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Rel B-modified dendritic cells possess tolerogenic phenotype and functions on lupus splenic lymphocytes *in vitro*

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Summary

Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterized by high morbidity and mortality and its treatment remains challenging. Dendritic cells (DCs) have been shown to participate in the initiation and perpetuation of lupus pathogenesis and the DCs that can induce tolerogenicity appear as potential cell-based therapy in this condition. In this study, we examined the *in vitro* tolerogenic properties of bone-marrow derived DCs (BMDCs) in the murine lupus setting. We used lentiviral transduction of RelB-silencing short hairpin RNA to modify the expression of RelB, a key transcription factor regulating DC maturation, in BMDCs from MRL/MpJ mice. Tolerogenic properties of RelB-modified DCs were compared with scrambled control (SC) -modified DCs. RelB expression was found to be significantly reduced in RelB-modified DCs derived from MRL/MpJ mice, wild-type of the same genetic background as MRL/lpr lupus-prone mice. These MRL/MpJ RelB-modified DCs displayed semi-mature phenotype with expression of lower levels of co-stimulatory molecules compared with SC-modified DCs. RelB-modified DCs were found to be low producers of interleukin-12p70 (IL-12p70) and could induce hyporesponsiveness of splenic T cells from MRL/MpJ and MRL/lpr mice. Furthermore, they down-regulated interferon-γ expression and induced IL-10-producing T cells in MRL/MpJ splenocytes, and attenuated interferon-γ and IL-17 expression in MRL/lpr splenic $CD4^+$ lymphocytes. Splenocytes primed by RelB-modified DCs demonstrated antigen-specific suppressive effects on allogeneic splenocytes. In conclusion, RelB-silencing in DCs generates DCs of tolerogenic properties with immunomodulatory function and appears as potential option of cell-targeted therapy.

Keywords: dendritic cells; immune tolerance; immunotherapy; systemic lupus erythematosus; transcription factor.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that affects predominantly women of child-bearing age and is associated with significant morbidity and mortality.[1] This disease is characterized by hyperactive autoreactive T and B lymphocytes with abundant production of autoantibodies forming immune complexes, leading to tissue inflammation and organ damage. The current treatment regimen of SLE involves systemic corticosteroids and immunosuppressants, which are associated with significant adverse effects.

Dendritic cells (DCs) are professional antigen-presenting cells that bridge innate and adaptive immunity and play a

Abbreviations: APC, allophycocyanin; BMDC, bone marrow derived dendritic cells; CFSE, carboxy-fluorescein diacetate, succinimidyl ester; DCs, dendritic cells; GFP, green fluorescent protein; IFN-γ, interferon-γ; IL-4, interleukin-4; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PE, phycoerythrin; SC, scrambled control; shRNA, short hairpinRNA; SLE, systemic lupus erythematosus; Th, T helper; TNF-α, tumour necrosis factor-α; Treg, regulatory T

key role in immune defence.[2] Activated DCs prime and induce differentiation of naive T cells into different T effector subsets including T helper type 1 (Th1), Th2, Th17, and regulatory T (Treg) cells, and determine the outcome of adaptive immune responses.[3] On the other hand, DCs also possess critical functions in the regulation of immune tolerance.[4] Immature DCs that express low levels of MHC class II and co-stimulatory molecules induce peripheral tolerance through different mechanisms such as T-cell anergy, clonal deletion, cytokine deviation and induction of Treg cells.[5] Tolerogenicity associated with the immaturity status of DCs[6] has subsequently been exploited in therapeutics by *ex vivo* generation of semi-mature DCs with tolerogenic function aiming to induce immune tolerance in tissue transplants and in various autoimmune conditions.[7]

RelB is a member of the nuclear factor-κB (NF-κB) family, which is involved in diverse biological processes in DC biology including cellular development, differentiation and apoptosis and is expressed in abundance in DCs.[8] RelB and other members of the NF-κB family including c-Rel, p50 and p52, with the exception of RelA (p65), are all up-regulated during DC maturation.[9] RelB is the key transcription factor that plays a predominant role in the regulation of DC differentiation, antigen presentation and priming of T-cell responses[10] by integrating the canonical and non-canonical NF-κB pathways.[11] RelB-deficient mice have defective DCs with impaired antigen-presenting function.[12] Hence, silencing RelB in DCs may present a method to generate DCs with less maturity that possess tolerogenic functions. In this study, we hypothesized that RelB-modified DCs had an immunomodulatory effect on T lymphocytes from a murine model of lupus. We generated RelB-modified DCs by lentiviral-mediated RelB-specific short hairpin (sh)RNA from MRL/MpJ wild-type mice and examined their effects on splenic T lymphocytes from MRL/lpr lupus-prone mice of the same genetic background. We found that RelB-modified DCs were semi-mature in phenotype and were capable of inducing T-cell hyporesponsiveness. In addition, these cells demonstrated immunomodulatory functions of attenuating pro-inflammatory cytokine production by lupus splenic T lymphocytes and inducing IL-10-producing $CD4^+$ T cells.

Materials and methods

Animals and cell lines

The project was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong under license issued by the Animal Control Unit of the Department of Health in Hong Kong. Only female mice aged 8–10 weeks were used in the experiment. BALB/c and C57BL/6 mice were obtained from the Laboratory Animal Unit of the University of Hong Kong. The lupus-prone mice MRL/lpr (MRL/MpJ-Fas^{lpr}/2J) and the wild-type of the same genetic background, MRL/MpJ mice, were offered by Dr Godfrey Chan, Department of Paediatrics. MRL/lpr is a widely used inbred lupus murine model that shows lupus-like features such as serum anti-nuclear antibodies, hypergammaglobulinaemia, arthritis, skin manifestations, lupus nephritis and increased numbers of double-negative T cells.[13] In this study, bone-marrow-derived DCs (BMDCs) were derived from MRL/MpJ mice and splenic lymphocytes were obtained from MRL/MpJ, MRL/lpr and C57BL/6 mice. Cell lines including 293T and NIH/3T3 were used for preparation of virus, transfection and transduction (offered by Dr G.R. Li, Division of Cardiology). All cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37° in a humidified atmosphere containing 5% CO_2.

Lentiviral constructs for RelB- and scrambled control-shRNA

Short hairpin RNAs were designed and chemically synthesized at the Centre for Genomic Sciences, University of Hong Kong. Sense shRNA sequences targeting RelB that were incorporated into recombinant lentiviral vectors (V211, V221, V231) included:

V211 – GATCCGGTTCTCTTTGAGCCCATTTCTCG-AGAAATGGGCTCAAAGAGAA CCGTTTTTG;
V221 – GATCCGACGAATACATTAAGGAGAACTCG-AGTTCTCCTTAATGTATTCGT CGTTTTTG; and
V231 – GATCCCACATGGAATCGAGAGCAAACTCG-AGTTTGCTCTCGATTCCATG TGGTTTTTG.

All shRNAs and non-silencing scrambled control (SC) shRNA (Santa Cruz Biotech, Dallas, TX) were cloned to lentiviral vector PLVX-shRNA2-green fluorescent protein (GFP) (offered by Dr V. Chan, Division of Rheumatology) that was linearized by restriction endonucleases *Bam*HI and *Eco*RI (Promega, Madison, WI). All constructs were verified by sequence analysis (Beijing Genomics Institute, Beijing, China). Recombinant work vector, psPAX2 packaging plasmid and pMD2.G envelope plasmid were co-transfected to 293T cells using lipofectamine 2000 (Invitrogen, Carlsbad, CA) to produce lentivirus in culture medium after 24 hr and stored at −80°. Optimal vector titre was selected based on GFP expression in 293T cells that were transduced with serial dilutions of vector. The NIH 3T3 cells were transduced with V211, V221 and V231 and examined for transfection efficiency by immunofluorescence.

