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Novel sinomenine derivative 1032 improves immune suppression in experimental autoimmune encephalomyelitis

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ABSTRACT

Sinomenine (SIN) is an alkaloid isolated from the Chinese medicinal plant *Sinomenium acutum*. It is widely used as an immunosuppressive drug for treating autoimmune diseases. Due to its poor efficiency, the large-dose treatment presents some side effects and limits its further applications. In this study, we used chemical modification to improve the therapeutic effect of SIN in vitro and in vivo. A new derivative of sinomenine, named 1032, demonstrates significantly improved immunosuppressive activity over that of its parent natural compound (SIN). In an experimental autoimmune encephalomyelitis (EAE) model, 1032 significantly reduced encephalitogenic T cell responses and induced amelioration of EAE, which outcome was related to its selective inhibitory effect on the production of IL-17. By contrast, SIN treatment only led to a moderate alleviation of EAE severity and the expression level of IL-17 was not significantly reduced. Furthermore, 1032 exhibited suppression of Th17, but not Treg, cell differentiation, a result probably related to its inhibitory effect on IκB-α degradation as well as on IL-6 and TNF-α secretion in BMDCs. We speculate that 1032 as a novel anti-inflammatory agent may target DC to block IL-6 production, which in turn would terminate Th17 cell development. Thus, SIN derivative 1032 presents considerable potential in new drug development for treating autoimmune and inflammatory disease.

Introduction

Experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS), is a chronic inflammatory disease of the central nervous system [1]. Evolution of EAE and MS processes is closely related to auto-reactive T cells and other inflammatory cells as well as pro-inflammatory cytokines, which play a central role in the pathogenesis of such diseases [2,3]. As EAE mediators, $CD4^+$ T cells, especially Th1, have received great attention. In addition, Th17 cell, a distinct T cell subset that produces high levels of IL-17, has been demonstrated to play crucial roles in EAE development recently [4,5]. The differentiation of Th17 cells from naïve T cells appears to involve signals from TGF-β, IL-6, IL-21, IL-1β and IL-23 [6–11]. Improved understanding of the factors governing the development of EAE will accelerate progress toward the development of rational strategies for treating patients with MS. Current disease modifying treatments of MS, including β-interferon (IFN-β), have been shown to be effective [12–14]. However, the treatment efficacy of these strategies is still not sufficient in the long term, and there is an unmet need for additional drug development [15].

Traditional Chinese medicine (TCM), which has been practiced for thousands of years, has influenced modern drug discovery in all therapeutic areas for many decades [16]. Numerous bioactive natural compounds have been isolated or characterized from various herbal sources [17]. Such an approach offers some unique advantages and provides a vast source of pharmaceutical materials for the development of modern small-molecule drugs. However, a significant portion of these was often found to show very weak potency under biological testing, and is usually insufficiently qualified for further development in pharmaceutical applications. It has been reported that weakly bioactive compounds can give rise to more potent derivatives through rational chemical modifications [18], which approach provides an innovative strategy for TCM modernization.

Abbreviations: SIN, sinomenine; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; TCM, traditional Chinese medicine; MOG, myelin oligodendrocyte glycoprotein; BMDCs, bone marrow-derived dendritic cells.

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Sinomenine (SIN), a naturally abundant alkaloid isolated from the Chinese medicinal plant *Sinomenium acutum*, has been demonstrated to have variety of pharmacological effects including anti-inflammation, immunosuppression, and anti-angiogenesis etc. [19–21]. It has been successfully used in China for centuries in the treatment of patients with rheumatoid arthritis [22,23]. Although SIN has been approved as a useful element of TCM in clinical treatment, few applications have been developed due to its weaker therapeutic effect. In order to improve its therapeutic efficiency, we synthesized hundreds of sinomenine derivatives by embedding nitrogen-containing heterocycles, into the skeleton of natural sinomenine [18,24].

In this study, we investigate the therapeutic effects of a novel derivative of SIN, termed 1032 (IUPAC name, 6S,6aS,14aR)-6,6a,7,14-tetrahydro-2-methoxy-10,11,17-trimethyl-5H-6,14a-(iminoethano) naphtha [1,2-b] phenazin-1-ol). Our data indicate that 1032 exhibits an improved immune suppression of Th17 cells and in turn reduces inflammatory symptoms in experimental autoimmune encephalomyelitis (EAE). These results provide an excellent example of the means by which therapeutic efficiency of a weakly bioactive pure compound used in traditional Chinese herbal medicine can be improved by proper chemical modification and may then be tested in the treatment of inflammatory response-mediated autoimmune disease.

