Original Paper

Crocetin Activates Foxp3 Through TIPE2 in Asthma-Associated Treg Cells

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Key Words

OVA • Asthma • Crocetin • Regulatory T cells (Treg) • Foxp3 • Tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 (TIPE2)

Abstract

Background/Aims: Regulatory T cells (Treg) are critical regulators of asthma. Crocetin is isolated from Chinese herb saffron and is a natural carotenoid dicarboxylic acid with anti-inflammatory potential. However, the effects of Crocetin on asthma as well as the underlying mechanisms have not been studied. **Methods:** We used Crocetin to treat mice with established ovalbumin (OVA)-induced asthma. We purified CD4+CD25+ Treg cells by flow cytometry and analyzed the levels of two immunoregulatory proteins Foxp3 and tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 (TIPE2) in Treg cells. We depleted either Foxp3 or TIPE2 in mouse lung through lentivirus-mediated delivery of shRNA, and analyzed their effects on severity of asthma and Treg cells after Crocetin treatment. **Results:** Crocetin treatment significantly reduced the severity of an ovalbumin (OVA)-induced asthma in mice. Moreover, Crocetin significantly increased the levels of TIPE2 and Foxp3 in Treg cells and the number of Treg cells. Depletion of Foxp3 abolished the increased in Treg cells, and the effects of Crocetin on the severity of asthma, without affecting TIPE2 levels in Treg cells. On the other hand, depletion of TIPE2 abolished both the increased in Treg cells and the effects of Crocetin on the severity of asthma, through suppressing Foxp3. **Conclusion:** Crocetin may activate Foxp3 through TIPE2 in asthma-associated Treg cells to mitigate the severity of asthma.

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Introduction

Typical pathological events in Asthma include bronchial inflammation, airway hyper-responsiveness and airflow obstruction. Allergic asthma makes up most asthma cases, J. Ding and J. Su contributed equally to this work.

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characterized by activation of type-2 T helper cells, IgE production, eosinophilia, and an inappropriate T cell response to environmental allergens [1-4].

Regulatory T cells (Treg) are T cells that suppress potentially harmful immune responses, and have been shown to suppress inflammatory responses and airway hyper-responsiveness to maintain peripheral immune tolerance [5-7]. Treg has been defined by surface markers of combined CD4 and CD25 and also characterized by expression of a member of the forkhead box transcription factor, Foxp3 [7-9]. Foxp3 is crucial for naive T cell differentiation towards the Treg phenotype and its expansion [7-14]. However, the molecular bases underlying the activation of Treg through Foxp3 are not completely understood.

Previous studies have shown that herb-drug could improve the therapeutic effects and reduce side effects during cancer treatment. Saffron is a Chinese traditional herb, and Crocetin is the major constituent of saffron, which has recently found to have multiple pharmacological effects including anticancer [15-17]. However, the effects of Crocetin on asthma as well as the underlying mechanisms have not been studied.

Tumor necrosis factor (TNF)-alpha-induced protein 8-like 2 (TIPE2) was first identified as a novel immune negative molecule that regulates the adaptive and innate immunity [18, 19]. TIPE2 was later found to inhibit inducible nitric oxide synthase (iNOS) and thus generation of nitric oxide, resulting in suppression of inflammation [20-27]. The dysregulation of TIPE2 has also been found to mediate diverse immunological diseases [28-32]. However, whether TIPE2 may be involved in the pathogenesis of asthma and regulation of Treg has not been reported.

In the current study, we studied the effects of Crocetin treatment on the severity of an ovalbumin (OVA)-induced asthma in mice, and in this model, we studied the relationship between TIPE2 and Foxp3 in Treg cells.

Materials and Methods

Mouse handling

All mouse experiments were approved by the IACUC of Shanghai Pulmonary Hospital. Only 10-week-old male C57BL/6 mice (Jackson lab, Bar Harbor, ME, USA) were used for *in vivo* experiments. Mice were housed in a specific pathogen-free environment.

Ovalbumin (OVA)-induced allergic asthma model and Crocetin treatment

Male C57BL/6 mice of 10 weeks of age were sensitized with an intraperitoneal injection of 50 µg OVA (OVA, grade V; Sigma-Aldrich, St. Louis, MO, USA) with 2 mg aluminum hydroxide gel (Alum; Sigma-Aldrich) once per week for 3 times (week 0-2). Then the mice were challenged with 50 µg OVA by intranasal administration under light anesthesia every other day for another 7 weeks (week 3-9). Control mice received PBS. After another week, the mice were examined, sampled and analyzed (week 10). Crocetin (C₂₀H₂₄O₄, molecular weight, 328.4, MP Biomedicals, Santa Ana, CA, USA) in 100 µl dimethyl sulfoxide (DMSO) was given by intranasal administration after OVA challenge daily at a dose of 100 µmol/l till the end of the experiment (week 9-10).

