Neutrophil extracellular traps drive lupus flares with acute skin and kidney inflammation triggered by ultraviolet irradiation

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Short title: NETosis and UVB-induced lupus flares

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ABSTRACT

Sunlight triggers lupus flares causing both local skin and systemic inflammation, including lupus nephritis, through poorly understood mechanisms. To address this knowledge gap, we found that UVB irradiation of asymptomatic, young female lupus-prone mice induced skin and kidney inflammation with proteinuria, accompanied by neutrophil infiltration and neutrophil extracellular trap (NET) formation. Furthermore, UVB irradiation induced co-expression of CXCR4 and cytokines/C3 by neutrophils *in vitro* and *in vivo*, in the skin and kidneys of lupus-prone mice, indicating their transmigratory and pro-inflammatory potentials. A causality study demonstrated that inhibiting CXCR4 attenuated renal neutrophil infiltration, accumulation of NETs, NET-associated cytokines/C3, and proteinuria in UVB-irradiated lupus-prone mice. Remarkably, inhibiting NETosis through a novel strategy targeting nuclear envelope integrity reduced deposition of NET-associated cytokines/C3 in skin and kidneys, attenuating proteinuria in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice. Our investigation unveils a new mechanism by which neutrophil NETs drive the early onset of lupus flares triggered by UVB-irradiation. Targeting neutrophil transmigration and NETosis could be promising therapeutic strategies.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototype systemic autoimmune disease with involvement of multiple organs, including skin and kidneys. SLE is characterized by the accumulation of immune cells, cytokines, immune complexes (ICs), and complement in affected organs, leading to their inflammatory responses and tissue damage (*1*). Although the etiology of SLE remains unclear, genetic, epigenetic, and environmental risk factors are believed to play a role (*1, 2*). Although genetic factors play a role in lupus susceptibility, twin studies have shown that only one-third of monozygotic twins develop SLE concordantly, highlighting the importance of environmental factors in lupus etiology (*1*). Since the 1960s, ultraviolet B (UVB) in sunlight has been recognized as an important environmental risk factor for lupus (*3*), but the mechanism by which UVB irradiation induces SLE remains unclear.

About 70% of patients with cutaneous lupus erythematosus (CLE) are photosensitive to sunlight (*4*). In addition, sun overexposure triggers lupus flare by exacerbating not only local skin inflammation but also systemic symptoms, such as lupus nephritis (*5, 6*). A clinical pathology study of renal biopsies reveals a seasonal pattern in lupus nephritis for SLE patients living in southern France, with more frequent lupus flares occurring during the season with longer sunshine duration (*7*). In basic studies, UVB overexposure induces epidermal keratinocyte apoptosis, in which apoptotic cells serve as a key source of clustered and concentrated autoantigens with immunogenic properties, potentially initiating and driving autoimmune responses in the development of systemic lupus (*5*). However, other studies questioned the role of UVB-induced apoptotic keratinocyte accumulation in systemic lupus development (*8, 9*). Therefore, further investigation is required to elucidate the mechanisms by which sunlight triggers lupus flares and the early onset of lupus kidney injury.

Following UVB overexposure, neutrophils are the first group of circulating leukocytes to infiltrate the skin, playing crucial roles in cutaneous inflammation (*10*). In a recent animal

study, neutrophils are the earliest infiltrating immune cells in skin lesions of a mouse model with spontaneous CLE (*11*). In humans, recent studies have shown a gradual enrichment of neutrophil transcripts during lupus progression (*12*), indicating a crucial role for neutrophils in lupus development (*13, 14*). In our recent studies, we demonstrated that UVB irradiation in wild-type (WT) mice triggers skin inflammation with infiltration of neutrophils, which release their nuclear DNA to form neutrophil extracellular traps (NETs) (*15, 16*). In literature, NETosis has been implicated in the pathogenesis of systemic lupus, including CLE (*13, 17*), and lupus nephritis (*18, 19*). Studies from different groups, including ours, have demonstrated that NETs induce production of cytokines, such as type I interferon (IFN-I), a primary driver of lupus pathogenesis (*20*), from various types of immune cells, including neutrophils (*16, 21*). Nuclear acids and other nuclear constituents released with NETs can serve as sources of autoantigens (*21*). Furthermore, both NETs and IFN-I can induce B cell activation and proliferation (*22, 23*), leading to adaptive immune responses, autoantibody production, and subsequent formation of ICs (*24*). In addition, NETs can be associated with complement (*25*) or activate complement (*25, 26*). The deposition of ICs and complement C3 in the skin (*27*) or kidneys (*28*) is believed to play a key role in autoimmune inflammation of the affected organs in lupus pathogenesis. Given its pathogenic importance in lupus, NETosis could be a novel therapeutic target for lupus treatment.

To investigate the role of NETosis in lupus skin inflammation triggered by UVB irradiation, we exposed asymptomatic young female lupus-prone mice to UVB irradiation following our published protocol (*15, 16*). Surprisingly, UVB irradiation induced not only skin inflammation, but also proteinuria with kidney inflammation in these originally asymptomatic lupus-prone mice. This closely mirrors the lupus flares associated with sunlight overexposure in humans (*6*). To understand the potential link between UVB-induced skin inflammation and proteinuria, we reviewed the existing literature, which has long established that patients with

skin thermal burns experience proteinuria and acute kidney injury (*29*) with the involvement of neutrophil activation (*30*). A recent study with single high dose UVB irradiation of WT mice induced acute skin inflammation and transient proteinuria with neutrophil involvement in kidney injury (*31*). Another recent report demonstrates that skin infection of staphylococcus aureus in *Nfkbiz*$^{\Delta K5}$ mice induces SLE-like symptoms with sustained epidermal inflammatory responses and kidney inflammation via neutrophil activation (*32*). All of the above studies highlight the involvement of neutrophils in the connection between skin inflammation and kidney injury in various settings. Our preliminary study showed the involvement of neutrophils, especially their released NETs, in lupus flares with skin and kidney inflammation in UVB-irradiated lupus-prone mice. This study investigated the relevant mechanisms by which neutrophils and their released proinflammatory NETs drive the skin inflammation and the early onset of kidney injury in lupus flares triggered by UVB irradiation.

Since its first description, the diverse array of signaling pathways regulating NETosis has posed a significant challenge in identifying specific molecular targets for therapeutic intervention in NETosis associated human diseases (*21, 33*). To overcome this challenge, we investigated the cellular mechanisms governing nuclear envelope rupture, a required step for extracellular release of nuclear chromatin during NET formation (*21, 33*). Our recent cellular mechanistic study uncovered that nuclear envelope rupture and NET formation are driven by PKCα-mediated phosphorylation and subsequent disassembly, not proteolytic degradation, of nuclear lamin B, an essential structural protein for nuclear envelope integrity (*15, 16, 21*). Based on the newly identified cellular mechanism, the current study investigated a novel strategy to control NETosis through nuclear envelope regulation, and explored its effect on UVB-induced lupus flares in lupus-prone mice (*15, 21, 33*).

About 50-60% of SLE patients develop lupus nephritis, one of the most severe organ manifestations and the leading cause of death in SLE patients. Given the importance of sunlight

in triggering lupus nephritis (*7*) and lupus flares (*6*), deciphering the mechanisms behind UVB-induced lupus flares with kidney injury could provide valuable insights for developing targeted therapeutics to prevent or delay the onset, or even alleviate lupus nephritis. In this study, we explored the effects of UVB irradiation on neutrophil expression of CXCR4, a chemokine receptor that mediates neutrophil transmigration between organs (*34, 35*). We also investigated the effects of UVB irradiation on neutrophil expression of cytokines/C3 *in vitro* and *in vivo*, as these proinflammatory molecules may contribute to inflammatory responses in the skin and kidneys during UVB-induced lupus flares. We further conducted a causal experiment to examine the effects of CXCR4 inhibition on neutrophil transmigration and infiltration into the kidneys during UVB-induced lupus flares. In addition, we investigated the crucial role of NETosis in driving skin and kidney inflammation in lupus flares. Moreover, the study also explored a novel therapeutic strategy to alleviate UVB-induced lupus flares by controlling NETosis through nuclear envelope regulation. Therefore, this work explored new mechanisms behind UVB-induced lupus flares, unveiling promising therapeutic targets for developing innovative therapeutics to prevent or delay the onset of lupus kidney inflammation.

RESULTS

UVB irradiation induced skin and kidney inflammation with proteinuria in asymptomatic young female MRL/lpr lupus-prone mice

By irradiation of asymptomatic young female lupus-prone mice, we found that UVB irradiation induced skin inflammation with increased thickness in the epidermal and dermal skin, and inflammatory cell infiltration (nucleated cell count) in skin (Fig 1A-D). Surprisingly, the UVB-irradiated lupus-prone mice also had significant proteinuria (Fig 1E) and glomerular

hypercellularity (Fig 1F,G) in these mice as compared to those in the sham controls. Furthermore, the nucleated cell count in the dermis was correlated with dermal skin thickness (Fig 1H). In addition, the dermal nucleated cell counts were correlated with dermal skin thickness and proteinuria (Fig 1H-I), indicating a role of inflammatory cell infiltration in skin inflammation, and a potential link between dermal inflammatory cells and kidney injury. Moreover, the observed glomerular hypercellularity was significantly correlated with proteinuria (Fig 1J), suggesting a role for renal inflammatory cell infiltration in proteinuria as leukocyte infiltration contributes to the glomerular hypercellularity in lupus kidneys (*36*). Therefore, our results suggested a potential connection between skin inflammation and kidney injury in UVB-irradiated asymptomatic lupus-prone mice. Several recent studies have demonstrated the involvement of neutrophils in linking of skin inflammation to kidney injury, observed in different settings (*30-32*). Previous studies from our team and others have shown the involvement of neutrophils and neutrophil NETosis in UVB-induced skin inflammation (*15, 16*) or lupus skin inflammation in the current study, and lupus nephritis (*18, 19*) in mice. To understand the above findings, the current study investigated the role of neutrophils and their released NETs in lupus flares with acute skin and kidney inflammation triggered by UVB irradiation of asymptomatic, young female lupus-prone mice.

UVB irradiation induced infiltration of neutrophils, and their expressions of cytokines, C3 and CXCR4, as well as NET formation in skin of UVB-irradiated lupus-prone mice

To understand the role of leukocyte infiltrates in UVB-triggered lupus flare, we found increased neutrophil infiltration and deposition of neutrophil NETs in the inflamed skin of UVB-irradiated MRL/lpr mice as compared to non-irradiated sham controls (Fig 2A-C). In addition, we detected increased expression of IFNα, IFNγ, IL-17A, and C3 in neutrophils and

their released proinflammatory NETs in dermal skin of UVB-irradiated MRL/lpr mice as compared to those in non-irradiated sham controls (Fig 2D-G), indicating their contributions to lupus skin inflammation triggered by UVB irradiation of asymptomatic lupus-prone mice. Furthermore, NET-associated C3 in skin was positively correlated with nucleated cell count in skin in the combined groups of MRL/lpr mice without or with UVB irradiation (Fig 2J), indicating a role of the NET-associated C3 in the skin inflammation.

CXCR4 is a chemokine receptor, which is crucial for reverse transmigration (RTM) of cells, including neutrophils, for crosstalk between organs (*34, 35*). In addition, the CXCR4/CXCL12 axis is important in lupus pathogenesis, with increased CXCR4 expression in immune cells and elevated CXCL12 expression in kidneys of lupus prone mice (*37*). Interestingly, UVB irradiation increased expression of CXCR4 in both intact non-netting neutrophils and NETotic neutrophils in the skin of MRL/lpr mice as compared to non-irradiated sham controls (Fig 2H,I). In addition, the distribution of NET-associated C3 in skin was significantly correlated with the nucleated cell count in the skin, indicating a role of C3 in skin inflammation (Fig 2J). Importantly, CXCR4-expressing neutrophils in skin was positively correlated with proteinuria in the combined groups of MRL/lpr mice without or with UVB irradiation (Fig 2K), suggesting a potential link between dermal CXCR4-expressing neutrophils and kidney injury in these mice.

Neutrophil infiltration, NET formation, and accumulation of NET-associated cytokines and C3 are involved in acute kidney inflammation in UVB-irradiated lupus-prone mice

In the kidneys, we found increased neutrophil infiltration, accumulation of citrullinated H3-positive NETs (Fig 3A-C), and NET-associated IFNα, IFNγ, IL-17A, and C3 (Fig 3D-G, Suppl Fig 1A) in glomeruli of UVB-irradiated lupus-prone mice as compared to those in sham

controls. In addition, the glomerular neutrophil infiltration was correlated with glomerular hypercellularity in the combined groups of MRL/lpr mice without or with UVB irradiation (Suppl Fig 1B), suggesting the contribution of glomerular neutrophil infiltration to the glomerular hypercellularity and renal inflammation (*36*). Importantly, the infiltrated neutrophils, NETs, and their associated IFNα, IFNγ, IL-17A, and C3 in glomeruli were positively correlated with proteinuria in the combined groups of MRL/lpr mice without or with UVB irradiation (Suppl Fig 1C-H). Moreover, NET-associated C3 in glomeruli was positively correlated with glomerular hypercellularity in the combined groups of MRL/lpr mice without or with UVB irradiation (Suppl Fig 1I), indicating a role of C3 in renal inflammation. The above results indicate a role for the infiltrated neutrophils, NETs, and their associated cytokines/C3 in the early onset of kidney inflammation and proteinuria in lupus flares triggered by UVB irradiation of asymptomatic lupus-prone mice.

Corresponding to the increased number of CXCR4-expressing neutrophils in the skin (Fig 2H-J), we found increased numbers of CXCR4-expressing non-netting neutrophils and NETotic neutrophils in the kidneys of UVB-irradiated MRL/lpr mice compared to sham controls (Fig 3H,I). Furthermore, CXCR4-expressing neutrophils and NETs in the kidneys were positively correlated with proteinuria (Fig 3J). Taken together, results from the skin and kidneys of UVB-irradiated MRL/lpr mice (Fig 2-3) demonstrate the link between the dermal CXCR4-expressing neutrophils and the kidney injury, and the role of renal CXCR4-expressing neutrophils in early onset of renal inflammation and lupus kidney injury triggered by UVB irradiation. Given that CXCR4 is known to contribute to neutrophil transmigration in the crosstalk between organs (*34*, *35*), our results suggest a potential role of CXCR4 in neutrophil transmigration from the inflamed skin to kidneys during the early onset of lupus kidney injury triggered by UVB irradiation of asymptomatic young lupus-prone mice.

