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Original Paper

TIPE2 Alleviates Systemic Lupus Erythematosus Through Regulating Macrophage Polarization

Feng Li Xiaohua Zhu Yongsheng Yang Lan Huang Jinhua Xu

Department of Dermatology, Affiliated Huashan Hospital of Fudan University, Shanghai, China

Key Words
Systemic lupus erythematosus (SLE) • Macrophage polarization • M1 macrophage • M2 macrophage • TIPE2 • ALD-DNA

Abstract
Background/Aims: We have recently shown that macrophage polarization may alter the pathogenesis and severity of systemic lupus erythematosus (SLE). However, a practical approach to modulate macrophage polarization *in vivo* is so far not available. In the current study, we aimed to use tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 (TIPE2) to regulate macrophage polarization *in vitro* and *in vivo*, and to study the effects on experimental SLE. ***Methods:*** We prepared adeno-associated virus carrying TIPE2 (AAV-TIPE2). We induced experimental SLE in mice with an activated lymphocyte-derived DNA (ALD-DNA) method. We examined the effects of TIPE2 overexpression on macrophage polarization *in vitro*, and *in vivo* in the SLE model. We also examined the effects of TIPE2 overexpression on the severity of SLE, by serum anti-dsDNA autoantibody, renal pathological changes, and urine protein levels. ***Results:*** ALD-DNA induced SLE-like features in mice, manifested by induction of serum anti-dsDNA autoantibody, renal pathological changes, and increases in urine protein levels. TIPE2 overexpression by AAV-TIPE2 induced macrophage polarization to a M2 phenotype, *in vitro* and *in vivo* in the SLE mouse model. TIPE2 overexpression significantly decreased SLE severity. ***Conclusion:*** TIPE2 alleviates experimental SLE through induction of macrophage polarization to a M2 phenotype, which may be used as a promising therapeutic strategy for treating SLE.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder that occurs in multiple organs characterized with autoantibody production [1-3]. In typical SLE, the immune system

F. Li and X. Zhu contributed equally to this work.

Jinhua Xu
Department of Dermatology, Affiliated Huashan Hospital of Fudan University, 12 Wulumuqizhong Road, Shanghai 200040, (China)
Tel. +862152887783, E-Mail xujinghua_hs@163.com

is aberrantly activated to attack tissue from self as autoantigens, resulting in production of autoantibody and generation of a vicious cycle of chronic inflammation and tissue damage [1-3]. Although it is traditionally believed that lymphocyte abnormalities are the major pathological bases for SLE [1-3], recent studies have shown the evidence of the involvement of monocytes/macrophages in the pathogenesis of the disease [4-6].

Monocyte dysfunction and abnormality have been associated with a variety of autoimmune disorders, including SLE [4-6]. The macrophages can be activated to different phenotypes, mostly commonly simplified as M1 and M2 macrophages, based on differential inductive effects of interleukin-4 (IL-4) and interferon gamma (IFN-ɤ) on macrophages *in vitro* [7-13]. This definition originated from the differential arginine regulation between macrophages from C57BL/6 mice and macrophages from Balb/c mice [12, 13]. Indeed, M1 macrophages respond to Th1 and subsequently produce reactive oxygen species and nitric oxide to kill pathogens and damaged or foreign cells, while M2 macrophages respond to Th2 to mediate humoral immunity and tissue regeneration [7-13]. The differential activation of macrophage phenotypes is termed polarization [12, 13].

Tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 (TIPE2) was first identified from inflamed spinal cords of mouse, as an immune negative molecule critical to homeostasis maintenance in the adaptive and innate immunity [14, 15]. The dysregulation of TIPE2 has been found to mediate diverse immunological diseases [16-20]. Moreover, TIPE2 is shown to inhibit inducible nitric oxide synthase (iNOS) and NO generation, and consequently suppress inflammation by switching arginine metabolism from nitric oxide synthase to arginase [21]. This feature suggests that TIPE2 may be able to modulate macrophage polarization [11, 22-27].

