

Tolerogenic Dendritic Cells Induced by BD750 Ameliorate Proinflammatory T cell Responses and Experimental Autoimmune Encephalitis in Mice

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BD750, a novel JAK3/STAT5 inhibitor, can inhibit T cell proliferation. This study aims to evaluate whether BD750 can induce tolerogenic dendritic cells (tolDC) and their function in experimental autoimmune encephalitis (EAE) in mice. Following BD750 treatment, lipopolysaccharide (LPS)-induced maturation of DCs, allogeneic T cell proliferation, Th1 and Th17 cell functional differentiation, and STAT5 and AKT activation were determined. The effect of tolDC loaded with antigen peptide on the development and severity of EAE and splenic Th1 and Th17 cell responses was determined. In comparison with LPS-induced mature DCs (mDCs), BD750 treatment induced tolDC with lower expression levels of costimulatory molecules and proinflammatory cytokines and lower levels of STAT5 phosphorylation. TolDC inhibited allogeneic T cell proliferation and reduced Th1 and Th17 responses. Adoptive transfer of tolDC loaded with myelin oligodendrocyte glycoprotein₃₅₋₅₅ inhibited the development and severity of EAE in mice, accompanied by reduced numbers of inflammatory infiltrates and decreased levels of demyelination in the spinal cord tissues. In addition, treatment with tolDC loaded with antigen peptide also significantly reduced the frequency of splenic Th1 and Th17 cells in EAE mice. The effects of tolDC were similar to that of JAK/STAT inhibitor CP690550-treated DCs. In conclusion, treatment with BD750 induced tolDC that inhibited proinflammatory T cell immunity *in vitro* and *in vivo*. BD750 and tolDC may be valuable for development of new therapies for EAE and other autoimmune diseases.

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease in the central nervous system (CNS), predominantly mediated by autoreactive CD4⁺ Th1 and Th17 cells. During the pathogenic process of MS, autoreactive CD4⁺ T cells pass through the blood-brain barrier and migrate into the brain and spinal cord to cause demyelination

in the CNS (1,2). Together with defects in the function of regulatory T cells (Tregs) in patients with MS, this enhances pathogenic Th1 and Th17 responses within the brain (3). Therefore, reestablishing antigen-specific tolerance will be of great significance in managing patients with MS (4).

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) and can

induce naïve T cell activation. Mature DCs present peptide of antigens by major histocompatibility complex (MHC) molecules and express high levels of costimulatory molecules, such as CD80, CD83, CD86 and CD40, on their surface to provide two necessary signals for full T cell activation. Inflammatory DCs can secrete IL-12, IL-6, TGF- β and IL-23, which promote Th1 or Th17 polarization (5). The levels of proinflammatory cytokines produced by DCs largely depend on the maturation status, ranging from fully mature immunogenic DCs to tolerogenic immature DCs, spanning a wide variety of intermediate semi-mature states. Tolerogenic DCs (tolDC) are usually resistant to maturation, with lower levels of costimulatory molecule expression and inflammatory cytokine production, but higher levels of inhibitory molecule expression. tolDC can induce anergy and apoptosis of effector T cells,

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but promote Treg responses (6,7). Adoptive transfer of tolDC is a new strategy to restore immune tolerance and may be a therapeutic strategy for autoimmune diseases (8,9). Treatment with type II collagen-pulsed tolDC (dexamethasone/vitamin D3-modified) decreases the percentages of pathogenic Th17 cells in arthritic mice and reduces the severity and progression of arthritis (10). Furthermore, H4₇₁₋₉₄ peptide-loaded tolDC augments suppressive function of Tregs, suppresses Th17 cells and benefits patients with lupus (11). In addition, tolDC generated *in vitro* inhibit the process of experimental autoimmune encephalitis (EAE) in mice (12). More importantly, tolDC from relapsing-remitting MS patients can induce stable antigen-specific hyporesponsiveness in myelin-reactive T cells (13).

Induction of tolDC has been experimentally achieved by suppressive agents, including IL-10 and TGF- β ; immunomodulatory drugs, such as dexamethasone and vitamin D; and genetic modification (14). Our previous study identified that a benzothiazole derivative, BD750 [2-(2-benzothiazolyl)-4,5,6,7-tetrahydro-2H-indazol-3-ol, C₁₄H₁₃N₃OS, MW: 271.3], is an inhibitor of JAK3/STAT5 signaling and can inhibit T cell proliferation (15). JAK3 is crucial for the maturation of DCs, and JAK3^{-/-} DCs fail to induce T cell proliferation (16,17). STAT5 is crucial for thymic stromal lymphopoietin-dependent DC activation and can up-regulate the expression of costimulatory molecules and chemokines (18). Accordingly, we hypothesize that BD750 may induce tolDC, which may inhibit antigen-specific Th1 and Th17 responses and the pathogenic process of EAE in mice. In the current study, we tested the effect of BD750 on the maturation and function of DCs *in vitro* and the impact of adoptive transfer of BD750-treated DCs on Th1 and Th17 responses in EAE mice. We found that BD750 induced tolDC that impaired allogenic antigen-stimulated T cell responses *in vitro*. Furthermore, adoptive transfer of tolDC that had been loaded with antigen peptide inhibited Th1 and Th17 responses and the development

of EAE in mice. Our findings may aid in the design of new therapies for MS.

MATERIALS AND METHODS

Animals and Animal Models

C57BL/6 and BALB/c mice were purchased from Dashuo Biotechnology (Chengdu, China). The experimental protocols were approved by the Animal Care and Use Committee at Chengdu Medical College. All mice were bred and maintained in a specific pathogen-free facility in the Experimental Animal Center of Chengdu Medical College at consistent room temperatures (20–26°C) and humidity (approximately 40–70%), with a 12 h/12 h light/dark cycle.

Female C57BL/6 mice (10 wks old, 19–21 g) were used for the experiments aimed at investigating the therapeutic effect of BD750-treated DCs on the development and progression of EAE. Animals were randomized by simple randomization into seven groups (n = 8 mice per group). The mice were immunized subcutaneously at the dorsal flanks with 200 μ g myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ peptide (China-Peptides, Shanghai, China) emulsified 1:1 with complete Freund's adjuvant (CFA) supplemented with 4 mg/mL heat-killed *Mycobacterium tuberculosis* (Chondrex, Redmond, WA, USA). Individual mice were injected intraperitoneally with 500 ng pertussis toxin (PTX) on d 0 and 2. One group of mice received adjuvant and PTX only and served as the control. The mice were monitored daily for clinical symptoms, which were scored as: 0, no clinical signs; 1, paralyzed tail; 2, loss of coordinated movement, hind limb paresis; 3, both hind limbs paralyzed; 4, forelimbs paralyzed; and 5, moribund (19).

