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The influence of cyclosporin A on lymphocyte attenuator expression

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Abstract

B and T lymphocyte attenuator (BTLA), a recently identified immune inhibitory receptor, has been demonstrated to have the ability to maintain self-tolerance and transplant-tolerance in mice. However, little is known about the effects of immunosuppressive drugs on the expression of BTLA. In the present study, we observed that the immunosuppressive drug cyclosporin A (CsA) could significantly reduce BTLA but not CD25 and CD69 expression on $CD4^+$ T cells during activation in vitro, while rapamycin (RPM) had little effect on it. Exogenous interleukin-2 (IL-2) failed to reverse the inhibitory effect that CsA had on BTLA expression. Furthermore, phorbol 12-myristate 13-acetate (PMA) or ionomycin alone could efficiently induce BTLA protein expression on $CD4^+$ and $CD8^+$ T cells, while CsA significantly suppressed BTLA expression in this system. The present data indicate that the regulation of BTLA expression on $CD4^+$ T cells does not depend on IL-2 and T cell activation but depends on calcineurin-dependent and calcineurin-independent pathways. The observation that CsA significantly inhibits BTLA expression on $CD4^+$ T cells during activation, suggests that CsA might block the immune tolerance induced by BTLA and potentially increase the susceptibility to autoimmune diseases and graft rejection.

Keywords: BTLA; Cyclosporin A; Immunosuppression; Immune tolerance; Autoimmunity

1. Introduction

Efficient and proper immune responses are achieved, at least in part, by the regulation of co-molecule signaling that provokes either positive or negative signals. Stimulatory signals are crucial for initiating, augmenting and maintaining the expansion and proliferation of T cells, whereas inhibitory signals can attenuate, dampen and terminate the responses [1–4]. Therefore, the balance of both signals will dictate the outcomes of immune responses. BTLA is a recently identified co-inhibitory receptor of the CD28 superfamily [5]. It is highly induced on activated T cells and also expressed on B cells, macrophages, and bone marrow-derived dendritic cells [6,7]. The ligand of BTLA is herpes virus entry mediator (HVEM) [8–10], a co-stimulator of the TNFR family through the interaction with LIGHT and lymphotoxin-α (LT-α) [11]. It has been reported that ligation of BTLA results in tyrosine phosphorylation of BTLA, recruited Src homology domain 2-containing protein tyrosine phosphotases-1 (SHP-1) and SHP-2 and subsequently generated signals that dampen T and B cell

Abbreviations: APC, antigen presenting cell; BTLA, B and T lymphocyte attenuator; ConA, Concanavalin A; Cpm, counts per minute; CsA, cyclosporin A; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; FCM, flow cytometry; FITC, fluorescein isothiocyanate; HVEM, herpes virus entry mediator; IL-2, interleukin-2; MFI, mean fluorescence intensity; PE, phycoerythrin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PI, propidium iodide; TNFR, tumor necrosis factor receptor.

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activation in vitro and in vivo [5,12]. BTLA or its ligand HVEM-deficient mice were more susceptible to autoimmune diseases with more severe pathological tissue changes, such as experimental autoimmune encephalomyelitis (EAE), autoimmune hepatitis and allergic airway inflammation, indicating that the BTLA pathway plays a critical role in self-tolerance [5,13–15]. In addition, BTLA promoted graft rejection in fully MHC-mismatched cardiac graft models, while it strongly prolonged heart-graft survival in the partially MHC-mismatched heart-grafted mouse model [16], which indicates that BTLA may have an important and unique role to play in allogeneic responses in the heart transplant mouse model.

Cyclosporin A (CsA) and rapamycin (RPM) are important immunosuppressive agents, which are widely used in clinics to block allograft rejection and to treat autoimmune diseases [17,18]. The mechanism for the immunosuppressive action of CsA involves initial binding to cyclophilin to form a complex that then inhibits calcineurin, leading to reduction of interleukin-2 (IL-2) production and T cell proliferation [19]. RPM binds to FKBP12 forming an immunosuppressive complex that inhibits rapamycin effector kinases (REK), RAFT/TOR, and FRAP, thereby blocking T cell transition from G0 to G1 [20]. Although these drugs can prevent graft rejection and the progress of autoimmune diseases, current studies have revealed that some of these drugs might have effects on some inhibitory co-molecules. For example, CsA and RPM can both inhibit expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), which is another inhibitory receptor of the CD28 family [21]. These findings suggest that these drugs may potentially inhibit negative co-molecules and thus, increase the risk of contracting an autoimmune disease and decrease the susceptibility to tolerance-induction by allo-antigens.

It is known that BTLA plays an important role in self-tolerance and chronic allo-graft rejection in mice. However, the effect of immunosuppressive drugs on the expression of BTLA has not yet been determined. In the present study, we observed that CsA, but not RPM, significantly reduced BTLA protein levels on $CD4^+$ T cells through calcineurin-dependent and calcineurin-independent pathways. This suggests that CsA might potentially block BTLA-mediated immune tolerance to auto- or allo-antigens.

2. Materials and methods

2.1. Mice

Six-week-old C57BL/6 female mice were purchased from the animal center of Beijing University (Beijing, China). All mice were maintained in a specific pathogen-free facility and were housed in micro-isolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the institutional guidelines for the care and use of laboratory animals.

2.2. Monoclonal antibodies (mAbs) and reagents

The following mAbs were purchased from BD Biosciences PharMingen (San Diego, CA, USA): fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 mAb (RM4-5; rat IgG2a), phycoerythrin (PE)-labeled anti-mouse CD25 mAb (PC61; rat IgG1), PE-labeled anti-mouse CD69 mAb (FN50; mIgG), hamster anti-mouse CD28 mAb, rat anti-mouse CD3 mAb. In addition, PE-labeled anti-mouse BTLA mAb (6F7; mouse IgG1) was obtained from eBioscience (San Diego, CA). Rat anti-mouse FcR mAb (2.4G2, IgG2b) was produced by 2.4G2 hybridoma (ATCC, Rockville, Maryland) in our laboratory.

In addition, ConA, Rapamycin, ionomycin and phorbol 12-myristate 13-acetate (PMA) were all purchased from Sigma Chemical Co. (St. Louis, MO). CsA was purchased from Novartis, China. Recombinant human IL-2 was purchased from R&D Systems (Minneapolis, MN). The culture medium was RPMI 1640 (GIBCO, USA) containing 10% fetal bovine serum, 25 mM Hepes, 2 mM L-glutamine, 100 IU/ml of penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO).

