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Interleukin-2 Protects Lupus-prone Mice from Multiple End-organ Damage by Limiting CD4^{-}CD8^{-} Interleukin-17-producing T-cells

Masayuki Mizui[*], **Tomohiro Koga**[*], **Linda A. Lieberman**[*], **Jessica Beltran**[*], **Nobuya Yoshida**[*], **Mark C. Johnson**[†], **Roland Tisch**[†,‡], and **George C. Tsokos**[*]

[*]Division of Rheumatology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA02215

[†]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC27599

[‡]UNC Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC27599

Abstract

Interleukin-2 (IL-2), a cytokine with pleiotropic effects, is critical for immune cell activation and peripheral tolerance. Although the therapeutic potential of IL-2 has been previously suggested in autoimmune diseases, the mechanisms whereby IL-2 mitigates autoimmunity and prevents organ damage remains unclear. Using an inducible recombinant adeno-associated virus (rAAV) vector we investigated the effect of low systemic levels of IL-2 in lupus-prone MRL/Fas$^{lpr/lpr}$ (MRL/lpr) mice. Treatment of mice after the onset of disease with IL-2-rAAV resulted in reduced mononuclear cell infiltration and pathology of various tissues including skin, lungs and kidneys. In parallel, we noted a significant decrease of IL-17-producing CD3^{+}CD4^{-}CD8^{-} double-negative T cells and an increase in CD4^{+}CD25^{+}Foxp3^{+} immunoregulatory T cells (Treg) in the periphery. We also show that IL-2 can drive DN T cell death through an indirect mechanism. Notably, targeted delivery of IL-2 to CD122^{+} cytotoxic lymphocytes effectively reduced the number of DN T cells and lymphadenopathy whereas selective expansion of Treg by IL-2 had no effect on DN T cells. Collectively, our data suggest that administration of IL-2 to lupus-prone mice protects against end-organ damage and suppresses inflammation by dually limiting IL-17-producing DN T cells and expanding Treg.

Introduction

Systemic lupus erythematosus is a complex autoimmune disease characterized by autoantibody production and tissue inflammation (1). Kidney damage through glomerular

Corresponding Authors: George C. Tsokos, Division of Rheumatology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Ave, CLS-937, Boston, MA 02215. Phone: 617-735-4160, Fax: 617-735-4170 gtsokos@bidmc.harvard.edu.

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inflammation in response to immune complexes and mononuclear cell infiltration of the interstitial and perivascular areas is associated with significant morbidity (2–4). Similarly, MRL/MpJ-Fas$^{lpr/lpr}$ (MRL/lpr) female mice, a model for SLE, develop systemic autoimmunity 10 to 12 weeks after birth characterized by autoantibody production, and T cell driven lymphadenopathy. Severe lymphadenopathy is largely attributed to an expanded pool of $CD3^+CD4^-CD8^-$ double negative T cells (5–7). In addition to kidney disease MRL/lpr mice display skin and lung injury characterized by infiltrating proinflammatory cells (8, 9).

IL-2 is produced by activated T cells and dendritic cells, and exhibits potent pleotropic effects. For instance, IL-2 is canonical growth factor for conventional $CD4^+$ and $CD8^+$ T cells, but also promotes activation and/or expansion of various immune effectors such as natural killer cells (10). Notably, IL-2 is critical for the survival, expansion, and function of Foxp3-expressing immunoregulatory T cells (Treg) (11). In addition, IL-2 plays an important role in activation-induced cell death (AICD), a process that regulates T cell expansion (12). Furthermore, IL-2-mediated signals block the differentiation of IL-17-producing $CD4^+$T helper cells (T_H17) (10, 13) and inhibit the generation of follicular helper T cells (Tfh)(14). Moreover, IL-2-deficient mice develop severe autoimmunity marked by reduced Treg numbers and systemic expansion of pathogenic T effectors (15) (10), indicating that IL-2 is crucial for the maintenance of T cell-mediated self-tolerance.

T cells from SLE patients and various lupus-prone mice, such as NZB x NZW F1 and MRL/lpr mice exhibit impaired IL-2 production (1, 16–19), which in turn correlates with reduced Treg and an increase in IL-17-producing cells (20, 21). Furthermore, we have shown that $CD3^+CD4^-CD8^-$ DN T cells are a major source of IL-17 in both human and murine lupus (22, 23). Importantly, DN T cells are found infiltrating the kidneys of SLE patients (22) and in aged MRL/lpr mice, and account for the severe lymphadenopathy and splenomegaly in murine models (24).

IL-2 immunotherapy has been applied in several murine tumor and infection models, resulting in reduced tumor size and elimination of the infectious pathogens, respectively. Mechanistically, this was shown to be the result of cytotoxic T cell and natural killer cell expansion and activation (25). Although high dose recombinant IL-2 has been used clinically for the treatment of renal carcinoma and melanoma, efficacy is limited due to severe toxicity, including the development of vascular, capillary leak syndrome (VLS) and/or a secondary inflammatory cytokine storm (26). On the other hand, low dose IL-2 therapy has recently been shown to be effective in the clinic for the treatment of systemic pathologies such as chronic graft-versus-host disease (GVHD) (27, 28) and chronic hepatitis C-mediated vasculitis (29) (30) by promoting Treg expansion. IL-2 administration also prolongs survival in MRL/lpr and NZB x NZW F1 mice (18, 31, 32). In the present study we investigated the effect of IL-2 on disease development in MRL/*lpr* mice using tetracycline-inducible recombinant adeno-associated virus (rAAV) vector encoding IL-2 (33). Induction of expression results in low, continuous serum IL-2 levels, which significantly reduce inflammatory cell infiltration of the kidney, lung and skin in MRL/*lpr* mice. Furthermore, suppression of pathology corresponds with reduced IL-17-producing DN cells and increased Treg. We show that IL-2 selectively reduced DN T cell population by

inducing cell death, but the effect of IL-2 could be elicited only when splenocytes besides DN T cells were present. Importantly, delivery of an IL-2/anti-IL-2 antibody complex and selective targeting of IL-2 to cytotoxic lymphocytes resulted in reduction of DN T cells and lymphadenopathy whereas targeting to Treg cells had no effect on DN T cells. We propose that proper administration of IL-2 limits organ inflammation by altering the distribution of T cell subsets and should be considered for clinical trials in patients with SLE.

Materials and Methods

Mice

Female MRL/MpJ (stock no 000486), MRL/MpJ-Tnfrsf6lpr (MRL/lpr, stock no 000485) mice were purchased from the Jackson Laboratories. MRL/lpr-Foxp3^{gfp} mice were generated by backcrossing the Foxp3^{gfp} reporter transgene cassette (C57BL/6) onto the MRL/lpr genetic background for more than 10 generations. Mice were maintained in the specific pyrogen free animal facility of Beth Israel Deaconess Medical Center. All animal procedures in the study were approved by the Institutional Animal Care and Use Committee (IACUC) of Beth Israel Deaconess Medical Center.

Recombinant-AAV vaccination

Full-length cDNA encoding murine *IL-2* subcloned into an AAV-Tet-on vector plasmid as previously described(33). Briefly, The AAV-Tet-on vector contains a bidirectional promoter with a tetracycline response element flanked by mini-CMV promoters that regulate expression of IL-2 and reverse transcriptional transactivator gene. MRL/lpr and/or MRL/lpr-Foxp3^{gfp} mice were vaccinated with 5×10^{10} viral particles (v.p.) of rAAV-Tet-on-IL-2 in contralateral hind limb muscles using an insulin syringe. Twenty-four hours after rAAV injection, mice were fed ad libitum chow containing 200 mg/kg doxycycline (BioServ) for induction of IL-2 or control diet. Blood samples were collected from tails and urine samples were collected using individual metabolic cages periodically.

Flow Cytometry

Splenocytes were isolated as previously described(23). PBS-perfused kidneys and lungs were minced and digested with collagenase IV (100 μg/ml) for 30 minutes at 37 °C to prepare single cells.

Isolated cells were stained with the following antibodies specific for: TCRβ (H57-597, BioLegend), CD3ε (145-2C11, eBiosciences), CD4(GK1.5, BioLegend), CD8α (53-6.7, eBiosciences), B220 (RA3-6B2, BioLegend), CD25(PC61, BioLegend), I-A/I-E (M5/114.15.2, BioLegend), Nkp46 (29A1.4, BioLegend) for 30min at 4 °C. For intracellular staining, cells were stimulated with 20 ng/ml of phorbol-myristate acetate (PMA, Sigma) and 1 mg/ml of ionomycin(Sigma) for 4 hours, washed and stained with TCRβ, CD4, CD8, I-A/I-E. BD cytofix/cytoperm plus with Golgi stop staining kits (BD Biosciences) were used according to the manufacturer's protocol. Antibodies of IFN-γ (XMG1.2, BioLegend) and IL-17A (TC11-18H10.1, BioLegend) were used for detecting intracellular cytokines.

Histological Analyses and Immunofluorescence

Kidneys, lungs and skin biopsies were fixed in 10% formaldehyde. Tissue sections were stained with H&E or Periodic acid-Schiff (PAS) and visualized by Nikon. Histological scores of skins and kidneys were evaluated as described(34, 35). Kidneys and skins were scored from 6 mice in each group. At least 20 glomeruli and 10 tubular and vessel area in low power field are scored and calculated an average in each tissue section. Briefly, severity of glomeluronephritis, interstitial nephritis and perivascular infiltrates were graded in a semiquantitative (0–3+) manner. For skin, the degree of acanthosis, none (0) to marked thickened dermis (27); hyperkeratosis, none (0) to markedly increased keratin (2); inflammation, sparse (0) to heavy lymphocytic infiltrates (27); fibrosis, dermal collagen with normal (0) to markedly thickened (2); vessels, normal (0) to diffuse dilated (2); ulcer, absence (0) ore presence (1). For immunofluorescence, frozen kidney sections (5 μm thickness) were fixed in acetone and blocked with 1% normal goat serum in PBS. Diluted FITC-anti-mouse IgG (Jackson Immunoresearch) and FITC-anti-mouse C3(36) antibodies were incubated overnight at 4 °C, and staining visualized with a Nikon C1 confocal microscope.

