has been implicated in SLE (497). In an EAE model, IL-18 was able to stimulate production of IL-17 from gamma-delta (γδ) T cells (498), which may contribute to LN. Further, IL-18 can induce expression of pro-apoptotic FasL, cell adhesion molecules, and chemokines and other pro-inflammatory cytokines (499, 500). These immunostimulatory effects support IL-18 being the mediator of the damaging effects of the inflammasome. To test this, a study similar to ours could be conducted using NZM-*Il18*$^{-/-}$ mice to determine if these mice are protected from SLE manifestations. It may be possible that loss of both IL-1β and IL-18 may be necessary to provide full protection so double knockout NZM-*Il1b*$^{-/-}$*Il18*$^{-/-}$ could be tested as well.

Interestingly, deletion of caspase-1 increases survival in animal models of endotoxin shock (501, 502). Similar to our study, this protective role for caspase-1 is independent of its involvement in activating pro-IL-1β as IL-1β-deficiency is not protective in this model either (502). This study went a step further and determined that IL-18 knockout mice and IL-1β/IL-18 double knockout mice were sensitive to sepsis. They determined that protection in caspase-1 knockout mice resulted from reduction in splenic B cell apoptosis. In fact, there is increasing evidence to suggest that cross talk can occur between caspase-1 and apoptotic caspases. For example, caspase-1 has been reported to cleave Bid (which is typically cleaved by caspase-8) triggering intrinsic apoptosis (503, 504). Therefore, it is possible that capsase-1-deficiency in a lupus-prone mouse model could be protective owing partly to a reduction in apoptosis, rather than pyroptosis during which IL-1β would be secreted.

Caspase-1 can also act as a scaffolding molecule that promotes RIP2-mediated NFκB activation (505). In the sepsis study described above, levels of IL-6 (used as a measure of NFκB activation) were decreased in the caspase-1 knockout mice, but not in the IL-1β or IL-1β/IL-18 knockout mice (502). It is possible that this scaffolding function of caspase-1 could be involved in the differences observed in disease severity between caspase-1 knockout and IL-1β knockout in lupus-prone murine backgrounds. While caspase-1 knockout has already been studied in a pristane-induced lupus model (383), a caspase-1 knockout could be generated on the same NZM lupus-prone background and used to compare directly with the NZM-*Il1b*$^{-/-}$ and NZM mice. Levels of IL-6 could be measured in each strain to determine relative amounts of NFκB activation. If the scaffolding function of caspase-1 is driving disease pathogenesis, you would expect to see increased levels of IL-6 in the NZM and NZM-*Il1b*$^{-/-}$ mice compared to the NZM-*Casp1*$^{-/-}$ mice. To further confirm this role, NZM mice could be generated that have a mutation that renders caspase-1 enzymatically inactive (NZM-*Casp1*^{C284A}) (506). Disease parameters and survival could be monitored in these mice and compared to the other strains. It would be expected that the NZM-*Casp1*^{C284A} mice would not be protected in a similar manner to the NZM-*Casp1*$^{-/-}$ mice if the important function of caspase-1 in this setting is its activation of NFκB rather than its processing of IL-1β/IL-18 and induction of pyroptotic cell death.

4.6 What mechanism is responsible for enriched IL-17 signaling in the absence of IL-1β?

We observed increased expression of TNF and IL-17 signaling pathways by RNA-seq in the kidneys of NZM-*Il1b*$^{-/-}$ mice compared to NZM controls that may be responsible for driving increased immune complex deposition and proteinuria. Future studies should further investigate the link between loss of IL-1β and enrichment of these inflammatory pathways. This will be of particular interest as IL-1β plays a critical role in differentiation of Th17 cells (507, 508), which

are the canonical producers of IL-17. While it is seemingly contradictory that loss of IL-1β would result in the increase of a signaling pathway for which IL-1β is known to be important, there are several hypotheses that may explain this.

IL-17 producing Th17 cells have been implicated in the pathogenesis of several autoimmune diseases (509-511), and high levels of IL-17 are reported in human SLE sera (512). In addition to Th17 cells, several cell types produce IL-17 including $CD8^+$ T cells (513), γδ T cells (514-518), double negative (DN) T cells (519), innate lymphoid cells (518), invariant natural killer T (iNKT) cells (520), lymphoid tissue inducer-like cells (521), and neutrophils (522). Few studies have investigated the specific sources of IL-17 in SLE and LN, but it may be that sources other than Th17 cells are important in disease pathogenesis.

Populations of DN T cells are expanded in SLE patients and in some lupus-prone mouse models (523, 524) and these cells have been shown to be major producers of IL-17 (519). Further, these IL-17-producing DN T cells have been identified in kidney biopsies from patients with LN (519). In a murine model of crescentic glomerulonephritis, the primary source of IL-17 in the kidney was shown to change over the course of the disease (525). Later in disease, most IL-17 was produced by Th17 cells. However, at early time points, γδ T cells were the major producers of IL-17 with additional contributions from DN T cells and NKT cells. These $IL\text{-}17^+$ γδ T cells were dependent on renal dendritic cell-derived IL-23 (another cytokine reported to be highly expressed in human SLE sera (512)) and the transcription factor RORγt. Absence of γδ T cells in this model resulted in less neutrophil and macrophage infiltration into the kidney as well as reduced levels of serum creatinine, suggesting a pathogenic role for the IL-17-producing γδ T cells (525). Intriguingly, and relevant to our work, IL-17 production from γδ T cells was not dependent on IL-

1β (525). This suggests a potential mechanism by which loss of IL-1β, an important cytokine for Th17 cells, can still result in enhanced IL-17 signaling.

To address this, kidneys from NZM and NZM-*Il1b*$^{-/-}$ mice should be assessed for levels of these other IL-17 producing cell types, especially DN T cells and γδ T cells. If loss of IL-1β altered any of these IL-17 producing populations, this cell type could be depleted to determine how these cells influence the differences in kidney disease we observed between mouse strains. While we did not observe differences in immune cell infiltration in the absence of IL-1β, it would be interesting to examine this at an earlier timepoint. If γδ T cell-derived IL-17 is more important earlier in disease and this promoted increased immune cell infiltration into the kidney, we may have missed this by only looking at renal histology at our study's endpoint. It may be that earlier cellular infiltrates contribute to the damage we observe at this later timepoint, but that the cells are no longer present in the tissue at this time.

A second mechanism by which IL-17 signaling pathways may be elevated in the absence of IL-1β is through IL-18 stimulation. IL-18 has been shown to promote IL-17 production from mucosal associated invariant T (MAIT) cells (526). Tissue-resident MAIT cells are found in both healthy and diseased kidneys and numbers of these cells correlate with the histologic severity of chronic kidney disease (527). Perhaps, in the absence of IL-1β, IL-18 stimulation of IL-17 production may serve as a compensatory mechanism. Generating an IL-1β /IL-18 double knockout NZM mouse would allow for investigation into any contributions from IL-18 to this phenotype, specifically any that occur to compensate for the loss of IL-1β. Levels of MAIT cells in the kidney could also be examined as a possible source of IL-17 in the NZM-*Il1b*$^{-/-}$ mice.

