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

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Short title: NETosis and UVB-induced acute lupus flares

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ABSTRACT

Sunlight overexposure triggers acute lupus flares, causing local skin and systemic inflammation and tissue injury, including the kidneys, through poorly understood mechanisms. To address this knowledge gap, we exposed asymptomatic, young female lupus-prone mice to UVB, inducing skin and kidney inflammation with proteinuria, accompanied by neutrophil infiltration in both organs and formation of neutrophil extracellular traps (NETs), known for their proinflammatory and immunogenic properties that drive lupus development. In both the skin and kidneys, infiltrated neutrophils expressed pro-inflammatory cytokines, complement C3, and CXCR4, a chemokine receptor facilitating immune cell transmigration between organs. When exposed *in vitro* to UVB or platelet-activating factor, a lipid mediator produced by UVB-irradiated keratinocytes, mouse neutrophils expressed cytokines/C3, which partially co-expressed with CXCR4, enabling them to exert proinflammatory and transmigratory effects. In a causality study, inhibiting CXCR4 significantly reduced neutrophil infiltration to the kidneys, resulting in ameliorated renal accumulation of NETs and NET-associated cytokines/C3, and decreased proteinuria, indicating a role of CXCR4 in the transmigration of neutrophils from the skin to kidneys in UVB-irradiated lupus-prone mice. Through a novel approach, we inhibited NETosis via nuclear envelope regulation in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice in which strengthening nuclear envelope integrity reduced NET formation, concomitantly decreasing exhibition of NET-associated cytokines/C3 in the skin and kidneys, thereby ameliorating proteinuria. Therefore, this study unveils a new mechanism by which NETosis drives UVB-triggered acute lupus flares, highlighting neutrophil transmigration and NET formation as potential therapeutic targets. Remarkably, nuclear envelope regulation holds significant potential for developing targeted therapies in lupus and other NETosis-related diseases.

SIGNIFICANCE

Sunlight overexposure triggers acute lupus flares through poorly understood mechanisms. NETosis is known important in lupus pathogenesis, but its contribution to UVB-induced lupus flares remains unexplored. UVB-irradiation triggered neutrophil infiltration and formation of NETs that exhibit cytokines/C3, leading to skin and kidney inflammation, and proteinuria in originally asymptomatic, young female lupus-prone mice. UVB-irradiation empowered skin neutrophils with proinflammatory and transmigratory capability, enabling them transmigration to the kidneys. Importantly, blocking renal neutrophil infiltration by CXCR4 inhibition, or inhibiting NETosis through a novel nuclear envelope regulation strategy, ameliorated UVB-induced acute lupus flares. These studies unveil a new mechanism linking sunlight exposure to lupus pathogenesis, emphasizing the importance of NETosis, and identifying neutrophil transmigration and NET formation as potential therapeutic targets.

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). The etiology of SLE remains unclear, while genetic, epigenetic, and environmental risk factors play crucial roles (1, 2). Studies of monozygotic twins, who share nearly identical DNA, reveal that only one-third develop SLE concordantly, highlighting a significant role of environmental factors in the etiology of lupus (1). Sunlight exposure has been recognized as an important environmental risk factor for lupus as early as the 1960s (3), the underlying mechanisms remain largely unexplained.

Over 70% of patients with cutaneous lupus erythematosus (CLE) are photosensitive to sunlight (4). A clinical-pathologic study of renal biopsies from SLE patients in southern France shows a seasonal pattern of lupus nephritis, with higher frequency of lupus flares occurring during the season with longer sunshine duration (5). Additionally, sun overexposure triggers acute lupus flares by exacerbating local skin inflammation and systemic symptoms, such as lupus nephritis (6, 7). Earlier studies demonstrate that UVB overexposure triggers apoptosis of keratinocytes, which enriched with clustered autoantigens, initiating and driving autoimmune responses and development of SLE (6). However, other studies questioned the role of UVB-induced apoptotic keratinocytes in SLE (8, 9). Further research is necessary to elucidate the mechanisms by which sunlight triggers lupus skin and systemic inflammation.

Following UVB overexposure, neutrophils are the first group of circulating leukocytes to infiltrate the skin, playing a crucial role in initiating inflammatory responses in the skin (10). A recent animal study reported that neutrophils are the earliest immune cells infiltrating to the skin before the clinical evident disease of spontaneous CLE (11). In humans, studies have

shown a gradual enrichment of neutrophil transcripts during lupus progression (12), indicating a crucial role for neutrophils in lupus development (13, 14). Recently, we demonstrated that UVB irradiation of wild-type (WT) mice triggers skin inflammation with infiltration of neutrophils and formation of neutrophil extracellular traps (NETs) (15, 16). To investigate the role of NETosis in lupus skin inflammation, we exposed asymptomatic, young female lupus-prone mice to UVB. Surprisingly, UVB irradiation induced not only skin inflammation, but also proteinuria with kidney inflammation in these originally asymptomatic lupus-prone mice. This study closely replicates the skin inflammation and kidney damage observed in human lupus flares triggered by sunlight overexposure (7). Intriguingly, we found that UVB irradiation triggered neutrophil infiltration and NET formation in the skin and kidneys of lupus-prone mice, suggesting a potential role of NETosis in acute lupus flares triggered by UVB. However, the impact of NETosis on UVB-induced acute lupus flares with both skin and kidney inflammation remains unexplored.

Neutrophil NETs with their proinflammatory and immunogenic properties play an important role in lupus pathogenesis, including CLE (13, 17), and lupus nephritis (18, 19). NETs can induce production of cytokines, including type I interferon (IFN-I), a primary driver of lupus pathogenesis (20), from several types of immune cells, including neutrophils (16, 21). Furthermore, both NETs and IFN-I can induce B cell activation and proliferation (22, 23), leading to autoantibody production and subsequent formation of ICs (24). NET-associated nuclear acids and other nuclear components also serve as sources of autoantigens (21). Furthermore, NETs can activate and associate with complement (25, 26), which contribute to tissue injury in autoimmune diseases. Deposition of ICs and complement C3 in the skin (27) or kidneys (28) contribute to lupus development by triggering inflammatory responses and tissue damage in these organs. Thus, NETosis has emerged as a novel therapeutic target for lupus.

Over the past two decades, diverse signaling pathways have been reported to regulate NETosis, including NOX-dependent or -independent, and ROS-dependent or -independent pathways (21, 29, 30). The diversity of these signaling pathways has challenged the field to identify molecular targets for controlling NETosis and treatment of NETosis-related diseases. Nuclear envelope rupture is a critical step, and essential for nuclear chromatin extracellular release and NET formation (21, 30). Our recent studies have identified a novel mechanism for NETosis, where nuclear envelope rupture and NET formation are driven by PKCα-mediated phosphorylation and disassembly of nuclear lamin B, rather than the previously assumed proteolytic cleavage (15). These findings highlight the highly regulated nature of NETosis and opens potential avenues for therapeutic interventions in NETosis-related diseases. The new mechanistic understanding of kinase-mediated nuclear lamina disassembly in NETotic nuclear envelope rupture has gained recognition by peers in NETosis research (21, 30).

Using an innovative model of UVB irradiation of asymptomatic, young female lupus-prone mice, we investigated the crucial role of neutrophil NETosis in driving acute lupus flares with skin and kidney inflammation. In addition, we elucidated the mechanism by which UVB irradiation empowered infiltrating neutrophils with proinflammatory and transmigratory capability, enabling them to transmigrate from the skin to the kidneys and contributing to skin and kidney inflammation by releasing their pre-expressed cytokines with NETs. Moreover, we studied the effects of inhibiting neutrophil transmigration and controlling NETosis on acute lupus flares triggered by UVB. Notably, we explored a novel strategy for controlling NETosis and mitigating UVB-induced acute lupus flares by targeting nuclear envelope integrity based on new mechanistic understanding of NETosis (15, 21, 30, 31). Therefore, this study unveiled a previously unknown mechanism by which sunlight exposure triggers acute lupus flares through neutrophil NETosis, and identified potential therapeutic targets, paving the way for developing targeted treatment for acute lupus flares.

RESULTS

UVB irradiation induced acute lupus flares with skin and kidney inflammation, and proteinuria in asymptomatic, young female lupus-prone mice. To understand the role of sunlight exposure in acute lupus flares, we exposed asymptomatic, young female lupus-prone mice to UVB irradiation (Fig 1A). Surprisingly, we found that UVB-irradiated mice exhibited significant proteinuria (Fig 1B) and glomerular hypercellularity (Fig 1C,D) in their kidneys as compared to sham controls. Glomerular hypercellularity was correlated with proteinuria (Fig 1E). As leukocyte infiltration in the kidneys contributes to the glomerular hypercellularity in lupus kidneys (32), our results suggest a potential role of renal inflammatory cell infiltration in kidney injury and proteinuria in UVB-irradiated lupus-prone mice. To explore the relevant mechanisms, we found UVB-induced skin inflammation with increased epidermal and dermal thickness, and inflammatory cell infiltration (nucleated cell count) in the skin of UVB-irradiated lupus-prone mice (Fig 1F-I). Importantly, the nucleated cell count in the skin correlated not only with dermal skin thickness (Fig 1J), but also proteinuria (Fig 1K), indicating a role of inflammatory cell infiltration in skin inflammation, and its potential link to kidney injury.

The above results suggest that skin inflammation might be connected to kidney inflammation, with the potential communication between inflammatory cells infiltrating the skin and the kidneys, in UVB-irradiated lupus-prone mice. In line, several studies have shown that neutrophils could be potential mediators of the link between skin inflammation and kidney injury in different scenarios, including skin thermal burn and UVB irradiation (33, 34). Moreover, ours and other studies have reported the role of neutrophils and/or NETosis in UVB-induced skin inflammation (15, 16) or lupus kidney inflammation (18, 19) in mice. To

understand the above interesting findings, we investigated the neutrophils and their released NETs in the skin and kidneys of UVB-irradiated lupus-prone mice.

UVB irradiation induced infiltration of neutrophils, their expressions of cytokines/C3 and CXCR4, and NET formation in the skin of UVB-irradiated lupus-prone mice. Further analyses demonstrated increased infiltration of Ly6G-neutrophils and NET formation in the inflamed skin of UVB-irradiated MRL/lpr mice as compared to non-irradiated sham controls (Fig 2A-C). In addition, we found increased expression of IFNα, IFNγ, IL-17A, and C3 in infiltrated neutrophils (including non-netting neutrophils and their released NETs) in dermal skin of UVB-irradiated MRL/lpr mice as compared to sham controls (Fig 2D-G), suggesting their contribution to lupus skin inflammation triggered by UVB irradiation. 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 in acute lupus flares.

CXCR4 is a chemokine receptor that plays a crucial role in reverse transmigration (RTM) of immune cells, including neutrophils, during communications between organs (35, 36). In addition, CXCR4/CXCL12 axis is important in lupus pathogenesis, with increased CXCR4 expression in immune cells and elevated CXCL12 expression in kidneys of different strains of lupus-prone mice (37). Interestingly, UVB irradiation increased expression of CXCR4 in infiltrated neutrophils (including non-netting neutrophils and their released NETs) in the skin of MRL/lpr mice as compared to sham controls (Fig 2H,I). Importantly, expression of CXCR4 in skin neutrophils positively correlated with proteinuria in the combined groups of MRL/lpr mice without or with UVB irradiation (Fig 2K), suggesting the potential association between CXCR4-expressing neutrophils in the skin and kidney inflammation and injury in UVB-irradiated lupus-prone mice.

