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Immunopathology and Infectious Disease

Decay-Accelerating Factor Ameliorates Systemic Autoimmune Disease in MRL/lpr Mice via Both Complement-Dependent and -Independent Mechanisms

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Decay-accelerating factor (DAF) is a glycosylphosphatidylinositol-anchored membrane protein that restricts complement activation on autologous cells. Previous studies have established a significant protective activity of DAF in the MRL/lpr murine model of human systemic lupus erythematosus. To dissect the mechanism of protection by DAF in this disease model, we evaluated the effect of C3 gene ablation on disease development in MRL/lpr-Daf-1$^{-/-}$ mice. We found no significant difference in lymphadenopathy, splenomegaly, or anti-chromatin autoantibody titer between complement-sufficient and complement-deficient MRL/lpr-Daf-1$^{-/-}$ mice. On the other hand, complement deficiency strikingly reduced the incidence and severity of dermatitis in MRL/lpr-Daf-1$^{-/-}$ mice. To assess the contribution of DAF expression on lymphocytes versus local tissues in suppressing dermatitis, we generated BM chimeric mice between MRL/lpr-Daf-1$^{-/-}$ and MRL/lpr-Daf-1$^{+/+}$ mice. Compared with MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{-/-}$ controls, MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{+/+}$ chimeras developed significantly attenuated dermatitis, suggesting that the protective effect of DAF in suppressing dermatitis is primarily attributable to its local expression. We conclude that DAF works as a complement regulator in the skin to protect MRL/lpr mice from skin inflammation, whereas its inhibitory role in the induction phase of MRL/lpr autoimmunity is complement-independent. Together, these results reveal multiple mechanisms of action for DAF in ameliorating systemic autoimmunity. ***(Am J Pathol 2007, 170:1258–1266; DOI: 10.2353/ajpath.2007.060601)***

Decay-accelerating factor (DAF, CD55), a membrane-anchored complement inhibitor, is widely expressed on cells both within and outside the vascular space such as blood cells, endothelial cells, and many kinds of epithelial and stroma cells.[1,2] In humans, deficiency of DAF and CD59, another glycosylphosphatidylinositol (GPI)-anchored membrane inhibitor of complement, on affected blood cells of paroxysmal nocturnal hemoglobinuria patients was responsible for the increased sensitivity of their erythrocytes and platelets to autologous complement injury.[3–6] Although anecdotal evidence has also linked germline DAF gene mutation to an intestinal inflammatory disorder,[7–9] the role of DAF in human autoimmune and inflammatory tissue injury is primarily unknown.

Using mice deficient in Daf-1, the murine homologue of human DAF, a number of recent studies have addressed the role of DAF in mouse models of inflammatory tissue injury and immune response.[10–15] DAF has been shown to be protective in complement-mediated renal ischemia reperfusion injury[16] and nephrotoxic serum nephritis,[14,15] as well as dextran sulfate-induced colitis.[17] In a previous study, we demonstrated a significant protective activity of DAF in the MRL/lpr murine model of human systemic lupus erythematosus.[18] MRL/lpr mice have the *fas^{lpr}* mutation, which inhibits the expression of Fas, a cell surface apoptosis receptor in the tumor necrosis factor receptor gene family.[19] In addition, the MRL background is autoimmune.[19] MRL/lpr mice spontaneously develop an au-

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toimmune syndrome characterized by elevated levels of immunoglobulin (Ig), multiple autoantibodies, nephritis, and dermatitis, in association with massive lymphoproliferation.[19–21] We found that compared with DAF-sufficient MRL/lpr littermate controls, female MRL/lpr-Daf-$1^{-/-}$ mice developed increased splenomegaly and lymphadenopathy, elevated autoantibody levels, and aggravated dermatitis with high incidence.[18]

Apart from Daf-1, another membrane inhibitor, complement receptor 1-related gene/protein y (Crry), also exists in the mouse.[22–24] Crry overlaps with Daf-1 both in its complement-regulating activity and tissue distribution.[23,24] Given this redundancy, it is not understood why Crry did not compensate for the lack of Daf-1 in the setting of systemic autoimmunity in MRL/lpr mice. Indeed, there is reason to wonder whether Daf-1 acted as a complement regulator or through other mechanisms in ameliorating autoimmunity in MRL/lpr mice. In addition to functioning as a complement regulator, DAF has also been identified as a ligand for CD97, an activation-associated lymphocyte antigen with seven transmembrane domains characteristic of type II G protein-coupled receptors.[25,26] Furthermore, as a GPI-anchored protein, DAF has been shown to function as a costimulatory molecule on lymphocytes.[27] Several studies have demonstrated enhanced T-cell activation by antibody cross-linking of DAF.[27–29] Cross-linking of DAF also caused its clustering with nonreceptor tyrosine kinases in specialized membrane domains.[29] A recent study further established that DAF acted as a co-stimulatory molecule on human $CD4^+$ T cells via its interaction with CD97. Its activity in this regard is complement-independent and associated with increased production of the inhibitory cytokine interleukin (IL)-10.[30]

In the present study, we have examined the mechanism of action of DAF in MRL/lpr mice. Specifically, we have attempted to address the following questions: is exacerbated autoimmune disease in MRL/lpr-Daf-$1^{-/-}$ mice mediated by complement? Is the inhibitory effect of DAF on dermatitis development conferred at the local or systemic level? Does the Daf-1 gene mutation require both the MRL background and the *fas*^{lpr} mutation to produce the observed disease exacerbation phenotype? These questions were addressed by studying MRL/lpr-Daf-$1^{-/-}$/$C3^{-/-}$ and MRL/MpJ-Daf-$1^{-/-}$ mice produced by genetic breeding and chimeric mice produced by bone marrow (BM) transfer between MRL/lpr-Daf-$1^{+/+}$ and MRL/lpr-Daf-$1^{-/-}$ mice. Our studies revealed both complement-dependent and -independent activities of DAF in ameliorating systemic autoimmune disease in MRL/lpr mice.

