Cytokine-induced alterations of BAMBI mediate the reciprocal regulation of human Th17/Treg cells in response to cigarette smoke extract

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Abstract. In $CD4^+$ T helper (Th) cells, transforming growth factor β (TGF-β) is indispensable for the induction of both regulatory T (Treg) and interleukin-17-producing effector T helper (Th17) cells. Although BMP and activin membrane-bound inhibitor (BAMBI) is part of a rheostat-like mechanism for the regulation of TGF-β signalling and autoimmune arthritis in mouse models, the underlying activity of BAMBI on the human Th17/Treg cell axis, particularly during exposure to cigarette smoke, remains to be elucidated. The present study aimed to further characterize BAMBI expression in human $CD4^+$ cells, as well as immune imbalance during activation and cigarette smoke exposure. Results from the present study indicated that exposure to cigarette smoke extract partially suppressed Treg differentiation and promoted Th17 cell generation under stimulation by anti-CD3/28 antibodies and TGF-β1. Additionally, exposure to cigarette smoke induced an inhibition of phosphorylated-Smad2/Smad3, which may have arisen from a concomitant enhancement of BAMBI expression. In conclusion, human BAMBI may function as a molecular switch to control TGF-β signalling strength and the Th17/Treg cell balance, which may be used not only as a biomarker but also as a target of new treatment strategies for maintaining immune tolerance and for the treatment of smoking-induced immune disorders.

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Abbreviations: BAMBI, BMP and activin membrane-bound inhibitor; COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; TGF-β, transforming growth factor β; Th, T helper cell; Treg, regulatory T cell

Key words: CSE, BAMBI, Treg, Th17

Introduction

Many autoimmune diseases are caused by autoreactive T helper (Th)1 clones, and dysregulated Th1/Th2 responses have long been associated with the induction and regulation of autoimmunity (1). However, the finding that a lack of interferon γ makes otherwise experimental autoimmune encephalomyelitis mice susceptible to disease (2) raised the question of whether another T cell subset, other than Th1, may be required for the induction of autoimmune diseases. Subsequently studies identified an independent subset of interleukin (IL)-17-producing effector T helper (Th17) cells, which are involved in the pathogenesis of numerous experimental autoimmune diseases and human inflammatory states (3). By contrast, regulatory T cells (Tregs) are a subset of $CD4^+$ T cells that express forkhead box P3 (FOXP3) and are crucial in controlling inflammation and maintaining self-tolerance (4).

Transforming growth factor (TGF)-β1 has been recognized as a link between Treg and Th17 cell subsets. Th17 cells are reciprocally related to Tregs given that TGF-β1 induces differentiation of naive T cells into $FOXP3^+$ Tregs, whereas IL-6 inhibits the TGF-β1-induced expression of FOXP3 and ultimately induces the generation of Th17 cells (3). By contrast, high concentrations of TGF-β1 prevent the induction and effector functions of Th17 cells by inducing FOXP3 (3). However, numerous questions remain concerning the integration effects of TGF-β signals and inflammatory stimuli on the plasticity between Tregs and Th17 cells. Notably, BMP and activin membrane-bound inhibitor (BAMBI) was identified as a TGF-β1 rheostat that controls Th17/Treg cell differentiation and the development of autoimmune arthritis in mice (5). BAMBI is structurally similar to TGF-β type I receptors (TGF-βRIs) but lacks an intracellular kinase domain, causing it to antagonize TGF-β family signalling (6). Although BAMBI has been shown to serve roles in human

lung inflammation associated with chronic obstructive pulmonary disease (COPD) and bacterial infection (7), the precise expression and activity of BAMBI in human immune cells, especially in the Th17/Treg cell paradigm, remains to be determined.

Although smoking has been well established as a central factor in many pathological conditions, such as neoplasms and lung and cardiovascular diseases, little is known about the processes and factors involved in changing immune arms in response to cigarette smoke (8). Although tobacco smoke was demonstrated to stimulate the production of pro-inflammatory cytokines (such as tumour necrosis factor α, IL-1 and IL-6) and to reduce anti-inflammatory cytokine levels (such as IL-10), local homeostasis and cytokine networks, as well as genetic predisposition, should be taken into account when analysing the influence of cigarette smoke (8). Our previous studies have demonstrated that cigarette smoking disrupts the survival of $CD4^+CD8^+$ Tregs in COPD, partially through muscarinic receptor-dependent mechanisms (9,10). However, the pathological processes and pathways underlying the Th17/Treg cell imbalance are still unknown.

Our previous study also demonstrated that an impaired TGF-β/BAMBI pathway promotes inflammation leading to Th17/Treg imbalance, which is a new mechanism in smokers who develop COPD (11). The present study aimed to further characterize BAMBI expression in human $CD4^+$ cells and immune imbalance during activation and cigarette smoke exposure. These data suggested that cigarette smoke may disturb TGF-β-dependent BAMBI expression in human TCR-stimulated $CD4^+$ T cells, which, in turn, may cause the dysfunction of Smad2/Smad3 phosphorylation following cigarette smoke exposure. The results indicated that BAMBI was functionally expressed in activated $CD4^+$ T cells and may act as a molecular switch to control TGF-β signalling and the Th17/Treg cell balance. The TGF-β1/BAMBI pathway in T cells may serve a role in immune dysfunction associated with cigarette smoke.

Materials and methods

Cell isolation. This study was conducted between January 2016 and May 2017. Blood samples (20-30 ml) were obtained from 25 healthy adults (age, 25-55 years; 15 male, 10 female) and immediately placed on ice. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of blood samples. All experiments were performed in accordance with relevant manufacturer's protocols. Naive $CD4^+$ T cells were isolated from PBMCs by MACS, based on negative selection using the naive $CD4^+$ T cell isolation kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The purity of $CD4^+CD45RA^+$ T cells was >95%, as measured by flow cytometry. This study was approved by the Ethics Committee of Union Hospital, Tongji Medical School, Huazhong University of Science and Technology (Wuhan, China); written informed consent was received from each donor.

Cigarette smoke extract (CSE) preparation. Aqueous CSE was prepared according to a modification of our previously published method (10). Briefly, the smoke from one cigarette (Huanghelou, Wuhan, China) was bubbled slowly into a tube containing 2.5 ml of sterile RPMI-1640 medium (Beijing Suolaibao Biotechnology Co., Ltd., Beijing, China). The resulting solution was further filtered through 0.22 μm filters and was defined as 100% CSE. The CSE was prepared fresh, within 30 min prior to each experiment.