If the primary cell type responsible for producing IL-17 in this model was identified and shown to be involved in increasing the severity of renal disease, it would also be informative to

generate a knockout strain of this cell type on the NZM background. The severity of kidney disease in these mice would help determine the overall significance of this cell type in LN. If the newly generated KO mice had less severe kidney disease compared to NZM, this would suggest that this identified cell type is pathogenic on its own. If the newly generated KO mice had comparable kidney disease to NZM, this would be indicative of this cell type only being pathogenic in the context of IL-1β depletion. This would be informative for use of IL-1β inhibitors in SLE patients. If you wanted to treat a patient with SLE with one of these IL-1β inhibiting drugs, it may be beneficial to simultaneously target the cell type/cytokine responsible for increasing IL-17 signaling in the absence of IL-1β (if possible with current therapeutic options).

4.7 Conclusions

In Chapter 2, we expand on our previous data showing that SLE keratinocytes undergo increased rates of cell death after exposure to UVB light compared to healthy controls (Fig. 4-2). Specifically, we identify that chronic exposure to type I IFNs primes keratinocytes for enhanced activation of apoptosis rather than inflammatory forms of programmed cell death. This apoptosis is caspase-8-dependent; however, it occurs independently of several known death ligands suggesting that caspase-8 is activated via ligand-independent death receptor activation. Loss of IRF1 abrogated the IFN-mediated increase in UVB-induced apoptosis. It is likely that type I IFNs drive expression of IRF1 which, in turn, enhances expression of pro-apoptotic factors such as caspase-8 and, perhaps, the nucleic acid sensor ZBP1 which further potentiates IFN signaling and apoptotic responses. We also show that overexpression of IFN-κ in the epidermis of UVB-exposed mice significantly increases keratinocyte apoptosis. Future studies should address the ways in which apoptosis, generally considered to be an anti-inflammatory pathway, generates an immunostimulatory environment in SLE skin. This may involve mechanisms such as altered

nucleic acid sensing, defective clearance of apoptotic debris, dysregulated plasma membrane rupture, and increased immune cell infiltration.

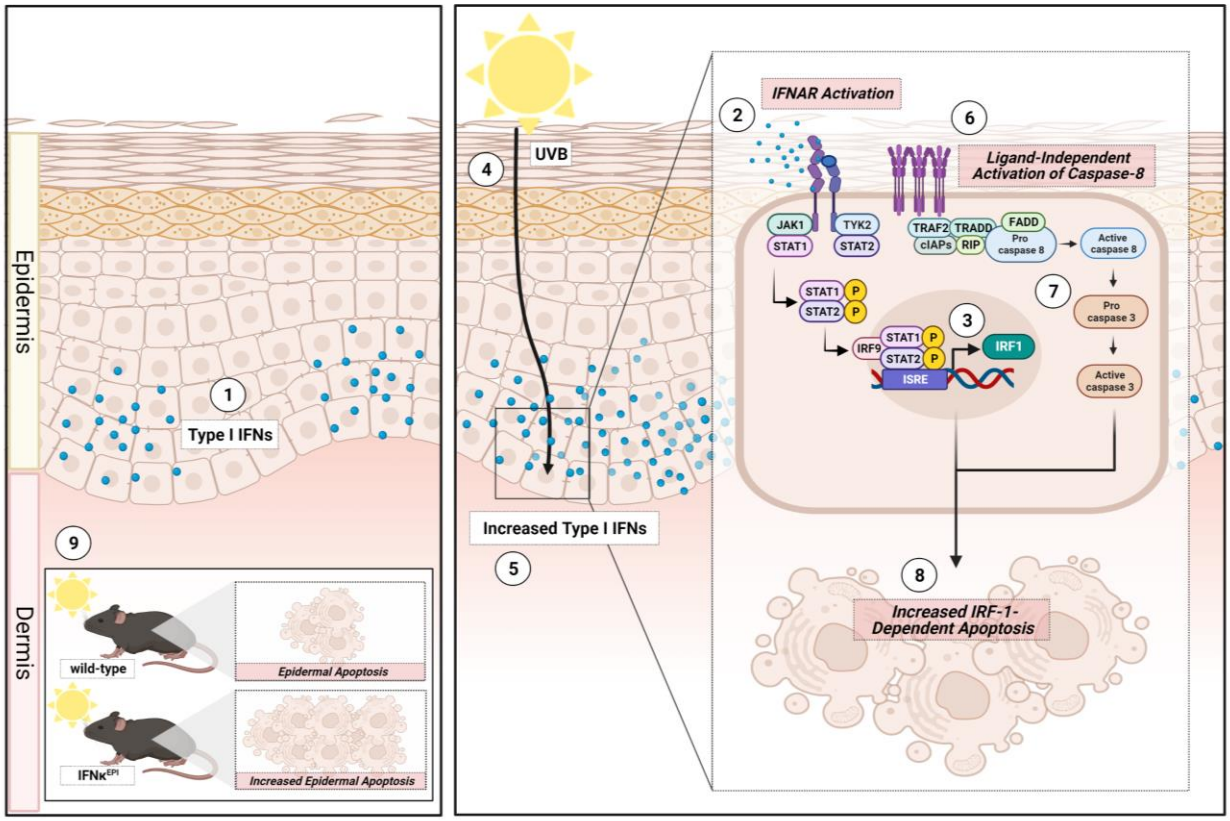


Figure 6 – Schematic of IFN-primed keratinocyte responses to UVB light.
(1) Levels of type I IFNs are enhanced in lupus skin at baseline. (2) Type I IFNs bind to the type I IFN receptor (IFNAR) on keratinocytes, activating a signaling cascade and (3) resulting in expression of the transcription factor IRF1. (4) Upon UVB exposure (5) keratinocytes produce increased levels of type I IFNs. (6) Death receptors on keratinocytes activate caspase-8 in a ligand-independent manner. (7) Activated caspase-8 cleaves pro-caspase-3 to its mature form (8) which triggers apoptotic death of the cell. This occurs in an IRF1-dependent manner. (9) In mice, UVB irradiation of mice that overexpress IFNκ- in the epidermis ($IFN\kappa^{EPI}$) results in increased rates of epidermal apoptosis compared to wild-type mice. Created with BioRender.com.

In Chapter 3, our data indicate that while IL-1β is a highly inflammatory cytokine that contributes to autoimmunity in several diseases, it may exert a protective effect in SLE, specifically in the kidneys of females with LN. While survival and autoantibody production were not significantly different in NZM-*Il1b*$^{-/-}$ mice compared to NZM controls, the absence of IL-1β increased immune complex deposition in the kidneys and levels of proteinuria. Interestingly, these differences in kidney disease were only present in female mice. We identified enrichment of IL-

17 and TNF signaling pathways in female NZM-*Il1b*$^{-/-}$ kidneys that may contribute to the inflammatory responses that drive nephritis. The upregulation of renal IL-17 signaling pathways in the absence of IL-1β may be the result of IL-17 secretion from non-Th17 cells such as γδ T cells and DN T cells, but future studies should address this. This study may indicate that precautions should be taken when treating SLE patients with inhibitors of IL-1β to prevent the possibility of aggravating nephritis.