Generation of Murine Bone Marrow-Derived Dendritic Cells

Murine bone marrow-derived dendritic cells (BMDCs) were isolated from female C57BL/6 mice (6 wks old, 16–18 g), as described previously (20). Briefly, the animals were anesthetized with intraperitoneal injection of sodium pentobarbital

and euthanized by cervical disconnection. Subsequently, their femur and tibia bones were cut with scissors and the marrow was flushed out, followed by passing through a nylon mesh to remove small pieces of bone and debris. The resulting single cells (1×10^6 cells/dish) were cultured in 10% fetal calf serum RPMI 1640 (complete medium) for 4 h. The suspended cells were removed and the adherent cells were cultured in complete medium containing 20 ng/mL of recombinant granulocyte-macrophage colony-stimulating factor and 10 ng/mL of IL-4 (PeproTech) for 6 d. The cells were exposed to fresh medium every 3 d. On d 6 post-incubation, the cells were harvested and some cells were stained with fluorescent-labeled antibodies, followed by flow cytometry analysis. The CD11c⁺ immature DCs in the remaining cells were purified by magnetic anti-CD11c beads (Miltenyi). The purified immature DCs were pretreated with BD750 or vehicle dimethyl sulfoxide for 12 h, ensuring that the dimethyl sulfoxide was <0.025%. The cells were then stimulated with 100 ng/mL lipopolysaccharide (LPS) to induce DC maturation for 24 h.

Allogenic Mixed Leukocyte Reaction

To test the effect of BD750-treated DCs on stimulating T cell proliferation, naïve splenic CD4⁺CD69⁻ T cells were isolated from female BALB/c mice (6 wks old, 17–19 g) using a T cell isolation kit (Miltenyi) and labeled with carboxyfluorescein diacetate succinimidyl ester (1.2 μ M; Invitrogen). The naïve T cells were cocultured in triplicate with allogenic BD750 pretreated DCs (BD750-mDC) at three DC:T cell ratios (1:10, 1:30, 1:100) for 72 h. The proliferation of activated T cells was analyzed by flow cytometry.

Flow Cytometry

The mature DCs were stained in duplicate with fluorescent-labeled antibodies against CD11c, CD69, CD83, CD40, CD80, CD86, MHC II or isotype-matched controls (BioLegend, San Diego, CA, USA). The maturation status of DCs was analyzed by flow cytometry.

Some mature DCs were analyzed for their apoptosis by flow cytometry using the PI Annexin V Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturers' instruction.

In addition, the functional phenotypes of CD4⁺ T cells were analyzed by flow cytometry. Briefly, after mixed leukocyte reaction (MLR) coculture, CD4⁺ T cells were separated from the cell culture, then treated with anti-CD16/32 (Mouse BD Fc Block™), stained with fluorescein isothiocyanate-anti-CD4, fixed, permeabilized and intracellularly stained with PE-conjugated anti-IFN- γ and Alexa Fluor® 647-conjugated anti-IL-17, followed by flow cytometry. Moreover, Tregs were analyzed by flow cytometry using the Transcription Factor Buffer Set (BD Pharmingen), after being stained with APC-anti-CD25 and intracellularly with PE-anti-Foxp3, according to the manufacturer's instructions. All flow cytometry was performed in the Accuri™ C6 (BD Biosciences) or Cytomics FC 500 (Beckman Coulter).

Enzyme-Linked Immunosorbent Assay

The supernatants of cultured DCs or DCs cocultured with T cells were harvested and the levels of IL-6, IL-10, IL-12, TNF- α , IL-1 β , IL-23, IFN- γ and IL-17 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using specific kits (eBioscience), according to the manufacturer's instructions. Briefly, the control and experimental samples were simultaneously tested in triplicate, and the plates were read at 450 nm (Powerwave XS, BioTek, Winooski, VT, USA).

Western Blotting

The purified immature DCs were pretreated with BD750 (1, 5 or 20 μ M), CP690550 (1 μ M), LY294002 (1 μ M) or vehicle for 12 h. The cells were stimulated with 100 ng/mL LPS to induce DC maturation for 24 h. The harvested mature DCs were lysed in radioimmunoprecipitation assay buffer (Millipore), and the protein concentrations in the lysates were determined using a BCA Protein

Assay Kit (BioTek). Individual cell lysate samples (20 μ g/lane) were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After being blocked with 5% bovine serum albumin, the membranes were incubated with specific antibodies against STAT5, phospho-STAT5, Akt, phospho-Akt or GAPDH (Cell Signaling Technology). The bound antibodies were detected with horseradish peroxidase-conjugated second antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) and visualized by enhanced chemiluminescence (Millipore). The relative levels of target proteins were normalized to the internal GAPDH and analyzed using ImageJ software.

Adoptive Transfer Experiments

Immature DCs at 6 d post-culture were pretreated with BD750 (20 μ M) for 12 h. The cells were pulsed with MOG₃₅₋₅₅ (20 μ g/mL) for 24 h and subsequently treated with LPS (100 ng/mL) to induce DC maturation for 24 h. The cells were harvested and injected intravenously into mice at -2, 2 and 6 or 7, 11 and 15 d post first PTX injection (1 \times 10⁶ cells/mouse). The control mice were injected with PBS.

Histological Analysis

At 22 d post-induction, the mice were anesthetized with intraperitoneal injection of sodium pentobarbital and perfused with PBS (pH 7.4), followed by 4% (w/v) paraformaldehyde. Spinal cord samples were then fixed in 4% (w/v) paraformaldehyde overnight and paraffin-embedded. The coronal sections (5 μ m) of cervicothoracic spinal cord tissues were stained with hematoxylin and eosin and examined for cellular infiltration or with Luxol fast blue/periodic acid-Schiff to determine demyelination.

Statistical Analysis

The data are expressed as mean \pm standard error of the mean. The differences among the groups were evaluated

using analysis of variance and *post hoc* least significant different *t* test or Kruskal-Wallis test using PRISM 6.0 software (GraphPad Software, San Diego, CA, USA). A *P* value < 0.05 was considered statistically significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

BD750 Treatment Induces tolDC

Mature dendritic cells (mDCs) possess numerous dendrites that may be of great advantage to interaction with T cells (21). In this study, we first examined the effect of BD750 treatment on the maturation and survival of DCs *in vitro*. Treatment with a low dose of BD750 (1 μ M) did not change the morphology of mDCs, and both mDCs that had been treated with low-dose BD750 and vehicle displayed large clusters of adherent cells with sharp cytoplasmic projections. In contrast, most mDCs that had been treated with BD750 (5 or 20 μ M) or CP690550, a JAK inhibitor, had fewer small dendrites and smaller clusters than typical mDCs (Figure 1A). However, treatment with BD750 did not change the frequency of spontaneous mDC apoptosis (Figures 1B, C). Hence, treatment with a higher dose of BD750 altered the morphology of mDC, but did not change the frequency of spontaneous mDC apoptosis *in vitro*.

