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Targeted TNF receptor 1 preligand assembly domain improves skin lesions in MRL/lpr mice

Guo-Min Deng, Lena Liu, and George C. Tsokos

Division of Rheumatology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115 USA

Abstract

Objective—Skin disease is the second most common manifestation in patients with systemic lupus erythematosus (SLE). TNF receptor (TNFR) preligand assembly domain (PLAD) has been found to block the effect of TNF- α and TNFR1 PLAD (P60 PLAD) inhibits inflammatory arthritis. We asked whether TNFR PLAD can limit inflammatory skin injury in SLE.

Methods—Female MRL/lpr mice received P60 PLAD (100 μ g/mouse, i.p.) or P80 PLAD (100 μ g/mouse, i.p.) or PBS (100 μ l/mouse, i.p.) three times a week starting at age of 6 weeks for 26 weeks.

Results—Immunohistochemistry studies demonstrated that TNFR1 but not TNFR2 is dominantly expressed in skin lesions in MRL/lpr mice. We found that TNFR1 PLAD but not TNFR2 PLAD (P80 PLAD) protein significantly inhibited skin injury in lupus MRL/lpr mice. P60 PLAD significantly inhibited NF- κ B, MCP-1 and iNOS expression in skin lesions. P60 PLAD reduced lupus serum-induced monocyte differentiation into dendritic cells. P60 PLAD did not reduce IgG deposition in the skin and improve kidney pathology progression in MRL/lpr mice.

Conclusion—Our results indicate that TNFR1 is involved in the expression of skin injury in lupus MRL/lpr mice and P60 PLAD or similar biologics may be of clinical value if applied locally.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by high levels of autoantibody and multi-organ tissue damage including kidney and skin (1,2). Keratinocyte apoptosis represents one of the histological features of skin lesions in SLE patients and may represent a source of autoantigens which in turn may be involved in the production of antibody. Antibody is deposited in the skin of patients with SLE even in non-affected areas (band test) and the presence of circulating anti-Ro (SS-A) antibodies has been associated with skin disease (3). Exposure to ultraviolet light is known to trigger disease (photosensitivity) but the exact mechanisms are not clear (4).

Correspondence, Correspondence should be addressed to G.M. Deng (gdeng@bidmc.harvard.edu) or George C. Tsokos (gtsokos@bidmc.harvard.edu) Division of Rheumatology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School.

Author Contributions

Dr. Deng and Tsokos had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis

Study design. Guo-Min Deng, George C. Tsokos

Acquisition of data. Guo-Min Deng, Lena Liu

Analysis and interpretation of data. Guo-Min Deng, George C. Tsokos

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Statistical analysis. Guo-Min Deng

Tumor necrosis factor α (TNF- α) is a proinflammatory cytokine mainly produced by macrophages/monocytes (5). TNF α exerts its effect by binding to extracellular domains of TNFR1 (P60) and TNFR2 (P80). Recently, TNFR PLAD has been found to exert a crucial role in TNFR signaling (6). PLAD protein block effects of TNF- α and P60 PLAD protein inhibits inflammatory arthritis induced by TNF- α , CpG DNA and collagen (7). Given that PLAD has a key role in TNFR signaling, and that TNF- α induces skin inflammation (8), we investigated potential inhibitory role of soluble PLAD proteins in skin inflammation of lupus MRL/lpr mice. We found that P60 not P80 PLAD protein inhibited skin injury in MRL/lpr mice. Our data demonstrates that P60 PLAD protein can ameliorate skin injury in MRL/lpr mice.

MATERIAL AND METHODS

Mice and materials

Female MRL/lpr/2J mice, TNFR1 and TNFR2 gene deficient mice and C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed in the animal facility of Beth Israel Deaconess Medical Center. Double strand (ds) DNA, mouse IgG antibodies were purchased from Sigma-Aldrich. Antibodies to TNFR1, TNFR2, MCP-1, iNOS and NF- κ B were purchased from Santa Cruz (CA).

PLAD protein preparation

PLAD60 and PLAD80 proteins were prepared by using GST-fusion protein as described previously (7). LPS was removed from the purified PLAD protein using Detoxi-Gel AffinityPak Columns (pierce) before use.

Treatment of MRL/lpr mice by P60 and P80 PLAD protein

Female MRL/lpr mice received P60 PLAD (100 μ g/mouse, i.p. n=8) or P80 PLAD (100 μ g/mouse, i.p. n=8) or PBS (100 μ l/mouse, i.p. n=8) three times a week starting at age of 6 weeks for 26 weeks. Skin and kidney from sacrificed mice were collected for histological examination and serum was collected for measurement of serum IgG and anti-dsDNA antibody. During the experimental period, urine protein content and mortality were monitored.