UVB irradiation induced co-expression of CXCR4 and cytokines/C3 *in vitro* in primary neutrophils from lupus-prone mice

To explore if neutrophil expression of CXCR4 and proinflammatory cytokines/C3 in inflamed skin was due to UVB irradiation, we isolated primary neutrophils from MRL/lpr mice and conducted UVB irradiation *in vitro*. We found that UVB irradiation induced upregulation of CXCR4, IFNα (in the presence of DNA-NETs), IFNγ, IL-17A, and C3 *in vitro* in dose-dependent (Suppl Fig 2A-E) manner. As platelet activating factor (PAF) is released from UVB-irradiated keratinocytes (*15, 38*), we also stimulated neutrophils with PAF as an indirect effect of UVB. Interestingly, PAF treatment of mouse neutrophils upregulated their expression of CXCR4, IFNα (in the presence of DNA-NETs), IFNγ, IL-17A, and C3 *in vitro* (Fig 4 A-L) in a dose-dependent (Suppl Fig 2A-E) manner. These results suggest that UVB irradiation can directly and/or indirectly upregulate expression of CXCR4 and cytokines/C3 in mouse neutrophils. Most importantly, a special group of neutrophils that were either irradiated by UVB or stimulated by PAF co-expressed CXCR4 and cytokines/C3 (Fig 4 A-L), facilitating their potential in transmigratory and proinflammatory activity for neutrophil infiltration and kidney inflammation. Previous studies from our team and others demonstrated that only portion of neutrophils can become NETotic *in vitro* (*39*) and *in vivo* in skin of UVB-irradiated WT mice (*16*). Therefore, the intact non-netting neutrophils, particularly those with co-expression of CXCR4 and cytokines/C3 (Figs 2,4) in skin may transmigrate to the kidneys which have elevated CXCL12 expression in either UVB-irradiated mice (*31*) or lupus-prone mice (*37*). Once the CXCR4-expressing neutrophils are recruited by the kidneys, they may become NETotic and release NETs and their-associated cytokines/C3, contributing to kidney inflammation and injury, as well as proteinuria in lupus flare triggered by UVB irradiation of lupus-prone mice (Figs 1,3).

Inhibition of CXCR4 attenuated acute kidney inflammation in lupus-prone mice with lupus flare triggered by UVB

Based on the above findings (Figs 2-4), we sought to explore the causal role of CXCR4 in kidney inflammation in UVB-triggered lupus flare. We found that inhibition of CXCR4, by intraperitoneal application of its selective antagonist IT1t, significantly ameliorated glomerular hypercellularity and proteinuria in lupus flare triggered by UVB irradiation of asymptomatic lupus-prone mice (Fig 5A-C). In addition, CXCR4 inhibition attenuated glomerular infiltration of Ly6G-positive and CXCR4-expressing neutrophils in the kidneys of UVB-irradiated lupus-prone mice (Fig 5D,E). To confirm the inhibitory effects of IT1t on neutrophil transmigration, we also found that IT1t reduced neutrophil transmigration *in vitro* in transwell migration experiments (Fig 5F). Furthermore, the reduced neutrophil recruitment in kidneys resulted in amelioration in glomerular deposition of NETs (Fig 5G), and NET-associated IFNα, IFNγ, IL-17A, and C3 (Fig 5H-L), in kidneys from UVB-irradiated lupus-prone mice with IT1t intraperitoneal application as compared to those in UVB-irradiated MRL/lpr mice with normal saline administration. Importantly, the glomerular deposition of NET-associated IFNα, IFNγ, IL-17A or C3 were positively correlated with proteinuria in the combined groups of UVB-irradiated MRL/lpr mice with IT1t or normal saline administration (Suppl Fig 3A-D), suggesting a significant role for NET-associated cytokines/C3 in the pathogenesis of proteinuria. Therefore, this animal experiment indicated that inhibition of CXCR4 attenuated the early onset of renal inflammation and kidney injury triggered by UVB irradiation of asymptomatic lupus-prone mice. Our findings suggest that CXCR4-mediated neutrophil transmigration played a key role in the initiation of UVB-induced lupus flares, and inhibition of neutrophil transmigration could be a promising therapeutic strategy to prevent or delay the onset of lupus kidney inflammation and injury during lupus flares.

In addition to the kidney inflammation, application of IT1t also attenuated dermal, but not epidermal, skin thickness and nucleated cell counts in the skin (Suppl Fig 3E-H), as well as infiltration of Ly6G neutrophils, NET formation, and NET-associated IFNα, and IL-17A (Suppl Fig 3I,K,L,N) in the skin. However, IT1t administration did not significantly suppress CXCR4-expressing neutrophils or NET-associated IFNγ and C3 in the skin of UVB-irradiated lupus-prone mice compared to those treated with normal saline (Suppl Fig 3J,M,O). These results suggest that CXCR4 also affect inflammatory cell infiltration into the inflamed skin of lupus-prone mice, which were subjected to repeated 5-day UVB irradiation regimens, accompanied by the daily intraperitoneal application of IT1t daily for 6 consecutive days, starting one day before the first UVB irradiation session. In line, Sarchio et al reported that CXCR4 inhibition attenuates sunlight-induced skin cancer by blocking infiltration of mast cells to the skin (*40*).

Controlling NETosis by strengthening nuclear envelope integrity attenuated lupus skin inflammation in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice

Given the importance of NETosis in lupus pathogenesis in previous works (*13, 14, 21*), the current study demonstrated that deposition of NETs and NET-associated cytokines/C3 is crucial to the lupus flares triggered by UVB irradiation (Fig 2-3). To target these troublemakers, we investigated how controlling NETosis through nuclear envelope regulation affects lupus flares based on our recently identified cellular mechanisms that PKCα-mediated nuclear lamin B disassembly drives nuclear envelope rupture, nuclear chromatin release, and NET formation in neutrophils (*15*). To explore our hypothesis, we generated lupus-prone mice with nuclear lamin B1 overexpression in which the strengthened nuclear envelope integrity could reduce NET release (*15*). However, overexpression of nuclear lamin B in cells does not necessarily affect their transmigration capacity as previous studies have demonstrated that the

ratio of lamin A to lamin B in the nuclear envelope influences nuclear stiffness (*15, 41*). Overexpression of lamin B does not lead to the increase in nuclear stiffness or decrease in transmigration in hematopoietic cells (*15, 41*).

In the MRL/lpr·lmnB1^{Tg} mice, we found no significant differences in the transmigration capacity of the primary neutrophils from these mice compared to control mice (Suppl Fig 4A). This result was in line with previous studies (*15, 41*). Importantly, the NETosis experiment *in vitro* using primary neutrophils from MRL/lpr·lmnB1^{Tg} mice revealed that strengthening nuclear envelope integrity through genetic overexpression of nuclear lamin B significantly attenuated NETosis compared to MRL/lpr mice (Suppl Fig 4B). The above experiments indicated that these mice are good tools for us to not only examine the effects of controlling NETosis on UVB-induced lupus flare, but also test a new strategy of inhibition of NETosis by nuclear envelope regulation. In the *in vivo* experiments, the UVB-irradiated MRL/lpr·lmnB1^{Tg} mice exhibited significantly decreased skin inflammation with reduced epidermal and dermal skin thickness, as well as significantly ameliorated nuclearated cell counts and neutrophil infiltration in their skin as compared to UVB-irradiated MRL/lpr mice (Fig 6A-D, Suppl Fig 4C). Given the role of nuclear envelope regulation, we saw reduced neutrophil NET formation, and accumulation of NET-associated IFNα, IFNγ, IL-17A, and C3 in the skin of UVB-irradiated MRL/lpr·lmnB1^{Tg} mice as compared to UVB-irradiated MRL/lpr mice (Fig 6E-J), indicating the protective effects of controlling NETosis, through nuclear envelope regulation, on lupus skin inflammation in MRL/lpr·lmnB1^{Tg} mice. Interestingly, the nucleated cell counts, NETs, and NET-associated IFNα, IFNγ, IL-17A, and C3 in skin were positively correlated with dermal skin thickness in the combined groups of MRL/lpr and MRL/lpr·lmnB1^{Tg} mice without or with UVB irradiation (Suppl Fig 4D-I). Furthermore, NET-associated C3 in skin was positively correlated with nucleated cell count in skin in the combined groups of MRL/lpr mice and MRL/lpr·lmnB1^{Tg} without or with UVB irradiation

(Suppl Fig 4J), indicating a role of NET-associated C3 in the skin inflammation. Our study further supports the role of NETs and NET-associated cytokines/C3 in lupus skin inflammation during lupus flares triggered by UVB irradiation of lupus-prone mice.

Controlling NETosis by strengthening nuclear envelope integrity ameliorated the early onset of lupus kidney injury and proteinuria in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice

To investigate the effect of controlling NETosis on the early onset of lupus kidney inflammation and injury triggered by UVB, we found that UVB-irradiated MRL/lpr·lmnB1^{Tg} mice showed decreased proteinuria, glomerular hypercellularity, and neutrophil infiltration in their kidneys compared to UVB-irradiated MRL/lpr mice (Fig 7A-D). In addition, glomerular Ly6G neutrophil infiltration and accumulation of NET-associated C3 were correlated with glomerular hypercellularity (Suppl Fig 5A,B), suggesting the contribution of renal neutrophil infiltration to glomerular hypercellularity (*36*) and the role of NET-associated C3 in renal inflammation. Importantly, UVB-irradiated MRL/lpr·lmnB1^{Tg} mice demonstrated reduced glomerular deposition of neutrophil NETs and NET-associated IFNα, IFNγ, IL-17A, and C3 in their kidneys compared to UVB-irradiated MRL/lpr mice (Fig 7E-J). These results indicated the protective effects of controlling NETosis on the early onset of lupus kidney inflammation and injury triggered by UVB irradiation of asymptomatic lupus-prone mice. In addition, the glomerular hypercellularity, neutrophil infiltration, deposition of NETs, and NET-associated IFNα, IFNγ, IL-17A, and C3 in kidneys were positively correlated with proteinuria in the combined groups of MRL/lpr and MRL/lpr·lmnB1^{Tg} mice without or with UVB irradiation (Fig 7K, Suppl Fig 5C-H). These results support the roles of glomerular neutrophil infiltration, deposition of proinflammatory NETs and NET-associated cytokines/C3 in renal inflammation and kidney injury in lupus flare. Therefore, our study suggested that controlling NETosis could

be a promising therapeutic target to prevent or delay the early onset of lupus kidney inflammation and injury triggered by UVB irradiation. Importantly, our experiments also demonstrated that nuclear envelope regulation could be a novel therapeutic target to control NETosis for treatment of lupus flares triggered by UVB irradiation of asymptomatic young female lupus-prone mice.

DISCUSSION

Following UVB irradiation, neutrophils, the first group of immune cells responding to injury, infiltrate the inflamed skin, where some of them undergo NETosis, as observed in WT (*15, 16*) and lupus-prone mice. Interestingly, UVB induced neutrophil expression of CXCR4 and cytokines/C3 both *in vitro* and *in vivo* in the skin and kidneys of UVB-irradiated lupus-prone mice. The co-expression of CXCR4 and cytokines/C3 empowered the infiltrated neutrophils with transmigratory (*34, 35*) and pro-inflammatory capacities, which enable them transmigrating from the inflamed skin to the kidneys where the recruited neutrophils released NETs and NET-associated cytokines/C3, propagating inflammatory responses in the kidneys of UVB-irradiated lupus-prone mice. Importantly, inhibiting CXCR4 suppressed infiltration of CXCR4-expressing neutrophils into the kidneys, consequently reducing the accumulation of neutrophil NETs and NET-associated cytokines/C3, thereby decreasing proteinuria in UVB-irradiated lupus-prone mice. On the other hand, directly targeting NETosis by controlling neutrophil NETosis reduced the accumulation of NETs, and NET-associated cytokines/C3 in the skin and kidneys, leading to decreased proteinuria in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice. Remarkably, this study identified that strengthening the nuclear envelope integrity in MRL/lpr·lmnB1^{Tg} mice significantly inhibited NETosis *in vitro* and *in vivo*, thereby ameliorating UVB-induced lupus flares. This finding suggests that nuclear envelope regulation

could be a potential therapeutic target for NETosis-related diseases, including lupus. Therefore, our investigation not only elucidates the critical role of neutrophil NETosis in lupus flares, but also unveils promising therapeutic avenues to prevent or delay the early onset of lupus kidney injury triggered by UVB irradiation.

Studies from different settings have demonstrated that either skin thermal burn in patients (*29, 30*), or acute UVB irradiation with a single-dose in WT mice (*31*), or local skin infections with staphylococcus aureus in mice (*32*) can induce systemic symptoms, including kidney inflammation, indicating a potential dermal-renal crosstalk in these scenarios. Over the past 60 years, numerous studies have reported proteinuria in patients with skin thermal burn (*29*), in which neutrophil gelatinase-associated lipocalin from activated neutrophils are implicated (*30*). A recent study using a single high dose of UVB irradiation on WT mice revealed transient proteinuria, accompanied by the transmigration of neutrophils from the inflamed skin to the kidneys, as monitored by a photoactivatable lineage tracking approach (*31*), highlighting the potential dermal-renal crosstalk in response to the acute skin injury. In addition, a previous study demonstrated that neutrophil depletion protects mice from developing UV-irradiation-induced lupus-like skin lesions (*42*). On the other hand, skin infection by staphylococcal aureus in *Nfkbiz*$^{\Delta K5}$ mice promotes keratinocyte apoptosis, neutrophil activation and NET release, leading to SLE-like autoimmune inflammation and kidney injury (*32*). Therefore, the studies discussed above indicate that neutrophils play a role in potential dermal-renal crosstalk during skin inflammation-triggered systemic symptoms, including kidney inflammation (*30-32*). The growing body of evidence suggests that skin inflammation may play a more important role in systemic autoimmune inflammation than previously thought.