In our recent report, we used an activated lymphocyte-derived DNA (ALD-DNA) method to induce SLE in mice [28]. We found that ALD-DNA induced SLE-like features in mice, manifested by induction of serum anti-dsDNA autoantibody, renal pathological changes, and increases in urine protein levels. Clodronate significantly decreased macrophages in mice, which significantly increased SLE severity. Adoptive transplantation of M2, but not M1 macrophages significantly reduced SLE severity in clodronate- and ALD-DNA-treated mice [28]. In the current study, we showed that TIPE2 overexpression by adeno-associated virus mediated TIPE2 gene transfer induced macrophage polarization to a M2 phenotype *in vitro*, and *in vivo* in the SLE mouse model. TIPE2 overexpression significantly decreased SLE severity.

Materials and Methods

Mouse handling

All animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, China, 1998) and the guidelines of the Fudan University Medical Laboratory Animal Care and Use Committee. Ten-week-old female Balb/c mice (Jackson lab, Bar Harbor, ME, USA) were housed in the pathogen-free mouse colony at our institution, and were used for the current study.

DNA preparation

Genomic DNAs from syngeneic Con A-activated or unactivated splenocytes were extracted and treated with S1 nuclease (Sigma-Aldrich, St. Louis, MO, USA) and proteinase K (Sigma-Aldrich) according to the manufacturer's instructions, and then purified using the UltraPureTM genomic DNA purification kit, as recommended by the manufacturer (Shanghai SBS Genetech, China). DNA concentrations were determined by absorbance measurement at 260 nm. The final A260/A280 for all the DNA preparations was > 1.8.

Animal immunization

BALB/c mice were divided into two groups of 10 mice and actively immunized by subcutaneous injection on the back with 0.2 ml of an emulsion containing the indicated doses of ALD-DNA in phosphate-

buffered saline (PBS). Mouse blood was taken from retro-orbital sinus prior to immunization and at 1-week internals until the end of the experiment.

ELISA

Anti-ssDNA and anti-dsDNA antibody titers were assessed using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well protamine sulphatepretreated polystyrene microtitre plates were coated with prepared DNA at 50μg/ml and placed overnight at 4˚C after incubation at 37˚C for 2 h. The plates were then washed three times with PBS containing 0.05% Tween-20 (PBST). HRP-conjugated goat anti-mouse polyvalent (IgG, IgM, IgA) Ab (Sigma-Aldrich) was used as the secondary Ab. The absorbance was measured at 490 nm on a microtiter plate reader (R&D System, Los Angeles, CA, USA). Results are presented as the mean of triplicate determinations on sera from individual mice.

Isolation, culture and differentiation of bone-marrow derived mouse macrophages

Isolation of bone-marrow derived mouse macrophages has been described before [28]. Marrow from female, 10 week-old Balb/c mice was flushed out with vehicle solution (PBS containing 20 mmol/l Tris and 100mmol/l NaCl, pH 7.5) through a 23G needle. Cells were pre-treated with FITC-conjugated F4/80 antibody (Becton-Dickinson Biosciences, San Jose, CA, USA) and then sorted for positive cells by flow cytometry. Purified F4/80-positive macrophages were cultured in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; Invitrogen, St. Louis, MO, USA) supplemented with 10mmol/l L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin and 100U/ml recombinant M-CSF (mouse M-CSF, R&D Systems). *In vitro* differentiation of macrophages has been performed as has been previously described [28-30].

Cell culture and AAV transduction

Human HEK293 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA). Primary GC cells were cultured in the same media. All cells were incubated in a humidified chamber with 5% CO_2 at 37°C. HEK293 cells or primary bone-marrow derived mouse macrophages were transduced with AAV. AAV (serotype 8)-pCMV-TIPE2 and control AAV-pCMV-scr (a scrambled sequence) were prepared as has been previously described [31-33]. Briefly, we used a pAAV-pCMV-GFP plasmid (Clontech, Mountain View, CA, USA), a packaging plasmid carrying the serotype 8 rep and cap genes, and a helper plasmid carrying the adenovirus helper functions (Applied Viromics, LLC. Fremont, CA, USA) in this study. The TIPE2 construct was prepared from human cDNA, amplified by PCR with EcoRI-restriction-endonuclease-forward and NheI-restriction-endonuclease-reverse primers. The construct was then subcloned into the 50-EcoRI and 30-NheI sites of the pAAV-CMV-GFP vector. Between TIPE2/scr and GFP, there was a 2A peptide-linked multicistronic vectors that are used to express multiple proteins from a single open reading frame (ORF). The small 2A peptide sequences, when cloned between genes, allow for efficient, stoichiometric production of discrete protein products within a single vector through a novel "cleavage" event within the 2A peptide sequence. Sequencing was performed to confirm the correct orientation of the prepared plasmid. AAV was prepared by triple transfection of the pAAV-pCMV-TIPE2 or control pAAV-pCMV-scr plasmid, R2C8 (containing AAV2 Rep and AAV8 capsid genes) and plAd5 (containing adenovirus helper genes) into HEK293 cells by Lipofectamine 2000 reagent (Invitrogen). The viruses were purified using CsCl density centrifugation and then titered by a quantitative densitometric dot-blot assay.

