

Immune Modulation and Tolerance Induction by RelB-Silenced Dendritic Cells through RNA Interference¹

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Dendritic cells (DC), the most potent APCs, can initiate the immune response or help induce immune tolerance, depending upon their level of maturation. DC maturation is associated with activation of the NF- κ B pathway, and the primary NF- κ B protein involved in DC maturation is RelB, which coordinates RelA/p50-mediated DC differentiation. In this study, we show that silencing RelB using small interfering RNA results in arrest of DC maturation with reduced expression of the MHC class II, CD80, and CD86. Functionally, RelB-silenced DC inhibited MLR, and inhibitory effects on alloreactive immune responses were in an Ag-specific fashion. RelB-silenced DC also displayed strong *in vivo* immune regulation. An inhibited Ag-specific response was seen after immunization with keyhole limpet hemocyanin-pulsed and RelB-silenced DC, due to the expansion of T regulatory cells. Administration of donor-derived RelB-silenced DC significantly prevented allograft rejection in murine heart transplantation. This study demonstrates for the first time that transplant tolerance can be induced by means of RNA interference using *in vitro*-generated tolerogenic DC. *The Journal of Immunology*, 2007, 178: 5480–5487.

Dendritic cells (DC)⁴ are the most potent APCs, largely bearing the responsibility of initiating protective immune responses (1). In contrast, DC also play a critical role in the induction of immune tolerance. It has been well demonstrated that the properties of DC depend on maturation status, phenotype, and source of origin, and that DC can be either immunostimulatory or immunoregulatory (2). In general, DC that inhibit immune responses have been described as immature (3), having plasmacytoid morphology (4), or being alternatively activated (5). Collectively, suppressive DC have been termed “tolerogenic DC.” Previous studies have demonstrated that donor-specific, allogeneic tolerogenic DC can enhance survival of transplanted grafts (6–8). Owing to the lack of one or more activation signals, immature DC (imDC) inhibit T cell responses via the mechanisms of apoptosis (9), anergy (10), and default Th2 differentiation (11, 12).

Recent research has revealed that DC maturation is associated with activation of the NF- κ B pathway (13, 14). NF- κ B represents a family of five Rel proteins: c-Rel, RelA/p65, RelB, NF- κ B1 (p50 and its precursor, p105), and NF- κ B2 (p52 and its precursor, p100). NF- κ B activation is essential for T cell activation by DC, and this occurs via the canonical NF- κ B pathway, a heterodimeric transcription factor that is predominantly composed of p65 and p50. Moreover, in the noncanonical pathway, the p100-RelB complex is simultaneously activated by phosphorylation of the C-terminal region of p100 by an I κ B kinase- α homodimer. This activation in turn leads to ubiquitination, followed by degradation of the p100 I κ B-like C-terminal sequences that generate p52-RelB. RelB is absent from the nucleus of precursor DC but is present in the nucleus of fully active, functional DC, suggesting that the molecular control of DC function occurs through a RelB mechanism. Direct evidence for a relationship between RelB and DC has been shown in studies in which the RelB gene was disrupted via either mutation or gene knockout, resulting in a dramatic reduction of NF- κ B activity with impaired DC function (15, 16). In contrast to the p65/p50 pathway, RelB plays a critical role coordinating the terminal stages of DC maturation and has the ability to induce optimal Th1 T cell responses. Maturation of DC, in response to stimuli, is prevented when NF- κ B activation is inhibited either pharmacologically or by overexpressing I κ B (17) as a result of modulating NF- κ B control gene expression (18). Inhibition of NF- κ B activation in DC results in subsequently reduced expression of T cell stimulatory molecules, such as the MHC class II, CD80, and CD86 molecules (14). In contrast to other NF- κ B family members such as RelA/p50, which chiefly contributes to cell survival, RelB is responsible for DC differentiation (19). Therefore, we hypothesize that RelB could be a potential target for manipulating tolerance-promoting imDC.

RNA interference (RNAi) is a newly discovered process in which dsRNA selectively inactivates homologous mRNA transcripts (20). It has been demonstrated that after a long dsRNA duplex enters the cytoplasm, a RNase III enzyme cleaves the duplex into smaller 21–23 bp. These cleaved components of dsRNA

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⁴ Abbreviations used in this paper: DC, dendritic cell; imDC, immature DC; mDC, mature DC; RNAi, RNA interference; siRNA, small interfering RNA; Treg, regulatory T cell; KLH, keyhole limpet hemocyanin.

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mediate the silencing of endogenous gene expression. Exogenous administration of small RNA duplexes, termed small interfering RNA (siRNA), is capable of blocking gene expression in mammalian cells without triggering the nonspecific panic response (21). We have demonstrated that siRNA effectively silences immune genes in DC (22), and that both *in vivo* and *in vitro* immune modulation can be achieved via induction of RNAi in DC using siRNA (23).