Materials and methods

SIN derivative 1032. 1032 was chemically synthesized by embedding a small nitrogen-containing heterocycle into the vicinal dicarbonyl functionality of the C-ring of SIN. Briefly, starting from a vicinal diketone, the hydrolysis product of sinomenine, one category of sinomenine derivative, was efficiently prepared by fusing with a pyrazine-ring to the C-ring [24]. The title compound 1032 ((6S,6aS,14aR)-6,6a,7,14-tetrahydro-2-methoxy-10,11,17-trimethyl-5H-6,14a-(iminoethano)naphtha[1,2-b]phenazin-1-ol) was one of these compounds. In this study, in order to improve the water-solubility, both sinomenine and 1032 were hydrochlorinated and were dissolved in PBS to provide stock solutions.

Induction and evaluation of EAE. Male C57BL/6 mice (6–8 wk; Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were immunized s.c. with the synthetic peptide (200 μg), myelin oligodendrocyte glycoprotein (MOG residues 35–55, MEVGWYRSPFSRVVHLYRNGK, 95% purity, GL Bio-Chem (Shanghai) Ltd.). Immunization was performed by mixing MOG peptide with CFA containing 5 mg/mL heat-killed H37Ra, a strain of Mycobacterium tuberculosis (Difco Laboratories). Pertussis toxin (200 μg) (Sigma–Aldrich) was administered i.v. on day 0 and day 2. Sinomenine, 1032 (at 15 mg/kg mice, about 375 μg per mouse) or PBS as vehicle control was administered i.p. daily from day 1 to 28. Mice were examined daily and scored for disease severity using the standard scale [25]: 0, no clinical signs; 1, limp tail; 2, paraparesis (weakness, incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with fore limb weakness or paralysis; 5, moribund or death. Animals were kept in conventional conditions and were handled in compliance with Chinese Academy of Sciences guidelines for Animal Care and Use.

Histopathology. Spinal cords from mice transcardially perfused with 4% paraformaldehyde were dissected and postfixed overnight. Paraffin-embedded 5–10 μm spinal cord sections were stained with H&E or Luxol fast blue and then examined by light microscopy.

Proliferation and cytokine assay. In proliferation assays, splenocytes (5×10^5 per well) derived from EAE mice were cultured in complete DMEM in 96-well plates. Cells were cultured in the presence or absence of the MOG peptide (30 μg/mL, respectively), at 37 °C in 5% CO_2 for 72 h. Cells were pulsed with 1 μCi of [^{3}H] thymidine during the last 14–18 h of culture before harvest. [^{3}H] Thymidine incorporation was measured as cpm detected by liquid scintillation counting (Beckman LS6500). For cytokine measurements, supernatants were collected from cell culture at 48 h and diluted for measurement of IFN-γ, TNF-α, IL-4, and IL-17 by ELISA (R&D Systems) according to the manufacturer's instructions. A standard curve was performed for each plate and used to calculate the absolute concentrations of the indicated cytokines.

Th17 and Treg differentiation in vitro. For in vitro Th17 and Treg differentiation, naive CD4$^+$ (CD4$^+$CD44^{low}CD62L^{high}) T Cells were purified routinely to >95% purity using a FACSAria (BD Biosciences). T cells were maintained in RPMI 1640 supplemented with 10% FCS and stimulated with 5 μg/mL plate-bound anti-CD3 and 2 μg/mL soluble anti-CD28 under conditions formulated to obtain cell types: Th17 (1 ng/mL TGF-β1, 20 ng/mL IL-6, 10 ng/mL IL-1α, 10 ng/mL IL-23, 10 μg/mL anti-IFN-γ, and 10 μg/mL anti-IL-4), or induced regulatory T cell (Treg) (10 ng/mL TGF-β1and 50 U/mL IL-2). In this stage, vehicle control (PBS) or sinomenine (Low dose, 6.33 μg/mL; High dose, 11.25 μg/mL) or 1032 (Low dose, 6.33 μg/mL; High dose 11.25 μg/mL) were added to inhibit T cell differentiation. Cells were then harvested for FACS analysis. Briefly, PE-labeled anti-mouse IL-17 and FITC-labeled anti-mouse Foxp3 were used to label the cells. Cells were analyzed on a FACSCalibur cytometer using CellQuest software (BD Biosciences).