For cell transduction *in vitro*, the HEK293 or primary mouse macrophages were incubated with AAV at a MOI of 100 for 12 hours. For cell transduction *in vivo*, 10^8 AAV were injected into the tail vein.

Fluorescence-activated cell sorting (FACS) for macrophage (subtype)

Treated bone-marrow derived cells were detached from the culture plates with 0.25% Trypsine solution (Invitrogen), washed three times with PBS, re-suspended, labeled with FITC-conjugated F4/80 antibody and/or APC-conjugated MGL antibody (Becton-Dickinson Biosciences), and then sorted for macrophages, or M1 and M2 subtypes. Data were analyzed and quantified using Flowjo software (Flowjo LLC, Ashland, OR, USA).

Assessment of stage score for lupus nephritis

Glomeruli in each mouse were scored blindly by an experienced pathologist for the stage score for lupus nephritis as following. Normal appearance: score 0; Class I (mild disease with small amount of swelling): score 1; Class II (still fairly mild disease but more swelling than Class I): score 2; Class III (moderate degree of swelling with less than 50% of the filtering glomeruli affected): score 3; Class IV (severe degree of swelling with greater than 50% filtering glomeruli affected): score 4; Class V (most of the swelling is confined to the outer layer surrounding the filter unit): score 5; Class VI (most of the filter glomeruli show scarring): score 6. In each mouse, 100 glomeruli were assessed.

Assessment of proteinuria

Proteinuria was measured with the Coomassie brilliant blue assay. Albumin (bovine serum, Sigma-Aldrich) was used as standard curves. Murine urine was centrifuged (20 min, 4500 rpm), and supernatants were taken and diluted at 1:10 with normal saline. After Coomassie brilliant blue solution was added, the sample was tested at 540 nm and the concentration of proteinuria could be calculated by standard curves.

RT-qPCR

RNA was extracted from purified macrophages after FACS with RNeasy (Qiagen, Hilden, Germany). Complementary DNA (cDNA) synthesis was performed by reserve transcription. Quantitative Real-time PCR (RT-qPCR) was subsequently performed in duplicate with a 1:4 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen). Primers are: TIPE2-Forward: 5'-gac tga cca cat acc cca ctc-3'; TIPE2-Reverse: 5'-tca cca aag cta agt gccgt-3'; CD163-Forward: 5'-TCC ACA CGT CCA GAA CAGTC-3'; CD163-Reverse: 5'-CCT TGG AAA CAG AGA CAGGC-3'; CD206-Forward: 5'-CAG GTG TGG GCT CAG GTAGT-3'; CD206-Reverse: 5'-TGT GGT GAG CTG AAA GGTGA-3'; iNOS-Forward: 5'-CAG AGG ACC CAG AGA CAAGC-3'; iNOS-Reverse: 5'-TGC TGA AAC ATT TCC TGTGC-3'; Arginase-Forward: 5'-GCT GTC TTC CCA AGA GTT GGG-3'; Arginase-Reverse: 5'-ATG GAA GAG ACC TTC AGC TAC-3'; α-tubulin-Forward: 5'-CCA AGC TGG AGT TCT CTA -3'; α-tubulin-Reverse: 5'-CAG AGT GCT CCAGG-3'. Data were collected and analyzed using 2^{-Ct} method for quantification of the relative mRNA expression levels. Values of genes were first normalized against α-tubulin, and then compared to the experimental controls.