Generation and transduction of BMDCs

Briefly, bone marrow cells were collected from murine femur and tibia and put into culture at a cell density of

1×10^6/ml in the presence of recombinant murine granulocyte–macrophage colony-stimulating factor and interleukin-4 (IL-4) (both 20 μg/ml; PeproTech, Rocky Hill, NJ). Interleukin-4 was omitted in some experiments to examine for differences in phenotype. Culture medium with cytokines was refreshed on Day 3. Lentiviral transduction was performed on day 5 of BMDC differentiation. Polybrene (4 μg/ml; Santa Cruz Biotech) and an appropriate volume of supernatant containing lentivirus with RelB- or SC-shRNAs were added to BMDC culture. Culture medium was refreshed with cytokines after 18 hr. GFP-positive cells were observed using fluorescence microscopy for transfection efficiency 3 days after transduction. On day 4 after transduction, all transduced and control BMDCs cultured in medium alone were stimulated by lipopolysaccharide (LPS; 1 μg/ml; Sigma, St Louis, MO) for 24 hr. The three treatment conditions tested in all experiments included LPS-matured DCs, RelB- and SC-shRNA DCs. In some experiments, DCs were rechallenged by CpG-B DNA (10 μg/ml, Hycult Biotech, Uden, the Netherlands) and recombinant soluble CD40 ligand (sCD40L) (1 μg/ml, PeproTech) for 24 hr.

Western blotting for RelB protein

RelB protein expression in BMDCs was examined by Western blot. Briefly, extracted proteins from cell lysates were obtained using lysis buffer (Thermo Fisher Scientific, Massachusetts, USA) at 4° and were separated by 12% SDS–PAGE and then transferred to nitrocellulose membrane (Thermo Fisher Scientific). The membrane was blocked by 5% non-fat milk in Tris-buffered saline for 1 hr and then incubated with rabbit anti-mouse RelB or rabbit anti-mouse β-actin polyclonal antibodies (Santa Cruz Biotech) for 1 hr at room temperature. This was followed by incubation with horseradish peroxidase-goat anti-rabbit IgG antibody (Cell Signaling, Danvers, MA). Protein bands were developed using an ECL Western Blot detection kit (GE Healthcare, Chalfont St Giles, UK). RelB was revealed as a protein band of 68 000 MW. Quantification was measured by densitometry and normalized to β-actin level.

DC–splenocyte co-culture

Intensive washing of transduced BMDCs was performed before DC–splenocyte co-culture experiments to eliminate free viral particles. Transduced BMDCs from MRL/MpJ mice were co-cultured with splenocytes from MRL/MpJ, MRL/lpr and C57BL/6 mice, aged 8–10 weeks, in a ratio of 1 : 10 in the presence or absence of anti-mouse CD3 and CD28 co-stimulation (eBioscience, San Diego, CA). Cell surface marker expression and intracellular cytokine production were detected by flow cytometry. Splenocyte proliferation was detected by carboxy-fluorescein diacetate, succinimidyl ester (CFSE) dilution. Splenocytes were incubated with 5 μM CFSE (Sigma) in DMSO (Sigma) for 7 min at 37° before being used for further co-culture experiments.

Flow cytometry analysis

The BMDCs were incubated with mouse Fc block (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°. Fluorochrome-labelled monoclonal antibodies used in the experiments included anti-mouse CD80-allophycocyanin (APC), CD86-phycoerythrin (PE), MHC-II-PE, CD11c-APC, CD40-APC, CD83-PE, CCR7-PE, CCR5-APC, CXCR3-APC, programmed death-ligand 1 (PD-L1) -PE, CD3e-FITC, CD4-PE, CD8-APC, IL-17-PE, IL-10-PE, interferon-γ (IFN-γ) -PE-Cy7, Foxp3-APC and CD25-PE (eBioscience) and their corresponding isotypic controls. For intracellular cytokine staining, cells were pre-treated with Golgi Stop (BD Biosciences, Franklin Lakes, NJ) and were then stained with fluorochrome-labelled surface staining monoclonal antibodies or isotypic control for 30 min. A Cytofix Cytoperm kit (BD Biosciences) was then used to permeabilize and fix cells at 4° for another 30 min. Perm wash buffer was used to wash the processed cells before staining them with anti-IFN-γ, anti-IL-17 and anti-IL-10 monoclonal antibodies and isotypic controls for 30 min at 4°. Anti-mouse Foxp3 staining set APC (eBioscience) was used for Foxp3 immunostaining. Data were captured by flow cytometry (Beckman Coulter, Brea, CA) and analysed using the FlowJo software (FlowJo, Ashland, OR).

ELISA for cytokine production in supernatant

Supernatant collected from cell culture was stored at −80° for cytokine measurement. Mouse IL-6, IL-10, IL-17, IFN-γ, tumour necrosis factor-α (TNF-α) and IL-12p70 production were measured by commercial sandwich ELISA kits according to the manufacturers' protocol (eBioscience). Briefly, 96-well plates were pre-coated with capture antibodies overnight. The plates were blocked for 1 hr. Standards and samples were pre-diluted and added into wells and incubated at room temperature for 2 hr followed by detection antibodies for a further 1 hr. These were then allowed to conjugate with avidin-horseradish peroxidase and substrate solution, followed by stop solution and the plates were read at a wavelength of 450 nm.

Quantitative real-time PCR

Freshly harvested BMDCs were preserved in Trizol reagent (Life Technologies, Carlsbad, CA). Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany) with subsequent cDNA conversion by standard reverse transcription by Superscript Reverse Transcriptase II (Life Technologies). Relative expression of mRNA of

RelB, NF-κB(p50) and NF-κB(p52) was determined by quantitative PCR with specific primers and SyBr Green PCR Reagent Mix (all Life Technologies) normalized to GAPDH. Quantitative PCRs were performed on StepOnePlus Real-Time PCR Systems (Thermo Fisher Scientific) according to the manufacturer's instruction.

Splenic lymphocytes suppression assay

To investigate the antigen-specific suppressive effect of RelB-shRNA DC-primed T cells, splenocytes from C57BL/6 mice were co-cultured with transduced BMDCs from MRL/MpJ mice at a ratio of 10 : 1. Non-adherent cells were collected after 6 days and rested in a new plate with IL-2 (50 ng/ml) for 4 days. These BMDC-primed C57BL/6 splenocytes enriched for lymphocytes were co-cultured with CFSE pre-labelled splenocytes (responder cells) from MRL/lpr (allogeneic), C57BL/6 (syngeneic), and BALB/c (third party) mice in the ratio of 1 : 1. Proliferation of CFSE-labelled responder cells gated on $CD4^+$ T lymphocytes was observed by flow cytometry.

Statistical analysis

Statistical analysis was performed using SPSS 16·0 (SPSS Inc., Chicago, IL). Comparisons between groups were performed by Student's *t* test or Mann–Whitney *U* tests depending on conformance of data to normal distribution. Difference between pre- and post-treatment was compared using paired tests. Results are expressed in diagrams as means ± standard error of the mean (SEM). *P*-value < 0·05 was considered statistically significant.

