Lactobacillus Acidophilus Supplementation Exerts A Synergistic Effect on Tacrolimus Efficacy by Restoring Th17/Treg Imbalance in Lupus-Prone Mice via the SIGNR3 Pathway

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Abstract

Background: Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterised by tissue-binding autoantibodies and immune complexes. Tacrolimus (Tac), also known as FK506, is an immunosuppressant used in the treatment of lupus; however, it induces T-cell imbalances. *Lactobacillus acidophilus* (LA) is reported to have therapeutic efficacy in immune-mediated diseases via T-cell regulation. This study investigated whether a combination therapy of LA and Tac improves the therapeutic efficacy of Tac by ameliorating T-cell imbalance in an animal model of SLE. Eight-week-old MRL/Mp-*Fas*^{lpr} (MRL/*lpr*) mice were orally administered with 5 mg/kg of Tac and/or 50 mg/kg of LA daily for 8 weeks. Caecal microbiota compositions, serum autoantibodies levels, the degree of proteinuria, histological changes in the kidney, and populations of various T-cell subsets in the spleen were analysed.

Results: MRL/*lpr* mice presented with significant gut microbiota imbalances, which were subsequently recovered by the combination treatment of Tac and LA. Double negative T-cells, a pathogenic subset of T-cells, in the peripheral blood and spleens of MRL/*lpr* mice were significantly decreased by the combination therapy. The combination treatment also reduced serum levels of anti-double-stranded DNA antibodies and immunoglobulin G2a, and renal pathology scores were markedly alleviated. The combination therapy induced Treg cells and decreased Th17 cells both *in vitro* and *in vivo*. *In vitro* treatment with LA induced the production of indoleamine-2,3-dioxygenase, programmed death-ligand 1, and inerleukin-10, which was partially mediated by the induction of the specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3 (SIGNR3) receptor.

Conclusions: The present findings indicate that LA augments the therapeutic effect of Tac and restores Th17/Treg balance in a murine model of lupus. Accordingly, the combination treatment of Tac with LA could be a promising therapeutic candidate for lupus.

Background

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterised by the presence of tissue-binding autoantibodies and the formation of immune complexes [1]. SLE can affect multiple organ systems concurrently, and can have fatal consequences due to damage to organs such as the kidneys or the central nervous system [1]. Its pathophysiology likely involves the innate and adaptive immune systems, as well as environmental and genetic factors; however, its clinical complexity and heterogeneity remain a challenge [2–4].

Currently, there is no cure for SLE; most therapeutic modalities focus on nonspecific immune suppression [1, 5–7]. Tacrolimus (Tac), also known as FK506, is one of the most widely used immunosuppressants in organ transplant recipients and patients with autoimmune diseases, including those with lupus nephritis (LN); it inhibits calcineurin, thereby suppressing T-cell development and proliferation [8, 9]. However, Tac also represses the production of interleukin (IL)-2, which is essential for regulatory T (Treg) cell function [10–12]. Reductions in Treg cell production can cause an imbalance in T-helper 17 (Th17) and Treg cell

numbers, leading to dysfunctions in immune regulation; in fact, an imbalanced Th17/Treg ratio has been suggested as a pathognomonic immune alteration of SLE [13]. For these reasons, the therapeutic role and efficacy of Tac is limited.

Changes in gut microbiota are observed in various autoimmune diseases [14, 15]. Bacterial dysbiosis is seen in SLE patients and, to some extent, in murine models of lupus [16–19]. Furthermore, the dysbiosis is not only detected in the intestinal microbiota, but also in the oral mucosa and skin of lupus-affected subjects [20–22]. Therefore, bacterial dysbiosis may be related to the clinical status, or even the dietary metabolism, of lupus patients [23–25]. Whether changes in the caecal bacterial composition of lupus patients are merely coincidental, or are genuine etiological factors of the disease, are unknown; however, probiotic implantation as a novel therapeutic modality in SLE shows some promise [26]. More specifically, the administration of probiotics has been demonstrated to improve lupus-related clinical features and reduce inflammatory cytokines in lupus-prone and lupus-induced mouse models [26, 27]. Probiotic treatment also affected the T-cell population, represented by a skewing of the Th17/Treg ratio towards an immune-regulatory phenotype. Moreover, this effect was not limited to the gut mucosa or its associated lymphoid tissues, but instead was systemic [27].

Against this background, probiotic supplementation is hypothesised to augment the therapeutic efficacy of Tac in SLE by limiting its destabilizing effects on the Th17/Treg ratio. To investigate whether Tac and probiotic supplementation is more effective for treating lupus when administered in combination rather than separately, the gut microbiota were assessed before and after the development of a lupus-like phenotype in an animal model of SLE. Then, Tac with and without probiotics was administered, and efficacy was compared for various measures.

Results

Gut dysbiosis of lupus-prone mice

MRL/Mp-*Fas*^{lpr} (MRL/*lpr*) mice were used in a murine model of lupus for this study because they show lupus-like features such as splenomegaly, lymphadenopathy, and glomerulonephritis [28]. These mice present with different caecal microbiota compositions depending on their age and the presence of lupus-mimicking phenotypes [17]. The proportion of gut bacteria from the order *Lactobacillales* has been reported to be decreased in lupus-prone mice [17, 18]. Here, the diversity and composition of gut microbiota were evaluated in mice before (6 weeks old) and after (16 weeks old) the development of a lupus-mimicking disease. The acquisition of a lupus-like phenotype was associated with significant reductions in gut bacterial diversity, and microbial compositions were different compared to the pre-disease state (Fig. 1A and 1B). Data from the 16 s ribosomal ribonucleic acid sequencing of caecal microbiota revealed that the relative abundancies of *Lactobacillaceae* and *Lactobacillus* at the family and genus level, respectively, were significantly reduced after disease onset (Fig. 1C and 1D).

Lactobacillus acidophilus (LA) improves gut dysbiosis and decreases the proportion of double negative T-cells

Given the evidence supporting *Lactobacillus* supplementation for regulating gut homeostasis and immunological imbalances in lupus, LA, a species from the genus *Lactobacillus* was chosen to supplement Tac treatment [17, 26, 27, 29]. Tac (5 mg/kg) with or without LA (50 mg/kg) was administered daily to 8-week-old MRL/*lpr* mice for 8 weeks. When given alone, Tac did not sufficiently restore the reduced diversity index of caecal microbiota in the 16-week-old lupus-prone mice. By contrast, in mice treated with LA and Tac, the Shannon diversity indices were significantly improved, indicating enrichment of the caecal bacterial composition (Fig. 2A).

SLE patients show increases in the population of cluster of differentiation (CD)4^- $CD8^-$ T-cells, also known as double negative T (DNT) cells [30]. DNT cells produce inflammatory cytokines and infiltrate target tissues in lupus patients [31]. Similar to humans, MRL/*lpr* mice also show an increase, of up to 80%, in the proportion of DNT cells [32]. Notably, the proportion of DNT cells correlates with the severity of lupus-like phenotypes in MRL/*lpr* mice. Thus, changes in DNT cell proportions were assayed biweekly for the duration of the *in vivo* experiment, and the effect of the different treatments on their proportions was investigated. The percentage of DNT cells in the peripheral blood of MRL/*lpr* mice increased to over 80%. Tac-alone treatment did not significantly change the percentage of DNT cells, but when combined with LA, the proportion of DNT cells were significantly reduced (Fig. 2B). The combination treatment also decreased the DNT cell population in splenocytes acquired from 16-week-old MRL/*lpr* mice (Fig. 2B). Moreover, the sizes and weights of spleens, which represent the severity of the lupus-like phenotype, in MRL/*lpr* mice were significantly lower in the combination-treated mice (Fig. 2C). These findings suggest that, in the murine model, oral administration of LA in addition to Tac could improve caecal microbial composition and alleviate lupus-like features by reducing the proportion of DNT-cells.

Impact of the LA and Tac combination treatment on renal inflammation of lupus-prone mice

Histopathological assessments of kidney tissue acquired from 16-week-old MRL/*lpr* mice, with and without treatment, were performed to determine whether the effects of oral probiotic administration on DNT cells also apply to renal tissue inflammation.