Cell viability assay. Purified naive $CD4^+$ T cells ($1x10^5$ cells/well) were cultured and incubated at 37˚C in 200 μl of complete medium containing 10% heat-inactivated (56˚C for 30 min) foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 50 U/ml IL-2 (PeproTech, Inc., Rocky Hill, NJ, USA) in 96-well plates and stimulated with plate-bound anti-CD3 (monoclonal, OKT3; 5 μg/ml) and anti-CD28 (5 μg/ml) antibodies (both purchased from eBioscience; Thermo Fisher Scientific, Inc.). Cells were stimulated for 5 days in the presence of serial dilutions of CSE at 0, 0.002, 0.02 and 0.2%. Cell viability was tested by Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. The safe concentrations of CSE at 0.002 and 0.02% used in subsequent experiments were chosen following a cytotoxicity assay (Fig. 1).

Development of naive and differentiating T cells in CSE-conditioned medium. To investigate the effects of cigarette smoke on the development of naive $CD4^+$ T cells, purified cells ($5x10^5$ cells/well) were incubated at 37˚C in 1 ml complete medium containing FBS and IL-2, as aforementioned, in 48-well plates with plate-bound anti-CD3/CD28, in the absence or presence of CSE (0.002 and 0.02%). In parallel experiments, naive T cells were cultured *in vitro* under Treg-polarizing conditions (2 ng/ml TGF-β1) or Th17 cell-polarizing conditions (2 ng/ml TGF-β1 and 30 ng/ml IL-6; or 10 ng/ml IL-1β, 30 ng/ml IL-6 and 50 ng/ml IL-23), combined with or without CSE at the initiation of culture. Recombinant human TGF-β1, IL-6, IL-1β and IL-23 were purchased from PeproTech, Inc. To confirm that the TGF-β1 produced and activated in T cell receptor (TCR)-stimulated cells was indeed responsible for BAMBI expression, a purified anti-TGF-β antibody (500 ng/ml; clone 19D8; BioLegend, Inc., San Diego, CA, USA) that is able to block human TGF-β1 activity was included in the culture. The involvement of Smad3 was determined by treating cells with 1 μM of the Smad3-specific inhibitor SIS3 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), which caused potent and selective inhibition of Smad3 function (12). After 5 days incubation, the expression of T cell subsets and related molecules was determined by flow cytometry.

Flow cytometry. The expression of T cells markers were analysed by flow cytometry as described previously (10); surface or intracellular proteins were stained with fluorescein isothiocyanate-conjugated anti-CD4 (Clone RPA-T4; BD Biosciences, San Jose, CA, USA), phycoerythrin (PE)-cyanine 7-conjugated anti-CD25 (Clone M-A251; BD Biosciences), PE-conjugated anti-FOXP3 (Clone 236A/E7; eBioscience; Thermo Fisher Scientific, Inc.), eFluor660-conjugated anti-IL-17A (Clone eBio64DEC17; eBioscience; Thermo Fisher Scientific, Inc.), Alexa Fluor (AF)-647-conjugated anti-BAMBI (Polyclonal; BIOSS, Beijing, China) and AF647-conjugated

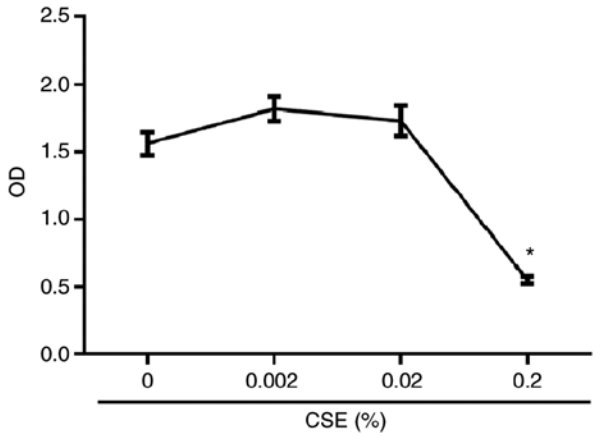

Figure 1. Effects of CSE on cell viability. Naive CD4+ T cells isolated from peripheral blood were stimulated with plate-bound α-CD3 and α-CD28 monoclonal antibodies in the presence of CSE at 0, 0.002, 0.02 and 0.2% for 5 days. Cell viability was examined by Cell Counting Kit-8 at an absorbance of 450 nm. The data are presented as the mean ± standard error of the mean (n=4), and are representative of three independent experiments; *P<0.05 vs. 0% CSE. CSE, cigarette smoke extract; OD, optical density.

anti-Smad2 (pS465/pS467)/Smad3 (pS423/pS425) (Clone O72-670; BD Biosciences) antibodies. Intracellular staining for FOXP3, IL-17 and Smad2/Smad3 was performed following fixation and permeabilization using the FOXP3 Staining Buffer Set (eBioscience; Thermo Fisher Scientific, Inc.), as described previously (10). Isotype controls were used to exclude non-specific staining and to identify the gates for negative/positive population selection. To identify Th17 cells, 50 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich; Merck KGaA), 1 μg/ml ionomycin (Sigma-Aldrich; Merck KGaA) and 1 μl/ml GolgiStop (BD Biosciences) were added to the culture medium on day 5 for 5 h. To measure intracellular levels of phosphorylated (p)-Smad2/Smad3, cells were treated with 2 ng/ml TGF-β1 for 30 min at 37˚C on day 5 prior to harvesting. Flow cytometry was performed using the BD LSRFortessa and analysed with FACSDiva Software v8 (BD Biosciences) and FlowJo v10 software (Tree Star, Inc., Ashland, OR, USA).

Statistics. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons tests. Data analysis was conducted with GraphPad Prism v7.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. No statistical method was used to predetermine sample size.

Results

Treg differentiation upon CSE exposure. To better understand the induction of Tregs, the expression levels of CD25 and FOXP3 were determined in naive CD4+ T cells stimulated with anti-CD3/28 antibodies and TGF-β1, in combination with or without the Smad3-specific inhibitor SIS3. According to classical *in vitro* Treg-polarizing conditions (with anti-CD3/28 antibodies in the presence of TGF-β1) (13,14), high levels of CD25+FOXP3+ Tregs were induced successfully during differentiation; whereas this induction was blocked by SIS3 treatment (Fig. 2).

To determine whether the stimulation of cigarette smoke was associated with a change in Treg induction, CSE was added to CD4+ T cell cultures at different non-cytotoxic concentrations (0.002 and 0.02%; Fig. 1). Exposure to CSE alone did not induce naive CD4+ T cells to become CD25+FOXP3+ suppressor cells (15). Under classical Treg-polarizing conditions, however, CSE treatment notably reduced the differentiation rate of Tregs (Fig. 2).

CD25 expression is one of the activation markers of T cells. During Treg cell differentiation, a high induction of CD25 was also observed in CD4+ T cells following activation with anti-CD3/28 antibodies in the presence of TGF-β1 (Fig. 2). Similar to the observed trend in Treg generation, CD25 induction was inhibited by SIS3 and 0.02% CSE treatment (Fig. 2).