Next, we analyzed whether BD750 could change the maturation of DCs. DCs were stimulated and generated at d 6 post stimulation as immature DCs (imDCs), and further stimulated with LPS for maturation as mDCs. Some imDCs were treated with or without BD750 or CP690550 alone. Subsequently, the supernatants of cultured DCs were harvested and the cells were stained with fluorescent-labeled antibodies to analyze the levels of CD69, CD40, CD80, CD83, CD86 and I-A^b expression by flow cytometry. ImDCs expressed very low levels of each molecule tested relative to mDCs (Figure 2). Furthermore, DCs that had been treated with BD750 at

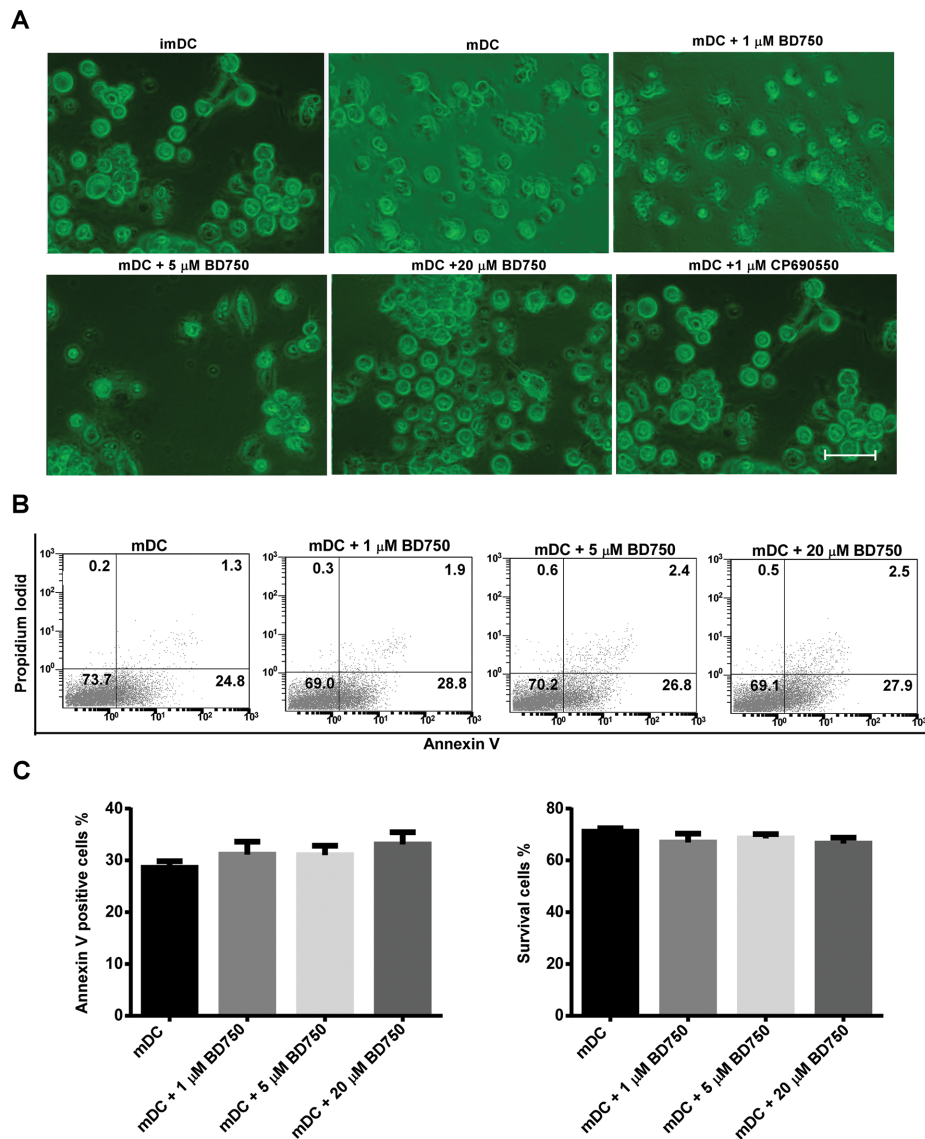


Figure 1. Morphological characteristics and survival of typical mDCs and BD750-treated DCs. mDCs were stimulated with cytokines and treated with or without BD750 at the indicated dose or CP690550 for 12 h and then further stimulated with LPS for 24 h. The imDCs, mDCs and BD750-treated DCs were observed by a light microscope, and the percentages of apoptotic DCs in different groups of cells were determined by flow cytometry analysis. Data are representative images or expressed as the mean \pm SEM of three independent experiments. (A) Light microscopic analysis of different groups of DCs. (B) Flow cytometry analysis of apoptotic cells. (C) Quantitative analysis of apoptotic cells. * $P < 0.05$, ** $P < 0.01$ versus the mDC group.

concentrations of 5 and 20 μM but not 1 μM displayed significantly reduced levels of each molecule relative to mDC. Similarly, treatment with CP690550 further significantly reduced the levels of each molecule in DCs.

Analysis of cytokines indicated that treatment with BD750 at a higher dose or with CP690550 significantly reduced the levels of LPS-stimulated IL-6, IL-12, TNF- α , IL-1 β and IL-23 production by DCs (Figures 3A, C–F). BD750 did not

modulate cytokine secretion of IL-10, even at a high concentration of 20 μM (Figure 3B). Western blot analysis revealed that while treatment with LPS significantly induced STAT5 and AKT phosphorylation in DCs, treatment with BD750 or CP690550 significantly inhibited STAT5 but not AKT phosphorylation in cultured DCs (Figures 3G, H). Hence, treatment with BD750 at a higher dose induced tolDC *in vitro*, which was associated with inhibiting the LPS-induced JAK-STAT5 signaling in DC.

BD750-Treated DC Functionally Impairs Allogenic T Cell Responses *In Vitro*

To investigate the immunoregulatory capacity of BD750-treated DCs, imDCs, mDCs and BD750-treated DCs were cocultured with allogenic naive CD4⁺CD69⁻ T cells for 3 d. The T cell proliferation induced by allogenic antigens was analyzed by flow cytometry. While mDC stimulated strong allogenic T cell proliferation, BD750-treated DC impaired allogenic antigen-stimulated T cell proliferation *in vitro* in a dose-dependent manner (Figure 4). A stronger inhibition of allogenic antigen-stimulated T cell proliferation was observed when mDC was treated with CP690550.