Although Tac is prescribed to patients with membranous LN, the Tac-alone treatment did not significantly reduce renal inflammation in the lupus-prone mice. By contrast, as with systemic autoimmunity, supplementation of LA with Tac significantly reduced inflammation of murine kidneys, as reflected in the representative images for renal pathology (Fig. 3A) and semiquantitative scores for glomerulonephritis, interstitial nephritis, and vasculitis (Fig. 3B). These findings imply that the addition of oral probiotics augments the effects of Tac on kidney inflammation in lupus-prone mice.

Tac and LA combination treatment improves systemic autoimmunity in lupus-prone mice

The impact of the combination treatment on systemic autoimmunity was assayed by measuring levels of immunoglobulin G (IgG) 2a and anti-double-stranded deoxyribonucleic acid (dsDNA) antibodies, which are the most pathognomonic autoantibodies in lupus, as well as their isotypes in the sera of MRL/*lpr* mice [33, 34]. Serum levels of total anti-dsDNA antibodies and their isotypes, IgG2a and IgG3, increased over time in the *in vivo* experiments. The Tac-only treatment did not significantly reduce their levels (Fig. 4A). The increase in autoantibodies was reduced in the combination-treated mice, but the reduction did not reach statistical significance. Meanwhile, as overall sera IgG2a levels increased over time in MRL/*lpr* mice, the Tac-alone treatment significantly inhibited IgG2a production, and its efficacy was increased by the addition of LA (Fig. 4B).

The immune complexes causing renal inflammation in lupus are mainly autoantibodies and immunoglobulins [35, 36]. The relationship between levels of circulating autoantibodies and immunoglobulins and the severity of proteinuria was assessed. Proteinuria severity in 16-week-old MRL/*lpr* mice, as indexed by the levels of creatinine and albumin in urine, was ameliorated by both treatments (Fig. 4C and 4D). Accordingly, the Tac and LA combination treatment may reduce circulating autoantibodies and the production of pathogenic immunoglobulins, thereby ameliorating the renal tissue damage caused by immune complex deposition.

Th17/treg Imbalance Was Alleviated By La Supplementation

SLE presents with an imbalance between Th17 and Treg cell populations. Despite its wide usage in lupus patients, Tac can negatively affect Treg cell differentiation [10, 11, 13]. Because previous studies have reported that LA administration can restore T-cell balance [27, 37], the therapeutic effects of LA when added to Tac may result from a restored Th17/Treg balance. Changes in the proportion of Th17 and Treg cells following Tac treatment, with and without LA supplementation, were evaluated *ex vivo* using cryosectioned spleen tissues from 16-week-old MRL/*lpr* mice treated as described above, and *in vitro* using total splenocytes from the same mice. Similar to the kidney, the spleen tissues from mice treated with the combination regimen show significantly lower inflammation (Fig. 5A). Confocal imaging of spleen tissue from the 16-week-old mice show reduced numbers of IL-17-expressing T-cells and increased numbers of forkhead box P3 (Foxp3)-expressing T-cells in the Tac + LA-treated mice only (Fig. 5B). In total splenocytes from lupus-prone mice that were stimulated *in vitro* with anti-CD3 antibodies and treated with Tac, LA, or Tac + LA, only the combination treatment significantly inhibited Th17 cell differentiation and showed a tendency to induce Treg cells, when compared to controls (Fig. 5C). Notably, cells treated with Tac alone showed slightly decreased Treg differentiation relative to the control. Cytokine quantification in the supernatants of the splenocytes obtained under the aforementioned conditions revealed that the levels of inflammatory IL-17 and regulatory IL-10 were significantly decreased and increased, respectively, in the Tac + LA treated cells compared to controls, and to the Tac- and LA-alone treated cells (Fig. 5D).

The specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3 (SIGNR3) receptor mediates the immune regulatory properties of LA

Gut microbiota express surface proteins that interact with specific receptors on the host's immune cells, potentially affecting the systemic immune system [38]. Surface layer protein (Slp) A is a unique protein expressed on the surface of LA [39]. It binds to a specific C-type lectin receptor of immune cells, SIGNR3 [40]. The interaction between SlpA and SIGNR3 can skew T-cell differentiation towards immune regulation, leading to an increased proportion of Treg cells [40].

To investigate the role of SIGNR3 in host-microbial interactions, the effect of probiotics on SIGNR3 expression in lupus-prone mice was first evaluated. Spleen tissues extracted from LA and Tac-treated MRL/*lpr* mice contained more SIGNR3-immunopositive cells than those from mice in the other treatment groups (Fig. 6A). In subsequent *in vitro* experiments, lipopolysaccharide (LPS)-stimulated splenocytes from MRL/*lpr* mice were cultured under Tac or Tac + LA. Only the splenocytes treated with Tac + LA showed significantly higher messenger ribonucleic acid (mRNA) expression levels of SIGNR3 and other immune-regulatory factors, including indoleamine-2,3-dioxygenase (IDO), programmed death-ligand 1 (PD-L1), and IL-10 (Fig. 6B). Next, SIGNR3 was silenced in splenocytes using small interfering ribonucleic acid (siRNA) transfection (Fig. 6C). The siRNA-transfected cells did not show increased mRNA expression of regulatory cytokines, even with the combination treatment (Fig. 6D). These results demonstrate that the immune regulatory effects of LA could be due to SIGNR3-mediated host-microbial interactions.

The efficacy of LA for restoring Th17/Treg balance in human cells

Lastly, the immune regulatory effects of LA were tested *in vitro* in human cells. Isolated peripheral blood mononuclear cells (PBMCs) from healthy humans were stimulated with anti-CD3 antibodies in the presence of Tac, LA, or Tac + LA. After 96 h of cell culture, the Tac + LA-treated cells showed the largest reduction and elevation in IL-17 + CD4 and CD25 + Foxp3 + CD4 T-cell numbers, respectively (Fig. 7A). Similar to the murine splenocytes, the Tac-alone-treated cells showed a lower proportion of Treg cells compared to the control. Enzyme-linked immunosorbent assay (ELISA) of the supernatants of the cells revealed that those treated with the combination of LA and Tac had the greatest decrease and increase in IL-17 and IL-10 levels, respectively (Fig. 7B). PBMCs from lupus patients displayed analogous results under the same conditions, although statistical significance was not reached (Fig. 7C). These findings show the potential immunomodulatory effects of adding LA to Tac for the treatment of lupus patients.

Discussion

In this study, probiotic supplementation of an immunosuppressant increased its efficacy in a lupus animal model by improving gut dysbiosis and restoring Th17/Treg balance. More specifically, LA supplementation restored the caecal microbial composition in lupus-prone mice. This probiotic significantly improved the efficacy of Tac, in terms of regional inflammation and systemic autoimmunity, in lupus-mimicking mice, where the proportion of DNT cells and levels of serum autoantibodies were reduced, and renal histology was improved. Based on the present results, LA appears to ameliorate

Th17/Treg imbalance, since silencing SIGRN3 blocks its regulatory effects. This report is the first to demonstrate the potential efficacy of a lupus treatment that combines probiotic supplementation with a general immunosuppressant.

Since its efficacy was first demonstrated in murine models [41], Tac has been suggested as a treatment option for lupus, and especially for membranous LN, in the guidelines of European countries [7]. However, the exact mechanisms underlying the clinical efficacy of Tac in lupus patients remain unclear. Tac is thought to globally inhibit T-cell proliferation and differentiation [8, 9]. IL-2, which is transcriptionally suppressed by Tac, is essential for Treg cell differentiation [42, 43], and pharmacological suppression thereof can cause an imbalance in the Th17/Treg ratio, leading to immune dysregulation [44]. The immune-dysregulating effects of Tac, exerted via the inhibition of Treg cells, have been described in studies on organ transplantation, for which Tac is more widely used than in lupus patients. Organ transplant recipients treated with Tac were more likely to experience acute rejection, possibly due to a Tac-induced reduction in Treg cells stemming from increased Treg apoptosis and attenuation of activity in IL-2-related pathways [45]. According to *in vitro* data, IL-2 supplementation could promote organ transplant survival [46]. In this study, Tac inhibited Treg cell differentiation and regulatory cytokine secretion in a murine lupus model, both *in vitro* and *in vivo*. Tac-induced immune suppression, likely due to its effects on Treg cell differentiation, has a modest inhibitory effect on immune effector cell function, but its overall efficacy is limited by Treg cell shortages related to IL-2. Overall, Tac did not display noteworthy immune-modulatory effects on systemic autoimmunity, as demonstrated by the DNT cell population and serological status, nor on regional inflammation, such as in the kidney, where Tac is mainly used in lupus patients.