Collectively, this dissertation highlights important mechanisms by which dysregulation of innate immunity contributes to SLE manifestations in the skin and kidneys. Continued investigation into the mechanisms underlying type I IFN-mediated skin inflammation and IL-1β-mediated kidney protection will help inform development of novel prophylactic options to prevent SLE flares and improve patient outcomes.

Appendix

Appendix A – Chapter 3 Supplementary Tables

Table A-1 – Lists of IL-17 signaling pathway genes expressed in each human dataset

KEGG IL-17 signaling pathway (https://www.genome.jp/entry/pathway+hsa04657)

List of the 77 genes expressed in the human glomerular discovery dataset and used for IL-17 pathway score calculation

Gene	Description
51433	ANAPC5; anaphase promoting complex subunit 5 [KO:K03352]
836	CASP3; caspase 3 [KO:K02187] [EC:3.4.22.56]
841	CASP8; caspase 8 [KO:K04398] [EC:3.4.22.61]
6356	CCL11; C-C motif chemokine ligand 11 [KO:K16597]
6361	CCL17; C-C motif chemokine ligand 17 [KO:K21083]
6347	CCL2; C-C motif chemokine ligand 2 [KO:K14624]
6364	CCL20; C-C motif chemokine ligand 20 [KO:K14625]
6354	CCL7; C-C motif chemokine ligand 7 [KO:K05509]
1051	CEBPB; CCAAT enhancer binding protein beta [KO:K10048]
1147	CHUK; component of inhibitor of nuclear factor kappa B kinase complex [KO:K04467] [EC:2.7.11.10]
1437	CSF2; colony stimulating factor 2 [KO:K05427]
1440	CSF3; colony stimulating factor 3 [KO:K05423]
2919	CXCL1; C-X-C motif chemokine ligand 1 [KO:K05505]
3627	CXCL10; C-X-C motif chemokine ligand 10 [KO:K12671]
2920	CXCL2; C-X-C motif chemokine ligand 2 [KO:K05505]
2921	CXCL3; C-X-C motif chemokine ligand 3 [KO:K05505]
6372	CXCL6; C-X-C motif chemokine ligand 6 [KO:K05506]
3576	CXCL8; C-X-C motif chemokine ligand 8 [KO:K10030]
1994	ELAVL1; ELAV like RNA binding protein 1 [KO:K13088]
8772	FADD; Fas associated via death domain [KO:K02373]
2353	FOS; Fos proto-oncogene, AP-1 transcription factor subunit [KO:K04379]
2354	FOSB; FosB proto-oncogene, AP-1 transcription factor subunit [KO:K09029]
8061	FOSL1; FOS like 1, AP-1 transcription factor subunit [KO:K04502]
2932	GSK3B; glycogen synthase kinase 3 beta [KO:K03083] [EC:2.7.11.26]
3320	HSP90AA1; heat shock protein 90 alpha family class A member 1 [KO:K04079]
3326	HSP90AB1; heat shock protein 90 alpha family class B member 1 [KO:K04079]
7184	HSP90B1; heat shock protein 90 beta family member 1 [KO:K09487]
3458	IFNG; interferon gamma [KO:K04867]
3551	IKBKB; inhibitor of nuclear factor kappa B kinase subunit beta [KO:K07209] [EC:2.7.11.10]
9641	IKBKE; inhibitor of nuclear factor kappa B kinase subunit epsilon [KO:K07211] [EC:2.7.11.10]
3596	IL13; interleukin 13 [KO:K05435]
27190	IL17B; interleukin 17B [KO:K05460]
23785	IL17RA; interleukin 17 receptor A [KO:K05164]
55540	IL17RB; interleukin 17 receptor B [KO:K05165]
84818	IL17RC; interleukin 17 receptor C [KO:K05166]
3553	IL1B; interleukin 1 beta [KO:K04519]
64806	IL25; interleukin 25 [KO:K05493]
3569	IL6; interleukin 6 [KO:K05405]
3725	JUN; Jun proto-oncogene, AP-1 transcription factor subunit [KO:K04448]
3727	JUND; JunD proto-oncogene, AP-1 transcription factor subunit [KO:K04449]
3934	LCN2; lipocalin 2 [KO:K21129]
6885	MAP3K7; mitogen-activated protein kinase kinase kinase 7 [KO:K04427] [EC:2.7.11.25]
5594	MAPK1; mitogen-activated protein kinase 1 [KO:K04371] [EC:2.7.11.24]
5602	MAPK10; mitogen-activated protein kinase 10 [KO:K04440] [EC:2.7.11.24]
5600	MAPK11; mitogen-activated protein kinase 11 [KO:K04441] [EC:2.7.11.24]
6300	MAPK12; mitogen-activated protein kinase 12 [KO:K04441] [EC:2.7.11.24]
5603	MAPK13; mitogen-activated protein kinase 13 [KO:K04441] [EC:2.7.11.24]
1432	MAPK14; mitogen-activated protein kinase 14 [KO:K04441] [EC:2.7.11.24]
5595	MAPK3; mitogen-activated protein kinase 3 [KO:K04371] [EC:2.7.11.24]
5597	MAPK6; mitogen-activated protein kinase 6 [KO:K06855] [EC:2.7.11.24]
5598	MAPK7; mitogen-activated protein kinase 7 [KO:K04464] [EC:2.7.11.24]
5599	MAPK8; mitogen-activated protein kinase 8 [KO:K04440] [EC:2.7.11.24]
5601	MAPK9; mitogen-activated protein kinase 9 [KO:K04440] [EC:2.7.11.24]
4312	MMP1; matrix metallopeptidase 1 [KO:K01388] [EC:3.4.24.7]
4322	MMP13; matrix metallopeptidase 13 [KO:K07994] [EC:3.4.24-.]
4318	MMP9; matrix metallopeptidase 9 [KO:K01403] [EC:3.4.24.35]
4586	MUC5AC; mucin 5AC, oligomeric mucus/gel-forming [KO:K21125]
727897	MUC5B; mucin 5B, oligomeric mucus/gel-forming [KO:K13908]
4790	NFKB1; nuclear factor kappa B subunit 1 [KO:K02580]
4792	NFKBIA; NFKB inhibitor alpha [KO:K04734]
5743	PTGS2; prostaglandin-endoperoxide synthase 2 [KO:K11987] [EC:1.14.99.1]
5970	RELA; RELA proto-oncogene, NF-kB subunit [KO:K04735]
6279	S100A8; S100 calcium binding protein A8 [KO:K21127]
6280	S100A9; S100 calcium binding protein A9 [KO:K21128]
6426	SRSF1; serine and arginine rich splicing factor 1 [KO:K12890]
23118	TAB2; TGF-beta activated kinase 1 (MAP3K7) binding protein 2 [KO:K04404]
29110	TBK1; TANK binding kinase 1 [KO:K05410] [EC:2.7.11.10]
7124	TNF; tumor necrosis factor [KO:K03156]
7128	TNFAIP3; TNF alpha induced protein 3 [KO:K11859] [EC:3.4.19.12]
8717	TRADD; TNFRSF1A associated via death domain [KO:K03171]
7186	TRAF2; TNF receptor associated factor 2 [KO:K03173] [EC:2.3.2.27]
7187	TRAF3; TNF receptor associated factor 3 [KO:K03174]
10758	TRAF3IP2; TRAF3 interacting protein 2 [KO:K21124]
9618	TRAF4; TNF receptor associated factor 4 [KO:K09848]
7188	TRAF5; TNF receptor associated factor 5 [KO:K09849]
7189	TRAF6; TNF receptor associated factor 6 [KO:K03175]
29761	USP25; ubiquitin specific peptidase 25 [KO:K11846] [EC:3.4.19.12]