CSE exposure in Th17 cell differentiation. Classical differentiation of pro-inflammatory Th17 cells was also examined. In naive CD4+ T cells incubated in the presence of TGF-β1 + IL-6 (the first protocol), Th17 cells were successfully detected (Fig. 3). Notably, this induction was further enhanced in the presence of SIS3, which indicated that weakened Smad3 signalling may act as a regulator of Th17 cell skewing and Treg suppression. Subsequently, the underlying effects of cigarette smoking on Th17 cell induction were further examined. A previous study reported that the addition of CSE alone was unable to induce IL-17 expression in naive CD4+ T cells (15). Noatbly, under Th17 cell-polarizing conditions (the first protocol), CSE induced the differentiation of Th17 cells (Fig. 3).

In addition, the combination of IL-1β, IL-6 and IL-23 stimulation (the second protocol) was also able to initiate *in vitro* Th17 cell differentiation (Fig. 4). The combination of IL-6, IL-1β and IL-23 stimulation induced similar production levels of IL-17 between Th17 cells co-treated with or without SIS3 (Fig. 4). Notably, by the second protocol, CSE induced statistically stronger expression of Th17 cells compared with SIS3 treatment (Fig. 4).

TGF-β/Smad3 signalling is attenuated by CSE exposure. As crucial downstream mediators in the TGF-β signalling pathway, the potential role of Smad2/Smad3 phosphorylation was further evaluated in TCR-stimulated naive cells. Results from flow cytometric analysis revealed a significant increase in Smad2/Smad3 phosphorylation TGF-β1-treated cells compared with expression in cells cultured in complete medium without stimulation (Fig. 5). Smad2/Smad3 phosphorylation was significantly inhibited in TCR-stimulated cells co-treated with SIS3, which corroborated the specificity of the inhibitor.

To identify the potential for TGF-β/Smad signalling as a possible mechanism for the differentiation of naive T cells by CSE, the role of CSE in Smad2/Smad3 phosphorylation was evaluated. Cells co-treated with 0.02% CSE exhibited a significant reduction of Smad2/Smad3 phosphorylation compared with TGF-β-treated cells (Fig. 5), but to a lesser extent compared with SIS3 treatment. It is noteworthy that CSE treatment did not completely inhibit p-Smad2/Smad3 expression to that level of untreated cells.

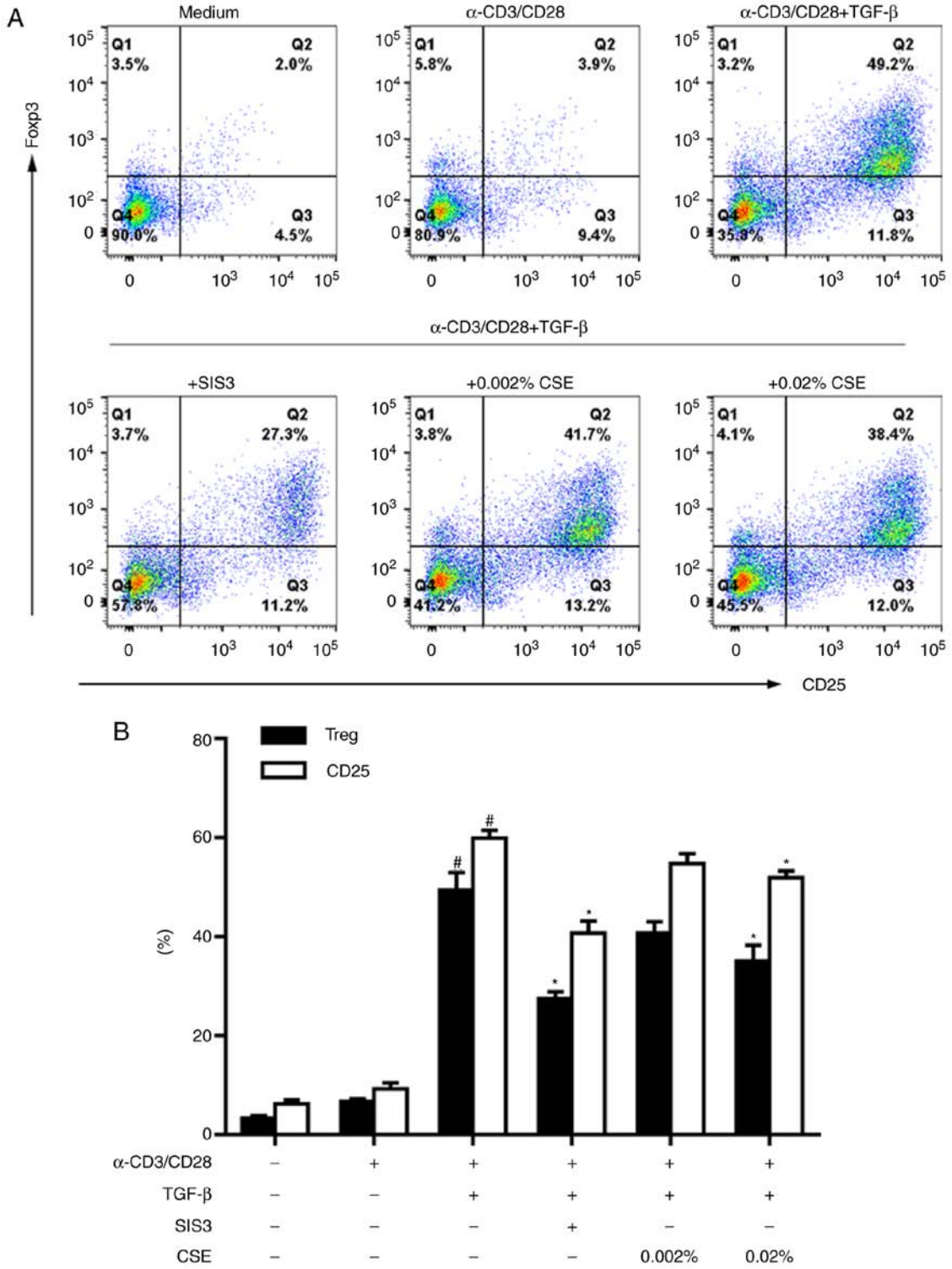

Figure 2. Effects of CSE on Treg differentiation. (A and B) Naive CD4+ T cells isolated from peripheral blood were cultured in complete medium and stimulated with plate-bound α-CD3 and α-CD28 monoclonal antibodies under the indicated conditions for 5 days. (A) Cells were co-stained for CD25 and FOXP3 expression and measured by flow cytometry; representative pseudocolour dot plots gated on CD4+ T cells are shown. (B) Summary data of CD25+ FOXP3+ Tregs and CD25+ T cells in each condition, from (A) Data are presented as the mean ± standard error of the mean (n=4), and are representative of three independent experiments; #P<0.05 vs. Untreated control or α-CD3/28; *P<0.05 vs. respective α-CD3/28 + TGF-β1. CSE, cigarette smoke extract; FOXP3, forkhead box P3; TGF-β1, transforming growth factor β1; Treg, regulatory T cell; SIS3, a Smad3-specific inhibitor.