Statistics

GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA) was used for statistical analyses. Unpaired two-tailed Student t test was applied for comparison between two groups, and one-way ANOVA with the Tukey posttest was applied for comparison between three or more groups. Data were represented as mean ± SD and were considered significant if $p < 0.05$.

Results

Preparation of TIPE2-overexpressing AAV

First, we prepared AAV carrying TIPE2 under the control of a CMV promoter. The control virus carried a scrambled sequence under the control of a CMV promoter. Both viruses also had a GFP reporter. In order to validate the virus, we infected HEK293 cells with either AAV, at a MOI of 100. The infection was analyzed by flow cytometry based on GFP and the transduced cells were purified (Fig. 1A). These purified transduced cells expressed strong GFP in culture (Fig. 1B). Then we analyzed TIPE2 levels in the purified transduced cells and found that TIPE2-tranduced cells increased their TIPE2 mRNA levels by 13.5±1.6 fold (Fig. 1C), and TIPE2 protein levels by 10.8 ± 1.6 fold (Fig. 1D). Thus, the quality of the TIPE2-overexpressing AAV was confirmed.

TIPE2 induces macrophage polarization in vitro

Next, we examined whether TIPE2 may modulate macrophage polarization *in vitro*. We isolated mouse macrophages from bone marrow based on F4/80 expression by flow cytometry (Fig. 2A) and then put these purified macrophages in culture (Fig. 2B). Macrophages were then transduced with either AAV-TIPE2 or AAV-scr, and then analyzed for

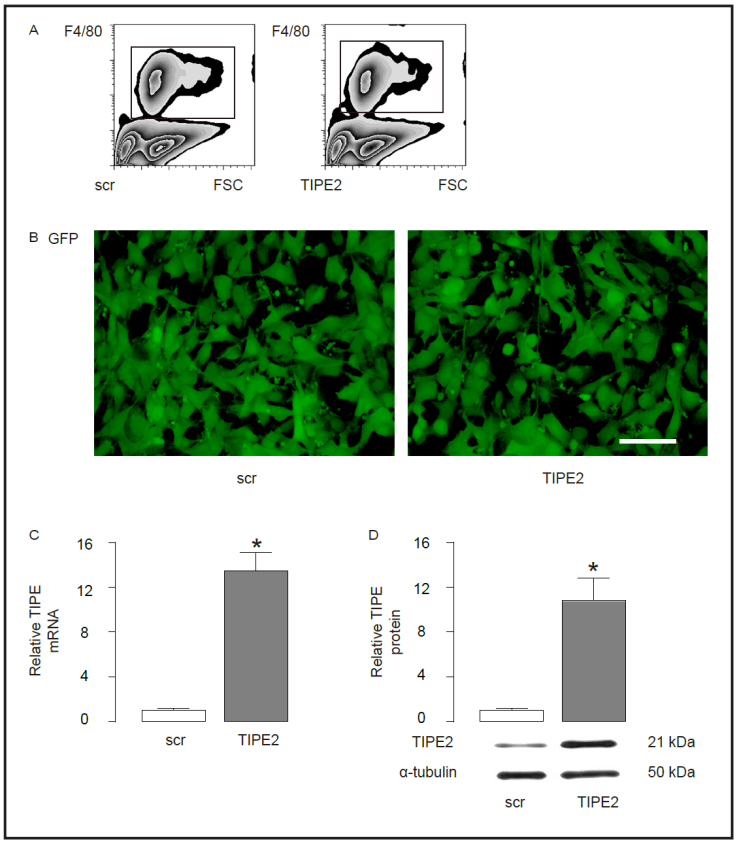

Fig. 1. Preparation of TIPE2-over-expressing AAV. We prepared AAV carrying TIPE2 under the control of a CMV promoter. The control virus carried a scrambled sequence under the control of a CMV promoter. Both viruses also had a GFP reporter. In order to validate the virus, we infected HEK293 cells with either AAV, at a MOI of 100. (A) The infection was analyzed by flow cytometry based on GFP and the transduced cells were purified. (B) These purified transduced cells expressed strong GFP in culture. (C-D) We analyzed TIPE2 levels in the purified transduced cells and found that TIPE2-tranduced cells increased their TIPE2 mRNA levels by 13.5 ± 1.6 fold (C), and TIPE2 protein levels by 10.8 ± 1.6 fold (D). Scale bar is 20μm. * $p < 0.05$. N = 5. Statistics: one-way ANOVA with the Tukey posttest.