Published studies have reported that CXCR4/CXCL12 axis play an important role in the pathogenesis of lupus by recruiting CXCR4-expressing immune cells, including

neutrophils, into kidneys with CXCL12 expression in both patients with lupus nephritis and lupus-prone mice (*37, 43*). Other studies has also demonstrated that CXCR4-expressing immune cells are recruited to kidneys with CXCL12 expression in mice with ischemic-renal injury (*44*) and mice with UVB-induced acute renal injury (*31*). Furthermore, CXCR4 is a known mediator of transmigration for various cell types, including neutrophils, facilitating crosstalk between organs (*34, 35*). In the current study, we found expression of CXCR4 and proinflammatory cytokines/C3 in the non-netting neutrophils and neutrophil NETs in the skin and kidneys of UVB-irradiated lupus-prone mice. In addition, UVB irradiation directly or indirectly (PAF) induced upregulation of CXCR4, IFNα, IFNγ, IL-17A, or C3 *in vitro* in the isolated mouse neutrophils. Importantly, portion of the UVB-irradiated neutrophils co-expressed CXCR4 and cytokines/C3, which equipped these neutrophils with both transmigratory and proinflammatory capabilities, enabling their transmigration to the kidneys. As demonstrated in previous studies by our team and others, only portion of neutrophils undergo NETosis *in vitro* (*39*) and *in vivo* (*16*). Therefore, one may expect the transmigration of the non-netting, CXCR4-expressing neutrophils with their expressed cytokines/C3 from the inflamed skin to kidneys of UVB-irradiated MRL/lpr mice, as upregulation of CXCL12 expression has been documented in the kidneys of lupus-prone mice (*37*) and UVB-irradiated mice (*31*). Once recruited by the kidneys, the infiltrated neutrophils can further propagate inflammatory responses with their expressed cytokines/C3, indicating the role of UVB irradiation in driving CXCR4-mediated dermal-renal crosstalk and consequent kidney inflammation. Furthermore, the causality experiments demonstrated that inhibition of CXCR4 attenuated the infiltration of CXCR4-expressing neutrophils to the kidneys, leading to reduced renal accumulation of neutrophil NETs and proinflammatory NET-associated cytokines/C3, and ameliorated proteinuria in UVB-irradiated lupus-prone mice. This causal experiment supports the hypothesis about the transmigration of the non-netting, CXCR4-expressing

neutrophils from the inflamed skin to the kidneys, where they undergo NETosis and exhibit their associated cytokines/C3, contributing to kidney inflammation and proteinuria observed in UVB irradiated lupus-prone mice. Therefore, inhibiting transmigration of neutrophils, particularly CXCR4-expressing neutrophils, could be a promising therapeutic strategy to prevent or delay the onset of lupus kidney inflammation in lupus flares triggered by UVB irradiation.

Over the last decade, neutrophils and NETosis have been implicated in lupus pathogenesis (*13, 14, 21*). A recent report demonstrated that neutrophils selectively infiltrate to the skin of PD-1H KO mice prior disease onset in CLE, indicating the importance of neutrophils in initiation of early-stage of cutaneous lupus (*11*). On the other hand, infiltration of immune cells, including neutrophils, in the kidneys is crucial for the initiation and development of lupus nephritis (*45*). A longitudinal analysis of blood transcriptional profiles of 158 SLE patients for up to 4 years demonstrates that neutrophil-related signatures are associated with the progression of lupus nephritis (*12*). In the current study, we observed a significant increase of neutrophil infiltration and deposition of NETs in the skin and kidneys of UVB-irradiated lupus-prone mice. In addition, the neutrophils and NETs in the skin or kidneys showed positive correlations with dermal skin thickness or proteinuria, respectively, indicating a crucial role of neutrophils and NETs in the skin and kidney inflammation with the early onset of lupus kidney injury and proteinuria triggered by UVB irradiation. In line, previous studies have shown the pathogenic roles of NETosis in CLE (*13, 17*) and lupus nephritis (*18, 19*). NETosis is characterized by the extracellular release of DNA and other immunogenic nuclear components, which are crucial to lupus pathogenesis (*13, 14, 21*). Thus, NETosis is a novel therapeutic target for lupus and other autoimmune diseases (*14*).

Over the past two decades, various signaling pathways have been reported to regulate NETosis, including NOX-dependent or -independent, and ROS-dependent or -independent

pathways (*21, 33, 39*). The diversity of these signaling pathways have challenged the field to identify molecular targets for controlling NETosis. As nuclear chromatin decondensation by peptidyl-arginine deaminase 4 (PAD4) is crucial in NETosis (*46*), PAD4 inhibition has been shown to control NETosis *in vitro* and *in vivo*, inhibiting lupus development both in the skin and kidneys of lupus-prone mice (*13*). However, conflicting results have emerged from another study in which neither genetic deletion nor pharmacological inhibition of PAD4 appeared to affect the development of lupus nephritis in lupus-prone mice (*47*). Therefore, a better understanding of the mechanisms of NET formation, and the development of innovative strategies to effectively inhibit NETosis, are crucial for treating NETosis-related various human diseases, including SLE (*21, 33*).

Given that the nuclear envelope is the first physical barrier for nuclear chromatin externalization and its extracellular release, nuclear envelope rupture is required for NET formation (*21, 33*). Recent studies from our team and others have demonstrated that kinase-mediated disassembly of the nuclear lamina is crucial to nuclear envelope rupture, which allows the extracellular release of nuclear chromatin and NET formation (*15, 16, 48*). In addition, our recent studies demonstrate that strengthening nuclear envelope integrity by overexpression of nuclear lamin B (*15, 33*), or deleting PKCα (*49*), or inhibiting PKCα nuclear translocation (*16, 50*) reduce neutrophil NETosis *in vitro* and *in vivo* in mice. These studies suggested that nuclear envelope regulation could be a novel strategy to inhibit NETosis *in vivo* for therapeutic use. In this study, we found that controlling NETosis by strengthening nuclear envelope integrity effectively alleviated skin and kidney inflammation in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice compared to UVB-irradiated MRL/lpr mice. These results highlight the crucial role of NETosis in skin inflammation, and the early onset of kidney injury triggered by UVB irradiation of asymptomatic young female lupus-prone mice. Therefore, targeting NETosis could be a promising therapeutic strategy to prevent or delay the onset of lupus kidney

inflammation in UVB-induced lupus flares. More importantly, our study demonstrates the protective effects of controlling NETosis on lupus flares through nuclear envelope regulation (*21, 33*). Therefore, targeting nuclear envelope regulation represents as a novel therapeutic strategy for controlling NETosis and treating lupus and other NETosis-related diseases.

As demonstrated in our study, UVB irradiation induced expression of proinflammatory cytokines (IFNα, IFNγ, IL-17A) and complement C3 in neutrophils, both in non-netting and NETotic neutrophils in the skin and kidneys, contributing to inflammatory responses in skin and kidneys, as well as proteinuria observed in UVB-irradiated lupus-prone mice. IFNα and IFNγ, members of the IFN family, are secreted cytokines and important regulators of immunity and inflammation (*20*). Studies from our team and others demonstrate that both IFNα and IFNγ can be expressed by neutrophils, and released with NETs (*16, 51, 52*). Extensive research in animal and human has revealed that the sustained activation of IFN-I pathway plays a crucial role in the pathogenesis of SLE, including cutaneous lupus and lupus nephritis (*20*). In addition, our published studies demonstrate an elevated IFN-regulated gene signature in patients with CLE (*53*), and a reduction in local IFN-I responses with subsequent improvement in skin lesions in SLE patients through downregulating plasmacytoid dendritic cells (*54*). Moreover, Mavragani et al reported elevated expression of IFN-I and defensin-α3 transcripts in renal tissues from patients with lupus nephritis compared to control kidneys (*55*). Evidence suggests that defensin-α3, a NET-associated neutrophil product, may induce local IFN-I expression in kidneys of lupus nephritis patients (*55*). A phase II randomised trial of type I interferon inhibitor anifrolumab has shown beneficial effects in patients with active lupus nephritis compared to placebo (*56*). On the other hand, a large body of evidence from animal and human studies supports the essential role of IFNγ in the development of lupus, including CLE and lupus nephritis (*20, 57, 58*). IFNγ has been shown to have pleiotropic roles in lupus nephritis, including recruitment and activation of neutrophils and other immune cells in the kidneys (*59*).

When IFN-I or IFNγ binds to its receptors, they activate JAK/STAT, a common proinflammatory signaling pathway in SLE (*20*). The limited information from ongoing clinical trials with inhibitors of JAKs or TYK2 have shown beneficial effects on both CLE (*60*) and lupus nephritis (*61*). Given that neutrophil NETs are an upstream target for IFNα and IFNγ, the existing literature suggests that controlling NETosis could be a promising therapeutic strategy for treating lupus flares with skin and kidney inflammation triggered by UVB irradiation in lupus-prone mice.

IL-17A plays a crucial role in lupus pathogenesis by recruiting immune cells to affected organs, including the skin and kidneys (*62*). Oh et al have reported increased IL-17A and IFNα expression in the skin lesions of lupus patients, and expression of IL-17A is correlated with IFNα in these patients, indicating the importance of both cytokines in pathogenesis of skin lupus (*63*). On the other hand, Ramani et al have reported that IL-17A plays an essential role in the development of lupus nephritis (*64*), and IL-17 receptor KO mice exhibit diminished influx of inflammatory cells to their kidneys (*65*). In addition, IL-17A can also regulate IFNγ-mediated neutrophil migration in mouse kidneys (*66*). Furthermore, NET-associated DNA (*67*) or histone H2a (*68*) also have chemotactic effects for leukocyte recruitment or cancer cell metastasis (*67*). We have demonstrated an increase in IL-17A expression in neutrophils and its release with proinflammatory NETs in the skin of UVB-irradiated non-lupus mice in our published works (*15, 16*). In the current study, UVB irradiation induced IL-17A expression in mouse neutrophils *in vitro* and in neutrophil/NETs *in vivo* in the skin and kidneys, contributing to lupus flares with skin and kidney inflammation in UVB-irradiated lupus-prone mice. Therefore, neutrophil NETs and their-associated IL-17A and chemotactic DNA contribute to the continuous recruitment of neutrophils to the inflamed skin and kidneys, propagating the ongoing inflammatory responses in these organs in UVB-irradiated lupus-prone mice.

Deposits of C3 have been found in both the skin (*27*) and kidneys (*69*) of lupus patients. Glomerular C3 deposition indicates ongoing immune responses and active disease in patients with lupus nephritis (*69*) or in animals with experimental glomerulonephritis (*70*). In addition, C3 deposition in the kidneys promotes immune cell infiltration, while C3 depletion results in reduced neutrophil infiltration, NET formation, and ameliorated renal injury in C3 KO mice with ischemia-reperfusion (*71*). In the current study, UVB irradiation induced C3 expression in neutrophils and their released NETs *in vitro* and *in vivo* in the skin and kidneys during lupus flare triggered by UVB irradiation. In line, a previous study reported that C3 can be synthesized and secreted by activated neutrophils (*72*). On the other hand, excessive NETs in SLE patients can activate complement, exacerbating the disease (*26*). Another study also demonstrates that neutrophil NETs can mediate activation of alternative complement pathway, endothelial damage, and glomerular injury, resulting in proteinuria in autoimmune vasculitis (*73*). Furthermore, Liu et al reported significantly increased renal levels of C3 in patients with lupus nephritis and at the onset of lupus nephritis in NZB/W mice (*74*), and C3 deposition in the kidneys may lead to albuminuria in lupus nephritis (*74*). In addition to the role of complement in kidney injury, neutrophil elastase released with NETs also contributes to glomerular vascular endothelial leakage of albumin by degrading the endothelial intercellular junction protein VE-cadherin (*19*). Our study also found significant correlations between the NET-associated C3 and skin or kidney inflammation, as well as proteinuria, indicating a role of NET-associated C3 in lupus flares with skin and kidney inflammation. Therefore, renal deposition of proinflammatory NETs and NET-associated C3 contribute to immune cell infiltration, and the early onset of kidney injury, as well as proteinuria in lupus flares triggered by UVB irradiation in lupus-prone mice.

Taken together, the current study unveiled that neutrophils, especially their released proinflammatory NETs, drive acute skin and kidney inflammation, and the early onset of lupus

kidney injury triggered by UVB irradiation in asymptomatic, young female lupus-prone mice. UVB-induced co-expression of CXCR4 and cytokines/C3 in skin neutrophils was crucial for their transmigration to the kidneys during UVB-induced lupus flares. In addition, the proinflammatory NETs and their-associated cytokines/C3 in the skin and kidneys amplified inflammatory responses, triggering the early onset of renal injury and proteinuria in UVB-induced lupus flares. Generally, freely secreted cytokines and C3 typically diffuse over wider areas, or dilute in the circulation by circulating blood. In contrast, NET-associated cytokines or C3 retain these molecules concentrated for extended periods in the skin or kidneys, thereby sustaining and amplifying inflammatory responses in both organs during UVB-induced lupus flares. Importantly, blocking neutrophil transmigration by CXCR4 inhibition, or directly controlling neutrophil NETosis through nuclear envelope regulation reduced skin and kidney inflammation with ameliorated proteinuria, providing promising therapeutic strategies to prevent or delay the onset of lupus kidney injury in lupus flares triggered by UVB irradiation. More studies are needed to identify pharmacologic inhibitors that can inhibit NETosis through nuclear envelope regulation for future treatment of lupus flares and other NETosis-related human diseases. Furthermore, UVB-induced programmed cell death of neutrophils, their released NETs, and NET-associated proinflammatory and immunogenic properties, contribute to lupus pathogenesis, providing a new mechanistic explanation between sunlight exposure and lupus development.

MATERIALS AND METHODS

Mice

MRL/lpr lupus-prone and Lamin B1 transgenic C57BL/6J-Tg (Lmnb1^{TG}) 1Yfu/J mice (JAX stock #023083) (*75*), and their corresponding littermate controls, were purchased from Jackson

Laboratory and housed in a pathogen-free environment, and given food and water *ad libitum*. To generate MRL/lpr·Lmnb1^{TG}, lupus-prone mice with nuclear lamin B overexpression, Lmnb1^{TG} mice have been backcrossed to MRL/lpr lupus-prone genetic background for at least 10 generations. All animal experiments were approved by the Animal Care and Use Committee of Philadelphia VA Medical Center.