List of the 80 genes expressed in the human glomerular validation dataset and used for IL-17 pathway score calculation

Gene	Description
51433	ANAPC5; anaphase promoting complex subunit 5 [KO:K03352]
836	CASP3; caspase 3 [KO:K02187] [EC:3.4.22.56]
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6356	CCL11; C-C motif chemokine ligand 11 [KO:K16597]
6361	CCL17; C-C motif chemokine ligand 17 [KO:K21083]
6347	CCL2; C-C motif chemokine ligand 2 [KO:K14624]
6364	CCL20; C-C motif chemokine ligand 20 [KO:K14625]
6354	CCL7; C-C motif chemokine ligand 7 [KO:K05509]
1051	CEBPB; CCAAT enhancer binding protein beta [KO:K10048]
1147	CHUK; component of inhibitor of nuclear factor kappa B kinase complex [KO:K04467] [EC:2.7.11.10]
1437	CSF2; colony stimulating factor 2 [KO:K05427]
1440	CSF3; colony stimulating factor 3 [KO:K05423]
2919	CXCL1; C-X-C motif chemokine ligand 1 [KO:K05505]
3627	CXCL10; C-X-C motif chemokine ligand 10 [KO:K12671]
2920	CXCL2; C-X-C motif chemokine ligand 2 [KO:K05505]
2921	CXCL3; C-X-C motif chemokine ligand 3 [KO:K05505]
6374	CXCL5; C-X-C motif chemokine ligand 5 [KO:K05506]
6372	CXCL6; C-X-C motif chemokine ligand 6 [KO:K05506]
3576	CXCL8; C-X-C motif chemokine ligand 8 [KO:K10030]
1994	ELAVL1; ELAV like RNA binding protein 1 [KO:K13088]
8772	FADD; Fas associated via death domain [KO:K02373]
2353	FOS; Fos proto-oncogene, AP-1 transcription factor subunit [KO:K04379]
2354	FOSB; FosB proto-oncogene, AP-1 transcription factor subunit [KO:K09029]
8061	FOSL1; FOS like 1, AP-1 transcription factor subunit [KO:K04502]
2932	GSK3B; glycogen synthase kinase 3 beta [KO:K03083] [EC:2.7.11.26]
3320	HSP90AA1; heat shock protein 90 alpha family class A member 1 [KO:K04079]
3326	HSP90AB1; heat shock protein 90 alpha family class B member 1 [KO:K04079]
7184	HSP90B1; heat shock protein 90 beta family member 1 [KO:K09487]
3458	IFNG; interferon gamma [KO:K04867]
3551	IKBKB; inhibitor of nuclear factor kappa B kinase subunit beta [KO:K07209] [EC:2.7.11.10]
9641	IKBKE; inhibitor of nuclear factor kappa B kinase subunit epsilon [KO:K07211] [EC:2.7.11.10]
8517	IKBKG; inhibitor of nuclear factor kappa B kinase regulatory subunit gamma [KO:K07210]
27190	IL17B; interleukin 17B [KO:K05460]
53342	IL17D; interleukin 17D [KO:K05462]
23785	IL17RA; interleukin 17 receptor A [KO:K05164]
55540	IL17RB; interleukin 17 receptor B [KO:K05165]
84818	IL17RC; interleukin 17 receptor C [KO:K05166]
132014	IL17RE; interleukin 17 receptor E [KO:K05168]
3553	IL1B; interleukin 1 beta [KO:K04519]
3569	IL6; interleukin 6 [KO:K05405]
3725	JUN; Jun proto-oncogene, AP-1 transcription factor subunit [KO:K04448]
3727	JUND; JunD proto-oncogene, AP-1 transcription factor subunit [KO:K04449]
3934	LCN2; lipocalin 2 [KO:K21129]
6885	MAP3K7; mitogen-activated protein kinase kinase kinase 7 [KO:K04427] [EC:2.7.11.25]
5594	MAPK1; mitogen-activated protein kinase 1 [KO:K04371] [EC:2.7.11.24]
5602	MAPK10; mitogen-activated protein kinase 10 [KO:K04440] [EC:2.7.11.24]
5600	MAPK11; mitogen-activated protein kinase 11 [KO:K04441] [EC:2.7.11.24]
6300	MAPK12; mitogen-activated protein kinase 12 [KO:K04441] [EC:2.7.11.24]
5603	MAPK13; mitogen-activated protein kinase 13 [KO:K04441] [EC:2.7.11.24]
1432	MAPK14; mitogen-activated protein kinase 14 [KO:K04441] [EC:2.7.11.24]
225689	MAPK15; mitogen-activated protein kinase 15 [KO:K19603] [EC:2.7.11.24]
5595	MAPK3; mitogen-activated protein kinase 3 [KO:K04371] [EC:2.7.11.24]
5596	MAPK4; mitogen-activated protein kinase 4 [KO:K06855] [EC:2.7.11.24]
5597	MAPK6; mitogen-activated protein kinase 6 [KO:K06855] [EC:2.7.11.24]
5598	MAPK7; mitogen-activated protein kinase 7 [KO:K04464] [EC:2.7.11.24]
5599	MAPK8; mitogen-activated protein kinase 8 [KO:K04440] [EC:2.7.11.24]
5601	MAPK9; mitogen-activated protein kinase 9 [KO:K04440] [EC:2.7.11.24]
4312	MMP1; matrix metallopeptidase 1 [KO:K01388] [EC:3.4.24.7]
4322	MMP13; matrix metallopeptidase 13 [KO:K07994] [EC:3.4.24-.]
4314	MMP3; matrix metallopeptidase 3 [KO:K01394] [EC:3.4.24.17]
4318	MMP9; matrix metallopeptidase 9 [KO:K01403] [EC:3.4.24.35]
4586	MUC5AC; mucin 5AC, oligomeric mucus/gel-forming [KO:K21125]
727897	MUC5B; mucin 5B, oligomeric mucus/gel-forming [KO:K13908]
4790	NFKB1; nuclear factor kappa B subunit 1 [KO:K02580]
4792	NFKBIA; NFKB inhibitor alpha [KO:K04734]
5743	PTGS2; prostaglandin-endoperoxide synthase 2 [KO:K11987] [EC:1.14.99.1]
5970	RELA; RELA proto-oncogene, NF-kB subunit [KO:K04735]
6279	S100A8; S100 calcium binding protein A8 [KO:K21127]
6280	S100A9; S100 calcium binding protein A9 [KO:K21128]
6426	SRSF1; serine and arginine rich splicing factor 1 [KO:K12890]
23118	TAB2; TGF-beta activated kinase 1 (MAP3K7) binding protein 2 [KO:K04404]
257397	TAB3; TGF-beta activated kinase 1 (MAP3K7) binding protein 3 [KO:K12793]
29110	TBK1; TANK binding kinase 1 [KO:K05410] [EC:2.7.11.10]
7124	TNF; tumor necrosis factor [KO:K03156]
7128	TNFAIP3; TNF alpha induced protein 3 [KO:K11859] [EC:3.4.19.12]
8717	TRADD; TNFRSF1A associated via death domain [KO:K03171]
7186	TRAF2; TNF receptor associated factor 2 [KO:K03173] [EC:2.3.2.27]
7187	TRAF3; TNF receptor associated factor 3 [KO:K03174]
10758	TRAF3IP2; TRAF3 interacting protein 2 [KO:K21124]
9618	TRAF4; TNF receptor associated factor 4 [KO:K09848]
7188	TRAF5; TNF receptor associated factor 5 [KO:K09849]
7189	TRAF6; TNF receptor associated factor 6 [KO:K03175] [EC:2.3.2.27]
29761	USP25; ubiquitin specific peptidase 25 [KO:K11846] [EC:3.4.19.12]