Histology of skin and kidney

Histopathological examination of skin and kidney was done after routine fixation and paraffin embedding of the tissue. Tissue sections from skin were cut and stained with hematoxylin and eosin. All slides were coded and evaluated in a 'blinded' to sample identity manner. Severity of skin inflammation will be scored 0–4. Grade 0: normal; Grade 1: hyperplasia of epidermis; Grade 2–4: different amounts of infiltrating inflammatory cells in the skin. Renal pathology is graded by glomerular, interstitial and perivascular inflammation. Scores from 0 to 4 are assigned for each of the features. A minimum of 100 glomeruli were assessed to determine the glomerular scoring index in each mouse.

Urine analysis

The mice in each group were placed overnight in a Nalgene metabolic cage to collect urine. Urine was measured with Multistix 10 SG and analyzed by Clinitek Status analyzer (Bayer Healthcare). Proteinuria is expressed as 0–4, 0 (none), 1 (30–100 mg/dl), 2 (100–300 mg/dl), 3 (300–2000 mg/dl), or 4 (> 2000 mg/dl).

Measurement of serum IgG and anti-DNA antibody

Serum IgG and anti-dsDNA antibody were detected by ELISA. 96-well plate was coated with double calf thymus DNA (1.5 mg/ml, Sigma) or mouse IgG antibody (0.1 mg/ml, Sigma) in PBS at 4°C overnight. After blocking with 1% BSA for 2 hours at room temperature (RT), serum samples were added to plate and incubated for 2 hours at RT. After washing 4 times with PBS, HRP-conjugated goat anti-mouse IgG antibody (Sigma) was added to plate and incubated for 1 hour at RT. Antibody binding was visualized using TMB (3', 3', 5', 5'- tetramethylbenzidine, Sigma), and plates were read the optical density at 450 nm.

Immunohistochemistry staining

After routine fixation and paraffin embedding of the tissue, tissue sections from skin were cut. After deparaffinization and antigen retrieval, samples were stained with antibody to TNFR1, TNFR2, NF-κB, MCP-1, iNOS (Santa Cruz, CA) followed by incubation with biotinylated secondary antibodies and avidin-biotin-peroxidase complexes and 3-amino-9-ethylcarbazole containing H₂O₂. All sections were counterstained with Mayer's hematoxylin.

Monocyte differentiation into DCs

Spleens were removed from C57BL/6, TNFR1 or TNFR2-deficient mice. Mononuclear cells were isolated from mouse spleens. Mononuclear cells were incubated in 24-well plates at 37°C. Monocytes were obtained after suspending lymphocytes were removed. Sera (40 μl) prior or after IgG depletion from lupus-prone mice (5 month old MRL/lpr mice) or normal C57BL/6 mice, TNF-α (2 ng) and M-CSF (10 ng) were added, as indicated, in plates (9). Differentiation of monocytes into DCs was examined by light microscopy. DCs were fixed and permeabilized and then stained with monoclonal anti-CD40 and CD80 conjugated to PE (Santa Cruz, CA). Expression of CD40, CD80 on DCs was examined using flow cytometry.

Statistics

Statistical evaluations of skin inflammation, renal pathology, serum IgG and anti-dsDNA antibody data were performed using the Student's *t*-test or the Mann-Whitney test. $P \leq 0.05$ was considered statistically significant.

RESULTS

TNFR1 but not TNFR2 is expressed in skin lesions of lupus MRL/lpr mice

MRL/lpr mice, an established lupus animal model, develop spontaneously lupus skin injury (10) (Fig. 1 A). We investigated the expression of TNFR1 and TNFR2 in skin lesions of MRL/lpr mice using immunohistochemistry and found significant expression of TNFR1 and much less TNFR2 expression (Fig. 1B). This data suggests the involvement of TNFR1 in the expression of inflammatory process in the skin of lupus prone mice. Immunofluorescent staining with an anti-CD11c antibody revealed the presence of dendritic cells in the skin lesions (Fig. 1C).

Treatment of MRL/lpr with P60 PLAD protein limits the appearance of skin lesions

We prepared soluble P60 and P80 PLAD proteins as described previously (7) and injected them in the peritoneum of 6 weeks old MRL/lpr mice for 26 weeks. We assumed that if TNFRs are involved in the expression of skin lesions of these mice then their inhibition should suppress their appearance. During the treatment period, we observed that skin lesions developed on both sides of the face of MRL/lpr mice treated with P80 PLAD or PBS but not in the face of mice treated with P60 PLAD (Fig. 2A–B). The skin lesions on the face

appeared earlier and the incidence of skin lesions was higher in mice treated with P80 PLAD than with control PBS (Fig. 2A–B). Histopathologic examination demonstrated a markedly thickened epidermis with inflammatory cell infiltration in the dermis in PBS and P80 PLAD-treated mice but not in mice treated with P60 PLAD (Fig. 2C–D). Also, one mouse treated with P80 PLAD developed skin injury on the back. In a group of mice we discontinued PLAD treatment and we observed the development of cutaneous injury on month later in mice previously treated with P60 PLAD (Fig. 2E). These data indicates that P60 PLAD protein can suppresses skin injury in MRL/lpr mice.