UVB exposure of mice and sample harvest

The dorsal skins of 8-week-old young female asymptomatic MRL/lpr or MRL/lpr·Lmnb1^{TG} mice were UVB (150 mJ/cm^2/day) or sham (control) irradiated for 5 consecutive days under anesthetization as described in our previous published animal model (*15, 16, 49*). In a separate experiment, CXCR4 selective antagonist IT1t (10 mg/kg/day) or sterile normal saline (control) was administered intraperitoneally daily for 6 consecutive days to 8-week-old asymptomatic female MRL/lpr mice, beginning one day before the first session of the 5-day UVB irradiation regimen. Each group of the above experiments included 5-10 mice. One day after the last UVB exposure, all mice will be anesthetized and the systemic circulation perfused with EDTA-PBS (50 units/ml) via left-sided cardiac puncture (*76*). Then the perfused kidneys and dorsal skin were harvested, portion of them will be snap frozen in liquid nitrogen for following experiments. For histologic studies, the skin or kidney tissues were fixed in 4% paraformaldehyde (PFA) for 24h before paraffin-embedded sectioning to 5 µm thick sections for all mice (*15, 16*). Proteinuria score was measured for all mice, before and after 5-day UVB irradiation, with Chemstrip 2GP (Roche, Indianapolis, IN). A scale of 0–3 was used to correspond to the following total urinary protein levels: 0 (negative), 5–20 mg/dL (trace), 30 mg/dL (+), 100 mg/dL (++), and 500 mg/dL (+++).

Cell culture and treatment

Mouse bone marrow (BM) neutrophils from the *femur* and *tibia* BM of either MRL/lpr or MRL/lpr·Lmnb1^{TG} mice were isolated as described in our previous publications (*15, 16*), and were purified with a neutrophil isolation kit (Miltenyl). All of the above primary mouse neutrophils were treated without or with 5 or10 μM platelet-activating factor (PAF), or irradiated without or with UVB at 30-60 mJ/cm^2, and cultured for 20h (*15, 16*) for analyses of neutrophil expression of IFNγ and IL-17A cytokines, complement C3 (C3c), or CXCR4. For induction of IFNα in neutrophils, the above treatments were co-incubated with isolated NETs that were prepared and isolated as described before (*15, 16*). Then, the neutrophils were fixed with 2% PFA and stored at -20 °C for later analysis.

Assessment of Neutrophil NET Formation

All of the above primary neutrophils from MRL/lpr or MRL/lpr·Lmnb1^{TG} mice were treated with 5 or 10 μM PAF for 3h. NET formation assessment and quantification was analyzed by immunofluorescent imaging quantification analysis through staining with impermeable DNA dye SYTOX Green for detection of NET formation, and the cell-permeable DNA dye SYTO Red to count the total number of neutrophils. Then, percentage of NETotic neutrophils were analyzed as described before (*15*).

Transmigration Assays

To study the effects of CXCR4-CXCL12 signaling pathway on neutrophil migration in the context of UVB, the transmigration ability of primary neutrophils from MRL/lpr or MRL/lpr·Lmnb1^{TG} mice was examined by using Boyden chamber as previously described (*15, 16, 37*). Firstly, the primary neutrophils from MRL/lpr or MRL/lpr·Lmnb1^{TG} mice were first stimulated by 10 μM PAF for 20h to induce their CXCR4 expression. To study the inhibitory effects of IT1t, cells (4 x 10^5) from MRL/lpr with PAF pretreatment were treated or not by 10

μM IT1t, a selective CXCR4 antagonist, for 30 min before adding to the upper chamber with 3 μm pore inserts of 24-well trans-well plates, followed by stimulation with CXCL12 (300 ng/ml) in the bottom well for 5h. To examine the effects of nuclear lamin B, neutrophils from MRL/lpr or MRL/lpr·Lmnb1^{TG} mice with PAF pretreatment were added to the upper chamber with 3 μm pore inserts of 24-well trans-well plates, followed by stimulation with CXCL12 (300 ng/ml) in the bottom well for 5h. The pore transmigration ability was expressed as relative transmigration compared to that in control neutrophils of the mice without treatment as described before (*16*).

Flow cytometry analysis

For detection of neutrophil expression of CXCR4, IFNα, IFNγ, IL-17A, and C3 (C3c), the fixed mouse neutrophils that were treated without or with PAF and/or UVB, were first permeabilized with 0.1% Tween-20 and co-stained by PE-labeled anti-mouse Ly6G, FITC-labeled anti-mouse IFNα, IFNγ, IF-17A, or C3, and APC-labeled anti-mouse CXCR4. Analyses of expressions of CXCR4, and/or cytokines, C3 in neutrophils were based on gated cells that were Ly6G positive cells. Flow cytometry analyses were conducted by FACSCanto, and data were analyzed by Flowjo software as described before (*16*).

Immunohistochemistry analyses of the skin and kidneys

The paraffin-embedded skin or kidney tissue sections of all mice were stained either by hematoxylin and eosin (H&E) or by fluorescent staining as described before (*15, 16*). Infiltration of inflammatory cells in skin of all mice was counted for the nucleated cells in the epidermis, dermis, or fat of the H&E stained skin sections as described in our published works (*15, 16*). The glomerular cellularity, including podocytes, endothelial cells, mesangial cells, and infiltrated cells of the kidney cross sections of all mice, was counted as described before

with modifications (*77*). In fluorescent staining, the neutrophil markers PE-labeled anti-ly6G or Alexa-647-labeled myeloperoxidase (MPO) were used for detection of neutrophils and NETosis in combination with FITC-labeled citrullinated histone H3 (citH3), and DNA staining with DAPI as described before (*15*). FITC-labeled anti-mouse antibodies against IFNα, IFNγ, IL-17A, C3, or CXCR4 were used to stain the corresponding molecules in the skin or kidneys. The fluorescent color matched isotype immunoglobulins were used as isotype control staining. Slides were mounted with Gold Antifade Mountant (Invitrogen). The fluorescent images were analyzed with an Olympus Fluoview 1000 confocal microscope or Leica fluorescent microscope. To analyze images for molecular quantification in IHC analyses, the 2D high resolution fluorescent images from at least 5-6 random non-adjacent areas of dermis per skin section, or 20 random glomeruli per mouse kidney section, were captured with an Olympus confocal or Leica fluorescent microscopes and saved as .tiff files. The images were analyzed with NIH ImageJ software by following step-by-step workflow according to the published protocols (*78, 79*). Quantification of the molecules of interest, i.e. neutrophils, NETs, cytokines, or C3, and CXCR4 were based on an automatic threshold analysis of immunofluorescence images to automatically identify the top brightest structures of each image as described in our published work (*15, 16*).

Statistical analyses

GraphPad Prism 6 was used to perform statistical analysis. Normally distributed data are shown as means ± standard deviations (SD). Comparisons between two groups were conducted with student T test. Comparisons amongst three or more groups were performed using ANOVA, followed by Student-Newman-Keuls (SNK) test. Pearson's correlation coefficient was used for analyses of a linear association between two variables. Comparison between two groups was

analyzed using Student's t-test. Statistical significance was considered at a level of P-value < 0.05.

AUTHOR CONTRIBUTIONS

The research was conducted at the University of Pennsylvania and VAMC in Philadelphia. Liu conceived and designed the study. Lyu, Li, Liu performed the laboratory experiments and acquired the experimental data. For the microscopic image analysis, Liu, Lyu took the images, Lyu and Li conducted further quantification and statistical analysis. Liu and Lyu analyzed and interpreted the data and finalized the paper. Lyu, Li, Zhang, Wei, Werth and Liu were involved in the investigation, preparation and revision of the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. UVB irradiation of asymptomatic young female MRL/lpr lupus-prone mice triggered lupus flares with skin and kidney inflammation as well as proteinuria

A-**D**. Summary analyses of skin thickness of epidermis (A), dermis (B), inflammatory cell infiltration (C, nucleated cells) in skin, representative images of H&E staining of skin (D). **E-G.** Summary analyses of proteinuria scores (E) and glomerular cellularity of kidneys (F), and representative images of H&E staining of glomeruli (G) in asymptomatic young female MRL/lpr mice without (sham) or with UVB irradiation. **H**-**J**. Correlations between inflammatory cell infiltration in skin and dermal skin thickness (H) or proteinuria scores (I), and correlation between glomerular cellularity and proteinuria scores (**J)** in the combined groups of mice without or with UVB. Scale bars, 100 µm. Results represent 5 biological replicates in each group. Panels A-C,E,F display means ± SD, student's t-test were conducted for two-group comparisons. Panels H-J were Pearson correlation analysis.

Figure 2. UVB irradiation induced infiltration of neutrophils, their expression of cytokines/C3 and CXCR4, and NETosis in skin of UVB-irradiated lupus-prone mice

A-**I.** Summary or representative images of neutrophils, citrullinated H3-positive NETs, NET-associated IFNα, IFNγ, IL-17A, C3 or CXCR4 in the skin of MRL/lpr mice without (sham) or with UVB irradiation. In panels A-I, neutrophils, NETs and NET-associated IFNα, IFNγ, IL-17A, C3, and CXCR4 in skin sections from all mice were stained with PE-labeled anti-ly6G, FITC-labeled antibodies against citH3, IFNα, IFNγ, IL-17A, C3, or CXCR4, and DAPI for DNA. In panels C and I, yellow arrows indicate neutrophil NETs, and light blue arrows indicate

intact non-netting neutrophils. Scale bars, 50 μm, **J**. Correlation analysis between C3 distribution and dermal nucleated cell count in combined groups of mice without or with UVB. **K**. Correlation analysis between CXCR4 expression in neutrophils and NETs in skin and proteinuria scores in combined groups of mice without or with UVB. Results represent 5 biological replicates in each group. Panels A-J display mean ± SD, student's t-test were conducted for two-group comparisons. Panel J was Pearson correlation analysis.

Figure 3. Neutrophil infiltration, NET formation, and NET-associated cytokines/C3 are involved in acute kidney inflammation in UVB-irradiated lupus-prone mice

A-I. Summary or representative images of neutrophils, citrullinated H3-positive NETs, NET-associated IFNα, IFNγ, IL-17A, C3 or CXCR4 in kidneys of MRL/lpr mice without (sham) or with UVB irradiation. In panels A-I, neutrophils, NETs and NET-associated IFNα, IFNγ, IL-17A, C3, and CXCR4 in kidney sections from all mice were stained with PE-labeled anti-ly6G, FITC-labeled antibodies against citH3, IFNα, IFNγ, IL-17A, C3, or CXCR4, and DAPI for DNA. In panels C and I, yellow arrows indicate neutrophil NETs and light blue arrows indicate intact non-netting neutrophils. Scale bars, 50 μm. **J**. Correlation analysis between CXCR4 expression in neutrophils and NETs in kidneys and proteinuria scores in the combined groups of mice without or with UVB. Results represent 5 biological replicates in each group. Panels A-J display mean ± SD, student's t-test were conducted for two-group comparisons. Panel J was Pearson correlation analysis.

Figure 4. UVB irradiation induced co-expression of CXCR4, and cytokines or C3 *in vitro* in primary neutrophils from lupus-prone mice

A-L. Summary or representative dotplots of flow cytometry analyses of expressions of cytokines IFNα (A-C), IFNγ (D-F), IL-17A (G-I), or C3 (J-L) and their co-expression with CXCR4 in primary neutrophils from MRL/lpr mice, which were stimulated without (control) or with either 10 μM PAF, or 60 mJ/cm^2 UVB irradiation, and incubated for 20h. The cells were fixed with 2% PFA, permeabilized with 0.1% Tween-20, and stained by PE-labeled anti-mouse ly6G, APC-labeled anti-mouse CXCR4, and FITC-labeled anti-mouse IFNα, IFNγ, IF-17A, or C3, followed by flow cytometry analyses. All neutrophils in panels A-L were based on gated cells that were ly6G positive cells. Results represent 5-6 biological replicates in different panels. Panels A,B,D,E,G,H,J,K display means ± SD, student's t-test were conducted for two-group comparisons.

Figure 5. Inhibition of CXCR4 attenuated acute kidney inflammation in lupus-prone mice with lupus flare triggered by UVB

A-C. Summary analyses or representative IHC images of proteinuria scores (A), glomerular cellularity (B,C) of kidneys of UVB-irradiated MRL/lpr mice without (control) or with pre-administration of IT1t (i.p.). **D-E**. Summary analyses of neutrophils or CXCR4-expressing neutrophils in glomeruli of kidneys of above mice without or with IT1t administration. **F**. Relative migration analysis of mouse neutrophils with CXCR4 expression, induced by 10 μM PAF stimulation, without or with IT1t pretreatment, was conducted with Boyden chamber by stimulation with CXCL12 at 300 ng/ml. **G-L**. Summary analyses or representative IHC images of deposition of NETs, and NET-associated IFNα, IFNγ, IL-17A, or C3 in glomeruli of kidneys of UVB-irradiated MRL/lpr mice without (control) or with pre-administration of IT1t. Neutrophils, CXCR4-expressing neutrophils, NETs, and NET-associated IFNα, IFNγ, IL-17A, or C3 in glomeruli of kidney sections from all mice were stained with PE-labeled anti-ly6G, FITC-labeled antibodies against citH3, IFNα, IFNγ, IL-17A, C3, or CXCR4, and DAPI for

DNA. Scale bars, 100 µm. Results represent 3-5 biological replicates in different panels. Panels A,B,D-G,I-L display means ± SD, student's t-test were conducted for two-group comparisons.

Figure 6. Controlling NETosis by strengthenning nuclear envelope integrity attenuated lupus skin inflammation in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice

A-C. Summary analyses of skin thickness of epidermis (A), dermis (B), and proinflammatory cell infiltration (C, nucleated cells) in skin of MRL/lpr vs MRL/lpr·lmnB1^{Tg} mice without or with UVB irradiation. **D-J**. Summary analyses or representative images of neutrophils (D), NETs (E), and NET-associated IFNα, IFNγ, IL-17A, and C3 (F-J) in skin of MRL/lpr vs MRL/lpr·lmnB1^{Tg} mice without (sham) or with UVB irradiation. Neutrophils, NETs and NET-associated IFNα, IFNγ, IL-17A, and C3 in skin sections (D-J) were stained with PE-labeled anti-ly6G, FITC-labeled antibodies against citH3, IFNα, IFNγ, IL-17A, or C3, and DAPI for DNA. Scale bars, 100 µm. Results represent 5-10 biological replicates in each group. Panels A-E,G-J display mean ± SD, $P<0.01$ by ANOVA; displayed are significance levels for individual pairwise comparisons by SNK test.