In this study, we generated tolerogenic DC by silencing RelB genes in DC. We demonstrate that RelB-silenced DC display an immature phenotype; these imDC, in turn, inhibit T cell responses in an Ag-specific manner. RelB-silenced DC initiated immune modulation by generating regulatory T cells (Treg). In a subsequent experiment, we demonstrate that administration of donor-derived RelB-silenced DC significantly prevented allograft rejection following murine heart transplantation, suggesting the potential clinical use of gene-silenced DC using RNAi.

Materials and Methods

Generation of bone marrow-derived DC

DC were generated from bone marrow progenitor cells, as previously described (24). Briefly, bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice (The Jackson Laboratory), and then washed and cultured in 24-well plates (2×10^6 cells/ml) in 2 ml of RPMI 1640 complete medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g of streptomycin, 50 μ M 2-ME, and 10% FCS (all from Invitrogen Life Technologies), supplemented with recombinant GM-CSF (10 ng/ml; PeproTech) and recombinant mouse IL-4 (10 ng/ml; PeproTech). All cultures were incubated at 37°C in 5% humidified CO₂. Nonadherent granulocytes were removed after 48 h of culture, and fresh medium was added. DC were cultured for 6 days (imDC) or for 8 days after being activated with CD40L (100 ng/ml) for 48 h (mature DC (mDC)).

siRNA synthesis and transfection

siRNA sequences were selected in accordance with the method described by Elbashir et al. (25). Four sequences specific to the RelB gene were selected: target 1 (UGGAAAUCAUCGACGAAUUAU), target 2 (GCUA CGGUGUGGACAAGAAUU), target 3 (GAAGAUCACGUGGGAAU UUU), and target 4 (GGGAAAGACUGCACGGACGUU). siRNA was synthesized and annealed by the manufacturer (Dharmacon). A pool consisting of four targeting siRNA was used to silence DC. siRNA specific to the luciferase gene GL2 Duplex (Dharmacon) was used as a sham-silencing control. Transfection was conducted as described previously (25). Briefly, 60 pmol of annealed siRNA was incubated with 3 μ l of GenePorter (Gene Therapy Systems) in a volume of 100 μ l of RPMI 1640 (serum-free) at room temperature for 30 min. This then was added to 400 μ l of DC cell culture, as earlier described. After 4 h of incubation, an equal volume of RPMI 1640, supplemented with 20% FCS, was added to the cells. At 24–48 h later, transfected DC were washed and used for subsequent experiments.

Flow cytometry

Phenotypic analysis of isolated or cultured DC was performed on a FACScan (BD Biosciences). All Abs were purchased from BD Pharmingen unless otherwise indicated. For T cells, we used Cy5-, FITC-, or PE-conjugated anti-mouse CD4, CD25, and FoxP3 (eBioscience). For DC, we used FITC- or PE-conjugated anti-mouse CD11c, I-A^b, CD80, and CD86 mAb. FoxP3 expression was assessed by intracellular cytokine staining using a cell permeabilization kit (eBioscience). T cell and DC subsets were analyzed by means of two- or three-color staining with various combinations of mAbs. All flow cytometric analyses were performed using appropriate isotype controls (Cedarlane Laboratories).

RT-PCR and real-time PCR

Total RNA was isolated from Treg or DC after gene silencing, using TRIzol (Invitrogen Life Technologies) according to the manufacturer's protocol. To remove DNA contamination, total RNA was processed further using the Message Clean kit (GenHunter). Briefly, 20 μ g of RNA was digested with 10 U of DNase I at 37°C for 30 min, extracted with phenol:chloroform (3:1), precipitated with ethanol, washed with 70% ethanol, and finally dissolved in 20 μ l of RNase-free water. To generate the first-strand cDNA, the SuperScript Preamplification System (Invitrogen Life Technologies) was used. Briefly, 0.5 μ g of oligo(dT) (12–18 bp) and 200 U of

SuperScript-2 reverse transcriptase were incubated with 2 μ g of DNA-free total RNA for 50 min at 42°C, in the presence of 0.5 mM dNTP, 10 mM DTT, and 1 \times first-strand buffer. For PCR amplification, reactions were conducted in a volume of 25 μ l of PCR Supermix High Fidelity (Invitrogen Life Technologies). Primers used in this study included the following: RelB (405 bp), sense 5'-CCGAGCTAGGGCCTTGGGTTCC-3' and antisense 5'-AGCTCGATGGCGGGCAGGGTCTTG-3'; FoxP3 (382 bp), sense 5'-CAGCTGCCTACAGTGCCTCCCTAG-3' and antisense 5'-CATTGCCAGCAGTGGGTAGCTG-3'; and GAPDH (249 bp), sense 5'-TGATGACATCAAGAAGGTGGTAA-3' and antisense 5'-TCCTTGGAGGC CATGTAGGCCAT-3'. PCR was conducted as DNA initially was denatured at 95°C for 3 min. This process was followed by 30 cycles consisting of denaturation at 95°C for 1 min, renaturation at 58°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. The PCR products were resolved by electrophoresis on a 2% agarose gel with 1 \times TAE buffer (40 mM Tris acetate, 2 mM Na₂EDTA 2H₂O (pH 8.5)) and visualized by ethidium bromide staining.