BAMBI expression is increased during activation and CSE exposure. BAMBI acts as a TGF-β1 rheostat that regulates Th17/Treg cell differentiation and the development of COPD and autoimmune arthritis (5,9). Therefore, the potential expression characteristics of BAMBI under the various activation levels and the cytokine milieu conditions were investigated. Naive CD4+ T cells from peripheral blood express low or undetectable levels of BAMBI protein (5). Although treatment with TGF-β1 alone was able to induce mild BAMBI expression compared with untreated cells, anti-CD3/CD28 stimulation

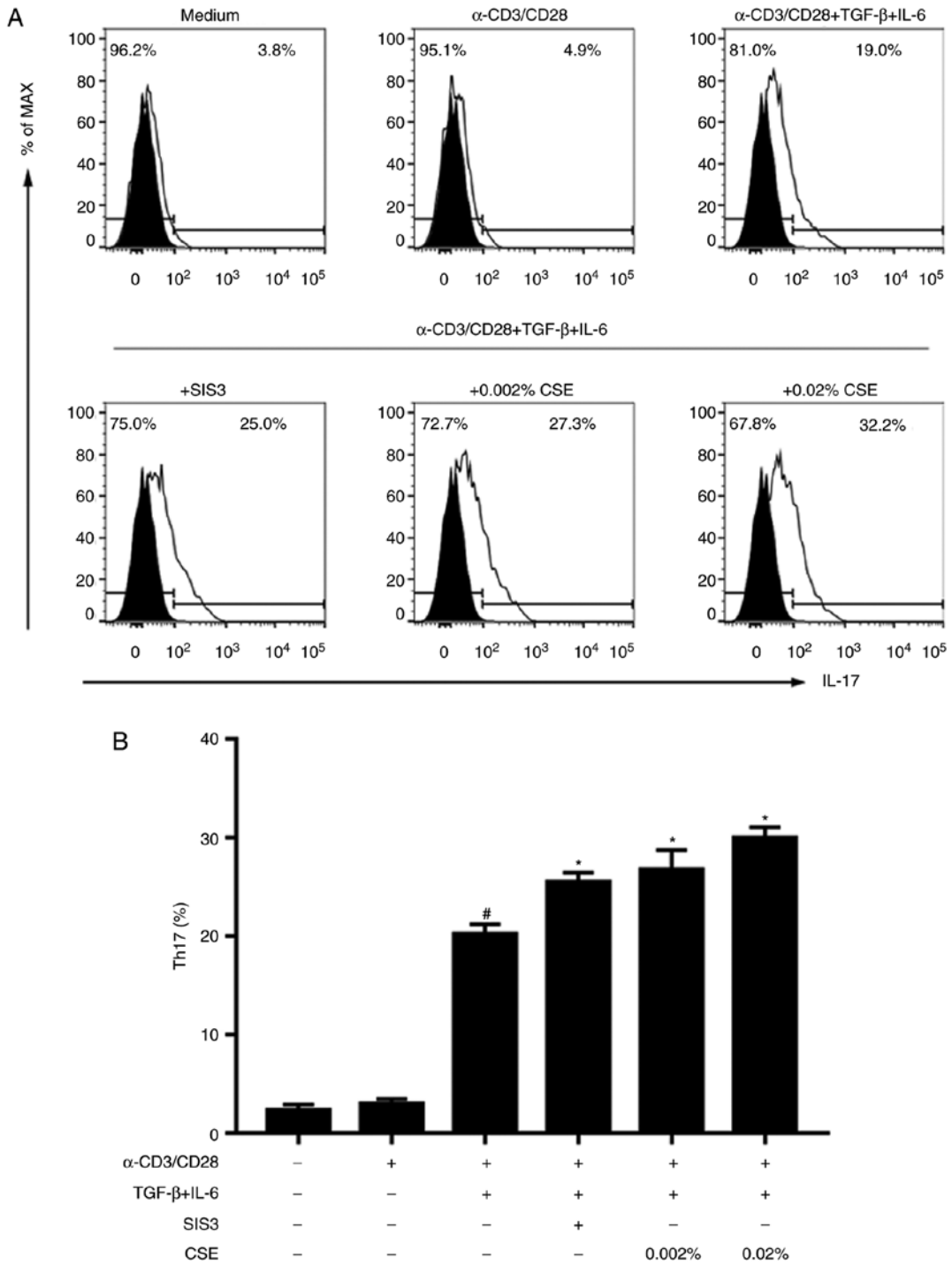

Figure 3. Effects of CSE on Th17 cell differentiation. (A and B) Naive CD4+ T cells isolated from peripheral blood were cultured in complete medium and stimulated with plate-bound α-CD3 and α-CD28 monoclonal antibodies under the indicated conditions for 5 days. (A) Th17 cell counts were determined by flow cytometry, and representative histograms gated on lymphocytes are presented. (B) Summary data of Th17 cells in each condition from (A) Data are presented as the mean ± standard error of the mean (n=4), and are representative of three independent experiments; #P<0.05 vs. Untreated or α-CD3/28; *P<0.05 vs. α-CD3/28 + TGF-β1 + IL-6. CSE, cigarette smoke extract; IL, interleukin; TGF-β1, transforming growth factor β1; Th17, IL-17-producing T cells; SIS3, a Smad3-specific inhibitor.

significantly increased BAMBI expression compared with untreated and TGF-β-treated cells (Fig. 6). In addition, the endogenous active TGF-β produced by the TCR-activated cells was necessary for sustained expression of BAMBI, since neutralization of TGF-β1 with anti-TGF-β antibody significantly reduced BAMBI induction in TCR-stimulated cells (Fig. 6). Furthermore, it was observed that the addition of exogenous TGF-β1 further enhanced TCR-induced BAMBI expression compared with TCR-induction alone.

The potential effects of CSE on BAMBI expression were also investigated. Under stimulation by anti-CD3/28 antibodies in the presence of TGF-β1, CSE treatment induced a significant increase of BAMBI expression at 0.02% concentration (Fig. 6).