Generation of bone marrow-derived DC. The method was modified from one initially described by Inaba et al. [26]. Briefly, bone marrow cells were flushed from the femurs and tibias of C57BL/6 mice and subsequently depleted of red cells with ammonium chloride. Cells were cultured at 2×10^6 cells/well in 24-well plates in medium supplemented with 20 μg/mL murine rGM-CSF. Nonadherent cells were removed carefully, and fresh medium was added every 2 days. On day 8, nonadherent cells released spontaneously from proliferating cell clusters were collected. For pro-inflammatory molecule detection, BMDCs were cultured at 2×10^6 cells/well in 24-well plates with sinomenine or 1032 or vehicle control under the condition of 0.5 μg/mL LPS stimulation. Supernatants were collected after 6 h and concentrations of TNF-α and IL-6 were measured by ELISA (R&D Systems) according to the manufacturer's instructions.

Immunoblotting. BMDCs were pretreated with sinomenine (0.2 mg/mL, 1 mg/mL or 5 mg/mL), 1032 (0.2 mg/mL, 1 mg/mL or 5 mg/mL) or vehicle control for 30 min and then LPS (0.5 μg/mL) was added for another 30 min except to the negative control. Cells were harvested and cell lysates were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were then transferred onto a nitrocellulose membrane (BIO-RAD). After 2 h of blocking, the membranes were incubated overnight at 4 °C with specific primary Abs: anti-IκB-α (Santa Cruz Biotechnology) and anti-β-actin (Santa Cruz Biotechnology) was used as control. The membranes were then stained with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Sigma) for 1 h. The blots were developed using ECL detection reagents (Pierce). For p-STAT3 and STAT3 detection, MOG-specific splenocytes treated with sinomenine or 1032 or vehicle control were collected and the same procedure was followed to test the samples. Anti-phospho-STAT3 and anti-STAT3 antibodies were obtained from Cell Signaling Technology.

Statistics. A Student *t* test was used to analyze the differences between the groups. One-way ANOVA was initially performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student *t* test. A p value of 0.05 was considered statistically significant.

Results

A new derivative of sinomenine shows a strong immune suppressive activity in vivo and in vitro

Based on the relatively poor immunosuppressive activity of sinomenine in clinical treatment, we set about to create a new therapeutic derivative of sinomenine by chemical synthesis [18,24]. At the initial stage, we had elaborated over 200 synthesized derivatives. Assays for T cell and B cell proliferation were used to screen the potential derivatives in vitro (data not shown). Sinomenine demonstrates relatively low biological activity in clinical treatment and experimental study [27,28]. After screening, a new active derivative was selected and named 1032 (Supplement 1). Compound 1032 had a high LD_{50} (368 mg/kg) as compared to that of sinomenine (289 mg/kg) when it was administered to mice by i.p. injection. The experiments show that the inhibitory effect of 1032 on spleen lymphocyte proliferation increased approximately 67-fold as compared to sinomenine (data not shown). To further confirm the biological activity of 1032 in vivo, EAE was selected as a model system. When mice were treated with 1032 daily at dose (15 mg/kg) from day 1 to day 40 after immunization, the EAE score was significantly reduced in the 1032-treated group as compared to sinomenine-treated mice in which only a moderate suppression was observed (Fig. 1A). The observed clinical effect of 1032 was consistent with markedly reduced inflammation and demyelination in affected spinal cord lesions as determined by histological analysis (Fig. 1B). Taken together, the data demonstrate that 1032 shows improved immune suppression in vivo.

Regulatory effects of 1032 on encephalitogenic T cell response

The therapeutic effect of 1032 in EAE prompted us to investigate its potential mechanisms of suppression of encephalitogenic T cells. To address this question, MOG-specific T cell proliferation and cytokine profiles were analyzed. The results demonstrate that T cell proliferation was significantly suppressed in sinomenine- and 1032-treated mice (Fig. 2A). Both drugs significantly reduced production of IFN-γ and TNF-α but not IL-4(Fig. 2B–D). In addition, it is interesting to note that IL-17 production was markedly reduced in the 1032-treated group as compared to the sinomenine-treated group or the control group (Fig. 2E), indicating that 1032 might have a suppressive effect on Th17 cells.