Neutrophil infiltration, NET formation, and accumulation of NET-associated cytokines and C3 were important in acute kidney inflammation in UVB-irradiated lupus-prone mice. Corresponding to the increased CXCR4-expressing neutrophils in the skin (Fig 2H,I) and their association with proteinuria (Fig 2K), we found increased infiltration of Ly6G-neutrophils, and CXCR4-expressing neutrophils, as well as accumulation of citrullinated H3-positive NETs in the kidneys of UVB-irradiated MRL/lpr mice compared to sham controls (Fig 3A-E). In addition, we also found increased accumulation of NET-associated IFNα, IFNγ, IL-17A, and C3 (Fig 3F-I, Suppl Fig 1A) in glomeruli of UVB-irradiated lupus-prone mice as compared to sham controls. In addition, 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 glomerular hypercellularity and renal inflammation (32). Importantly, the infiltrated CXCR4-expressing neutrophils, Ly6G-neutrophils, NETs, and their associated IFNα, IFNγ, IL-17A, and C3 in glomeruli positively correlated with proteinuria in the combined groups of MRL/lpr mice without or with UVB irradiation (Fig 3J, Suppl Fig 1C-H). Moreover, NET-associated C3 in glomeruli 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 the acute renal inflammation.

The above results indicate a role of infiltrated CXCR4-expressing neutrophils, NETs, and their associated cytokines/C3 in the kidney inflammation and injury in acute lupus flares in UVB-irradiated asymptomatic lupus-prone mice. Taken together, results from the skin and kidneys of UVB-irradiated MRL/lpr mice (Fig 2-3) suggest a potential link between CXCR4-expressing neutrophils in the skin and kidney injury, as well as the role of renal CXCR4-expressing neutrophils in kidney inflammation and injury triggered by UVB irradiation. Given

that CXCR4 is known for neutrophil transmigration during the communications between organs (35, 36), our results suggest a potential role of CXCR4 in neutrophil transmigration from the inflamed skin to kidneys during acute lupus flares in UVB-irradiated asymptomatic lupus-prone mice.

UVB irradiation induced co-expression of CXCR4 and cytokines/C3 *in vitro* in primary neutrophils from lupus-prone mice. To explore the causal effects of UVB irradiation on CXCR4 and cytokines/C3 expression by neutrophils in inflamed skin, we stimulated isolated neutrophils from MRL/lpr mice with UVB irradiation that induced neutrophil expression of CXCR4, IFNα (in the presence of DNA-NETs), IFNγ, IL-17A, and C3 *in vitro* in dose-dependent (Suppl Fig 2A-E) manner. It's known that UVB irradiation induces keratinocytes to release platelet activating factor (PAF) (38), which could serve as a natural stimulus of NETosis in UVB-irradiated mice (15). Here, we found that PAF treatment mouse neutrophils upregulated their expression of CXCR4, IFNα (in the presence of DNA-NETs), IFNγ, IL-17A, and C3 *in vitro* (Fig 4 B-M) in a dose-dependent (Suppl Fig 2A-E) manner, suggesting the indirect effects of UVB. Therefore, UVB irradiation can directly and/or indirectly upregulate expression of CXCR4 and cytokines/C3 in mouse neutrophils.

Most importantly, subsets of neutrophils stimulated by UVB or PAF co-expressed CXCR4 and cytokines/C3 (Fig 4 B-M), enabling their migration from inflamed skin to the kidneys, where they promote inflammatory responses by releasing pre-expressed cytokines/C3 with NETs. Previous studies from us and others demonstrated that only portion of neutrophils undergo NETosis *in vitro* (29) and *in vivo* in the skin of UVB-irradiated mice (16). Therefore, one may expect that the non-netting neutrophils with CXCR4 expression in the inflamed skin may transmigrate to the kidneys which may have elevated expression of CXCL12 in either UVB-irradiated mice (34) or lupus-prone mice (37).

Inhibition of CXCR4 attenuated acute lupus flares with kidney inflammation in UVB-irradiated lupus-prone mice. To explore the causal role of CXCR4 in neutrophil transmigration and kidney inflammation, we found that inhibition of CXCR4, by intraperitoneal application of its selective antagonist IT1t (Fig 5A), significantly attenuated glomerular infiltration of CXCR4-expressing neutrophils, Ly6G-positive neutrophils, glomerular hypercellularity, and proteinuria in acute lupus flares triggered by UVB irradiation of asymptomatic lupus-prone mice (Fig 5B-F). To confirm the inhibitory effects of IT1t on neutrophil transmigration, we found that IT1t reduced neutrophil transmigration *in vitro* in transwell migration experiments (Fig 5G). Furthermore, inhibition of neutrophil infiltration resulted in amelioration in glomerular accumulation of NETs (Fig 5H), and NET-associated IFNα, IFNγ, IL-17A, and C3 (Fig 5I-M), in the kidneys of UVB-irradiated lupus-prone mice with IT1t intraperitoneal application as compared to UVB-irradiated MRL/lpr mice with normal saline administration. Importantly, glomerular accumulation of NET-associated IFNα, IFNγ, IL-17A or C3 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 kidney inflammation, kidney injury, and proteinuria. Therefore, our findings indicate that CXCR4-mediated neutrophil transmigration plays a crucial role in kidney inflammation, and blocking CXCR4-mediated neutrophil transmigration could be a promising therapeutic strategy for lupus kidney inflammation and injury in acute lupus flares triggered by UVB irradiation.

In addition, CXCR4 inhibition 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 in the skin of UVB-irradiated mice with IT1t administration as compared to those treated with normal saline (Suppl

Fig 3I,K,L,N). However, no significant differences were observed in CXCR4-expressing neutrophils or NET-associated IFNγ and C3 in the skin between IT1t-treated or non-treated mice (Suppl Fig 3J,M,O). These results suggest that CXCR4 inhibition by repeated systemic IT1t administration alongside UVB irradiation effectively reduces inflammatory cell infiltration in the skin of lupus-prone mice. Sarchio et al reported that CXCR4 inhibition attenuates sunlight-induced skin cancer by blocking infiltration of mast cells to the skin (39).

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 (13, 14, 21), we investigated the effects of controlling NETosis on UVB-induced acute 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 (15), we sought to study the effects of nuclear envelope regulation on NETosis inhibition by modulation of nuclear lamin B content. Therefore, we generated lupus-prone mice with nuclear lamin B1 overexpression in which the strengthened nuclear envelope integrity could reduce neutrophil NETosis (15). However, overexpression of lamin B does not increase nuclear stiffness or decrease cell transmigration in hematopoietic cells as the nuclear stiffness is determined by the ratio of lamin A to lamin B in the nuclear envelope (40). Therefore, overexpression of nuclear lamin B in neutrophils does not affect their transmigration capacity (15, 40). Our study revealed no significant differences in the transmigration capacity of the primary neutrophils isolated from MRL/lpr·lmnB1^{Tg} mice compared to control mice (Suppl Fig 4A). Importantly, primary neutrophils from MRL/lpr·lmnB1^{Tg} mice with genetically overexpressed nuclear lamin B exhibited significantly reduced NETosis compared to those from MRL/lpr mice (Suppl Fig 4B). Therefore, these mice

are valuable to explore the effects of controlling NETosis *in vivo* on UVB-induced acute lupus flares through nuclear envelope regulation.

In *in vivo* studies, the UVB-irradiated MRL/lpr·lmnB1^{Tg} mice exhibited significant decreased skin inflammation with reduced epidermal and dermal skin thickness, as well as significantly ameliorated nucleated cell counts and neutrophil infiltration in their skin as compared to UVB-irradiated MRL/lpr mice (Fig 6A-E, Suppl Fig 4C). Importantly, UVB-irradiation triggered less neutrophil NETosis in MRL/lpr·lmnB1^{Tg} mice than in MRL/lpr mice, resulting in ameliorated accumulation of NET-associated IFNα, IFNγ, IL-17A, and C3 in their skin (Fig 6F-K), indicating the protective effects of controlling NETosis by nuclear envelope regulation on lupus skin inflammation in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice. Moreover, 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). In addition, NET-associated C3 in skin positively correlated with nucleated cell count in skin in these mice, indicating a role of NET-associated C3 in lupus skin inflammation (Suppl Fig 4J). This study highlights the therapeutic potential of controlling NETosis through nuclear envelope regulation in lupus skin inflammation triggered by UVB-irradiation.

Controlling NETosis by strengthening nuclear envelope integrity ameliorated lupus kidney inflammation and proteinuria in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice. Compared to MRL/lpr mice, UVB-irradiated MRL/lpr·lmnB1^{Tg} mice exhibited decreased proteinuria, accompanied by reduced glomerular hypercellularity and attenuated neutrophil infiltration in their kidneys (Fig 7A-D). In addition, controlling NETosis through nuclear envelope regulation lead to reduced glomerular deposition of neutrophil NETs and NET-associated IFNα, IFNγ, IL-17A, and C3 in the kidneys of MRL/lpr·lmnB1^{Tg} mice as compared

to MRL/lpr mice with UVB-irradiation (Fig 7E-J). These results demonstrated therapeutic potential of controlling NETosis through nuclear envelope regulation in acute lupus flares with lupus kidney inflammation triggered by UVB irradiation.

In addition, glomerular neutrophil infiltration and NET-associated C3 accumulation correlated with glomerular hypercellularity, indicating a contribution of renal neutrophil infiltration to glomerular hypercellularity (32) and the role of NET-associated C3 in renal inflammation (Suppl Fig 5A,B). Moreover, the glomerular hypercellularity, neutrophil infiltration, deposition of NETs, and NET-associated IFNα, IFNγ, IL-17A, and C3 in kidneys 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 NETs and NET-associated proinflammatory cytokines/C3 in kidney inflammation, kidney injury, and proteinuria in UVB-induced acute lupus flares. Our study indicates that controlling NETosis could be a promising therapeutic target for lupus kidney inflammation triggered by UVB-irradiation. Furthermore, nuclear envelope regulation could be a novel therapeutic strategy to control NETosis for developing targeted treatment for acute lupus flares triggered by UVB irradiation.

DISCUSSION

Sunlight overexposure induces acute lupus flares with not only skin inflammation, but also systemic inflammation, with poorly understood mechanisms. Our study reveals a novel mechanism by which UVB exposure triggered neutrophil infiltration and formation of NETs that exhibited proinflammatory cytokines and C3 in the skin and kidneys, resulting in skin and kidney inflammation and proteinuria, in originally asymptomatic, young female lupus-prone mice. Interestingly, UVB irradiation empowered skin-infiltrating neutrophils with pro-

inflammatory and transmigratory capabilities by upregulating expression of cytokines/C3 and CXCR4, enabling the transmigration of non-netting neutrophils from the skin to the kidneys (35, 36). To explore the causality, we found that CXCR4 inhibition suppressed neutrophil infiltration to the kidneys, consequently reducing glomerular accumulation of NETs and NET-associated cytokines/C3, thereby decreasing proteinuria in UVB-irradiated lupus-prone mice. By directly targeting NETosis through nuclear envelope regulation, a novel strategy based on new mechanistic understanding (15, 16, 21), we found significantly reduced NETosis and deposition of NET-associated cytokines/C3 in the skin and kidneys, with effectively suppressed inflammatory responses in both organs and proteinuria in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice. Therefore, our studies provide compelling evidence for the critical role of neutrophil NETosis in UVB-induced acute lupus flares with skin and kidney inflammation. Building on our findings, the study also suggests potential therapeutic targets for acute lupus flares triggered by UVB irradiation.