List of the 74 genes expressed in the human tubular discovery dataset and used for IL-17 pathway score calculation

Gene	Description
51433	ANAPC5; anaphase promoting complex subunit 5 [KO:K03352]
836	CASP3; caspase 3 [KO:K02187] [EC:3.4.22.56]
841	CASP8; caspase 8 [KO:K04398] [EC:3.4.22.61]
6356	CCL11; C-C motif chemokine ligand 11 [KO:K16597]
6361	CCL17; C-C motif chemokine ligand 17 [KO:K21083]
6347	CCL2; C-C motif chemokine ligand 2 [KO:K14624]
6364	CCL20; C-C motif chemokine ligand 20 [KO:K14625]
1051	CEBPB; CCAAT enhancer binding protein beta [KO:K10048]
1147	CHUK; component of inhibitor of nuclear factor kappa B kinase complex [KO:K04467] [EC:2.7.11.10]
1437	CSF2; colony stimulating factor 2 [KO:K05427]
1440	CSF3; colony stimulating factor 3 [KO:K05423]
2919	CXCL1; C-X-C motif chemokine ligand 1 [KO:K05505]
3627	CXCL10; C-X-C motif chemokine ligand 10 [KO:K12671]
2920	CXCL2; C-X-C motif chemokine ligand 2 [KO:K05505]
6372	CXCL6; C-X-C motif chemokine ligand 6 [KO:K05506]
3576	CXCL8; C-X-C motif chemokine ligand 8 [KO:K10030]
1994	ELAVL1; ELAV like RNA binding protein 1 [KO:K13088]
8772	FADD; Fas associated via death domain [KO:K02373]
2353	FOS; Fos proto-oncogene, AP-1 transcription factor subunit [KO:K04379]
2354	FOSB; FosB proto-oncogene, AP-1 transcription factor subunit [KO:K09029]
8061	FOSL1; FOS like 1, AP-1 transcription factor subunit [KO:K04502]
2932	GSK3B; glycogen synthase kinase 3 beta [KO:K03083] [EC:2.7.11.26]
3320	HSP90AA1; heat shock protein 90 alpha family class A member 1 [KO:K04079]
3326	HSP90AB1; heat shock protein 90 alpha family class B member 1 [KO:K04079]
7184	HSP90B1; heat shock protein 90 beta family member 1 [KO:K09487]
3458	IFNG; interferon gamma [KO:K04867]
3551	IKBKB; inhibitor of nuclear factor kappa B kinase subunit beta [KO:K07209] [EC:2.7.11.10]
9641	IKBKE; inhibitor of nuclear factor kappa B kinase subunit epsilon [KO:K07211] [EC:2.7.11.10]
3596	IL13; interleukin 13 [KO:K05435]
23785	IL17RA; interleukin 17 receptor A [KO:K05164]
55540	IL17RB; interleukin 17 receptor B [KO:K05165]
84818	IL17RC; interleukin 17 receptor C [KO:K05166]
3553	IL1B; interleukin 1 beta [KO:K04519]
64806	IL25; interleukin 25 [KO:K05493]
3569	IL6; interleukin 6 [KO:K05405]
3725	JUN; Jun proto-oncogene, AP-1 transcription factor subunit [KO:K04448]
3727	JUND; JunD proto-oncogene, AP-1 transcription factor subunit [KO:K04449]
3934	LCN2; lipocalin 2 [KO:K21129]
6885	MAP3K7; mitogen-activated protein kinase kinase kinase 7 [KO:K04427] [EC:2.7.11.25]
5594	MAPK1; mitogen-activated protein kinase 1 [KO:K04371] [EC:2.7.11.24]
5602	MAPK10; mitogen-activated protein kinase 10 [KO:K04440] [EC:2.7.11.24]
5600	MAPK11; mitogen-activated protein kinase 11 [KO:K04441] [EC:2.7.11.24]
6300	MAPK12; mitogen-activated protein kinase 12 [KO:K04441] [EC:2.7.11.24]
5603	MAPK13; mitogen-activated protein kinase 13 [KO:K04441] [EC:2.7.11.24]
1432	MAPK14; mitogen-activated protein kinase 14 [KO:K04441] [EC:2.7.11.24]
5595	MAPK3; mitogen-activated protein kinase 3 [KO:K04371] [EC:2.7.11.24]
5596	MAPK4; mitogen-activated protein kinase 4 [KO:K06855] [EC:2.7.11.24]
5597	MAPK6; mitogen-activated protein kinase 6 [KO:K06855] [EC:2.7.11.24]
5598	MAPK7; mitogen-activated protein kinase 7 [KO:K04464] [EC:2.7.11.24]
5599	MAPK8; mitogen-activated protein kinase 8 [KO:K04440] [EC:2.7.11.24]
5601	MAPK9; mitogen-activated protein kinase 9 [KO:K04440] [EC:2.7.11.24]
4312	MMP1; matrix metallopeptidase 1 [KO:K01388] [EC:3.4.24.7]
4322	MMP13; matrix metallopeptidase 13 [KO:K07994] [EC:3.4.24-.]
4318	MMP9; matrix metallopeptidase 9 [KO:K01403] [EC:3.4.24.35]
4586	MUC5AC; mucin 5AC, oligomeric mucus/gel-forming [KO:K21125]
727897	MUC5B; mucin 5B, oligomeric mucus/gel-forming [KO:K13908]
4790	NFKB1; nuclear factor kappa B subunit 1 [KO:K02580]
4792	NFKBIA; NFKB inhibitor alpha [KO:K04734]
5743	PTGS2; prostaglandin-endoperoxide synthase 2 [KO:K11987] [EC:1.14.99.1]
5970	RELA; RELA proto-oncogene, NF-kB subunit [KO:K04735]
6279	S100A8; S100 calcium binding protein A8 [KO:K21127]
6280	S100A9; S100 calcium binding protein A9 [KO:K21128]
6426	SRSF1; serine and arginine rich splicing factor 1 [KO:K12890]
23118	TAB2; TGF-beta activated kinase 1 (MAP3K7) binding protein 2 [KO:K04404]
29110	TBK1; TANK binding kinase 1 [KO:K05410] [EC:2.7.11.10]
7124	TNF; tumor necrosis factor [KO:K03156]
7128	TNFAIP3; TNF alpha induced protein 3 [KO:K11859] [EC:3.4.19.12]
8717	TRADD; TNFRSF1A associated via death domain [KO:K03171]
7186	TRAF2; TNF receptor associated factor 2 [KO:K03173] [EC:2.3.2.27]
7187	TRAF3; TNF receptor associated factor 3 [KO:K03174]
10758	TRAF3IP2; TRAF3 interacting protein 2 [KO:K21124]
9618	TRAF4; TNF receptor associated factor 4 [KO:K09848]
7188	TRAF5; TNF receptor associated factor 5 [KO:K09849]
7189	TRAF6; TNF receptor associated factor 6 [KO:K03175] [EC:2.3.2.27]
29761	USP25; ubiquitin specific peptidase 25 [KO:K11846] [EC:3.4.19.12]