Results

Lentiviral RelB shRNA-modified RelB expression in BMDCs

We designed three lentiviral vectors encoding shRNAs to target RelB cDNA sequence V211, V221, V231 as well as non-silencing SC. Figure 1(a) shows a representative diagram of RelB expression on lentiviral-mediated transduction of NIH3T3 cells by Western blot and a summary of RelB expression measured by densitometry. Lentiviral transduction by the vectors V221 [mean ± standard deviation (SD) 0·44 ± 0·06, $P = 0·04$] and V231 (0·31 ± 0·08, $P = 0·02$), but not V211 (1·02 ± 0·27), were shown to significantly reduce RelB expression compared with SC (0·66 ± 0·05). Expression of $CD11c^+$ cells was observed to be > 90% on day 5 of BMDC differentiation. Transfection of various lentiviral vectors was satisfactory in BMDCs, as reflected by GFP-positive cells under a fluorescence microscope and by flow cytometry (Fig. 1b). Next, we tested the RelB-silencing effect of lentiviral vectors V221, V231 and a combination of V221 and V231 in MRL/MpJ BMDCs. The mean ± SD (median) levels of RelB expression on untransduced BMDCs and BMDCs transduced with SC, V221, V231 and a combination of V221 and V231 were 0·65 ± 0·10 (0·59), 0·61 ± 0·10 (0·57), 0·40 ± 0·04 (0·39), 0·37 ± 0·05 (0·38) and 0·16 ± 0·11 (0·13), respectively. There was significantly lower RelB expression from transduction by V231 by 35·6% ($P = 0·03$) and combination of V221 and V231 by 78% ($P = 0·04$) (Fig. 1c). Hence, combination of V221 and V231 was used in all subsequent experiments. RelB-shRNA DCs were also found to express significantly lower RelB mRNA compared with SC-shRNA DCs (median 0·37 versus 0·94, $P = 0·02$) (Fig. 1d). However, mRNA levels of NF-κB (p50) and NF-κB(p52) were not different.

RelB shRNA-DCs showed semi-mature phenotype

RelB- and SC-shRNA DCs were derived from MRL/MpJ BMDCs and were stimulated by LPS for 24 hr with viability over 80%. The LPS-matured BMDCs without lentiviral transfection were used as controls in all experiments. Derivation of BMDCs was performed in some experiments with or without recombinant mouse IL-4 to examine for difference in phenotype of the derived BMDCs. Maturation and activation markers were examined by flow cytometry. Expression of MHC-II, a surface marker that is highly expressed in DCs, and co-stimulatory molecules including CD80, CD86, CD40 and CD83 were measured at baseline and at 24 hr after LPS-induced maturation. At baseline, there was no difference in co-stimulatory molecule expression between immature and transduced BMDCs (Fig. 2a). After LPS stimulation for 24 hr, mature BMDCs expressed high levels of MHC-II and co-stimulatory molecules (Fig. 2b). The SC-shRNA DCs also expressed high-level co-stimulatory molecules comparable to LPS-matured BMDCs regardless of derivation with or without IL-4. RelB-shRNA DCs expressed significantly lower MHC-II ($P < 0·001$), CD86 and CD40 (both $P < 0·05$) but the levels of CD80 or CD83 were not different compared with SC-shRNA DCs, suggesting that RelB-shRNA DCs had a semi-mature phenotype.

We then rechallenged these DCs with CpG-DNA (Fig. 2c) and sCD40L (Fig. 2d) to examine their resistance to maturation. Mature DCs and SC-shRNA DCs showed further up-regulation of co-stimulatory molecules including CD83 and CD86 upon rechallenge. On the other hand, RelB-shRNA DCs did not show augmentation in any co-stimulatory molecule expression upon rechallenge, suggesting that RelB-shRNA DCs expressed a stable semi-mature phenotype.

Next, we examined cytokine production including IL-12, IL-10, IL-6 and TNF-α by the transduced BMDCs.

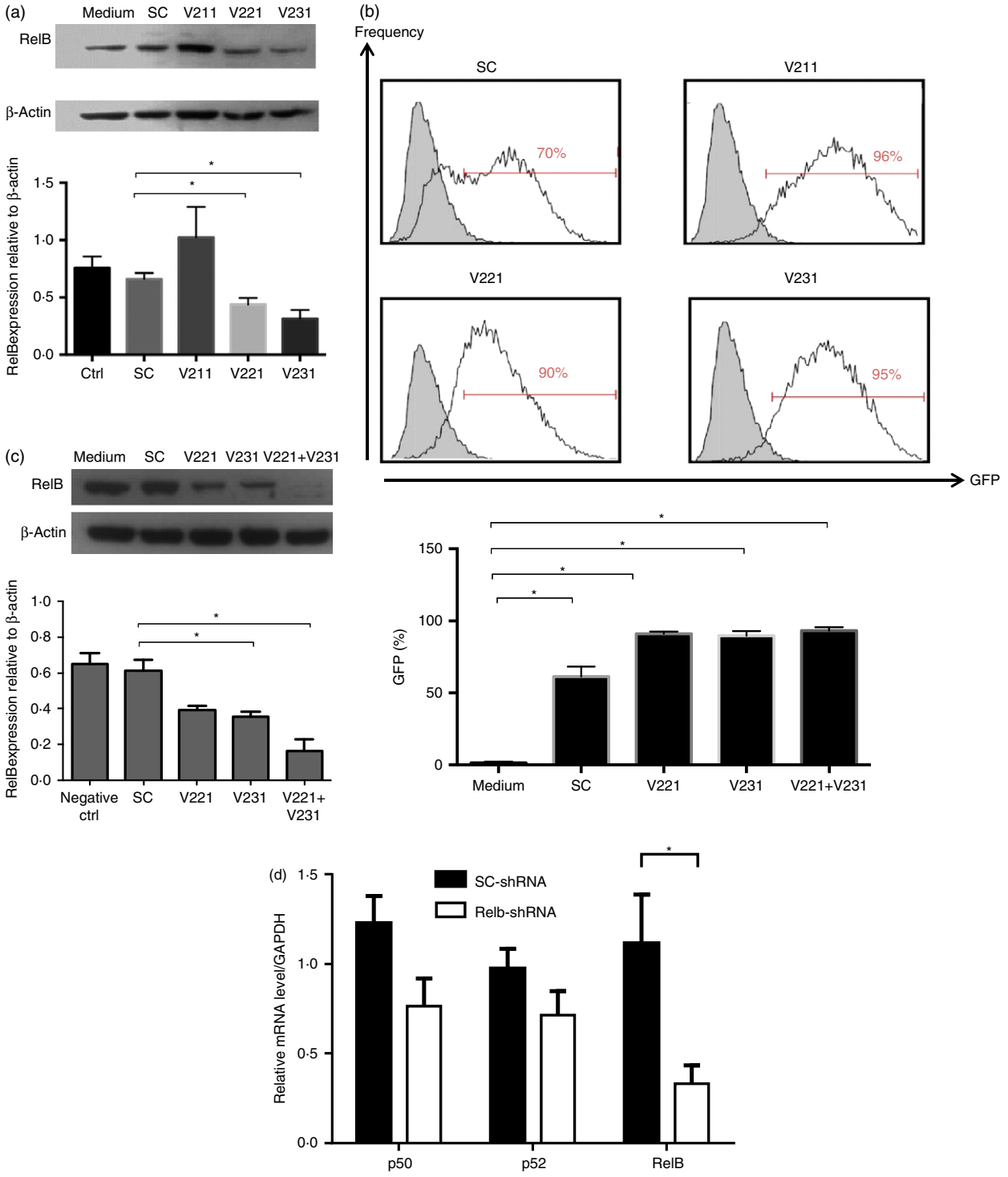

Figure 1. Lentiviral-mediated transduction of RelB short hairpin RNA (shRNA) on NIH3T3 cells and MRL/MpJ BMDCs. (a) RelB expression on lentiviral-mediated transduction of NIH3T3 cells by shRNAs of scrambled control (SC), V211, V221 and V231 by Western blot. (b) Transfection efficiency of transduced bone-marrow-derived dendritic cells (BMDCs) was examined by flow cytometry. (c) RelB expression on MRL/MpJ BMDCs transduced by shRNAs of SC, V221, V231 and V221 + V231 by Western blot. (d) mRNA expression of RelB, nuclear factor-κB (NF-κB) (p50), NF-κB(p52) in SC- and RelB-shRNA DCs. Data were obtained from three to six independent experiments. *$P < 0{\cdot}05$.