P60 PLAD protein reduces lupus MRL/lpr serum-induced monocyte differentiation into dendritic cells (DCs)

Monocytes contribute to skin injury in MRL/lpr mice (11). Monocytes not only become macrophages but also represent an important source of DCs during an inflammatory process (12–14). We investigated whether P60 PLAD protein can regulate lupus MRL/lpr serum-induced monocyte differentiation into DCs (9). Serum from 5-month old MRL/lpr mice induced monocyte differentiation into DCs but serum from normal C57BL/6 mice did not (Fig. 3A and supplementary figure 1A). Flow cytometry analysis demonstrated that lupus serum, but normal serum, caused enhanced expression of CD40 and CD80 on the surface membrane of monocytes (Fig. 3B). Depletion of IgG from lupus MRL/lpr serum abolished lupus serum-mediated monocyte differentiation into DCs (Fig. 3C). Immunofluorescent staining demonstrated the cells identified as DCs by light microscopy were CD11c positive cells (Supplementary figure 1B). In addition, we found the accumulated CD11c positive cells in skin sites followed intradermal injection of lupus serum from MRL/lpr mice not from C57BL/6 mice (Fig. 3D).

To understand the role of TNFR1 in lupus MRL/lpr serum-induced monocyte differentiation into DCs, we investigated whether P60 PLAD regulates lupus serum-induced monocyte differentiation into DCs. We observed that P60 PLAD not P80 PLAD reduced lupus MRL/lpr serum-induced monocyte differentiation into DCs (Fig. 3E). To further confirm the role of TNFR1 in lupus MRL/lpr serum-induced monocyte differentiation into DCs, we repeated experiments using monocytes from TNFR1 or TNFR2 knockout mice. We observed that lupus MRL/lpr serum-induced monocyte differentiation into DCs was markedly limited in TNFR1 but not TNFR2-deficient mice (Fig. 3F). These data indicates that P60 PLAD regulates lupus MRL/lpr serum containing IgG-induced monocyte differentiation into DCs.

P60 PLAD protein inhibited the expression of MCP-1, iNOS, NF- κ B in skin lesions of MRL/lpr mice

Immune cells migrate to inflammatory sites from blood vessels in response to increased expression of chemokines (15). MCP-1 has been shown to contribute to the attraction of immune cells to the inflamed skin in MRL/lpr mice (11,16). MCP-1 expression was significantly reduced in the skin lesions in mice treated with P60 PLAD protein compared to PBS treatment (Fig. 4A).

Nitric oxide is involved in tissue inflammation (17). We investigated whether P60 PLAD regulates iNOS expression in skin lesions in MRL/lpr mice. iNOS expression was markedly decreased in skin lesions in mice treated with P60 PLAD protein (Fig. 4B).

NF- κ B is an important intracellular regulator of genes involved in the inflammatory response (7,18,19), we investigated whether NF- κ B is downregulated in the skin lesions of MRL/lpr mice treated with P60 PLAD protein. NF- κ B expression was markedly decreased in inflammatory lesions of MRL/lpr mice treated with P60 PLAD protein compared to the MRL/lpr mice treated with PBS (Fig. 4 C).

Effect of TNFR PLAD protein on autoantibody production and skin deposition in MRL/lpr mice

We determined the effect of TNFR PLAD protein treatment on the production of autoantibodies in MRL/lpr mice. We measured levels of IgG, anti-dsDNA antibodies in the sera of MRL/lpr mice at age of 16 weeks by ELISA. We noted a tendency for higher IgG levels in mice treated with P60 PLAD protein and also a similar tendency for higher levels of anti-ds-DNA antibody production (Fig. 5A–B) suggesting that blockade of TNFR does not improve systemic autoimmunity. Next we also investigated whether PLAD protein treatment alters IgG deposition in the skin of MRL/lpr mice. Immunohistochemistry staining demonstrated that PLAD protein treatment does not significantly inhibit IgG deposition in the skin of MRL/lpr mice compared to control PBS treatment (Fig. 5C) suggesting again that the effect of PLAD P60 relies on its ability to limit the local skin inflammatory response.