2.3. Cell preparation and stimulation

C57BL/6 mouse spleen single-cell suspensions were prepared by grinding the tissue with glass slides and passing it through a nylon mesh. Red blood cells were removed with a hemolysis buffer (17 mM Tris–HCl and 140 mM NH_4Cl, pH 7.2). Splenocytes (at 2×10^6 cells/ml) were stimulated with ConA (2.5 μg/ml), plate-bounded anti-CD3 mAb (1 μg/ml) and anti-CD28 mAb (5 μg/ml), PMA (10 ng/ml), ionomycin (1 μM), or IL-2 (1000 or 200 U/ml) in the absence or presence of a given concentration of CsA in a 24-well or 96-well plate (Costar). They were then incubated for a given time at 37 °C in 5% CO_2 atmosphere.

2.4. Immunofluorescence staining and flow cytometry (FCM)

Splenocytes (5×10^5) from C57BL/6 mice were washed once with a FACS buffer (PBS, pH 7.2, containing 0.1% NaN_3 and 0.5% bovine serum albumin (BSA)) and stained with FITC-labeled anti-mouse CD4 mAb or anti-mouse CD3 mAb versus PE-labeled anti-BTLA, CD25 or CD69 mAb or the non-specific staining control mAb, respectively. Non-specific FcR binding was blocked by anti-mouse FcR mAb 2.4G2. At least 10,000 cells were assayed by a FASCalibur flow cytometry (Becton Dickinson, CA), and data were analyzed with CellQuest software (Becton Dickinson, Mountain View, CA). Non-viable cells were excluded using the vital nucleic acid stain propidium iodide (PI). The percentage of cells stained with a particular reagent or reagents was determined by subtracting the percentage of cells stained non-specifically with the negative control mAb, from those that stained in the same dot-plot region with the anti-mouse mAbs. Specific molecule-expression levels were determined as the median

fluorescence intensity (MFI) of the cells that stained positively with the specific mAb.

2.5. T cell proliferation

C57BL/6 splenocytes (4×10^5 cells/well) were cultured in flat-bottomed, 96-well plates with 2.5 μg/ml ConA and/ or 1000 U/ml IL-2 in the absence or presence of 10 ng/ml of CsA for 72 h at 37 °, 5%CO_2. 0.5 μCi [^{3}H]thymidine was added to each well for the last 16–18 h of incubation. Cells were harvested onto glass fiber filters with an automatic cell harvester (Tomtec, Toku, Finland). Samples were assayed in a liquid scintillation analyzer (Beckman Instruments, USA) and values presented as counts per minute (cpm) of triplicate wells.

2.6. Statistical analysis

All data were presented as the mean ± SD. Student's unpaired *t* test for comparison of means was used to compare groups. A *P*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Up-regulation of BTLA expression on CD4$^+$ T cells after activation

To investigate the effects of immunosuppressive drugs CsA and RPM on BTLA expression, we first examined the kinetics of BTLA induction on CD4$^+$ T cells upon ConA stimulation. As shown in Fig. 1, non-activated CD4$^+$ T cells expressed low or undetectable levels of BTLA. After mouse splenocytes were cultured with ConA for 24 h, CD4$^+$ T cells expressed high levels of BTLA protein, as well as increased expression of CD25 and CD69 (Fig. 1A). Furthermore, the up-regulated BTLA expression occurred as early as 12 h after stimulation (Fig. 1B, C, D).

3.2. CsA but not RPM remarkably inhibited ConA-induced BTLA expression on T cells

CsA and RPM both significantly inhibited T cell proliferation (data not shown). CsA and RPM were added separately to C57BL/6 mouse splenocytes, which were stimulated with ConA. After 24 h of incubation, the expression of BTLA,