Interestingly, studies in different settings have demonstrated that either skin thermal burn in patients (33, 41), or acute single high-dose UVB irradiation of WT mice (34) can induce systemic symptoms, including kidney inflammation, indicating potential dermal-renal communications in different scenarios. Over the past 60 years, numerous studies have reported proteinuria in patients with skin thermal burn (41), in which neutrophil gelatinase-associated lipocalin from activated neutrophils are implicated (33). A recent study using a single high-dose UVB irradiation of WT mice led to transient proteinuria, accompanied by neutrophil transmigration from the inflamed skin to the kidneys tracked by a photoactivatable lineage tracing approach (34). The above studies highlight a critical role for neutrophils in communication between the skin and kidneys, linking acute skin injury or inflammation to subsequent kidney damage (33, 34).

CXCR4/CXCL12 axis plays an important role in lupus pathogenesis by recruiting CXCR4-expressing immune cells, including neutrophils, into the kidneys with CXCL12 expression in patients or mice with lupus nephritis (37, 42). In addition, CXCR4-expressing immune cells have also been shown to be recruited to kidneys with CXCL12 expression in mice with ischemic-renal injury (43) or with UVB-induced acute kidney injury (34). Furthermore, CXCR4 is a known mediator of transmigration for various cell types, including neutrophils, facilitating communication and interaction between different organs (35, 36). Jamaly et al reported that infiltration of immune cells, including neutrophils, in the kidneys is crucial for the initiation and development of lupus kidney inflammation and nephritis (44). In the current study, we found expression of CXCR4 and cytokines/C3 in infiltrated neutrophils and their released NETs in both the skin and kidneys of UVB-irradiated lupus-prone mice. Interestingly, expression of CXCR4, IFNα, IFNγ, IL-17A, or C3 in mouse neutrophils *in vitro* can be upregulated by UVB irradiation directly or by PAF, released by UVB-irradiated keratinocytes, implying a causal role of UVB in neutrophil expression of CXCR4 and cytokines/C3 in the skin under UVB-irradiation. As demonstrated by us and others, only a portion of neutrophils undergo NETosis *in vitro* (29) and *in vivo* (16) in previous studies. Therefore, one may expect that some infiltrated neutrophils underwent NETosis in the skin, releasing their pre-expressed cytokines/C3 with NETs to contribute to skin inflammation (16). Meanwhile, the non-netting neutrophils with CXCR4 expression may migrate from the inflamed skin to the kidneys of UVB-irradiated MRL/lpr mice, as upregulation of CXCL12 has been documented in the kidneys of lupus-prone mice (37) and UVB-irradiated mice (34). Once recruited, the infiltrated neutrophils in the kidneys may undergo NETosis to release their pre-expressed cytokines/C3 with NETs, propagating inflammatory responses and kidney injury in acute lupus flares triggered by UVB.

To examine the causal role of CXCR4 in transmigration, our study demonstrated that inhibition of CXCR4 with its selective antagonist attenuated neutrophil infiltration to the kidneys, resulting in reduced glomerular accumulation of neutrophil NETs and NET-associated proinflammatory cytokines/C3, ultimately attenuating kidney injury and proteinuria in UVB-irradiated lupus-prone mice. This study indicates that the non-netting neutrophils with CXCR4 expression might migrate from inflamed skin to the kidneys, contributing to kidney inflammation in acute lupus flares. Thus, our study suggests that UVB irradiation empowered skin infiltrating neutrophils by upregulation of CXCR4 expression, enabling them migration from the inflamed skin to the kidneys, where they subsequently underwent NETosis and released their pre-expressed cytokines/C3 with NETs, leading to kidney inflammation and injury during acute lupus flares. Therefore, inhibiting neutrophil transmigration by CXCR4 inhibition could be a promising therapeutic target for lupus kidney inflammation in acute lupus flares triggered by UVB irradiation.

Neutrophil NETosis plays an important role in lupus pathogenesis over the last decade (13, 14, 21). In the current study, we observed a significant increase of neutrophil NET formation in the skin and kidneys of UVB-irradiated lupus-prone mice. Importantly, NET accumulation in the skin or kidneys were positively correlated with dermal skin thickness or proteinuria, respectively, indicating a crucial role of neutrophil NETosis in skin and kidney inflammation during acute lupus flares triggered by UVB irradiation. Furthermore, previous studies have shown the pathogenic roles of NETosis in CLE (13, 17) and lupus nephritis (18, 19). Given that NETosis is characterized by extracellular release of nuclear DNA and other immunogenic nuclear components which are crucial to lupus pathogenesis, NETosis has emerged as a novel therapeutic target for lupus and other autoimmune diseases (13, 14, 21).

According to new mechanistic understanding, nuclear chromatin decondensation, nuclear envelope rupture, and plasma membrane breakdown are key cellular events that are

crucial to nuclear chromatin extracellular release and NET formation (21, 30). Nuclear chromatin decondensation in NETosis is achieved by peptidyl-arginine deaminase 4 (PAD4)-mediated histone citrullination (21, 30). Thus, PAD4 inhibition has been employed to control NETosis *in vitro* and *in vivo*, attenuating lupus skin and kidney inflammation in 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 (45). A better mechanistic understanding in NETosis is key for developing targeted therapeutics for NETosis-related various human diseases, including lupus (21, 30).

The nuclear envelope is the first physical barrier that holds nuclear chromatin inside the nucleus, nuclear envelope rupture is a required step for nuclear chromatin release and NET formation (21, 30). Recently, we reported that nuclear envelope rupture and NET formation is driven by PKCα-mediated phosphorylation and disassembly of nuclear lamin B, not the previously assumed proteolytic cleavage (15, 16, 21). Based on the new mechanistic understanding in NETosis, we explored the effects of a novel nuclear envelope regulation strategy on controlling NETosis and acute lupus flares by modulating lamin B content in UVB-irradiated MRL/lpr·Lmnb1^{TG} mice, which were generated by backcrossing Lmnb1^{TG} mice (46) to MRL/lpr lupus-prone mice for at least 10 generations.

The Lmnb1^{TG} mice were originally generated for studying adult onset autosomal-dominant leukodystrophy and the mice do not show any abnormal pathology before 12 months of age (46). In the current study, MRL/lpr·Lmnb1^{TG} mice was used to conduct experiments between 8 to 9 weeks of age when they were asymptomatic. Young female MRL/lpr·Lmnb1^{TG} mice served as a valuable model to study how UVB irradiation, an environmental risk factor, triggers acute lupus flares in lupus-prone mice prior to the onset of any spontaneous symptoms. Building on the well-established MRL/lpr mouse model, the MRL/lpr·Lmnb1^{TG} mice, tailored

to the new mechanistic understanding of NETosis (15, 16, 21), allowed us to investigate how regulating nuclear envelope integrity by modulation of nuclear lamin B content affects NETosis and UVB-induced acute lupus flares *in vivo*. However, overexpression of lamin B does not increase nuclear stiffness or decrease cellular transmigration in hematopoietic cells as the nuclear stiffness depends on the ratio of lamin A to lamin B in the nuclear envelope, not simply on lamin B levels alone (40). Thus, overexpression of nuclear lamin B does not affect the transmigration capacity of neutrophils in MRL/lpr·lmnB1^{Tg} mice in the current study, consistent with observations in previous studies (15, 40). Furthermore, alterations of cellular lamin B1 expression have been shown in various pathophysiological conditions (46, 47).

In the current study, we found that controlling NETosis by strengthening the nuclear envelope integrity effectively alleviated skin and kidney inflammation in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice compared to UVB-irradiated MRL/lpr mice. Our findings underscore the importance of NETosis in skin and kidney inflammation, suggesting that NETosis could be an important therapeutic target for UVB-induced acute lupus flares. On the other hand, our study also indicates that regulating nuclear envelope integrity might be a promising strategy for controlling NETosis and treating UVB-induced acute lupus flares. In this study, we investigated the effects of nuclear envelope regulation on controlling NETosis by modulation of nuclear lamin contents in MRL/lpr·lmnB1^{Tg} mice with genetic overexpression of nuclear lamin B. Further research exploring additional approaches to inhibit NETosis via nuclear envelope regulation, such as targeting kinases that mediated nuclear lamina disassembly, offers significant potential for developing targeted therapies for lupus and other NETosis-related diseases (15, 16, 21, 30).

As demonstrated in our study, UVB irradiation induced expression of proinflammatory cytokines (IFNα, IFNγ, IL-17A) and complement C3 in neutrophils, and their released NETs in the skin and kidneys, contributing to inflammatory responses in these organs, as well as

kidney injury and proteinuria in acute lupus flares. IFNα and IFNγ are secreted cytokines and important regulators of immunity and inflammation (20). Studies from us and others demonstrate that both IFNα and IFNγ can be expressed by neutrophils (16, 48, 49), which can further release these pre-expressed cytokines with NETs (16, 48). Extensive research in animal and human has revealed that the sustained activation of IFN-I pathway plays a crucial role in lupus pathogenesis, driving inflammatory responses in multiple organs, including the skin and kidneys (20). In addition, our previous studies demonstrate elevated IFN-regulated gene signature in CLE patients (50), while downregulating plasmacytoid dendritic cells in SLE patients result in reduced local IFN-I responses and subsequent improvement in skin lesions (51). Moreover, Mavragani et al reported elevated expression of IFN-I and defensin-α transcripts in kidney tissues from patients with lupus nephritis, while defensin-α is a NET-associated neutrophil component that is involved in induction of IFN-I expression in the kidneys of lupus patients (52).

A large body of evidence from animal and human studies supports an important role of IFNγ in the development of lupus, including CLE and lupus nephritis (20, 53, 54). IFNγ has been shown to have pleiotropic roles in lupus nephritis, including recruitment and activation of neutrophils and other immune cells in the kidneys (55). 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 CLE (56) and lupus nephritis (57). Since neutrophil NETs are an upstream target for IFNα and IFNγ, controlling NETosis might be a promising therapeutic target for treating acute lupus flares with skin and kidney inflammation triggered by UVB irradiation.

IL-17A plays a crucial role in lupus pathogenesis by recruiting immune cells to affected organs, including the skin and kidneys (58, 59). Oh et al have reported increased expression of

IL-17A and IFNα in the skin lesions of lupus patients, with a significant correlation between IL-17A and IFNα in these patients, suggesting their potential synergistic contribution to pathogenesis of skin lupus (60). Ramani et al have reported an essential role of IL-17A in the development of lupus nephritis (61), while IL-17 receptor KO mice exhibit diminished infiltration of inflammatory cells to their kidneys (58), indicating a role of IL-17A in immune cell recruitment to the kidneys of lupus-prone mice. In addition, IL-17A can also regulate IFNγ-mediated neutrophil migration in mouse kidneys (62). Moreover, NET-associated DNA or histone H2a (63) also have chemotactic effects for leukocyte recruitment. Our published studies have demonstrated increased neutrophil expression of IL-17A and release of NET-associated IL-17A in the skin of UVB-irradiated non-lupus mice (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 acute lupus flares with skin and kidney inflammation in UVB-irradiated lupus-prone mice. Furthermore, a recent study reported that skin infection by staphylococcal aureus in *Nfkbiz*$^{\Delta K5}$ mice promotes keratinocyte apoptosis, neutrophil NETosis and release IL-17A, leading to SLE-like autoimmune inflammation and kidney injury (64). Therefore, NETs and their-associated IL-17A drive inflammatory responses in the skin and kidneys during acute lupus flares.