Materials and Methods

Mice

The generation of MRL/lpr-Daf-$1^{-/-}$ mice[18] and MRL/lpr-$C3^{-/-}$ mice[31] have been described previously. To generate MRL/lpr mice deficient in both Daf-1 and C3, MRL/lpr-Daf-$1^{-/-}$ mice were crossed with MRL/lpr-$C3^{-/-}$ mice to produce F1 generation mice. F1 mice (MRL/lpr-Daf-$1^{+/-}$-$C3^{+/-}$) were then backcrossed with MRL/lpr-Daf-$1^{-/-}$ mice to generate MRL/lpr-Daf-$1^{-/-}$-$C3^{+/-}$ mice as breeders. The latter (MRL/lpr-Daf-$1^{-/-}$-$C3^{+/-}$) were intercrossed to produce MRL/lpr-Daf-$1^{-/-}C3^{+/+}$ and MRL/lpr-Daf-$1^{-/-}C3^{-/-}$ mice as littermates.

To determine whether Daf-1 deficiency in MRL/MpJ mice (without the *fas*^{lpr} mutation) also leads to exacerbated autoimmunity, we produced MRL/MpJ-Daf-$1^{-/-}$ mice as follows. First, MRL/lpr-Daf-$1^{-/-}$ mice were crossed with MRL/MpJ mice (Jackson Laboratory, Bar Harbor, ME) to generate MRL/MpJ-$^{+/lpr}$-Daf-$1^{+/-}$ mice. These mice were then backcrossed with MRL/MpJ mice to produce MRL/MpJ-Daf-$1^{+/-}$ mice as breeders. Next, MRL/MpJ-Daf-$1^{+/-}$ mice were intercrossed to generate MRL/MpJ-Daf-$1^{+/+}$ and MRL/MpJ-Daf-$1^{-/-}$ mice as littermates.

Throughout the breeding steps, mutated Daf-1, C3, and lpr genes were screened by polymerase chain reaction (PCR) of tail DNA and/or fluorescence-activated cell sorting (FACS) analysis of erythrocyte DAF expression. Genotyping of the lpr allele was performed by using the following primers: 5′-AGCACACTTGCTTCTGTTTA-3′, 5′-AGCTAGGGTCAGTGAGTA-3′, and 5′-CTGTCCGTTGCTCCGATGT-3′.[32] The wild-type (WT) allele produced a 240-bp fragment, and Fas-deficient allele, a 445-bp fragment. The C3 allele was typed with the following primers: 5-GATCCCCAGAGCTAATG-3′, 5′-TCGTCCTGCAGTTCATTCAG-3′, and 5′-AGGGACCAGCCCAGGTTCAG-3′.[33] The WT allele produced a 373-bp fragment, and the mutant C3 allele, a 503-bp fragment. To genotype the Daf-1 alleles, mouse erythrocytes, obtained by tail vein bleeding, were stained with a phycoerythrin (PE)-conjugated hamster anti-mouse DAF monoclonal antibody (mAb) (Pharmingen, San Diego, CA). Cells were analyzed by FACScan (Becton Dickinson, San Jose, CA).

Mice were monitored for autoimmune disease development until they were 5 months old (for MRL/lpr-Daf-$1^{-/-}C3^{+/+}$ and MRL/lpr-Daf-$1^{-/-}C3^{-/-}$ mice) or 6 months old (for MRL/MpJ and MRL/MpJ-Daf-$1^{-/-}$ mice), at which time they were sacrificed for pathological evaluation. All mice were kept in a specific pathogen-free barrier facility. Experiments with mice were conducted by following established guidelines for animal care, and all protocols were approved by the appropriate institutional committees.

Assessment of Dermatitis

Mice were inspected monthly for the development of dermatitis, and the age at which open skin lesions developed was recorded. The dimension of the skin lesion was measured using a ruler. At the time of sacrifice, skin samples from the dorsal neck area (lesional and perilesional when present) were fixed in 10% buffered formalin and processed for paraffin embedding and sectioning, followed by hematoxylin and eosin (H&E) staining and histological evaluation.

Assessment of Lymphoproliferation

At the time of sacrifice, spleen and lymph nodes were weighed, meshed, and cleared of erythrocytes by ACK lysis buffer (0.15 mol/L NH_4Cl, 1 mmol/L $KHCO_3$, and 0.1 mmol/L Na_2EDTA). After the spleen and lymph node cells were counted by a cell counter (Beckman Coulter, Fullerton, CA), the cell suspension (1.5×10^6 cells) was incubated with the following antibodies: Thy1.2-PE, CD4-PE, CD8-PE, B220-FITC, CD69-FITC, CD44-PE, CD19-FITC, CD21-FITC, CD80-PE, CD23-PE, and CD24-PE (Pharmingen). Flow cytometry analysis was performed by using FACScan (Becton Dickinson Immunocytometry Systems).

Enzyme-Linked Immunosorbent Assay (ELISA) for Autoantibodies and Their Isotypes

Serum was collected by tail vein bleeding at monthly intervals starting at 3 months until sacrifice. Samples were frozen at −20°C until use, when they were diluted to predetermined concentrations for ELISA. Quantification was achieved with either a standard curve (total IgM, IgG, IgG2a) or a positive serum sample from 5-month-old MRL/lpr mice as an internal standard (Ag-specific autoantibodies) for interplate comparison. Sandwich ELISA was used for determining the titers of antibodies (Abs). For total serum IgM, IgG, and IgG2a, plates were coated with the appropriate capture Ab. Diluted sera, as well as serially diluted IgM, IgG, and IgG2a standards, were added to the plates and allowed to bind overnight. The plate was incubated with biotin-conjugated anti-mouse IgM, IgG, and IgG2a Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours at 4°C. Avidin-alkaline phosphatase (Sigma, St. Louis, MO) was added, followed by alkaline phosphatase yellow liquid substrate (Sigma) for color development. OD_{405} was measured on a microplate reader (Molecular Devices, Sunnyvale, CA).