Quantitative real-time PCR analysis

Total mRNA was purified from muscle, lung and kidney using TRIZol (Invitrogen). Messenger RNA from splenocyte was purified using RNeasy Mini Kit (QIAGEN). Complementary DNA (cDNA) was synthesized using cDNA EcoDry Premix (Clontech) for PCR amplification. Taqman primers were purchased from Applied Biosystems for Gapdh (Mm99999915_g1), Il17a (Mm00439619_m1), IFN-γ (Mm01168134_m1), TNFα (Mm00443260_g1), MCP1 (mm00441242_m1), CCL20 (Mm001268754_m1), CXCL11(Mm00444662_m1) and IL8(Mm04208136_m1). Gene expression was assessed by comparative CT method.

T Cell Labeling and Transfer experiment

$CD3^+$ T cells were isolated from spleen and lymph nodes of MRL/lpr mice at 12 weeks of age using MACS pan T cell isolation kit (Miltenyi Biotec), then labeled with CellTrace Violet (Life Technologies) or carboxyfluorescein succinimidyl ester (CFSE) according to the manufacture's protocol. In brief, cells were washed and resuspended to 10^7 cells/ml in pre-warmed PBS containing 5 μM CellTrace Violet or 5μM CFSE and incubated for 20 minutes at 37 °C, then washed three times with RPMI containing 10% FCS and resuspended with PBS. Aliquots of 1.5×10^8 stained cells were injected i.v. into MRL/MpJ mice at 12 weeks of age or MRL/lpr mice at the age of 14 weeks. For IL-2 treatment, mice were injected with 4 μg/body of mouse rIL-2 (BioLegend) twice a day for 5 days. Fluorescence-positive cells in spleens were counted and analyzed by flow cytometry. For in vitro IL-2 treatment, Isolated DN T cells were labeled with 1 μM CFSE and 5×10^6 cells were incubated with or without 5×10^6 of spleen cells for 5 days in the presence of indicated concentrations of IL-2.

Treatment with IL-2/anti-IL-2 monoclonal antibody complex

1.5 μg of mouse rIL-2 and 15 μg of anti-IL-2 monoclonal antibody clone S4B6-1 and the same amount of IL-2 and JES6-1A12 (BioXcell) were mixed and incubated at room temperature for 30 minutes, respectively. MRL/lpr mice at the age of 14 weeks were received i.p. injection of 15 μg of isotype control rat IgG, IL-2/S4B6-1 and IL-2/JES6-1 complexes every other day for 2 weeks.

ELISA

IL-2, IFN-γ and IL-17 were measured in mice sera using Ready-set-go ELISA system (eBiosciences). Anti-dsDNA antibody was detected using mouse anti-ds DNA IgG ELISA kit (Alpha Diagnostic International).

Statistical analyses

Unpaired two-tailed Student t test and Mann Whitney tests were used for analyzing the differences between two groups. For time-series experiments, paired t-test or two-way ANOVA with Bonferoni's post-test were used. Differences between three data sets were analyzed by one-way ANOVA. A p value of < 0.05 was considered significant. Statistical analyses were performed in GraphPad Prism 5.01 software.

Results

IL-17-producing DN T cells increase with advancing age in MRL/lpr mice

We previously reported that $CD3^+CD4^-CD8^-$ DN T cells from patients with SLE and lupus-prone mice produce IL-17. Similarly, a significant increase in the percentage of DN versus $CD4^+$ T cells that have the potential to produce IL-17 (IL-17-DN T cells) was detected in 19 week-old MRL/*lpr* mice, whereas the frequency of IFN-γ^+ DN T cells was reduced relative to IFN-γ^+ $CD4^+$ T cells (CD4 vs DN of IFN-γ-positive cell percentage; 14.8 ± 1.33 vs 1.51 ± 0.0338 %, p<0.0001, IL-17; 1.37 ± 0.134 vs 2.85 ± 0.320 %, p= 0.0028, Figure 1A). Furthermore, the total number of splenic IL-17-DN T cells rather than IL-17-$CD4^+$ T cells increased markedly in an age-dependent manner ($5.34 \pm 0.52 \times 10^5$ for 15 weeks and $20.1 \pm 3.67 \times 10^5$ for 19 weeks, p= 0.0434, Figure 1B). Interestingly, the number of IFN-γ-DN T cells also increased in aged mice ($4.86 \pm 0.05 \times 10^5$ for 15 weeks and $11.6 \pm 1.72 \times 10^5$ for 19 weeks, p= 0.0455); in contrast the number of IFN-γ-$CD4^+$ T cell remained constant during disease development. These results indicate that DN T cells represent the preferential source of IL-17 in aged MRL/*lpr* mice and could contribute to disease development.

Treatment of MRL/lpr mice with IL-2-rAAV increases Treg and decreases DN T cells but does not affect autoantibody production

IL-2 has a short half-life in serum, which typically necessitates frequent and/or continuous injections to mediate a therapeutic effect. On the other hand, long-term administration and doses of IL-2 too high induce adverse effects including systemic activation of T cells and other immune effectors, and vascular or capillary leak syndrome (VLS) leading to severe pulmonary edema and volume depletion. To overcome these obstacles a rAAV vector encoding inducible IL-2 was employed(33). MRL/lpr mice were injected intramuscularly

(i.m.) with 5×10^{10} viral particle (v.p.) of rAAV1-Tet-IL-2 and fed doxycycline (Dox)-containing chow for 4 weeks. The expression of IL-2 mRNA in the muscle and IL-2 protein in the serum was highest on day 7 after infection and gradually decreased after 14 and 28 days (Figure 2A,B). Notably, IL-2 mRNA was 3-fold higher even on day 28 as compared with that of anti-CD3/CD28-activated splenocytes and more than 5000-fold higher than control (day 0), indicating elevated and sustained expression of IL-2 in the muscle. Serum IL-2 concentration was significantly increased on day 7 (334.4 ± 35.7 pg/ml, p= 0.0119) and day 14 (66.5 ± 5.39 pg/ml, p= 0.0225) and showed no significance on day 28 (56.5 ± 12.5 pg/ml p= 0.0508) but still tended to be increased, as compared with controls (15.6 ± 9.13 pg/ml) (Figure 2B).

To test whether IL-2-rAAV therapy affected disease development, peripheral lymphocyte populations and serum autoantibody production were examined in MRL/lpr mice injected i.m. with IL-2-rAAV at 8 weeks of age and fed Dox. Consistent with a previous report, DN T cells were significantly decreased in the peripheral blood by IL-2-rAAV (percentages of DN T cells in total T cells of control vs IL-2: day 14; 26.9 ± 2.948 vs 13.78 ± 0.917. p= 0.0046, day 21; 33.83 ± 7.062 vs 18.40 ± 1.699. p= 0.0463, day 28; 51.83 ± 7.248 vs 26.95 ± 3.160. p= 0.0174, Figure 2C). Moreover, an increased percentage of $CD4^+CD25^+Foxp3^+$ Treg was observed 14 days post infection and sustained through day 21 (percentage of Treg cells in $CD4^+$ T cells of control vs IL-2: day 7; 4.91± 0.193 vs 6.22± 0.027, day 14; 4.13 ± 0.403 vs 7.89 ± 0.403, p<0.0001, day 21; 4.14 ± 0.517 vs 6.79 ± 0.451, p<0.001, Figure 2D). However, the titer of anti-dsDNA, the representative autoantibody in MRL/lpr mice, increased gradually with age and was comparable between control- and IL-2-rAAV-treated mice (Figure 2E). These results clearly demonstrate that IL-2 reduces the DN T cell pool and expands Treg without affecting autoreactive antibody responses in MRL/lpr mice.

IL-2-rAAV administration into MRL/lpr mice with established disease reduces DN T cells and cytokine production while expanding Treg

In considering the translational potential of IL-2 therapy, 12 week-old MRL/lpr-$Foxp3^{gfp}$ mice, a time point at which disease is ongoing were treated with IL-2-rAAV. The Foxp3-GFP reporter cassette facilitates identification of Treg in the periphery and various organs. Eight weeks after treatment IL-2-treated mice exhibited reduced spleen weights (control vs IL-2: 0.9433 ± 0.1094 g vs 0.6117 ± 0.0686 g, p=0.028)(Figure 3A). The percentage of DN T cells among total T cells and the absolute number in the spleen were significantly reduced (Figure 3C) (cell number of control vs IL-2: $1.30 \pm 0.197 \times 10^8$ vs $0.590 \pm 0.108 \times 10^8$, p=0.0098). Treg ($GFP^+$) were significantly increased in IL-2-treated mice (control vs IL-2: $2.54 \pm 0.144 \times 10^6$ vs $4.32 \pm 0.566 \times 10^6$, p=0.021) (Figure 3D). The fact that $CD4^+$ and $CD8^+$ T cell numbers were comparable between the control- and IL-2-treated groups indicated that the splenomegaly in MRL/l*pr* mice was mainly due to expansion of DN T cells. We also found that serum concentrations of IFN-γ (control vs IL-2: 976.8 ± 423.6 pg/ml vs 147.2 ± 20.40 pg/ml, p= 0.0359) and IL-17 (control vs IL-2: 3106 ± 418.1 pg/ml vs 1595 ± 423.8 pg/ml, p= 0.0294) were significantly decreased in IL-2-rAAV-treated mice (Figure 3B). These results suggest that systemic inflammatory responses were reduced by IL-2-rAAV treatment, at least in part, by the reduction of DN T cells.

IL-2-rAAV suppresses skin and lung inflammation in MRL/lpr mice

The typical skin lesions of diseased MRL/*lpr* mice are characterized by dermal hyperplasia and marked inflammatory cell infiltrate. Alopecia on the back of the neck and partial shedding and scabbing of the ear which were observed in the control rAAV-treated mice were diminished significantly in IL-2-rAAV-treated mice (Figure 4A, left). As seen in Fig. 4A IL-2-rAAV significantly decreased skin injury (37) (control vs IL-2: acanthosis; 1.83 ± 0.477 vs 0.667 ± 0.333. p= 0.0364, hyperkeratosis; 1.17 ± 0.307 vs 0.500 ± 0.224. p= 0.0550, inflammation; 1.833 ± 0.477 vs 0.833 ± 0.307. p= 0.0543, fibrosis; 1.17 ± 0.307 vs 0.333 ± 0.211. p= 0.0247, vessel cellular infiltration; 1.00 ± 0.365 vs 0.167 ± 0.167. p= 0.0323, ulcer; 0.333 ± 0.211 vs 0.167 ± 0.167 p= 0.275, n=6, Figure 4A, right).