Several attempts have been made to compensate for Tac-related immune-dysregulation, including IL-2 replacement [46], which can lead to flare-ups of lupus-like symptoms, and inhibition of intracellular signals [47]. However, the efficacy of these approaches have not been demonstrated sufficiently in humans. The immune-regulatory properties of probiotic supplementation have been widely reported in animal models of various diseases [27]. Given the ease of administration in humans and lupus patients, probiotics are used as immune modulators. Species from the *Lactobacillus* family were considered as candidates for promoting Treg cell expression, based on gut microbiome profiles in lupus-prone murine models [16–18]. While some *Lactobacillus* species could induce Treg cell expression [26, 27, 29, 40, 48], other strains, such as *L. reuteri*, have been suggested to confer a protective effect in murine models, but also act as potent mediators in lupus patients [49]. Therefore, the choice of bacteria should be carefully considered at the species level. Ultimately, LA, a probiotic consistently reported to have Treg-inducing effects in animal models, was used as a complementary factor for Tac [29, 37, 40, 50, 51]. Peterson et al. previously reported that LA could promote Treg activity in a mouse model of colitis [29]. The immune-regulatory effects of LA are not limited to simply enhancing Treg cell functional activity, but also include affecting the Th17/Treg balance by increasing the number of Treg cells. In this study, an immune-modulatory effect of LA was observed *in vitro*, and the *in* vivo results showed that it led to a systemic reduction in autoimmunity.

Trillions of microbiota reside within the gut mucosa and interact with the host [38]. After colonization, supplemented probiotics exert their regional and systemic effects in the same manner. LA possesses three types of Slps: SlpA, SlpB, and SlpX [39]. These mainly interact with pattern recognition receptors on host cells, such as dendritic cells (DCs) and macrophages. Lightfoot et al. reported that SlpA is associated with immune regulation; it binds to SIGNR3 [40], which resembles the human DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) [52]. Whereas the human DC-SIGN is mainly involved in allergic reactions and fungal immunity [53, 54], SIGNR3, a murine homolog, confers its regulatory properties by increasing the expression of protective cytokines, such as IL-10 [40]. To confirm whether the Slps-SIGNR3 interaction is central to the immune-regulatory functions of LA, SIGNR3 was deleted in murine cells in this study, which resulted in repression of LA-mediated immune regulation. Notably, the immune-regulatory mechanisms of LA supplementation seemed to be separate from the IL-2-mediated T-cell suppression of Tac, possibly accounting for its effects on Tac efficacy. In support of this, the *in vivo* results indicated that probiotic immune regulation is independent from Tac immunosuppression, as does the gradually increasing efficacy observed in the *in vitro* experiments. However, whether *Lactobacillus* species are reduced in the gut microbiota of lupus patients according to the disease state, and whether supplementation with other bacteria possessing the same surface protein (i.e., SlpA) exert the same immune-regulatory effects in lupus, needs further study. Also, whether human DC-SIGN, a human homolog of SIGNR3, is the same target receptor of LA in lupus patients remains to be determined.

Conclusion

The high heterogeneity of lupus manifestations precludes the use of general immunosuppressants as a treatment. Although disease phenotype clustering and the application of specific treatments are increasing, numerous challenges remain. Given the importance of Th17/Treg imbalance in lupus pathogenesis, suppression of Treg cells by Tac, a currently available treatment for lupus, limits its therapeutic efficacy and application. This study attempted to compensate for Tac-induced Treg depletion via probiotic supplementation. In a murine lupus model, oral administration of LA effectively ameliorated gut dysbiosis and improved Tac efficacy by rebalancing the Th17/Treg ratio. In future trials, combination treatment with immunosuppressants and probiotics should be considered as a potential therapeutic modality for lupus.

Methods

Animals

MRL/*lpr* mice were purchased from SLC Inc. (Shizuoka, Japan). They were housed in groups of five in polycarbonate cages in a specific-pathogen-free environment. The mice had access to standard mouse chow (Ralston Purina Co., St. Louis, MO, USA) and water *ad libitum*. Eight-week-old MRL/*lpr* mice were orally administered 5 mg/kg Tac (MedChemExpress, Monmouth Junction, NJ, USA) and/or 50 mg/kg LA (CNS Pharm Korea Co., Ltd., Jincheon, Korea), daily for 8 weeks. The LA were heat-killed at 80°C for 30

min prior to administration. All experimental procedures were approved by the Animal Research Ethics Committee of the Catholic University of Korea (approval number: 2020-0151-04).

Caecal DNA extraction, PCR amplification and sequencing

Total DNA was extracted using the Maxwell® RSC PureFood GMO and Authentication Kit (Promega, USA), in accordance with the manufacturer's instruction. PCR amplification was performed using fusion primers targeting from V3 to V4 regions of the 16S rRNA gene with the extracted DNA. For bacterial amplification, fusion primers of 341F (5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3'; underlining sequence indicates the target region primer) and 805R (5'- CAAGCAGAAGACGGCATACGAGAT-XXXXXXXX-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3'). The Fusion primers are constructed in the following order which is P5 (P7) graft binding, i5 (i7) index, Nextera consensus, Sequencing adaptor, and Target region sequence. The amplifications were carried out under the following conditions: initial denaturation at 95 °C for 3min, followed by 25 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec, with a final elongation at 72 °C for 5 min. The PCR product was confirmed by using 1% agarose gel electrophoresis and visualized under a Gel Doc system (BioRad, Hercules, CA, USA). The amplified products were purified with the CleanPCR (CleanNA). Equal concentrations of purified products were pooled together and removed short fragments (non-target products) with CleanPCR (CleanNA). The quality and product size were assessed on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip. Mixed amplicons were pooled and the sequencing was carried out at Chunlab, Inc. (Seoul, Korea), with Illumina MiSeq Sequencing system (Illumina, USA) according to the manufacturer's instructions.

Caecal microbiome data analysis pipeline

Processing raw reads started with quality check and filtering of low quality (<Q25) reads by Trimmomatic ver. 0.32 [55]. After QC pass, paired-end sequence data were merged together using fastq_mergepairs command of VSEARCH version 2.13.4 [56] with default parameters. Primers were then trimmed with the alignment algorithm of Myers & Miller [57] at a similarity cut off of 0.8. Non-specific amplicons that do not encode 16S rRNA were detected by nhmmer [58] in HMMER software package ver. 3.2.1 with hmm profiles. Unique reads were extracted and redundant reads were clustered with the unique reads by derep_fulllength command of VSEARCH [56]. The EzBioCloud 16S rRNA database [59] was used for taxonomic assignment using usearch_global command of VSEARCH [56] followed by more precise pairwise alignment [57]. Chimeric reads were filtered on reads with <97% similarity by reference based chimeric detection using UCHIME algorithm [60] and the non-chimeric 16S rRNA database from EzBioCloud. After chimeric filtering, reads that are not identified to the species level (with <97% similarity) in the EzBioCloud database were compiled and cluster_fast command [56] was used to perform de-novo clustering to generate additional OTUs. Finally, OTUs with single reads (singletons) are omitted from further analysis. The secondary analysis which includes diversity calculation and biomarker discovery

was conducted in EzBioCloud 16S-based MTP, which is a Chunlab, Inc (Seoul, South Korea) bioinformatics cloud platform.