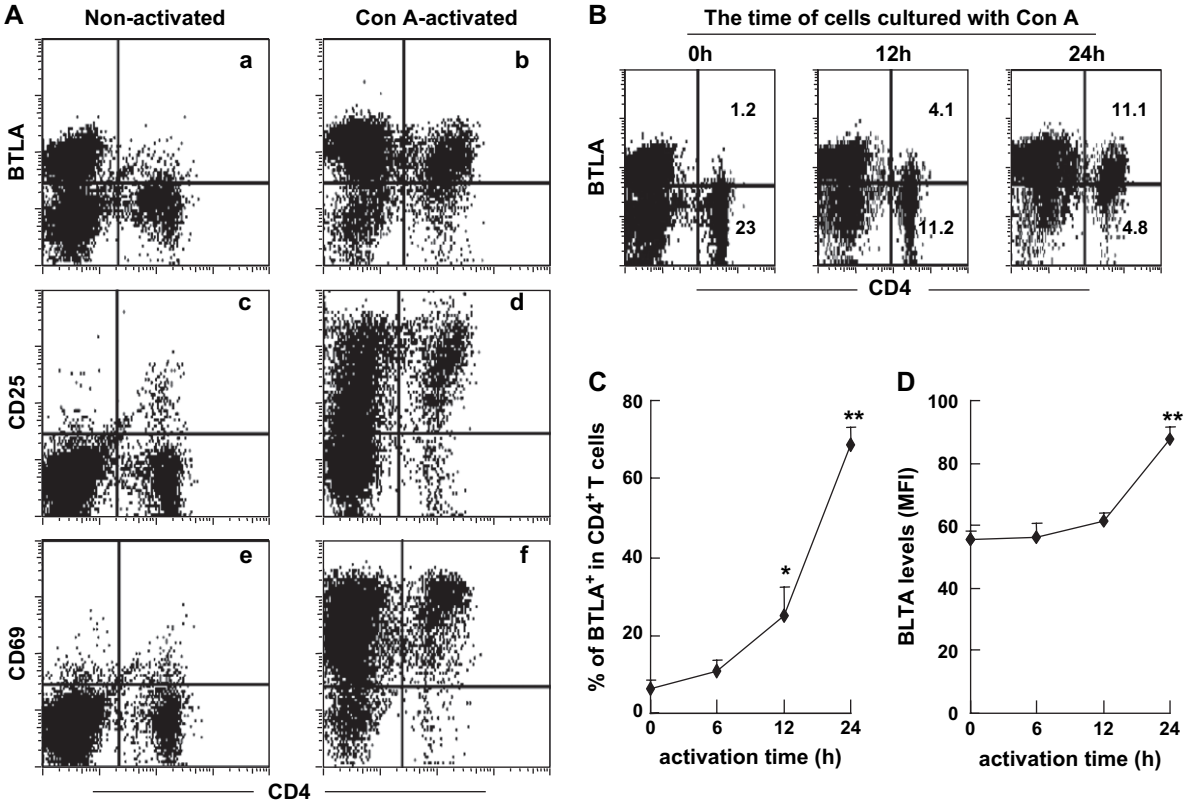

Fig. 1. The up-regulation of BTLA expression during CD4$^+$ T cell activation. (A) C57BL/6 mouse splenocytes were stimulated with ConA (2.5 μg/ml) for 24 h, and the expression of BTLA, CD25 and CD69 on CD4$^+$ cells were detected by two-color FCM. (B) Representative flow cytometric profiles of CD4 and BTLA expression on splenocytes at different time points after stimulation with ConA has been shown here. (C) The percentage of BTLA$^+$ cells in CD4$^+$ T cells during activation. (D) The amount of BTLA expression on CD4$^+$ T cells during activation was indicated by MFI (mean fluorescence intensity). Mouse splenocytes were stimulated with ConA (2.5 μg/ml) for the indicated time points, and the kinetics of BTLA expression on gated CD4$^+$ population were analyzed by FCM. The data are representative of one of three independent experiments with similar data. *$P < 0.05$, **$P < 0.01$ compared with the cells without ConA stimulation.

CD25 and CD69 on $CD4^+$ T cells were determined by FCM. The results from these three independent experiments are shown in Fig. 2. CsA but not RPM significantly decreased the percentages of $BTLA^+$ cells in $CD4^+$ T cells during activation (Fig. 2A, B). CsA decreased BTLA expression on $CD4^+$ T cells in a dose-dependent manner. However, MFI of BTLA expression on $CD4^+$ T cells was unchanged by the treatment of CsA or RPM (Fig. 2C). In addition, CsA

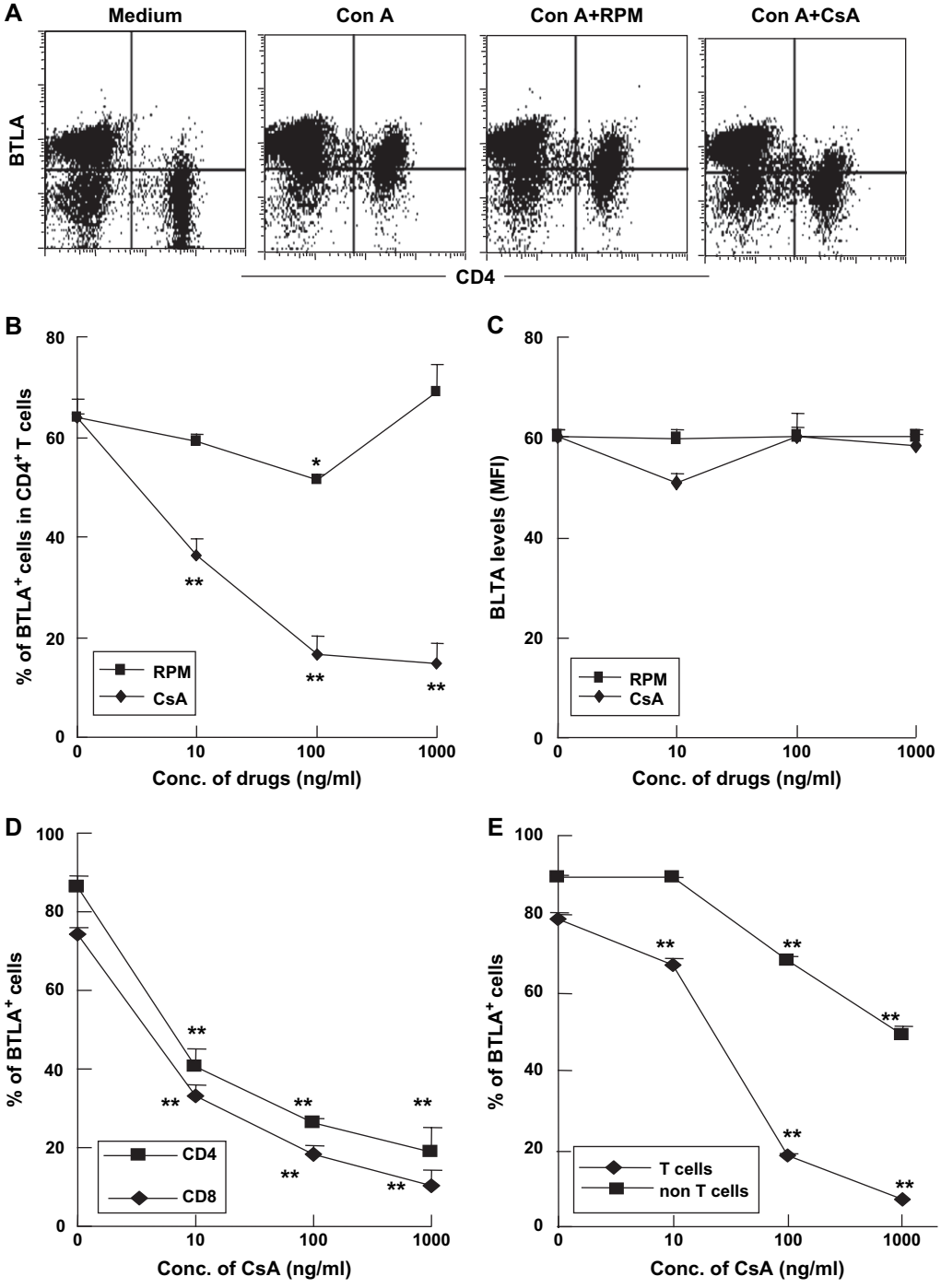

Fig. 2. CsA but not RPM significantly inhibited ConA-induced BTLA expression on $CD4^+$ and $CD8^+$ T cells during activation. Splenocytes of C57BL/6 mice were stimulated with ConA (2.5 μg/ml) in the presence of various concentrations of CsA (10–1000 ng/ml) or RPM (10–1000 ng/ml) for 24 h. (A) One representative flow cytometric profile of BTLA, CD25 and CD69 expressions on the gated $CD4^+$ population after stimulation with ConA in the presence of CsA(10 ng/ml), RPM(10 ng/ml) or none were shown here. (B) CsA but not RPM significantly decreased the percentage of $BTLA^+$ cells in $CD4^+$ T cells during ConA stimulation. (C) BTLA expression on activating $CD4^+$ T cells in the presence of CsA or RPM was indicated by MFI. (D) CsA significantly inhibited BTLA expression on either $CD4^+$ or $CD8^+$ T cells during T cell activation by ConA. (E) The expression of BTLA on TCR^+ and TCR^- splenocytes during ConA stimulation in the presence of CsA. One of three independent experiment results is shown as mean ± SD ($n = 4$ in each group). $^{**}P < 0.01$ when compared to the groups without CsA.