1032 selectively inhibits the differentiation of Th17

To further confirm whether 1032 suppresses Th17 development, naive $CD4^+$ T Cells were purified and then the Th17 and Treg cell differentiation were induced in vitro. The results demonstrate that Th17 differentiation was significantly suppressed after 1032 treatment at high dose when compared to sinomenine treatment (Fig. 3). Furthermore, the development of Treg ($Foxp3^+/CD4^+$ cells) remained unaltered under the same condition of sinomenine or 1032 treatment (Fig. 3), which indicated the inhibitory selectivity

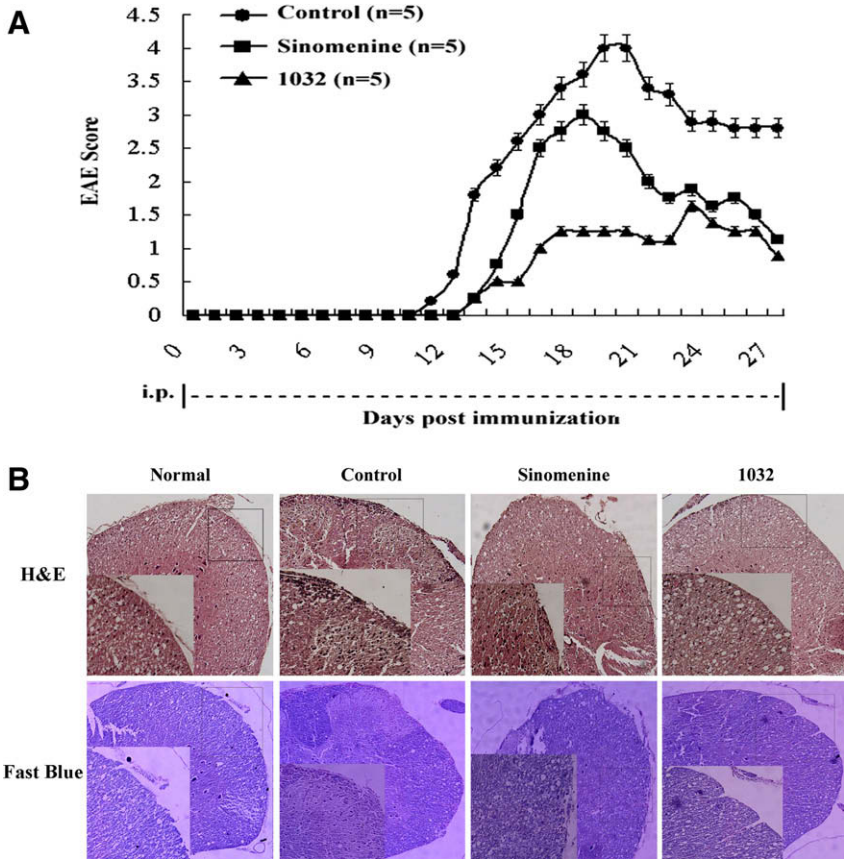

Fig. 1. Clinical course and severity of EAE in mice treated with sinomenine and 1032. C57BL/6 mice were immunized with MOG_{35-55} peptide to induce EAE and were administered daily i.p. injections of sinomenine (15 mg/kg, –■–), 1032(15 mg/kg, –▲–) or vehicle control(–●–) from day 1 postimmunization onwards (A). Mice were monitored and scored daily as described in *Materials and methods*. (B) Histopathology of spinal cord tissue sections of EAE mice treated with sinomenine, 1032 or vehicle control. Spinal cord was removed at day 21 followed by H&E staining (upper panels) and Fast blue staining (lower panels). H&E staining shows the filtration of encephalitogenic T cells, which were indicated by dark blue dots in the magnified portion of the panel. Fast blue staining showed the integrality of the myelin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