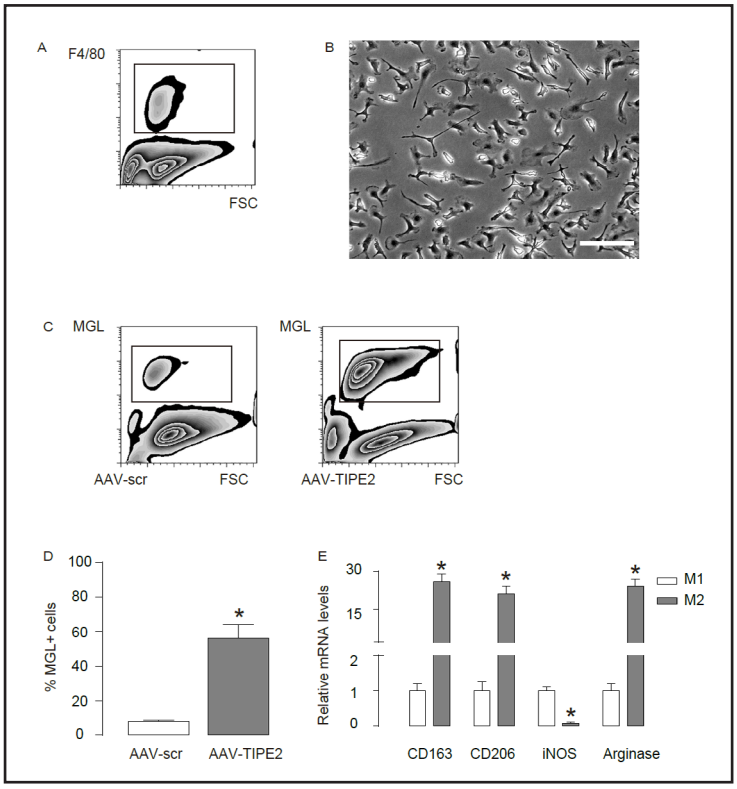

Fig. 2. TIPE2 induces macrophage polarization *in vitro*. (A) We isolated mouse macrophages from bone marrow based on F4/80 expression by flow cytometry. (B) The purified macrophages in culture. (C-D) Macrophages were then transduced with either AAV-TIPE2 or AAV-scr, and then analyzed for macrophage polarization by expression of a M2-specific marker MGL on the cells using flow cytometry (C), showing that TIPE2 increased M2 vs M1 ratio by about 7 fold (D). (E) M1 or M2 macrophages were then isolated and further confirmed of the phenotype by RT-qPCR examination of CD163, CD206, iNOS and Arginase. Scale bar is 20μm. * $p < 0.05$. N = 5. Statistics: one-way ANOVA with the Tukey posttest.

macrophage polarization by expression of a M2-specific marker MGL on the cells using flow cytometry (Fig. 2C), showing that TIPE2 increased M2 vs M1 ratio by about 7 fold (Fig. 2D),