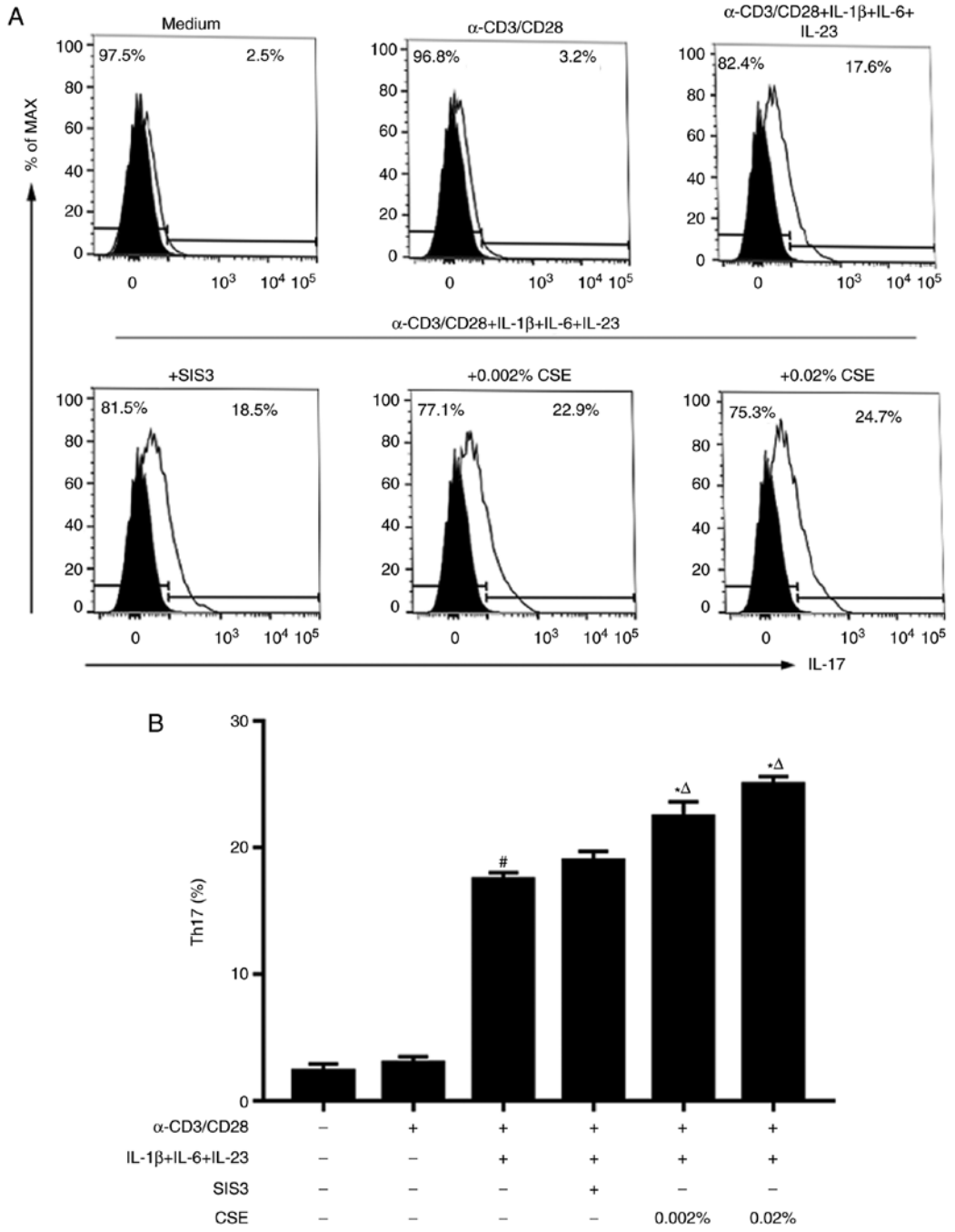

Figure 4. Effects of cigarette smoke extract (CSE) on Th17 cell differentiation. (A and B) Naive CD4+ T cells isolated from peripheral blood were cultured in complete medium and stimulated with plate-bound α-CD3 and α-CD28 monoclonal antibodies under the indicated conditions for 5 days. (A) Th17 cell counts were determined by flow cytometry, and representative histograms gated on lymphocytes are presented. (B) Summary data of Th17 cells in each condition from (A). Data are presented as the mean ± standard error of the mean (n=4), and are representative of three independent experiments; #P<0.05 vs. Untreated or α-CD3/28; *P<0.05 vs. α-CD3/28 + IL-1β/IL-6/IL-23; ΔP<0.05 vs. α-CD3/28 + IL-1β/IL-6/IL-23 + SIS3. CSE, cigarette smoke extract; IL, interleukin; Th17, IL-17-producing T cells; SIS3, a Smad3-specific inhibitor.

Discussion

Although previous studies have demonstrated key roles for tobacco smoke in immunity and inflammation, the cytokine milieu as well as genetic susceptibility should also be taken into account during analyses of effects of cigarette smoke (8). A previous study indicated that CSE treatment alone does not induce differentiation of naive CD4+ T cells (15). Under specific differentiation conditions, however, CSE substantially interfered with Treg development and concomitantly promoted the differentiation of Th17 cells. Results from the present study demonstrated that cigarette smoke-induced dysfunction in pro/anti-inflammation may be associated with Smad-mediated canonical TGF-β signalling pathway and functionally expressed BAMBI levels. BAMBI functions as a molecular switch to regulate the balance between the development of Tregs and Th17 cells (5). These data may aid in guiding future clinical strategies for maintaining immune tolerance and for the treatment of smoking-induced immune disorders.