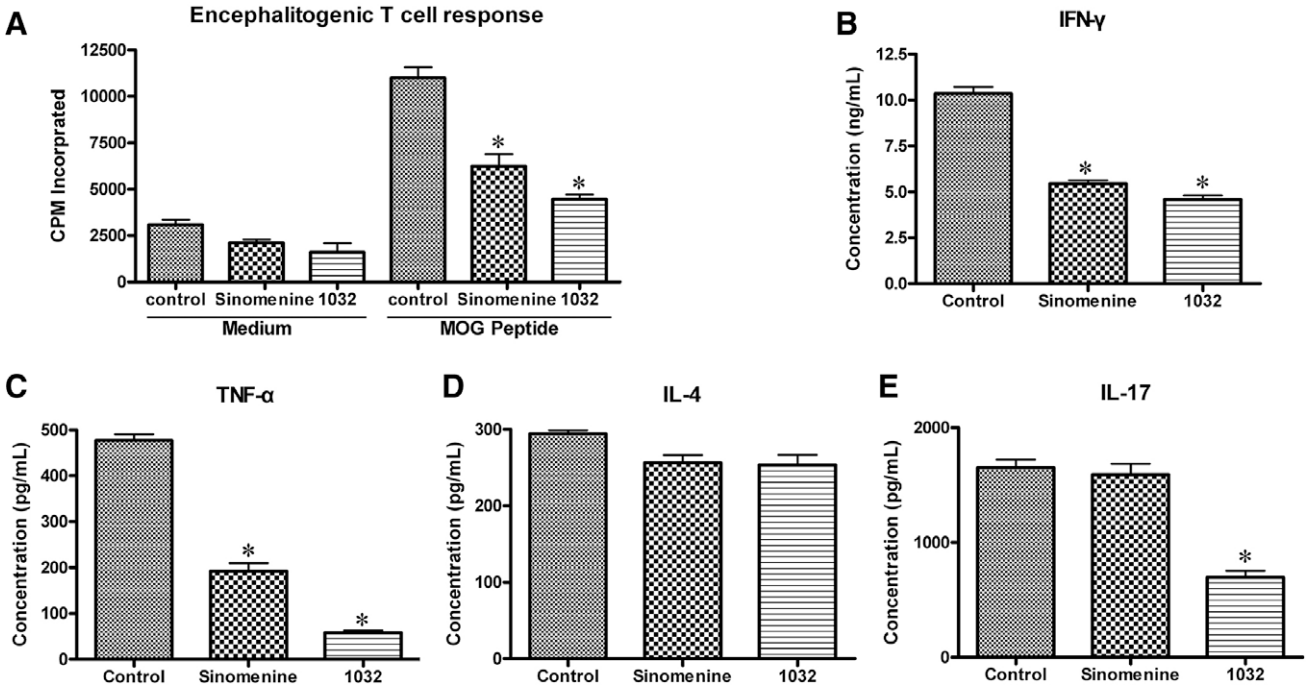

Fig. 2. Ex vivo analysis of the encephalitogenic T cell response and cytokine profile in response to MOG peptide in EAE mice treated with sinomenine, 1032 or vehicle control. (A) Mice were treated with sinomenine, 1032 or vehicle control according to the treatment protocol. Splenocytes were isolated 12 days postimmunization and examined ex vivo for proliferation in the presence (MOG) or absence (Med) of the MOG peptide. Data are presented as mean cpm ± SEM of triplicates. (B–E), Supernatants were collected from above mentioned cultures after 72 h, and concentrations of the indicated cytokines were measured using ELISA. The values represent mean concentrations (ng/mL ± SEM) of triplicate samples. Experiments were repeated at least twice. *Statistical significance between the groups ($p < 0.05$).

of 1032. However, 1032 did not change the expression of ROR-γT, the transcription factor known to regulate Th17 immune responses (data not shown), thus pointing to alternative explanations.

1032 is capable of suppressing IκB-α degradation and then reducing IL-6 production in bone marrow dendritic cells

Since 1032 cannot suppress Th17 differentiation directly, we wondered whether 1032 may inhibit antigen-presenting cells. NF-κB has been shown in multiple systems to have an impact on both survival and cytokine secretion in DCs [29–31], so we were particularly interested in the possibility that initially 1032 might affect DC maturation and then might eliminate T cell polarization through the NF-κB signal pathway. To address this question, bone marrow-derived DC (BMDC) were isolated and the activity of the NF-κB signaling pathway was analyzed in 1032-treated BMDC. The data show that 1032 appears to markedly increase the expression of IκB-α, a known inhibitory protein in the NF-κB signaling pathway (Fig. 4A) in LPS stimulated BMDC. Furthermore, we examined down-stream signaling production of IL-6 and TNF-α in LPS