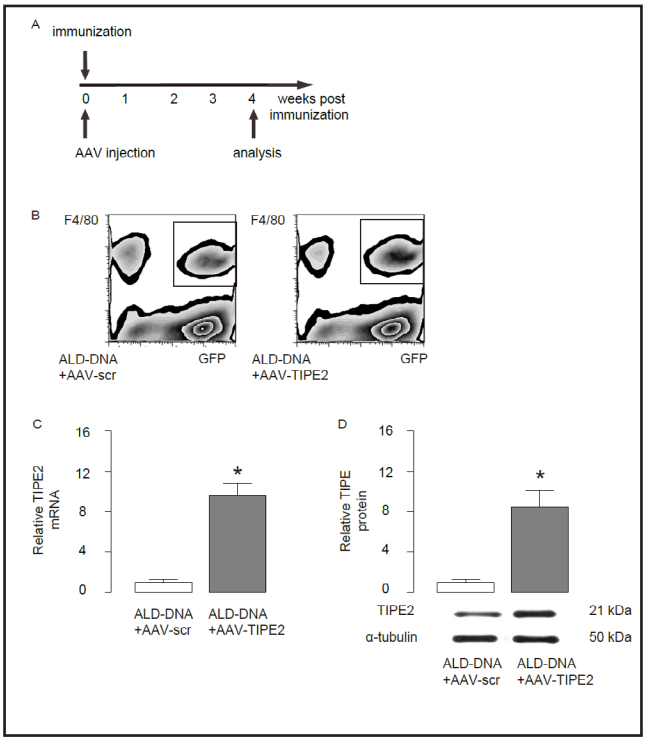

Fig. 3. AAV-TIPE2 increases TIPE2 levels in macrophages *in vivo* in SLE model. (A) Schematic of the experiment: We used a previously established SLE model, in which anti-dsDNA antibodies were induced by immunization with ALD DNA. Two groups of 10 BALB/c mice were first administered 50μg of ALD DNA, and received either tail vein injection of AAV-TIPE2 or AAV-scr, and then were followed for 4 weeks. (B) At 4 weeks, the macrophages from the kidney digests that were analyzed for viral transduction by flow cytometry based on GFP. (C-D) We found that the GFP+F4/80+ macrophages from AAV-TIPE2-treated mice had significantly higher levels of TIPE2 mRNA (C) and TIPE2 protein (D), compared to those from AAV-scr-treated mice. * $p < 0.05$. N = 10. Statistics: one-way ANOVA with the Tukey posttest.

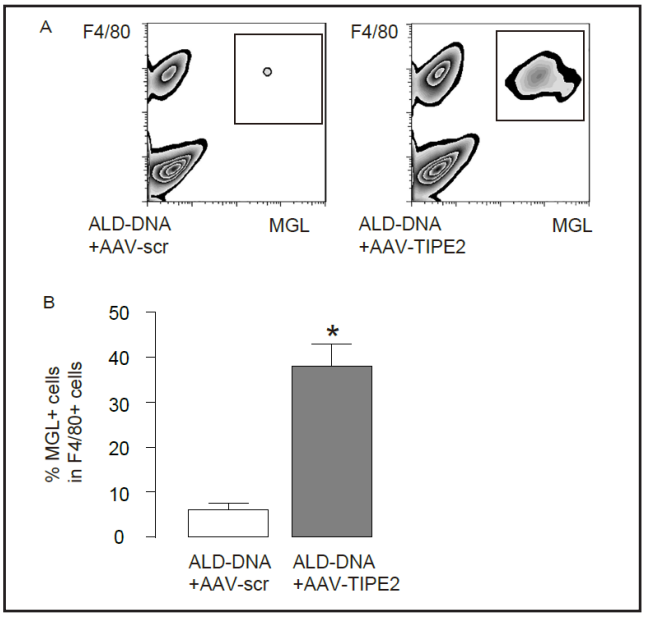

Fig. 4. AAV-TIPE2 induces macrophage polarization *in vivo* in SLE model. (A-B) We analyzed macrophage polarization from GFP+F4/80+ cells, and found that the GFP+F4/80+ macrophages from AAV-TIPE2-treated mice had significantly higher ratio of MGL+ vs MGL- cells, by representative flow charts (A), and by quantification (B). * $p < 0.05$. N = 10. Statistics: one-way ANOVA with the Tukey posttest.

which suggests that TIPE2 may induce macrophage polarization to a M2 phenotype. M1 or M2 macrophages were then isolated and further confirmed of the phenotype by RT-qPCR examination of CD163, CD206 and Arginase, which are predominantly expression by M2 macropahges, and of iNOS, which is predominantly expressed by M1 macrophages (Fig. 2E). Thus, these data suggest that TIPE2 induces macrophage polarization *in vitro*.