Quantitative PCR was performed on an ABI 7900 PCR Instrument (PerkinElmer) in a 10- μ l volume, using 2 \times Universal SYBR Green PCR Master mix (PerkinElmer). A relative quantitative assay was adopted. Mouse β -actin mRNA was used for normalization to ensure equal amounts of starting RNA. Each sample was tested in triplicate. Samples were obtained from at least three independent experiments to calculate the mean and SD.

MLR analysis

Splenic DC isolated from tolerant or rejecting recipients (BALB/c) were irradiated at 3000 rad. T cells ($1-5 \times 10^5$ /well) from C57BL/6 mice were added to the DC cultures, with the final MLR taking place in 200 μ l of complete RPMI 1640 medium (Invitrogen Life Technologies). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ for 3 days, and pulsed with 1 μ Ci of [³H]thymidine (Amersham Biosciences) for the last 16 h of culture. Cells were harvested onto glass fiber filters, and incorporated radioactivity was quantified using a Wallac Betaplate liquid scintillation counter. Results were expressed as mean \pm SEM cpm of triplicate cultures.

To test the Ag specificity of T cell responses, secondary MLRs were performed. T cells from primary MLR previously described were isolated using a T cell enrichment column and used as responders. Spleen cells from donor-specific mice (C57BL/6), third-party mice (C3H), and syngeneic mice (BALB/c) were used as stimulators in secondary MLR. The experimental procedures used to incubate and harvest cells were the same as described.

To determine the ability of Treg to inhibit MLR, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were isolated from tolerant or control BALB/c mice. These cells were added to an MLR that used normal BALB/c T cells as responders (2×10^5 /well) and irradiated (3000 rad) C57BL/6 spleen cells as stimulators (2×10^5 /well). Experimental procedures used to incubate and harvest cells were the same as described.

Immunization of mice with peptide-pulsed DC and Ag-specific T cell response

Six-day cultured DC were transfected with RelB-siRNA pool or with reagent alone, as described, and then pulsed with 10 μ g/ml keyhole limpet hemocyanin (KLH; Sigma-Aldrich) for 24 h. DC were then activated with CD40L for 48 h, washed extensively and used for subsequent transfer experiments. Ag-pulsed DC (5×10^5 cells/mouse) were injected s.c. into syngeneic mice. Mice were sacrificed after 10 days, and cell suspensions were prepared from the draining lymph nodes. These cells were cultured in 96-well plates at a concentration of 4×10^5 cells/well in the presence or absence of Ag for 3 days. An [³H]thymidine incorporation assay was performed as described for the MLR.

In some experiments, mice were immunized with OVA for testing Ag specificity. Two days before RelB-silenced DC or control DC immunization as described, the mice were s.c. immunized with 10 μ g of OVA. After 10 days, T cells from lymph nodes were isolated from the recipient mice. Ag-specific recall responses were performed in the presence of KLH or OVA, as described.

Heterotopic cardiac transplantation

The 8- to 12-wk-old male C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from The Jackson Laboratory and used as donors and recipients, respectively. BALB/c recipient mice were treated i.v. with RelB-silenced or nonsilenced DC (5×10^6 cells), 3 days before transplantation. Treated and untreated BALB/c mice were subjected to allogeneic cardiac transplantation, using organs from C57BL/6 donors. Heterotopic heart transplantation was performed according to our laboratory's routine procedures