Effect of TNFR PLAD proteins on kidney pathology in MRL/lpr mice

During treatment period, we also investigated effects of PLAD protein on kidney damage. It appeared that mice treated with PLAD proteins had a tendency to develop more proteinuria (Fig. 6A). Kidney pathology worsened in the mice treated with PLAD P60 (Fig. 6B). This data suggest that like systemic autoimmunity, lupus kidney pathology does not benefit from TNFR inhibition.

Discussion

Our results demonstrate that P60 PLAD but not P80 PLAD protein can suppress skin injury in lupus MRL/lpr mice. The fact that skin lesions express TNFR1 rather than TNFR2 may explain the mitigating effect of the TNFR1 inhibitor P60 PLAD. Our findings are in agreement with previous reports that TNF inhibition does not affect systemic autoimmunity, or it may, as it has been observed in patients receiving anti-TNF treatment (20), instigate systemic autoimmunity manifested by the appearance of anti-nuclear antibodies. Treatment of MRL/lpr mice with P60 or P80 PLAD proteins did not improve systemic autoimmunity, but instead, it appeared to provoke a tendency towards worsening. Treatment with P60 PLAD did not even affect the deposition of immunoglobulin to the skin of MRL/lpr mice despite the fact that the inflammatory process was suppressed. This antithetic effect signifies the independence of local organ injury from systemic autoimmunity and calls for attention to local events which may execute tissue damage and which may become targets of treatment.

Keratinocyte apoptosis is a histologically recognizable feature of skin lesions in patients with lupus. It has been well established that apoptotic material may flood the immune system with autoantigens which may promote the appearance of high levels of autoantibodies (21). On the other hand, since Fas-induced apoptosis is defective in MRL/lpr mice, blocking of TNF- α -induced apoptosis(5) may enhance severity of defective apoptosis in MRL/lpr mice. TNFR1 represents the main receptor in the TNF- α -induced apoptosis (5) and although TNFR2 lacks a death domain it activates NF- κ B (5) and is involved in T cell apoptosis, especially CD8+ T cells (22). Thus, blockade of TNFR1 or TNFR2 using P60 PLAD or P80 PLAD may enhance defective apoptosis in T cells and promote the production of autoantibodies in MRL/lpr mice. This data indicates that TNF may protect against nephritis by reducing the production of autoantibodies and by promoting T cell apoptosis in MRL/lpr mice.

Tissue damage in SLE is characterized by autoantibody production and immune complex formation and deposition (2). Antibody-nucleosome complexes and IgG have been found deposited in the renal glomerular basement membrane in lupus nephritis in humans and mice (23–25). These immune complexes activate complement and initiate the glomerulonephritis

which has been demonstrated in animal models (26,27). The anti-dsDNA and anti-nucleosome antibodies cross-react with proteins such as α -actinin in the kidney and may have a direct pathogenic effect on renal cells (28,29). The absence of TNFR1 in the Fas deficient mouse resulted in greatly accelerated glomerulonephritis and production of IgG and anti-dsDNA antibodies (30). Although the levels of anti-dsDNA antibody in mice treated with P60 or P80 PLAD did not rise significantly more than in mice treated with PBS, both levels of IgG and anti-dsDNA antibody displayed a trend towards higher values (Fig. 5). It is possible that other autoantibodies including anti-histone, anti-phospholipid antibodies might be elevated in MRL/lpr mice treated with P60 or P80 PLAD protein and make contribution to development of proteinuria and kidney damage.

DCs are important resident cells in skin and the first to react to pathogen in the skin inflammation (13). Activated DCs release cytokines and move to the draining lymph nodes where mature DCs activate T cells (14). Monocytes from blood vessels are thought to be an important resource of DCs (15). Serum from lupus patients promotes monocyte differentiation into DCs (9) and serum IgG from MRL/lpr mice was shown here to induce monocyte differentiation into DCs. Although IFN- α has been shown to exert important role in lupus patient serum-induced monocyte differentiation into DCs (9), our results demonstrate that lupus MRL/lpr serum-induced monocyte differentiation into DCs also requires TNFR1 signaling. Blocking TNFR1 signaling by P60 PLAD protein inhibited lupus MRL/lpr serum-induced monocyte differentiation into DCs. Lupus MRL/lpr serum-induced monocyte differentiation into DCs was markedly reduced in monocytes from TNFR1 but not TNFR2 knockout mice. TNFR1 has been shown to be required for differentiation of follicular DCs (31). TNFR1 knockout mice are resistant to TNF- α -induced skin inflammation (32).