Flow cytometry

Splenocytes and peripheral blood were immunostained with surface eFluor780-conjugated fixable viability dye (eBioscience, San Diego, CA, USA), Pacific Blue-conjugated anti-CD90.2 (Biolegend), peridinin-chlorophyll-protein-cyanine5.5-conjugated anti-CD4 (eBioscience), phycoerythrin (PE)-conjugated anti-CD8 (Biolegend), and allophycocyanin (APC)-conjugated anti-CD2 (Biolegend). After fixation and permeabilization, cells were stained with fluorescein isothiocyanate (FITC)-conjugated IL-17 (eBioscience) and PE-conjugated Foxp3 (eBioscience). For intracellular staining, cells were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate and 250 ng/mL ionomycin (Sigma, St. Louis, MO, USA) for 4 h in the presence of GolgiStop (BD Biosciences, San Diego, CA, USA). The data were analysed using FlowJo software (Tree Star, Ashland, OR, USA).

Histological analysis

Histological analyses were performed to quantify spleen and kidney inflammation. Kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Spleen tissue cryosections were fixed in methanol-acetone. Kidney and spleen sections were stained with haematoxylin and eosin, examined under a photomicroscope (Olympus, Tokyo, Japan), and scored [61].

Immunohistochemistry

Immunohistochemistry was performed using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Briefly, tissue sections were incubated overnight at 4°C with a primary antibody against SIGNR3 (R&D Systems, Minneapolis, MN, USA), followed by a biotinylated secondary antibody, and then reacted with a streptavidin-peroxidase complex for 1 h. 3,3’-Diaminobenzidine (Dako, Carpinteria, CA, USA) was added as a chromogen, and the samples were visualised using a microscope (Olympus).

Confocal microscopy

Tissue cryosections (7-μm-thick) were fixed in methanol-acetone and stained with FITC-conjugated anti-CD4, APC-conjugated anti-CD25, PE-conjugated anti-IL-17, and -Foxp3 (eBioscience). After incubation at 4°C overnight, the stained sections were analysed using a Zeiss microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany) at 200× magnification.

ELISA

Blood was collected from the orbital sinus, and serum samples were stored at –20°C until use. Serum levels of anti-dsDNA antibodies were measured using poly-L-lysine, dsDNA-cellulose (Sigma), and mouse IgG detection antibody (Bethyl Laboratories, Montgomery, TX, USA). IgG2a levels were measured using ELISA kits (Bethyl Laboratories). The levels of IL-10 and IL-17 in the cultured supernatants from MRL/*lpr*

splenocytes were measured using sandwich ELISA (R&D Systems). Absorbances were determined using an ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Urine albumin and creatinine assays

Urine albumin and creatinine concentrations were measured using a mouse albumin ELISA assay (Bethyl Laboratories) and a creatinine assay (R&D systems), respectively, according to the manufacturer's instructions.

Real-time polymerase chain reaction (PCR)

mRNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) as per the manufacturer's instructions. Complementary DNA was synthesised using a Super Script reverse transcription system (TaKaRa, Shiga, Japan). A Light-Cycler 2.0 Instrument (software v4.0; Roche Diagnostics, Indianapolis, IN, USA) was used for the PCR amplification. All reactions were performed using the LightCycler FastStart DNA Master SYBR Green I Mix (TaKaRa) following the manufacturer's instructions. The following primers were used: SIGNR3, 5'-TCA-AGA-GTT-TGG-CAG-AGT-ATA-CG-3' (sense) and 5'-TTG-TTC-TGA-ACC-TCT-GAG-CTG-3' (antisense); IDO, 5'-GAC-GGA-CTG-AGA-GGA-CAC-AG-3' (sense) and 5'-GGC-AGC-ACC-TTT-CGA-ACA-TC-3' (antisense); PD-L1, 5'-AAA-GTC-AAT-GCC-CCA-TAC-CG-3' (sense) and 5'-TTC-TCT-TCC-CAC-TCA-CGG-GT-3' (antisense); IL-10, 5'-GGC-CCA-GAA-ATC-AAG-GAG-CA-3' (sense) and 5'-AGA-AAT-CGA-TGA-CAG-CGC-CT-3' (antisense); β-actin, 5'-GAA-ATC-GTG-CGT-GAC-ATC-AAA-G-3' (sense) and 5'-TGT-AGT-TTC-ATG-GAT-GCC-ACA-G-3' (antisense). All mRNA levels were normalised to β-actin.

siRNA transfection

siRNA for SIGNR3 was purchased from Cosmo Genetech (Seoul, Korea). Before transfection, murine non-T-cells were cultured with LPS (100 ng/mL; Sigma) from *Escherichia coli* O111:B4. The next day, the cells were transfected using the Amaxa 4D-nucleofector X unit with a primary cell kit, as per the manufacturer's recommendations (Lonza, Cologne, Germany).

PBMC isolation and stimulation

PBMCs of healthy volunteers and SLE patients were prepared from heparinised blood using a standard Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). Cells were cultured in RPMI-1640 medium (Gibco BRL, Carlsbad, CA, USA) and stimulated with anti-CD3 (0.5 μg/mL) for 3 days. All procedures were approved by the ethics committee of Seoul St. Mary's Hospital (Seoul, Republic of Korea).

Statistics

Statistical analyses were performed using Prism software (v5.0; GraphPad Software Inc., San Diego, CA, USA). Differences between groups were evaluated using t-tests (two-tailed) for two groups and one-way

analysis of variance for three or more groups. $P < 0.05$ was considered statistically significant.

List Of Abbreviations

SLE: Systemic lupus erythematosus

Tac: Tacrolimus

LA:*Lactobacillus acidophilus*

MRL/*lpr*: MRL/Mp-*Fas*^{lpr}

SIGNR3: specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3

LN: lupus nephritis

IL: interleukin

Treg: regulatory T

Th17: T-helper 17

CD: cluster of differentiation

DNT: double negative T

IgG: immunoglobulin G

dsDNA: double-stranded deoxyribonucleic acid

Foxp3: forkhead box P3

Slp: Surface layer protein

LPS: lipopolysaccharide

mRNA: messenger ribonucleic acid

IDO: indoleamine-2,3-dioxygenase

PD-L1: programmed death-ligand 1

siRNA: small interfering ribonucleic acid

PBMCs: peripheral blood mononuclear cells

ELISA: Enzyme-linked immunosorbent assay

DCs: dendritic cells

DC-SIGN: DC-specific ICAM-3-grabbing non-integrin

Declarations

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Author's contributions

DSK, SHP, MLC, and SKK conceived and designed the experiments. DSK conducted the experiments. JWC performed the histochemical study. DSK, YP, SHP, MLC, and SKK analyzed the data. DSK, YP, MLC, and SKK wrote and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

All data generated or analysed during this study are available.

Ethics approval and consent to participate

See ethics paragraph in the "Materials and methods" section.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