Deposition of C3 has been found in the skin (27) and kidneys (65) of lupus patients. Glomerular C3 deposition indicates ongoing immune responses, tissue damage, and active disease in patients with lupus nephritis (65) and in mice with experimental glomerulonephritis (66). In addition, C3 deposition in the kidneys promotes immune cell infiltration, while C3 deletion results in reduced neutrophil infiltration and NET formation, and ameliorated kidney injury in C3 KO mice with ischemia-reperfusion (67). 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 acute lupus flares. A previous study reported that C3 can be synthesized

and secreted by activated neutrophils (68). On the other hand, excessive NETs in SLE patients can activate complement, exacerbating lupus development (26). In addition, neutrophil NETs can mediate activation of alternative complement pathway and glomerular endothelial damage, resulting in proteinuria in autoimmune vasculitis (69). Furthermore, Liu et al reported increased renal C3 levels in patients with lupus nephritis and in NZB/W mice at disease onset (70), and C3 deposition in the kidneys contributes to albuminuria in lupus nephritis (70). Importantly, our study found significant correlations between NET-associated C3 and skin or kidney inflammation, as well as proteinuria, indicating roles of NET-associated C3 in acute lupus flares with skin and kidney inflammation, and kidney injury. On the other hand, neutrophil elastase released with NETs also contributes to glomerular albumin leakage by proteolytically cleaving VE-cadherin, a key protein maintaining the integrity of endothelial intercellular junctions (19). Therefore, renal deposition of NETs and NET-associated C3 contribute to lupus kidney inflammation, kidney injury, and proteinuria in acute lupus flares in UVB-irradiated lupus-prone mice.

Taken together, the current study unveiled that neutrophil NETosis drives acute lupus flares with skin and kidney inflammation in UVB-irradiated asymptomatic, young female lupus-prone mice. Following UVB irradiation, the skin infiltrated neutrophils expressed cytokines/C3 and CXCR4, enabling them with proinflammatory and transmigratory capability. Some neutrophils underwent NETosis and released their pre-expressed cytokines/C3, contributing to lupus skin inflammation in UVB-induced acute lupus flares. While non-netting neutrophils with CXCR4 expression migrated to the kidneys, where they subsequently became NETotic and released their pre-expressed cytokines/C3 with NETs, leading to kidney inflammation, kidney injury, and proteinuria in UVB-irradiated lupus-prone mice. In general, secreted cytokines and C3 from immune cells diffuse over large areas, getting diluted in the big pool of circulating blood. In contrast, NET-associated cytokines or C3 captured by the NET

meshwork remain concentrated for extended periods in the skin and kidneys, thus sustaining and amplifying skin and kidney inflammation, and consequently contributing to kidney injury in UVB-induced acute lupus flares. Importantly, our study demonstrated that either blocking neutrophil transmigration or directly inhibiting neutrophil NETosis significantly reduced skin and kidney inflammation and ameliorated proteinuria, providing potential therapeutic targets for acute lupus flares triggered by UVB. Remarkably, our study also indicates that nuclear envelope regulation presents a promising avenue for developing targeted treatments for lupus and other NETosis-related diseases. Therefore, UVB-induced NETosis, a programmed cell death in neutrophils, might be a new mechanistic link 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) (46), 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 the Philadelphia VA Medical Center.

UVB exposure 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, 71). 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 (72). 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 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. See the supplemental methods

Flow cytometry analysis. For detection of UVB-induced neutrophil expression of CXCR4, IFNα, IFNγ, IL-17A, and C3 (C3c), the paraformaldehyde 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. See supplemental methods

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. Asymptomatic young female MRL/lpr mice were irradiated by UVB (150 mJ/cm^2/day) for 5 days, then urine, skin and kidney samples were collected at day 6 following whole body cardiac perfusion. **B**-**D**. Summary analyses of proteinuria scores (B) and glomerular cellularity of kidneys (C), and representative H&E staining images of glomeruli (D) in asymptomatic young female MRL/lpr mice without (sham) or with UVB irradiation. **E**. correlation between glomerular cellularity and proteinuria scores in the combined groups of mice without or with UVB. **F-I**. Representative of H&E staining images of skin (F) and summary analyses of skin thickness of epidermis (G), dermis (H), inflammatory cell infiltration (I, nucleated cells) in the skin of MRL/lpr mice without (sham) or with UVB irradiation. **J**-**K**. Correlations between inflammatory cell infiltration in skin and dermal skin thickness (J) or proteinuria scores (K). Scale bars, 100 µm. Results represent 5 biological replicates in each group. Panels B,C,G,H display means ± SD, student's t-test were conducted for two-group comparisons. Panels E,J,K 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 their released NETs in the 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 were important in acute kidney inflammation in acute lupus flares triggered by UVB irradiation. **A-I**. Summary or representative images of neutrophils, CXCR4 expressing neutrophils and their released NETs, citrullinated H3-positive NETs, NET-associated IFNα, IFNγ, IL-17A, or C3 in kidneys of MRL/lpr mice with UVB irradiation and their controls. In panels A-I, neutrophils, NETs, and their associated CXCR4, IFNα, IFNγ, IL-17A, or C3 in kidney sections from all experimental mice were stained with PE-labeled anti-ly6G, FITC-labeled antibodies against CXCR4, citH3, IFNα, IFNγ, IL-17A, or C3, and DAPI for DNA. In panels C,D, yellow arrows indicate neutrophil NETs, and light blue arrows indicate non-netting neutrophils. Scale bars, 50 µm. **J**. Correlation analysis between CXCR4 expressing neutrophils and their released NETs in the kidneys and proteinuria scores in the combined groups of mice without or with UVB. Results represent 5 biological replicates in each group. Panels A,B,E-I 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. Isolated mouse neutrophils were stimulated

by PAF or UVB to simulate the effects of UVB on infiltrated neutrophils in skin. B-M. Summary or representative dotplots of flow cytometry analyses of expressions of cytokines IFNα (B-D), IFNγ (E-G), IL-17A (H-J), or C3 (K-M) 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 B-M were based on gated cells that were ly6G positive cells. Results represent 5-6 biological replicates in different panels. Panels B,C,E,F,H,I,K,L 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 flares triggered by UVB. A. Asymptomatic young female MRL/lpr mice were irradiated by UVB (150 mJ/cm^2/day) for 5 days, with intraperitoneal administration of CXCR4 antagonist IT1t (10 mg/kg/day) daily for 6 consecutive days beginning one day before the first session of UVB-irradiation, then urine, skin and kidney samples were collected at day 6 following whole body cardiac perfusion. B-F. Summary analyses of CXCR4-expressing neutrophils (B), Ly6G-neutrophils (C), glomerular cellularity (E), or proteinuria scores (F), and representative glomeruli IHC images (D) of kidneys in UVB-irradiated MRL/lpr mice without (control) or with pre-administration of IT1t. G. 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. H-M. Summary analyses or representative IHC images of deposition of neutrophil 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 CXCR4, citH3, IFNα, IFNγ, IL-17A, or C3, and DAPI for DNA. Scale bars, 100 µm. Results represent 3-5 biological replicates in different panels. Panels B,C,E-H,J-M display means ± SD, student's t-test were conducted for two-group comparisons.

Figure 6. Controlling NETosis by strengthening nuclear envelope integrity attenuated lupus skin inflammation in UVB-irradiated MRL/lpr·lmnB1^{Tg} mice. A. Asymptomatic young female MRL/lpr or MRL/lpr·lmnB1^{Tg} mice were irradiated by UVB (150 mJ/cm^2/day) for 5 consecutive days, then urine, skin and kidney samples were collected at day 6 following whole body cardiac perfusion. B-D. Summary analyses of skin thickness of epidermis (B), dermis (C), and proinflammatory cell infiltration (D, nucleated cells) in skin of MRL/lpr vs MRL/lpr·lmnB1^{Tg} mice without or with UVB irradiation. E-K. Summary analyses or representative images of neutrophils (E), NETs (F), and NET-associated IFNα, IFNγ, IL-17A, and C3 (H-K) 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 (E-K) 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 B-F,H-K 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 UVB-irradiated MRL/lpr·lmnB1^{Tg} mice. 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

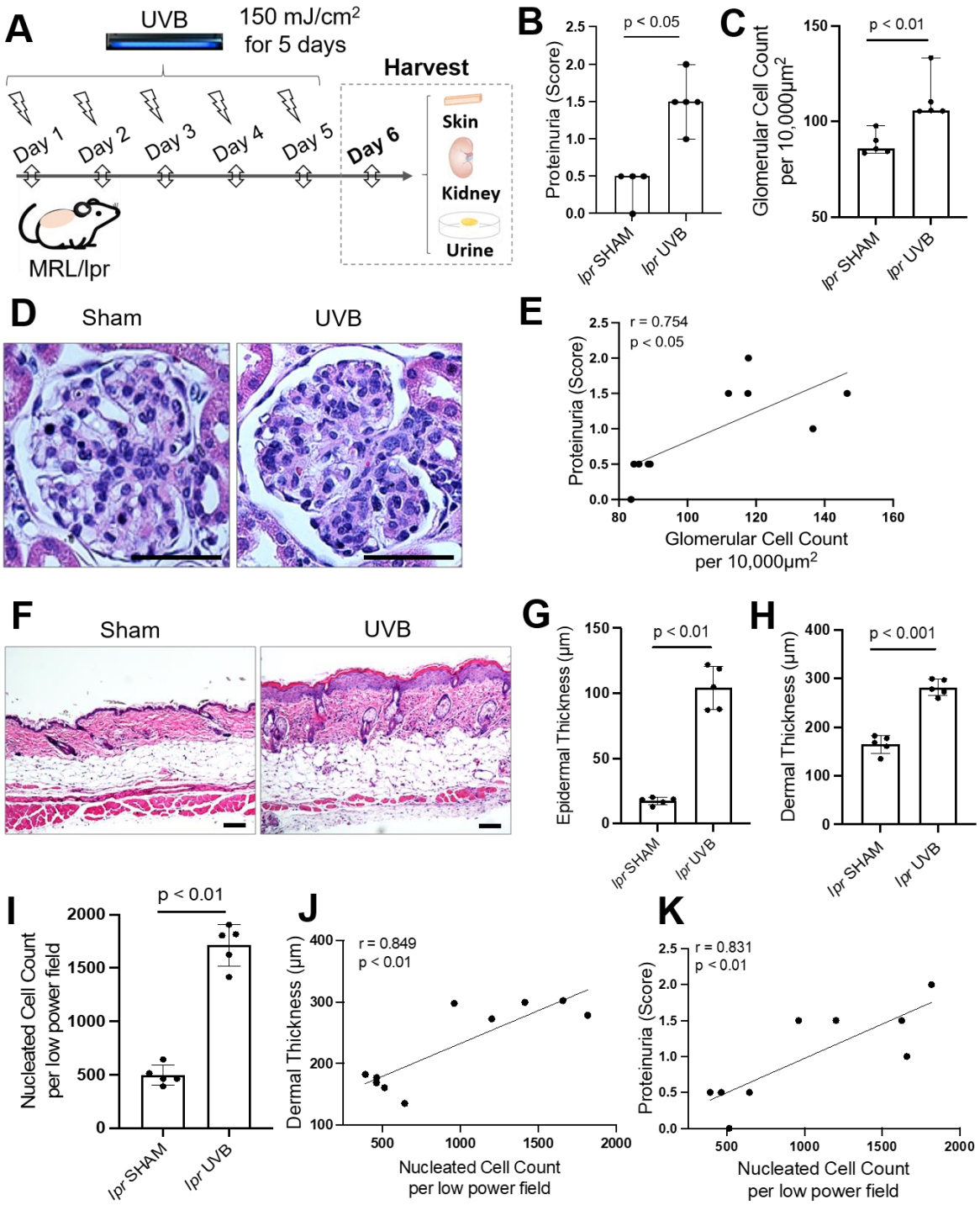

A
UVB
150 mJ/cm2
for 5 days
Harvest
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Skin
Kidney
Urine
MRL/lpr
B
p < 0.05
Proteinuria (Score)
lpr SHAM
lpr UVB
C
p < 0.01
Glomerular Cell Count per 10,000μm2
D
Sham
UVB
E
r = 0.754
p < 0.05
Proteinuria (Score)
Glomerular Cell Count per 10,000μm2
F
Sham
UVB
G
p < 0.01
Epidermal Thickness (μm)
H
p < 0.001
Dermal Thickness (μm)
I
p < 0.01
Nucleated Cell Count per low power field
J
r = 0.849
p < 0.01
Dermal Thickness (μm)
Nucleated Cell Count per low power field
K
r = 0.831
p < 0.01
Proteinuria (Score)
Nucleated Cell Count per low power field

Figure 2.