Flow cytometry characterization indicated that while mDC induced high frequency of Th1 and Th17 cells, DCs that had been treated with BD750 or CP690550 induced significantly lower frequency of Th1 and Th17 cells and slightly higher frequency of Tregs relative to mDC (Figures 5A–D). Further analysis revealed that high levels of IL-17A and IFN- γ were secreted by activated T cells cocultured with mDC and significantly lower levels of IL-17A and IFN- γ were detected in the supernatants of cocultured T cells with DC treated with a high dose of BD750 or with CP690550 (Figure 5E, F). The BD750-treated and CP690550-treated DCs also significantly increased the level of IL-10 in the supernatants cocultured with T cells, compared with mDCs. Together, BD750-treated DCs impaired allogenic

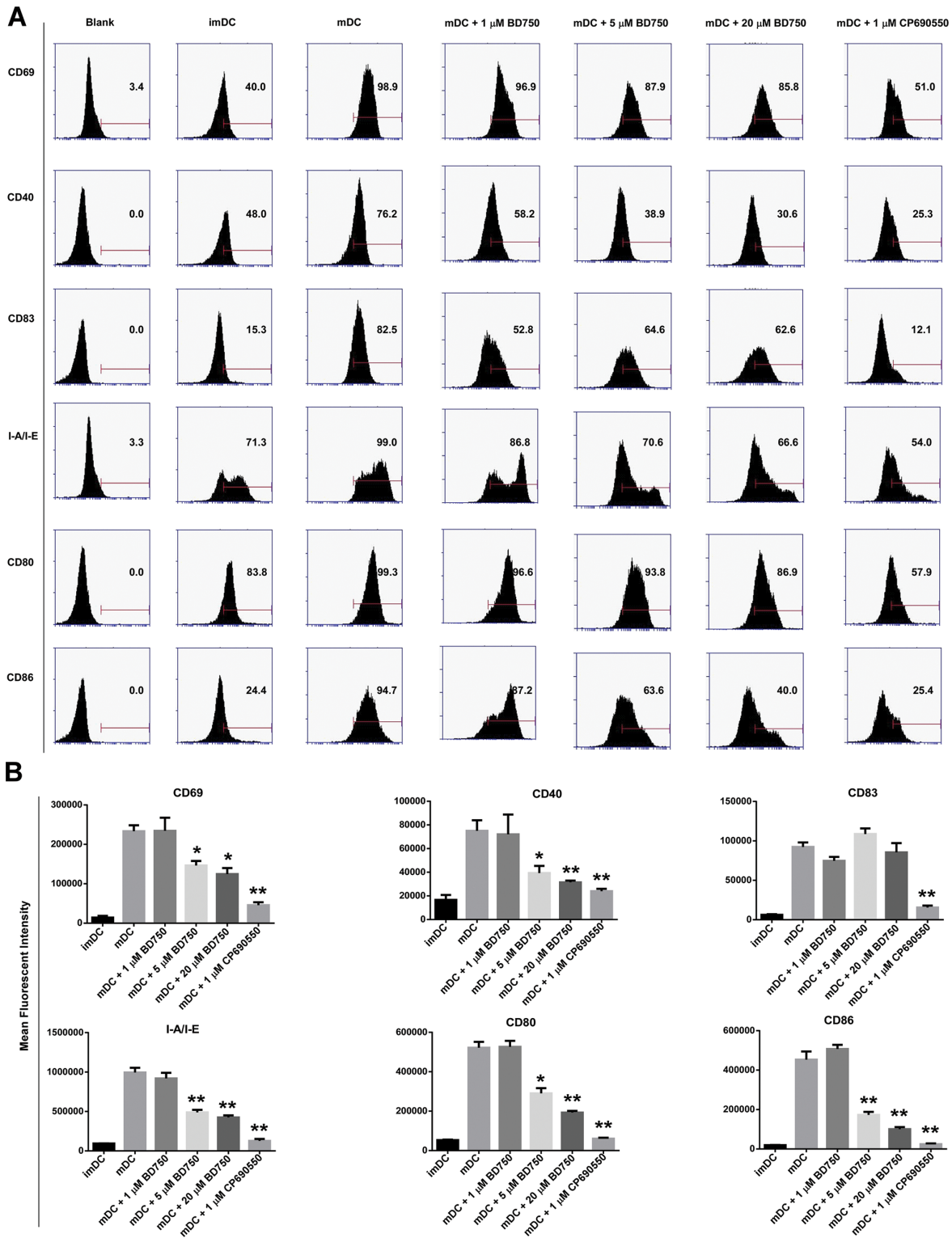


Figure 2. Analysis of maturation status of BD750-treated DCs by flow cytometry. After different treatments, the different groups of DCs were stained with the indicated antibodies and the levels of surface molecule expression were determined by flow cytometry. Data are representative histograms or expressed as the mean \pm SEM of three independent experiments. (A) Flow cytometry analysis. (B) Quantitative analysis. * $P < 0.05$, ** $P < 0.01$ versus the mDC group.