Assessment of Nephritis

Urine was collected in metabolic cages at monthly intervals starting at 3 months until sacrifice. Urinary albumin concentration was measured by a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX) and normalized to urinary creatinine measured by a colorimetric microplate assay kit (Oxford Biomedical Research, Oxford, MI). At the time of sacrifice, one kidney was fixed in 10% buffered formalin and processed for paraffin embedding and sectioning, followed by H&E and periodic acid-Schiff staining and histological evaluation. A blinded observer (K.Y.) evaluated and scored independently the severity of glomerular injury and inflammatory infiltrates (periglomerular, perivascular, and medulla, scored separately) by light microscopy. Glomerular injury was graded as follows: 0 = normal, 1 = glomerular changes (hypercellularity, crescents, or sclerotic glomeruli in 0 to 25% of total glomeruli), 2 = 25 to 50% glomeruli, 3 = 50 to 75% glomeruli, and 4 = >90% glomeruli. Infiltrative disease was graded as follows: 0 = no infiltrates visible, 1 = few infiltrates in some fields, 2 = moderate infiltrates easily found in most fields, 3 = moderate infiltrates in all fields, and 4 = severe infiltrates with loss of normal surrounding histology.

BM Transfer

BM cells were flushed out from the femurs and tibias of MRL/lpr/Daf-$1^{-/-}$ mice in cell culture medium (RPMI 1640, 15 mmol/L HEPES). After meshing and clearing of erythrocytes by treating with ACK buffer, the isolated BM cells were processed for depletion of mature T and B cells. To do this, cells were first incubated with anti-CD4, -CD8, -CD19, and -CD90 antibody-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) and then processed on a fully automated Clinimacs device (Miltenyi) equipped with TS separation columns using the program DepleteS by following the manufacturer's instructions. Recipient mice, MRL/lpr-Daf-$1^{+/+}$, and MRL/lpr-Daf-$1^{-/-}$ mice, were lethally irradiated with two 525-rad doses spaced 3 hours apart (totally 1050 rads). T/B-depleted BM cells were injected into recipient mice through the tail vein (1×10^7 cells per mouse).

Repopulation of the immune system was monitored by flow cytometric analysis of the blood cells using PE-conjugated hamster anti-mouse DAF. In the chimeric mice, ~90% of the T cells and more than 95% of the B cells and myeloid cells were derived from donor BM. Mice were monitored for 5 months after BM transfer. Similar procedures as described above were used to assess autoimmune disease phenotype in the chimera mice.

Statistical Analysis

Data were analyzed by Student's *t*-test or Mann-Whitney test as specified in the text.

Results

In previous studies, we generated MRL/lpr-C3$^{-/-}$ and MRL/lpr-Daf-$1^{-/-}$ mice by backcrossing.[18,31] There was no apparent disease modification by C3 deficiency except increased glomerular IgG deposition.[31] In contrast, Daf-1 deficiency significantly exacerbated autoimmune disease in MRL/lpr mice, particularly in females.[18] Compared with Daf-1-sufficient littermate controls, MRL/lpr-Daf-$1^{-/-}$ mice had increased lymphadenopathy and splenomegaly and elevated anti-chromatin autoantibody levels and, most strikingly, developed severe dermatitis with high incidence. To determine whether this phenotype was caused by the lack of Daf-1 as a complement regulator, we deleted the C3 gene from the MRL/lpr-Daf-$1^{-/-}$ mouse by crossing it with the MRL/lpr-C3$^{-/-}$ mouse.

By genotyping, we identified a total of 56 MRL/lpr-Daf-$1^{-/-}$C3$^{+/+}$ and 31 MRL/lpr-Daf-$1^{-/-}$C3$^{-/-}$ female mice. At monthly intervals, they were bled and their skin lesions evaluated. Figure 1, A–D, shows that C3 deficiency strikingly ameliorated dermatitis development in MRL/lpr-Daf-