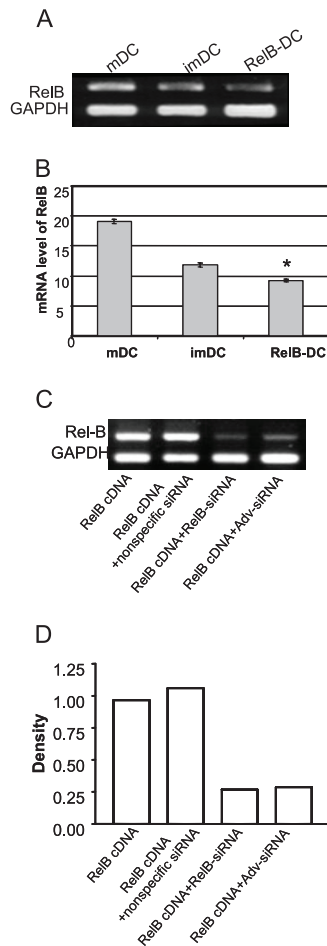


FIGURE 1. Silencing DC with RelB siRNA. *A*, RelB expression determined by RT-PCR. imDC were cultured from C57BL/6 mice for 6 days in the presence of GM-CSF and IL-4. mDC were cultured for 8 days following activation with CD40L (100 ng/ml) for 48 h. mDC were transfected with RelB siRNA using GeneSilencer. At 24 h after gene silencing, total RNAs were extracted from imDC, mDC, and RelB-silenced DC. RT-PCR was performed using primers specific to RelB and GAPDH, as described in *Materials and Methods*. *B*, RelB expression was determined by real-time quantitative PCR. DC culture and gene silencing were performed using the same protocols described in *A*. mRNA amounts were normalized to levels of GAPDH mRNA. The mean value and SD of triplicate measurements of samples are shown. *, $p < 0.05$. *C*, Gene silencing in RelB up-regulated DC. The 8-day cultured DC were cotransfected with a RelB expression vector together with RelB-siRNA to mDC. Gene silencing and RT-PCR were performed using the same protocols described in *A*. *D*, Quantification of RelB expression was performed by densitometry after cotransfection of RelB-cDNA and RelB-siRNA shown in *C*.

(26). Pulsation of heart grafts was monitored daily by two independent observers who were blinded to the treatment protocol. Direct abdominal palpation was used to assess graft viability, and the degree of pulsation was scored as: A, beating strongly; B, noticeable decline in the intensity of pulsation; or C, complete cessation of pulsation. Graft recipients surviving >100 days were classified as "tolerant" and used for subsequent in vitro experiments. Untreated BALB/c recipients that had rejected allografts were used as "rejecting controls."

Statistical analysis

Graft survival was compared between experimental groups using the log-rank test. MLR data were analyzed using one-way ANOVA and followed, if necessary, by the Newman-Keuls test. Differences for the value of $p < 0.05$ were considered significant.

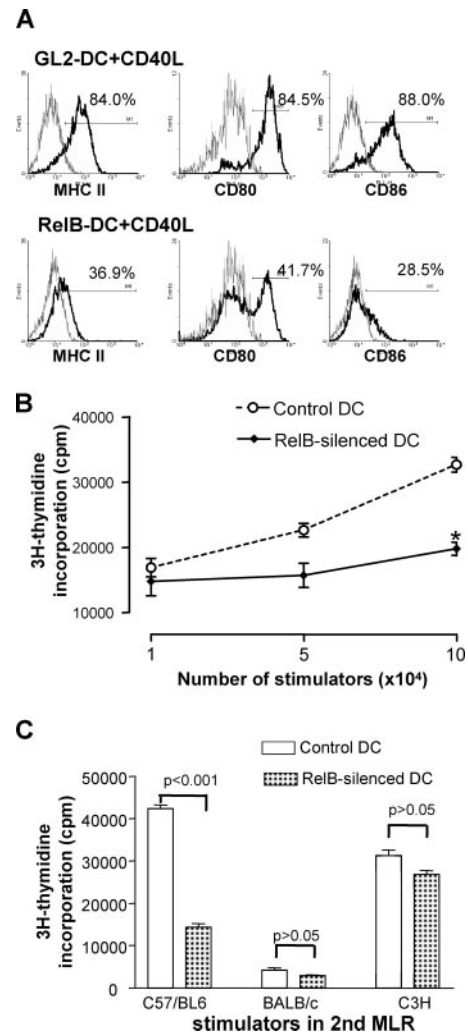


FIGURE 2. RelB-silenced DC are immature and tolerogenic. *A*, Phenotypic analysis of DC after silencing RelB. Bone marrow-derived DC (1×10^6) were transfected with 60 pmol of RelB-siRNA (RelB-DC) or control GL2-siRNA (GL2-DC) on day 6. The transfected DC subsequently were activated for 48 h with 100 ng/ml CD40L. Maturation was assessed in terms of expression of MHC class II, CD80, and CD86 by flow cytometry using FITC- or PE-conjugated Abs (thick line histogram) and isotype controls (dashed line histogram). *B*, RelB-siRNA transfection inhibits DC allostimulatory ability. C57BL/6-derived DC were transfected with 60 pmol of GL2 siRNA (control siRNA) or with 60 pmol of RelB-siRNA for 24 h. Allogeneic (BALB/c) T cells (2×10^5 /well) were incubated with siRNA-treated DC at the indicated concentrations for 72 h. Proliferation was determined using [³H]thymidine incorporation. *, $p < 0.05$. *C*, Allogeneic T cell response in secondary MLR. T cells from the primary MLR in *B* were isolated and used as responders (2×10^5 /well). The spleen cells from C57BL/6 (allogeneic), BALB/c (syngeneic), and C3H (third-party) mice were used as stimulators (2×10^5 /well). The experimental procedure of MLR was the same as described in *B*. Data are representative of three independent experiments.