RelB-shRNA DCs produced significantly lower IL-12p70 compared with SC-shRNA DCs regardless of the presence ($26{\cdot}7 \pm 11{\cdot}9$ versus $5{\cdot}1 \pm 1{\cdot}7$ pg/ml, $P = 0{\cdot}02$) or absence ($139{\cdot}9 \pm 23{\cdot}9$ versus $70{\cdot}3 \pm 20{\cdot}7$ pg/ml, $P = 0{\cdot}03$) of IL-4 in BMDC derivation (Fig. 2e). RelB-shRNA DCs also produced significantly less TNF-α

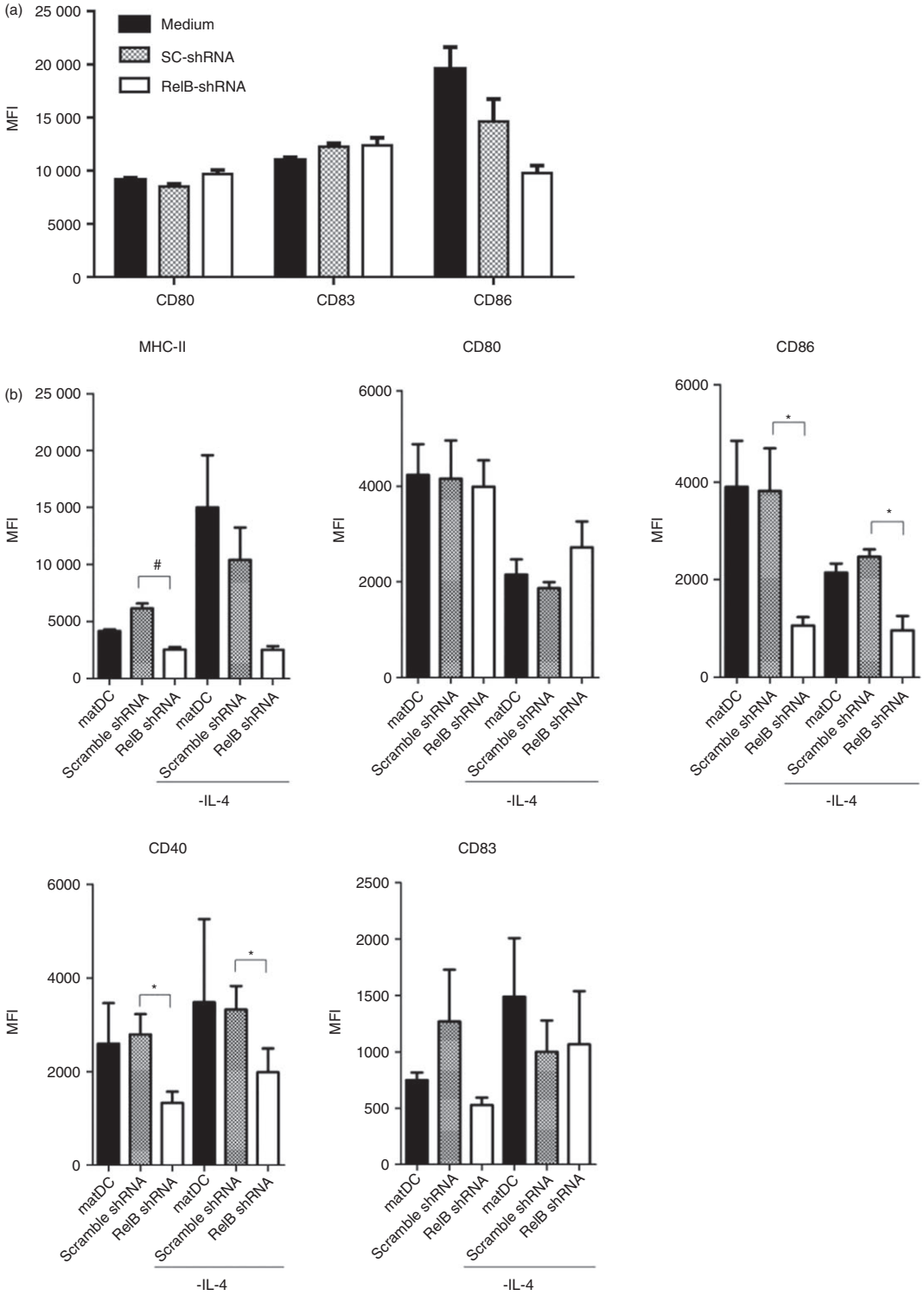

Figure 2. Expression of co-stimulatory molecules and cytokine production from bone-marrow-derived dendritic cells (BMDCs). The BMDCs were generated from MRL/MpJ bone marrow cells in the presence of mouse recombinant granulocyte–macrophage colony-stimulating factor with or without interleukin-4 (IL-4). On Day 5 of BMDC differentiation, cells were transduced with scrambled control (SC) or RelB short hairpin RNA (shRNA) followed by stimulation with lipopolysaccharide (LPS) for a further 24 hr. (a) Expression of co-stimulatory molecules in untransduced DCs, SC-shRNA and RelB-shRNA DCs before LPS-induced maturation. (b) Expression of co-stimulatory molecules after LPS-induced maturation in LPS-matured DCs, SC- (SC) and RelB-shRNA (RelB) DCs. (c,d) Expression of co-stimulatory molecules upon rechallenge by CpG-DNA (10 μg/ml) and sCD40L (1 μg/ml) for 24 hr. (e) Levels of IL-12p70, IL-10, IL-6 and tumour necrosis factor-α (TNF-α) production by BMDCs. Data were obtained from three or four independent experiments.*$P < 0{\cdot}05$, #$P < 0{\cdot}001$.