Lung inflammation is common in MRL/lpr mice and is manifested by peribronchiolar and perivascular mononuclear cell infiltration. Perivascular infiltration of mononuclear cells was readily seen in control but not IL-2-rAAV-treated mice (Figure 4B). $CD45^+$ cells infiltrating the lungs were increased in control mice as compared to IL-2-rAAV treated mice. More than 80% of $CD45^+$ leukocytes were $TCR\beta^+$ T cells, which in turn were substantially decreased in IL-2-rAAV treated mice (control vs IL-2: $CD45^+$ $5.05 \pm 0.97 \times 10^5$ vs $2.68 \pm 0.49 \times 10^5$; p = 0.045, Total T $4.14 \pm 0.82 \times 10^5$ vs $1.86 \pm 0.38 \times 10^5$; p= 0.032, $CD4^+$ $8.71 \pm 2.76 \times 10^4$ vs $3.52 \pm 0.25 \times 10^4$; p= 0.078, $CD8^+$ $3.90 \pm 1.35 \times 10^4$ vs $1.60 \pm 0.22 \times 10^4$; p= 0.094, DN $2.53 \pm 0.47 \times 10^5$ vs $1.32 \pm 0.45 \times 10^5$; p= 0.046, Figure 4B, right). Because IL-17 is well-known inducer of inflammatory cytokines and chemokines in the lungs, expression of TNFα, MCP-1 and IL-8 in the lung tissues was assessed. Expression of all of these molecules were upregulated in control mice, but significantly reduced in the lungs of IL-2-treated animals (TNFα of control vs IL-2: p= 0.0806, MCP1; p= 0.0105, IL-8; p= 0.0434, Figure 4C), indicating that the inflammatory response in the lung is reduced by ectopic IL-2.

IL-2-rAAV treatment reduces kidney damage by reducing the inflammatory immune response

Early mortality in MRL/*lpr* mice is often a result of renal failure due to inflammation. As expected severe mononuclear cell infiltration into the perivascular, tubulointerstitial and periglomerular areas of the kidney were observed in control-rAAV-treated mice (Figure 5A). In contrast, cellular infiltration in these areas was significantly reduced in IL-2-rAAV-treated mice. Notably, hypercellularity and expansion of the mesangial area in the glomeruli were significantly reduced in IL-2-rAAV-treated mice (glomeruli score of control vs IL-2: 1.47 ± 0.358 vs 0.587 ± 0.0926; p= 0.0092, tubulointerstitum 1.37 ± 0.405 vs 0.531 ± 0.140; p= 0.0477, perivascular 1.37 ± 0.295 vs 0.565 ± 0.100; p= 0.0172, Supplementary Figure 1A, Figure 5B), while C3 and IgG deposition were observed irrespective of the treatment group (Supplementary Figure 1A). The decrease of proteinuria in IL-2-rAAV-treated mice (urine albumin/ creatinine ratio on day 55 of control vs IL-2: $15.1 \pm 7.74 \times 10^5$ vs $1.44 \pm 0.39 \times 10^5$ mg/g; $p < 0.05$) clearly reflected the attenuation of glomerulitis, tubulointerstitial and perivascular inflammation (Figure 5C). $CD4^+$ and DN T cells predominated among $CD45^+$ cells in the kidneys, and all subsets of T cells including Treg were decreased by IL-2 treatment ($CD45^+$ cells of control vs IL-2: $11.8 \pm 1.11 \times 10^5$ vs $5.12 \pm 0.318 \times 10^5$; p= 0.0034, Total T cells $8.87 \pm 1.00 \times 10^5$ vs $4.27 \pm 0.30 \times 10^5$; p= 0.0045, CD8 $1.47 \pm 0.258 \times 10^5$ vs $0.565 \pm 0.119 \times 10^5$; p= 0.019, CD4 $3.53 \pm 0.367 \times 10^5$ vs $1.72 \pm 0.225 \times 10^5$; p=

0.0028, DN 3.85 ± 1.00 × 10^5 vs 1.84 ± 0.143 × 10^5, p= 0.0468, Treg 2.74 ± 0.014 × 10^4 vs 1.39 ± 0.02 × 10^4; p= 0.0063, Figure 5D). Gene expression levels of IFN-γ, IL-17, chemokines and chemokine ligands including CXCL9, IL-8, and CCL20 that promote recruitment of inflammatory cells were also reduced in the whole kidney of IL-2-rAAV-treated mice (IFN-γ; p= 0.0418, IL-17; p= 0.0366, CXCL9; p= 0.0304, IL-8; p= 0.0157, CCL20; p=0.0016, Figure 5E). These results demonstrate that IL-2-treatment decreases kidney injury by reducing the local recruitment of inflammatory cells.

IL-2 selectively decreases the DN T cell population by affecting its viability

The above results prompted us to consider whether IL-2 treatment controled DN T cell viability or generation in diseased mice. Accordingly, Calcein-violet labeled T cells from MRL/*lpr* mice were transferred to MRL/MpJ mice and rIL-2 was administrated for 5 days. Labeled T cells were not detected in the kidneys or the lungs regardless of IL-2 treatment (data not shown), indicating that T cell migration was not influenced by IL-2 treatment. Yet, the number of Calcein -Violet$^+$ DN, but not CD4$^+$ and CD8$^+$ T cells, was found decreased in the spleens of the recipient mice (Figure 6A). This observation suggests that IL-2 negatively affects survival of DN T cells.

To address whether IL-2 has an influence on DN T cell viability, we cultured spleen cells for 5 days in the presence of IL-2 and survived CD4$^+$, CD8$^+$ and DN T cells were counted. The number of survived CD4$^+$ and CD8$^+$ T cells were sustained or slightly increased in high concentrations of IL-2. However, survived DN T cell number was dramatically decreased in low concentration (1.25 ng/ml) of IL-2 and IL-2-mediated decrease of DN T cell viability was concentration-dependent (Figure 6B). Next we examined whether IL-2 directly affects DN T cell survival, CFSE-labeled DN T cells alone were cultured or co-cultured with splenocytes in the presence of IL-2. We found that the viability of DN T cells was increased in the presence of splenocytes as compared with in the absence of them (Figure 6C). When DN T cells were singly cultured with IL-2, the number of live cells did not change or rather increased slightly in higher concentrations of IL-2. However, when co-cultured with splenocytes, IL-2 significantly induced DN T cell death in a dose-dependent manner (Figure 6C). We also co-cultured DN T cells with CD4$^+$ T cells or with CD8$^+$ T cells in the presence of IL-2 and the viability of DN T cells was decreased when co-cultured with CD8$^+$ T cells but not with CD4$^+$ T cells (data not shown and supplementary Figure 2B). These results suggest that IL-2 can act on non-DN T cell that drives DN T cell death. We checked the expression of IL-2 receptors by each subset of T cells. Neither IL-2 receptor alpha chain (CD25) nor beta chain (CD122) was detectable on DN T cells, whereas CD122 was highly expressed on CD8$^+$ T cells. Moreover, phosphorylation of STAT5 by IL-2 was significantly obsereved in NK cells and CD8$^+$ T cells but not in non-CD8$^+$ T cells (data not shown and Supplementary Figure 2A). These results indicate that IL-2 indirectly affects DN T cell viability, possibly through modulating other lymphocyte function.

IL-2 targeting to non-Treg cells decreases the number of DN T cells and leads to reduced lymphadenopathy

It is well known that administration of IL-2 both in humans and mice results in expansion of Treg. Treg suppress several functions of conventional T cells directly and/or indirectly.

Activated T cells can be suppressed by molecules such as IL-10, TGFβ and IL-35, or CTLA-4, or can be killed directly by perforin-expressing Treg cells (38). IL-2 also contributes to the generation and proliferation of cytotoxic T lymphocytes (CTLs) and natural killer (10) cells. Functional impairment of cytotoxic lymphocytes in lupus has been reported and found to correlate with disease activity both in humans and mice (39, 40). Therefore, we examined the effect of IL-2/anti-IL-2-complexes that can be used to selectively target different immune effector subsets. We used IL-2 complexed with two different clones of anti-IL-2 monoclonal antibodies (mAbs), S4B6 and JES6-1. These two types of IL-2/mAb complexes have distinct biological activities. IL-2/S4B6-1 complex preferentially binds to memory $CD8^+$ T cells and NK cells expressing high levels of CD122 and expands these cells *in vivo*. By contrast, IL-2/JES6-1 complex binds to CD25 and induces selective expansion of $CD4^+CD25^+$ Treg (41). Isolated T cells from MRL/*lpr* mice were labeled with CFSE, transferred to recipient MRL/lpr mice, which were then treated with IL-2/ anti-IL-2 mAb complexes every other day for 7 days. $CD8^+$ T cells and $CD4^+Foxp3^+$ Treg were significantly increased in IL-2/S4B6 and IL-2/JES6-treated mice, respectively, compared to control rat IgG-treated mice (Figure 7A and Supplementary Figure 2B). Notably, the frequency (Supplementary Figure 2B) and number (cell number of rat IgG; $6.08 \pm 0.39 \times 10^4$, IL-2/JES6 $6.32 \pm 0.784 \times 10^4$, IL-2/S4B6 $3.00 \pm 0.305 \times 10^4$, $p<0.05$, Figure 7B) of DN T cells were significantly decreased only in IL-2/S4B6-treated mice, indicating that IL-2-delivered to cytotoxic lymphocytes affect the DN T cell population. Consistent with previous reports (25, 42), CFSE-labeled $CD8^+$ T cells and $CD4^+Foxp3^+$ cells underwent significant proliferation upon transfer into IL-2/S4B6-treated and IL-2/JES6-treated mice, respectively (Supplementary Figure 2C). Notably, the frequency of proliferating DN T cells was significantly decreased in IL-2/S4B6-treated but not IL-2/JES6-treated mice (percentage of proliferating cells of rat IgG; 40.77 ± 1.78, IL-2/JES6 42.03 ± 0.584, IL-2/S4B6 31.4 ± 1.40, $p<0.01$, Figure 6C). These results suggest that the direct effects of IL-2 on cytotoxic lymphocytes results in inhibition of DN T cell expansion.