AAV-TIPE2 increases TIPE2 levels in macrophages in vivo in SLE model

Next, we used a previously established SLE model, in which anti-dsDNA antibodies were induced by immunization with ALD DNA. Two groups of 10 BALB/c mice were first administered 50μg of ALD DNA, and received either tail vein injection of AAV-TIPE2 or

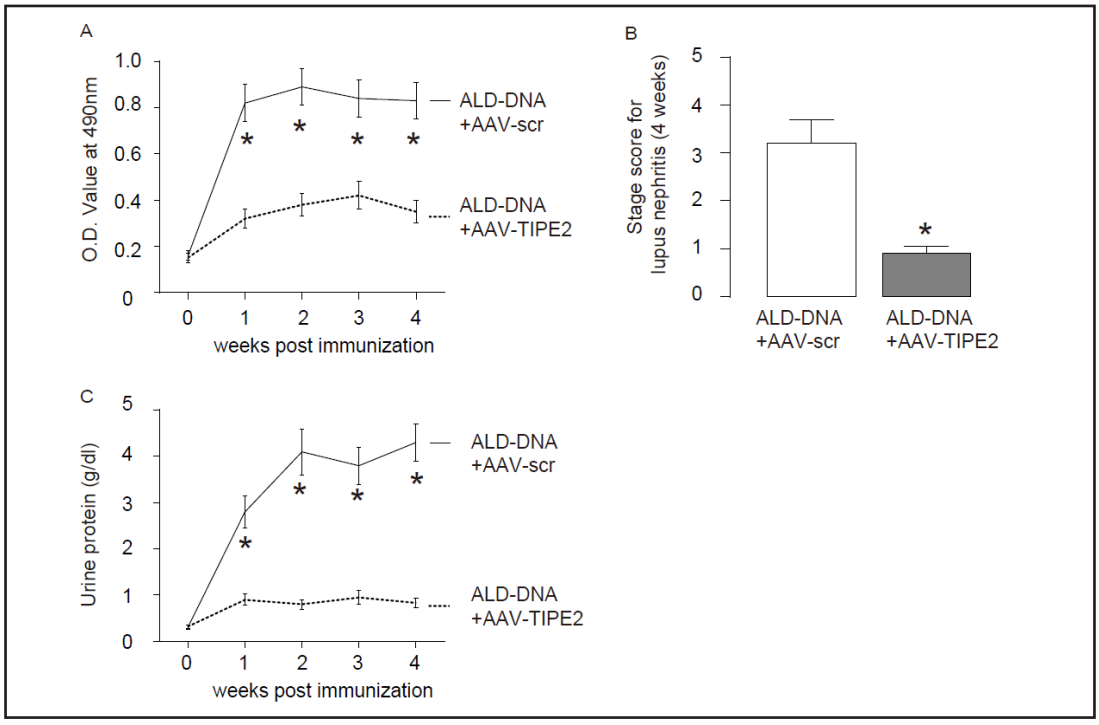

Fig. 5. TIPE2 alleviates SLE severity *in vivo*. We examined the effects of TIPE2 on SLE severity *in vivo*. (A) We detected significantly lower levels of IgG anti-dsDNA antibodies in the sera from the AAV-TIPE2-treated, ALD-DNA-immunized mice, compared to the AAV-scr-treated, ALD-DNA-immunized mice. (B) The pathological changes in kidneys were then assessed, showing significantly lower score for lupus nephritis in the AAV-TIPE2-treated, ALD-DNA-immunized mice, compared to the AAV-scr-treated, ALD-DNA-immunized mice. (C) AAV-TIPE2-treated, ALD-DNA-immunized mice had significantly lower urine protein, compared to the AAV-scr-treated, ALD-DNA-immunized mice. * $p < 0.05$. N = 10. Statistics: one-way ANOVA with the Tukey posttest.

AAV-scr, and then were followed for 4 weeks (Fig. 3A). At the end of the experiment, the macrophages from the kidney digests that were analyzed for viral transduction by flow cytometry based on GFP (Fig. 3B). We found that the GFP+F4/80+ macrophages from AAV-TIPE2-treated mice had significantly higher levels of TIPE2 mRNA (Fig. 3C) and TIPE2 protein (Fig. 3D), compared to those from AAV-scr-treated mice. Thus, the AAV-TIPE2 worked *in vivo*.