In conclusion, this study shows that TNFR1 is important for the expression of skin lesion in the lupus prone MRL/lpr mouse. Blockade of TNFR1 signaling mitigates skin lesions despite the fact that it does not affect skin immunoglobulin deposition. In agreement with previous information and clinical experience, TNF inhibition with PLAD proteins displayed a tendency towards aggravated systemic autoimmunity and kidney pathology. The value of our results lies with the establishment of the importance of local factors in the expression of local organ injury and the possibility of considering the application of local TNFR1 inhibitors in the treatment of lupus skin lesions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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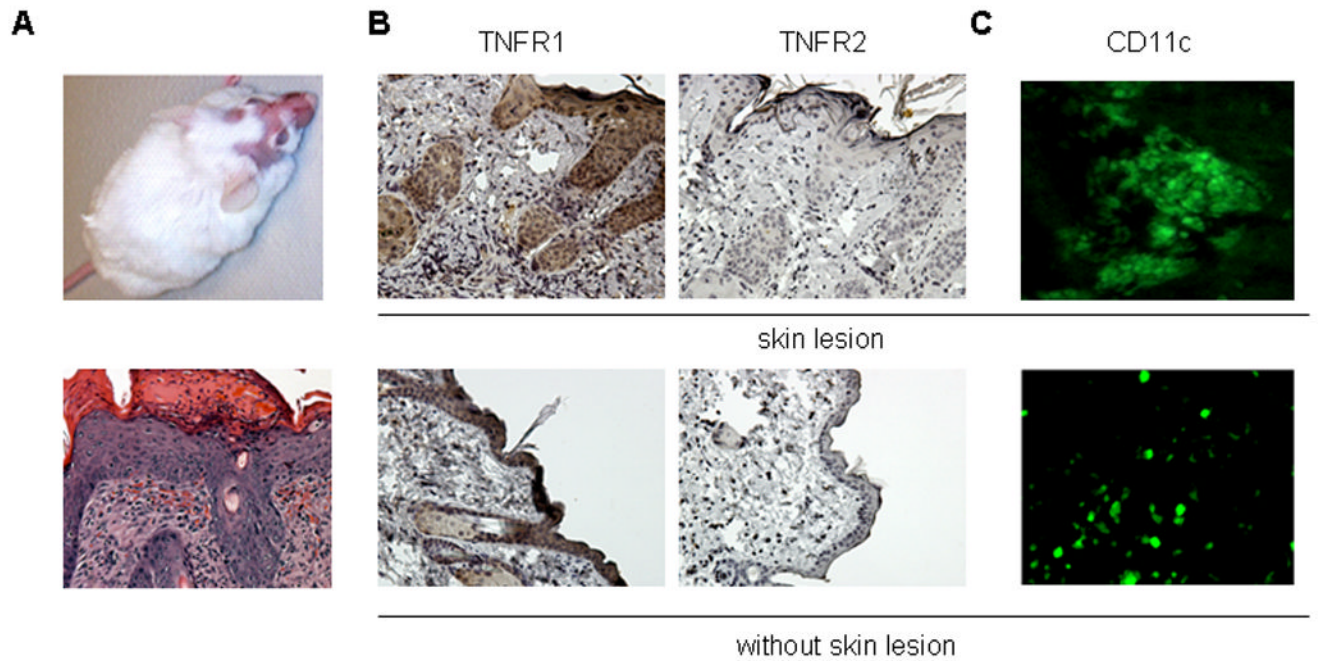


Figure 1.

Expression of TNFR and CD11c in skin lesions of MRL/lpr mice. (A) Representative pictures of skin lesions and histopathology in MRL/lpr mice. (B) Representative figures of immunohistochemistry staining of expression of TNFR1, TNFR2 (upper rows). Arrow represents staining of TNFR1 in the skin. Lower rows show TNFR1 and TNFR2 staining in the skin without injury. (C) Representative immunofluorescent staining of CD11c in skin injury of MRL/lpr mice (upper). Lower row shows staining without skin injury.