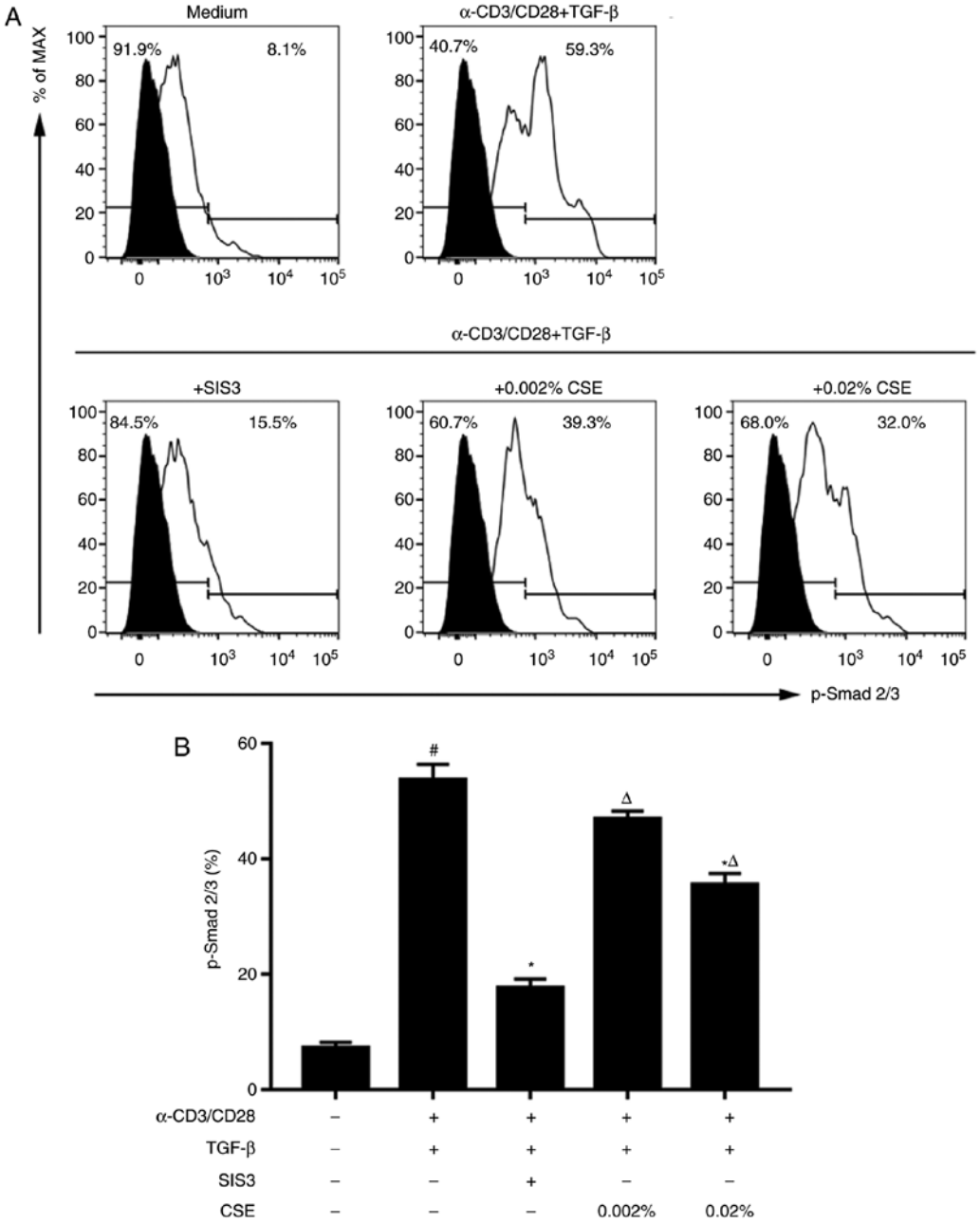

Figure 5. Effects of CSE on Smad2/Smad3 phosphorylation. (A and B) Naive CD4+ T cells isolated from peripheral blood were cultured in complete medium and stimulated with plate-bound α-CD3 and α-CD28 monoclonal antibodies under the indicated conditions for 5 days. (A) Phosphorylation levels of Smad2/Smad3 were determined by flow cytometry, and representative histograms gated on CD4+ T cells are shown. (B) Summary data of Smad2/Smad3 phosphorylation in each condition from (A). Data are presented as the mean ± standard error of the mean (n=4), and are representative of three independent experiments; #P<0.05 vs. Untreated control; *P<0.05 vs. α-CD3/28 + TGF-β1. ΔP<0.05 vs. α-CD3/28 + TGF-β1 + SIS3. CSE, cigarette smoke extract; p, phosphorylated; TGF-β1, transforming growth factor β1; SIS3, a Smad3-specific inhibitor.

Although considerable progress has been made in understanding the mechanisms by which tobacco smoking exerts immunomodulatory functions in inflammation and autoimmunity (8), these mechanisms are still not fully understood. A balance of Tregs and Th17 cells is crucial for the treatment of smoking-induced pathological conditions. To determine the potential mechanisms for immune response following cigarette smoke exposure, the present study examined the role of CSE in freshly isolated naive CD4+ T cells from healthy donors. In line with a previous study (15), *in vitro* CSE treatment alone did not induce the development and differentiation of naive CD4+ T cells, which indicated that the development of Tregs and Th17 cells may be regulated by the cytokine milieu in addition to cigarette smoke alone.

To better understand the induction process of Treg development, the expression of CD25 and FOXP3 in CD4+ T cells induced by specific-Treg differentiation conditions, treatment with TGF-β1 were analysed. This induction was blocked by a specific Smad3 inhibitor, SIS3. Notably, under Treg-polarizing conditions, the higher concentration of CSE remarkably inhibited Treg differentiation. In keeping with the observed trend in Treg generation, CD25 induction was also inhibited by SIS3