IL-2 delivery to cytotoxic lymphocytes reduces lymphadenopathy

DN T cells make up ~90% of the cells residing in the massive lymph nodes of diseased MRL/lpr mice. To determine if IL-2/S4B6 treatment reduced lymphadenopathy, 14 week-old MRL/lpr mice were treated with control rat-IgG, IL-2/JES6-1 or IL-2/S4B6-1, every other day for 3 weeks. No difference in the number of splenic DN T cells was seen in IL-2/JES6-1- and control rat-IgG-treated mice. On the other hand, $CD8^+$ T cells and NK cells in spleens from IL-2/S4B6-treated mice were significantly increased and DN T cells decreased, although the total cell number was comparable in all groups (Figure 7C). However, size and weight of axillary and mesenteric lymph nodes were significantly reduced in IL-2/S4B6-treated mice as compared with control mice (lymph node weight of control vs IL-2/S4B6: axillary 0.290 ± 0.015 g vs 0.165 ± 0.035 g, $p<0.05$, mesenteric 1.003 ± 0.096 g vs 0.523 ± 0.048 g, $p<0.05$, Figure 7D). These results suggest that activated/expanded cytotoxic lymphocytes, but not Treg, lead to the reduction of DN T cells resulting in the amelioration of lymphadenopathy in MRL/lpr mice.

Discussion

Our results demonstrate that systemic expression of low and continuous levels of IL-2 in MRL/*lpr* mice decreased the numbers of DN T cells and effectively suppressed ongoing inflammation in the kidneys, lungs and the skin. Delivery of IL-2 to lupus-prone mice has been reported using a vaccinia virus (31, 32). While vaccinia virus can escape early immunity, the recombinant is eventually neutralized thereby affecting long-term efficacy. rAAV-mediated gene therapy is clinically feasible for gene supplementation and is widely used for human gene therapy because AAV infects non-dividing cells, survives in the host nucleus and is stable for a long period with low immunogenicity. In our study IL-2 expression was detectable for at least for 28 days after a single injection; notably reports have demonstrated that rAAV-mediated gene expression in muscles can persist for more than a year (43, 44). Although IL-2 expression in muscle was decreased 2 weeks after injection possibly due to elimination of viruses to some extent, increased numbers of Treg were still observed in spleens 8 weeks after IL-2-rAAV injection (Figure 3C), indicating that IL-2 production in muscles could be persistent at a low levels even after 28 days post injection.

Consistent with the previous reports, autoantibody production was unchanged by IL-2 therapy (31). Recently, Ballesteros-Tato *et al.* showed that IL-2 administration impairs germinal center formation and high affinity IgG production upon influenza virus infection by restricting follicular helper T cell differentiation (14). In MRL/*lpr* mice extrafollicular plasmablasts are the main antibody forming cells whose differentiation is helped by T extrafollicular helper cells (Tefh) in the spleen (45). No difference, however, was detected in the number of $CD4^{+}B220^{-}PSGL1^{low}CD62L^{-}$ Tefh cells and $TCR\beta^{-}CD44^{high}B220^{+}CD138^{+}$ plasmablasts in control-versus IL-2-rAAV-treated mice (data not shown), indicating that IL-2 under the conditions being used did not alter plasmablast cell development and Tefh cell differentiation. Moreover, autoantibody production and tissue inflammation do not seem to be correlated in lupus nephritis. In mice, ICOS-deficient MRL/lpr mice showed significant decrease of each isotype of autoantibody as compared with wild-type mice, but severe renal pathology including glomerulonephritis and interstitial inflammation was comparable(46). There is also evidence that tubulointerstitial inflammation aside from glomerulonephritis is an important prognostic factor for renal outcome(47). Taken together, it is presumable that tissue infiltration and inflammation mediated by pathogenic lymphocytes rather than deposition of immune complex in glomeruli is important for disease progression. In this point of view, IL-2-mediated suppression of aberrant T cell expansion and activation could be beneficial for protecting from tissue damage.

A novel finding of our study is that IL-2 delivery in an autoimmune setting resulted in inhibition of IL-17-producing DN T cell and parallel expansion of Treg. The proinflammatory effects of IL-17 are thought to be in part dependent on the induction of cytokines and chemokines such as CXCL8 and CCL20 by adjacent cells. In view of work by our group and others showing that DN T cells are an important source of IL-17 in human SLE and lupus-prone mice, local release of IL-17 by DN T cells may drive infiltration of inflammatory cells including neutrophils, monocytes and T cells. Furthermore, a recent

report showed that IL-17 and IL-17R signaling plays a critical role in the development of glomerulonephritis in Fc-gamma receptor 2b (Fcγr2b)-deficient lupus-prone mice, and that infiltration of DN T cells and $CD4^+$ T cells into kidneys is decreased in the absence of IL-17 signaling (48). Therefore, reduction in the number of IL-17 producing DN T cell is expected to prevent of organ inflammation in MRL/*lpr* mice. Indeed, IL-2 treatment concomitant with a diminished pool of DN T cells limited infiltration of the kidneys, lungs and the skin of MRL/*lpr* mice. Considering a recent report that IL-2 expands polarized Th1 and Th17 cells in vitro (49), IL-2 treatment may drive the expansion of pathogenic effector cells under certain conditions. This scenario, however, was not observed. Although splenic IFN-γ- and IL-17-producing $CD4^+$ T cells were not affected by IL-2 treatment (data not shown), serum IFN-γ and IL-17 were both decreased. Here, Treg expanded by IL-2 treatment may block differentiation and/or function of effector T cells. Furthermore, low levels of IL-2 are expected to have only a minimal if any effect on conventional $CD4^+$ T cells which in the spleen of MRL/lpr mice are largely $CD25^-$ and $CD122^-$.

Another key finding was that the effects of IL-2 on DN T cells were indirect. DN T cells for instance expressed low if any CD25 and CD122. Phosphorylation of STAT5 in DN T cells by stimulation of IL-2 was minimal as compared with that in NK cells and $CD8^+$ T cells. IL-2 does not seem to have a direct effect on DN T cell, but have a significantly negative impact on DN T cell viability in the presence of other spleen cells. Furthermore the number of DN T cells was decreased by injection of the IL-2/S4B6 complex, which selectively targets IL-2 to $CD8^+CD122^+$ and NK cells (50). Direct $CD8^+$ T cell or NK cell-mediated killing of DN T cells, however, was not detectable (data not shown). Nevertheless a role for these cytotoxic effectors is suggested by evidence demonstrating that perforin-deficient MRL/lpr mice show greater accumulation of DN T cells and accelerated mortality with higher infiltrates in multiple end-organs as compared with perforin-intact MRL/lpr mice (40). Another possibility is that TNF-related apoptosis inducing ligand (TRAIL) produced by CTLs in the presence of IL-2 may induce DN T cell apoptosis. Given that $CD8^+$ T cell function is impaired in both human and lupus-prone mice (51), IL-2 may restore CTL function that contributes to the elimination of pathogenic lymphocytes including DN T cells in MRL/*lpr* mice. Taken together, our results suggest that CTLs treated with IL-2 can affect the pathogenic DN T cell viability and expansion.

The targeted IL-2-delivery experiments revealed that the selective Treg expansion did not affect the DN T cell population. It is accepted that IL-2 is necessary for Treg survival, expansion and function, and leads to the amelioration of several autoimmune diseases(33). However, unlike type 1 diabetes (52), transfer of Treg in lupus murine models is insufficient to block disease progression (18). Therefore, IL-2-mediated suppression of DN T cells represents a critical mechanism to reduce or even reverse end organ damage in SLE.

In conclusion, we show that treatment with rAAV-IL-2 via an indirect mechanism decreases DN T cell numbers, and limits the infiltration of inflammatory cells into organs thereby minimizing tissue damage. An expanded Treg pool may also contribute to efficacy by suppressing the expansion and function of pathogenic effector T cells. Our results strongly support the therapeutic potential of IL-2 in preventing or even reverting clinical manifestations in patients with SLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

SLE	systemic lupus erythematosus
rAAV	recombinant adeno-associated virus
DN T cells	$CD4^{-}CD8^{-}$ double-negative T cells
Treg	regulatory T cells
CFSE	carboxyfluorescein succinimidyl ester

References

1. Tsokos GC. Systemic lupus erythematosus. The New England journal of medicine. 2011; 365:2110–2121. [PubMed: 22129255]
2. Liu Z, Davidson A. Taming lupus-a new understanding of pathogenesis is leading to clinical advances. Nature medicine. 2012; 18:871–882.
3. Lewis JE, Fu SM, Gaskin F. Autoimmunity, end organ damage, and the origin of autoantibodies and autoreactive T cells in systemic lupus erythematosus. Discovery medicine. 2013; 15:85–92. [PubMed: 23449110]
4. Ortega LM, Schultz DR, Lenz O, Pardo V, Contreras GN. Review: Lupus nephritis: pathologic features, epidemiology and a guide to therapeutic decisions. Lupus. 2010; 19:557–574. [PubMed: 20089610]
5. Fossati L, Takahashi S, Merino R, Iwamoto M, Aubry JP, Nose M, Spach C, Motta R, Izui S. An MRL/MpJ-lpr/lpr substrain with a limited expansion of lpr double-negative T cells and a reduced autoimmune syndrome. Int Immunol. 1993; 5:525–532. [PubMed: 8318455]
6. Kinjyo I, Gordon SM, Intlekofer AM, Dowdell K, Mooney EC, Caricchio R, Grupp SA, Teachey DT, Rao VK, Lindsten T, Reiner SL. Cutting edge: Lymphoproliferation caused by Fas deficiency is dependent on the transcription factor eomesodermin. Journal of immunology. 2010; 185:7151–7155.
7. Nowling TK, Gilkeson GS. Mechanisms of tissue injury in lupus nephritis. Arthritis Res Ther. 2011; 13:250. [PubMed: 22192660]
8. Theofilopoulos AN, Kofler R, Singer PA, Dixon FJ. Molecular genetics of murine lupus models. Advances in immunology. 1989; 46:61–109. [PubMed: 2675565]
9. Davidson A, Aranow C. Lupus nephritis: lessons from murine models. Nature reviews Rheumatology. 2010; 6:13–20.
10. Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L, Shevach EM, O'Shea JJ. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007; 26:371–381. [PubMed: 17363300]
11. Liao W, Lin JX, Leonard WJ. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013; 38:13–25. [PubMed: 23352221]
12. Refaeli Y, Van Parijs L, London CA, Tschopp J, Abbas AK. Biochemical mechanisms of IL-2-regulated Fas-mediated T cell apoptosis. Immunity. 1998; 8:615–623. [PubMed: 9620682]