remarkably inhibited the BTLA expression on $CD8^+$ T cells, much as it did on $CD4^+$ T cells (Fig. 2D), whereas CsA decreased the BTLA expression on non-T cells in a weaker manner (Fig. 2E).

Furthermore, an inhibitory effect of CsA on BTLA expression on $CD4^+$ T cells during activation induced by antiCD3/anti-CD28 mAbs was observed (Fig. 3). Ligation of CD3/CD28 by plate-bound mAbs induced significantly high levels of BTLA expression on $CD4^+$ T cells at 24 or 48 h after incubation. CsA significantly decreased the BTLA expression on $CD4^+$ T cells as well ($P < 0.01$ or $P < 0.05$; Fig. 3B).

To determine when CsA acts on the BTLA expression of $CD4^+$ T cells during activation, we added CsA into the culture system at different times after ConA stimulation. As shown in Fig. 4, CsA significantly decreased the percentages of $BTLA^+$ cells in $CD4^+$ T cells as late as 12 h after T cell activation. However, similar BTLA expression was observed when CsA was added 1, 3, or 6 h after T cell activation, whereas the

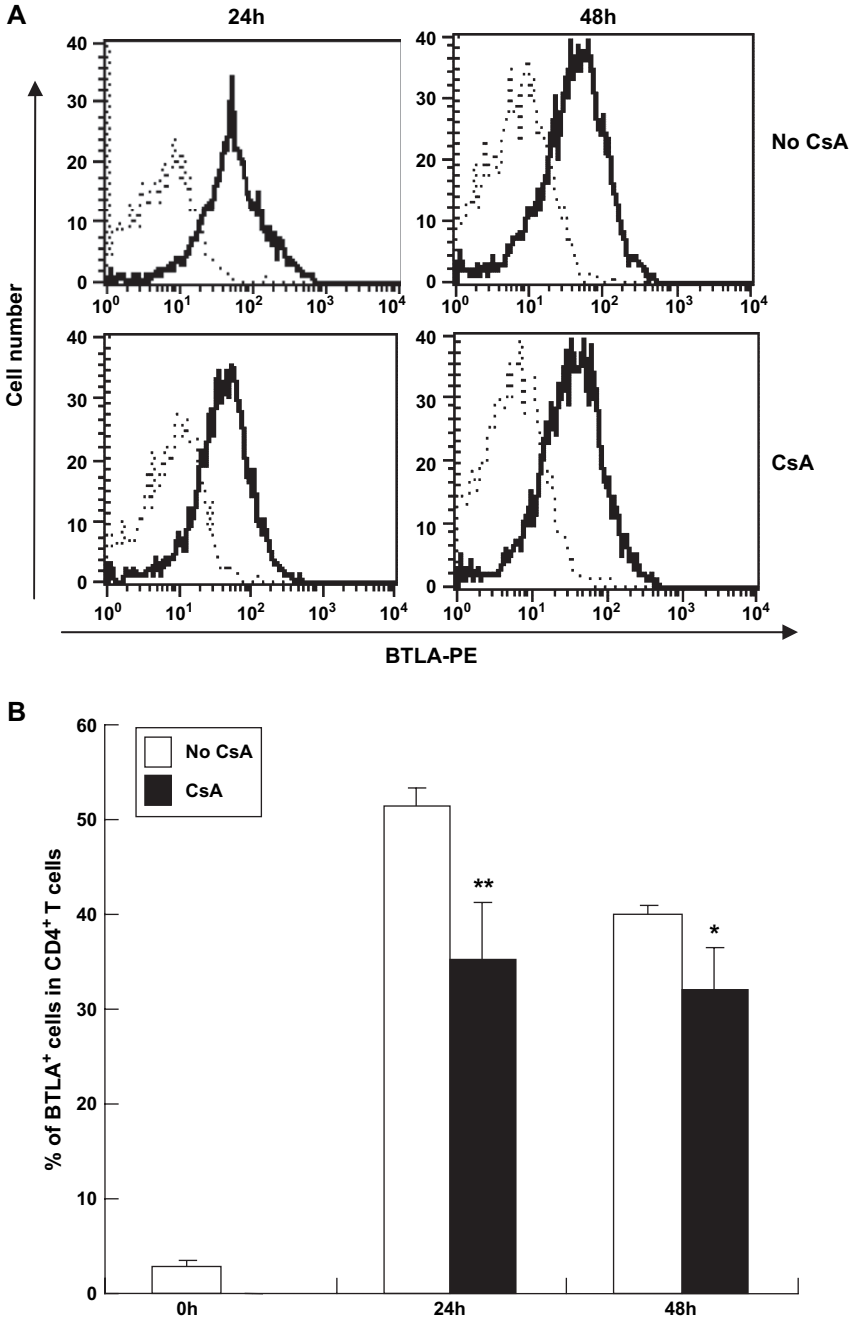

Fig. 3. CsA significantly inhibited BTLA expression on $CD4^+$ T cells during activation by anti-CD3/anti-CD28 mAbs. Splenocytes were cultured in anti-CD3/anti-CD28 mAbs-coated plates for 24 or 48 h in the presence of CsA or not. The expression of BTLA on $CD4^+$ T cells was determined by FCM. (A) One representative flow cytometric profile of BTLA expression on the gated $CD4^+$ population is shown here. The solid line presents BTLA expression, the dotted line presents the control mAb staining. (B) CsA significantly inhibited BTLA expression on $CD4^+$ T cells during activation by anti-CD3/anti-CD28 mAbs. One of two independent experiments with similar results is shown as mean ± SD ($n = 4$ in each group). *$P < 0.05$, **$P < 0.01$ when compared to the groups without CsA.

inhibitory effect of CsA added 12 h after activation was significantly lower than the effect of CsA added 6 h after activation ($P < 0.01$; Fig. 4). These data indicate that CsA may act on the expression of BTLA of $CD4^+$ T cells 6 h after activation of the proteins.