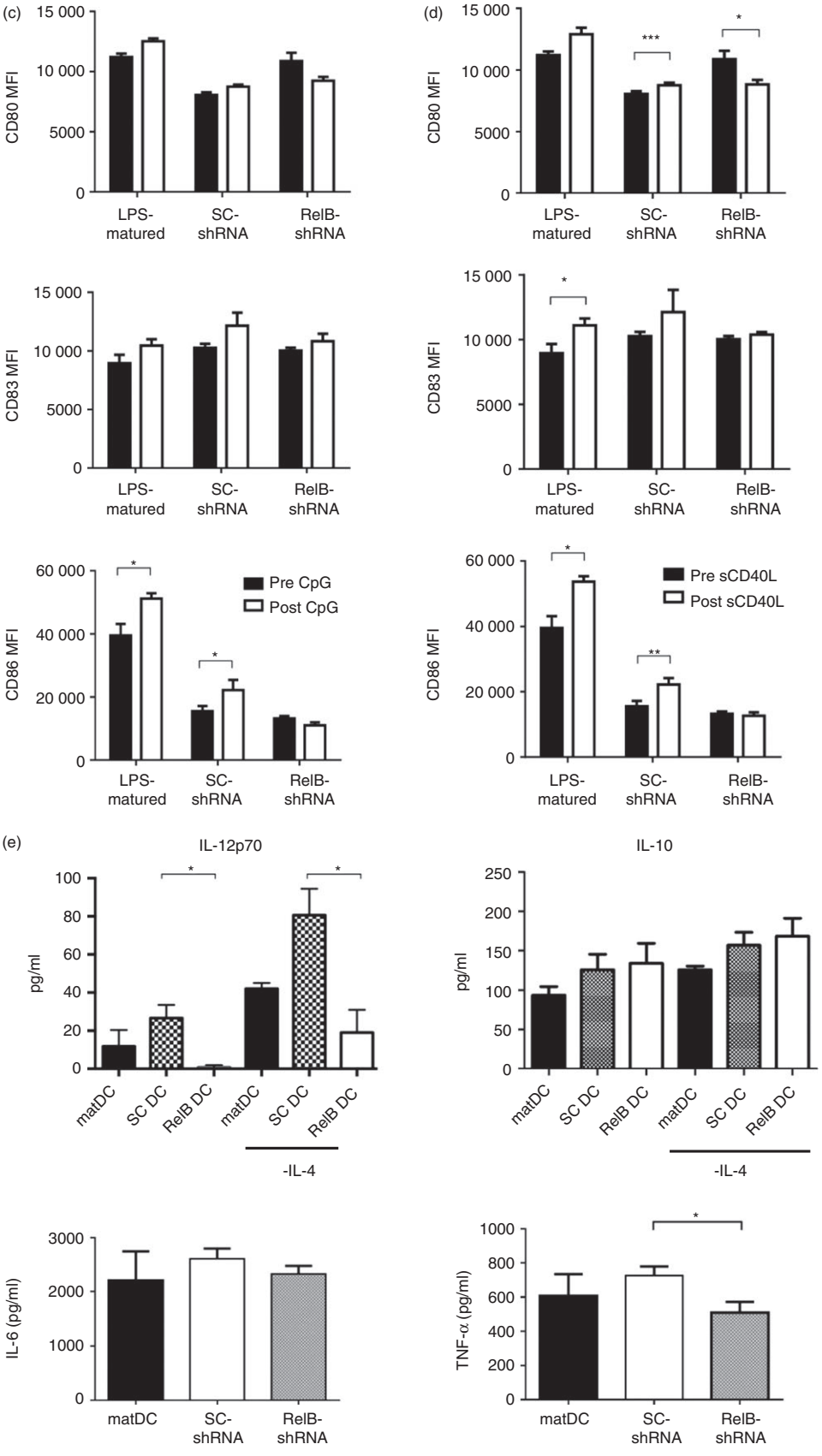

Figure 2. Continued.

(725·9 ± 108·2 versus 509·8 ± 124·7 pg/ml, $P = 0·04$) but comparable levels of IL-10 and IL-6 to SC-shRNA DCs. Overall, the IL-12p70 : IL-10 ratio was significantly lower in RelB-shRNA DCs compared with SC-shRNA DCs derived with ($P = 0·001$) and without ($P = 0·03$) IL-4.

RelB-shRNA DCs suppressed splenic $CD4^+$ lymphocyte proliferation

Lipopolysaccharide-matured BMDCs, SC-shRNA and RelB-shRNA DCs derived from MRL/MpJ mice were co-cultured with CFSE-labelled splenic $CD4^+$ T lymphocytes from MRL/MpJ (H2-k), MRL/lpr (H2-k) and C57BL/6 mice (H2-b) for 5 days. Proliferation and expression of intracellular cytokines in splenic T cells were measured by CFSE dilution in the presence or absence of CD3/CD28 co-stimulation. As expected, LPS-matured DC-primed splenic lymphocytes from MRL/MpJ, MRL/lpr and C57BL/6 mice induced higher proliferation compared with SC-shRNA and RelB-shRNA DCs in an unstimulated condition, the effect of which was remarkably augmented in the presence of CD3/CD28 co-stimulation (Fig. 3a). SC-shRNA DC-primed splenic T lymphocytes of these different mouse strains also demonstrated high proliferation under stimulated conditions. On the other hand, splenic T lymphocytes primed by RelB-shRNA DCs did not induce significant proliferation despite stimulation, suggesting that RelB-shRNA DCs induced hyporesponsiveness of splenic T cells.

RelB-shRNA DCs showed immunomodulatory effect on cytokine profile of splenic $CD4^+$ T cells

Next, we examined the intracellular cytokine profile including IFN-γ (Fig. 3b), IL-17 (Fig. 3c) and IL-10 (Fig. 3d) of proliferating splenic $CD4^+$ T lymphocytes in co-culture with various transduced BMDCs. Splenic T lymphocytes primed by RelB-shRNA DCs showed significantly reduced IFN-γ expression among proliferating splenic population from MRL/MpJ and MRL/lpr mice compared with those in co-cultured with SC-shRNA DCs ($P < 0·05$), and had significantly reduced IL-17 expression among proliferating splenic population from MRL/lpr mice ($P < 0·05$). On the other hand, RelB-shRNA DCs induced significantly higher IL-10 level in proliferating splenic $CD4^+$ T lymphocytes from MRL/MpJ mice ($P < 0·05$) but not in those from MRL/lpr and C57BL/6 mice. This suggested that RelB-shRNA DCs had an immunomodulatory effect on MRL/MpJ splenic T cells, leading to down-regulation of IFN-γ and increased IL-10-producing cells, and on MRL/lpr splenic T cells leading to down-regulation of both IFN-γ and IL-17 expression.

RelB-shRNA DCs did not induce $CD4^+$ $CD25^{hi}$ $Foxp3^+$ splenic population

To examine whether RelB-shRNA DCs may expand the Treg cell population or induce further Treg differentiation, the $CD4^+$ $CD25^{hi}$ $Foxp3^+$ population was examined by flow cytometry after 5 days in the presence of CD3/CD28 co-stimulation. There was no difference in the $CD4^+$ $Foxp3^+$ populations among gated $CD4^+$ splenic lymphocytes primed with LPS-matured DCs, SC-shRNA and RelB-shRNA DCs (Fig. 4). However, there were significantly fewer $CD4^+$ $CD25^{hi}$ $Foxp3^+$ lymphocytes in RelB-shRNA DCs compared with SC-shRNA DCs in BMDCs derived from MRL/MpJ mice ($P < 0·05$).

Splenic lymphocytes primed by RelB-shRNA DCs displayed an antigen-specific suppressive effect on allogeneic $CD4^+$ T-cell proliferation

T-cell suppression assay was performed by co-culturing splenic lymphocytes primed by various transduced DCs to responder splenocytes from allogeneic, syngeneic and third-party mouse strains so as to examine whether these DC-primed splenocytes demonstrated immunosuppressive effect, and in an antigen-specific fashion. Splenocytes from C57BL/6 mice were primed by transduced MRL/MpJ BMDCs and rested for 4 days in the presence of IL-2. Non-adherent cells were co-cultured with CFSE-labelled splenocytes from MRL/lpr (allogeneic), C57BL/6 (syngeneic), and BALB/c (third-party) mice. LPS-matured DC-primed splenocytes from C57BL/6 mice stimulated proliferation of $CD4^+$ splenic T cells from MRL/lpr and BALB/c but not C57BL/6 mice (Fig. 5). The SC-shRNA DCs demonstrated a similar effect. On the other hand, splenocytes primed by RelB-shRNA DCs showed a suppressive effect on proliferation of splenic T cells from MRL/lpr but not BALB/c mice ($P < 0·05$) suggesting that RelB-shRNA DCs induced splenocytes with immunosuppressive effect on allogeneic T cells in an antigen-specific manner.

PD-L1 is a negative regulatory molecule expressed by some DCs mediating their tolerogenicity.[14,15] There was no difference in the expression of PD-L1 among LPS-matured BMDCs, SC-shRNA and RelB-shRNA DCs derived in the presence or absence of IL-4 (Fig. 6a).