Figure 7. Inhibition of NETosis by nuclear envelope regulation attenuated acute kidney inflammation in MRL/lpr·lmnB1^{Tg} mice irradiated by UVB

A-C. Summary analysis of proteinuria scores (A) or glomerular cellularity (B) and representative glomerular images (C) of MRL/lpr vs MRL/lpr·lmnB1^{Tg} mice without (sham) or with UVB irradiation. **D-J**. Summary analysis or representative images of neutrophils (D), NETs (E), NET-associated IFNα, IFNγ, IL-17A, C3 (F-J) in glomeruli of kidneys of MRL/lpr vs MRL/lpr·lmnB1^{Tg} mice without (sham) or with UVB irradiation. Neutrophils, NETs, and NET-associated IFNα, IFNγ, IL-17A, and C3 in kidney sections (D-J) were stained with PE-labeled anti-ly6G, FITC-labeled antibodies against citH3, IFNα, IFNγ, IL-17A, or C3, and

DAPI for DNA. Scale bars, 50 µm. **K**. Correlation analysis between NET-associated IFNα and proteinuria scores in the combined groups of mice without or with UVB. Results represent 5-10 biological replicates in each group. Panels A,B,D-F,H-J display mean ± SD, $P<0.01$ by ANOVA; displayed are significance levels for individual pairwise comparisons by SNK test. Panels K was Pearson correlation analysis.

Figure 1

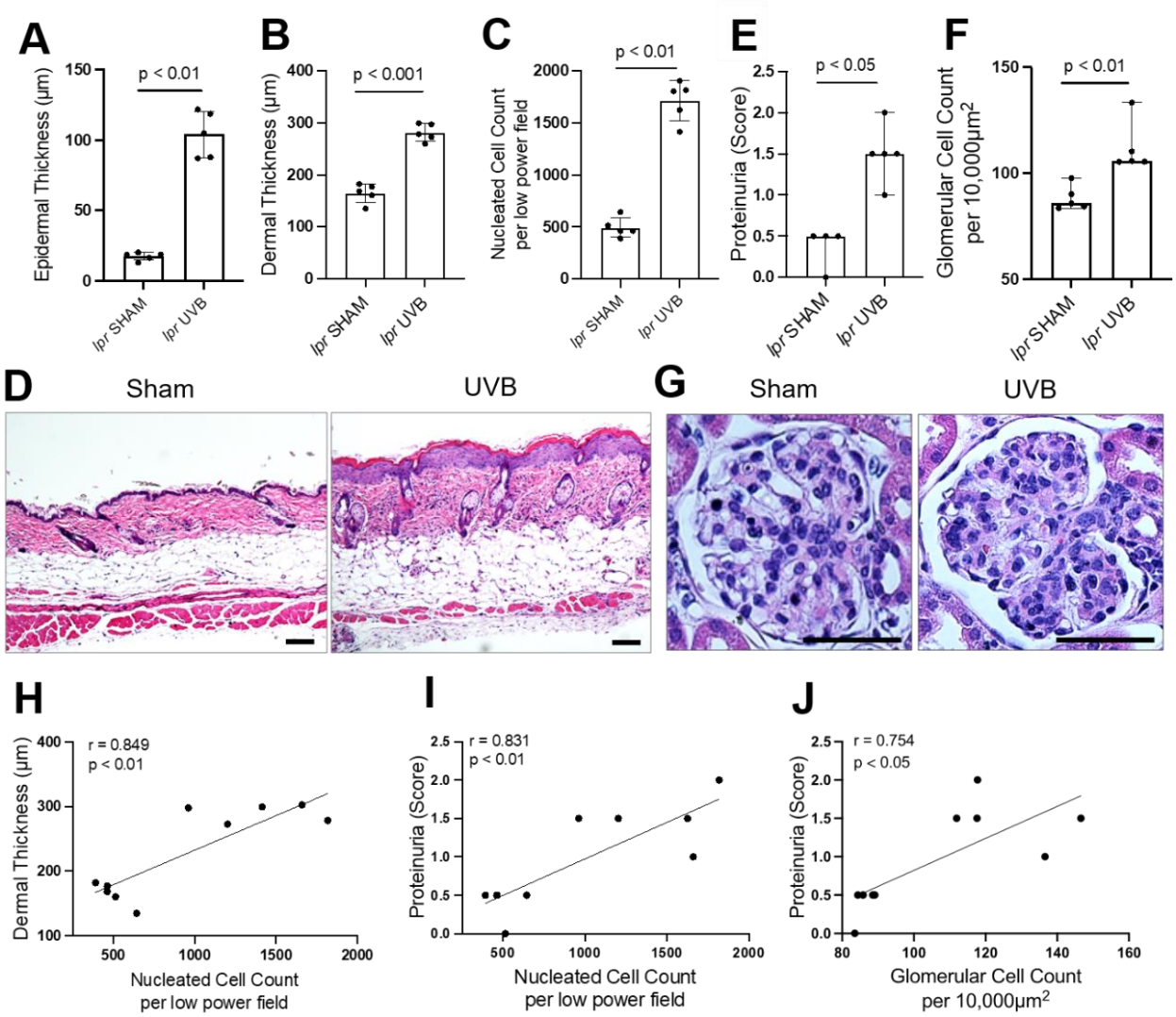

A
Epidermal Thickness (µm)
p < 0.01
lpr SHAM
lpr UVB
B
Dermal Thickness (µm)
p < 0.001
C
Nucleated Cell Count per low power field
p < 0.01
E
Proteinuria (Score)
p < 0.05
F
Glomerular Cell Count per 10,000µm²
p < 0.01
D
Sham
UVB
G
Sham
UVB
H
Dermal Thickness (µm)
r = 0.849
p < 0.01
Nucleated Cell Count per low power field
I
Proteinuria (Score)
r = 0.831
p < 0.01
Nucleated Cell Count per low power field
J
Proteinuria (Score)
r = 0.754
p < 0.05
Glomerular Cell Count per 10,000µm²

Figure 2.

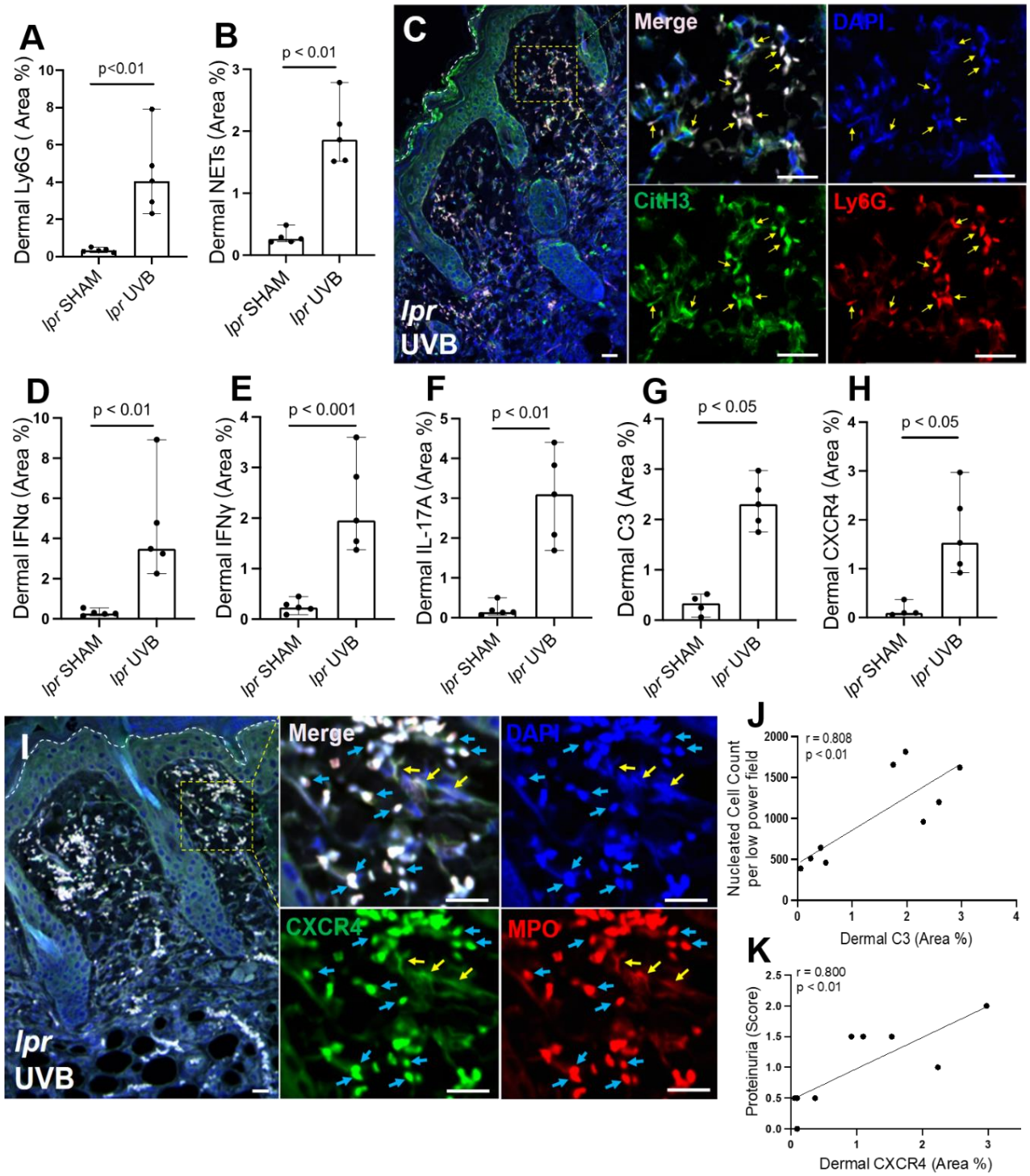

A
p<0.01
Dermal Ly6G (Area %)
lpr SHAM
lpr UVB
B
p < 0.01
Dermal NETs (Area %)
lpr SHAM
lpr UVB
C
Merge
DAPI
CitH3
Ly6G
lpr
UVB
D
p < 0.01
Dermal IFNα (Area %)
E
p < 0.001
Dermal IFNγ (Area %)
F
p < 0.01
Dermal IL-17A (Area %)
G
p < 0.05
Dermal C3 (Area %)
H
p < 0.05
Dermal CXCR4 (Area %)
I
Merge
DAPI
CXCR4
MPO
lpr
UVB
J
r = 0.808
p < 0.01
Nucleated Cell Count per low power field
Dermal C3 (Area %)
K
r = 0.800
p < 0.01
Proteinuria (Score)
Dermal CXCR4 (Area %)

Figure 3.

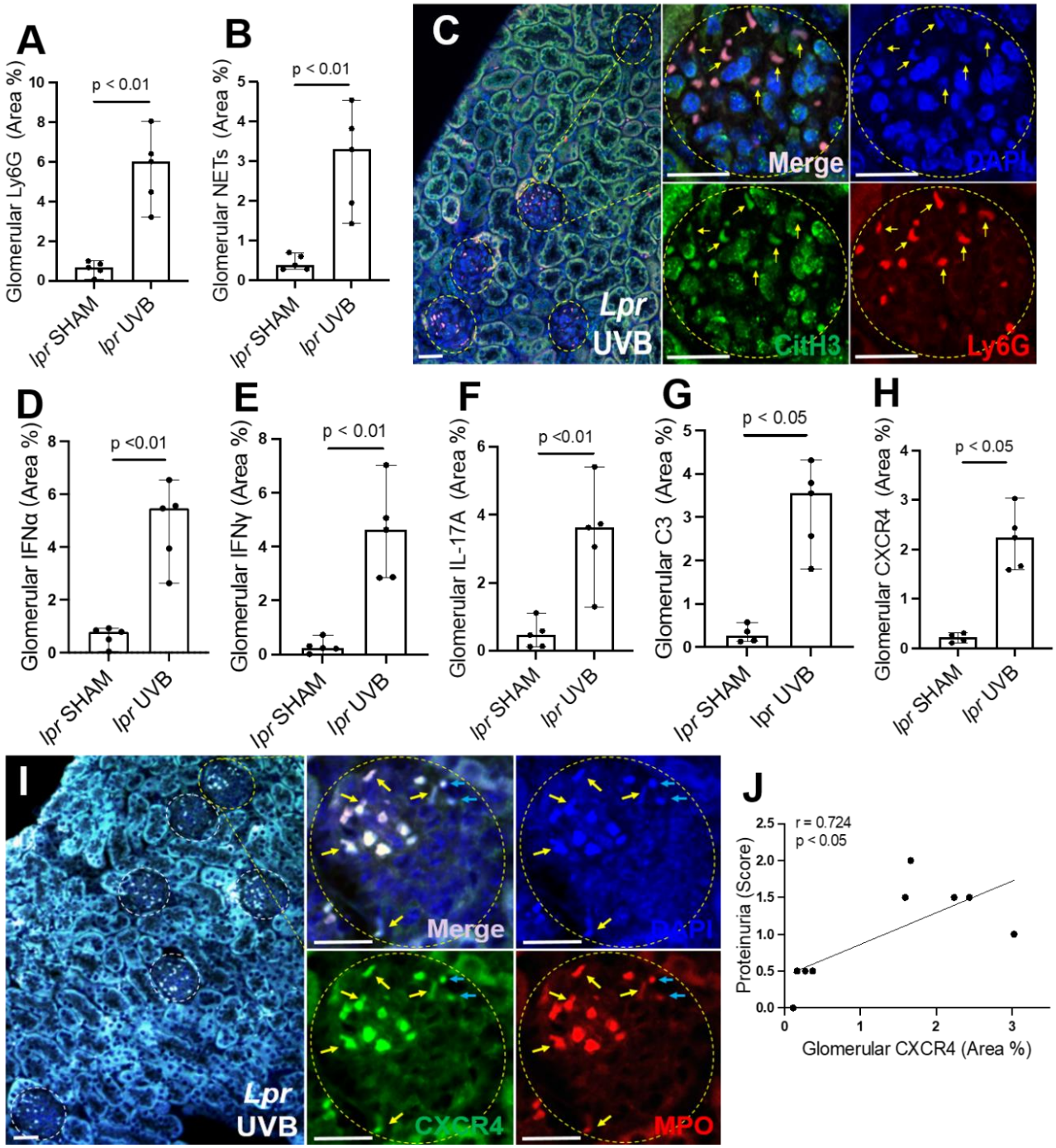

A
Glomerular Ly6G (Area %)
p < 0.01
lpr SHAM
lpr UVB
B
Glomerular NETs (Area %)
p < 0.01
lpr SHAM
lpr UVB
C
Lpr
UVB
Merge
DAPI
CitH3
Ly6G
D
Glomerular IFNα (Area %)
p <0.01
lpr SHAM
lpr UVB
E
Glomerular IFNγ (Area %)
p < 0.01
lpr SHAM
lpr UVB
F
Glomerular IL-17A (Area %)
p <0.01
lpr SHAM
lpr UVB
G
Glomerular C3 (Area %)
p < 0.05
lpr SHAM
lpr UVB
H
Glomerular CXCR4 (Area %)
p < 0.05
lpr SHAM
lpr UVB
I
Lpr
UVB
Merge
DAPI
CXCR4
MPO
J
r = 0.724
p < 0.05
Proteinuria (Score)
Glomerular CXCR4 (Area %)

Figure 4.