Results

Silencing RelB in DC

To validate gene silencing in DC, we transduced RelB-siRNA into in vitro-cultured bone marrow-derived DC. To compare mRNA levels of RelB between imDC and mDC, we determined RelB expression in DC using conventional RT-PCR (Fig. 1*A*) and real-time PCR (Fig. 1*B*). Although 6-day cultured imDC expressed low levels of RelB, an elevated expression was observed in 8-day cultured mDC that had been activated with CD40L. Silencing RelB

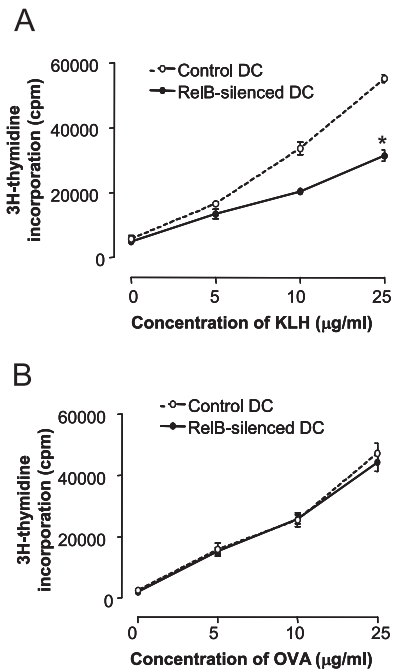


FIGURE 3. Immune modulation by siRNA-silenced DC. *A*, KLH-specific T cell response. Bone marrow-derived DC cultured in GM-CSF and IL-4 were transfected with RelB-siRNA (RelB-silenced DC) or GL2-siRNA (control DC). Subsequently, cells were pulsed with 10 µg/ml KLH for 24 h and s.c. injected (5×10^5 cells/mouse) into syngeneic C57BL/6 mice. *, $p < 0.05$. Two days before RelB-silenced DC or control DC immunization, the mice were immunized with 10 µg of OVA s.c. After 10 days, T cells from the lymph nodes were isolated from the recipient mice. Ag-specific recall responses were performed in the presence of KLH (*A*) or OVA (*B*), as described in *Materials and Methods*. Data are representative of three independent experiments.

using siRNA inhibited the up-regulation of RelB that is otherwise observed in mDC (Fig. 1, *A* and *B*).

To assess the potency of gene silencing using siRNA, we cotransfected RelB-cDNA and RelB-siRNA into DC. As shown in Fig. 1C, RelB-siRNA significantly inhibited RelB expression after gene transfection. To confirm this finding, we also cotransfected DC with an adenoviral siRNA specific for RelB. Similar efficacy in silencing was observed (Fig. 1C). Gene silencing produced a 4-fold decrease in RelB expression (Fig. 1D). Collectively, these results suggest that gene silencing can effectively block RelB expression during DC maturation, following transduction of exogenous RelB.

RelB-silenced DC are immature and tolerogenic

The critical role of NF-κB signaling in DC maturation is illustrated by the absence of mature DC in knockout mice that lack components of this pathway (27). We accordingly assessed the maturation of DC after gene silencing. Bone marrow-derived DC were matured by activation of CD40-CD40L via signaling RelB/p50 (28). A mature phenotype, expressing high levels of MHC-II, CD40 and CD86, was confirmed. Gene silencing of RelB resulted in arrest of maturation, even in the presence of maturation stimuli. After gene silencing, DC demonstrated decreased levels of MHC molecules CD80 and CD86 (Fig. 2A).

Immune activation signals (29, 30) stimulate TLRs on DC and cause NF-κB activation, leading to maturation, up-regulation of costimulatory molecules (14, 18), and cytokine secretion (31). These events culminate in mature DC that are capable of stimulating T cell responses. In contrast, DC in the steady state are