RelB silencing had no effect on chemokine receptor profile

The chemokine receptor expression profile of DCs changes and facilitates migration to lymphoid tissue during the DC maturation process. As DCs mature, they lose CCR5 and express CCR7, which enables them to migrate towards their ligands expressed in lymphoid tissue so that

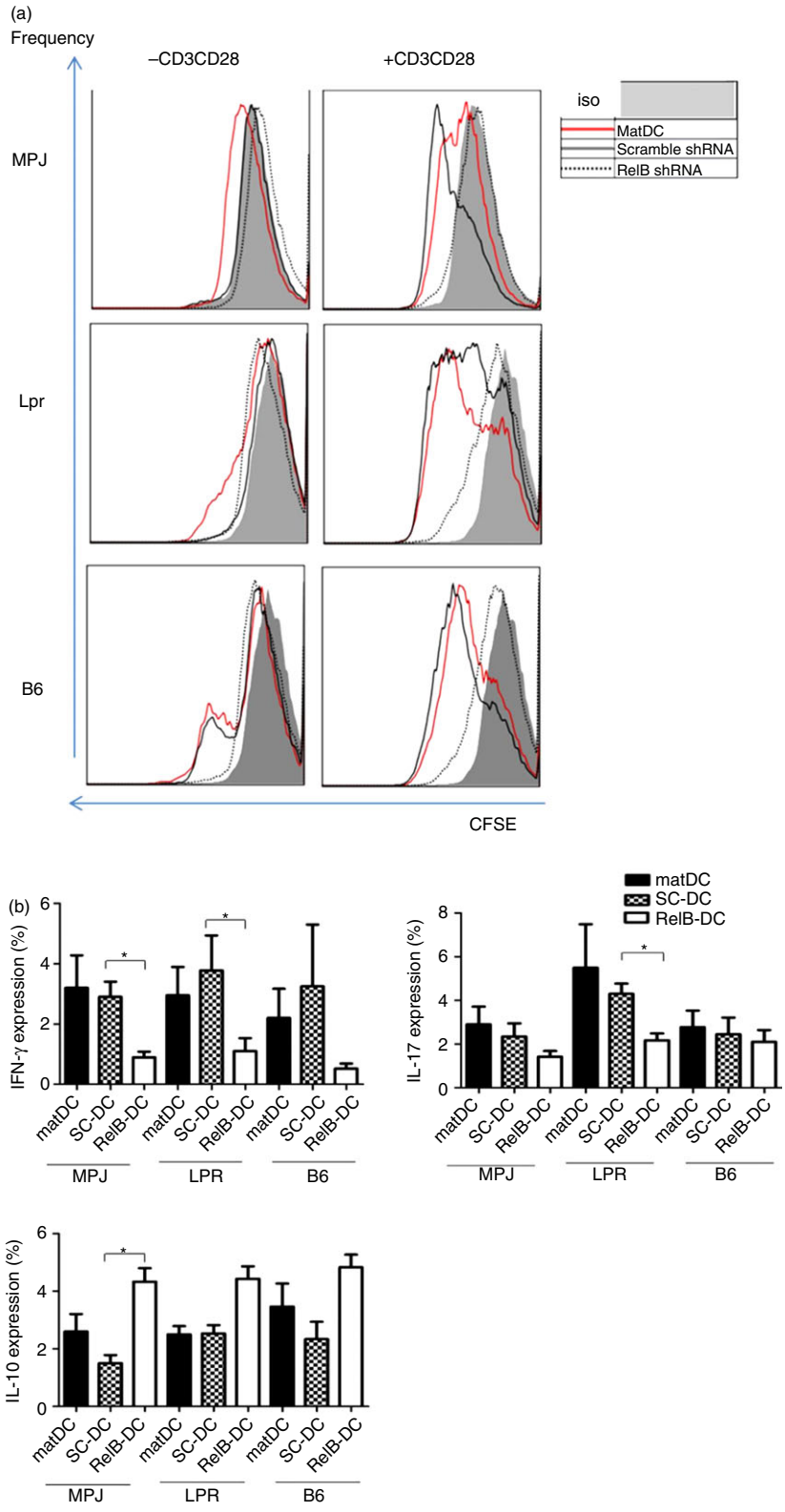

Figure 3. Proliferation and intracellular cytokine profile of transduced dendritic cell (DC) -primed splenic $CD4^+$ T cells. Splenocytes harvested from MRL/MpJ, MRL/lpr and C57BL/6 mice were stained with CFSE and co-cultured with lipopolysaccharide (LPS) -matured DCs, scrambled control (SC) and RelB short hairpin RNA (shRNA) DCs (DC : splenocyte ratio 1 : 10) in the presence or absence of anti-CD3 and CD28 for 5 days. Proliferation and cytokine production by splenocytes were detected by flow cytometry with gating on $CD4^+$ T cells. (a) Proliferation of transduced DC-primed splenic $CD4^+$ T cells and (b) intracellular expression of interferon-γ (IFN-γ), interleukin-17 (IL-17) and IL-10. Data were obtained from three independent experiments. $^*P < 0{\cdot}05$.

they can interact and activate antigen-specific T cells.[16] CXCR3 is expressed on plasmacytoid DCs and assists their homing to lymph nodes.[17] To examine if RelB-shRNA DCs expressed a distinct chemokine receptor profile, we measured CCR5, CCR7 and CXCR3 expression on these transduced BMDCs by flow cytometry. There

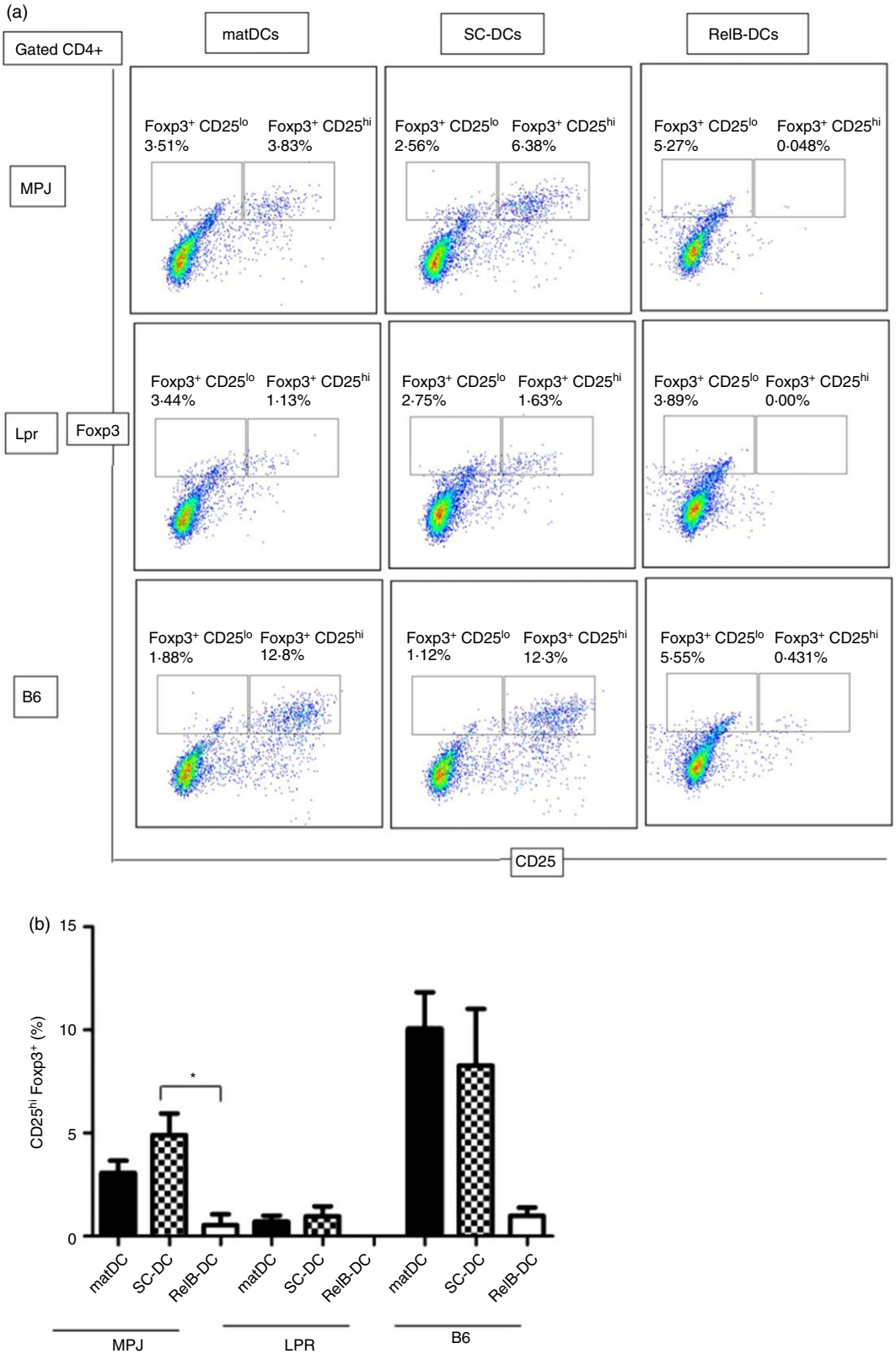

Figure 4. The effect of transduced dendritic cells (DCs) on induction of $CD4^+$ $CD25^{hi}$ and Foxp3-expressing splenic T cells. Splenocytes harvested from MRL/MpJ, MRL/lpr and C57BL/6 mice were co-cultured with lipopolysaccharide (LPS) -matured DCs, scrambled control (SC) and RelB short hairpin RNA (shRNA) DCs (DC : splenocyte ratio 1 : 10) for 5 days. Proportion of $CD4^+$ $CD25^{hi}$ T cells among gated splenic $CD4^+$ cells was detected by flow cytometry (a) with graphical presentation (b). Data were obtained from three independent experiments. $*P < 0{\cdot}05$.