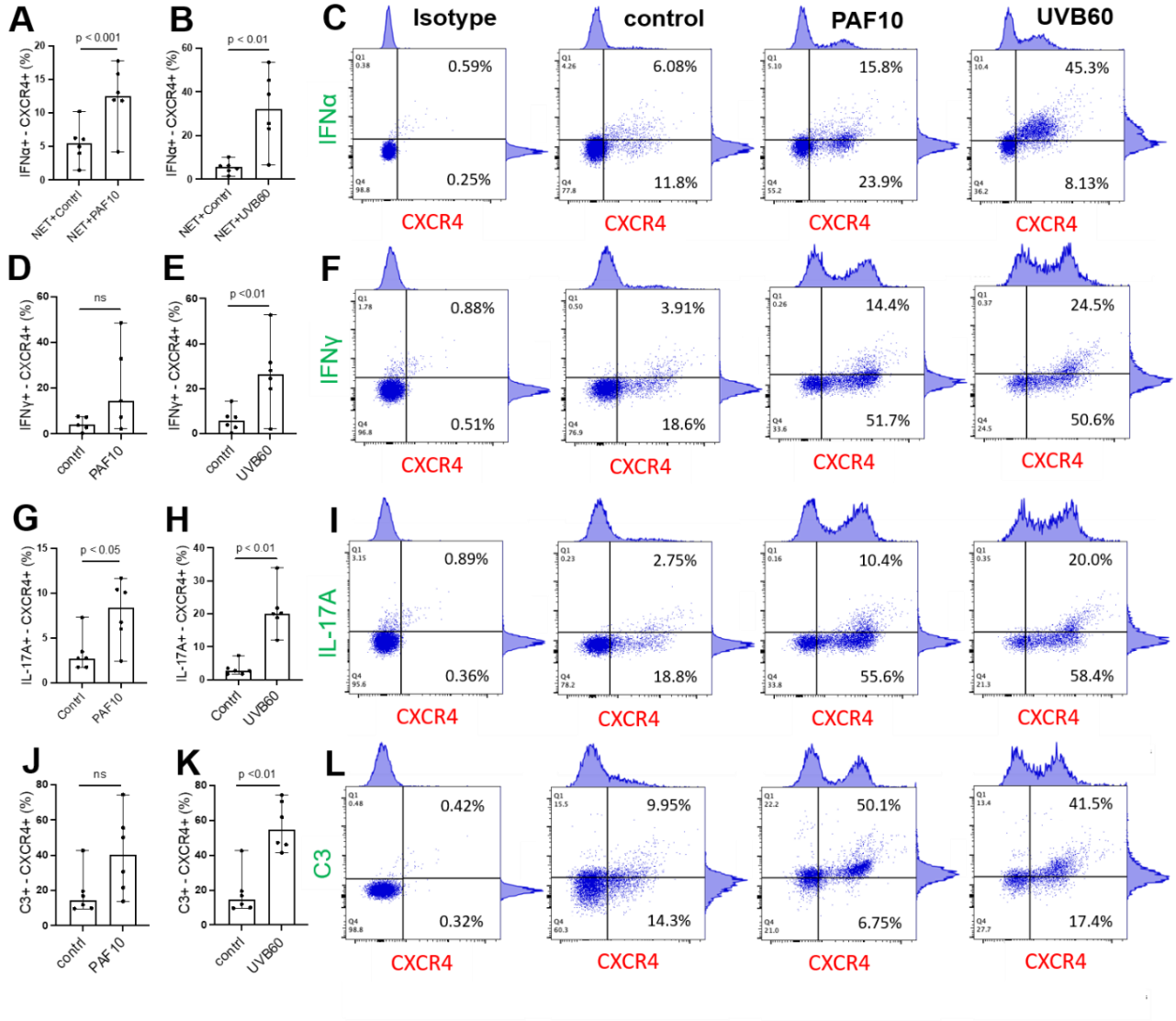
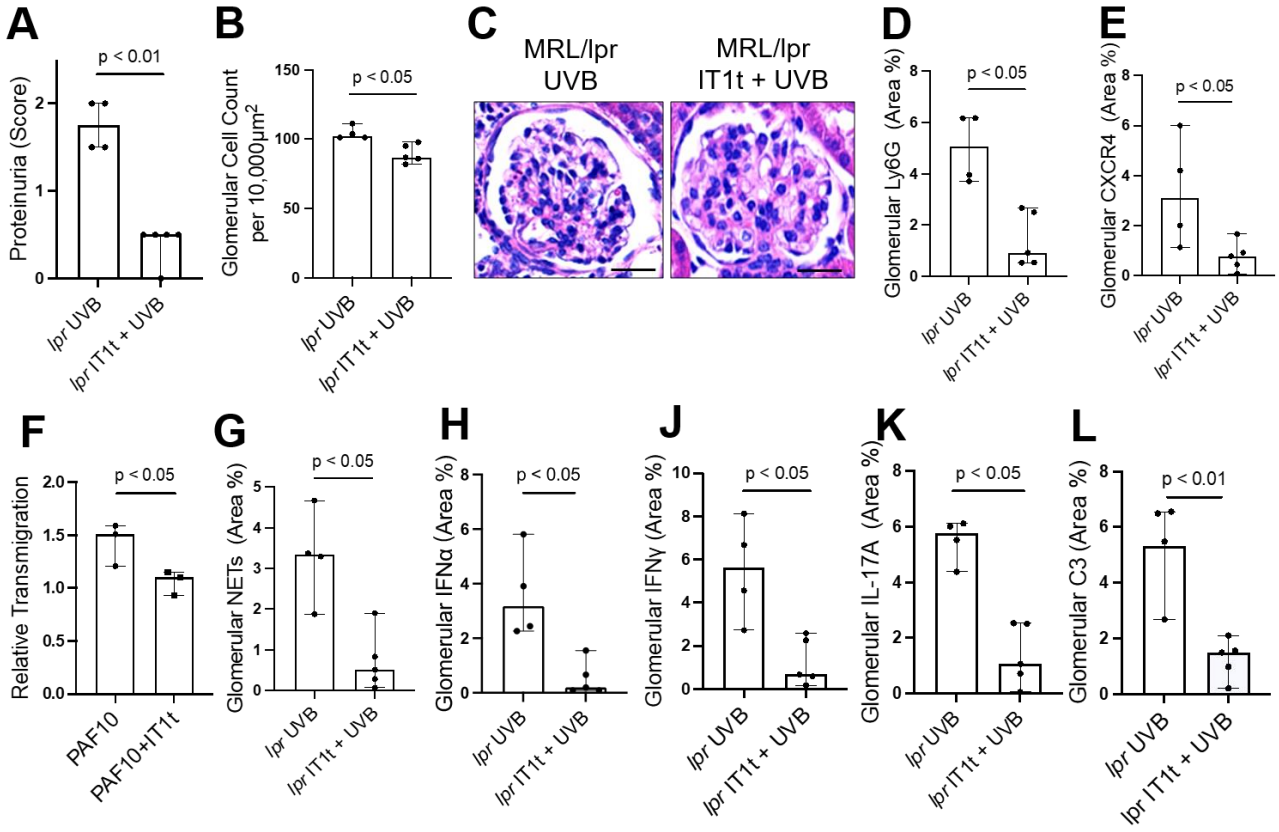

A
p < 0.001
IFNα+ - CXCR4+ (%)
NET+Contrl
NET+PAF10
B
p < 0.01
IFNα+ - CXCR4+ (%)
NET+Contrl
NET+UVB60
C
Isotype
control
PAF10
UVB60
IFNα
0.59%
0.25%
6.08%
11.8%
15.8%
23.9%
45.3%
8.13%
CXCR4
D
ns
IFNγ+ - CXCR4+ (%)
contrl
PAF10
E
p <0.01
IFNγ+ - CXCR4+ (%)
contrl
UVB60
F
IFNγ
0.88%
0.51%
3.91%
18.6%
14.4%
51.7%
24.5%
50.6%
CXCR4
G
p < 0.05
IL-17A+ - CXCR4+ (%)
Contrl
PAF10
H
p < 0.01
IL-17A+ - CXCR4+ (%)
Contrl
UVB60
I
IL-17A
0.89%
0.36%
2.75%
18.8%
10.4%
55.6%
20.0%
58.4%
CXCR4
J
ns
C3+ - CXCR4+ (%)
contrl
PAF10
K
p <0.01
C3+ - CXCR4+ (%)
contrl
UVB60
L
C3
0.42%
0.32%
9.95%
14.3%
50.1%
6.75%
41.5%
17.4%
CXCR4

Figure 5.


A
p < 0.01
Proteinuria (Score)
lpr UVB
lpr IT1t + UVB
B
p < 0.05
Glomerular Cell Count per 10,000μm²
C
MRL/lpr UVB
MRL/lpr IT1t + UVB
D
p < 0.05
Glomerular Ly6G (Area %)
E
p < 0.05
Glomerular CXCR4 (Area %)
F
p < 0.05
Relative Transmigration
PAF10
PAF10+IT1t
G
p < 0.05
Glomerular NETs (Area %)
H
p < 0.05
Glomerular IFNα (Area %)
J
p < 0.05
Glomerular IFNγ (Area %)
K
p < 0.05
Glomerular IL-17A (Area %)
L
p < 0.01
Glomerular C3 (Area %)

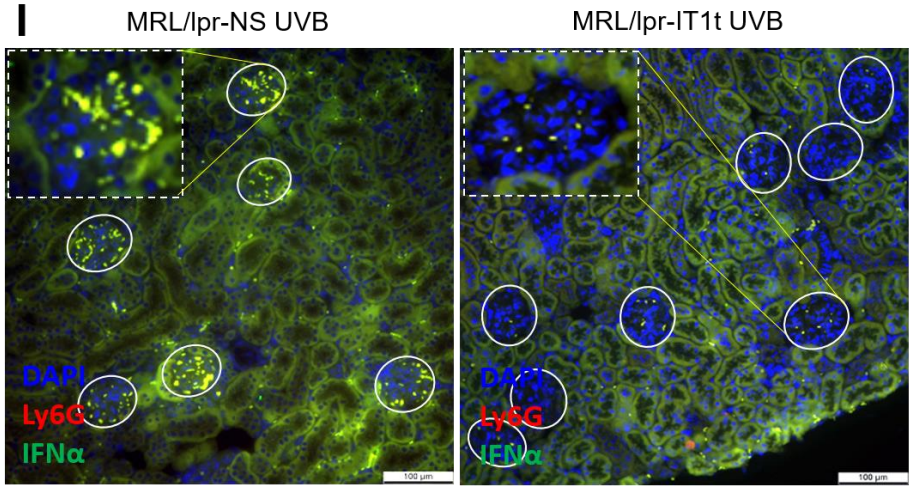

I
MRL/lpr-NS UVB
MRL/lpr-IT1t UVB
DAPI
Ly6G
IFNα

Figure 6.