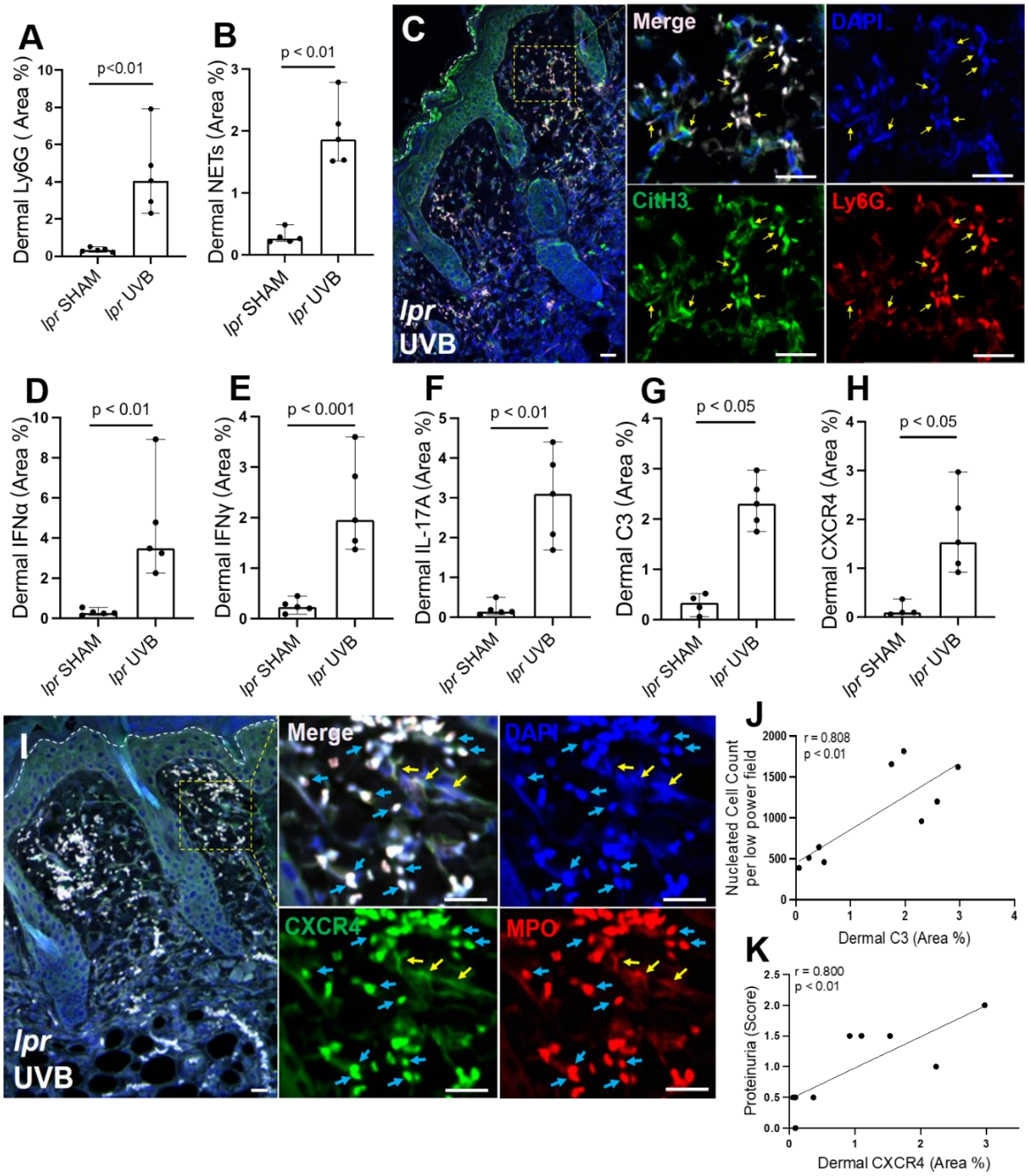

A
Dermal Ly6G (Area %)
p<0.01
lpr SHAM
lpr UVB
B
Dermal NETs (Area %)
p < 0.01
lpr SHAM
lpr UVB
C
Merge
DAPI
CitH3
Ly6G
lpr
UVB
D
Dermal IFNα (Area %)
p < 0.01
E
Dermal IFNγ (Area %)
p < 0.001
F
Dermal IL-17A (Area %)
p < 0.01
G
Dermal C3 (Area %)
p < 0.05
H
Dermal CXCR4 (Area %)
p < 0.05
I
Merge
DAPI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

Figure 3.

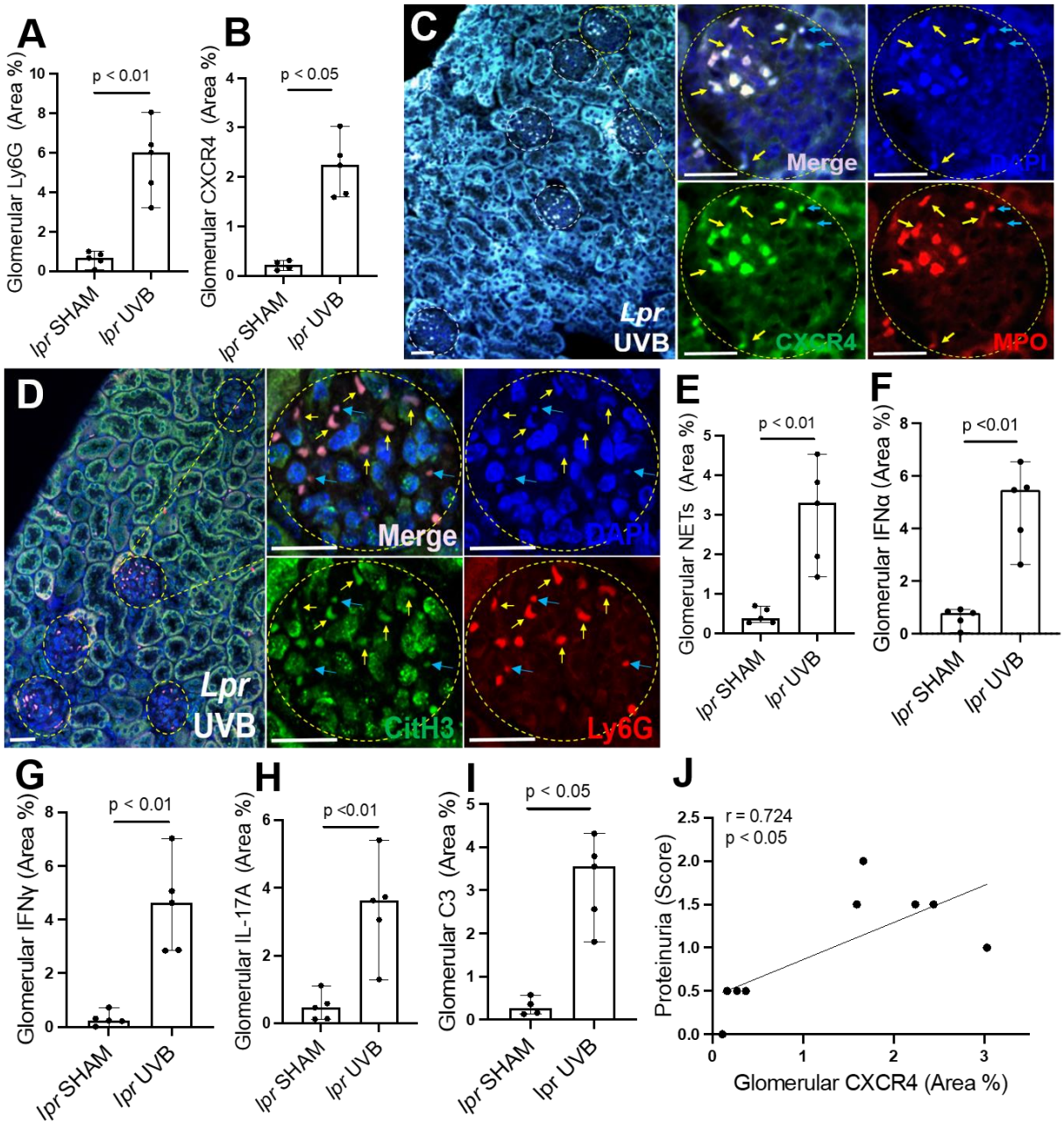

A
Glomerular Ly6G (Area %)
p < 0.01
lpr SHAM
lpr UVB
B
Glomerular CXCR4 (Area %)
p < 0.05
lpr SHAM
lpr UVB
C
Lpr
UVB
Merge
DAPI
CXCR4
MPO
D
Lpr
UVB
Merge
DAPI
CitH3
Ly6G
E
Glomerular NETs (Area %)
p < 0.01
lpr SHAM
lpr UVB
F
Glomerular IFNα (Area %)
p <0.01
lpr SHAM
lpr UVB
G
Glomerular IFNγ (Area %)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

Figure 4.