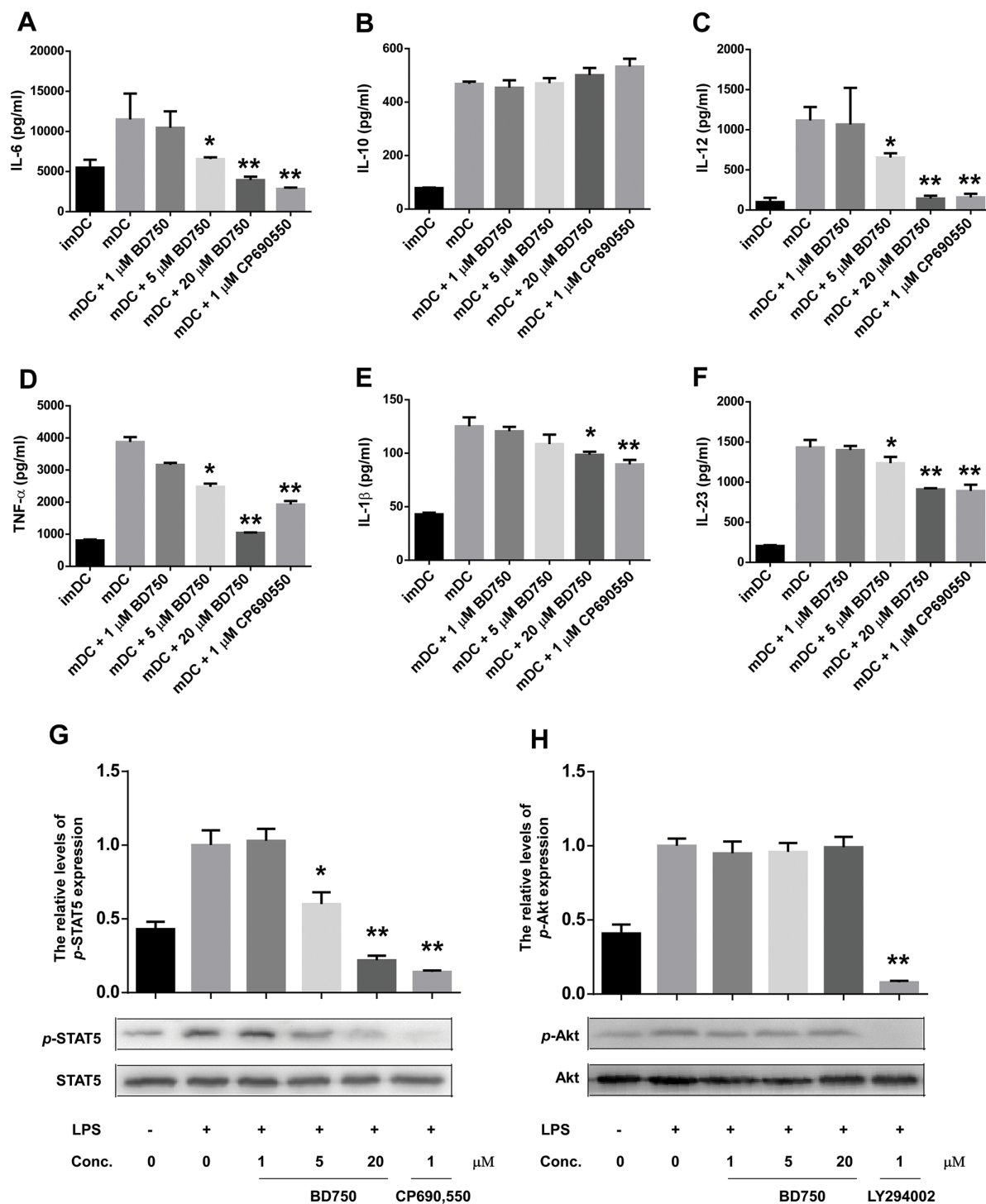


Figure 3. BD750 inhibits proinflammatory cytokine secretion and STAT5 phosphorylation in DCs. BMDCs were stimulated with cytokines for expansion in the presence or absence of BD750 for 12 h and then stimulated with LPS for 24 h. The supernatants of cultured cells were harvested and the levels of IL-6, IL-10, IL-12, TNF- α , IL-1 β and IL-23 in the supernatants were determined by ELISA (A–F). In addition, the relative levels of STAT5 and AKT expression and phosphorylation in the different groups of DCs were determined by Western blot assays (G–H). The relative levels of STAT5 and AKT phosphorylation to total STAT5 and AKT were determined. The relative density for bands of p-STAT5 or p-AKT activity was normalized to the total STAT5 or AKT, respectively. Data are representative images or expressed as the mean \pm SEM of three independent experiments. * P < 0.05, ** P < 0.01 versus the mDC group.

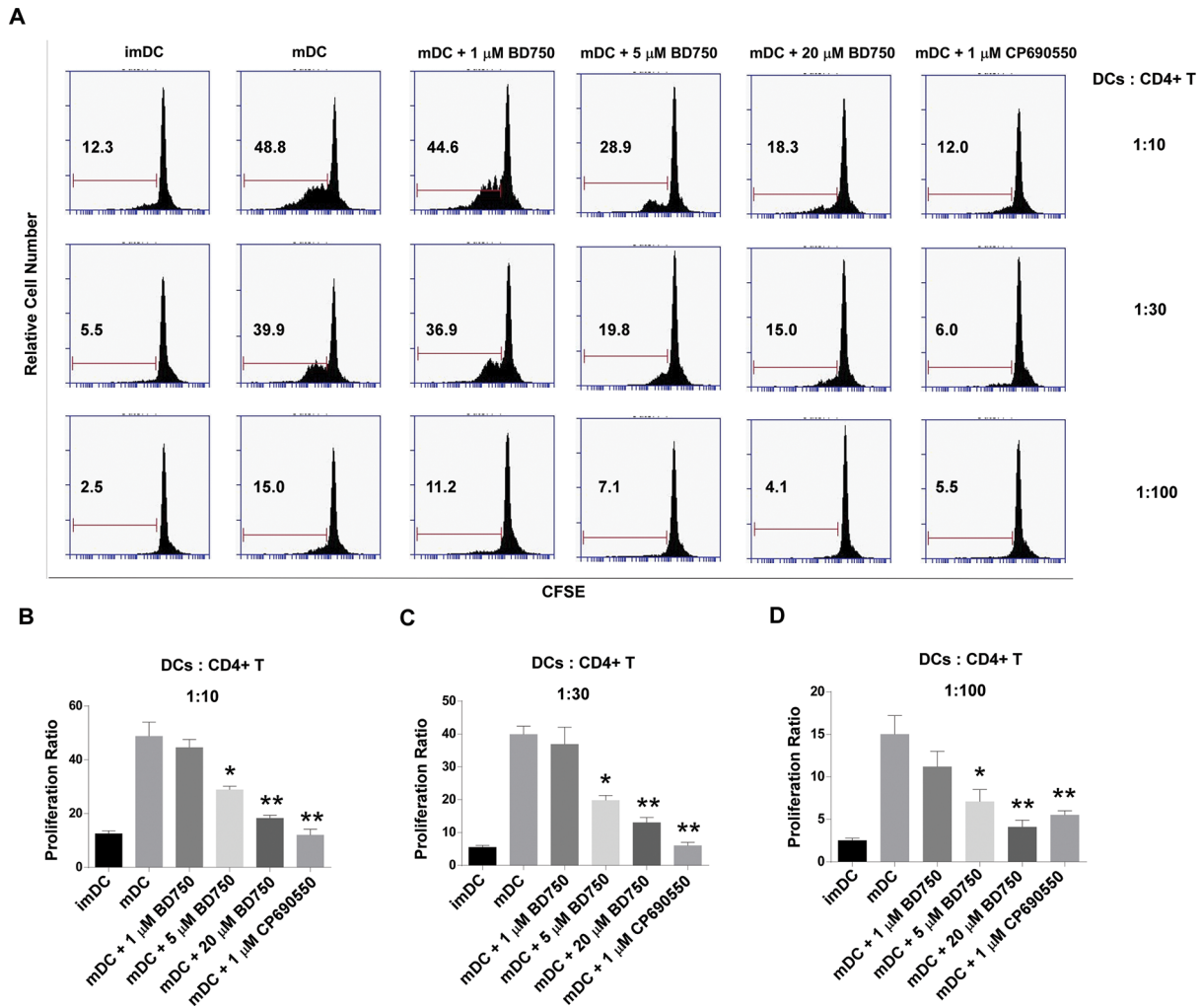


Figure 4. BD750-treated DC impairs allogeneic antigen-stimulated T cell proliferation *in vitro*. ImDCs, mDCs and BD750-pretreated DCs (12 h) were mixed in triplicate with carboxyfluorescein succinimidyl ester-labeled allogeneic naive CD4⁺CD25⁻ T cells for 72 h at different ratios (DC:CD4⁺ T, 1:10, 1:30 and 1:100), and the proliferation of T cells was determined by flow cytometry. Data are representative histograms or expressed as the mean ± SEM of three independent experiments. (A) Flow cytometry analysis. (B–D) Quantitative analysis. **P* < 0.05, ***P* < 0.01 versus the mDC group.

antigen-stimulated T cell proliferation and functional differentiation *in vitro*.