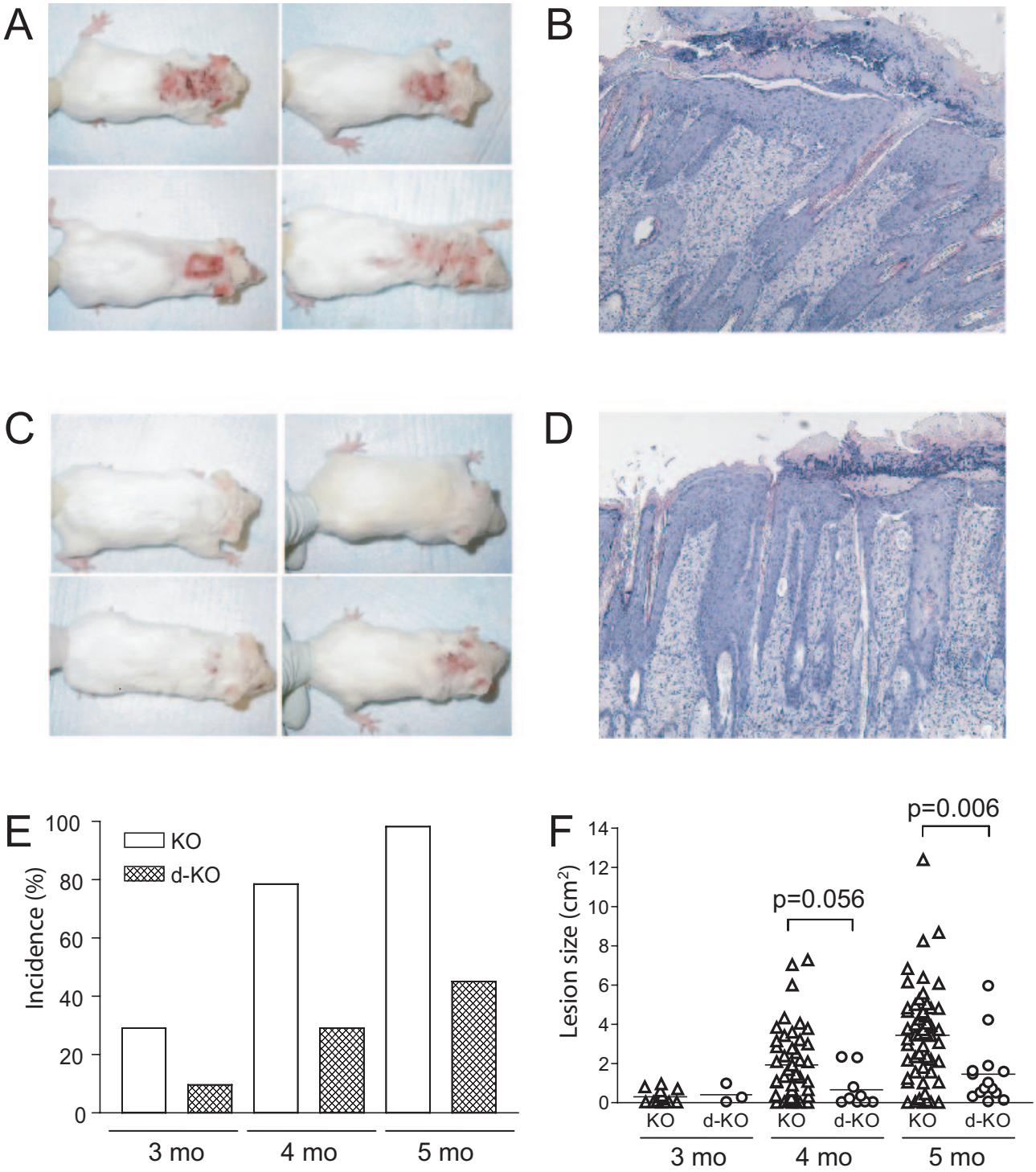

Figure 1. C3 deficiency ameliorates dermatitis in MRL/lpr-Daf-1$^{-/-}$ mice. **A:** Representative appearance of severe dermatitis in 5-month-old MRL/lpr-Daf-1$^{-/-}$C3$^{+/+}$ mice. **B:** Representative appearance of 5-month-old MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice. Most of them had no or very mild skin disease. Only a few mice developed severe skin lesions. **C** and **D:** There was no qualitative difference in lesion histology between MRL/lpr-Daf-1$^{-/-}$C3$^{+/+}$ mice (**B**) and the most severe cases of MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice (**D**). Both are characterized by skin thickening, mostly attributable to acanthosis of the epidermis, and the presence of scattered inflammatory cells as well as some dermal fibrosis. **E:** Percentage of mice with visible open skin lesions at 3, 4, and 5 months for MRL/lpr-Daf-1$^{-/-}$C3$^{+/+}$ and MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice. **F:** Scatter plot of open lesion size in dermatitis-positive MRL/lpr-Daf-1$^{-/-}$C3$^{+/+}$ and MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice. KO, MRL/lpr-Daf-1$^{-/-}$C3$^{+/+}$ mice; d-KO, MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice. *P* value refers to Student's *t*-test. Histology shown was by H&E staining. Original magnifications, ×40 (**B**, **D**).

1$^{-/-}$ mice. The percentage of mice with visible open skin lesions in the MRL/lpr-Daf-1$^{-/-}$ cohort was 25, 79, and 98% at 3, 4, and 5 months, respectively (Figure 1E). The corresponding percentages, at 10, 29, and 49%, were significantly lower in the MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ group (Figure 1E). Furthermore, in those MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice that did develop dermatitis, the lesions were smaller and milder (Figure 1, A, C, and F).

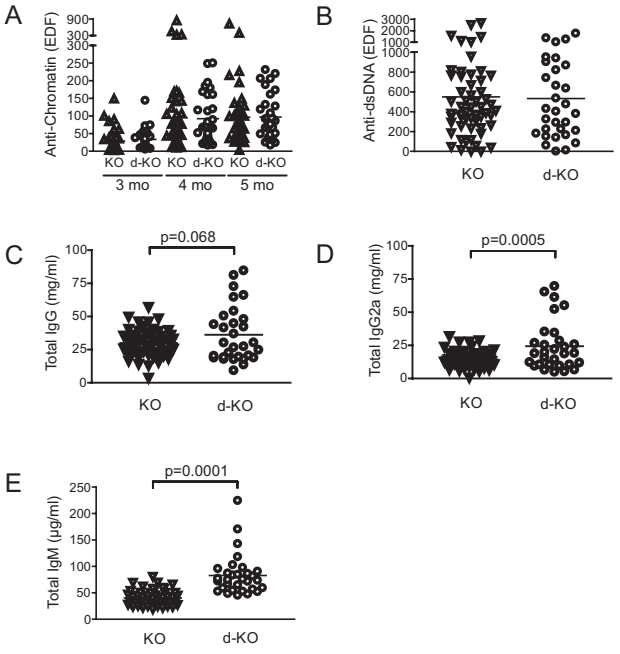

Figure 2. Effect of C3 deficiency on serum antibody levels. C3 deficiency had no impact on serum anti-chromatin (**A**) and anti-dsDNA (**B**) autoantibody titers. On the other hand, C3 deficiency increased serum total IgG (**C**), IgG2a (**D**), and IgM (**E**) levels, although only the elevations in IgG2a and IgM were statistically significant. KO, MRL/lpr-Daf-1$^{-/-}$C3$^{+/+}$; d-KO, MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$. Data shown in **B–E** are for 5 months. *P* values refer to Student's *t*-test.