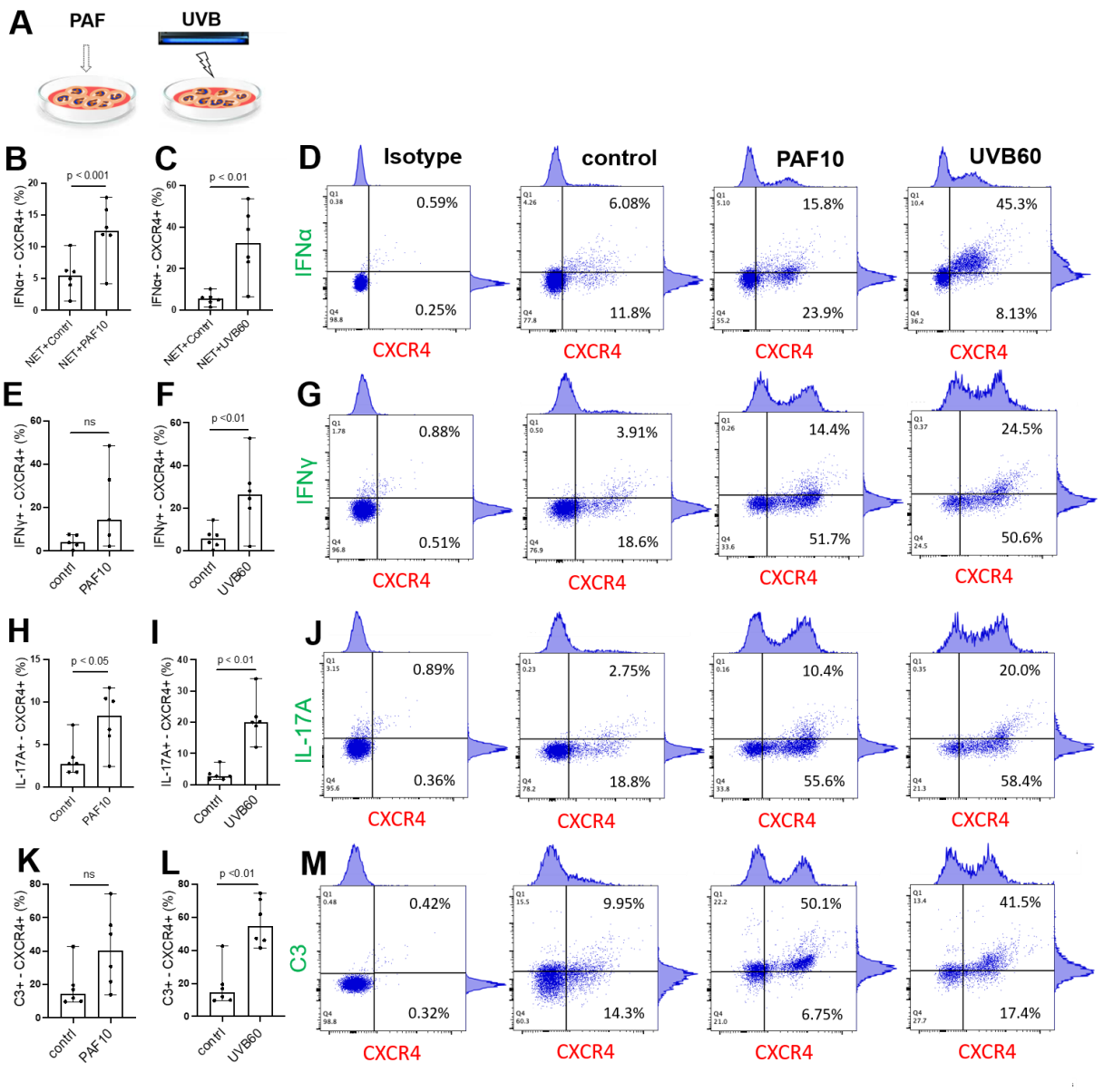

A
PAF
UVB
B
p < 0.001
IFNα+ - CXCR4+ (%)
NET+Contrl
NET+PAF10
C
p < 0.01
IFNα+ - CXCR4+ (%)
NET+Contrl
NET+UVB60
D
Isotype
control
PAF10
UVB60
IFNα
0.59%
0.25%
6.08%
11.8%
15.8%
23.9%
45.3%
8.13%
CXCR4
E
ns
IFNγ+ - CXCR4+ (%)
contrl
PAF10
F
p <0.01
IFNγ+ - CXCR4+ (%)
contrl
UVB60
G
IFNγ
0.88%
0.51%
3.91%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

Figure 5.

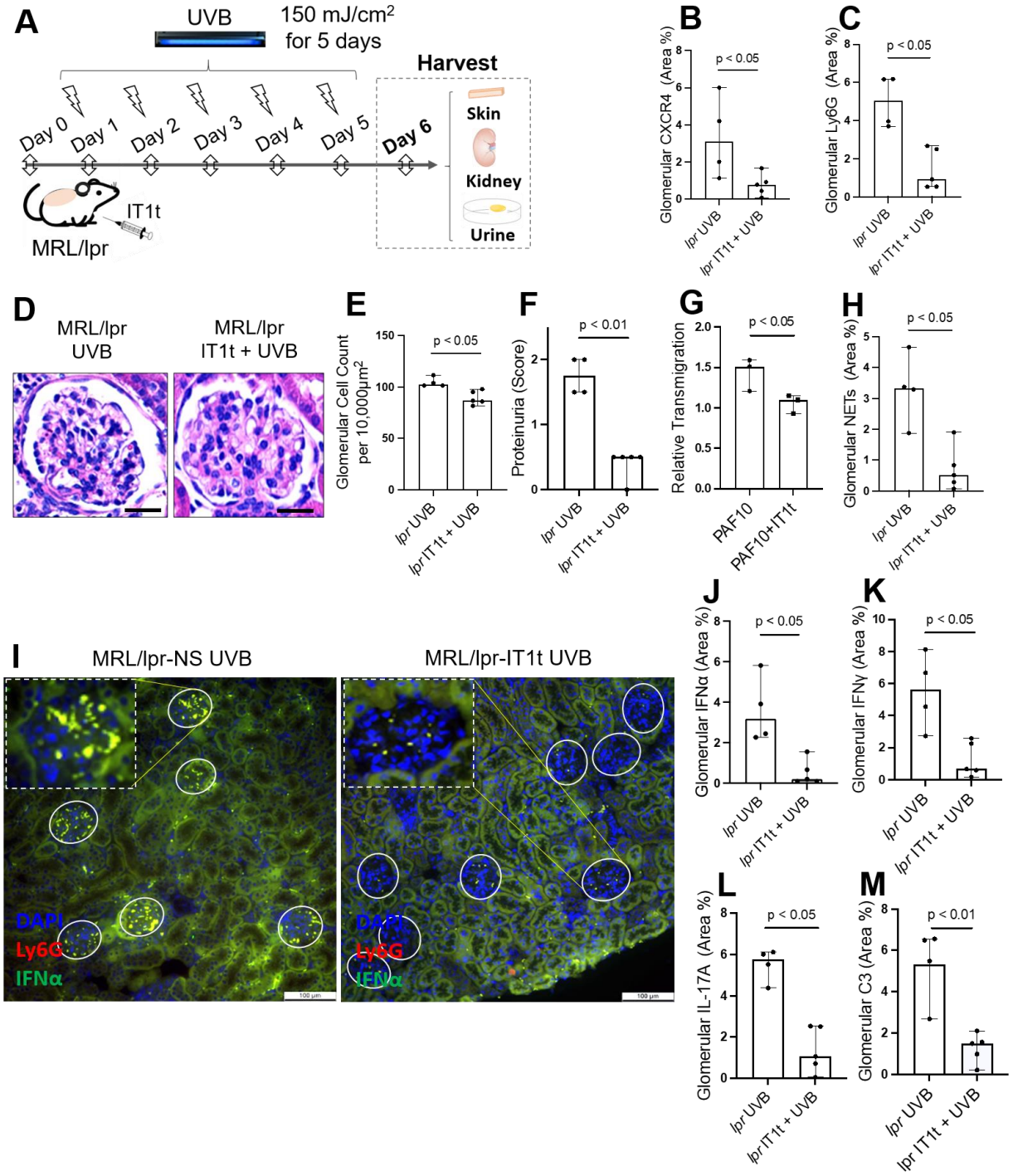

A
UVB
150 mJ/cm2
for 5 days
Harvest
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Skin
Kidney
Urine
IT1t
MRL/lpr
B
Glomerular CXCR4 (Area %)
p < 0.05
lpr UVB
lpr IT1t + UVB
C
Glomerular Ly6G (Area %)
p < 0.05
D
MRL/lpr
UVB
MRL/lpr
IT1t + UVB
E
Glomerular Cell Count
per 10,000μm2
p < 0.05
F
Proteinuria (Score)
p < 0.01
G
Relative Transmigration
p < 0.05
PAF10
PAF10+IT1t
H
Glomerular NETs (Area %)
p < 0.05
I
MRL/lpr-NS UVB
MRL/lpr-IT1t UVB
DAPI
Ly6G
IFNα
J
Glomerular IFNα (Area %)
p < 0.05
K
Glomerular IFNγ (Area %)
p < 0.05
L
Glomerular IL-17A (Area %)
p < 0.05
M
Glomerular C3 (Area %)
p < 0.01

Figure 6.