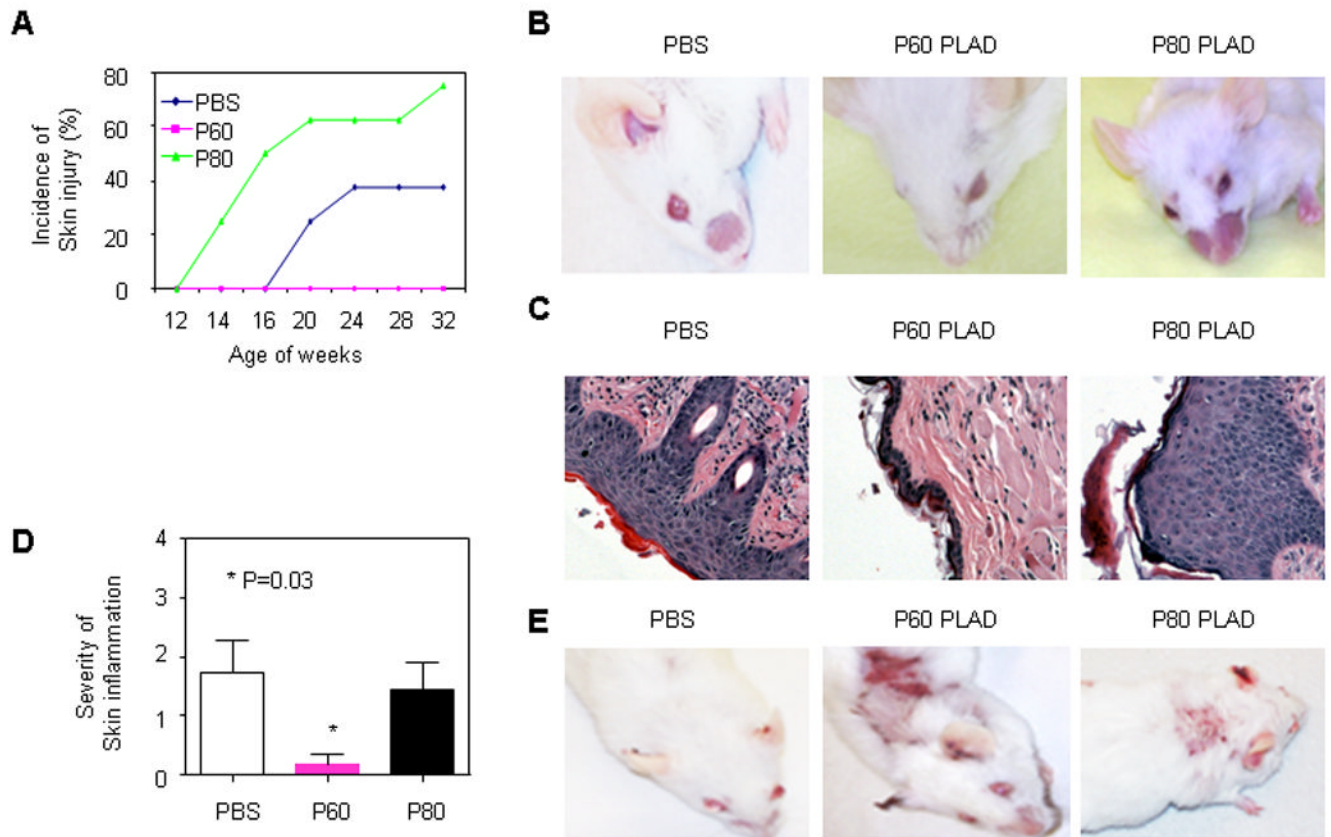


Figure 2.

Effect of P60 and P80 PLAD proteins on skin injury of MRL/lpr mice. MRL/lpr mice were treated with P60 PLAD (n= 8, 100 μ g/mouse, three times a week, i.p.) or P80 PLAD protein (n= 8, 100 μ g/mouse, three times a week, i.p.) or PBS (n= 8, 100 μ l/mouse, three times a week, i.p.) starting at 6 week of age for 26 weeks. Skin injury was assessed by incidence and histopathological examination. (A) Incidence of skin injury on the mouse face. (B) Representative picture of MRL/lpr with PBS or P60 or P80 treatment. (C) Representative histopathologic pictures of skin in MRL/lpr mice treated with PBS or P60 or P80 PLAD protein at age of 32 weeks. (D) Severity of skin inflammation. P value= 0.03 (PBS vs P60 PLAD) (E) Pictures of MRL/lpr mice 4 weeks after discontinued treatment.

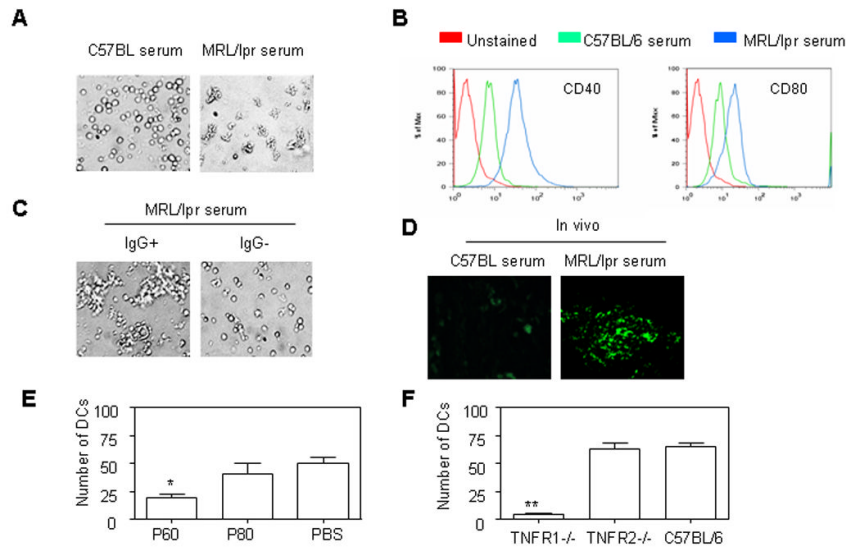


Figure 3.