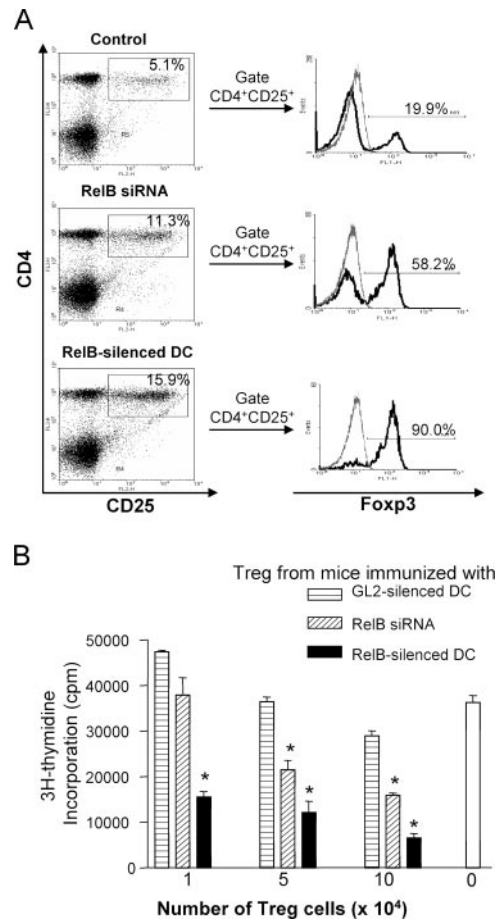


FIGURE 4. RelB-silenced DC generate Treg in vivo. Bone marrow-derived DC, cultured in GM-CSF and IL-4, were transfected with RelB-siRNA (RelB-silenced DC) or GL2-siRNA (control DC). Subsequently, cells were pulsed with 10 µg/ml KLH for 24 h and s.c. injected (5×10^5 cells/mouse) into syngeneic C57BL/6 mice. Alternatively, mice were not treated with DC, but immunized with KLH together with 50 ng of RelB siRNA (RelB-siRNA). After 10 days, T cells from lymph nodes were isolated from recipient mice. *A*, Phenotypic analysis of in vivo-generated Treg. T cells were triple-stained with Cy5-labeled anti-CD4 mAb, PE-labeled anti-CD25 mAb, and FITC-labeled anti-FoxP3 mAb. Cells were sorted and analyzed by flow cytometry. *B*, Inhibition of KLH-specific T cell response by Treg. CD4⁺CD25⁺ positive T cells were isolated by FACS sorting, and were added at indicated numbers to KLH-specific recall response as described in Fig. 3. Data are representative of three independent experiments. *, $p < 0.05$.

immature and tolerogenic, and they do not possess activated NF-κB (2). To evaluate the capacity of DC to stimulate T cell responses after RelB silencing, we performed MLR. Although control DC (nonspecific silencing) initiated a vigorous reaction, RelB-silenced DC failed to stimulate allogeneic T cell responses (Fig. 2B), suggesting that RelB-silenced DC are immunosuppressive or tolerogenic DC.

To determine the Ag specificity of the inhibited T cell responses, we performed a secondary MLR using T cells from the primary MLR. T cells that were previously primed by allogeneic RelB-silenced DC failed to respond to alloantigen (C57BL/6) stimulation in the secondary MLR. However, T cells that were previously primed with RelB-silenced or nonsilenced DC, however, retained equally vigorous responses to third-party (C3H) stimulation (Fig. 2C).

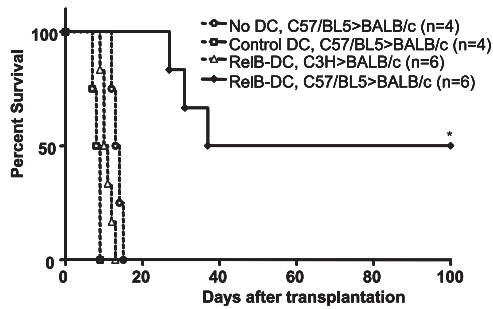


FIGURE 5. Induction of tolerance in allogeneic heart transplantation by RelB-silenced DC. The 6-day cultured donor (C57BL/6)-derived DC were transfected with RelB-siRNA (RelB-silenced DC) or GL2-siRNA (control DC). Subsequently, cells were injected i.v. (5×10^5 cells/mouse) into recipient (BALB/c) mice. At 7 days after DC treatment, allogeneic (C57BL/6 to BALB/c) and third-party control (C3H to BALB/c) heart transplants were performed. Data demonstrate survival rate of grafts after transplantation. *, $p < 0.001$.

Immune modulation by RelB-silenced DC

We next investigated whether RelB-silenced DC could function as immune modulators in vivo. To test this possibility, we cultured bone marrow-derived DC, silenced them with RelB siRNA, and then pulsed with KLH Ag. After immunization with these Ag-pulsed and RelB-silenced DC, the KLH-specific recall response was assessed in vitro. Immunization with control DC induced a strong KLH recall response, while administration of RelB-silenced DC inhibited T cell response to KLH rechallenge (Fig. 3A). To further test the Ag specificity of immune modulation by RelB-silenced DC, we coimmunized the mice with nonrelated OVA Ag. As shown in Fig. 3B, T cells retained normal proliferative responses to OVA. These data imply that RelB-silenced DC induce Ag-specific immune suppression in vivo.

RelB-silenced DC generate Ag-specific Treg in vivo

A recent study revealed that RelB^{-/-} DC, generated from NF- κ B-deficient mice, promoted production of Treg (32). To determine whether RelB-silenced DC are capable of generating Treg in vivo, we immunized mice with KLH-pulsed syngeneic DC. Significant increases in CD4⁺CD25⁺FoxP3⁺ Treg were observed in mice immunized with RelB-silenced DC, as compared with mice immunized with GL2-silenced control DC (Fig. 4A). Treg, isolated from RelB-silenced DC-immunized mice, could inhibit KLH-specific T cell responses (Fig. 4B). Direct delivery of RelB-siRNA to KLH-immunized mice also generated Treg in vivo, but this was less effective than using RelB-silenced DC (Fig. 4).