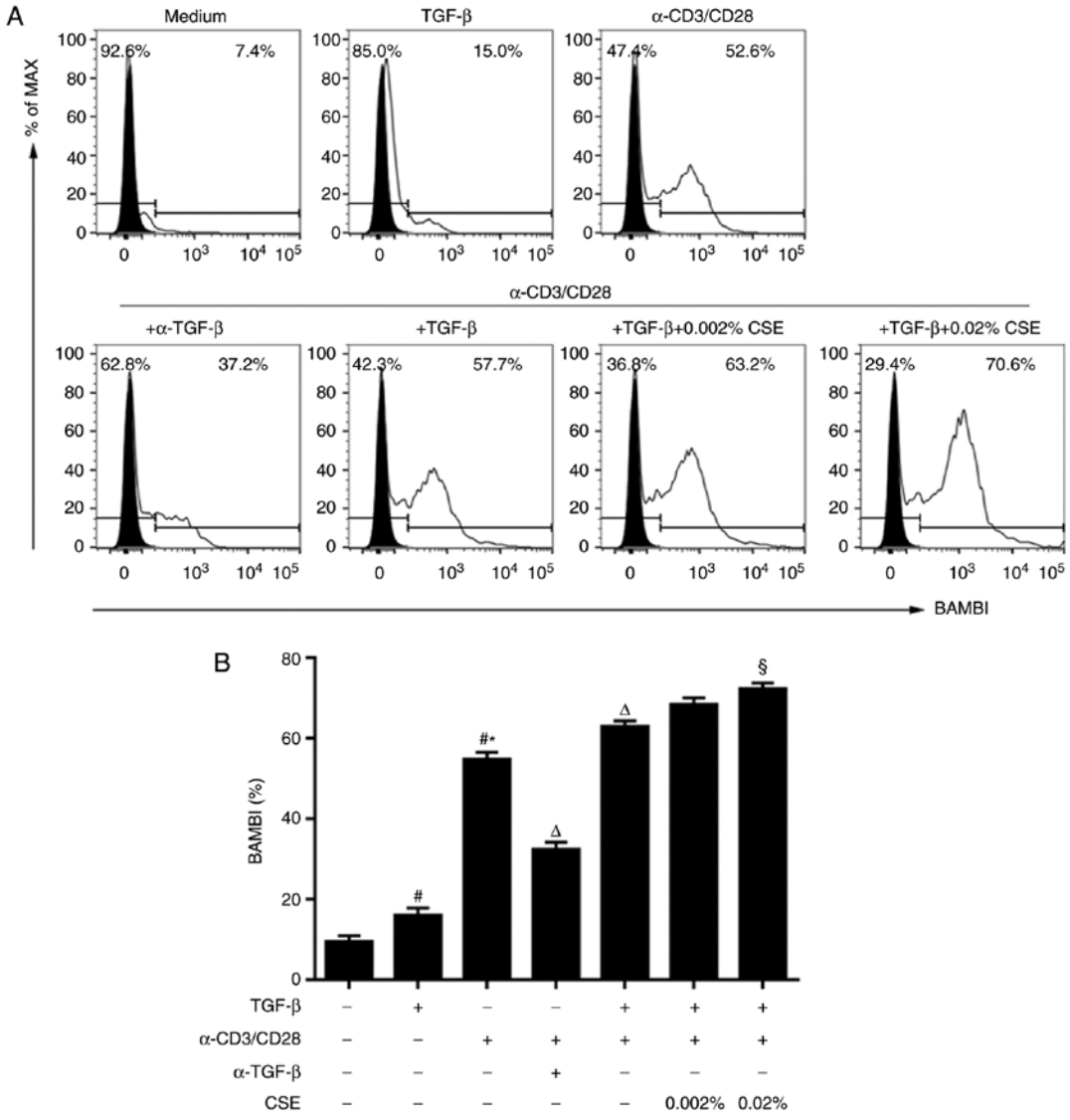

Figure 6. BAMBI expression increases during activation and CSE exposure. (A and B) Naive CD4+ T cells isolated from peripheral blood were cultured in complete medium and stimulated with plate-bound α-CD3 and α-CD28 monoclonal antibodies under the indicated conditions for 5 days. (A) Expression levels of BAMBI were determined by flow cytometry, and representative histograms gated on CD4+ T cells are provided. (B) Summary data of BAMBI expression in each condition from (A). Data are presented as the mean ± standard error of the mean (n=4), and are representative of three independent experiments; #P<0.05 vs. Untreated control; *P<0.05 vs. TGF-β1; ΔP<0.05 vs. α-CD3/28; §P<0.05 vs. α-CD3/28 + TGF-β1. BAMBI, BMP and activin membrane-bound inhibitor; CSE, cigarette smoke extract; TGF-β1, transforming growth factor β1.

and by CSE during Treg cell differentiation. In this regard, it has been demonstrated previously that TGF-β1 collaborates with TCR signalling to upregulate CD25 expression and that CD25 gene regulatory region contains a Smad3 binding site that is required for TGF-β1-mediated upregulation (16). Thus, the effects of CSE on Treg differentiation depend not only on the CSE concentration, but also on the differentiation milieu and activation status.

Th17 cells were also successfully induced using two different *in vitro* Th17 cell differentiation protocols: One used TGF-β1 and IL-6, and the other used a combination of IL-1β, IL-6 and IL-23. Notably, low levels of endogenous TGF-β1 are still indispensable for the second Th17 cell-polarizing condition (17). Smad3 inhibition by SIS3 can promote Th17 cell differentiation in the presence of exogenous TGF-β1 (the first protocol) but not endogenous TGF-β1 (the second protocol). However, CSE induced Th17 cell generation under both Th17 cell-polarizing conditions, and induced stronger Th17 expression compared with SIS3 treatment in the second protocol, which suggested that cigarette smoke may affect Th17 cells by unknown mechanisms (such as by reactive oxygen species) in addition to Smad signalling. Thus, although CSE itself does not induce the development of naive T cells, under Th subtype-specific stimulating conditions CD4+ T cells had a decreased capacity to differentiate into Tregs, and a corresponding increased capacity to differentiate into Th17 cells upon CSE treatment.

A previous study reported that phosphorylation status induces opposing roles of Smad2/Smad3 as cofactors of signal transducer and activator of transcription 3 in Th17 differentiation (18); however, the molecular mechanisms by which context regulates phosphorylation status remain unclear. Flow cytometric analysis suggested that Smad2/Smad3 is phosphorylated following TCR stimulation with exogenous TGF-β1.

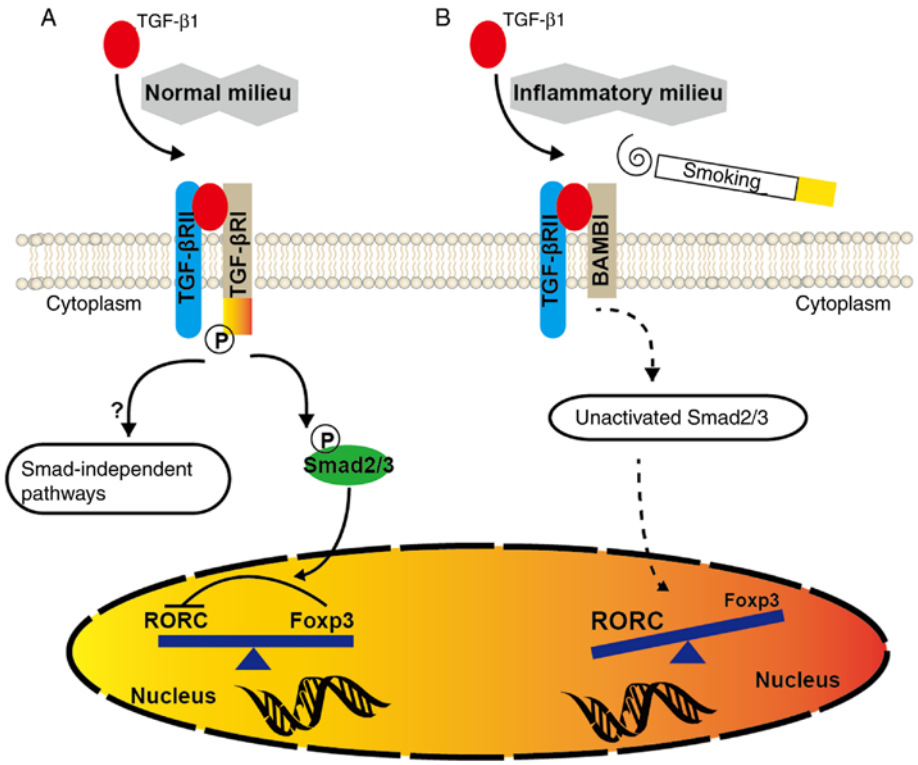

Figure 7. Schematic representation of the TGF-β/BAMBI pathway. (A) In the presence of TGF-β1, TCR-activated CD4+ T cells express both RORC and FOXP3. Enough Tregs keep effector T cells in check since RORC (a Th17-specific transcription factor) activity is antagonized by FOXP3. (B) However, upon inflammatory insult or CSE exposure, BAMBI overexpression by the activated immune system in susceptible individuals will suppress the generation of TGF-β-induced Tregs and evoke pro-inflammatory responses dominated by Th17 cells. BAMBI, BMP and activin membrane-bound inhibitor; FOXP3, forkhead box P3; TCR, T cell receptor; TGF-β, transforming growth factor β; RORC, retinoic acid-related orphan receptor C.