13. Yang XP, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, Hirahara K, Sun HW, Wei L, Vahedi G, Kanno Y, O'Shea JJ, Laurence A. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. Nature immunology. 2011; 12:247–254. [PubMed: 21278738]
14. Ballesteros-Tato A, Leon B, Graf BA, Moquin A, Adams PS, Lund FE, Randall TD. Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. Immunity. 2012; 36:847–856. [PubMed: 22464171]
15. Sadlack B, Lohler J, Schorle H, Klebb G, Haber H, Sickel E, Noelle RJ, Horak I. Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells. European journal of immunology. 1995; 25:3053–3059. [PubMed: 7489743]
16. Gomez-Martin D, Diaz-Zamudio M, Crispin JC, Alcocer-Varela J. Interleukin 2 and systemic lupus erythematosus: beyond the transcriptional regulatory net abnormalities. Autoimmunity reviews. 2009; 9:34–39. [PubMed: 19269352]
17. Lieberman LA, Tsokos GC. The IL-2 defect in systemic lupus erythematosus disease has an expansive effect on host immunity. Journal of biomedicine & biotechnology. 2010; 2010:740619. [PubMed: 20625413]
18. Humrich JY, Morbach H, Undeutsch R, Enghard P, Rosenberger S, Weigert O, Kloke L, Heimann J, Gaber T, Brandenburg S, Scheffold A, Huehn J, Radbruch A, Burmester GR, Riemekasten G. Homeostatic imbalance of regulatory and effector T cells due to IL-2 deprivation amplifies murine lupus. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107:204–209. [PubMed: 20018660]
19. Koga T, Ichinose K, Mizui M, Crispin JC, Tsokos GC. Calcium/calmodulin-dependent protein kinase IV suppresses IL-2 production and regulatory T cell activity in lupus. Journal of immunology. 2012; 189:3490–3496.
20. Mellor-Pita S, Citores MJ, Castejon R, Tutor-Ureta P, Yebra-Bango M, Andreu JL, Vargas JA. Decrease of regulatory T cells in patients with systemic lupus erythematosus. Annals of the rheumatic diseases. 2006; 65:553–554. [PubMed: 16531555]
21. Wong CK, Lit LC, Tam LS, Li EK, Wong PT, Lam CW. Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity. Clinical immunology. 2008; 127:385–393. [PubMed: 18373953]
22. Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, Kyttaris VC, Juang YT, Tsokos GC. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. Journal of immunology. 2008; 181:8761–8766.
23. Zhang Z V, Kyttaris C, Tsokos GC. The role of IL-23/IL-17 axis in lupus nephritis. Journal of immunology. 2009; 183:3160–3169.
24. Crispin JC, Tsokos GC. Human TCR-alpha beta+ CD4- CD8- T cells can derive from CD8+ T cells and display an inflammatory effector phenotype. J Immunol. 2009; 183:4675–4681. [PubMed: 19734235]
25. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science. 2006; 311:1924–1927. [PubMed: 16484453]
26. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. Nature reviews Immunology. 2012; 12:180–190.
27. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP 3rd, Armand P, Cutler C, Ho VT, Treister NS, Bienfang DC, Prasad S, Tzachanis D, Joyce RM, Avigan DE, Antin JH, Ritz J, Soiffer RJ. Interleukin-2 and regulatory T cells in graft-versus-host disease. The New England journal of medicine. 2011; 365:2055–2066. [PubMed: 22129252]
28. Matsuoka K, Koreth J, Kim HT, Bascug G, McDonough S, Kawano Y, Murase K, Cutler C, Ho VT, Alyea EP, Armand P, Blazar BR, Antin JH, Soiffer RJ, Ritz J. Low-dose interleukin-2 therapy restores regulatory T cell homeostasis in patients with chronic graft-versus-host disease. Science translational medicine. 2013; 5:179ra143.
29. Saadoun D, Rosenzwajg M, Joly F, Six A, Carrat F, Thibault V, Sene D, Cacoub P, Klatzmann D. Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. The New England journal of medicine. 2011; 365:2067–2077. [PubMed: 22129253]

30. Bluestone JA. The yin and yang of interleukin-2-mediated immunotherapy. The New England journal of medicine. 2011; 365:2129–2131. [PubMed: 22129258]

31. Gutierrez-Ramos JC, Andreu JL, Revilla Y, Vinuela E, Martinez C. Recovery from autoimmunity of MRL/lpr mice after infection with an interleukin-2/vaccinia recombinant virus. Nature. 1990; 346:271–274. [PubMed: 1973822]

32. Gutierrez-Ramos JC, Andreu JL, Marcos MA, Vegazo IR, Martinez C. Treatment with IL2/vaccinia recombinant virus leads to serologic, histologic and phenotypic normalization of autoimmune MRL/lpr-lpr mice. Autoimmunity. 1991; 10:15–25. [PubMed: 1742421]

33. Goudy KS, Johnson MC, Garland A, Li C, Samulski RJ, Wang B, Tisch R. Inducible adeno-associated virus-mediated IL-2 gene therapy prevents autoimmune diabetes. Journal of immunology. 2011; 186:3779–3786.

34. Kulkarni OP, Ryu M, Kantner C, Sardy M, Naylor D, Lambert D, Brown R, Anders HJ. Recombinant chaperonin 10 suppresses cutaneous lupus and lupus nephritis in MRL-(Fas)lpr mice. Nephrol Dial Transplant. 2012; 27:1358–1367. [PubMed: 21987536]

35. Benihoud K, Bonardelle D, Bobe P, Kiger N. MRL/lpr CD4- CD8- and CD8+ T cells, respectively, mediate Fas-dependent and perforin cytotoxic pathways. European journal of immunology. 1997; 27:415–420. [PubMed: 9045912]

36. Clementi R, Chiocchetti A, Cappellano G, Cerutti E, Ferretti M, Orilieri E, Dianzani I, Ferrarini M, Bregni M, Danesino C, Bozzi V, Putti MC, Cerutti F, Cometa A, Locatelli F, Maccario R, Ramenghi U, Dianzani U. Variations of the perforin gene in patients with autoimmunity/lymphoproliferation and defective Fas function. Blood. 2006; 108:3079–3084. [PubMed: 16720836]

37. Lahoud MH, Proietto AI, Gartlan KH, Kitsoulis S, Curtis J, Wettenhall J, Sofi M, Daunt C, O'Keeffe M, Caminschi I, Satterley K, Rizzitelli A, Schnorrer P, Hinohara A, Yamaguchi Y, Wu L, Smyth G, Handman E, Shortman K, Wright MD. Signal regulatory protein molecules are differentially expressed by CD8- dendritic cells. Journal of immunology. 2006; 177:372–382.

38. Yamaguchi T, Wing JB, Sakaguchi S. Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions. Semin Immunol. 2011; 23:424–430. [PubMed: 22055883]

39. Tsokos GC, Balow JE. Cytotoxic responses to alloantigens in systemic lupus erythematosus. J Clin Immunol. 1981; 1:208–216. [PubMed: 19338096]

40. Peng SL, Moslehi J, Robert ME, Craft J. Perforin protects against autoimmunity in lupus-prone mice. Journal of immunology. 1998; 160:652–660.

41. Shevach EM. Application of IL-2 therapy to target T regulatory cell function. Trends in immunology. 2012; 33:626–632. [PubMed: 22951308]

42. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, Grey ST, Sprent J. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. The Journal of experimental medicine. 2009; 206:751–760. [PubMed: 19332874]

43. Penaud-Budloo M, Le Guiner C, Nowrouzi A, Toromanoff A, Cherel Y, Chenuaud P, Schmidt M, von Kalle C, Rolling F, Moullier P, Snyder RO. Adeno-associated virus vector genomes persist as episomal chromatin in primate muscle. J Virol. 2008; 82:7875–7885. [PubMed: 18524821]

44. Flierl A, Chen Y, Coskun PE, Samulski RJ, Wallace DC. Adeno-associated virus-mediated gene transfer of the heart/muscle adenine nucleotide translocator (ANT) in mouse. Gene Ther. 2005; 12:570–578. [PubMed: 15647764]

45. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, Flavell RA, Craft J. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. The Journal of experimental medicine. 2008; 205:2873–2886. [PubMed: 18981236]

46. Zeller GC, Hirahashi J, Schwarting A, Sharpe AH, Kelley VR. Inducible co-stimulator null MRL-Faslpr mice: uncoupling of autoantibodies and T cell responses in lupus. J Am Soc Nephrol. 2006; 17:122–130. [PubMed: 16291836]

47. Hsieh C, Chang A, Brandt D, Guttikonda R, Utset TO, Clark MR. Predicting outcomes of lupus nephritis with tubulointerstitial inflammation and scarring. Arthritis Care Res (Hoboken). 2011; 63:865–874. [PubMed: 21309006]

48. Pisitkun P, Ha HL, Wang H, Claudio E, Tivy CC, Zhou H, Mayadas TN, Illei GG, Siebenlist U. Interleukin-17 cytokines are critical in development of fatal lupus glomerulonephritis. Immunity. 2012; 37:1104–1115. [PubMed: 23123062]

49. Amadi-Obi A, Yu CR, Liu X, Mahdi RM, Clarke GL, Nussenblatt RB, Gery I, Lee YS, Egwuagu CE. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. Nat Med. 2007; 13:711–718. [PubMed: 17496900]

50. Kayagaki N, Yamaguchi N, Nakayama M, Takeda K, Akiba H, Tsutsui H, Okamura H, Nakanishi K, Okumura K, Yagita H. Expression and function of TNF-related apoptosis-inducing ligand on murine activated NK cells. Journal of immunology. 1999; 163:1906–1913.