3.3. BTLA expression was more sensitive to CsA inhibition than CD25 and CD69 during $CD4^+$ T cell activation

The effects of CsA on the expression of T cell activation markers (CD25 and CD69) and BTLA were observed simultaneously. As shown in Fig. 5, the expression of CD25 and CD69 on $CD4^+$ T cells induced by ConA was not remarkably altered by CsA (Fig. 5), although the high dose of CsA (100 ng/ml) slightly but significantly decreased the percentages of $CD25^+$ cells and $CD69^+$ cells in $CD4^+$ T cells during activation (Fig. 5B). However, the percentage of $BTLA^+$ cells in $CD4^+$ T cells was significantly decreased when the cells were treated with as low as 1 ng/ml of CsA (Fig. 5B). The surface level of BTLA expression of $CD4^+$ T cells was not markedly changed, except for cells cultured with 10 ng/ml of CsA ($P < 0.05$; Fig. 5C), as indicated by the MFI of BTLA on $CD4^+$ T cells. The levels of CD25 and CD69 expression of $CD4^+$ T cells were somewhat increased in the presence of CsA, compared with control cells stimulated with ConA alone (Fig. 5C).

3.4. IL-2 failed to reverse the CsA-mediated inhibition on BTLA expression during T cell activation

It is well known that CsA markedly inhibits IL-2 production during T cell activation. To determine if decreased IL-2 production caused by CsA is involved in the CsA-mediated inhibition of BTLA expression during T cell activation, we performed add-back experiments with exogenous IL-2 in vitro. Recombinant human IL-2 (1000 U/ml) alone was able to promote the proliferation of T cells (Fig. 6A) and CD25 expression (data not shown), but it alone had no detectable effect on BTLA expression (Fig. 6B). Furthermore, IL-2 failed to block the effect of CsA on BTLA expression in activated T cells (Fig. 6B). Furthermore, similar data were observed when a low dose of IL-2 (200 U/ml) was used and IL-2 was found to be sufficient to promote T cell proliferation (Fig. 6C; data not shown). Thus, the failure to produce IL-2 does not cause a reduction in BTLA expression in this system.

3.5. CsA significantly inhibited BTLA expression on $CD4^+$ T cells induced by either calcium or MAPK pathway

To investigate how CsA reduced BTLA expression of $CD4^+$ T cells during activation, we stimulated splenocytes of C57BL/6 mice with PMA (a PKC activator) or ionomycin (a calcium activator) alone for 24 h in the absence or presence of CsA. We found that PMA or ionomycin alone was sufficient to induce BTLA expression of $CD4^+$ T cells, whereas the non-activated $CD4^+$ T cells expressed low or undetectable levels of BTLA as determined by FCM (Fig. 7). CsA significantly inhibited BTLA expression of $CD4^+$ T cells regardless of whether it was induced by calcium or the PKC pathway (Fig. 7).

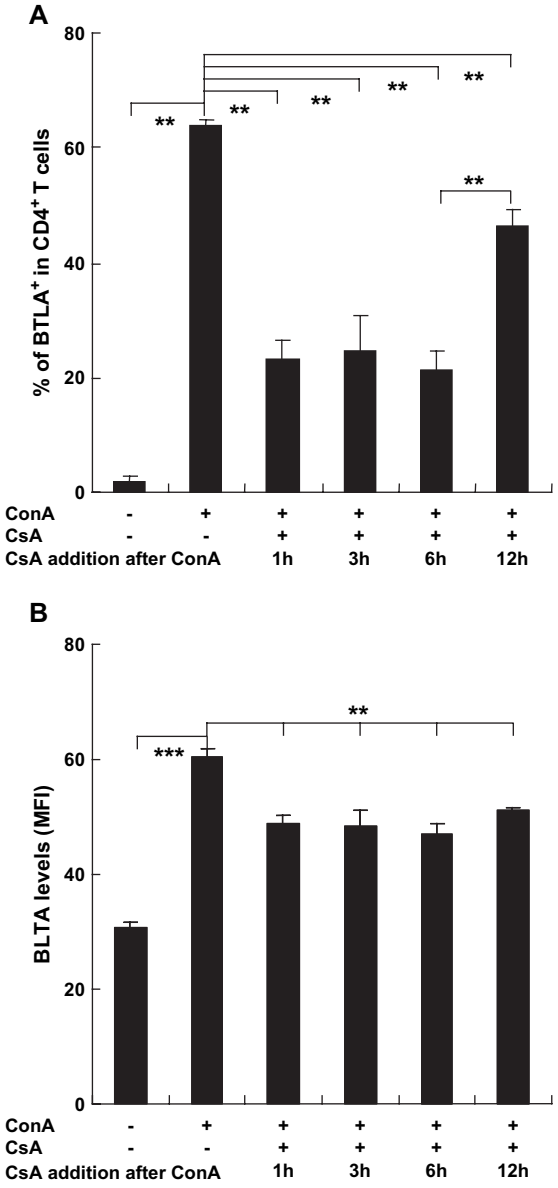

Fig. 4. No obvious effect of CsA on BTLA expression during the early stage of T cell activation During T cell activation by ConA, CsA was added at the indicated time points. (A) Adding CsA 6 h after T cell activation is early enough to block BTLA expression on $CD4^+$ T cells. The percentage of $BTLA^+$ cells in $CD4^+$ T cells was shown. (B) BTLA expression on $CD4^+$ T cells was shown by MFI. Results were shown as mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the indicated groups. Data are representative of three independent experiments with similar data.