P60 PLAD protein inhibited lupus serum-induced monocyte differentiation into dendritic cells (DCs). (A). Representative pictures of monocytes from C57BL/6 mice cultured with serum from lupus MRL/lpr mice, normal C57BL/6 mice for 40 hours. (B) Expression of CD40 and CD80 on DCs measured by flow cytometry. (C) Representative pictures of monocytes from C57BL/6 mice cultured with serum from lupus MRL/lpr mice with or without IgG depletion for 40 hours. (D) Immunofluorescent staining of DCs in C57BL/6 mice skin with FITC conjugated anti-CD11c Ab 3d after intradermal injection of serum (100 μ l) from lupus MRL/lpr or C57BL/6 mice. (E) Cumulative data of DCs differentiated from monocytes of C57BL/6 mouse treated with lupus MRL/lpr serum for 40 hours in the presence of PBS, P60 PLAD (100 μ g) or P80 PLAD (100 μ g). (F) Cumulative data of DCs differentiated from monocytes of mice with TNFR1 or TNFR2 gene deficiency and wild type mice were cultured with serum from lupus MRL/lpr mice for 40 hours.

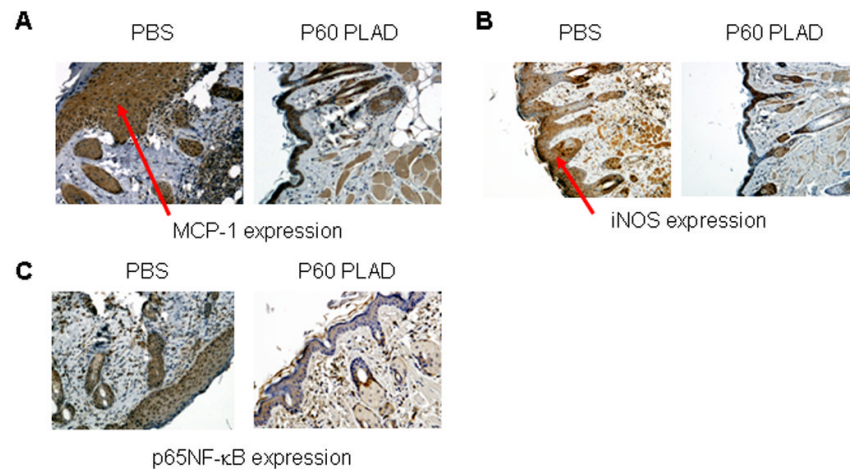


Figure 4. Expression of MCP-1, iNOS and NF- κ B in skin lesions of MRL/lpr mice. Expression of MCP-1, iNOS and NF- κ B (A–C) in skin lesions of MRL/lpr mice treated with PBS or P60 PLAD at age of 32 weeks. Arrow represents staining of MCP-1, iNOS and NF- κ B in the skin.