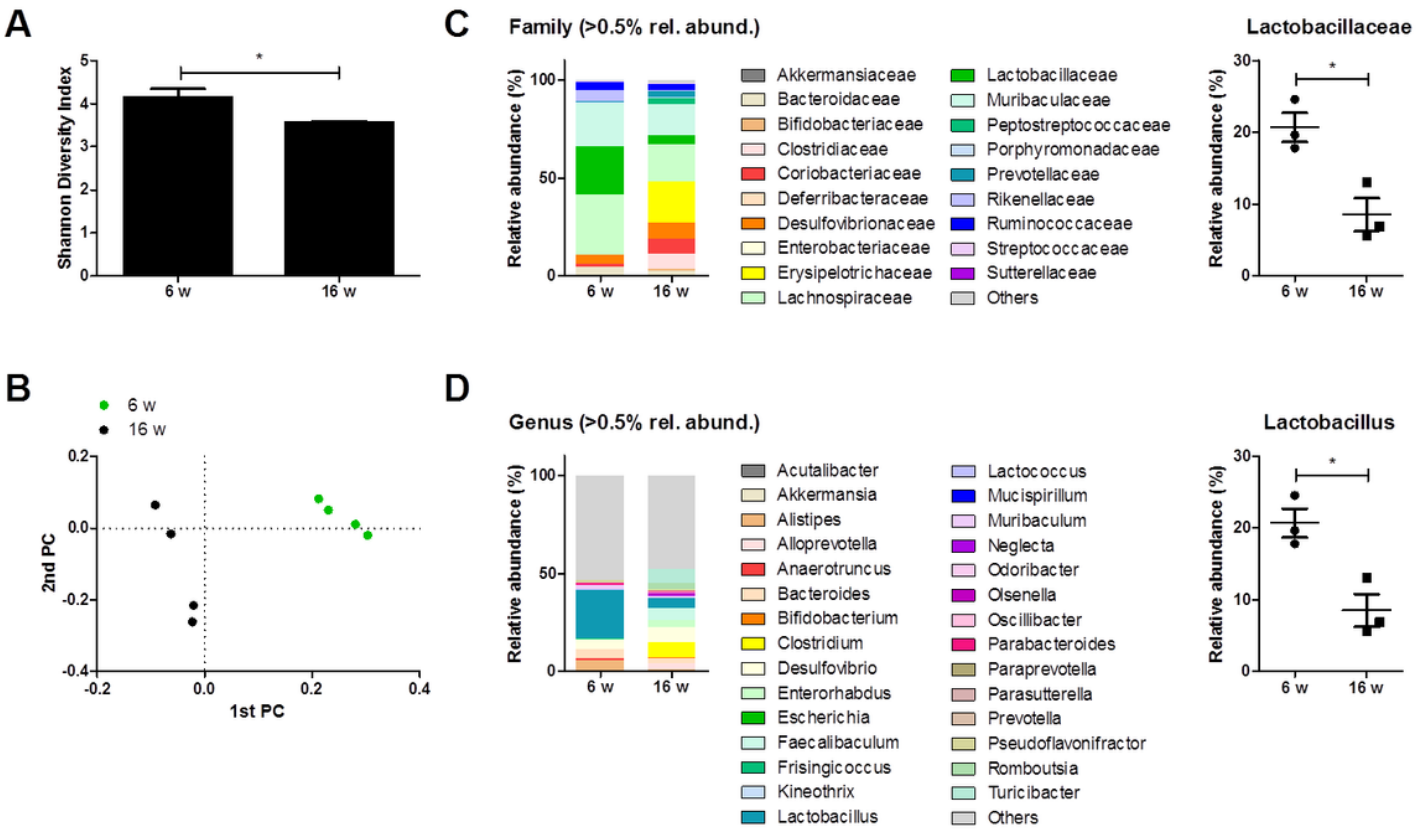

Figure 1

Lupus-prone mice exhibit gut dysbiosis with a reduction in Lactobacillus species. a Shannon diversity indices of MRL/Mp-Faslpr (MRL/lpr) mice at 6 and 16 weeks old (n = 4). The diversity index is lower in 16-week-old MRL/lpr mice. *p < 0.05. b The beta-diversity graph shows that the caecal bacterial composition of 16-week-old MRL/lpr mice differed from that of the 6-week-old mice. The relative abundance of cabal microbiota at the family and genus levels in MRL/lpr mice (n = 3) at 6 and 16 weeks old (c-d). The relative abundance of c Lactobacillaceae and d Lactobacillus was decreased after acquisition of the lupus phenotype (i.e., at 16 weeks old). *p < 0.05.

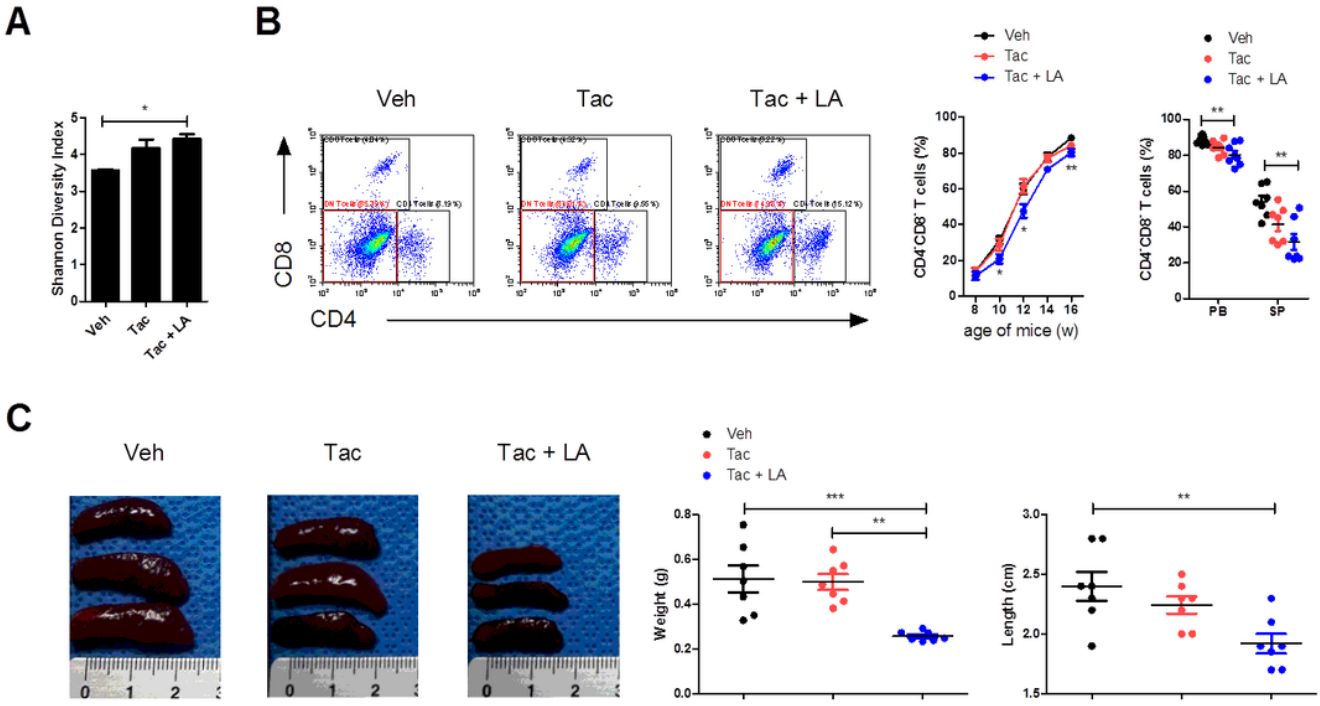

Figure 2

The combination of tacrolimus (Tac) and Lactobacillus acidophilus (LA) improves gut dysbiosis and reduces the proportion of double negative T (DNT) cells in lupus mice. Eight-week-old MRL/lpr mice (n = 5 in each group) were orally administered Tac (5 mg/kg, daily for 8 weeks) alone, or Tac + LA (50 mg/kg, daily for 8 weeks). a Shannon diversity indices of the caecal microbiota from each group at 16 weeks old. Tac + LA-treated mice showed significantly higher Shannon diversity indices. *p < 0.05. Peripheral blood samples and spleen tissues were acquired during (from 8–16 weeks, biweekly) and after (at 16 weeks, after sacrifice) the treatments, respectively (b-c). b The Tac + LA treatment significantly reduced the proportion of DNT (CD4− CD8−) cells in the peripheral blood of MRL/lpr mice compared to the Tac-alone treatment during and after the in vivo experiments (left and middle panels). *p < 0.05, **p < 0.01. The percentage of DNT cells in both the peripheral blood and spleens of 16-week-old MRL/lpr mice was significantly decreased by the Tac + LA treatment (right panel). **p < 0.01. c The weights and lengths of spleens from 16-week-old MRL/lpr mice were lower in the Tac + LA-treated group than in the vehicle and Tac-alone groups. **p < 0.01, *** p < 0.001.