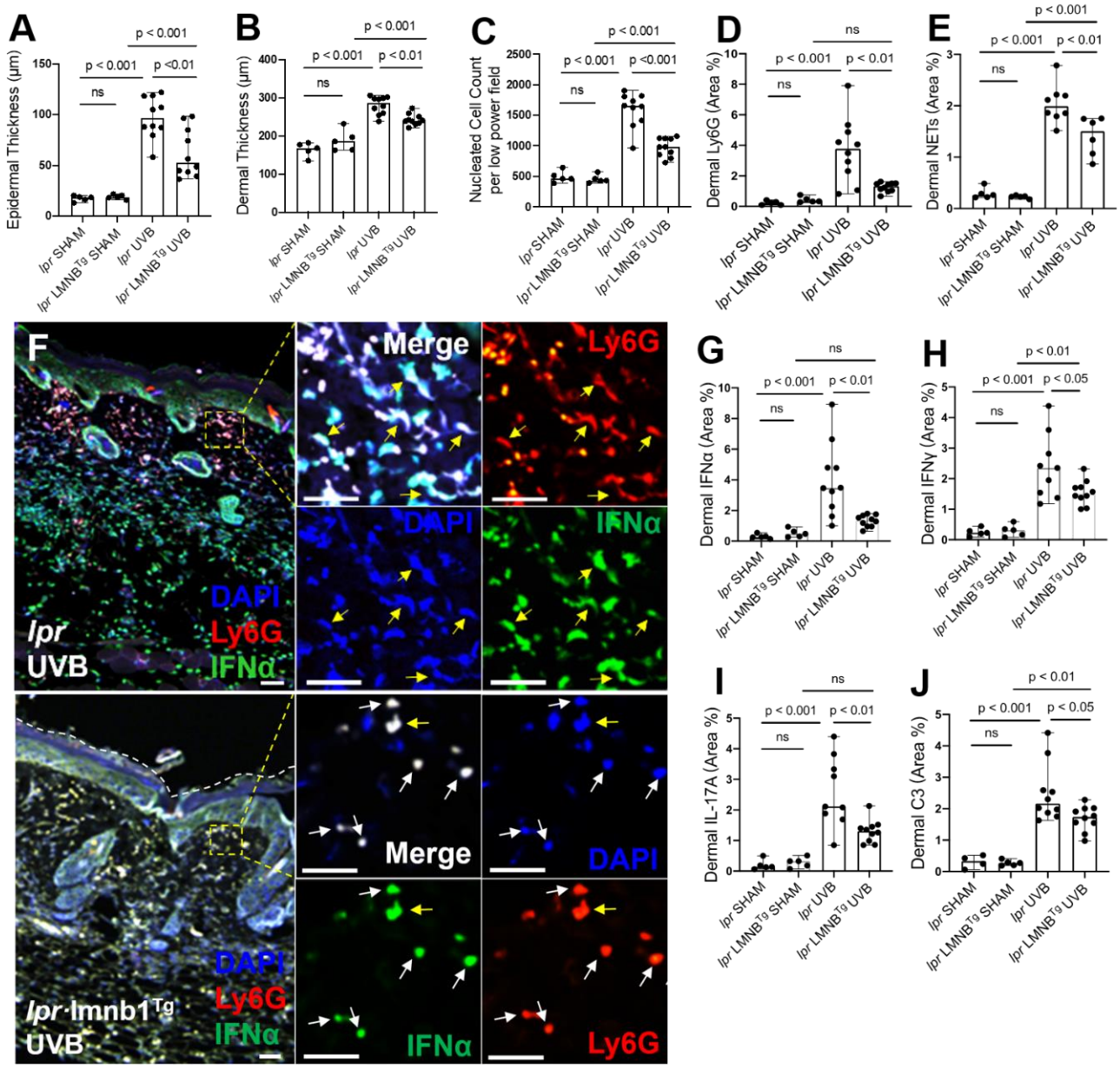
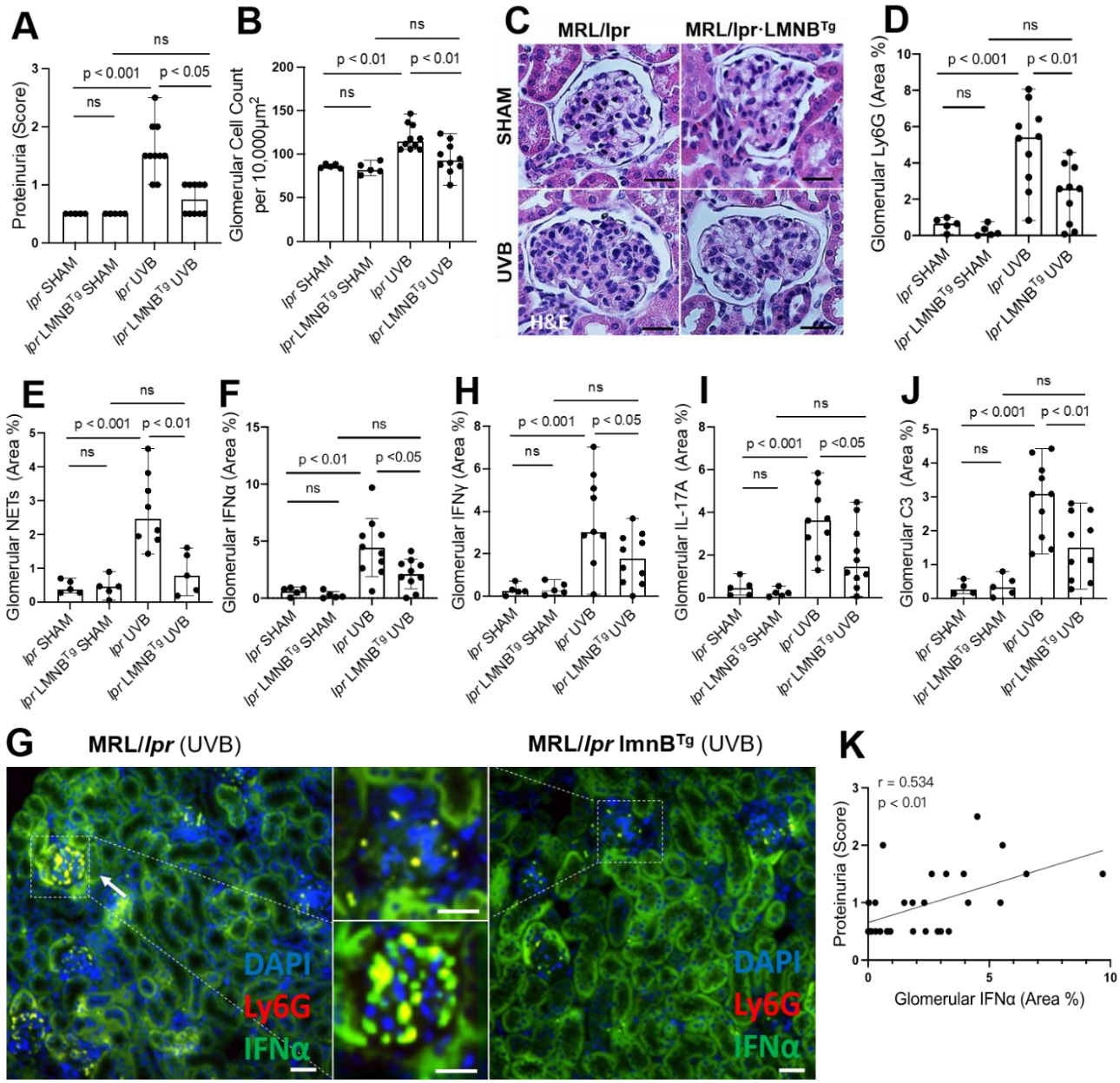

A
Epidermal Thickness (μm)
p < 0.001
p < 0.001
p <0.01
ns
lpr SHAM
lpr LMNB Tg SHAM
lpr UVB
lpr LMNB Tg UVB
B
Dermal Thickness (μm)
C
Nucleated Cell Count per low power field
D
Dermal Ly6G (Area %)
E
Dermal NETs (Area %)
F
Merge
Ly6G
DAPI
IFNα
lpr UVB
lpr·lmnb1Tg UVB
G
Dermal IFNα (Area %)
H
Dermal IFNγ (Area %)
I
Dermal IL-17A (Area %)
J
Dermal C3 (Area %)

Figure 7.


A
Proteinuria (Score)
ns
p < 0.001
p < 0.05
ns
lpr SHAM
lpr LMNBTg SHAM
lpr UVB
lpr LMNBTg UVB
B
Glomerular Cell Count per 10,000μm2
ns
p < 0.01
p < 0.01
ns
C
MRL/lpr
MRL/lpr·LMNBTg
SHAM
UVB
H&E
D
Glomerular Ly6G (Area %)
ns
p < 0.001
p < 0.01
ns
E
Glomerular NETs (Area %)
ns
p < 0.001
p < 0.01
ns
F
Glomerular IFNα (Area %)
ns
p < 0.01
p <0.05
ns
H
Glomerular IFNγ (Area %)
ns
p < 0.001
p < 0.05
ns
I
Glomerular IL-17A (Area %)
ns
p < 0.001
p <0.05
ns
J
Glomerular C3 (Area %)
ns
p < 0.001
p < 0.01
ns
G
MRL/lpr (UVB)
MRL/lpr lmnBTg (UVB)
DAPI
Ly6G
IFNα
K
r = 0.534
p < 0.01
Proteinuria (Score)
Glomerular IFNα (Area %)

Supplemental Data

For

Neutrophil extracellular traps drive lupus flares with acute skin and kidney inflammation triggered by ultraviolet irradiation

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Short title: NETosis and UVB-induced lupus flares

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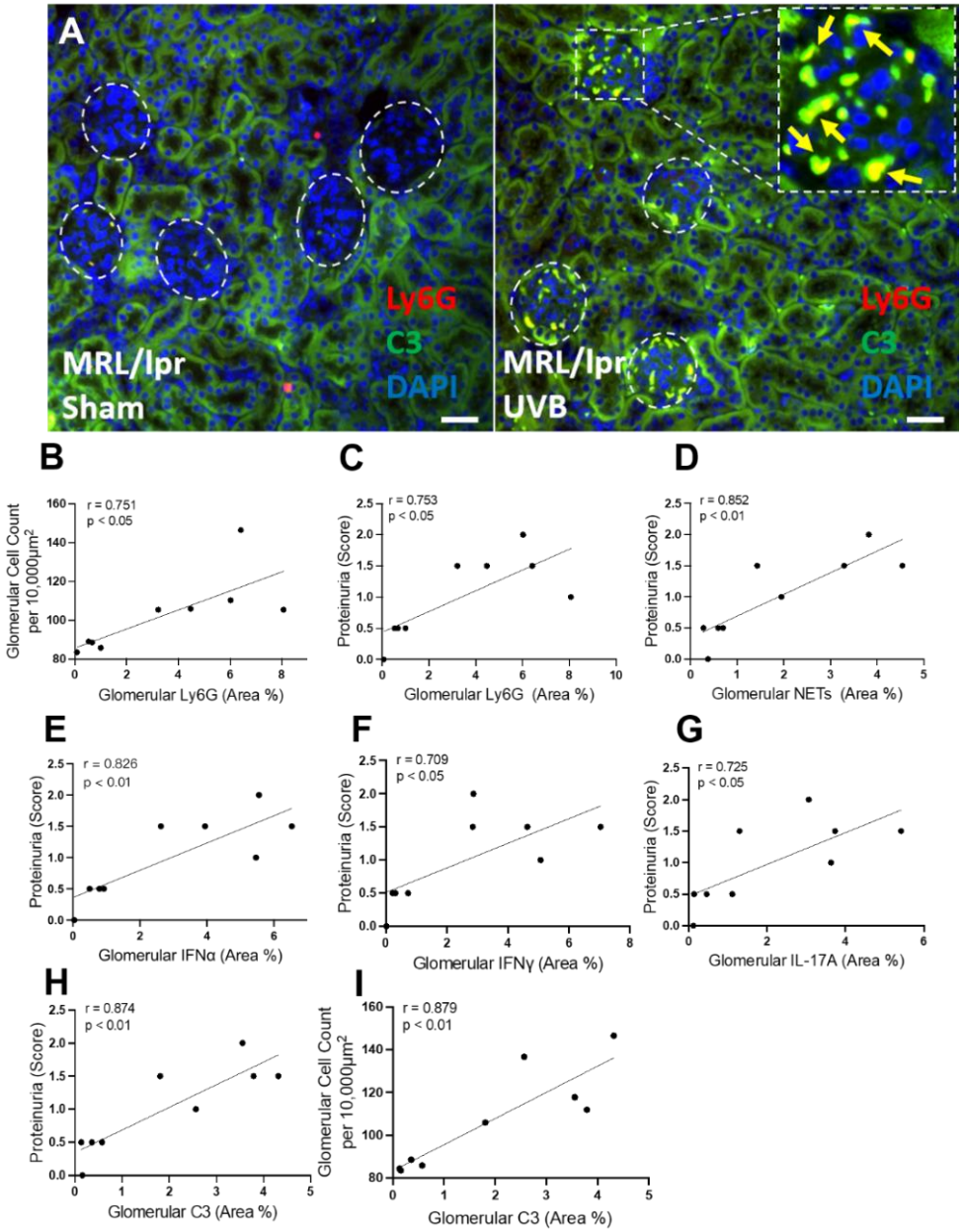
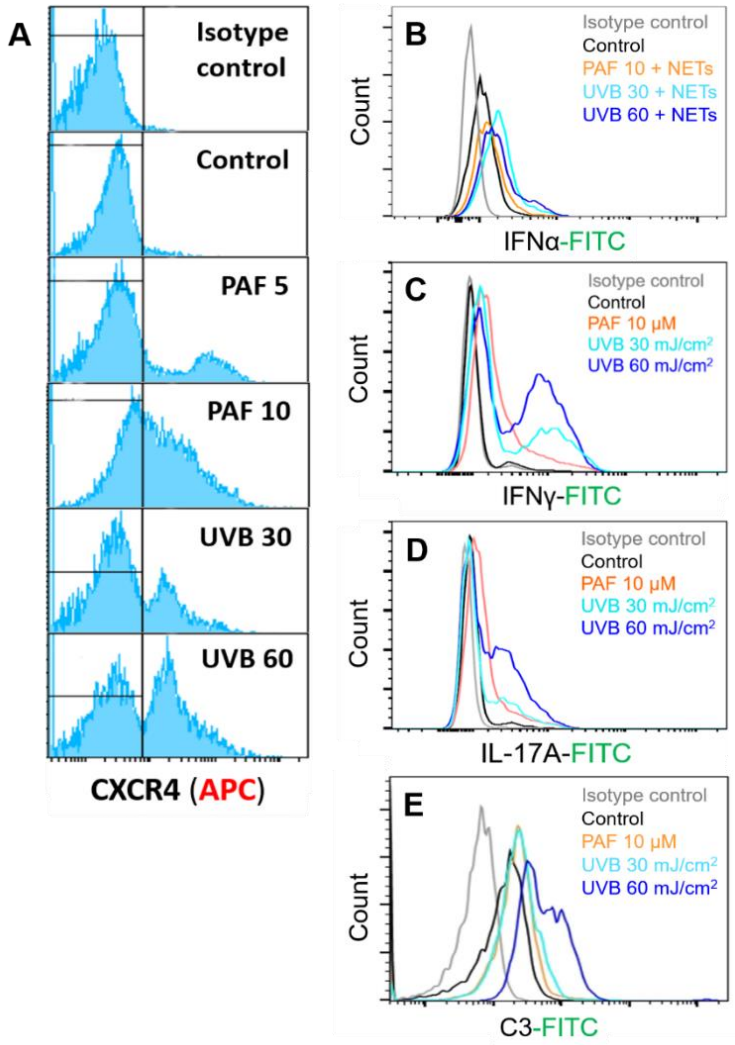
Key words: NETosis, lupus flare, skin inflammation, kidney inflammation, UVB