Airway hyper-responsiveness

Airway hyper-responsiveness (AHR) was measured by restrained invasive plethysmography 1 day after the last intranasal OVA challenge. Mice were anesthetized, after which a small incision was made to expose the trachea, and a cannula was inserted to connect to an inline nebulizer and ventilator. Mice were then challenged with aerosolized PBS followed by increasing doses of methacholine (Sigma-Aldrich). Airway resistance and dynamic compliance (C_{dyn}) were determined by analysis of pressure and flow waveforms.

Bronchoalveolar lavage, lung digestion and isolation of Treg

Mice were euthanized by pentobarbitone overdose after AHR examination. Bronchoalveolar lavage fluid (BALF) was obtained by instilling three washes of 0.4ml PBS with 0.1% BSA. BALF was centrifuged at 470g for 5 min, and cells were enumerated and labeled for further analysis. For lung digestion, lungs were

perfused with PBS, after which 0.8 ml 300 U/ml collagenase type I (Sigma-Aldrich), and 50 U/ml DNase I (Roche, Nutley, NJ, USA) in RPMI 1640 was injected into the trachea. Lungs were then removed, minced into small pieces, and digested at 37°C for 30 min. Lung pieces were disrupted with a syringe plunger, filtered through nylon mesh, and centrifuged. The cell pellet was resuspended in RBC lysis buffer, washed, enumerated, and labeled for flow cytometric analysis. Treg was analyzed and purified by fluorescence-activated cell sorting (FACS) with specific fluorescence-conjugated antibodies (CD4 and CD25, all from Becton-Dickinson Biosciences, San Jose, CA, USA) on a FACSaria cell sorter (Becton-Dickinson Biosciences). Data were analyzed using Flowjo software (Flowjo LLC, Ashland, OR, USA).

Lentivirus production and in vitro and in vivo transduction

We used a pcDNA3.1-CAG-GFP plasmid as backbones (all from Clontech, Mountain View, CA, USA). The sequence for shTIPE2 is 5'-CCG TGT AAA CCA CTC AAC TCA TCTT-3', and the sequence for shFoxp3 is 5'-GCA CAT TCC CAG AGT TCCT-3'. The plasmids and target shRNA (or control scrambled sequence-scr) were digested with XhoI and BamHI and subcloned with a 2A into a pcDNA3.1-CAG-luciferase, resulting in a construct named pCAG-shTIPE2-2A-GFP or pCAG-shFoxp3-2A-GFP. The small 2A peptide sequences, when cloned between genes, allow for efficient, stoichiometric production of discrete protein products within a single vector through a novel "cleavage" event within the 2A peptide sequence. Sequencing was performed to confirm the correct orientation of the new plasmid. To generate lentiviral particles, HEK293T cells were seeded in a 100 mm dish at 50,000 cells/cm² and co-transfected with 10 µg of target plasmids and 5 µg each of packaging plasmids (REV, pMDL and VSV-G) using Lipofectamine-2000 (Invitrogen). The supernatant containing lentiviral particles was collected 48 hours after transfection and filtered through a 0.45 µm syringe filter. The viruses were purified using CsCl density centrifugation and then titered by a quantitative densitometric dot-blot assay. For cell transduction *in vitro*, Treg were seeded in 100mm plates at 15,000 cells/cm² one day prior to lentiviral infection. The lentiviral particles were added along with 10 µg/ml polybrene (Sigma-Aldrich) to the cell culture at a multiplicity of infection (MOI) of 100 for 48 hours. Then the cells were washed twice with complete media and the transduction efficiency (> 90%) was evaluated by GFP. For *in vivo* transduction, the 10⁶ virus was injected via tail vein.

RT-qPCR

Total RNA was extracted from purified Treg by FACS or mouse lung, using RNeasy (Qiagen, Hilden, Germany), and cDNA synthesis was performed by reverse transcription. Quantitative PCR (RT-qPCR) was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed using 2^{-ΔΔCt} method for quantification of the relative mRNA expression levels. Values of genes were first normalized against β-actin, and then compared to experimental controls.

Statistics

Statistical analysis was performed with the unpaired two-tailed Student t test (for comparison between two groups), one-way ANOVA with the Tukey posttest (for comparison between three or more groups), or repeated-measures ANOVA with the Dunnett posttest (for AHR dose-response curves), using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA). Data were represented as mean ± SD and were considered significant if p < 0.05.