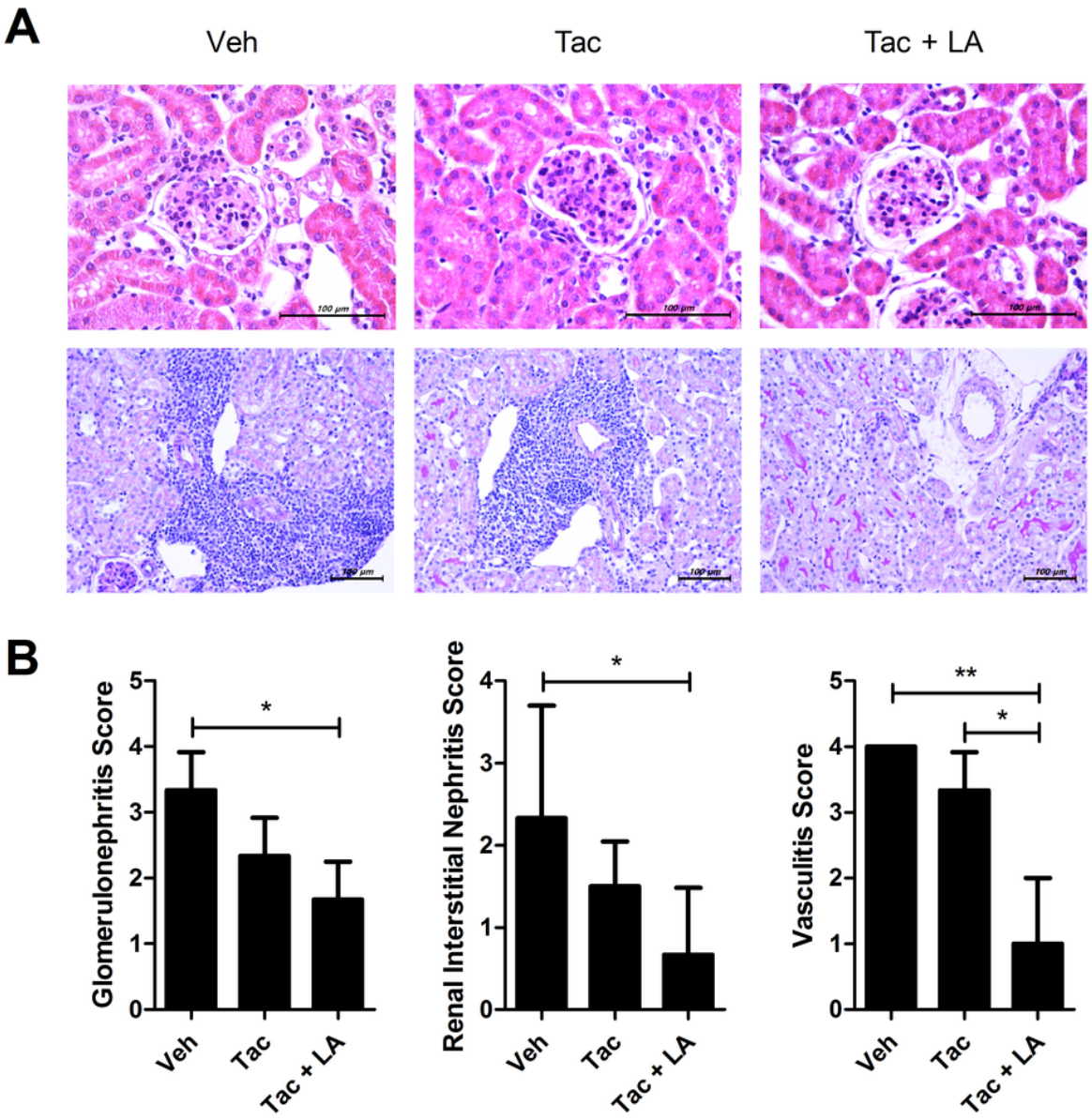

Figure 3

The combination of Tac and LA mitigates kidney inflammation in lupus-prone mice. The renal tissue of 16-week-old MRL/lpr mice treated with Tac alone or Tac + LA, as shown in Figure 2, was sectioned and stained. a Representative photomicrographs of haematoxylin and eosin stained renal tissues. Glomeruli and tubules (upper panels, 400× magnification) and vascular pathology (lower panels, 200× magnification) were evaluated. b Semiquantitative inflammation scores calculated from the renal tissue sections were significantly lower in the combination treatment group. *p < 0.05, **p < 0.01.

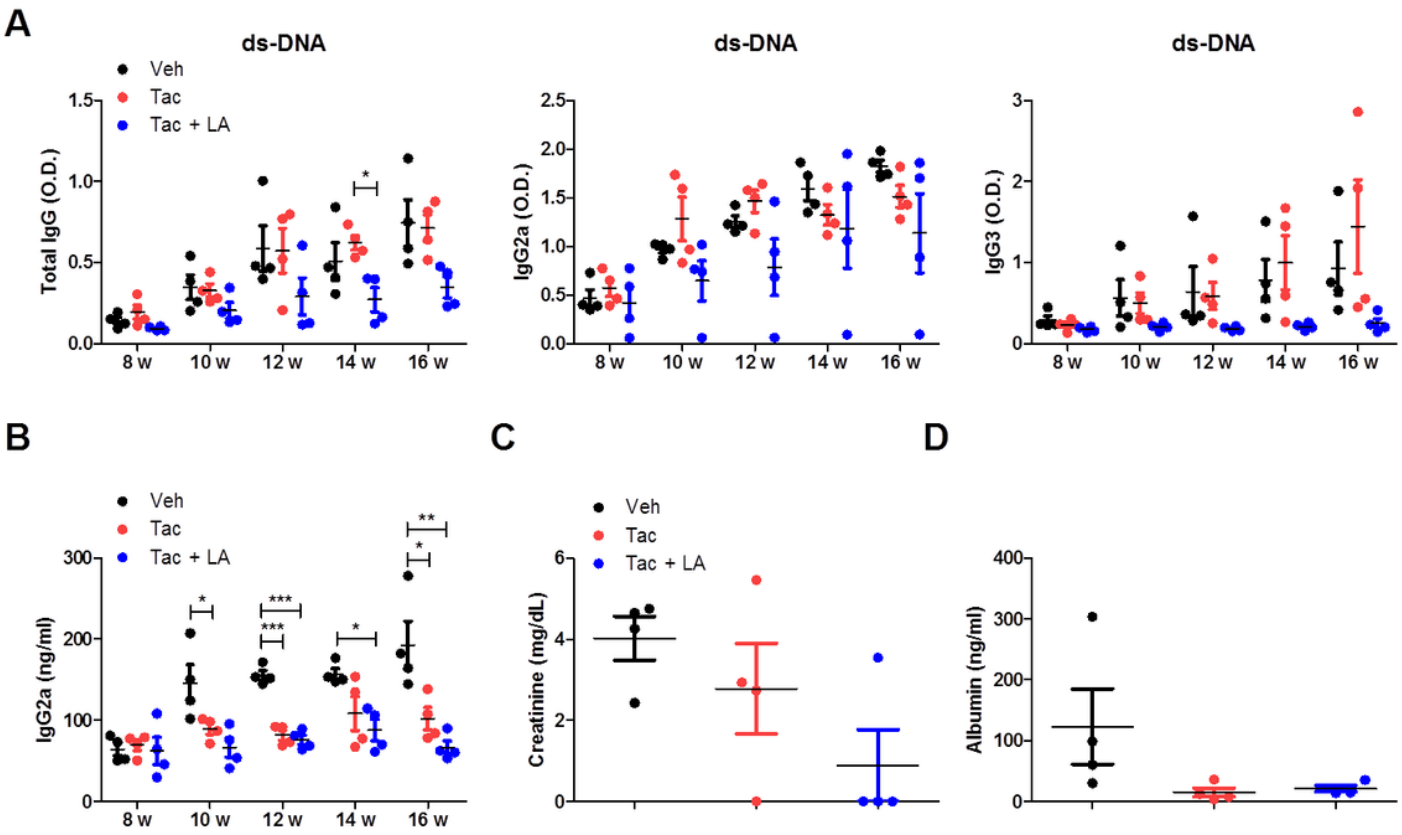

Figure 4

The combination of Tac and LA inhibits the production of autoantibodies in lupus-prone mice. After initiation of the in vivo experiments, as described in Figure 2, serum samples were acquired biweekly. a The combination treatment reduced the production of anti-double-stranded DNA (dsDNA) antibodies (total immunoglobulin G [30]) and their isotypes (IgG2a and IgG3) in the sera of lupus mice compared to those treated with vehicle or Tac alone. The amount of anti-dsDNA antibodies, and the subclasses thereof, was measured using enzyme-linked immunosorbent assay (ELISA). *p < 0.05. b Overall serum levels of IgG2a in MRL/lpr mice were significantly decreased by the combination treatment. *p < 0.05, **p < 0.01, *** p < 0.001. c Creatinine and d albumin levels in urine samples were reduced in both treatment groups at the time of sacrifice (i.e., at 16 weeks old).