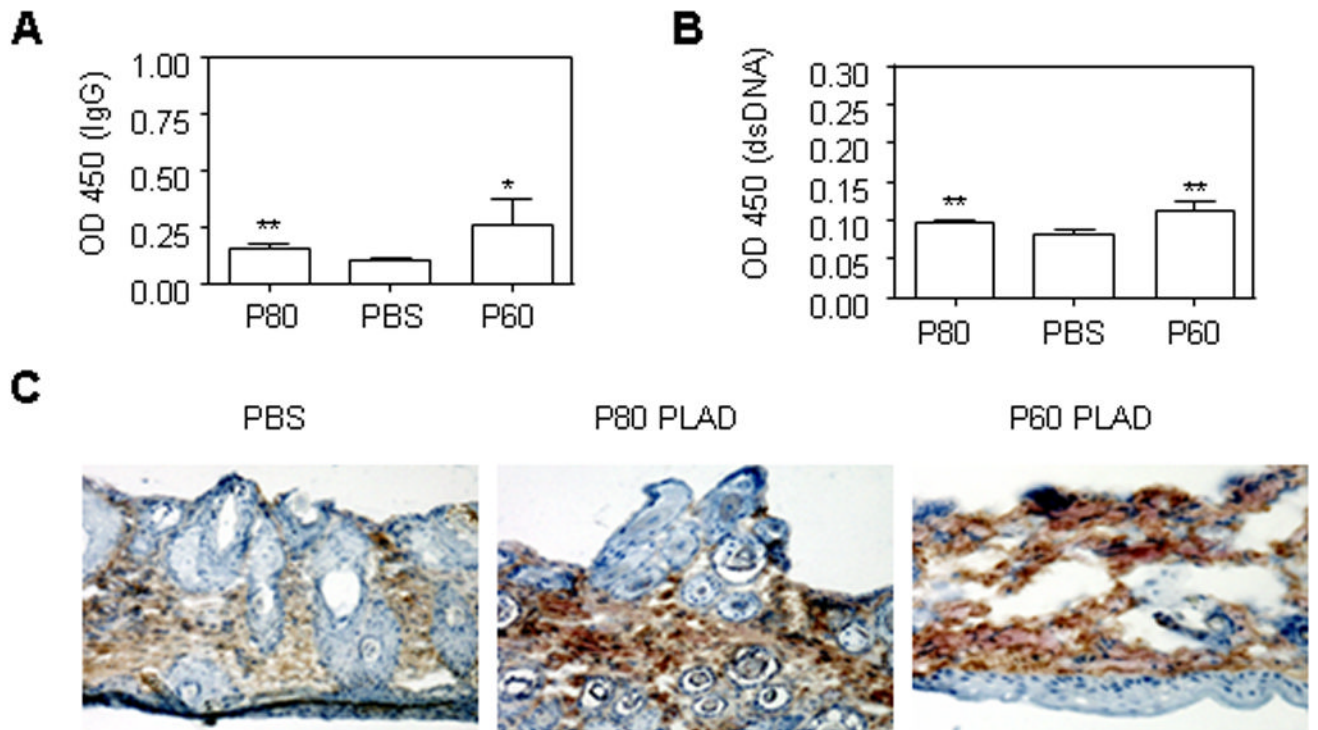


Figure 5.

Effects of P60 and P80 PLAD protein on autoantibody production and skin deposition in MRL/lpr mice. At 16 weeks of age, OD450 values of serum IgG (1:4) (A) and serum anti-dsDNA antibody (1:4) in MRL/lpr mice treated with PBS or P60 or P80 PLAD protein. * $P < 0.01$ and ** $P > 0.05$ (P value represents P60 or P80 PLAD vs PBS treatment). (C) Representative immunohistochemistry staining of IgG deposition in face skin injury of MRL/lpr mice. Skin from MRL/lpr mice treated with PBS or P80 PLAD or P60 PLAD at the age of 32 weeks.

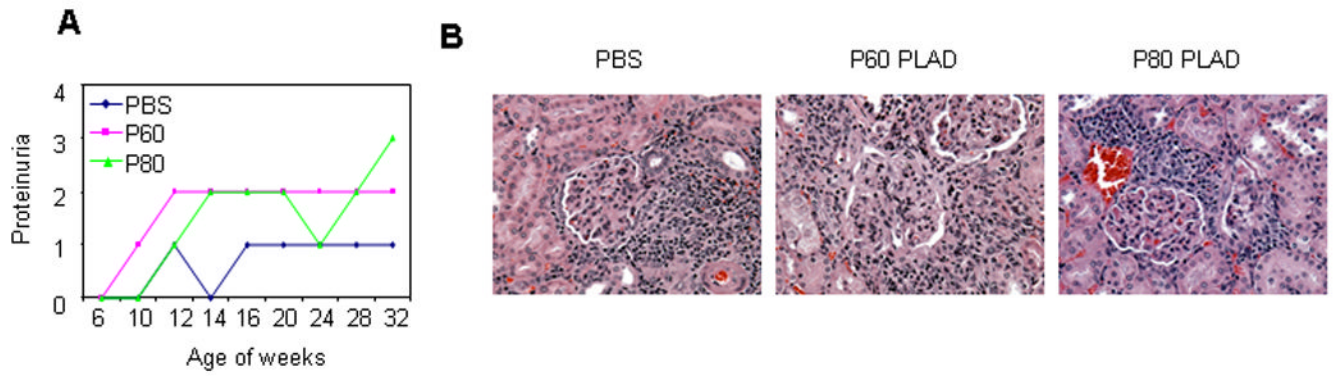


Figure 6.

Effect of PLAD proteins on kidney pathology in MRL/lpr mice. MRL/lpr mice were treated with P60 PLAD (n= 8, 100 μ g/mouse, three times a week, i.p.) or P80 PLAD protein (n= 8, 100 μ g/mouse, three times a week, i.p.) or PBS (n= 8, 100 μ l/mouse, three times a week, i.p.) starting at age of 6 weeks for 26 weeks. (A) Proteinuria from MRL/lpr mice treated with PBS or P60 or P80 PLAD protein. (B) Representative images of H&E stained tissue sections of kidney.