Results

Crocetin attenuates OVA-induced hallmarks of the asthma

We used OVA-induced mouse asthma model in our study. In this model, mice were first sensitized to alum-adsorbed OVA for 2 weeks, and then exposed to repeated airway provocation for 7 weeks to develop AHR. Crocetin was given after OVA challenge for one week, and then the mice were analyzed (Fig. 1A). The establishment of asthma model was demonstrated by a dose-dependent increase in lung resistance (RI) and decrease in Cdyn in response to a cholinergic stimulus (methacholine), which were significantly attenuated by Crocetin treatment (Fig. 1B-C). Another hallmark feature of OVA-induced allergic asthma

Fig. 1. Crocetin attenuates OVA-induced hallmarks of the asthma. (A) Experimental schematic for OVA sensitization, inhalation challenge, and Crocetin treatment. (B-C) RI (B) and Cdyn (C) in response to increasing doses of methacholine. (D) Percentage of leukocytes in BALF. * $p < 0.05$, $N = 10$. Statistics: one-way ANOVA with a Bonferroni Correction. CRO: Crocetin. CTL: Control.

Fig. 2. Crocetin increases Treg cells in OVA-treated mouse lung. (A-B) Treg cells were purified from the lung digests of OVA/Crocetin-treated mice based on CD4 and CD25 positivity by flow cytometry, shown by representative flow charts (A), and by quantification (B). (C) Foxp3 and TIPE2 levels in Treg cells by RT-qPCR. * $p < 0.05$. NS: non-significant, $N = 10$. Statistics: one-way ANOVA with a Bonferroni Correction. CRO: Crocetin. CTL: Control.

-eosinophilic accumulation in the pulmonary airways- was also evaluated. We found that the influx of inflammatory eosinophils increased to about 60% of total BALF cells by OVA-treatment, which was also significantly attenuated by Crocetin treatment (Fig. 1D). These data suggest that Crocetin attenuates OVA-induced hallmarks of the asthma in mice.

Fig. 3. Depletion of Foxp3 abolishes the effects of Crocetin on OVA-asthma without affecting TIPE2. We generated a lentivirus carrying shFoxp3 or a scrambled sequence control (scr). (A-B) These viruses were first used to transduce primary mouse CD4⁺CD25⁺ Treg *in vitro*. RT-qPCR for Foxp3 (A) and TIPE2 (B) were performed. (C) Experimental schematic for OVA sensitization, inhalation challenge, Crocetin treatment, and viral treatment. (D-E) Treg cells were purified from the lung digests based on CD4 and CD25 positivity by flow cytometry,

shown by representative flow charts (D), and by quantification (E). (F-G) RI (F) and Cdyn (G) in response to increasing doses of methacholine. *p < 0.05. NS: non-significant, N = 10. Statistics: one-way ANOVA with a Bonferroni Correction. CRO: Crocetin. CTL: Control.

Crocetin increases Treg cells in OVA-treated mouse lung

Then we aimed to find out the molecular mechanisms underlying the therapeutic effects of Crocetin. We hypothesized that Treg cells may be regulated by Crocetin, since Treg cells are potent inflammation suppressor in asthma. We used CD4 and CD25 to purify Treg from the lung digests of the mice by flow cytometry (Fig. 2A). We found that OVA itself did not significantly increase Treg, but we detected a significant increase in Treg cell number in the Crocetin-treated mouse lung (Fig. 2B). Interestingly, we found that the levels of both Foxp3 and TIPE2 significantly increased in CD4⁺CD25⁺ Treg cells from OVA+Crocetin treated mouse lung, compared to control or OVA-only-treated mice (Fig. 2C). Thus, Crocetin increases Treg in OVA-treated mouse lung, and the levels of Foxp3 and TIPE2 in Treg.

Depletion of Foxp3 abolishes the effects of Crocetin on OVA-asthma without affecting TIPE2

In order to examine the role of Foxp3 and TIPE2 in Crocetin-treated OVA-asthma, we generated a lentivirus carrying shFoxp3 or a scrambled sequence control (scr). These viruses were first used to transduce primary mouse CD4⁺CD25⁺ Treg *in vitro*. First, the knock-down of Foxp3 in Treg by shFoxp3 was confirmed by RT-qPCR (Fig. 3A). We found that depletion of Foxp3 in Treg did not alter TIPE2 levels (Fig. 3B). Then, these viruses were given to mice at 9 week, immediately before Crocetin treatment (Fig. 3C). We found that depletion of Foxp3 by lentivirus abolished the increases in Treg by Crocetin (Fig. 3D-E), resulting in abolishment of the effects of Crocetin on RI (Fig. 3F), and Cdyn (Fig. 3G). These data suggest that depletion of Foxp3 abolishes the effects of Crocetin on OVA-asthma and Treg, without affecting TIPE2.