AAV-TIPE2 induces macrophage polarization in vivo in SLE model

Then, we analyzed macrophage polarization from GFP+F4/80+ cells, and found that the GFP+F4/80+ macrophages from AAV-TIPE2-treated mice had significantly higher ratio of MGL+ vs MGL- cells, by representative flow charts (Fig. 4A), and by quantification (Fig. 4B). Thus, AAV-TIPE2 induces macrophage polarization *in vivo* in SLE model.

TIPE2 alleviates SLE severity in vivo

Finally, we examined the effects of TIPE2 on SLE severity *in vivo*. We detected significantly lower levels of IgG anti-dsDNA antibodies in the sera from the AAV-TIPE2-treated, ALD-DNA-immunized mice, compared to the AAV-scr-treated, ALD-DNA-immunized mice (Fig. 5A). The pathological changes in kidneys were then assessed, showing significantly lower score for lupus nephritis in the AAV-TIPE2-treated, ALD-DNA-immunized mice, compared to the AAV-scr-treated, ALD-DNA-immunized mice (Fig. 5B). Moreover, AAV-TIPE2-treated, ALD-DNA-immunized mice had significantly lower urine protein, compared to the AAV-scr-treated, ALD-DNA-immunized mice (Fig. 5C). Together, these data suggest that TIPE2-induced macrophage polarization alleviates SLE severity *in vivo*.

Discussion

Macrophage is a type of leukocytes that engulf and digest cellular debris, foreign substances, microbes and neoplastic cells [34-37]. Moreover, recent studies have shown that besides these macrophages with a classical phenotype termed "M1" macrophages, there is another macrophage phenotype termed "M2" that mediates humoral immunity and tissue repair [34-37]. Interestingly, a given macrophage can respond to the extracellular stimulation and then shift between M1 and M2 phenotype, which is termed "polarization" [34-37]. The M1 macrophages express nitric oxide synthase (iNOS) and some pro-inflammatory cytokines and toxin like reactive oxygen species (ROS) [34-37]. M2 macrophages are specified as expression of arginase, and surface markers CD163, CD206 and CD301 that are not expressed by M1 macrophages [11, 23, 24, 26, 34-40]. Macrophage polarization has been shown to regulate a variety of biological processes, as recently nicely reviewed [41-43]. TIPE2 was recently found to inhibit inducible nitric oxide synthase (iNOS) and thus generation of nitric oxide, resulting in suppression of inflammation [11, 21-27]. Hence, TIPE2 appears to be a potent regulator for M1/M2 polarization. However, whether TIPE2 may be used to regulate SLE has not been reported.

Here, we address this question using a well-established SLE model, in which anti-dsDNA antibodies were induced by immunization with ALD DNA. In one of our recent reports, we evaluated the roles of macrophages/macrophage subtypes in SLE, and used a specific macrophage eliminator clodronate to deplete all macrophages. We found that clodronate significantly aggravated the SLE-associated lupus nephritis and proteinuria, while adoptive transplantation of M2 macrophages, but not M1 macrophages, abolished clodronate-induced augment in SLE severity. These data suggest that M1 and M2 macrophages play different roles in development of SLE. M1 macrophages appear to increase the severity of SLE, while M2 macrophages reduce it. Thus, in our study, we tried to use TIPE2 to induce M1-to-M2 polarization and evaluate its effects on SLE severity. Indeed, our results confirmed the validity of our hypothesis, showing that ALD-DNA induced SLE-like features in mice were significantly alleviated by overexpression of TIPE2 in macrophages. TIPE2 overexpression by AAV-TIPE2 induced macrophage polarization to a M2 phenotype, *in vitro* and *in vivo* in the SLE mouse model.

The AAV virus induced relatively stable gene transfer, compared to adenovirus. On the other hand, AAV also induced very limited systemic inflammation [31-33, 44-46]. These merits were taken as an advantage in the current study. We also checked the effects of TIPE2 depletion on SLE in a loss-of-function experiment. However, since the initial level of TIPE2 in macrophages was too low, this loss-of-function experiment did not give a pronounced result.

To summarize, our study showed that TIPE2 alleviated experimental SLE through induction of macrophage polarization to a M2 phenotype, which may be used as a promising therapeutic strategy for treating SLE.

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Disclosure Statement

The authors have declared that no competing interests exist.

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