The results also demonstrated that higher concentrations of CSE were able to partially inhibit TCR- and CD28-induced Smad2/Smad3 phosphorylation. However, CSE did not completely inhibit p-Smad2/Smad3 to the level of non-activated cells, which may partially be due to TCR-stimulated cells producing biologically active TGF-β1 that was responsible for Smad2/Smad3 phosphorylation (19). The effects of CSE on p-Smad2/Smad3 in T cells were inconsistent with previous studies in human bronchial epithelial cells (20) and foetal lung fibroblasts (21), which reported that exposure to CSE increased Smad3 phosphorylation. This discrepancy may be due to differences of CSE doses and cell types.

Although a recent study defined the cytokine TGF-β1 to serve a crucial role in governing whether $CD4^+$ T cells differentiate into tolerogenic Tregs or pro-inflammatory Th17 cells (22), the molecular mechanisms underlying the mutually exclusive differentiation of the two lineages remain incompletely understood. Using a BAMBI-knockout mouse model of collagen-induced arthritis, BAMBI was identified as part of a rheostat-like mechanism for the control of TGF-β availability and signalling strength (5). However, the response of human T cells to polyclonal mitogens is more complicated compared with that of mouse T cells (23). For instance, human $CD4^+$ cells activated without exogenous TGF-β1 will still express FOXP3 through endogenous TGF-β1 activation by reactive oxygen species (19). In this regard, although previous studies have demonstrated that BAMBI expression may be involved in human lung inflammation associated with COPD and bacterial infection (7), the present study is the first, to the best of our knowledge, to report the *in vitro* expression and role of BAMBI in human immune cells.

BAMBI is a 260-amino-acid-long transmembrane glycoprotein that is structurally related to TGF-βRI, but lacking the intracellular kinase domain (6). Although BAMBI expression may be upregulated by TGF-β1 (24,25), bone morphogenetic protein (BMP)-4 (26) and Wnt/β-catenin (27) in various cell types, these effects have not been observed in human preadipocytes (28), which indicated that the modulation of BAMBI expression is cell type-dependent. Consistent with previous reports using C57BL/6 mouse T cells (5), increased expression of BAMBI was observed in activated $CD4^+$ T cells from human peripheral blood. Endogenous TGF-β1 produced by TCR-activated T cells may serve a crucial role in BAMBI induction in human $CD4^+CD25^-$ T cells, and the addition of exogenous TGF-β1 further enhanced this effect. Taken together, these data demonstrated that endogenous and/or exogenous TGF-β1 may serve as a link in BAMBI induction in naive human $CD4^+$ T cells following TCR stimulation. Additionally, we have provided the first evidence that CSE needs to cooperate with TGF-β1 to provoke induction of BAMBI expression. In summary, the effects of CSE on BAMBI induction and expression depend not only on cell activation status but also on local inflammatory stimuli, such as endogenous/exogenous TGF-β1 and the concentration of CSE.

Although BAMBI has been reported to negatively regulate TGF-β-family signalling mainly through Smad-dependent pathways in various cells lines (29-31), it serves a positive

role in triggering Smad2/Smad3 phosphorylation in human preadipocytes (28), which suggested cell type-specific differences. Given its high expression in activated $CD4^+$ T cells, the present study hypothesized that BAMBI also serves a crucial haemostatic role by controlling TGF-β signalling in human T cells. The results demonstrated that CSE caused a enhancement of BAMBI expression under stimulation by anti-CD3/28 antibodies and exogenous TGF-β1, which may result in a concomitant inhibition of p-Smad2/Smad3. Additionally, BAMBI upregulation by CSE may lead to the suppression of Treg differentiation and the promotion of Th17 generation in response to TGF-β1 stimulation, partially via Smad-dependent pathways. Thus, as in mouse cells, BAMBI may function as a crucial regulator of Th17/Treg plasticity through TGF-β/Smad-dependent pathways in human $CD4^+$ T cells. It was proposed that at the steady state or in the absence of any inflammatory insult, TGF-β1 produced in the immune system will facilitate the generation of Tregs, which keep effector T cells in check (Fig. 7). However, upon inflammation or CSE exposure, BAMBI overexpression by the activated immune system in susceptible individuals may suppress the generation of TGF-β-induced Tregs and evoke pro-inflammatory responses dominated by Th17 cells.

BAMBI is an inhibitor or regulator of TGF-β superfamily members, including BMP and activin, in addition to TGF-β (6). The present study focussed solely on TGF-β, as activin-A or BMP-2/4 seem to have only synergistic effects on endogenous and/or exogenous TGF-β-induced Treg generation (32,33). However, the present study has several limitations that warrant mention; the focus was primarily on TGF-superfamily signalling without considering the Wnt/β-catenin pathway, although BAMBI also functions as a positive regulator of Wnt signalling (28,34), which further negatively modulates Treg function (35). In addition, further work is needed to demonstrate the supplemental role of non-Smad TGF-β signals by mitogen-activated protein kinases, including c-Jun N-terminal kinase, p38 and, in particular, extracellular signal-regulated kinase (ERK) (36), as previous studies have demonstrated that BAMBI is able to facilitate ERK1/2 phosphorylation (37) and ERK inactivation mediates the TGF-β1-induced expression of FOXP3 (38). Furthermore, functional studies such as BAMBI knockdown and/or overexpression in human T cells *in vitro* are warranted to clarify the mechanism by which CSE exerts its effects.

In conclusion, results from the present study confirmed that BAMBI may be able to regulate the balance between protective Tregs and pathogenic Th17 cells through a rheostat-like mechanism in the human immune system. CSE, together with TGF-β1, was demonstrated to effect BAMBI expression and reduce Smad3 phosphorylation, which may act as a master regulator of Th17 induction and Treg suppression. These results may be beneficial in improving our understanding of the reciprocal regulation of these two cell lineages, and may aid in the development of better approaches for the treatment of smoking-associated inflammation or other T cell-mediated autoimmune diseases.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HJL and GC contributed to data analysis and interpretation. LC and MZ contributed to cell isolation and culture. XZX contributed to the design of the study. XZX and XNT were involved in volunteer recruitment. HJL, GC, ZJM and SWS wrote the manuscript and critically revised the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, and informed written consent was obtained from each subject.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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