For example, the average open lesion size in MRL/lpr-Daf-1$^{-/-}$ and MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice at 5 months of age were 3.44 ± 0.33 cm^2 (n = 55) and 1.46 ± 0.45 cm^2 (n = 14), respectively. On the other hand, we detected no substantive difference in the histology of fully developed lesions between the two strains of mice (Figure 1, B and D). In each strain, the lesions were characterized by acanthosis, infiltration of inflammatory cells, fibrosis, and loss of epidermal integrity (Figure 1, B and D).

In contrast to the clear amelioration of dermatitis, C3 deficiency did not significantly affect the degree of lymphadenopathy and splenomegaly caused by DAF deficiency. The spleen and lymph node weights (g) for a cohort of MRL/lpr-Daf-1$^{+/+}$ mice (n = 11) maintained in our colony were 0.182 ± 0.009 and 1.171 ± 0.069, respectively. In comparison, spleen and lymph node weights (g) for the MRL/lpr-Daf-1$^{-/-}$ and MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice were 0.264 ± 0.001 versus 0.234 ± 0.014 and 1.965 ± 0.079 versus 2.178 ± 0.147, respectively ($P < 0.05$ between DAF-sufficient and DAF-deficient groups; no significant difference between C3-sufficient and C3-deficient groups, Student's *t*-test). Likewise, C3 deficiency did not significantly influence the levels of serum anti-chromatin and anti-ds-DNA autoantibody titers in MRL/lpr mice (Figure 2, A and B). There was also no significant difference in the number and composition of cell subpopulations (CD4$^+$, CD8$^+$, B200$^+$, and so forth) and activation markers (CD69, CD44, CD80, CD23, CD24) (data not shown). Unexpectedly, C3 deficiency increased serum total IgG, IgG2a, and IgM levels, although the elevation in total serum IgG did not reach statistically significance (Figure 2, C–E). The development of nephritis in the two groups of mice showed no significant differences when assessed by morphological scoring of glomerular inflammation and injury (5.6 ± 0.4 versus 6.5 ± 0.7 for MRL/lpr-Daf-1$^{-/-}$ and MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice). On the other hand, urinary albumin excretion (μg/mg creatinine) in MRL/lpr-Daf-1$^{-/-}$C3$^{-/-}$ mice was significantly higher than in MRL/lpr-Daf-1$^{-/-}$ mice (1097 ± 999.5 versus 4438 ± 2889, P = 0.0225, Mann-Whitney test). These results suggested a dissociation in mechanisms between exacerbated local inflammation in the skin and systemic autoimmunity in MRL/lpr-Daf-1$^{-/-}$ mice.

DAF is highly expressed on lymphocytes and other BM-derived blood cells, as well as on virtually all peripheral tissues including the skin.[1,34,35] Although the C3 ablation experiment implied that DAF acted as a complement regulator in suppressing dermatitis development in MRL/lpr mice, it did not reveal where DAF exerted its role in such a capacity. To address this question, we generated BM chimera mice between MRL/lpr-Daf-1$^{-/-}$ and MRL/lpr mice. Mature T- and B-lymphocyte-depleted BM cells from MRL/lpr-Daf-1$^{-/-}$ mice were harvested and adoptively transferred via tail vein injection into either MRL/lpr-Daf-1$^{-/-}$ (group I, control group) or MRL/lpr mice (group II, experimental group). In the latter group of mice, all tissues except lymphocytes and other BM-derived cells expressed DAF. Figure 3A shows that 2 months after BM transfer, the donor mouse BM cells successfully reconstituted the hematopoietic system of the recipient mice, and greater than 95% of the circulating erythrocytes in the recipient mice had the donor mouse genotype. We found that both groups of chimera mice were susceptible to dermatitis development, with the incidence of visible skin lesions in group II mice (MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr chimera) being slightly reduced compared with group I mice (MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{-/-}$) (Figure 3D). However, the lesion size was strikingly smaller in group II mice than in group I mice (Figure 3, B, C, and E). We observed a trend of increased spleen weight, elevated serum anti-chromatin autoantibody titer, and urinary albumin excretion in group I mice (Figure 4, A, C, and D). However, none of the differences reached statistical significance.

Because no obvious autoimmune phenotype was seen with Daf-1$^{-/-}$ mice on other genetic backgrounds at a comparable age,[36] the MRL background and/or the *fas^{lpr}* mutation must have acted as critical modifiers to reveal the proautoimmune consequences of Daf-1 gene deficiency. To determine whether daf-1 gene deficiency in combination with the MRL background alone, without the *fas^{lpr}* mutation, was sufficient to recapitulate the exacerbated autoimmune phenotype of MRL/lpr-Daf-1$^{-/-}$ mice, we produced and studied 21 female MRL/MpJ-Daf-1$^{+/+}$ and 23 female MRL/MpJ-Daf-1$^{-/-}$ mice as littermates. Figure 5, A and B, shows that on the MRL/MpJ background, Daf-1 deficiency also increased the incidence and severity of dermatitis. However, only ~30% of MRL/MpJ-Daf-1$^{-/-}$ mice developed open skin lesions as op-