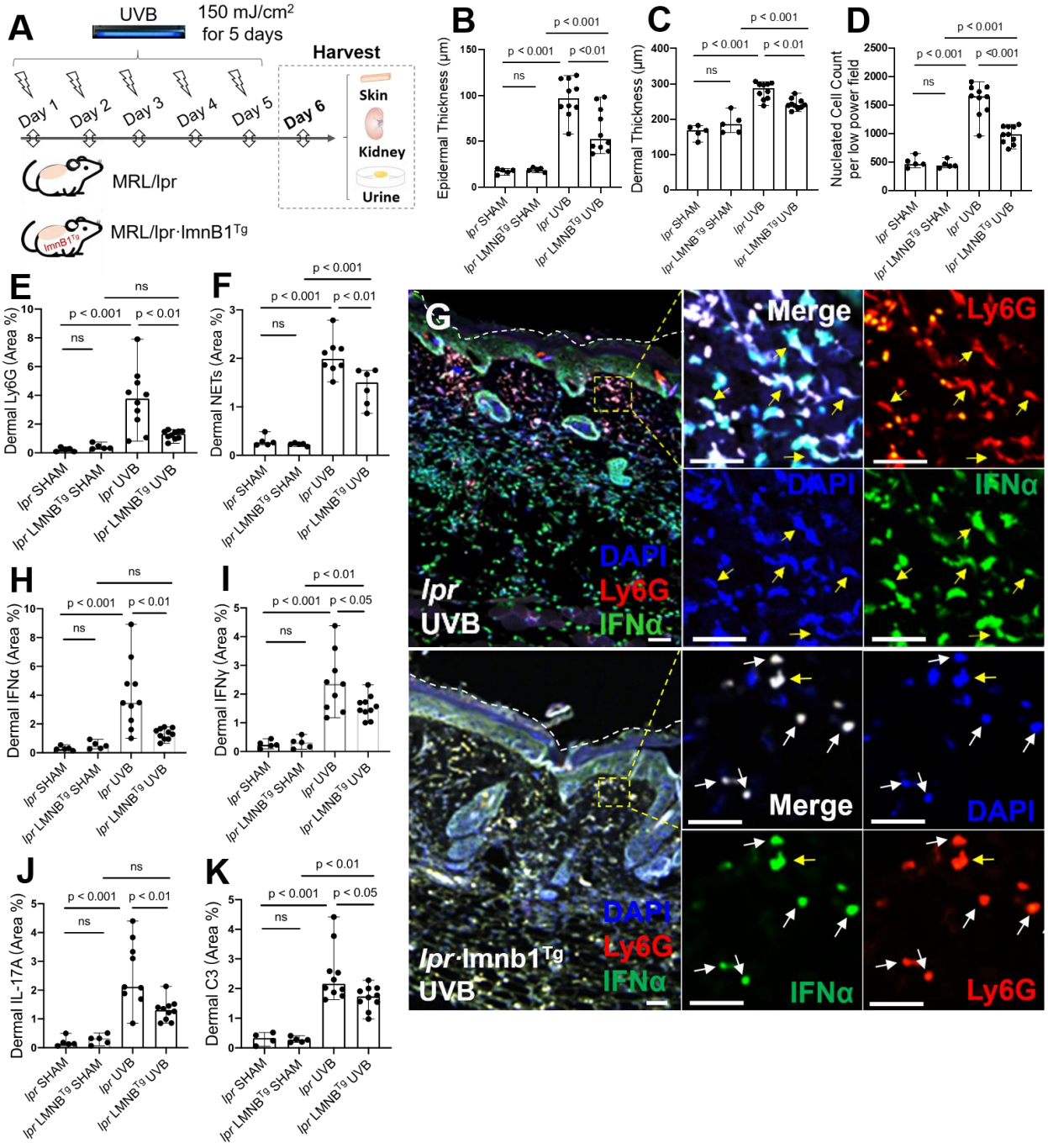
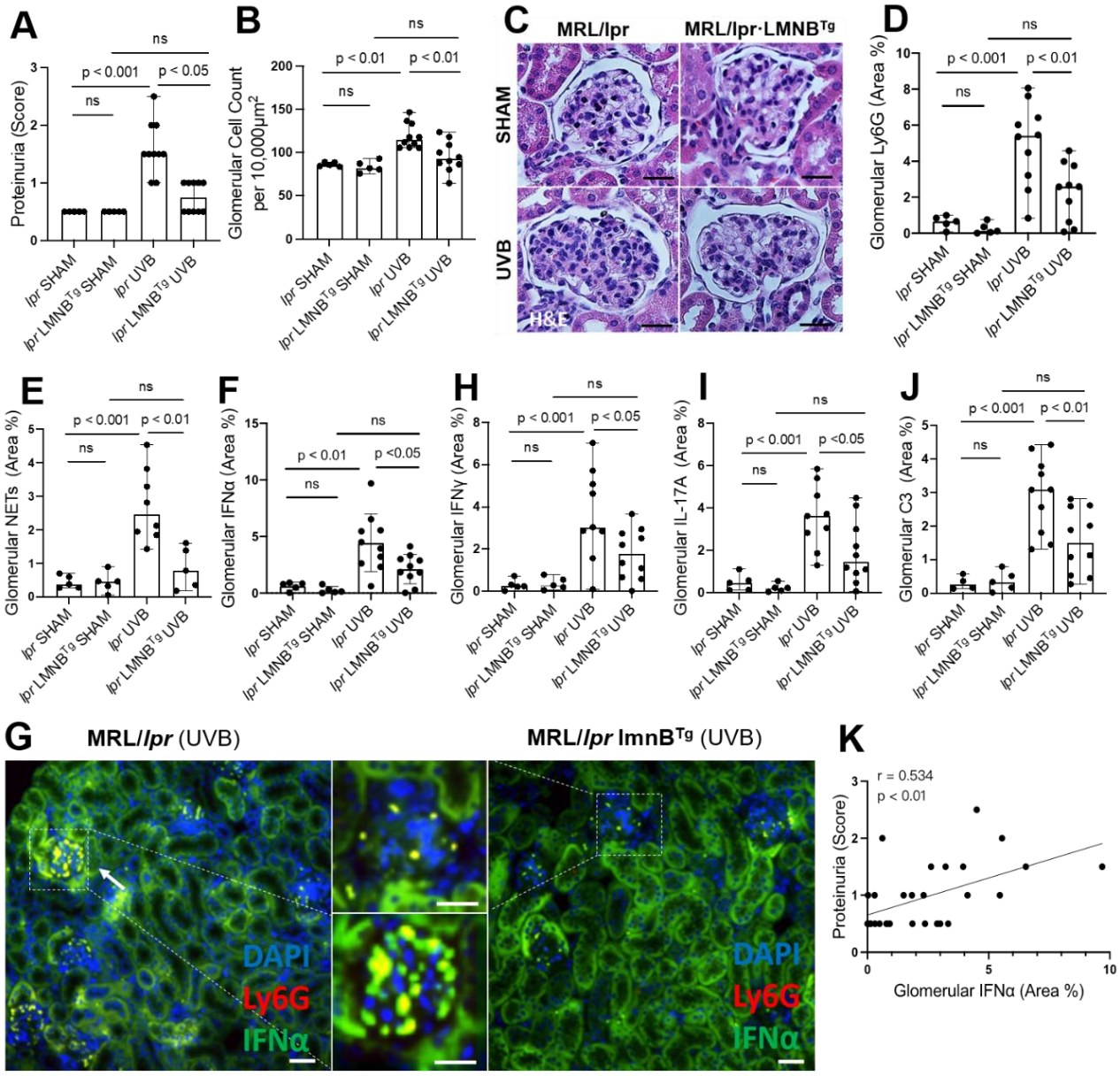

A
UVB
150 mJ/cm2
for 5 days
Harvest
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Skin
Kidney
Urine
MRL/lpr
MRL/lpr·lmnB1Tg
B
Epidermal Thickness (μm)
C
Dermal Thickness (μm)
D
Nucleated Cell Count per low power field
E
Dermal Ly6G (Area %)
F
Dermal NETs (Area %)
H
Dermal IFNα (Area %)
I
Dermal IFNγ (Area %)
J
Dermal IL-17A (Area %)
K
Dermal C3 (Area %)
lpr SHAM
lpr LMNBTg SHAM
lpr UVB
lpr LMNBTg UVB
ns
p < 0.001
p < 0.01
p < 0.05
G
Merge
Ly6G
DAPI
IFNα
lpr UVB
lpr·lmnb1Tg UVB

Figure 7.


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

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Supplemental Methods and Data

for

Neutrophil extracellular traps drive acute lupus flares with 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: neutrophil extracellular traps, acute lupus flare, UVB, skin inflammation, kidney inflammation

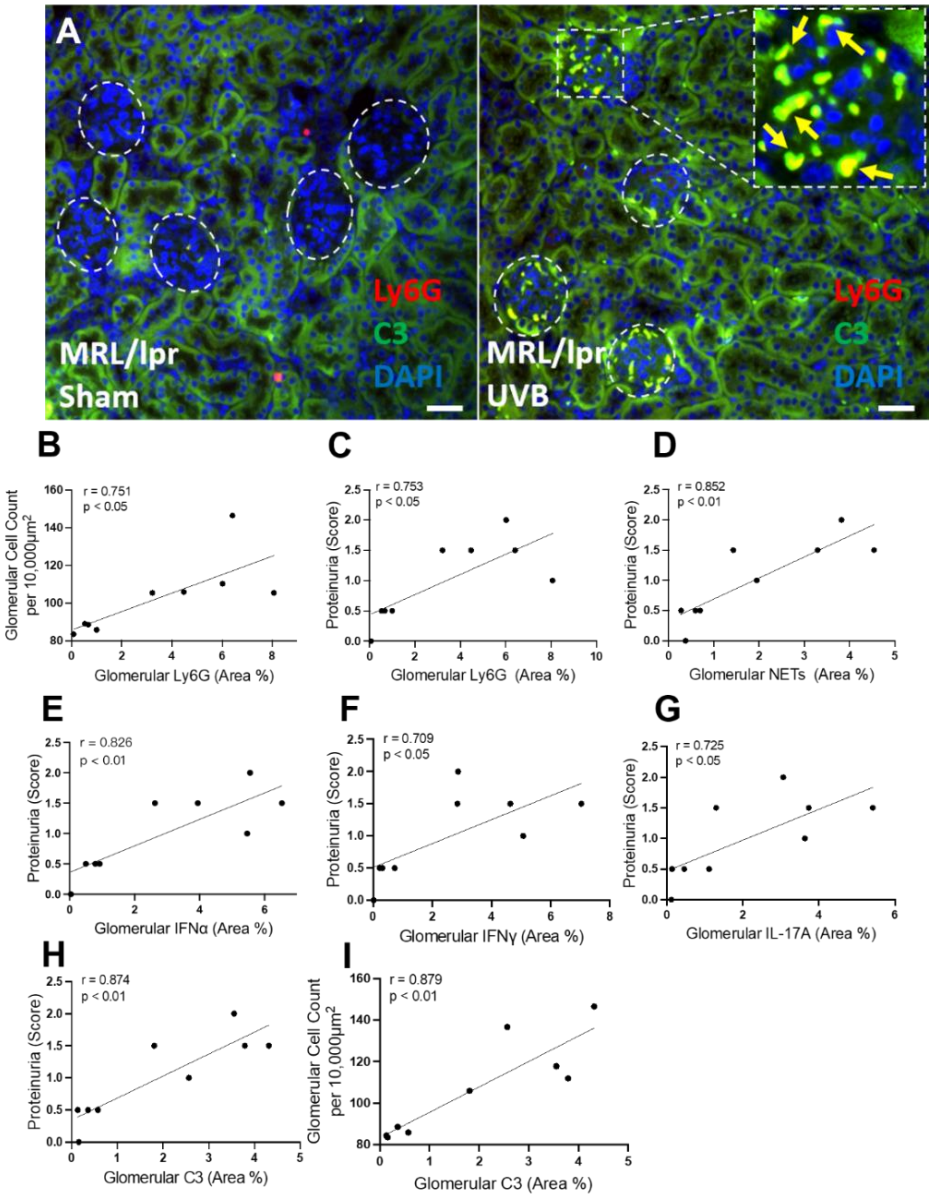
Supplemental Methods

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 (1-3). 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 (3).

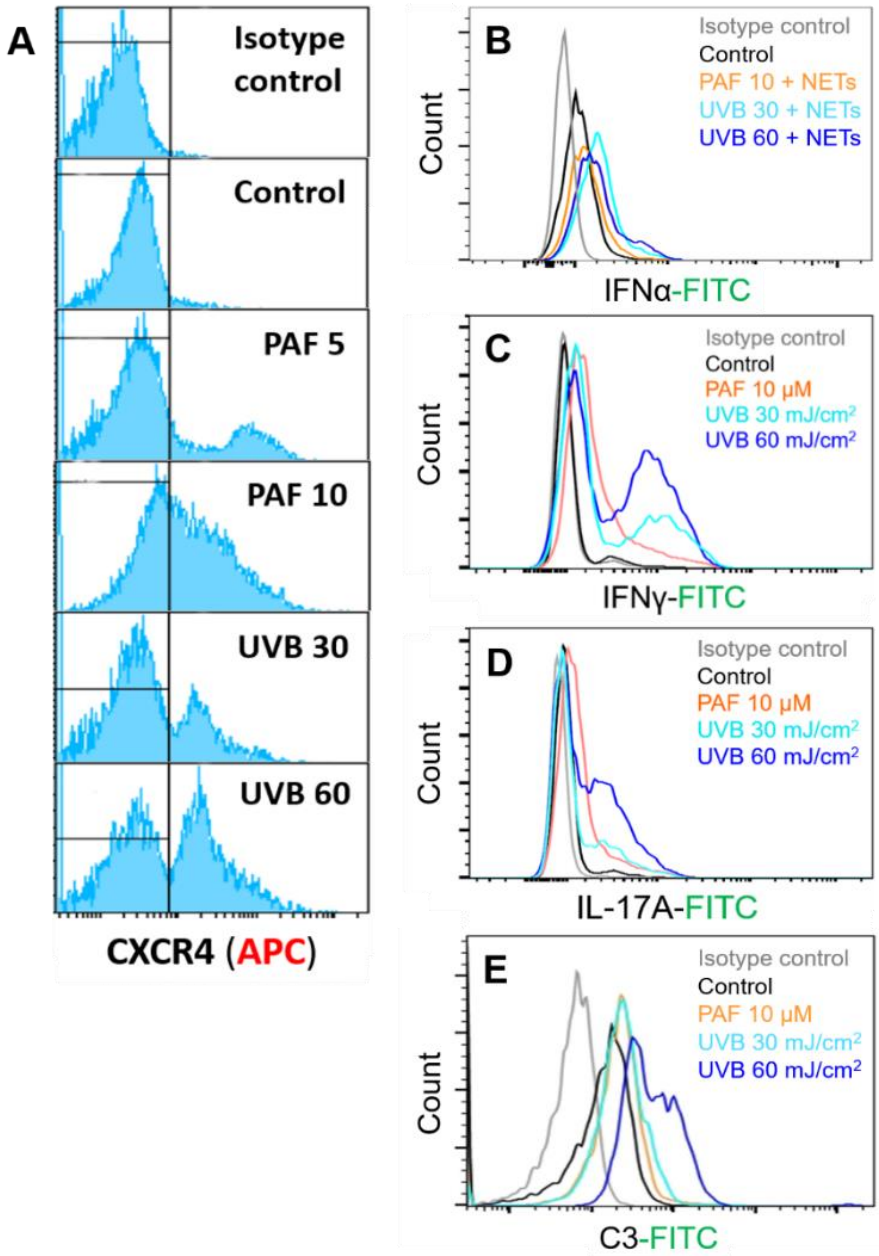
Immunohistochemistry analyses of the skin and kidneys. The paraffin-embedded skin or kidney tissue sections of all mice were stained either by hematoxylin and eosin (H&E) or by fluorescent staining as described before (2, 3). Infiltration of inflammatory cells in skin of all mice was counted for the nucleated cells in the epidermis, dermis, or fat of the H&E stained skin sections as described in our published works (2, 3). 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 (4). 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 (2). 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 (5, 6). 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 (2, 3).