4. Discussion

BTLA plays a unique role in the regulation of immune responses, autoimmunity, and graft rejection. However, the

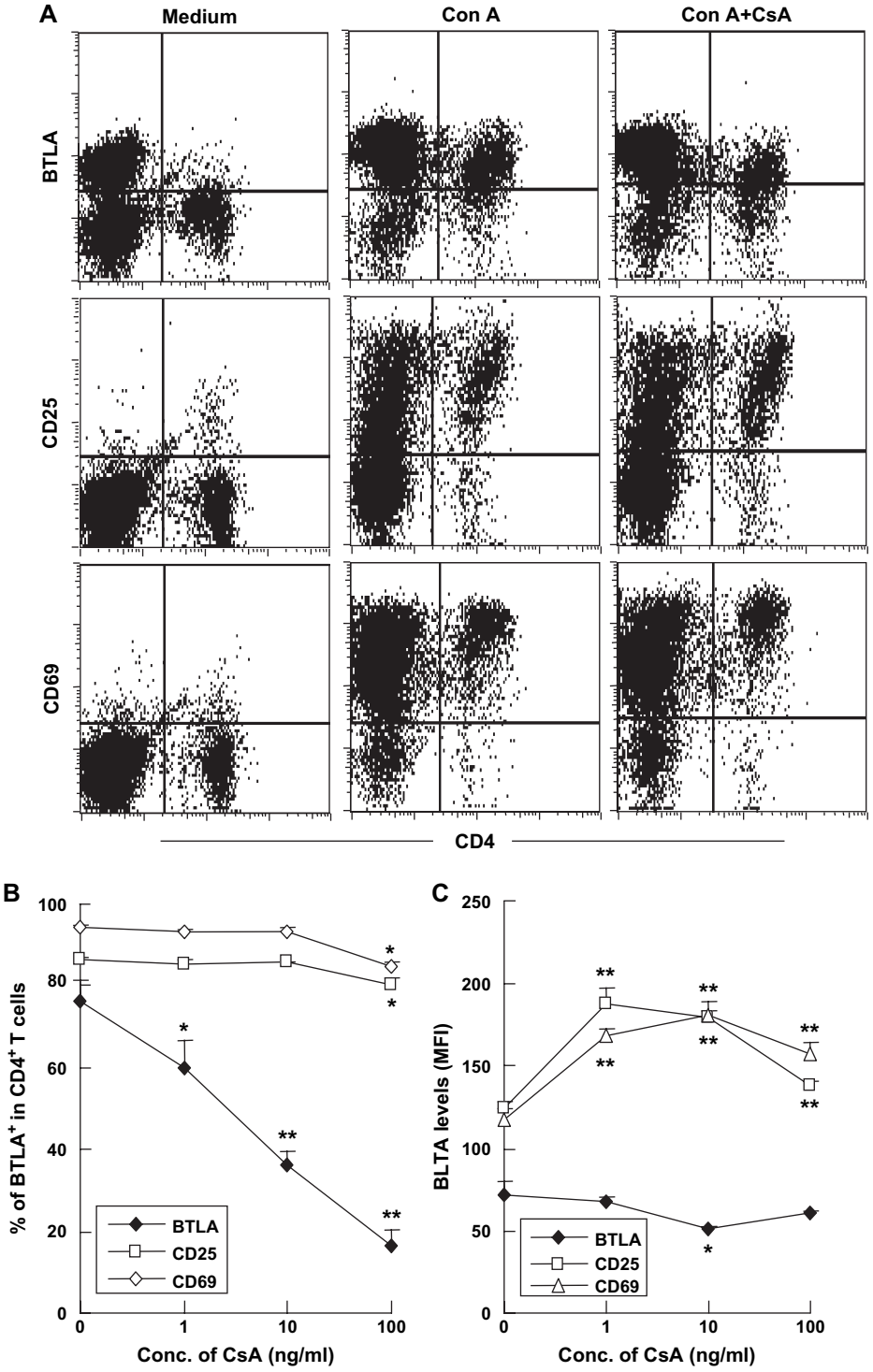

Fig. 5. Different sensitivity to CsA of BTLA, CD25 and CD69 expression during $CD4^+$ T cell activation. Splenocytes of C57BL/6 mice were stimulated with ConA (2.5 μg/ml) in the absence or presence of various concentrations of CsA (1−100 ng/ml) for 24 h, and then stained with FITC-labeled anti-CD4 mAb versus PE-labeled anti-BTLA, CD25, CD69 or control mAb, respectively. (A) Mouse splenocytes were cultured in the presence of 1 ng/ml CsA. One representative flow cytometry profile of BTLA, CD25 and CD69 expression on $CD4^+$ cells is shown here. (B) The percentages of BTLA, CD25 or CD69-positive cells in $CD4^+$ T cells during activation in the presence of CsA. Less than 10% of $BTLA^+$ cells in $CD4^+$ T cells were detected without ConA stimulation. (C) The MFIs of BTLA, CD25 and CD69 expression on $CD4^+$ T cells in the presence of CsA. Results were calculated and expressed as mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$ compared with groups treated with no CsA. Data are representative of four independent experiments.

effect of immunosuppressive drugs on BTLA expression after T cell activation was not fully understood. In the present study, we investigated whether CsA or RPM can induce BTLA expression of T cells by FCM. Our data shows that CsA could markedly inhibit the expression of BTLA of $CD4^+$ and $CD8^+$ T cells, but RPM had little effect. The CsA-sensitive pathway, but not the RPM-sensitive pathway, may be the main regulator of BTLA expression of T cells, based on our