List of the 82 genes expressed in the human tubular validation dataset and used for IL-17 pathway score calculation

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836	CASP3; caspase 3 [KO:K02187] [EC:3.4.22.56]
841	CASP8; caspase 8 [KO:K04398] [EC:3.4.22.61]
6356	CCL11; C-C motif chemokine ligand 11 [KO:K16597]
6361	CCL17; C-C motif chemokine ligand 17 [KO:K21083]
6347	CCL2; C-C motif chemokine ligand 2 [KO:K14624]
6364	CCL20; C-C motif chemokine ligand 20 [KO:K14625]
6354	CCL7; C-C motif chemokine ligand 7 [KO:K05509]
1051	CEBPB; CCAAT enhancer binding protein beta [KO:K10048]
1147	CHUK; component of inhibitor of nuclear factor kappa B kinase complex [KO:K04467] [EC:2.7.11.10]
1440	CSF3; colony stimulating factor 3 [KO:K05423]
2919	CXCL1; C-X-C motif chemokine ligand 1 [KO:K05505]
3627	CXCL10; C-X-C motif chemokine ligand 10 [KO:K12671]
2920	CXCL2; C-X-C motif chemokine ligand 2 [KO:K05505]
2921	CXCL3; C-X-C motif chemokine ligand 3 [KO:K05505]
6374	CXCL5; C-X-C motif chemokine ligand 5 [KO:K05506]
6372	CXCL6; C-X-C motif chemokine ligand 6 [KO:K05506]
3576	CXCL8; C-X-C motif chemokine ligand 8 [KO:K10030]
1994	ELAVL1; ELAV like RNA binding protein 1 [KO:K13088]
8772	FADD; Fas associated via death domain [KO:K02373]
2353	FOS; Fos proto-oncogene, AP-1 transcription factor subunit [KO:K04379]
2354	FOSB; FosB proto-oncogene, AP-1 transcription factor subunit [KO:K09029]
8061	FOSL1; FOS like 1, AP-1 transcription factor subunit [KO:K04502]
2932	GSK3B; glycogen synthase kinase 3 beta [KO:K03083] [EC:2.7.11.26]
3320	HSP90AA1; heat shock protein 90 alpha family class A member 1 [KO:K04079]
3326	HSP90AB1; heat shock protein 90 alpha family class B member 1 [KO:K04079]
7184	HSP90B1; heat shock protein 90 beta family member 1 [KO:K09487]
3458	IFNG; interferon gamma [KO:K04867]
3551	IKBKB; inhibitor of nuclear factor kappa B kinase subunit beta [KO:K07209] [EC:2.7.11.10]
9641	IKBKE; inhibitor of nuclear factor kappa B kinase subunit epsilon [KO:K07211] [EC:2.7.11.10]
8517	IKBKG; inhibitor of nuclear factor kappa B kinase regulatory subunit gamma [KO:K07210]
27190	IL17B; interleukin 17B [KO:K05460]
53342	IL17D; interleukin 17D [KO:K05462]
23785	IL17RA; interleukin 17 receptor A [KO:K05164]
55540	IL17RB; interleukin 17 receptor B [KO:K05165]
84818	IL17RC; interleukin 17 receptor C [KO:K05166]
132014	IL17RE; interleukin 17 receptor E [KO:K05168]
3553	IL1B; interleukin 1 beta [KO:K04519]
3569	IL6; interleukin 6 [KO:K05405]
3725	JUN; Jun proto-oncogene, AP-1 transcription factor subunit [KO:K04448]
3727	JUND; JunD proto-oncogene, AP-1 transcription factor subunit [KO:K04449]
3934	LCN2; lipocalin 2 [KO:K21129]
6885	MAP3K7; mitogen-activated protein kinase kinase kinase 7 [KO:K04427] [EC:2.7.11.25]
5594	MAPK1; mitogen-activated protein kinase 1 [KO:K04371] [EC:2.7.11.24]
5602	MAPK10; mitogen-activated protein kinase 10 [KO:K04440] [EC:2.7.11.24]
5600	MAPK11; mitogen-activated protein kinase 11 [KO:K04441] [EC:2.7.11.24]
6300	MAPK12; mitogen-activated protein kinase 12 [KO:K04441] [EC:2.7.11.24]
5603	MAPK13; mitogen-activated protein kinase 13 [KO:K04441] [EC:2.7.11.24]
1432	MAPK14; mitogen-activated protein kinase 14 [KO:K04441] [EC:2.7.11.24]
225689	MAPK15; mitogen-activated protein kinase 15 [KO:K19603] [EC:2.7.11.24]
5595	MAPK3; mitogen-activated protein kinase 3 [KO:K04371] [EC:2.7.11.24]
5596	MAPK4; mitogen-activated protein kinase 4 [KO:K06855] [EC:2.7.11.24]
5597	MAPK6; mitogen-activated protein kinase 6 [KO:K06855] [EC:2.7.11.24]
5598	MAPK7; mitogen-activated protein kinase 7 [KO:K04464] [EC:2.7.11.24]
5599	MAPK8; mitogen-activated protein kinase 8 [KO:K04440] [EC:2.7.11.24]
5601	MAPK9; mitogen-activated protein kinase 9 [KO:K04440] [EC:2.7.11.24]
4312	MMP1; matrix metallopeptidase 1 [KO:K01388] [EC:3.4.24.7]
4322	MMP13; matrix metallopeptidase 13 [KO:K07994] [EC:3.4.24-.]
4314	MMP3; matrix metallopeptidase 3 [KO:K01394] [EC:3.4.24.17]
4318	MMP9; matrix metallopeptidase 9 [KO:K01403] [EC:3.4.24.35]
4586	MUC5AC; mucin 5AC, oligomeric mucus/gel-forming [KO:K21125]
727897	MUC5B; mucin 5B, oligomeric mucus/gel-forming [KO:K13908]
4790	NFKB1; nuclear factor kappa B subunit 1 [KO:K02580]
4792	NFKBIA; NFKB inhibitor alpha [KO:K04734]
5743	PTGS2; prostaglandin-endoperoxide synthase 2 [KO:K11987] [EC:1.14.99.1]
5970	RELA; RELA proto-oncogene, NF-kB subunit [KO:K04735]
6279	S100A8; S100 calcium binding protein A8 [KO:K21127]
6280	S100A9; S100 calcium binding protein A9 [KO:K21128]
6426	SRSF1; serine and arginine rich splicing factor 1 [KO:K12890]
23118	TAB2; TGF-beta activated kinase 1 (MAP3K7) binding protein 2 [KO:K04404]
257397	TAB3; TGF-beta activated kinase 1 (MAP3K7) binding protein 3 [KO:K12793]
29110	TBK1; TANK binding kinase 1 [KO:K05410] [EC:2.7.11.10]
7124	TNF; tumor necrosis factor [KO:K03156]
7128	TNFAIP3; TNF alpha induced protein 3 [KO:K11859] [EC:3.4.19.12]
8717	TRADD; TNFRSF1A associated via death domain [KO:K03171]
7186	TRAF2; TNF receptor associated factor 2 [KO:K03173] [EC:2.3.2.27]
7187	TRAF3; TNF receptor associated factor 3 [KO:K03174]
10758	TRAF3IP2; TRAF3 interacting protein 2 [KO:K21124]
9618	TRAF4; TNF receptor associated factor 4 [KO:K09848]
7188	TRAF5; TNF receptor associated factor 5 [KO:K09849]
7189	TRAF6; TNF receptor associated factor 6 [KO:K03175] [EC:2.3.2.27]
29761	USP25; ubiquitin specific peptidase 25 [KO:K11846] [EC:3.4.19.12]