Adoptive Transfer of BD750-Treated DC Inhibits the Development and Reduces the Severity of EAE in Mice

To examine the potent immunosuppressive ability of BD750-treated DC, C57BL/6 mice were immunized subcutaneously with MOG₃₅₋₅₅ in CFA (4 mg/mL *Mycobacterium tuberculosis*) and injected with PTX. One week after induction, the mice were randomized and injected intravenously with PBS as

the control, MOG₃₅₋₅₅-pulsed and BD750 (20 μM)-treated DC on d 7, 11 and 15 post the first PTX injection (*dpi* 7, 11 and 15) (Figure 6A). The development and severity of EAE in individual groups of mice were monitored daily. In comparison with the mice receiving PBS, the mice receiving DC treated with 20 μM BD750 and loaded with antigen peptide displayed delayed EAE development and reduced severity of EAE (Figure 6A). In addition, we found that adoptive transfer of tolDC without MOG₃₅₋₅₅ did not improve EAE (Figure 6B). Moreover,

delayed adoptive transfer of tolDC loaded with antigen peptide at *dpi* 19, 23 and 27 or adoptive transfer of tolDC loaded with antigen peptide for 1–2 times failed to effectively inhibit EAE development in mice (Figures 6C, D). More interestingly, adoptive transfer with BD750-treated DC at *dpi* –2, 2 and 6 greatly inhibited development of EAE in mice (Figure 6C). Histological analysis of the spinal cord sections at peak disease (*dpi* 22) also indicated that treatment with BD750-treated and antigen-loaded tolDC, but not tolDC without antigen,

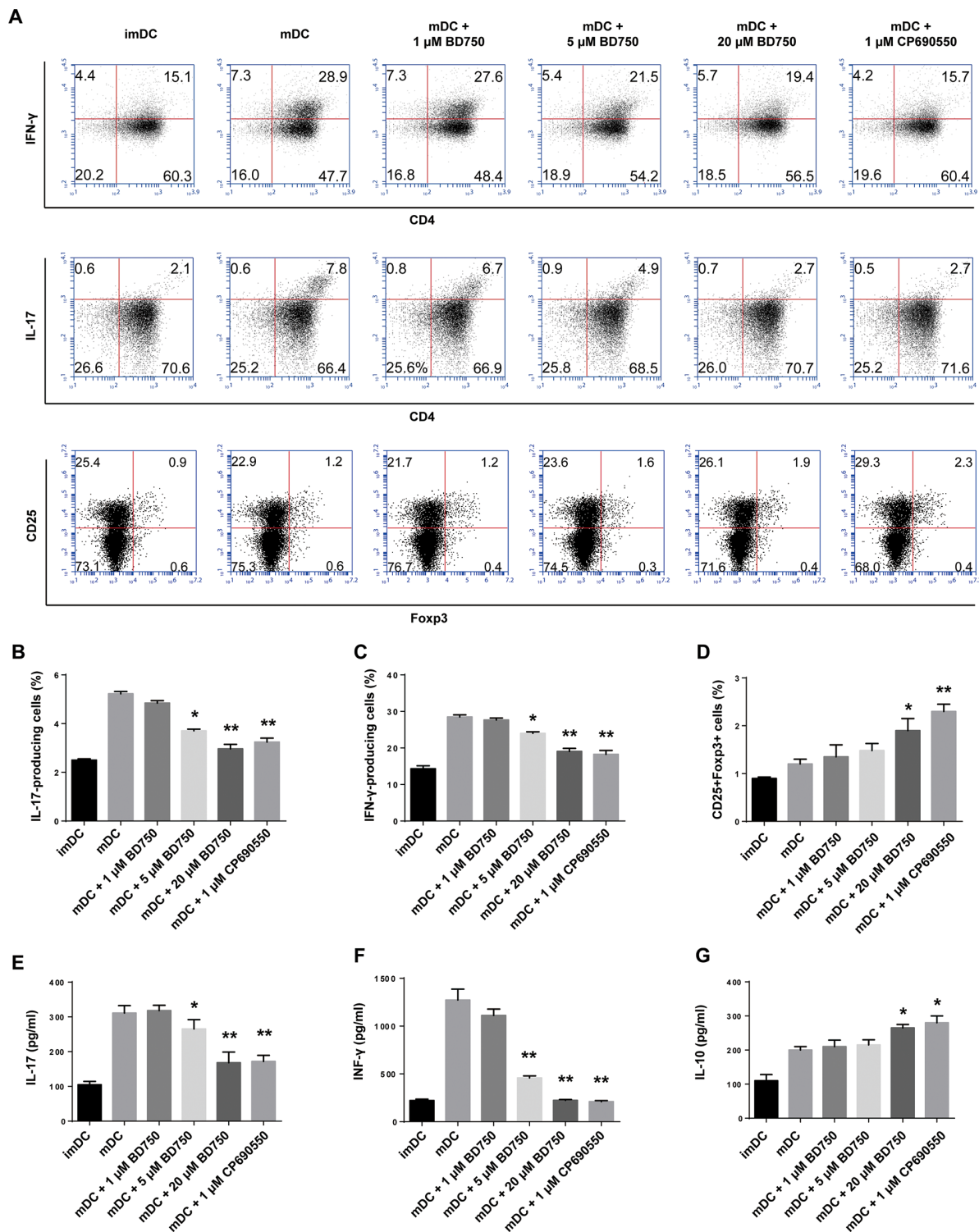


Figure 5. BD750-treated DC impairs allogeneic antigen-stimulated Th1 and Th17 responses *in vitro*. Following MLR *in vitro*, the frequency of Th1 and Th17 cells and Tregs was determined by flow cytometry. The levels of IL-17A and IFN- γ in the supernatants of cocultured T and DC cells were determined by ELISA. Data are representative FACS charts or expressed as the mean \pm SEM of three independent experiments. (A) Flow cytometry analysis of Th1 and Th17 cells and Tregs. (B–D) Quantitative analysis of Th1 and Th17 cells and Tregs. (E–G) ELISA analysis of the levels of IL-17A, IFN- γ and IL-10. * $P < 0.05$, ** $P < 0.01$ versus the mDC group.