Induction of tolerance by RelB-silenced DC

Previous studies have applied decoy oligonucleotides to inhibit NF- κ B activity, revealing the critical role of this transcription factor in graft rejection (33). As RelB-silenced DC demonstrated strong abilities to inhibit immune responses in vitro (Fig. 2) and in vivo (Fig. 3), we further assessed their capacity to induce tolerance in a murine transplantation model. As expected, in this MHC fully mismatched allogeneic heart transplantation model, control recipients had rapid graft rejection by day 12 posttransplantation. As well, administration of donor-derived and nonsilenced DC further accelerated graft rejection. In contrast, administration of donor-derived and RelB-silenced DC significantly prevented graft rejection; 50% of these recipients achieved tolerance to allogeneic cardiac grafts (Fig. 5). However, RelB-silenced C57BL/6 DC failed to protect third-party (C3H to BALB/c) rejection, suggesting the tol-

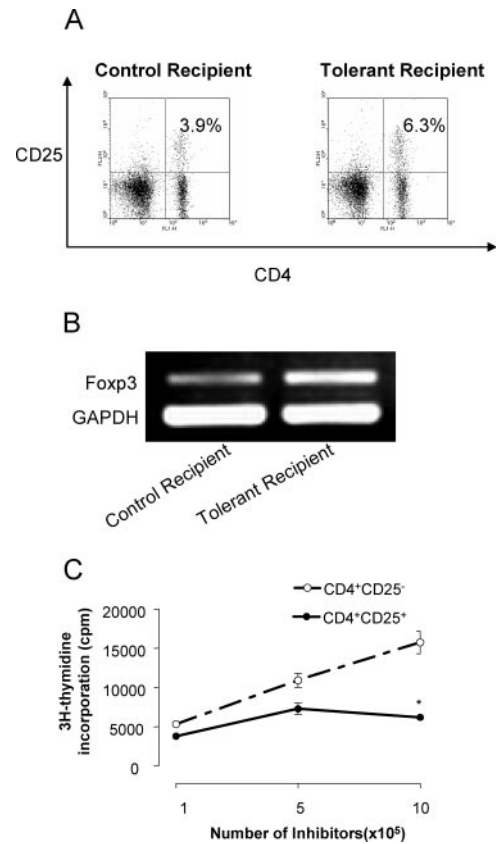


FIGURE 6. RelB-silenced DC-mediated tolerance is associated with Treg. *A*, Increase of Treg in tolerant recipients after RelB-silenced DC treatment. Splenic T cells were isolated from tolerant (treated with RelB-silenced DC) or control (treated with GL2-siRNA) mice after transplantation as described in Fig. 5. T cells were stained with anti-CD4 FITC and anti-CD25 PE, and analyzed by flow cytometry. *B*, Up-regulated expression of FoxP3 genes. RNA from the T cells described in *A* was extracted by means of the TRIzol method. RT-PCR was performed to assess expression of FoxP3 and GAPDH, using primers described in *Materials and Methods*. Data are representative of three independent experiments. *C*, Treg inhibit ongoing MLR. MLR was performed using BALB/c T cells as responders (5×10^5 cells/well) and C57BL/6 spleen cells (irradiated at 3000 rad) as stimulators (5×10^5 cells/well). CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from tolerant recipients in described in *A* were isolated by FACS analysis, then added to each well as inhibitors (1 – 10×10^5 cells/well). *, $p < 0.05$.

erance induced by RelB-silenced DC occurs in an alloantigen-specific manner.

Induction of tolerance by RelB-silenced DC is associated with Treg formation

It has been reported that DC from NF- κ B-deficient mice facilitate generation of Treg, which can transfer Ag-specific tolerance to primed recipients (32). We therefore tested for Treg in long-term survived recipients who had been treated with RelB-silenced DC. Compared with observations in rejecting mice that had received nonsilenced control DC, the CD4⁺CD25⁺ T cell population was significantly increased in the tolerant mice treated with RelB-silenced DC (Fig. 6A). CD4⁺CD25⁺ T cells isolated from tolerant recipients expressed high levels of the FoxP3 gene (Fig. 6B) and suppressed ongoing MLR (Fig. 6C). These data suggest that the tolerance induced by RelB-silenced DC may be associated with in vivo Treg generation.

Discussion

Experimental manipulation of DC to induce tolerogenic function has been achieved by inhibiting molecules involved in DC maturation and activation (2). Several approaches have been attempted to generate tolerogenic DC in vitro. For example, imDC have been generated by modifying culture conditions (34), by blocking transcription factors such as NF- κ B (33), and by pharmacologically preventing DC maturation (35). Tolerogenic DC have been generated by blocking costimulatory molecules through the use of Abs (36), fusion proteins (37, 38), and antisense oligonucleotide (33). Current research indicates that traditionally used immunosuppressants, such as tacrolimus and cyclosporin A, may indirectly result in immune suppression by stimulating production of tolerogenic DC (39). In addition, genetically engineering DC with the Th2/Th3 cytokines IL-10, TGF- β , Fas ligand (24), CTLA-4, or Srrate (a ligand of Notch proteins) (40) results in Ag-specific tolerance (24, 41) as well as the induction of Treg (42). Many previous studies have demonstrated the ability of tolerogenic DC to induce transplantation tolerance in various transplant models (24, 43–52).