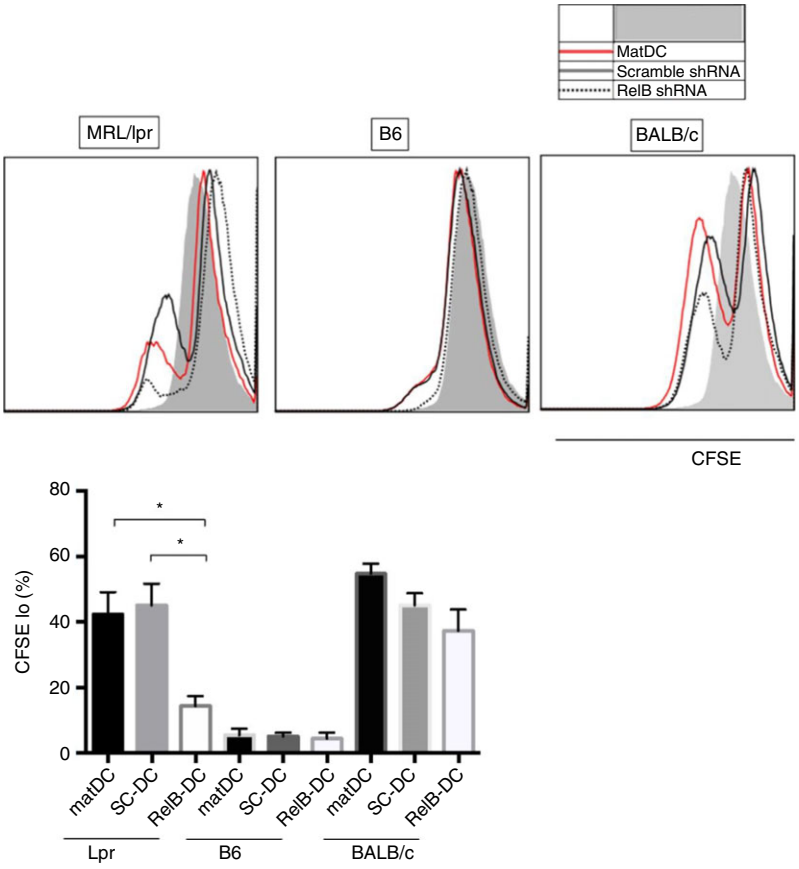

Figure 5. Splenocytes primed by RelB short hairpin RNA (shRNA) showed suppressive effects on allogeneic $CD4^+$ T-cell proliferation. Splenocytes from C57BL/6 were co-cultured with MRL/MpJ RelB-shRNA dendritic cells (DCs) at a 10 : 1 ratio for 6 days and then rested for 4 days. These DC-primed splenocytes were then co-cultured with CFSE pre-labelled responder splenocytes with gating on $CD4^+$ T cells from MRL/lpr (allogeneic), C57BL/6 (syngeneic), and BALB/c (the third party) mice in 1 : 1 ratio. (a) Proliferation of CFSE-labelled responder $CD4^+$ T cells was measured by flow cytometry. Data were obtained from three independent experiments.

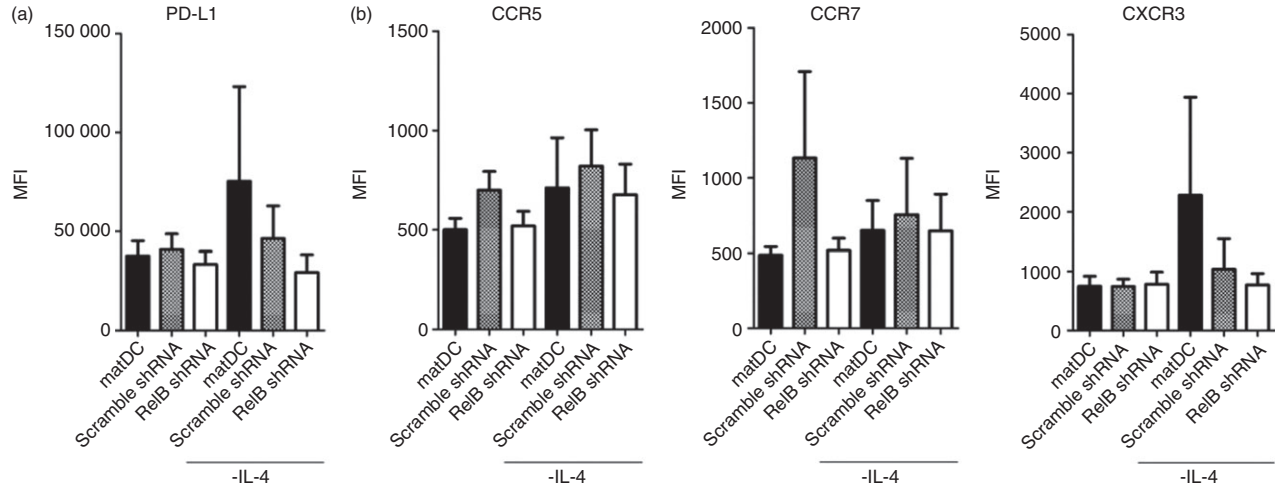

Figure 6. Expression of programmed death ligand 1 (PD-L1) and chemokine receptors on transduced dendritic cells (DCs). (a) Expression of PD-L1 and (b) chemokine receptors on lipopolysaccharide (LPS) -matured DCs, scrambled control (SC) and RelB short hairpin RNA (shRNA) DCs by flow cytometry. Data are obtained from three independent experiments.

were no differences in expression of these chemokine receptors between LPS-matured BMDCs, SC-shRNA and RelB-shRNA DCs derived in the presence or absence of IL-4 (Fig. 6b).

Discussion

Previous studies showed that DCs from the lupus-prone mouse model, FcγRIIb-deficient mice, were more mature

in phenotype and function than those from wild-type mice. These DCs expressed higher levels of co-stimulatory molecules and stimulated allogeneic T-cell activation, IL-2 production and proliferation.[18] Modulation of NF-κB by inhibitors in these mice can alleviate lupus disease activity that is accompanied by a less mature DC phenotype. Hence, alteration of NF-κB function in DCs may generate DCs that have an immunomodulatory effect on autoimmune diseases such as SLE. In this study, we generated BMDCs derived from MRL/MpJ mice with tolerogenic properties by modulating RelB, the key transcription factor in the NF-κB pathway[19] that regulates the maturation of DCs.[10] Although RelB expression in BMDCs can be modified to a reduced level by lentiviral transduction using these shRNA vectors, RelB expression cannot be completely abolished. Although DC development and function such as cytokine production are regulated by subunit-specific functions of NF-κB proteins including p50, RelA and cRel,[20] RelB has a distinct role in haematopoiesis[21] and cellular immunity[22] that cannot be functionally compensated by other members of the NF-κB family. $RelB^{-/-}$ $p50^{-/-}$ double knockout mice have severe defects in innate and adaptive immunity, major multifocal inflammation and organ damage and this knockout is not compatible with life whereas heterozygous $RelB^{+/-}$ mice were disease-free.[23] Furthermore, RelB selectively regulates the myeloid-related DC lineage.[24] $RelB^{-/-}$ mice had absent thymic and splenic DC populations and defective T-cell–macrophage interactions. Hence, low-level RelB expression on DCs is essential for their regulatory and anti-inflammatory functions.[8] RelB homodimers form heterodimers with p50 and p52,[25] but we found no difference in the expression of p50 and p52 mRNA despite a reduction in RelB mRNA in RelB shRNA DCs.