51. Puliaeva I, Puliaev R, Via CS. Therapeutic potential of CD8+ cytotoxic T lymphocytes in SLE. Autoimmunity reviews. 2009; 8:219–223. [PubMed: 18725326]

52. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, Masteller EL, McDevitt H, Bonyhadi M, Bluestone JA. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. The Journal of experimental medicine. 2004; 199:1455–1465. [PubMed: 15184499]

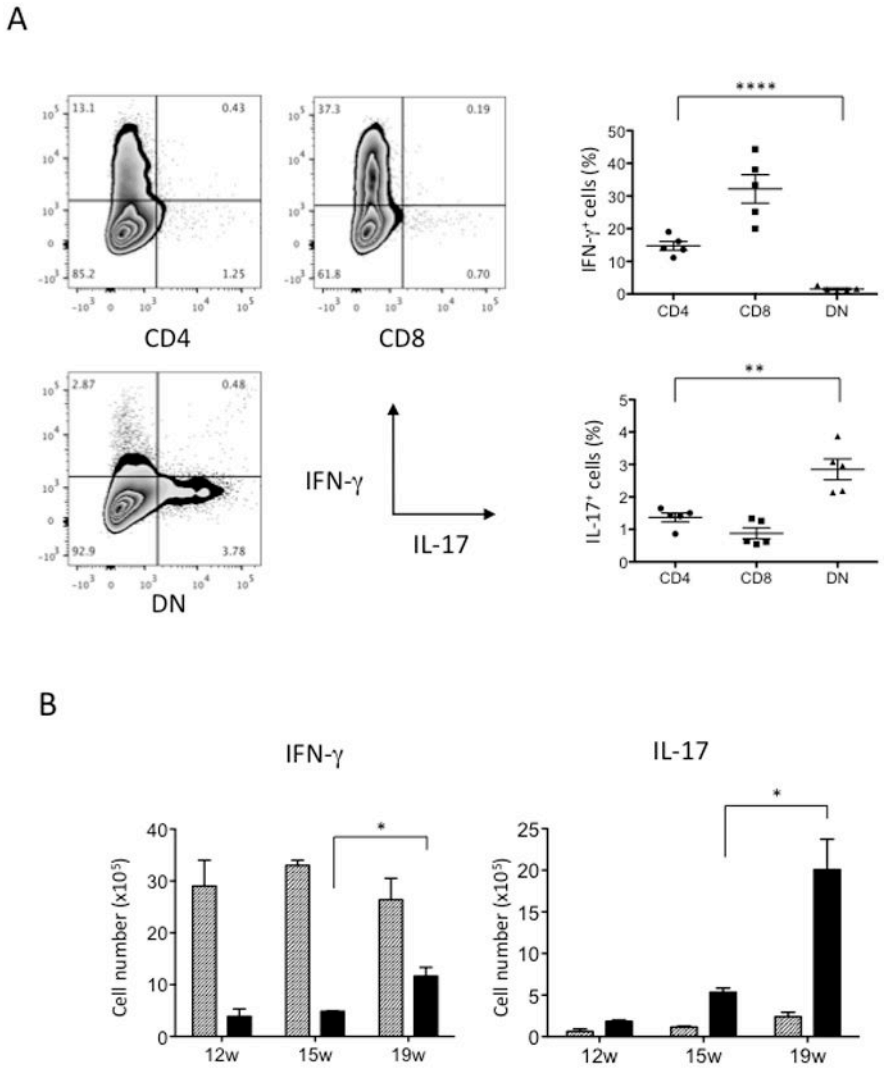

Figure 1. TCRβ$^+$CD4$^-$CD8$^-$ DN T cells preferentially produce IL-17 with advancing age

(A) Intracellular staining of IFN-γ and IL-17 in each subset of TCRβ$^+$ T cells from the spleen of 19 weeks-old MRL/lpr mice (left). Splenocytes were stimulated with phorbol-myristate acetate (PMA) and ionomycin. Percentages of IFN-γ$^+$ or IL-17A$^+$ cells in each T cell subsets are shown in scatter plot graph (right, ****p<0.0001, **p<0.01; mean ± SEM; n=5). (B) Total cell numbers of IFN-γ$^+$ and IL-17$^+$ CD4$^+$ (shaded bars) or DN T cells (black bars) from the spleens of each indicated age of MRL/lpr mice were counted (*p<0.05; mean ± SEM; n=5).

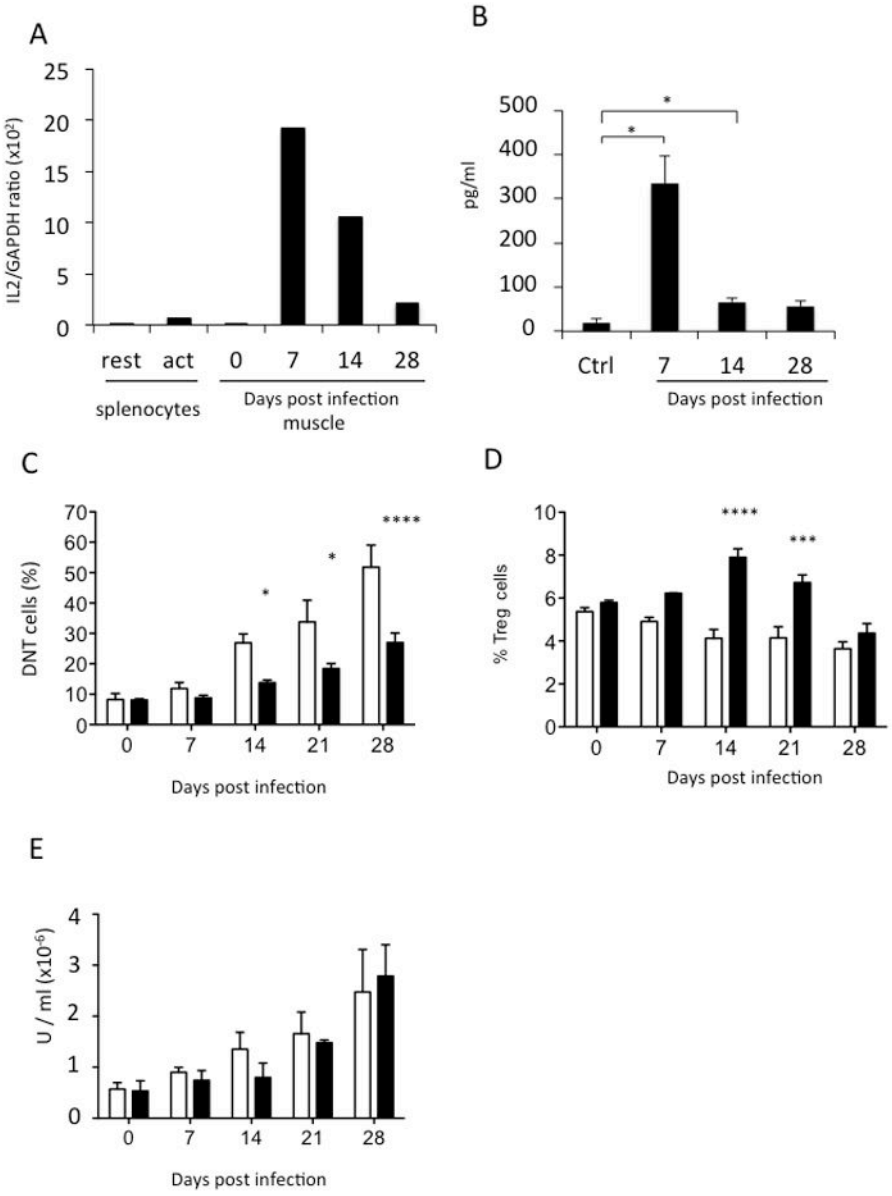

Figure 2. The effect of intramuscular injection of IL-2-rAAV on MRL/lpr disease development

(A) IL-2-Tet-on-rAAV was injected intramuscularly to MRL/lpr mice. Messenger RNA was isolated from the muscles on the indicated period after injection and IL-2 expression was detected by quantitative-PCR. The expression levels of resting and anti-CD3 and anti-CD28-activated (act) splenocytes are shown for references. (B) Serum IL-2 concentrations on indicated time points after IL-2-rAAV injection were detected by ELISA (*$p<0.05$; mean ± SEM, n=3). (C, D) The frequency of $TCR\beta^+CD4^-CD8^-$ DNT cells in total T cells (C) and $CD4^+CD25^+Foxp3^+$ T cells in total $CD4^+$ T cells (D) in the peripheral blood after the indicated time points of control-(white bars) or IL-2-rAAV (black bars) injection was shown (*$p<0.05$, ***$p<0.001$, ****$p<0.0001$; mean ± SEM; n=3). (E) Serum anti-dsDNA IgG were quantified in the sera of MRL/lpr mice treated with control-(white bars) or IL-2-rAAV (black bars) by ELISA. Data are shown on the indicated days after the injection into 8 weeks

old MRL/lpr mice (mean ± SEM; n=3). Statistics were determined using paired t-test (B) and two-way ANOVA with Bonferoni's post-test (C, D).