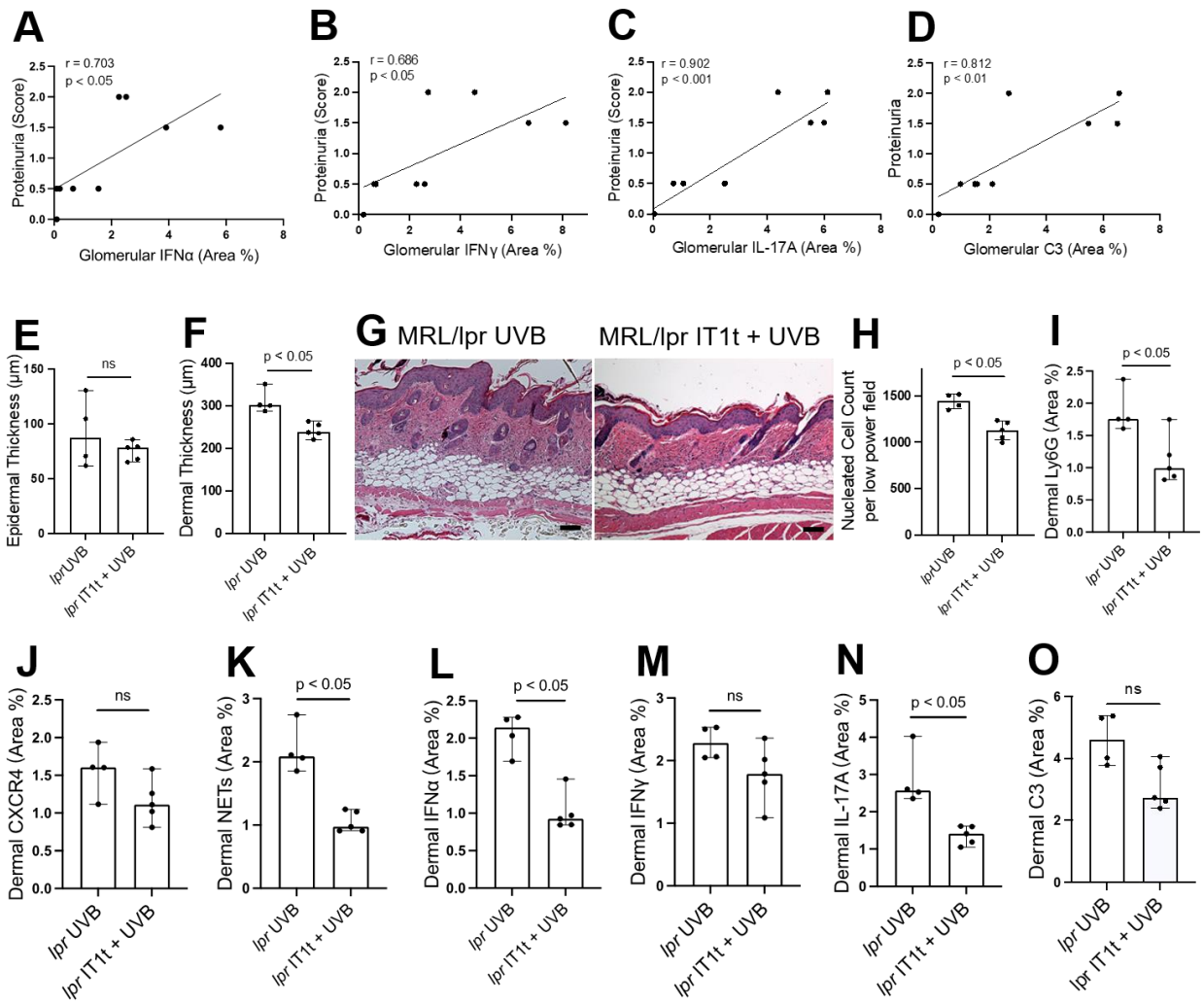
Supplemental Data



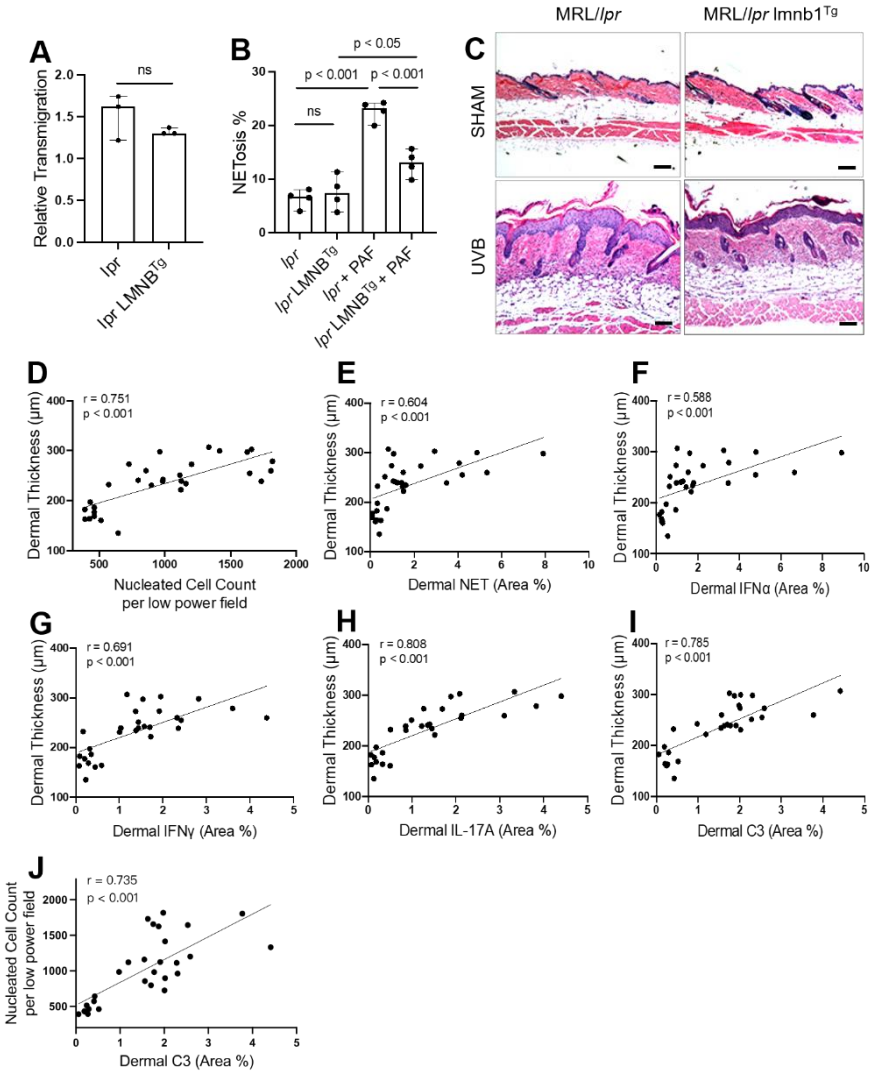
Suppl Fig. 1. Neutrophil infiltration, NET formation, and NET-associated cytokines/C3 are involved in acute kidney inflammation in UVB-irradiated lupus-prone mice. A. Representative images of neutrophils, NETs, NET-associated C3 in kidneys of asymptomatic young female MRL/lpr mice without (sham) or with 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.



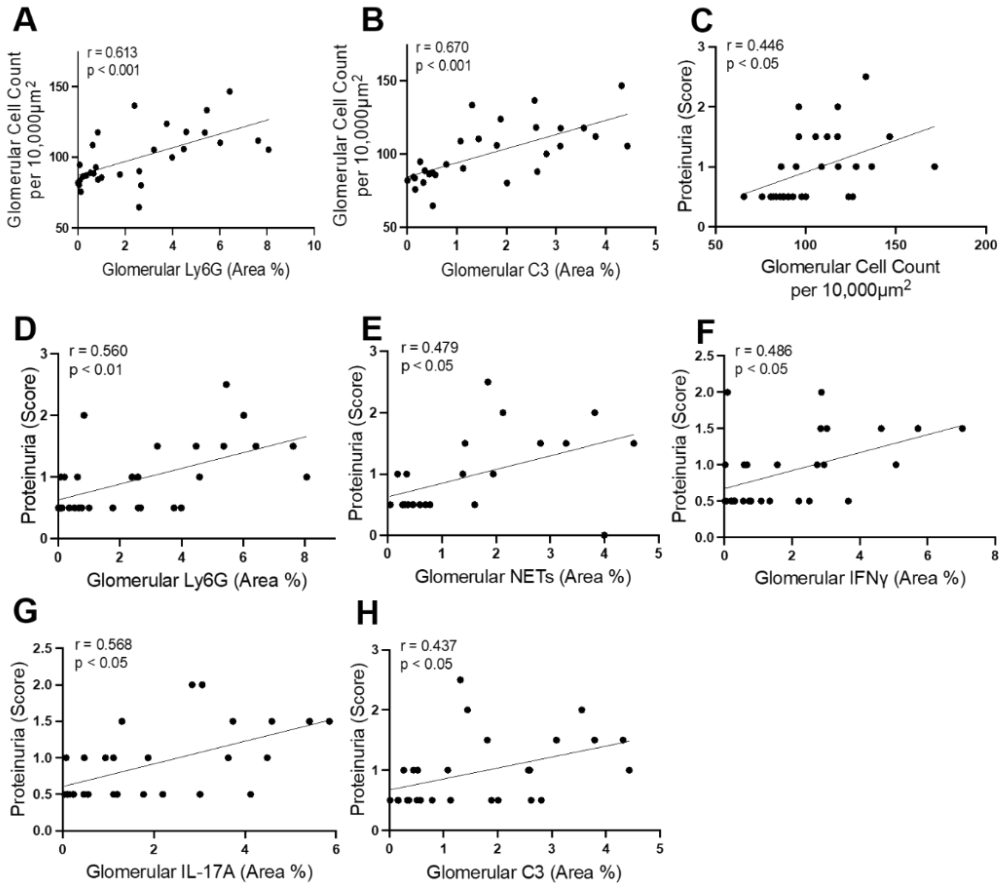
Suppl Fig. 2. UVB irradiation induced co-expression of CXCR4, cytokines, and C3 *in vitro* in primary neutrophils from lupus-prone mice. A-E. Representative histogram of expressions of CXCR4 or cytokines IFNα, IFNγ, IL-17A, or C3 in primary neutrophils from lupus-prone mice, which were stimulated without (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 lupus kidney inflammation 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.

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