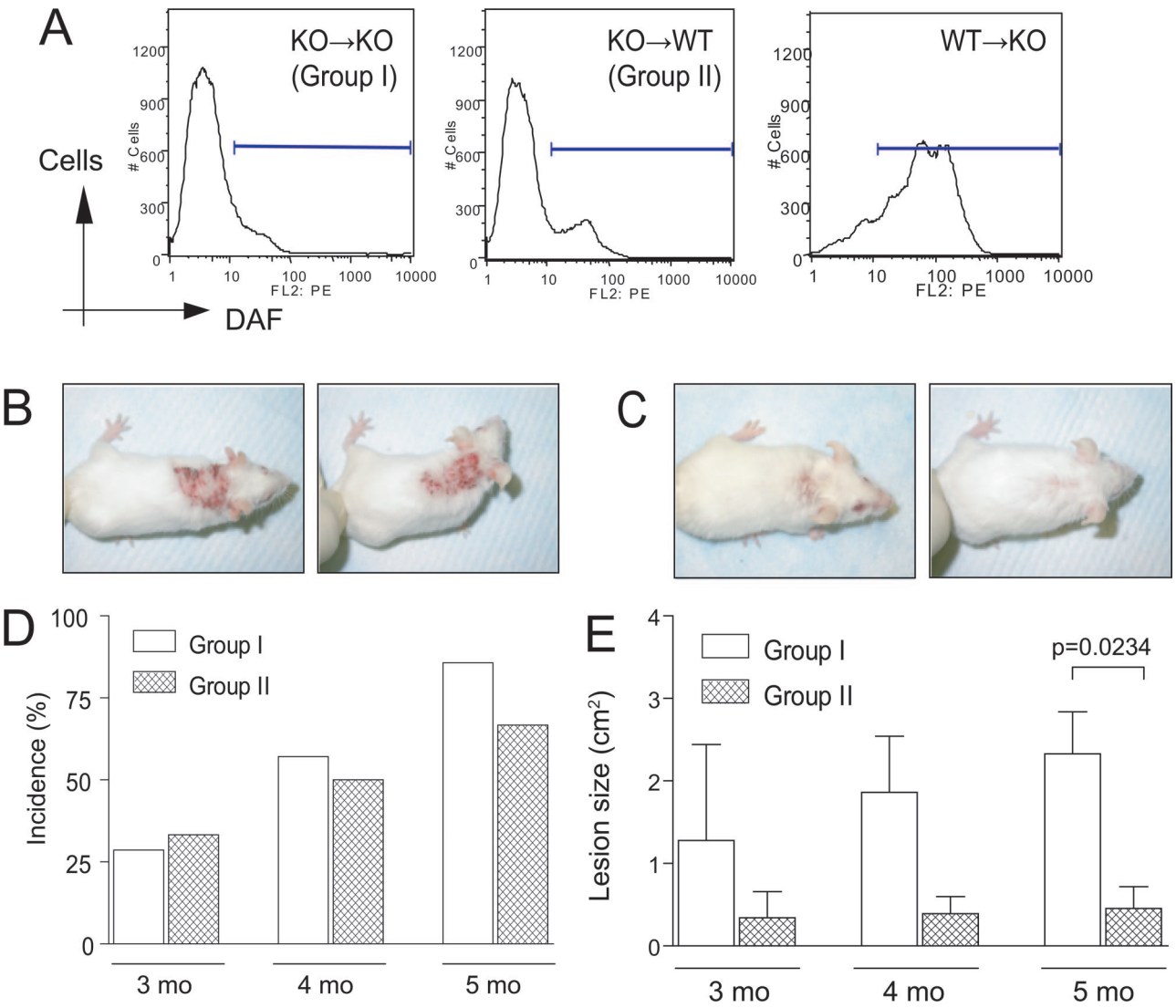

Figure 3. DAF ameliorates dermatitis mainly by acting locally in peripheral tissues. **A:** FACS analysis of erythrocyte DAF expression in chimeric mice 2 months after BM transfer. KO, MRL/lpr-Daf-1$^{-/-}$; WT, MRL/lpr-Daf-1$^{+/+}$. The result demonstrates that donor mouse BMs have successfully reconstituted the hematopoietic system of the recipient mice. The WT → KO chimera was used as a control for the efficiency of BM reconstitution. **B** and **C:** Representative pictures at 5 months after BM transfer showing that MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{+/+}$ chimeras (**C**) developed significantly attenuated dermatitis compared with MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{-/-}$ chimeras (**B**). **D** and **E:** Although the incidence of dermatitis (**D**) was only slightly lower in MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{+/+}$ chimeras (group II, $n = 6$) than in MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{-/-}$ chimeras (group I, $n = 7$), the dermatitis was significantly less severe in group II mice as measured by the size of open skin lesions (**E**). *P* value refers to Student's *t*-test.

posed to nearly 100% in MRL/lpr-Daf-1$^{-/-}$ mice (Figure 1). Both the lymph node and spleen weights were also higher in MRL/MpJ-Daf-1$^{-/-}$ mice, although the difference in spleen weight was not statistically significant (Figure 5, C and D). Thus, the *fas*^{lpr} mutation was not necessary for exposing the proautoimmune consequence of Daf-1 gene deficiency, but it clearly amplified the autoimmune phenotypes of MRL/MpJ-Daf-1-deficient mice.