Although some success in tolerance induction has been achieved in animal models using artificially manipulated tolerogenic DC, a clinically applicable method has not yet been developed. Several drawbacks have been documented with the use of conventional methods: production of anti-idiotypic Abs (53), inefficiency and instability in suppressing costimulatory molecules by antisense oligonucleotide (54), drug toxicity (55), and so on. Consequently, there is a great need for a more potent and physiologically acceptable method of specifically blocking gene expression. siRNA would be an ideal approach, owing to its potency, specificity, simplicity, and relative safety (56, 57). We previously have silenced DC using siRNA to achieve immune modulation (23, 56, 58). In this study, we generated tolerogenic DC by silencing their RelB genes. We demonstrated that RelB-silenced DC display an immature phenotype, and they inhibit T cell responses in an Ag-specific manner. RelB-silenced DC initiate immune modulation by stimulating the generation of Treg. Administration of donor-derived RelB-silenced DC significantly prevented allograft rejection ($p < 0.001$) following murine heart transplantation, suggesting the potential clinical use of gene-silenced DC.

A critical step in the maturation of DC involves activation of NF- κ B, as illustrated by the absence of mature DC in knockout mice that lack components of this pathway (27). Upon activation, NF- κ B translocates into the nucleus, where it turns on expression of the MHC class II molecules, CD80, CD86, and CD40 genes by binding to κ B sites (14, 18). Thus, NF- κ B is a key factor controlling DC maturation. In the context of transplantation, we and others have reported that inhibition of NF- κ B, using pharmacological inhibitors, endows DC with the ability to induce Treg formation and Th2 polarization (17, 59).

Specific family members of the NF- κ B pathway possess varying effects on DC maturation. The primary NF- κ B protein involved in DC maturation is RelB (19). Although individually knocking out NF- κ B p50, RelA or c-Rel does not affect DC maturation; only double knockout of p50 and RelA leads to arrest of DC maturation (27). In contrast, DC generated from mice lacking RelB do not up-regulate MHC class II and costimulatory molecules upon activation, and they are tolerogenic in vivo (32). Recent advances in DC research have improved our understanding about the NF- κ B-dependent mechanism of DC maturation via a canonical RelA/p50 pathway that requires the noncanonical RelB-p52 complex. This understanding has established that RelB plays a vital role in regulating the terminal stages of maturation. Our data together with other findings suggest that RelB silencing confers DC tolerance

and stimulates Treg development. Furthermore, RelB-silenced DC affected T cell infiltration into the transplant graft. Thus, RelB could be a potential target for promoting tolerance induction.

We previously have demonstrated that transplant tolerance is associated with the existence of imDC (59–61). Induction of immune tolerance by imDC is mediated via T cell apoptosis (62), anergy (63), or Treg differentiation (64). Classically, the lack of costimulatory molecules on tolerogenic DC (58), as well as the presence of inhibitory molecules such as PD-1L (65), has been identified as central to this immune suppression. imDC have been used therapeutically in transplantation. Treatment of DC progenitors with low-dose GM-CSF induces formation of a distinct imDC subset that possesses poor T cell costimulatory capacity (34). In the present study, we reported a novel method to generate imDC by inhibiting NF- κ B signaling in DC using RNAi. RelB-silenced DC express similar surface markers as imDC. It has been reported that imDC prolong donor-specific allograft survival (34). However, imDC may become mature after encountering maturation stimuli, such as Ags or activated T cells in vivo (2). In contrast to this possibility, RelB-silenced DC result from gene interference and they cannot engage in maturation even in the presence of maturation stimuli in vitro (Fig. 2). Thus, RelB-silenced DC would have therapeutic potential. DC, as professional APC, can potentially initiate naive T cells to differentiate into effector T cells in the immune response. However, DC can also initiate tolerance through generation of Treg (66). Whether DC stimulate a T cell response or tolerance depends on their biological properties, localization state, and maturation state. Basically, mDC provoke an immune response, whereas imDC induce tolerance (66). Previous studies have demonstrated that Treg differentiation can be driven by imDC (67). Treg generation by imDC has been demonstrated both in murine (68) and human systems (42). The precise mechanisms underlying DC-mediated Treg generation, however, have not been fully elucidated. Initial reports suggest that a lack of costimulation, together with the presence of inhibitory molecules on imDC, provides an environment suitable for Treg generation (65). Evidence that DC from RelB knockout mice induce Treg formation in vivo (32) provides a rationale for targeting the RelB gene. Indeed, imDC generated through the pharmacological inhibition of NF- κ B were able to induce Treg formation in our previous study (60). The present study demonstrates that RelB-silenced DC facilitates formation of Treg cells both in vitro and in vivo.

In summary, we describe a novel method to generate therapeutic DC using RNAi. Tolerogenic DC were generated by silencing the RelB gene in DC. Administration of donor-derived RelB-silenced DC significantly prevented allograft rejection following murine heart transplantation, suggesting the potential clinical use of gene-silenced DC.

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Disclosures

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