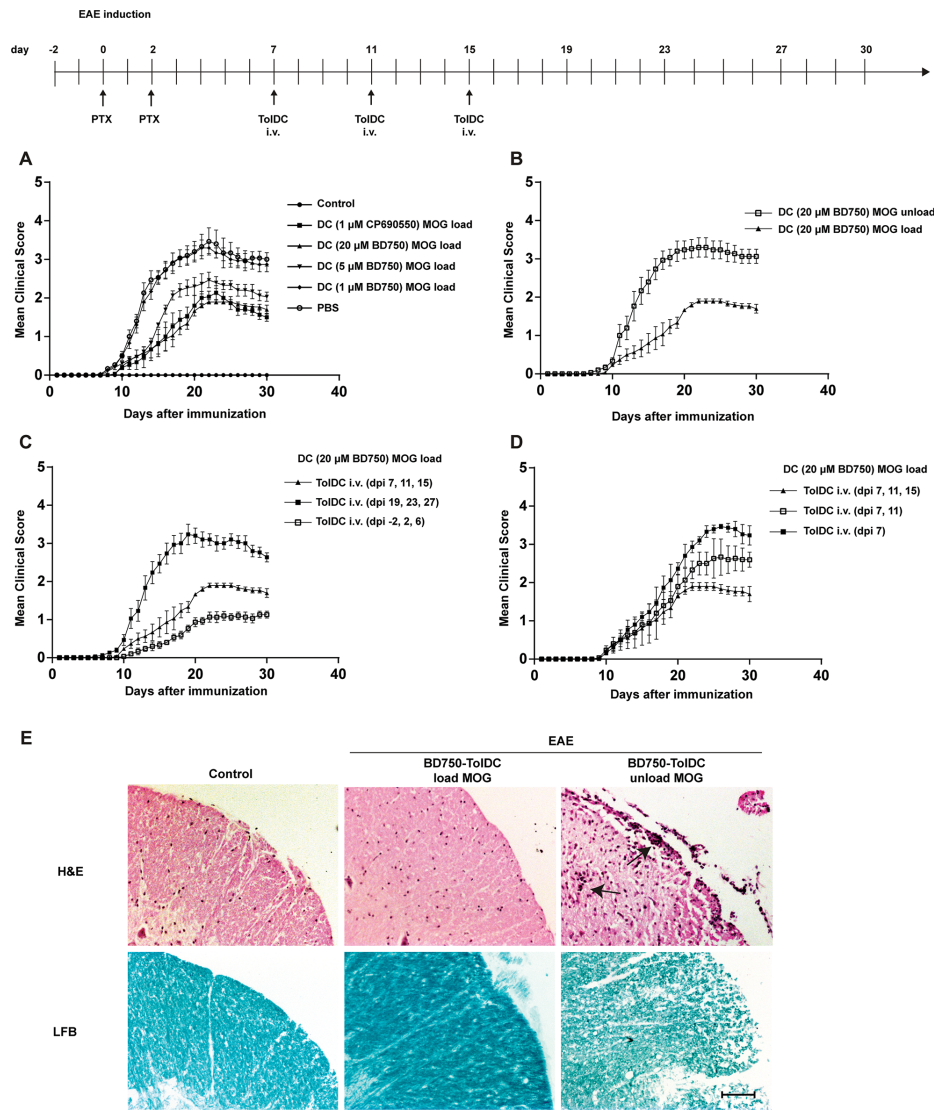


Figure 6. Treatment with BD750-treated DC ameliorates EAE. C57BL/6 mice were immunized with MOG₃₅₋₅₅ peptide in CFA and PTX. (A) The mice were randomized and treated intravenously with 20 μM BD750 pretreated DC (12 h) loaded with MOG₃₅₋₅₅ on d 7, 11 and 15 post the first PTX injection. Data are shown as the mean ± SEM of each group (n = 8 per group). *P < 0.05 versus the PBS group. (B) The EAE mice were treated intravenously with 20 μM BD750-treated DCs loaded with or without MOG₃₅₋₅₅ on d 7, 11 and 15 post the first PTX injection. Data are shown as the mean ± SEM of each group (n = 8 per group). *P < 0.05 versus the DC (20 μM BD750) without MOG₃₅₋₅₅ loading group. (C) The EAE mice were treated intravenously with 20 μM BD750-treated DCs loaded with MOG₃₅₋₅₅ on d 7, 11 and 15 or delayed on 19, 23 and 27 d or earlier on d -2, 2 and 6 post the first PTX injection. Data are shown as the mean ± SEM of each group (n = 8 per group). *P < 0.05 versus the tolDC intravenous (dpi 19, 23 and 27) group. (D) The EAE mice were treated intravenously with 20 μM BD750-treated DC loaded with MOG₃₅₋₅₅ on d 7, 11 and 15 post induction only for 1–2 times. Data are shown as the mean ± SEM of each group (n = 8 per group). *P < 0.05 versus the tolDC intravenous (dpi 7) group. The dynamic progression of EAE in individual groups of mice was monitored daily by two researchers without knowing the groups in a blinded manner, and some mice from some groups of mice were euthanized at 22 d post induction for analysis of inflammation and demyelination in the spinal cord tissues. (E) Histological examinations. Arrows indicate inflammatory infiltrates. Scale bar, 100 μm (magnification 100 ×).

reduced inflammatory infiltrates and demyelination intensity in mice (Figure 6E). Further analysis of splenic T cells indicated that adoptive transfer with tolDC that had been treated with 20 μM BD750 significantly reduced the frequency of Th1 and Th17 cells and increased the percentage of Tregs compared with mice receiving PBS (Figure 7). Thus, adoptive transfer with BD750-treated DC significantly inhibited T cell autoimmunity and EAE development in mice.