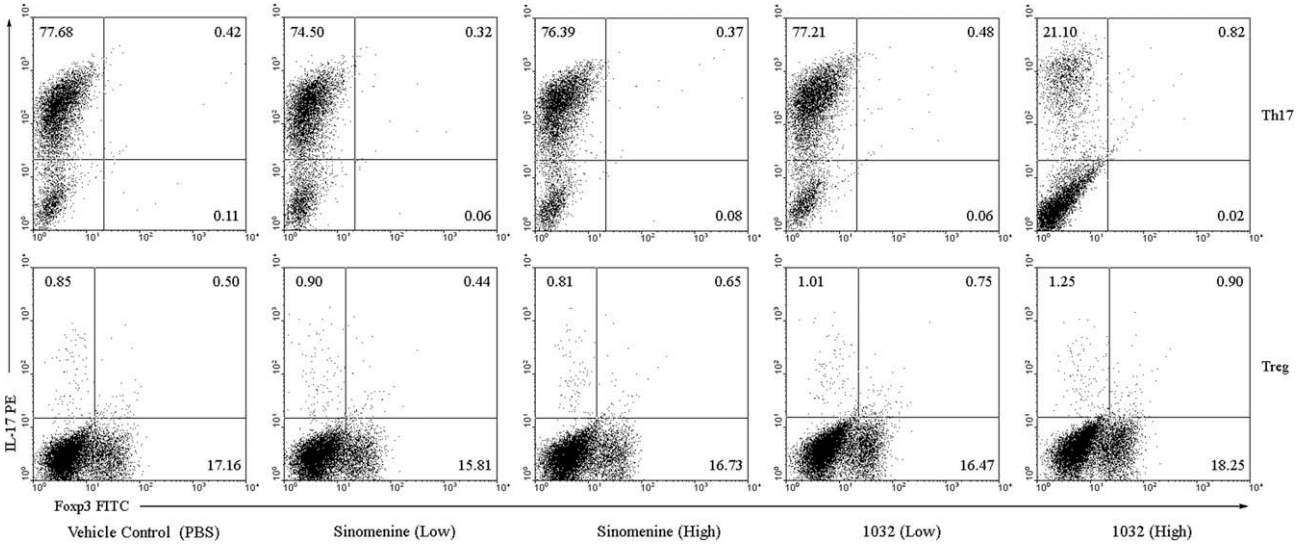

Fig. 3. 1032 selectively inhibits the differentiation of Th17 cells. Th17 and Treg cell differentiation were induced from Naive $CD4^+$ T cell as described in Material and Methods. During the last 48 h, vehicle control (PBS) or sinomenine (Low dose, 6.33 μg/mL; High dose, 11.25 μg/mL) or 1032 (Low dose, 6.33 μg/mL; High dose 11.25 μg/mL) were added to inhibit T cell differentiation. $IL\text{-}17^+$ and $Foxp3^+$ T cells were measured by FACS analysis. Experiments were repeated at least twice.