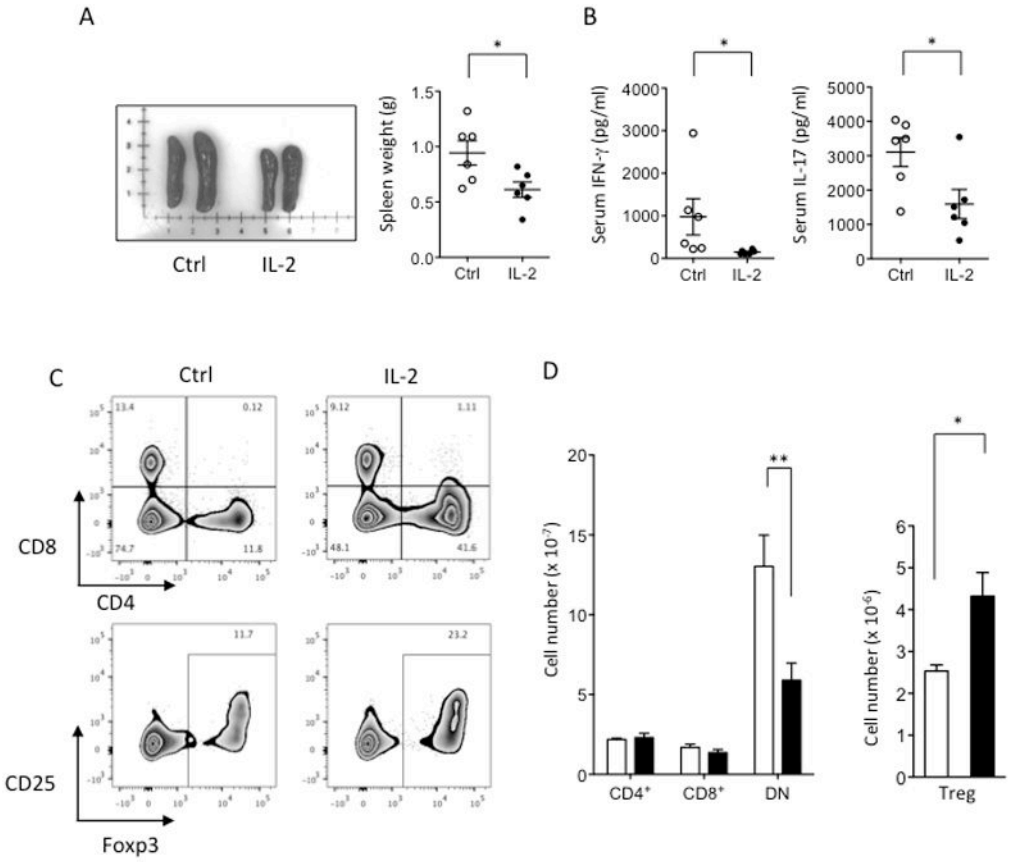

Figure 3. Reduction of DNT cells and cytokine production in IL-2-rAAV-treated mice

(A) Representative images of the spleens and spleen weight from control-rAAV-(circle dots) and IL-2-rAAV-(square dots) treated mice (*p<0.05, mean ± SEM; n=6). (B) Serum IFN-γ and IL-17 concentrations measured by ELISA (*p<0.05, mean ± SEM; n=6). (C) Representative dot plots of splenocytes. TCRβ$^+$MHC class II$^-$ cells and TCRβ$^+$ CD4$^+$ cells (lower) are shown. (D) Absolute numbers of splenic CD4$^+$, CD8$^+$, DN and Treg cells in control-(white bars) and IL-2-(black bars) treated mice (*p<0.05, **p<0.01; mean ± SEM; n=6).

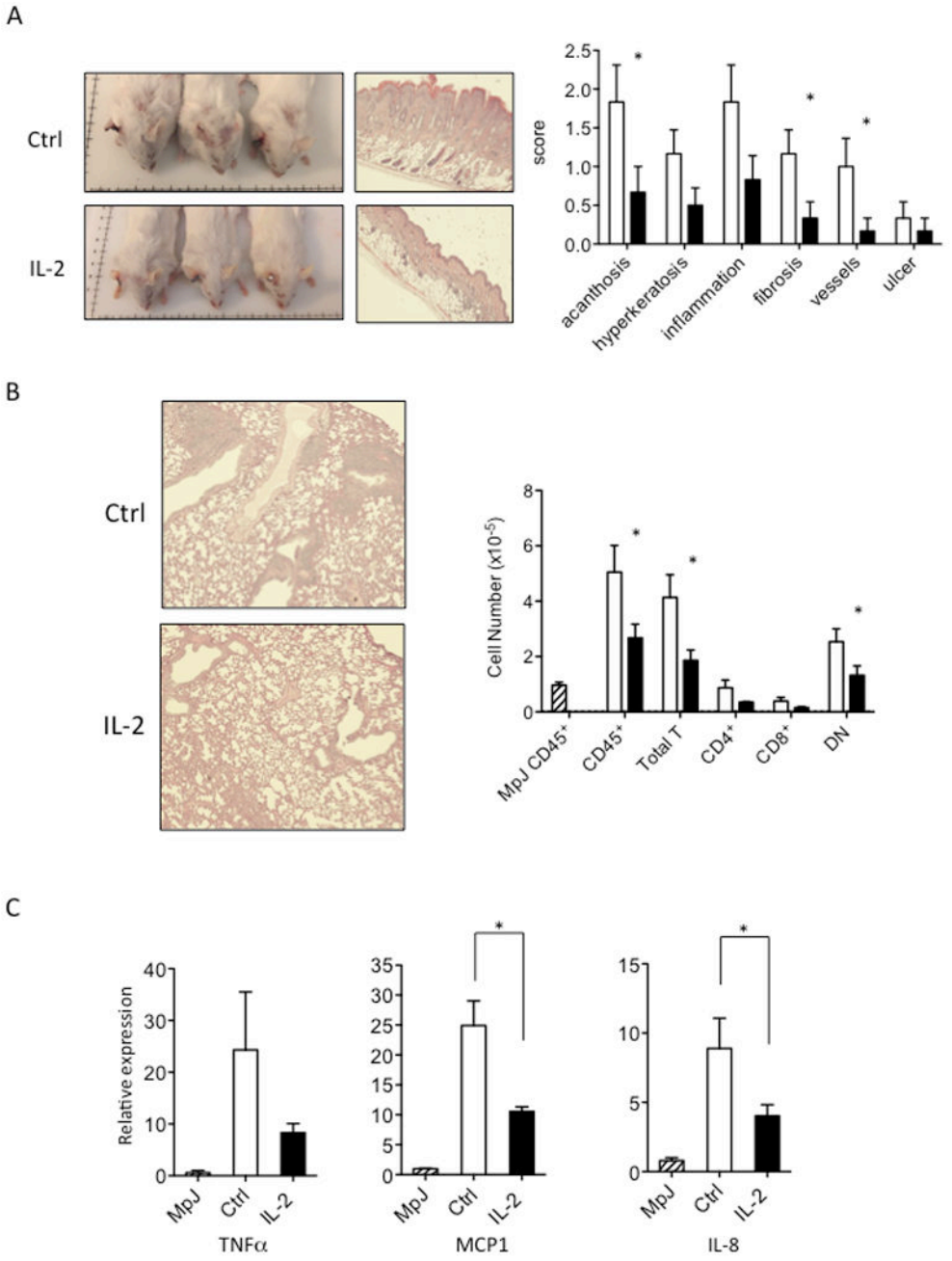

Figure 4. Inflammation of skin and lungs was ameliorated in IL-2-rAAV-treated mice

(A) Representative images of mice and skin lesions (original magnification 20×, left). Ear and skin lesions were severe in control mice. The semiquantitative assessment of skin injury score derived from the scores of six different criteria (right, control; white bars, IL-2-treated; black bars, *p<0.05, mean ± SEM; n=6). (B) Representative images of lung tissue section (20×, left) and absolute number of each subset of immune cells infiltrating into the lungs were counted (right, shaded bar, $CD45^+$ cells from MRL/MpJ mice; control; white bars, IL-2-treated; black bars, *p<0.05, mean ± SEM; n=6). (C) Relative mRNA expression (normalized by GAPDH) of TNFβ, MCP-1 and IL-8 in lung tissue of MRL/MpJ, control-

rAAV-treated and IL-2-rAAV-treated MRL/lpr mice (*$p<0.05$; mean ± SEM; n=6). Data were shown in control-rAAV-treated mice (white bars) and IL-2-rAAV-treated mice (black bars). Results are representative of three independent experiments.

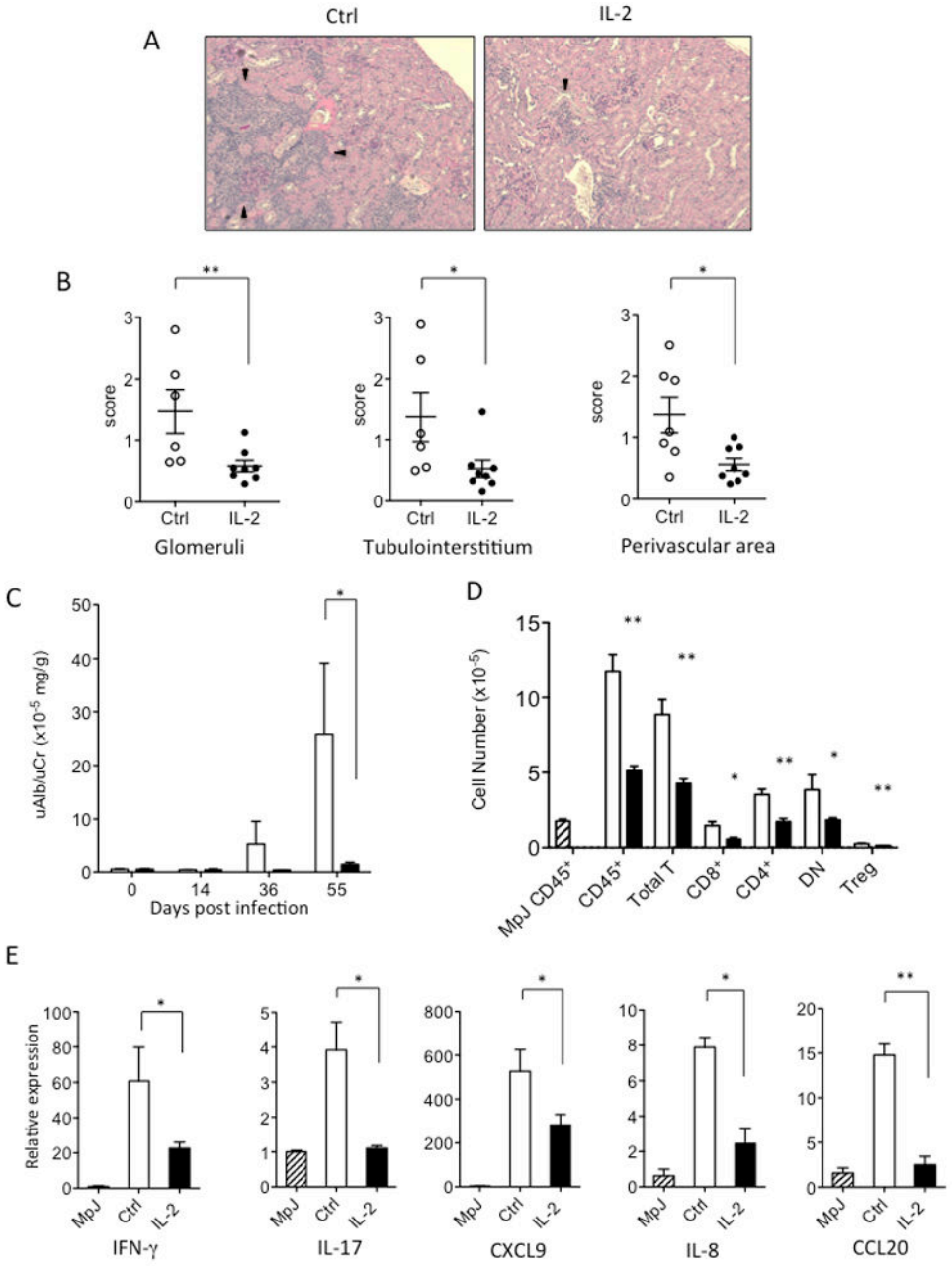

Figure 5. IL-2-rAAV treatment attenuates tissue damage of kidney

(A) PAS staining of renal cortex (10× magnification) Mononuclear cell infiltration into cortical tubulointerstitial and perivascular areas is observed (arrows). (B) Renal histopathological assessment was shown in glomelular, tubulointerstitial and perivascular lesion (*$p<0.05$; mean ± SEM; n=6). (C) Urine albumin and creatinine concentration were measured by ELISA. Albumin-creatinine ratio of control-(white bars) and IL-2-(black bars)-rAAV-treated mice is shown at the indicated days after rAAV injection (two-way ANOVA, *$p<0.05$; mean ± SEM; n=6). (D) Absolute number of each fraction of leukocytes in kidneys was counted. $CD45^+$ cell number from the kidney of MRL/MpJ mice (MpJ $CD45^+$) is shown as a control (37). (E) Relative mRNA expression of indicated cytokines