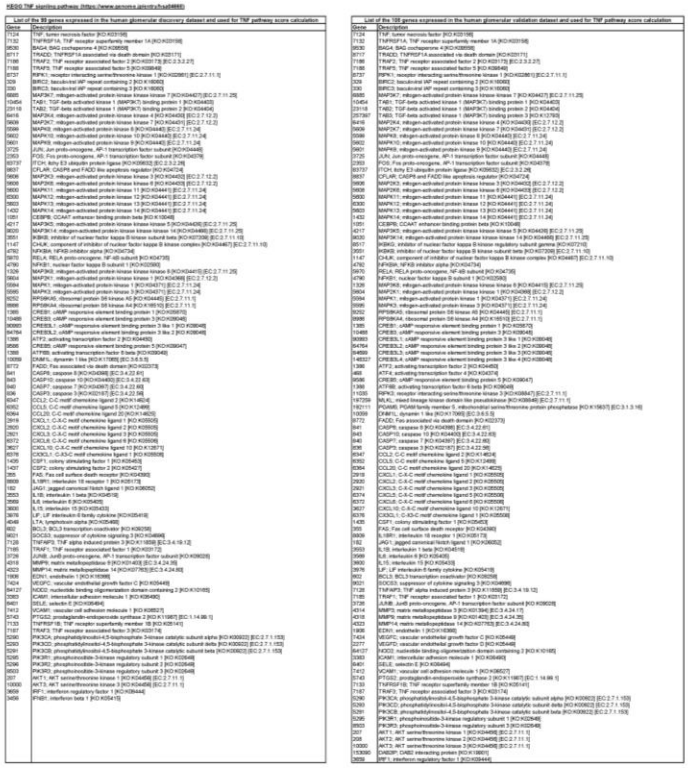
List of the 17 IL-17 signaling pathway genes not expressed in the human glomerular discovery dataset

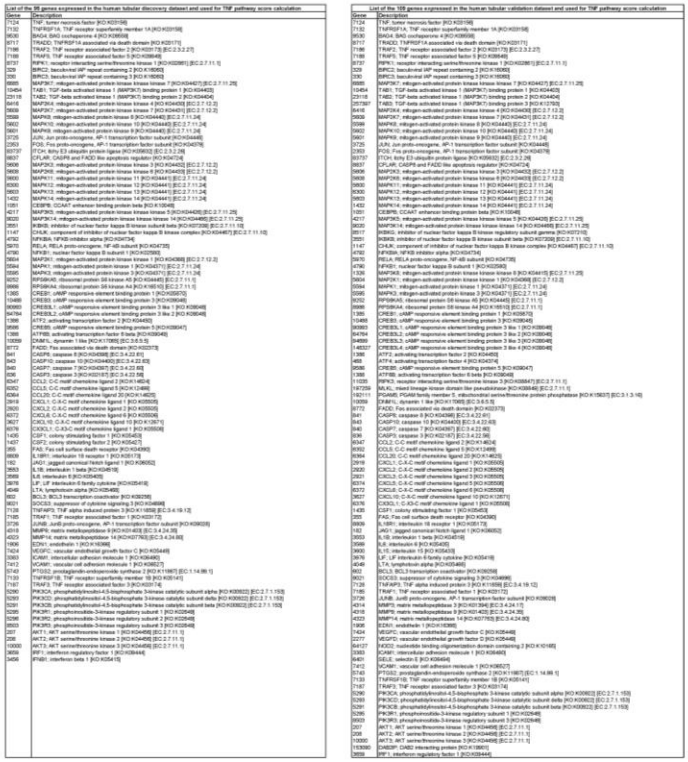
Gene	Description
6374	CXCL5; C-X-C motif chemokine ligand 5 [KO:K05506]
1673	DEFB4A; defensin beta 4A [KO:K21100]
1E+08	DEFB4B; defensin beta 4B [KO:K21100]
8517	IKBKG; inhibitor of nuclear factor kappa B kinase regulatory subunit gamma [KO:K07210]
3605	IL17A; interleukin 17A [KO:K05489]
27189	IL17C; interleukin 17C [KO:K05491]
53342	IL17D; interleukin 17D [KO:K05462]
112744	IL17F; interleukin 17F [KO:K05494]
132014	IL17RE; interleukin 17 receptor E [KO:K05168]
3565	IL4; interleukin 4 [KO:K05430]
3567	IL5; interleukin 5 [KO:K05428]
225689	MAPK15; mitogen-activated protein kinase 15 [KO:K19603] [EC:2.7.11.24]
5596	MAPK4; mitogen-activated protein kinase 4 [KO:K06855] [EC:2.7.11.24]
4314	MMP3; matrix metallopeptidase 3 [KO:K01394] [EC:3.4.24.17]
6278	S100A7; S100 calcium binding protein A7 [KO:K21126]
338324	S100A7A; S100 calcium binding protein A7A [KO:K21126]
257397	TAB3; TGF-beta activated kinase 1 (MAP3K7) binding protein 3 [KO:K12793]