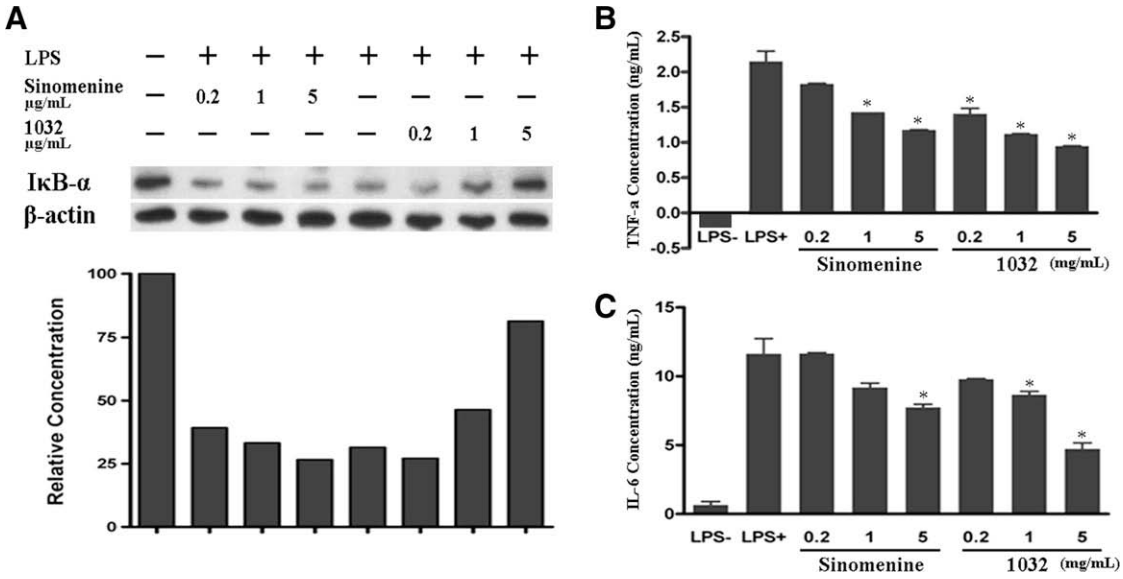

Fig. 4. Inhibition of IκB-α degradation and production of pro-inflammatory molecules after 1032 treatment. (A) BMDCs were pretreated as indicated for 30 min and 0.5 μg/mL LPS was added for another 30 min to all but the negative control. Shown is representative Western blot analysis for IκB-α as well as its densitometric analysis. The supernatants of the same treated BMDCs were collected at 6 h after LPS stimulation and the concentrations of TNF-α (B) and IL-6 (C) were measured. Experiments were repeated at least twice. *Statistical significance between the groups ($p < 0.05$).

stimulated BMDC. As expected, the treatment with 1032 resulted in a significant reduction of TNF-α (Fig. 4B) and IL-6 (Fig. 4C) in a dose-dependent manner. Our data suggest that, as compared to sinomenine, its novel derivative, 1032, exhibits a better inhibition of NF-κB activity as well as reduced TNF-α and IL-6 production, which in turn dampen DC-driven T cell polarization.

Discussion

Chemical modification by integration of a particular drug fragment [18], such as small heterocycles, into the skeleton of natural products is a technique frequently employed in drug design and is a useful protocol in improving biological activity. In this study, we demonstrate that 1032, a novel derivative of a naturally abundant and weakly bioactive alkaloid, sinomenine, has unique immunomodulatory properties and therapeutic potential with regard to autoimmune inflammatory diseases.

The study has addressed several important issues. First, our initial series of experiments showed that new derivative 1032 was more effective than natural sinomenine in amelioration of EAE as evidenced by the markedly reduced inflammation and demyelination in affected spinal cord lesions of 1032-treated mice. It is noteworthy that both 1032 and SIN show some suppression of disease onset. Secondly, in the mechanism studies, it was shown that both 1032 and SIN could inhibit MOG-specific T cell proliferation and IFN-γ and TNF-α production, but that neither influenced IL-4 production. Furthermore, only 1032-treated mice showed a significant reduction in IL-17 production. Consistent with that, 1032, but not sinomenine, exhibited suppression of Th17 cell differentiation in vitro. Meanwhile, the development of Treg remained unaltered with 1032 treatment, indicating the inhibitory selectivity of 1032. However, 1032 did not change the expression level of RORγT, a transcription factor known to regulate Th17 cell development, thus indicating additional mechanisms involved in regulating Th17 cell development. Further studies showed that 1032 inhibited the activity of NF-κB in DCs by preventing the degradation of IκB-α and in turn eliminating the production of pro-inflammatory cytokines, IL-6 and TNF-α. Since IL-6 has been reported to be essential for regulation of Th17 cell development [11], it is suspected that 1032 may suppress DC maturation and thus eliminate Th17-mediated EAE disease.

In conclusion, by simple introduction of a drug-like heterocyclic moiety into the skeleton of sinomenine, a new potent derivative of sinomenine 1032 has been identified. It presents a significant increase in potency in reducing disease severity, and exhibits much stronger immune-regulatory properties than its naturally occurring parent. These findings make this new derivative more suitable for entry into a modern drug discovery program and provide a successful example of converting a weakly bioactive ingredient of traditional Chinese herbal medicines into a more potent new drug candidate and a potentially useful molecule for probing novel signaling targets required for effective blocking of autoimmune T cell activation and function.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.028.

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