Discussion

We previously demonstrated a significant anti-autoimmune activity of DAF in MRL/lpr mice.[18] Although an apparent explanation for the observed effect of DAF in MRL/lpr mice was its complement regulatory activity, DAF has additional physiological functions that may be relevant.[26–30] In the present study, we have combined the C3 deletion with Daf-1 deletion in MRL/lpr mice to evaluate the mechanisms of action of DAF in MRL/lpr mice. By intercrossing MRL/lpr-Daf-1$^{-/-}$/C3$^{+/-}$ mice, we obtained 56 female MRL/lpr-Daf-1$^{-/-}$/C3$^{+/+}$ and 31 MRL/lpr-Daf-1$^{-/-}$/C3$^{-/-}$ mice. The apparent deviation from Mendelian inheritance was notable but not understood. We observed no significant mortality in the MRL/lpr-Daf-1$^{-/-}$/C3$^{-/-}$ group by the experimental end point (5 months). However, the possibility cannot be excluded that MRL/lpr-Daf-1$^{-/-}$/C3$^{-/-}$ mice had a higher mortality postnatally (ie, before genotyping at ~4 weeks old), even though these mice were kept in a specific pathogen-free facility and a similar phenotype has not been observed in C57/BL6 C3$^{-/-}$ mice.[33] It should also be noted that mild

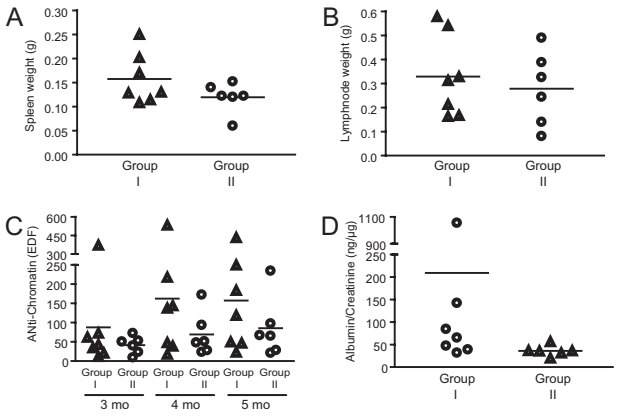

Figure 4. Effect of peripheral DAF expression on lymphoproliferation, anti-chromatin autoantibody production, and proteinuria in MRL/lpr mice. Spleen (**A**) and lymph node (**B**) weights, anti-chromatin autoantibody titer (**C**), and proteinuria (**D**) were not significantly different between MRL/lpr-Daf-$1^{-/-}$ → MRL/lpr-Daf-$1^{-/-}$ chimeras (group I, $n = 7$) and MRL/lpr-Daf-$1^{-/-}$ →MRL/lpr-Daf-$1^{+/+}$ chimeras (group II, $n = 6$). There seemed to be a trend of increased spleen weight, higher anti-chromatin titer, and increased proteinuria in group I mice, but the difference did not reach statistical significance (Student's *t*-test). Data in **A**, **B**, and **D** are 5 months after BM transfer.

renal disease and lack of significant dermatitis and mortality at 5 months of age were characteristics of the MRL/lpr mice in our colony. These features contrasted with those observed in other MRL/lpr mouse colonies[37,38] and presumably reflected genetic drifting in our MRL/lpr mouse line after prolonged in-house breeding.

Our results suggested that the aggravated dermatitis in MRL/lpr-Daf-$1^{-/-}$ mice was mainly caused by the lack of DAF as a complement inhibitor because it was ameliorated in DAF/C3 double-deficient mice. Nevertheless,

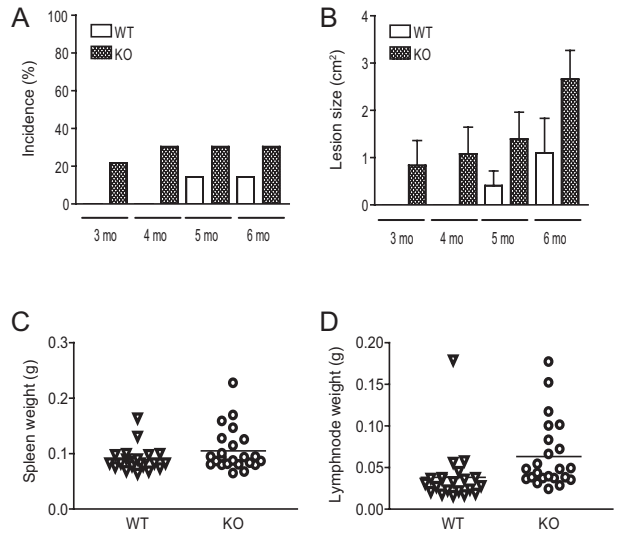

Figure 5. Effect of Daf-1 gene deficiency on dermatitis development and lymphoproliferation in MRL/MpJ mice. **A:** Dermatitis incidence was higher in MRL/MpJ-Daf-$1^{-/-}$ mice (KO, $n = 23$, 5 of 23, 7 of 23, 7 of 23, and 7 of 23 for 3, 4, 5, and 6 months, respectively) than in MRL/MpJ mice (WT, $n = 21$, 0 of 21, 0 of 21, 3 of 21, and 3 of 21 for 3, 4, 5, and 6 months, respectively). **B:** In those mice that developed skin disease, the average size of open skin lesions in MRL/MpJ-Daf-$1^{-/-}$ mice was bigger than in MRL/MpJ mice at 5 and 6 months, although the differences did not reach statistical significance. **C** and **D:** MRL/MpJ-Daf-$1^{-/-}$ mice (KO, $n = 23$) also had increased spleen (**C**) and lymph node (**D**) weights than MRL/MpJ mice (WT, $n = 21$) ($P = 0.1656$ for spleen, $P = 0.0012$ for lymph node; Mann-Whitney test).

the development of skin injury in some MRL/lpr-Daf-$1^{-/-}$-C$3^{-/-}$ mice was still a notable observation, as it contrasted with MRL/lpr mice, which rarely develop comparable dermatitis at that age. This observation implied the existence of a secondary, complement-independent effect of DAF in ameliorating skin inflammation. Such an effect may be exerted at the systemic level on T/B cells, in which the influence of DAF on lymphoproliferation was shown to be independent of complement (see discussion below).