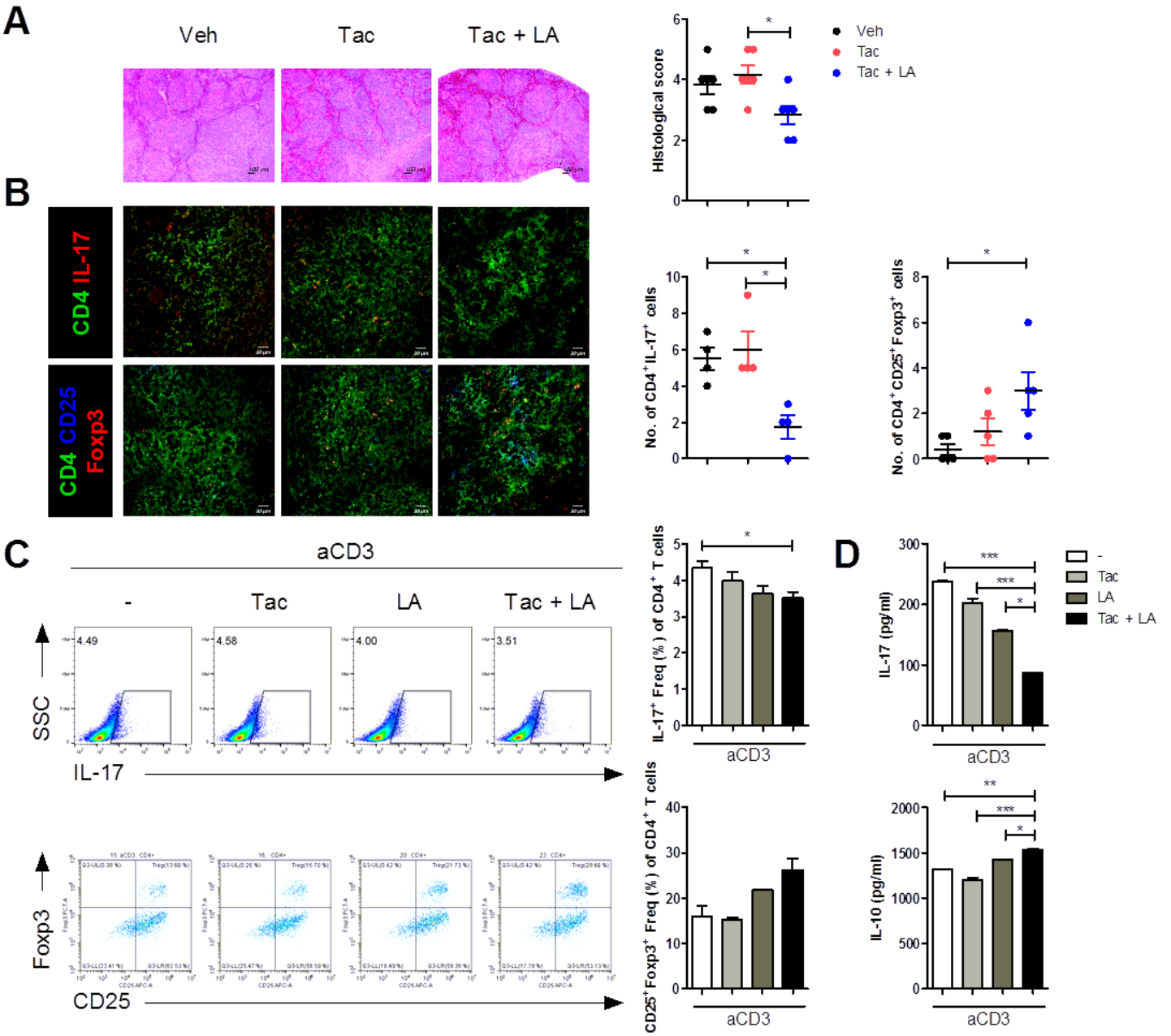

Figure 5

The imbalance between Th17 and Treg cells in lupus mice was alleviated by the combination treatment of Tac and LA. After the in vivo experiments, as described in Figure 2, cryosectioned spleen tissues were acquired at the time of sacrifice. a Photomicrographs of spleen tissues stained with haematoxylin and eosin (left panels, 40× magnification) and their histological scores (right panel). *p < 0.05. b Confocal photomicrographs of CD4 and interleukin (IL)-17-positive (Th17) cells; and CD4, CD25, and forkhead box P3 (Foxp3)-positive (Treg) cells in spleen tissues (left panels, 200× magnification); cell counts are shown in the right panels. Compared to the vehicle and Tac-alone groups, Th17 and Treg cell counts were decreased and elevated, respectively, in the combination treatment group. *p < 0.05. c Total splenocytes extracted from the spleens of MRL/lpr mice were cultured with Tac (0.1 nM), alone or with LA (100

μg/ml), under stimulation with anti-CD3 antibodies (0.5 μg/ml) for 96 h. Flow cytometry was used to analyse the proportion of IL-17- and Foxp3-expressing cells. The dot plots (left panels) and bar graphs (right panels) demonstrate that the percentages of IL-17-expressing (upper panels) and Foxp3-expressing (lower panels) cells were decreased and increased, respectively, by the combination treatment of Tac and LA. *p < 0.05. d ELISA cytokine quantification data for IL-17 and IL-10 from cultured splenocytes under the same conditions as c. Only the combination treatment of Tac and LA elevated the levels of IL-17 and reduced the levels of IL-10. *p < 0.05, **p < 0.01, *** p < 0.001.