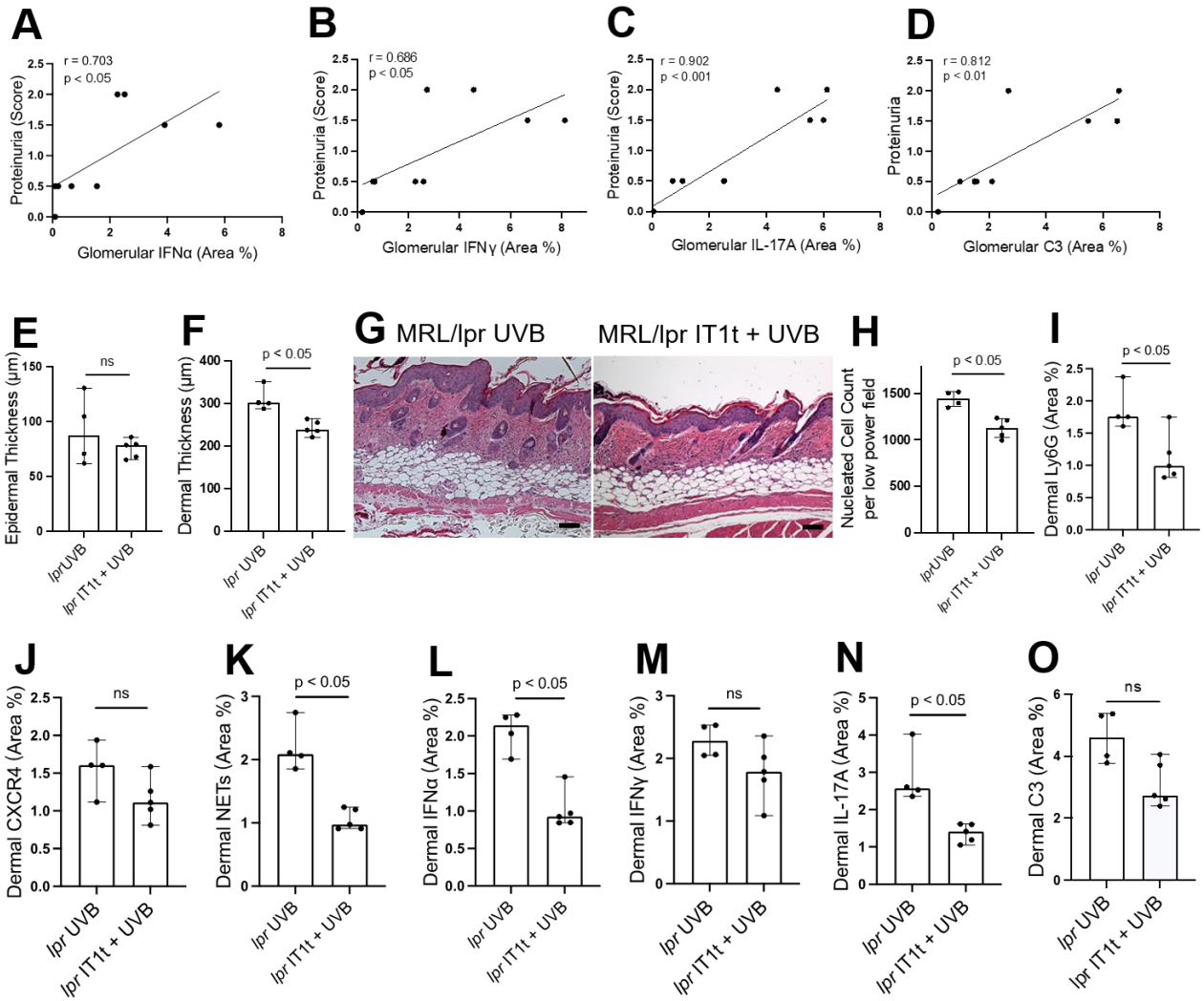
Suppl Fig. 1. Neutrophil infiltration, NET formation, and NET-associated cytokines/C3 are involved in acute kidney inflammation in UVB-irradiated lupus-prone mice

A. Representative images of neutrophils, NETs, NET-associated C3 in kidneys of asymptomatic young female MRL/lpr mice without (sham) or with UVB irradiation. Neutrophils, NETs and NET-associated C3 in kidney sections from all mice were stained with PE-labeled anti-ly6G, FITC-labeled antibodies against C3, and DAPI for DNA. Insets of panel A show enlarged images of representative glomerulus with neutrophils and NETs with staining of Ly6G, C3 and DAPI as indicated. Scale bars, 50 µm. **B**. Correlation analysis between glomerular neutrophil infiltration and glomerular cellularity. **C**-**H**. Correlation analyses between glomerular neutrophils, NETs, NET-associated IFNα, IFNγ, IL-17A, or C3 in kidneys and proteinuria in combined groups of mice without or with UVB. **I**. Correlation analysis between glomerular C3 distribution and glomerular cellularity. Suppl panels B-I were Pearson correlation analyses.



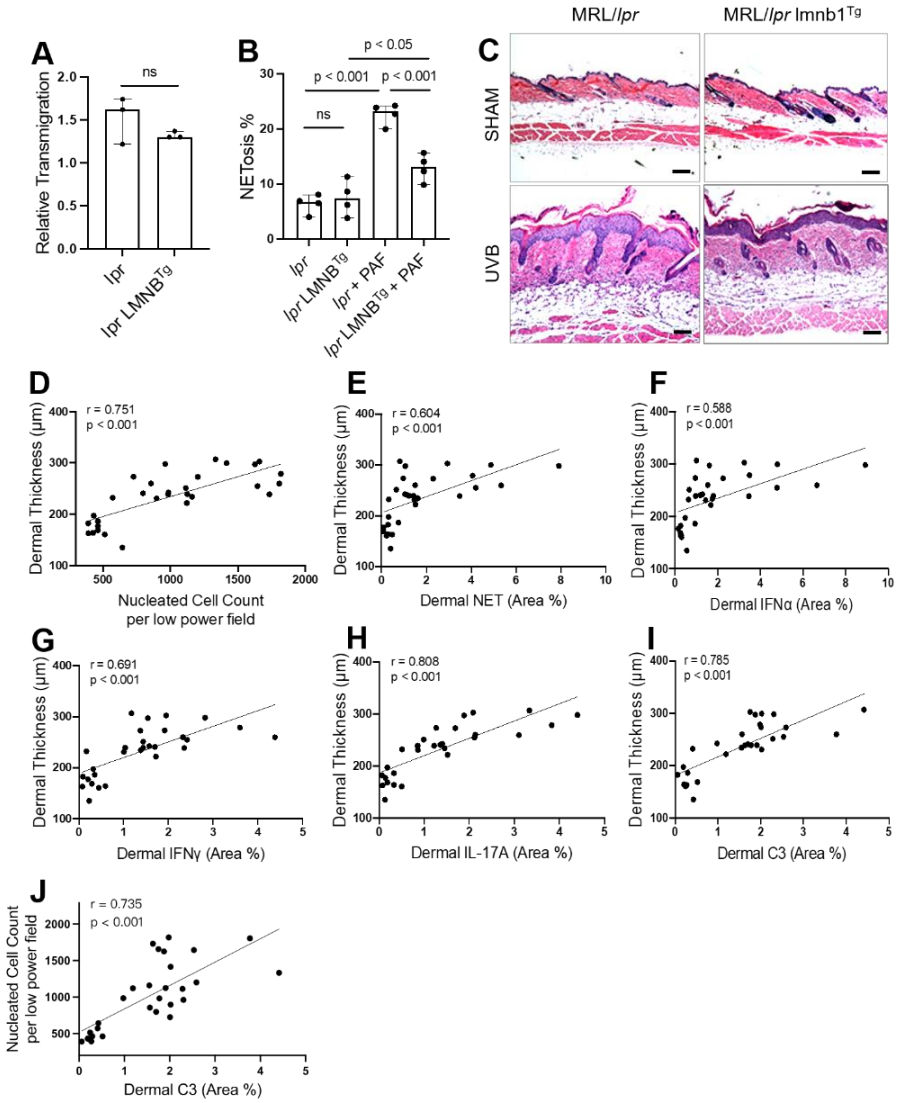
Suppl Fig. 2. UVB irradiation induced co-expression of CXCR4, cytokines, and C3 *in vitro* in primary neutrophils from lupus-prone mice

A-E. Representative histogram of expressions of CXCR4 or cytokines IFNα, IFNγ, IL-17A, or C3 in primary neutrophils from lupus-prone mice, which were stimulated without (control) or with either 5-10 μM PAF, or 30-60 mJ/cm^2 UVB irradiation, and incubated for 20h, then processed and stained as described in the methods.



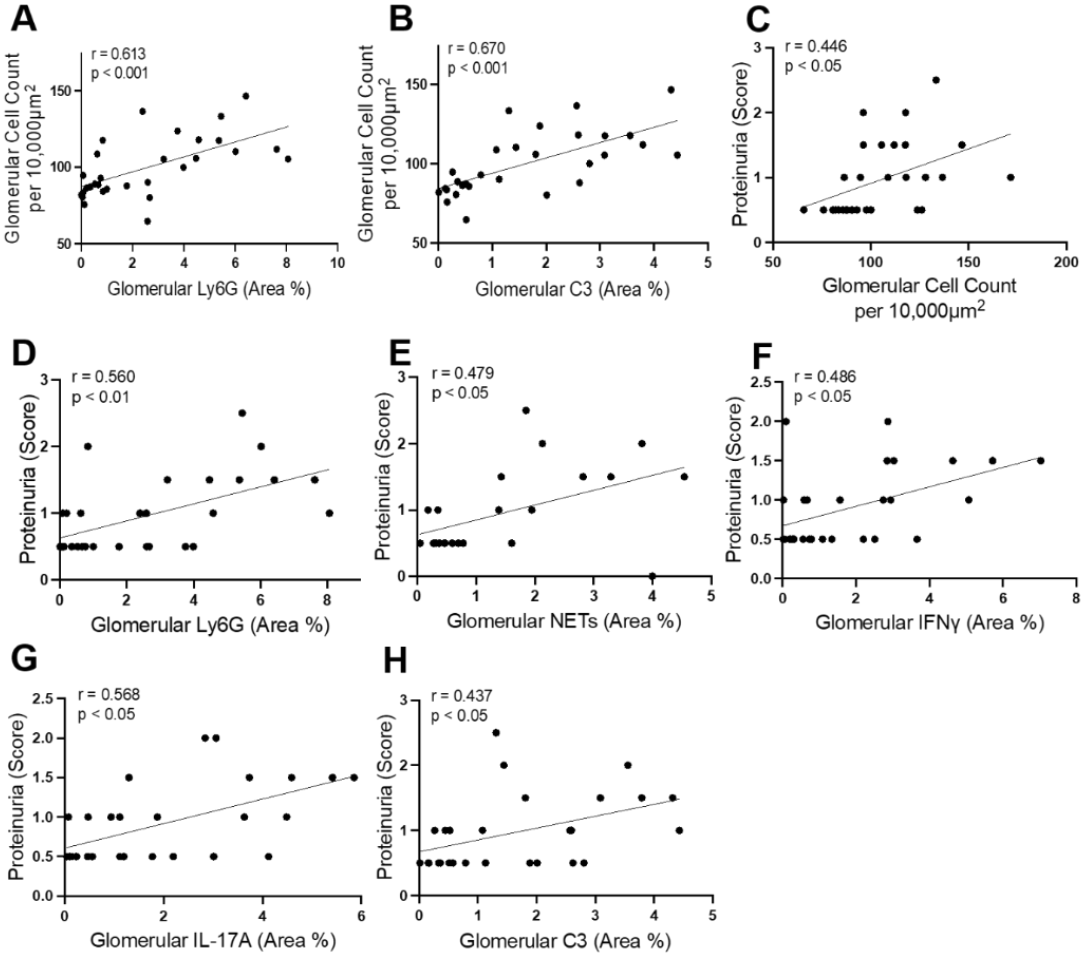
Suppl Fig. 3. Inhibition of CXCR4 attenuated acute kidney inflammation in lupus-prone mice irradiated by UVB

A-D. Correlation analyses between glomerular NET-associated IFNα, IFNγ, IL-17A, C3 in kidneys and proteinuria in combined groups of UVB-irradiated MRL/lpr mice without or with IT1t administration. **E-O**. Summary analyses or representative H&E images of skin thickness of epidermis (E), dermis (F), inflammatory cell infiltration (G-H, nucleated cells), neutrophils, CXCR4-expressing neutrophils, NETs, NET-associated IFNα, IFNγ, IL-17A, or C3 in the skin of UVB-irradiated MRL/lpr mice without (lpr UVB) or with IT1t administration (lpr IT1t+UVB). Scale bars, 100 μm. Results represent 4-5 biological replicates in different panels. Panels E,F,H-O display means ± SD, student's t-test were conducted for two-group comparisons.



Suppl Fig. 4. Controlling NETosis by strengthenning nuclear envelope integrity attenuated lupus skin inflammation in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice

A. Relative migration analyses of primary neutrophils from MRL/lpr vs MRL/lpr·lmnB1^{Tg} mice were conducted with Boyden chamber by stimulation with CXCL12 at 300 ng/ml. **B**. Summary analysis of NET formation of primary neutrophils from MRL/lpr vs MRL/lpr·lmnB1^{Tg} mice, stimulated without or with 10 μM PAF for 3 h, and stained with both cell permeable SYTO Red dye and cell impermeable Sytox Green dye. Images of NETosis *in vitro* were taken by fluorescent microscopy, followed by automated quantification of NETs on 5-6 non-overlapping area per well using ImageJ for calculation of percentage of cells with NET formation. **C**. Representative H&E staining images of skin sections of MRL/lpr vs MRL/lpr·lmnB1^{Tg} mice without or with UVB irradiation. **D-I**. Correlation analyses between nucleated cells in skin, NETs, NET-associated anti-IFNα IFNγ, IL-17A, or C3 and dermal thickness in combined groups of mice without or with UVB. **J**. Correlation analysis between dermal C3 distribution and dermal nucleated cell count. Scale bars, 100 μm. Results represent 5-10 biological replicates in each group or each condition. Panel A displays mean ± SD, P<0.01 by ANOVA; displayed are significance levels for individual pairwise comparisons by SNK test. Panel C, student's t-test was conducted for two-group comparisons. Panels D-I were Pearson correlation analysis.



Suppl Fig. 5. Controlling NETosis by strengthening nuclear envelope integrity ameliorated the early onset of lupus kidney injury and proteinuria in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice

A-B. Correlation analyses between glomerular neutrophil infiltration or C3 distribution and glomerular cellularity. **C-H**. Correlation analysis between glomerular cellularity, neutrophils, NETs, and NET-associated IFNγ, IL-17A, or C3 and proteinuria in the combined groups of mice without or with UVB. Results represent 5-10 biological replicates in each group. Panels A-F were Pearson correlation analysis.