DISCUSSION

DCs are the most powerful APCs and induce antigen-specific T cell responses (22,23). Mature DCs are powerful for inducing T cell activation, and immature DCs usually promote T cell tolerance. Previous studies have shown that tolDC can inhibit autoimmune diseases, such as rheumatoid arthritis and EAE, in rodents (24,25). Indeed, pharmacological modification is a new strategy to induce tolDC (10,26). It is well known that the JAK/STAT pathway is crucial for the maturation and tolerance of DCs (16). Inhibition of STAT5 can impair the maturation of monocyte-derived DCs with downregulated expression of costimulatory molecules and reduced secretion of IL-12 (27,28). Our previous study identified that BD750 is an inhibitor of JAK3/STAT5 and can inhibit T cell proliferation (15). In this study, we found that BD750 induced tolDC. Evidentially, BD750-treated DCs displayed typically immature morphology. Second, BD750-treated DCs had lower levels of costimulatory molecule and MHC II expression and secreted lower levels of proinflammatory cytokines following LPS activation *in vitro*. Furthermore, BD750-treated DCs impaired allogeneic antigen-stimulated T cell proliferation, accompanied by lower frequency of proinflammatory Th1 and Th17 cells and STAT5 phosphorylation. Its inhibitory effect was similar to that of CP690550. Given that tolDC are important to induce T cell tolerance, BD750 may be a valuable agent for inducing T cell tolerance. Notably, BD750 inhibited STAT5 activation

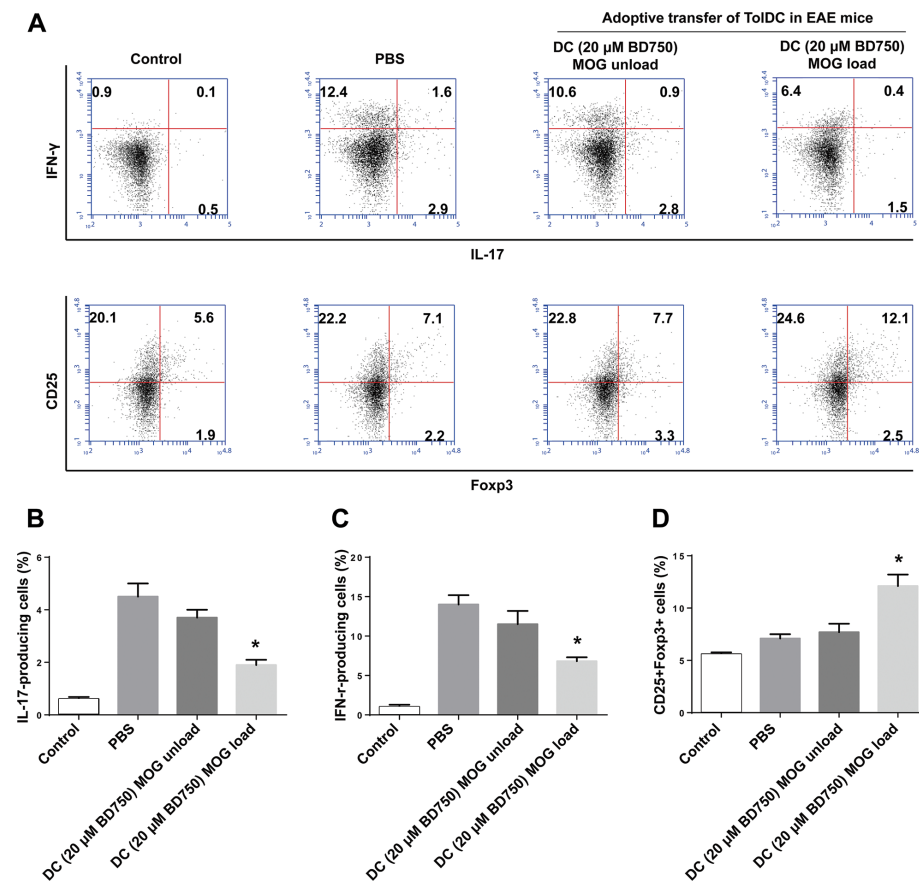


Figure 7. Adoptive transfer of BD750-treated DC decreases the proportion of splenic Th1 and Th17 cells and Tregs in EAE mice. On d 22 post the first PTX injection, some mice from each group were euthanized and their splenic T cells were stained with anti-CD4/anti-CD25, intracellularly stained with anti-IL-17A, anti-IFN- γ or anti-Foxp3. The percentages of splenic Th1 and Th17 cells and Tregs were determined by flow cytometry. Data are representative FACS charts or expressed as the mean \pm SEM of each group of mice ($n = 8$ per group). (A) Flow cytometry analysis. (B) Quantitative analysis of the percentages of Th1 and Th17 cells and Tregs. * $P < 0.05$, ** $P < 0.01$ versus the PBS group.

but not AKT phosphorylation, consistent with our previous findings (15). This observation suggests that BD750 may be a specific inhibitor of STAT5. We are interested in further examining the molecular mechanisms underlying the action of BD750 in regulating T cell immunity.

Myelin-specific Th1 and Th17 cells are responsible for the pathogenesis of EAE and MS. To understand the potential regulatory role of BD750-induced tolDC, we adoptively transferred antigen peptide-loaded tolDC during the development of EAE in mice. We found that adoptive transfer with antigen peptide-loaded tolDC, particularly at the early stage of

inducing EAE, significantly delayed the development of EAE and reduced its severity in mice. Furthermore, treatment with tolDC also reduced inflammatory infiltrates and the degree of demyelination in the spinal cords of mice. Given that tolDC are relatively stable in phenotype and function (13), autologous tolDC may be valuable for inducing T cell tolerance (29). Although it might be difficult for tolDC to pass the CNS (26), they did inhibit splenic Th1 and Th17 responses in EAE mice, similar to a previous report (26). In addition, although BD750 did not affect IL-10 production by LPS-stimulated DCs, BD750-DC secreted a low level of

IL-12. A previous study reported that decreased IL-12 production of tolDC plays an important role in inducing Treg differentiation (25), so BD750-DC secreted a low level of IL-12, which could enhance tolDC-mediated Tregs differentiation in MLR and subsequently expand the Treg population.

tolDC induced T cell tolerance, dependent on antigen peptide loading. Therefore, tolDC loaded with antigen peptide may be promising for inhibiting T cell autoimmunity and EAE progression. Our data indicate that adoptive transfer with antigen peptide-loaded tolDC should start at the early stage of the disease process. We speculate that this was because antigen-specific T cell response in the CNS was possibly the first demyelinating event. Similarly, we found that continual transfer of tolDC loaded with antigen peptide was necessary for the control of disease progression. Because MS is a chronic disease, it is also very difficult for us to speculate the long-term efficacy of passive transfer therapy and how frequently therapy is necessary to control disease progression. Therefore, further investigations are warranted to determine the safety and effectiveness of tolDC-based immunotherapies for MS.

CONCLUSION

Our data indicate that BD750 induced tolDC by downregulating their costimulatory molecule expression and cytokine production. BD750-treated DCs impaired allogeneic antigen-stimulated T cell proliferation. BD750-modified DCs induced hyporesponsiveness and impaired proinflammatory cytokine secretion by allogeneic T cells in MLR. In addition, the tolDC pulsed with MOG₃₅₋₅₅ effectively attenuated autoantigen-specific Th1/Th17 differentiation, and adoptive transfer of tolDC loaded with antigen peptide inhibited autoimmune responses in a mouse model of MS. Therefore, our findings may provide new insights into inducible immune tolerance and aid in the design of new therapies for MS.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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