and chemokines in kidneys was detected by quantitative real-time PCR (*p<0.05, **p<0.01; mean ± SEM; n=6). Data were shown in control-rAAV-treated mice (white bars) and IL-2-rAAV-treated mice (black bars). Results are representative of three independent experiments.

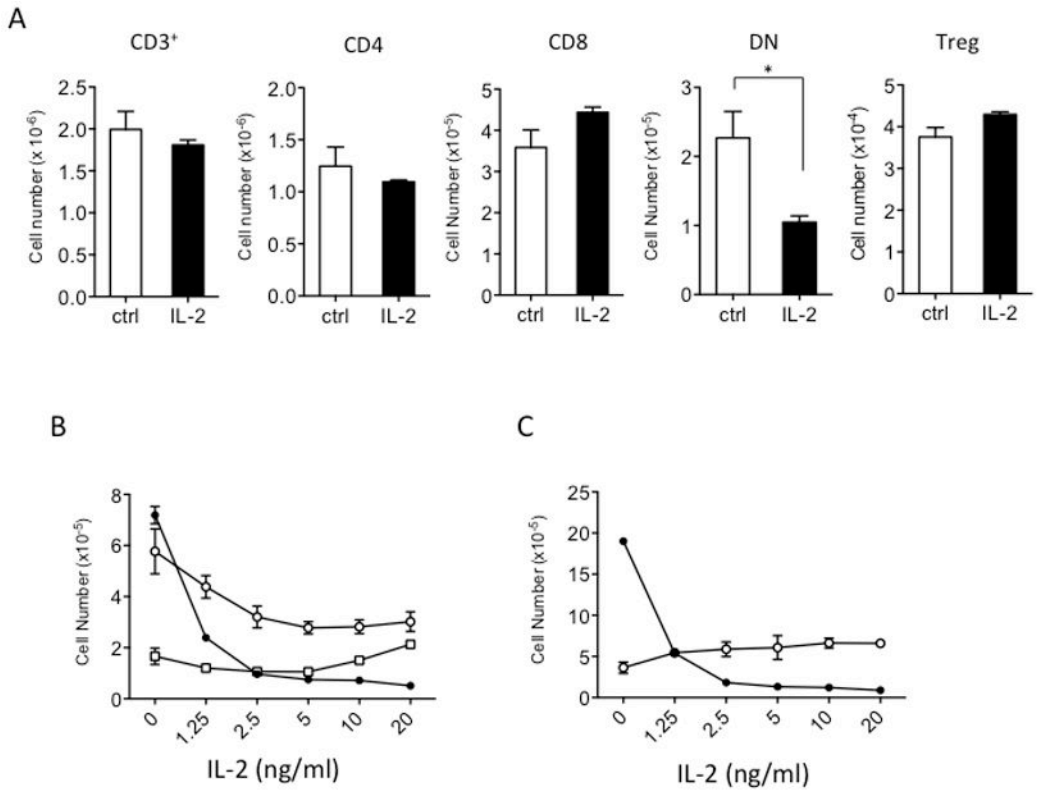

Figure 6. IL-2 indirectly induces a decreased DN T cell pool

(A) Calcein-Violet labeled T cells isolated from MRL/lpr mice were transferred to MRL/MpJ mice and mice were treated with PBS or recombinant IL-2 for 5 days. Calcein-Violet-positive each T cell subset in spleens was counted (*$p<0.05$; mean ± SEM, n=3). (B) Splee cells were incubated in the presence of various concentrations of IL-2 for 5 days. Live $CD4^+$ (white circle), $CD8^+$ (white square) and DN (black circle) T cells were counted. (C) CFSE-labeled DN T cells were cultured with IL-2 in the absence (white cirles) or in the presence (black circles) of splenocytes. The number of CFSE-positive live DN T cells was counted.

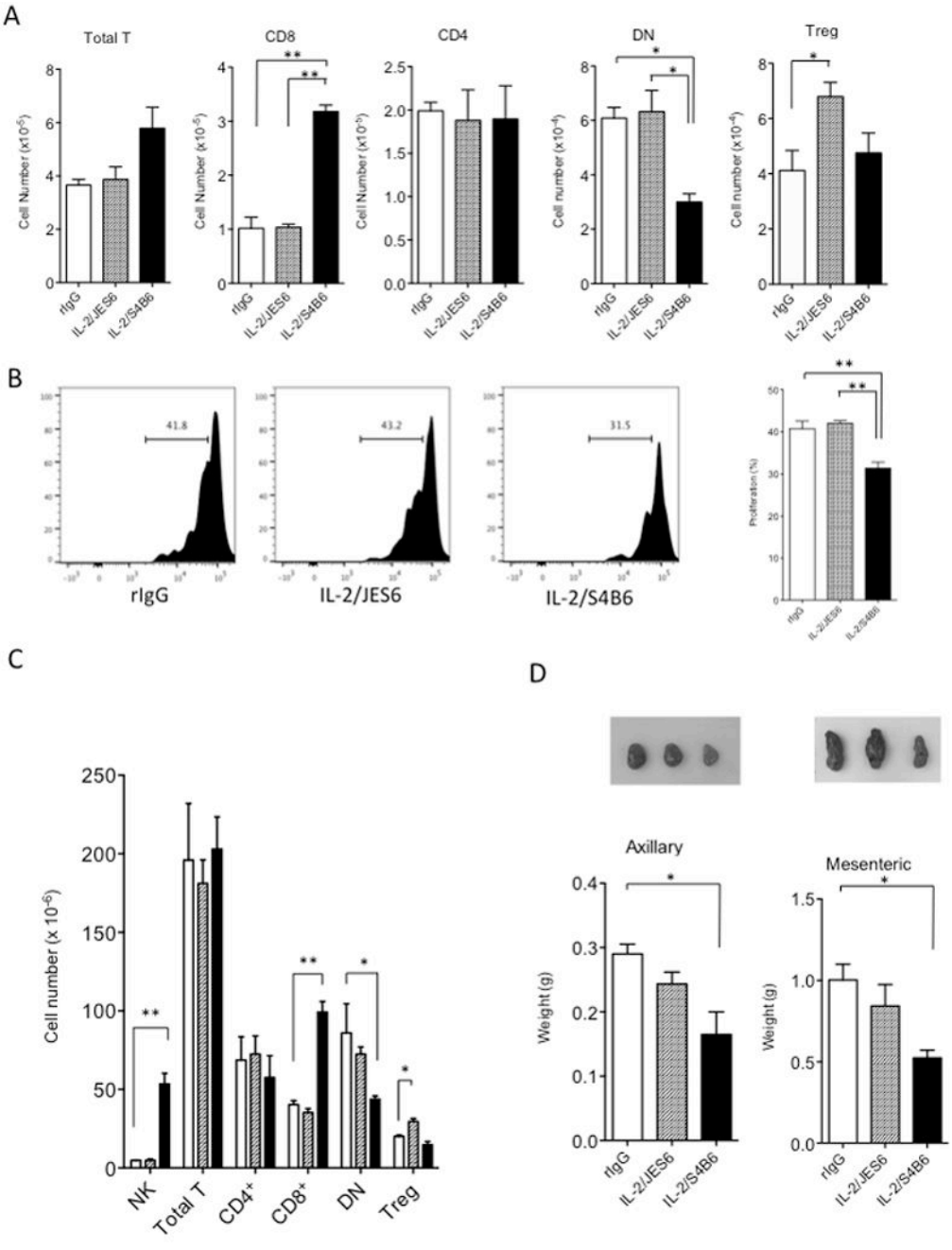

Figure 7. In vivo targeted delivery of IL-2 to cytotoxic lymphocytes reduces DN T cells and lymphadenopathy

(A) CFSE-labeled T cells isolated from diseased MRL/lpr mice were transferred to recipient MRL/lpr mice, and then mice were treated with rat IgG, IL-2/JES6 or IL-2/S4B6 every other day for 7 days. Absolute number of CFSE-positive each T cell subset in spleen was counted (*p<0.05, **p<0.01; mean ± SEM, n=3). (B) CFSE-dilution of DN T cells was detected by flow cytometry. Percentages of proliferating cells were counted and shown in the graph (**p<0.01; mean ± SEM, n=3). Results are representative of two independent experiments. (C) MRL/lpr mice at the age of 14 weeks were treated with rIgG (white bars), IL-2/JES6-1

(shaded bars) and IL-2/S4B6 (black bars) every other day for three weeks. Absolute cell number of each subset of leukocytes from the spleens was counted (*$p<0.05$, **$p<0.01$; mean ± SEM; n=4). (D) Representative picture of axillary lymph nodes and mesenteric lymph nodes from rIgG-, IL-2/JES6- or IL-2/S4B6-treated mice. The weight of lymph nodes was measured (*$p<0.05$; mean ± SEM, n=4). Results are representative of two independent experiments.