We previously showed that DAF deficiency in MRL/lpr mice exacerbated lymphadenopathy and splenomegaly and elevated serum anti-chromatin titers.[18] To determine the contribution of complement to these phenotypes, we examined the spleen and lymph nodes and measured anti-chromatin titers in MRL/lpr-Daf-$1^{-/-}$ and MRL/lpr-Daf-$1^{-/-}$-C$3^{-/-}$ mice. Because complement plays a role in B-cell function[39] and is important for immune complex processing,[40] we also measured several other antibodies to determine the potential effect of C3 deletion in the context of DAF deficiency. We found that C3 deficiency had no impact on the degree of lymphadenopathy and splenomegaly or on anti-chromatin and anti-ds-DNA autoantibody titers of MRL/lpr-Daf-$1^{-/-}$ mice. This implied that DAF worked to suppress T/B cell autoimmunity in MRL/lpr mice via a pathway that is independent of complement. This result contrasted with findings of recent studies of C57BL/6-Daf-$1^{-/-}$ mice, wherein increased T-cell recall responses were observed in Daf-$1^{-/-}$ mice and shown to be primarily complement-dependent in models of active immunization, using ovalbumin or myelin oligodendrocyte glycoprotein peptide as model antigens.[11] Thus, DAF could inhibit adaptive immunity via both complement-dependent and -independent mechanisms depending on the context. The complement-independent mechanism by which DAF inhibits lymphoproliferation and autoantibody production in MRL/lpr mice remains unknown. Antibody cross-linking of DAF during anti-CD3-inducd human $CD4^+$ T-cell activation increased IL-10 production.[30] In the active immunization model, C57BL/6-Daf-$1^{-/-}$ mouse T cells secreted significantly less IL-10 in response to antigen restimulation, and this defect in IL-10 production was not rescued by C3 deficiency.[11] Whether diminished production of IL-10, an inhibitory cytokine, was involved in the complement-independent exacerbation of lymphoproliferation and autoantibody production in MRL/lpr-Daf-$1^{-/-}$ mice remains to be determined. Another possibility that cannot be discounted at this time is the influence of 129J mouse strain-derived genes. The Daf-1 gene is localized to the distal region of chromosome 1, a region where several lupus susceptibility genes are known to reside.[41–43]

C3 deficiency also did not influence kidney disease development in MRL/lpr-Daf-$1^{-/-}$ mice as assessed by pathology scores. On the other hand, MRL/lpr-Daf-$1^{-/-}$-C$3^{-/-}$ mice developed more severe proteinuria than MRL/lpr-Daf-$1^{-/-}$ mice. These results were not unexpected because neither Daf-1 gene deficiency nor C3 deficiency was previously shown to affect kidney pathol-

ogy in MRL/lpr mice, and C3 deficiency likewise worsened proteinuria in these animals, possibly reflecting a role of C3 in immune complex solubilization in the kidney.[31] Surprisingly, we found that C3 deficiency significantly increased serum total IgM and IgG2a levels in MRL/lpr-Daf-1$^{-/-}$ mice. It is possible that this was a reflection of the failure by the C3-deficient MRL/lpr-Daf-1$^{-/-}$ mice to clear persistent infections, although the animals were housed in a specific pathogen-free facility. On the other hand, elevated IgG2a may have reflected impaired immune complex handling in the MRL/lpr-Daf-1$^{-/-}$-C3$^{-/-}$ mice. Whatever the underlying mechanism may be, it seems to be related to the Daf-1$^{-/-}$ background because elevated serum IgM or IgG2a was not observed in either MRL/lpr-C3$^{-/-}$[31] or C57BL/6-C3$^{-/-}$ mice.[44]

BM transplantation experiments showed that MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{+/+}$ chimeras developed significantly attenuated dermatitis compared with MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{-/-}$ chimeras. Our interpretation of this result is that DAF mainly acted as a complement inhibitor in peripheral tissues (most likely in the skin) to suppress skin inflammation. This conclusion was consistent with our previous demonstration of C3 deposition, often used as a surrogate to track tissue complement activation, along the dermal-epidermal junction in prelesional MRL/lpr-Daf-1$^{-/-}$ mouse skins.[18] Nevertheless, the fact that MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{+/+}$ chimeras, unlike MRL/lpr mice, did develop dermatitis, implied that exacerbated systemic autoimmunity at the T/B lymphocyte level also contributed to dermatitis development in MRL/lpr-Daf-1$^{-/-}$ mice. It is notable that although not statistically significant, there seemed to be a trend for increased spleen weight and serum anti-chromatin autoantibody titer and elevated proteinuria in MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{-/-}$ chimeras compared with MRL/lpr-Daf-1$^{-/-}$ → MRL/lpr-Daf-1$^{+/+}$ chimeras, suggesting that peripheral DAF expression may also impact these parameters.

Breeding experiment with MRL/MpJ mice showed that *fas*^{lpr} mutation was not necessary to expose the autoimmune consequence of Daf-1 gene deficiency (Figure 5). This finding suggests that DAF may be a significant disease-modifying gene in other animal and human autoimmune disease settings where the *fas*^{lpr} mutation does not occur. Nevertheless, the *fas*^{lpr} mutation clearly had an amplifying effect on the phenotype of Daf-1$^{-/-}$ mice, because only ~30% of female MRL/MpJ-Daf-1$^{-/-}$ mice developed dermatitis as compared with close to 100% observed in MRL/lpr-Daf-1$^{-/-}$ mice (Figure 1).

In summary, we have revealed multiple mechanisms of action for DAF in ameliorating systemic autoimmunity. We conclude that DAF works as a complement regulator locally to protect MRL/lpr mice from skin inflammation, whereas its inhibitory role in the induction phase of MRL/lpr autoimmunity is complement-independent. The proautoimmune consequence of DAF dysfunction does not require a *fas*^{lpr} mutation and may manifest in broad settings of systemic autoimmunity in animals and human patients. This further characterizes the complex role of complement and its regulators in the development of both systemic and organ-specific autoimmunity.

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