Fig. 4. Depletion of TIPE2 abolishes the effects of Crocetin on OVA-asthma via Foxp3. We generated a lentivirus carrying shTIPE2 or a scrambled sequence control (scr). (A-B) These viruses were first used to transduce primary mouse CD4+CD25+ Treg *in vitro*. RT-qPCR for TIPE2 (A) and Foxp3 (B) were performed. (C) Experimental schematic for OVA sensitization, inhalation challenge, Crocetin treatment, and viral treatment. (D-E) Treg cells were purified from the lung digests based on CD4 and CD25 positivity by flow cytometry, shown by representative flow charts (D), and by quantification (E). (F-G) RI (F) and Cdyn (G) in response to increasing doses of methacholine. *p < 0.05, N = 10. Statistics: one-way ANOVA with a Bonferroni Correction. CRO: Crocetin. CTL: Control.

flow charts (D), and by quantification (E). (F-G) RI (F) and Cdyn (G) in response to increasing doses of methacholine. *p < 0.05, N = 10. Statistics: one-way ANOVA with a Bonferroni Correction. CRO: Crocetin. CTL: Control.

Depletion of TIPE2 abolishes the effects of Crocetin on OVA-asthma via Foxp3

Next, we generated a lentivirus carrying shTIPE2 or a scrambled sequence control (scr). These viruses were first used to transduce primary mouse CD4+CD25+ Treg *in vitro*. First, the knock-down of TIPE2 in Treg by shTIPE2 was confirmed by RT-qPCR (Fig. 4A). We found that depletion of TIPE2 in Treg significantly decreased Foxp3 levels (Fig. 4B). Then, these viruses were given to mice at 9 week, immediately before Crocetin treatment (Fig. 4C). We found that depletion of TIPE2 by lentivirus abolished the increases in Treg by Crocetin (Fig. 4D-E), resulting in abolishment of the effects of Crocetin on RI (Fig. 4F), and Cdyn (Fig. 4G). These data suggest that depletion of TIPE2 abolishes the effects of Crocetin on OVA-asthma and Treg, possibly through Foxp3. Together, our data suggest that Crocetin may increase Foxp3 through TIPE2 to activate Treg to suppress the severity of asthma (Fig. 5).

Fig. 5. A schematic model. Crocetin may increase Foxp3 through TIPE2 to activate Treg to suppress the severity of asthma.

Discussion

Crocetin is a sort of carotenoids that have been reported of a lot of biomedical properties including anticancer [33, 34]. Nevertheless, its regulation on inflammatory responses has not been extensively studied. Especially, a role of Crocetin in asthma treatment is not acknowledged. Here, we sought to determine the role of Crocetin in asthma control, as well as the critical cellular and molecular signaling that mediate the activation of Treg cells by Crocetin to alleviate allergic asthma.

TIPE2 is a recently detected molecular with anti-inflammatory potential. TIPE2 is an essential negative regulator of inflammation and immune homeostasis. TIPE2-deficient mice suffer from chronic inflammatory diseases and TIPE2-deficient T cells and macrophages produce significantly increased levels of inflammatory cytokines. The inflammatory disease in TIPE2-deficient mice shares many similarities with those that have Foxp3 deficiency [35]. Thus, a regulatory relationship between TIPE2 and Foxp3, although never examined, deserves exploration.

We used a well-established mouse allergic asthma model [23], and purified CD4+CD25+ Treg. First, we showed that Crocetin attenuated OVA-induced hallmarks of the asthma. Next, we found that the number of Treg was increased by Crocetin. Since Foxp3 is a regulator of Treg activation and amplification, we examined the levels of Foxp3 in Treg, and found that Foxp3 was significantly increased by Crocetin. Moreover, we also examined the levels of TIPE2 in Treg, and found that it similarly upregulated after Crocetin treatment. Hence, we thought that both TIPE2 and Foxp3 may be involved in the effects of Crocetin on Treg. To prove it, we knocked out either TIPE2 or Foxp3 in Treg, and found that the Foxp3 levels were regulated by TIPE2, but not vice versa. Thus, it seemed that TIPE2 was upstream of Foxp3 and this pathway was activated upon Crocetin treatment. Furthermore, the block of this pathway at either TIPE2 or Foxp3 level completely abolished the activation of Treg by Crocetin.

Together, our data suggest that Crocetin may increase Foxp3 through TIPE2 to activate Treg to suppress the severity of asthma, and reveal a previously unrecognized cross-talk between TIPE2 and Foxp3 in the regulation of Treg to affect the severity of asthma, specifically after Crocetin treatment. Thus, our studies may shed light on TIPE2 as a novel therapeutic target for treating allergic asthma.

Disclosure Statement

The authors have declared that no competing interests exist.

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