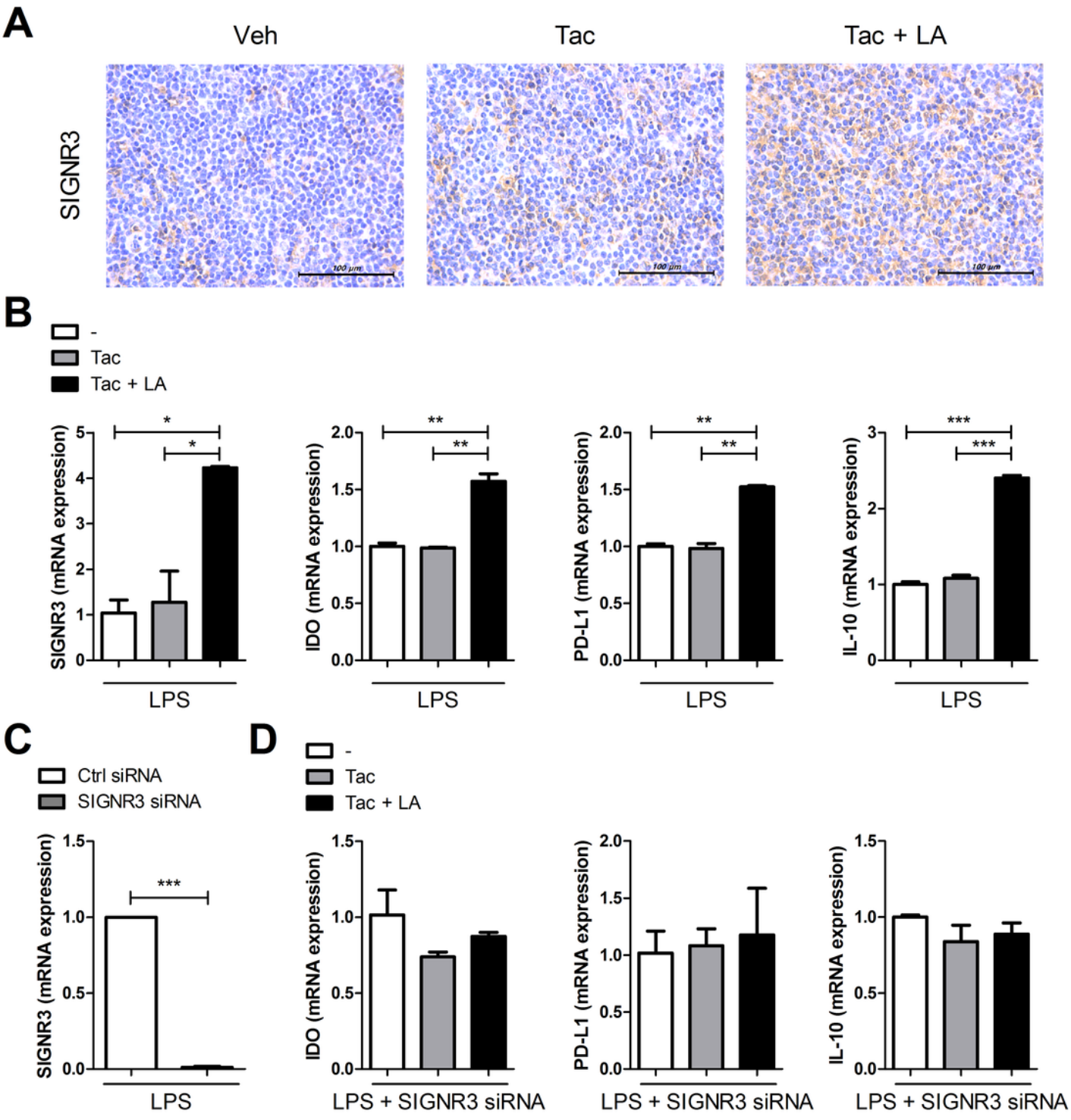

Figure 6

The specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3 (SIGNR3) receptor mediates immune regulation in lupus mice treated with the combination of Tac and LA. a Immunohistochemical staining of spleen tissues from 16-week-old MRL/lpr mice from the same in vivo experiments shown in Figure 2. SIGNR3-positive cells are more abundant in the Tac + LA treated group compared to the vehicle and Tac-alone groups. b Non-T cells from MRL/lpr mice spleen treated with Tac (0.1 nM), alone or with LA (100 μg/ml), were cultured and stimulated with lipopolysaccharide (LPS) for 24 h. Then, messenger RNA (mRNA) levels of SIGNR3, indoleamine-2,3-dioxygenase (IDO), programmed death-ligand 1 (PD-L1), and IL-10 were determined using real-time polymerase chain reaction (qPCR). Only the Tac + LA combination treatment elevated mRNA levels of SIGNR3, IDO, PD-L1, and IL-10. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. c Non-T cells from MRL/lpr mice spleen were transfected with SIGNR3 small interfering RNA (siRNA) after pre-treatment with LPS for 24 h. mRNA expression of SIGNR3 was suppressed in SIGNR3 siRNA-transfected cells. d The siRNA-transfected cells were stimulated with LPS and treated with Tac alone or Tac + LA for 24 h. mRNA expression levels of IDO, PD-L1, and IL-10 did not increase under any treatment, as measured by qPCR.

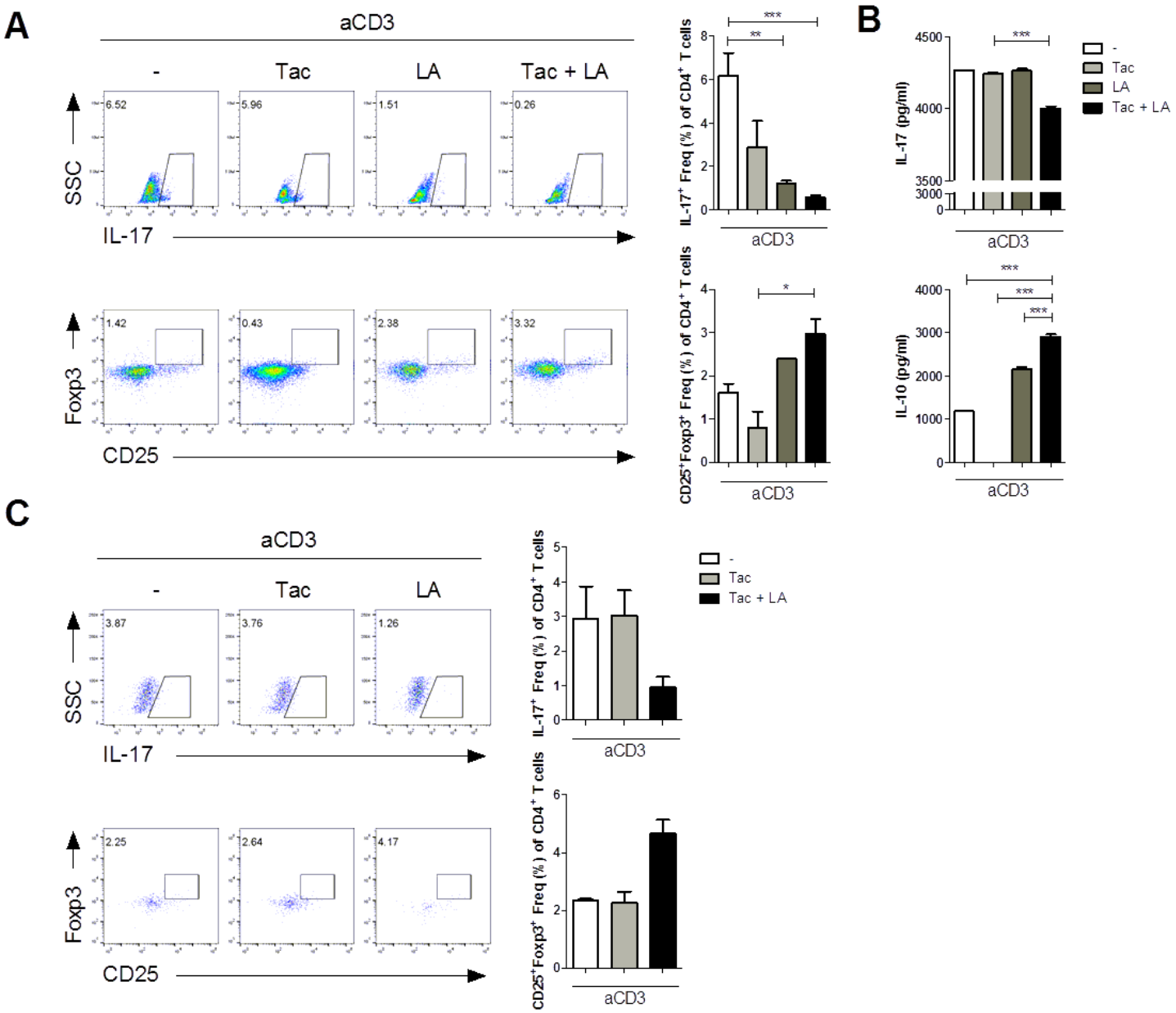

Figure 7

In vitro treatment with the combination of Tac and LA restores the Th17/Treg balance in human peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human controls. The cells were stimulated with anti-CD3 antibodies and treated with Tac and/or LA for 72 h. a Flow cytometry analysis of IL-17-expressing cells (Th17 cells) and Foxp3-expressing cells (Treg cells) indicated that the Tac + LA combination treatment led to the greatest elevation and reduction, respectively, of Treg and Th17 cells. *p < 0.05, **p < 0.01, *** p < 0.001. b Cytokine quantification with ELISA of the supernatants of cultured cells from the healthy controls indicated that, compared to the other groups, only the combination treatment group showed decreased levels of IL-17 and increased levels of IL-10. *** p < 0.001. c PBMCs from lupus patients were also isolated and cultured under anti-CD3 stimulation and treatment with Tac alone, or Tac + LA, for 96 h. Flow cytometry data indicated that only

the combination treatment group increased and reduced the proportions of Treg and Th17 cells, respectively.