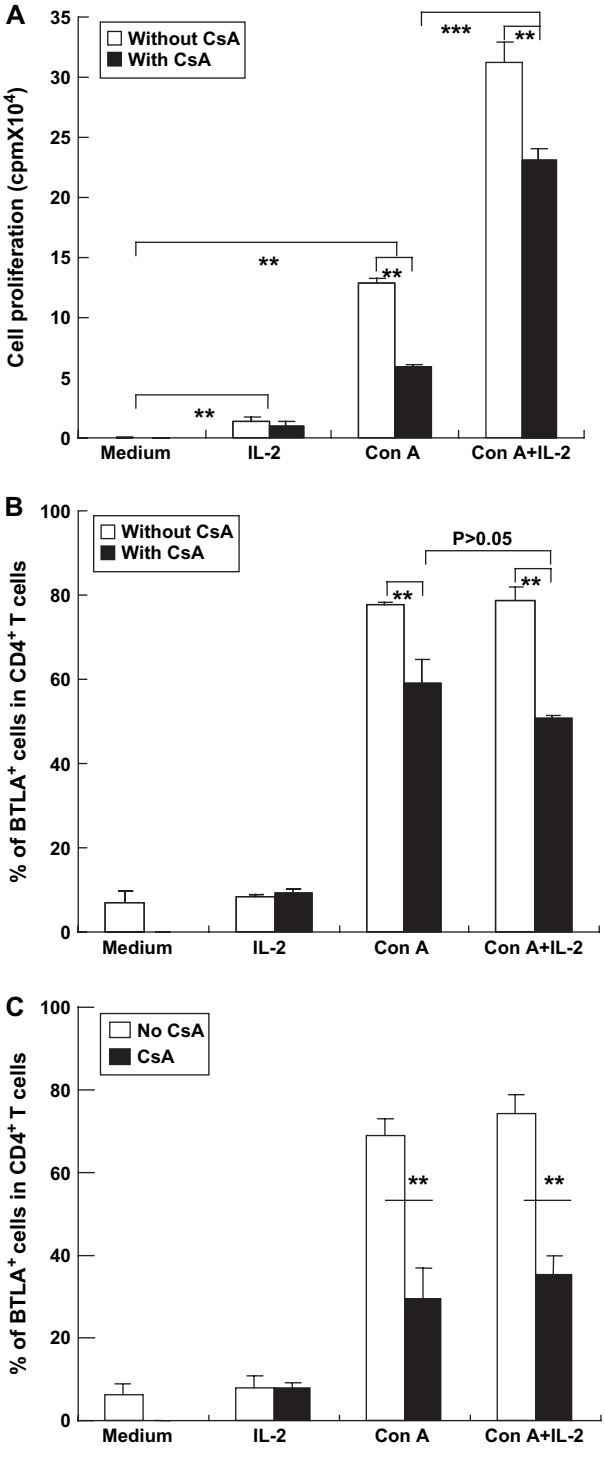

Fig. 6. CsA inhibited BTLA expression on $CD4^+$ T cells in an IL-2 independent pathway. (A) Exogenous IL-2 partially reversed the inhibitory effect of CsA on T cell proliferation induced by ConA stimulation. Splenocytes of C57BL/6 mice were cultured in the presence of the indicated agents for 72 h. The cell proliferation was determined by [^{3}H]thymidine incorporation. $^{**}P < 0.01$, compared the groups without CsA to the indicated groups. Data shown here are representative of three independent experiments. (B) High dose of exogenous IL-2 failed to reverse the inhibitory effect of CsA on BTLA expression induced by ConA stimulation. C57BL/6 splenocytes were stimulated with ConA (2.5 μg/ml) in the absence or presence of CsA (10 ng/ml), with the addition of 1000 U/ml hIL-2 in some cultures. At 24 h after culture, cells were harvested and analyzed for the expression of BTLA on $CD4^+$ T cells by FCM. (C) Exogenous IL-2 failed to reverse the inhibitory effect of CsA on BTLA expression induced by ConA stimulation. C57BL/6 splenocytes were stimulated with ConA (2.5 μg/ml) in the absence or presence of CsA (10 ng/ml), 200 U/ml hIL-2 or both respectively for 24 h. The cells were analyzed for the expression of BTLA on $CD4^+$ T cells by FCM. Results were shown as mean ± SD ($n = 4$ in each group). $^{**}P < 0.01$ compared between the indicated groups. One representative experiment of three is shown.

results. Although CsA remarkably inhibited BTLA expression of $CD4^+$ and $CD8^+$ T cells, CsA inhibited BTLA expression of immune cells (except T cells) to a much weaker degree. These data indicate that the regulation of BTLA of T cells and non-T cells may be mediated by different pathways.

CsA is widely used to treat autoimmune diseases and graft rejection in clinics and can potentially increase the susceptibility to autoimmune diseases [22,23]. Our present results may partially explain this phenomenon. The effects of CsA in blocking the occurrence of T cell anergy in the periphery has been previously reported [24–26]. It has also been reported that accelerated GVHD and allograft rejection might occur after withdrawal of CsA. CsA has been shown to aggravate and/or induce relapse in several autoimmune diseases including collagen-induced arthritis, EAE, and autoimmune thyroiditis [24–26].

Previous studies have shown that BTLA expression was primarily controlled by T cell activation [7]. However, our experiments showed that low doses of CsA efficiently suppressed the expression of BTLA, without a significant effect on the expression of T cell activation markers, CD25 and CD69. These data suggest that the decreased expression of BTLA by CsA might not be related to T cell activation. However, a high dose of CsA could slightly inhibit the expression of CD25 and CD69 during T cell activation, which is consistent with previous reports [27]. Our data also indicate that BTLA might be more sensitive to CsA than to CD25 and CD69, and BTLA expression might not be closely related to T cell activation. In addition, similar inhibition of BTLA expression in T cells was observed when CsA was added either at the same time as ConA stimulation or 6 h later. These data indicate that CsA may affect the expression of BTLA in $CD4^+$ T cells 6 h after activation of the protein levels and that BTLA expression is a relative event during T cell activation. This speculation is supported by the observation that significant levels of BTLA expression in $CD4^+$ T cells were detected 12 h after ConA stimulation, as assayed by FCM.

The immunosuppressive function of CsA has traditionally been explained on the basis of its inhibition of the IL-2 product, which is a crucial cytokine for T cell activation and proliferation. However, IL-2 alone failed to induce BTLA expression, while it significantly stimulated T cell proliferation. Addition of exogenous IL-2 into the culture system could not restore the expression of BTLA in $CD4^+$ T cells cultured with ConA and CsA. These results indicate that IL-2 is not involved in the inhibition of BTLA expression by CsA. Other researchers have shown that IL-2 was not required for the induction of CTLA-4, although CTLA-4 expression was also regulated by CsA [21]. The findings suggest that decreased BTLA and CTLA-4