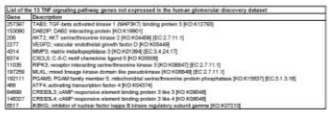
List of the 14 IL-17 signaling pathway genes not expressed in the human glomerular validation dataset

Gene	Description
6361	CCL17; C-C motif chemokine ligand 17 [KO:K21083]
1437	CSF2; colony stimulating factor 2 [KO:K05427]
1440	CSF3; colony stimulating factor 3 [KO:K05423]
1673	DEFB4A; defensin beta 4A [KO:K21100]
1E+08	DEFB4B; defensin beta 4B [KO:K21100]
3596	IL13; interleukin 13 [KO:K05435]
3605	IL17A; interleukin 17A [KO:K05489]
27189	IL17C; interleukin 17C [KO:K05491]
112744	IL17F; interleukin 17F [KO:K05494]
64806	IL25; interleukin 25 [KO:K05493]
3565	IL4; interleukin 4 [KO:K05430]
3567	IL5; interleukin 5 [KO:K05428]
6278	S100A7; S100 calcium binding protein A7 [KO:K21126]
338324	S100A7A; S100 calcium binding protein A7A [KO:K21126]

Table A-2 – Lists of TNF signaling pathway genes expressed in each human dataset







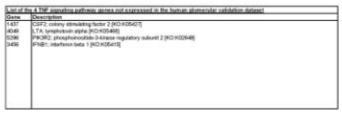


Table A-3 – Top 15 upstream regulators in female and male NZM-*Il1b*$^{-/-}$ compared to NZM mice as assessed with Ingenuity Pathway Analysis (IPA).

Top 15 upstream regulators in Female NZM-*Il1b*$^{-/-}$ compared to NZM mice				
Upstream Regulator	**Log2 fold-change**	**Molecule Type**	**Predicted Activation State**	**Activation z-score**
TNF	1.03	cytokine	Activated	3.002
TP53	0.38	transcription regulator	Activated	2.286
CTNNB1		transcription regulator		-0.779
TGFB1		growth factor	Activated	2.182
ESR2	-1.91	ligand-dependent nuclear receptor		1.515
APP		other		0.811
CG		complex		-0.948
AGT	3.11	growth factor		-0.163
IFNG	-2.18	cytokine	Activated	2.253
OGA	0.44	enzyme		-0.907
IL4		cytokine		0.613
PPARGC1A		transcription regulator	Inhibited	-4.163
Lh		complex	Activated	4.019
MLXIPL		transcription regulator	Activated	6.114
Immunoglobulin		complex		0.725

Top 15 upstream regulators in Male NZM-*Il1b*$^{-/-}$ compared NZM mice				
Upstream Regulator	**Log2 fold-change**	**Molecule Type**	**Predicted Activation State**	**Activation z-score**
SLC27A2		transporter	Inhibited	-3.959
PPARA	1.21	ligand-dependent nuclear receptor	Activated	4.747
ADRB3		G-protein coupled receptor		-0.791
PPARG		ligand-dependent nuclear receptor	Activated	3.765
mir-144		microRNA	Inhibited	-2.355
mir-8		microRNA	Inhibited	-2.666
miR-155-5p (miRNAs		mature microRNA	Inhibited	-3.378
ESR1		ligand-dependent nuclear receptor	Activated	4.028
CPT1B	-1.57	enzyme		-1.134
LIPE		enzyme		1.857
SRSF2	-0.61	transcription regulator		0.816
Fus	-0.57	transcription regulator		-1.89
BDNF		growth factor		-1.693
CIDEC		other		-1.648
PTP4A1	0.63	phosphatase		-1.47

Table A-4 – Clinical characteristics of reference biopsies analyzed by Affymetrix microarray gene expression profiling (528). All patients are Caucasian. Data are presented as mean ± SEM. NA: not available.

	Characteristic	Living donors (LD) female	Living donors (LD) male	Lupus nephritis (LN) female	Lupus nephritis (LN) male
Glomerular compartment	Patient number	4	9	19	3
	Age (years)	61.0 ± 3.2	48.4 ± 3.9	32.7 ± 2.3	43.9 ± 14.7
	Serum creatinine (mg/dL)	NA	1.1 ± 0.1	1.7 ± 0.2	3.0 ± 0.0
	Proteinuria (g/day)	absent	absent	3.9 ± 0.9	1.3 ± 0.5
	CKD-Epi GFR (ml/min/1.73m^2)*	NA	79.0 ± 7.7	55.6 ± 7.7	73.0 ± 9.8
Tubulointerstitial compartment	Patient number	6	4	19	3
	Age (years)	59.2 ± 3.0	42.5 ± 6.2	32.7 ± 2.3	43.9 ± 14.7
	Serum creatinine (mg/dL)	NA	1.0 ± 0.1	1.7 ± 0.2	3.0 ± 0.0
	Proteinuria (g/day)	absent	absent	3.9 ± 0.9	1.3 ± 0.5
	CKD-Epi GFR (ml/min/1.73m^2)*	NA	88.1 ± 12.4	55.6 ± 7.7	73.0 ± 9.8

Table A-5 – Clinical characteristics of reference biopsies analyzed by RNA-sequencing gene expression profiling (528). All patients are Caucasian. Data are presented as mean ± SEM. NA: not available.

	Characteristic	Living donors (LD) female	Living donors (LD) male	Lupus nephritis (LN) female	Lupus nephritis (LN) male
Glomerular compartment	Patient number	4	1	10	3
	Age (years)	50.4 ± 4.6	30.6	32.8 ± 4.9	46.9 ± 14.3
	Serum creatinine (mg/dL)	NA	NA	1.2 ± 0.2	1.0 ± 0.2
	Proteinuria (g/day)	absent	absent	5.2 ± 1.9	NA
	CKD-Epi GFR (ml/min/1.73m^2)*	NA	NA	69.4 ± 10.0	81.1 ± 15.7
Tubulointerstitial compartment	Patient number	4	1	18	6
	Age (years)	50.4 ± 4.6	30.6	37.1 ± 3.3	37.8 ± 8.1
	Serum creatinine (mg/dL)	NA	NA	1.3 ± 0.2	1.2 ± 0.2
	Proteinuria (g/day)	absent	absent	4 ± 1.1	3.4 ± 1.7
	CKD-Epi GFR (ml/min/1.73m^2)*	NA	NA	69.1 ± 8.9	81.3 ± 13.4

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