Silencing RelB in DCs can be achieved by small interfering RNA, which interferes with the expression of specific genes with complementary nucleotide sequences.[26] In this study, we used lentiviral mediated RelB-specific shRNA that can induce stable and durable gene silencing[27] without affecting other cellular functions or excess cytotoxicity.[28] It is an important rationale for cell-based therapy to generate semi-mature DCs with a stable phenotype to avoid conversion to immunogenic status under inflammatory environments *in vivo*.[29] RelB-silenced DCs were previously demonstrated to have a less mature phenotype with expression of low-level co-stimulatory molecules and exhibited inhibitory effects on mixed lymphocyte reaction and allogeneic immune responses.[30,31] RelB shRNA-silenced DCs also showed immunomodulatory effects *in vivo* in the prevention of graft rejection in murine heart[30] and liver transplantation[32] and in the treatment of a murine model of myasthenia gravis.[33,34]

Here, we generated semi-mature MRL/MpJ RelB-shRNA DCs that expressed lower levels of co-stimulatory molecules compared with SC-shRNA DCs. The lentiviral transduction procedure was followed by a step of LPS-induced maturation, which was previously established to generate alternatively activated DCs that are maturation-resistant with tolerogenic properties.[35,36] Lipopolysaccharide has also been suggested to be essential for the regulatory function and migratory capability of these DCs.[37] In accordance with previous reports on the effect of RelB-shRNA DCs on co-cultured T cells,[38] we found that MRL/MpJ RelB-shRNA DCs can induce hyporesponsiveness in both MRL/MpJ and MRL/lpr splenic T cells. These DCs attenuated T-cell proliferation, down-regulated IFN-γ expression and induced IL-10-producing splenic $CD4^+$ T lymphocytes from MRL/MpJ mice. They were also shown to suppress allogeneic T-cell proliferation, and reduce expression of IFN-γ and IL-17 in splenic T lymphocytes from MRL/lpr lupus-prone mice of the same genetic background. Furthermore, splenocytes primed by MpJ RelB-shRNA DCs demonstrated a suppressive effect on allogeneic T-cell proliferation in an antigen-specific fashion. The different outcome of MRL/MpJ and MRL/lpr splenic T lymphocytes primed by MRL/MpJ RelB-shRNA DCs may reflect a differential effect of these tolerogenic DCs on naive and memory T cells that exist in different proportions among splenocytes in these mouse models. Hence, naive T cells, which are predominant in young MRL/MpJ mice, are likely to be more responsive to induction by RelB-shRNA DCs and differentiate into IL-10-producing T cells. On the other hand, memory T cells that are present in higher numbers in MRL/lpr mice may only demonstrate attenuation of pro-inflammatory cytokine production in response to the immunomodulatory effect of MRL/MpJ RelB-shRNA DCs. Indeed, previous studies showed that tolerogenic DCs can show differential immunomodulatory effects on naive and memory T cells.[36] We have also previously shown that tolerogenic DCs generated by treating human monocyte-derived DCs by vitamin D3 and dexamethasone can induce IL-10-producing Treg cells in naive T cells and attenuate a pro-inflammatory profile in memory T cells derived from patients with SLE.[35]

Different mechanisms for induction of peripheral tolerance by tolerogenic DCs have been reported, including T-cell anergy, clonal deletion, cytokine deviation and induction of Treg cells.[5] Interleukin-10[39] and transforming growth factor-β[40] are cytokines that mediate the suppressive effect of some tolerogenic DCs on other immune cells. RelB-shRNA DCs were found to express significantly lower levels of co-stimulatory molecules and produce less IL-12p70 with, overall, a significantly lower IL-12p70 : IL-10 ratio compared with SC-shRNA DCs. The imbalance in IL-12p70 and IL-10 may partly contribute to the tolerogenicity of these DCs as low level of IL-12p70 production has previously been described as a distinct feature of tolerogenic DCs.[41] Low-level IL-12

production from tolerogenic DCs has been suggested to mediate their inducing effect on the hyporesponsiveness of T cells as exogenous IL-12p70 has been shown to revert T-cell anergy induced by tolerogenic DCs.[36] The inhibitory molecule PD-L1 is expressed by some DCs that are involved in the maintenance of peripheral tolerance,[14,15] but was not found to be expressed by MRL/MpJ RelB-shRNA DCs. Although some tolerogenic DCs had an effect of expansion[42] or induction of $CD25^{hi}$ $Fopx3^{+}$ Treg cells[43] or other T effector cells with regulatory functions,[44] we showed that MRL/MpJ RelB-shRNA DCs induced IL-10-producing T cells that possessed suppressive functions on allogeneic T lymphocytes despite a lack of increased Foxp3 expression. Indeed, Treg cells that are induced in the periphery (Tr-1) do not constitutively express Foxp3, unlike natural Treg cells that develop in the thymus.[45] Low-level CD40 expressed by RelB shRNA-silenced DCs has been suggested to mediate the induction of Treg cells.[46] Other studies have shown Th2 polarization to be the underlying mechanism for RelB shRNA-silenced DCs.[34]

As cell-based therapy, it is an important rationale for tolerogenic DCs to migrate to T-cell areas in secondary lymphoid tissue. As DCs mature, they lose CCR5[47] and express CCR7 and CXCR6, which enable them to migrate towards chemokines, including CCL21, CCL19 and CXCL13 expressed in lymphoid tissue so that they can interact and activate antigen-specific T cells.[16,48] Similar to most pharmacological agent-induced tolerogenic DCs,[44] we observed only modest expression of CCR7 in RelB-shRNA DCs.

Extensive research has been dedicated to modulating monocyte-derived DCs *ex vivo* to acquire tolerogenicity, such as by application of cytokines and growth factors, pharmacological agents and genetic engineering. Tolerogenic DCs generated using these methods demonstrated immunomodulatory effects of induction of T-cell anergy, expansion of natural Treg cells, or differentiation of peripheral Treg cells.[41] Tolerogenic DCs have been shown to have potential therapeutic uses in murine models of some autoimmune diseases such as collagen-induced arthritis,[49] experimental autoimmune encephalomyelitis,[50] myasthenia gravis[33,34] and antiphospholipid syndrome.[51] As DCs play an important role in the initiation and perpetuation of disease pathogenesis in SLE, tolerogenic DCs appear to be a potential cell-based therapy in this condition.[52,53]

Conclusions

In conclusion, MRL/MpJ RelB-shRNA DCs were shown to induce T-cell hyporesponsiveness, down-regulate IFN-γ expression and induce IL-10 producing splenic $CD4^{+}$ T lymphocytes in wild-type mice, and to suppress allogeneic T-cell proliferation and reduce IFN-γ and IL-17 expression in splenic T lymphocytes derived from MRL/lpr lupus-prone mice. Induction of T-cell anergy, low IL-12 production and induction of IL-10-producing Treg cells are probably underlying mechanisms that mediate their tolerogenicity. RelB-modified DCs provide a method in the generation of tolerogenic DCs that may have potential therapeutic implications in the treatment of SLE.

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Disclosures

The authors declare no conflict of interest.

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