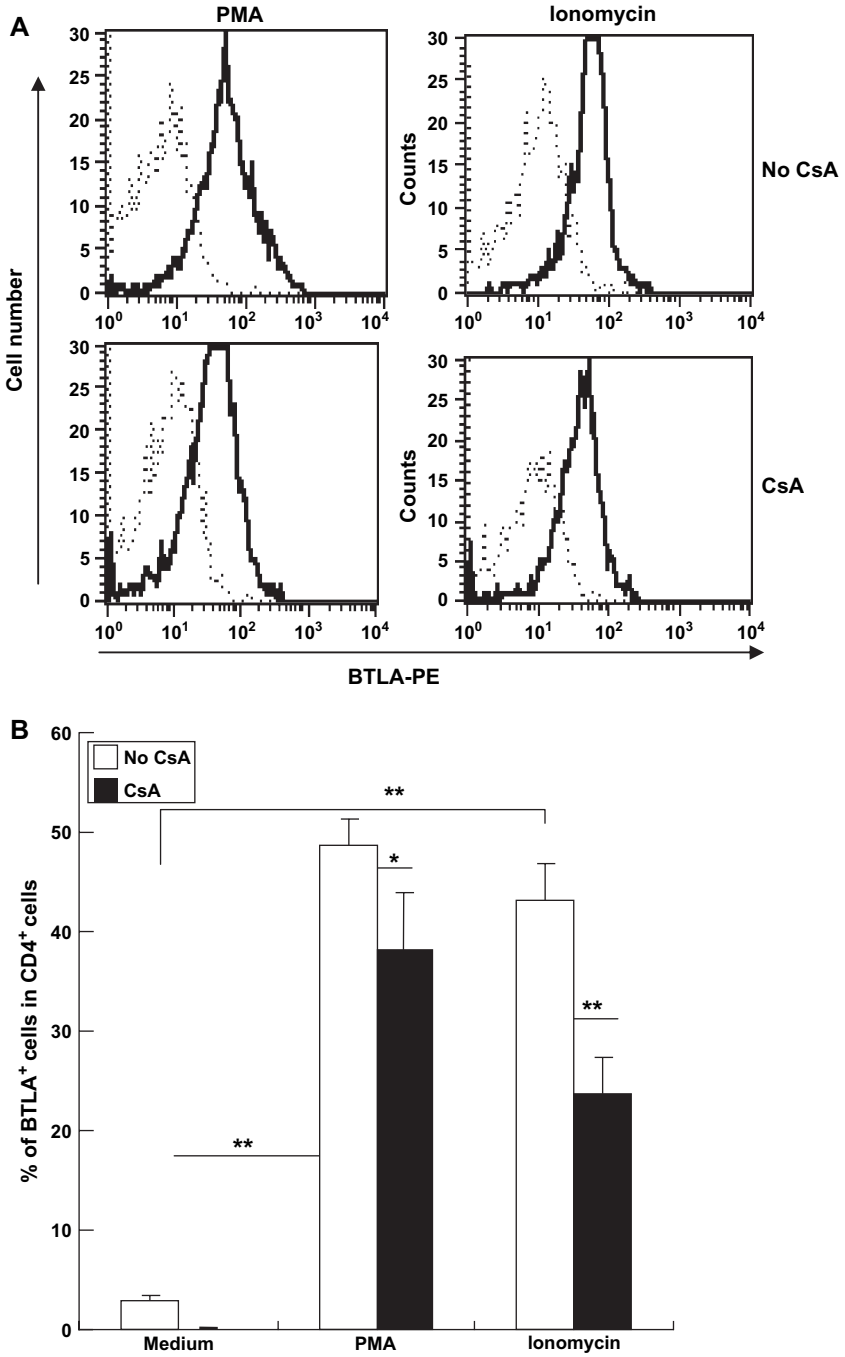

Fig. 7. CsA significantly inhibited PMA- or ionomycin-induced BTLA expression on $CD4^+$ T cells. (A) Splenocytes of C57BL/6 mice were stimulated with PMA (10 ng/ml) or ionomycin (1 μM) in the presence or absence of CsA (10 ng/ml) for 24 h. Histogram of BTLA expression on gated $CD4^+$ T cells is shown here. (B) CsA significantly inhibited PMA or ionomycin-induced BTLA expression on $CD4^+$ T cells. The percentages of $BTLA^+$ cells on $CD4^+$ T cells were determined by FCM. Results were calculated and expressed as mean ± SD. $^*P < 0.05$; $^*P < 0.01$ compared with the indicated groups. Data are representative of three independent experiments.

by CsA was not a consequence of the reduced IL-2, but might occur simultaneously with decreased production of IL-2.

The combination of PMA and ionomycin is usually used to mimic the TCR signal, but each one alone can not fully activate T cells. We stimulated splenocytes with PMA or ionomycin alone, and observed the induction of BTLA expression in $CD4^+$ T cells. These findings strongly support the impression that the expression of BTLA is not related to T cell activation. It has been reported that BTLA is up-regulated in thymocytes by the activation of the mitogen-activated protein kinases (MAPK) signaling pathway, and we found that the same process occurred in splenocytes [6]. In addition, we observed that increased calcium also gave rise to the expression of BTLA in $CD4^+$ T cells. CsA has been shown to target two distinct pathways: the calcineurin/NFAT pathway and JNK/p38 pathway [28]. Our experimental data show that CsA suppresses either

PMA or ionomycin-induced BTLA expression, which indicates that CsA might inhibit BTLA expression by interfering with both pathways.

The IC50 of CsA in vitro is about 10–20 μg/ml, and this dose of CsA only leads to partial inhibition of calcineurin [19]. It has been reported that the treatment with CsA does not prevent an increase in intracellular Ca^{2+} or kinase activation, but that the transcriptional activations of certain genes are inhibited [29]. Our data showed that the inhibitory effect of CsA on BTLA expression was decreased when CsA was added after ConA stimulation for more than 12 h, implying that CsA may affect the transcriptional level or the early events for BTLA expression. Our preliminary study showed that BTLA mRNA expression increased after splenocytes were stimulated with ConA, while addition of CsA significantly suppressed the mRNA level of BTLA (data not shown). Our results place the action of CsA proximal to the transcription factors that are involved in gene activation, but distal to the known cytoplasmic events that occur in the pathway.

In summary, our data show that CsA, but not RPM, has a significant effect on the expression of BTLA in $CD4^+$ and $CD8^+$ T cells, but not on other immune cells. CsA suppresses BTLA expression, neither by the influence of T cell activation or by the decreased production of IL-2. CsA might act on the transcriptional activation of BTLA through the calcineurin/NFAT pathway and the JNK/p38 pathway. The inhibition of BTLA expression by CsA may be one of the reasons for the occurrence of autoimmune diseases in CsA-treated patients. These findings provide important insights for the use of CsA in clinics.

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