Epigallocatechin-3-gallate (EGCG) Suppresses the Trafficking of Lymphocytes to Epidermal Melanocytes via Inhibition of JAK2: Its Implication for Vitiligo Treatment

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Vitiligo is an inflammatory skin disorder in which activated T cells play an important role in its onset and progression. Epigallocatechin-3-gallate (EGCG), the major chemical constituent of green tea, exhibits remarkable anti-oxidative and anti-inflammatory properties. EGCG administration has been confirmed to decrease the risk of vitiligo; however, the underlying mechanism is undetermined. In this study, we proved that EGCG directly inhibited the kinase activity of Janus kinase 2 (JAK2). In primary cultured human melanocytes, EGCG pre-treatment attenuated interferon (IFN)-γ-induced phosphorylation of JAK2 and its downstream signal transducer and activator of transcription (STAT)1 and STAT3 in a dose-dependent manner. We further examined the chemotactant expression in melanocytes and demonstrated that EGCG significantly inhibited IFN-γ-induced expression of intracellular adhesion molecule (ICAM)-1, CXCL10, and monocyte chemotactic protein (MCP)-1 in human melanocytes. In addition, EGCG reduced the protein levels of the corresponding receptors including CD11a, CXCR3, and CCR2 in human T lymphocytes. As a consequence, adhesion of human T cells to melanocytes induced by IFN-γ was effectively suppressed by EGCG. Taken together, our results provided new evidence for the effectiveness of EGCG in vitiligo treatment and supported JAK2 as a molecular target for vitiligo medicine development.

Key words vitiligo; epigallocatechin-3-gallate; melanocyte; Janus kinase 2; chemotactant

Epigallocatechin-3-gallate (EGCG), a major catechin in green tea, is considered beneficial for human health, especially as an anti-oxidative agent. In addition, EGCG was confirmed to directly inhibit protein kinases by working as an ATP analog. Previously, we reported the therapeutic effect of EGCG in vitiligo induced by monobenzone in mice. Our results suggested that it could contribute to the suppression of CD8+ T cell migration and the inflammatory cytokine expression. However, the mechanism regarding the EGCG regulation of immune responses in vitiligo is still unclear.

The aim of study is to elucidate the inhibitory effects of EGCG on inflammatory signaling pathways and to identify the target of EGCG inhibition.

RESULTS AND DISCUSSION

EGCG Inhibited Janus Kinase (JAK2) Activity in Vitro

The JAKs are a family of four non-receptor tyrosine kinases including JAK1, JAK2, JAK3 and tyrosine kinase (TYK)2. Recruitment of stimuli to cell surface receptors activates JAKs which, in turn, phosphorylates and stimulates latent cytoplasmic signal transducer and activator of transcription (STAT) proteins to an active dimer, leading to nuclear translocation and DNA binding and subsequently modulating gene transcription. The JAK/STAT signal pathway controls a number of important biological responses, including immune functions, cellular growth, cellular differentiation and hematopoiesis. Dysregulation of JAK signaling has been identified in multiple autoimmune disorders. Due to their ability to selectively modulate immune function, targeted JAK inhibitors are attractive candidates for some skin diseases such as psoriasis and atopic dermatitis.

Our computer simulation result suggested that EGCG may bind to JAK2 protein (Fig. 1C). To determine the effect of EGCG binding on JAK2 kinase activity, EGCG and its derivative, peracyetylated EGCG (AcEGCG, Fig. 1A) were tested.

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JAK2 inhibition (2126 nM) (Fig. 1B). These results indicated that EGCG could bind to JAK2 and directly inhibited its kinase activity.

**EGCG Inhibited IFN-γ-Induced Phosphorylation of JAK2, STAT1 and STAT3 in Human Melanocytes** Various cytokines elicit their biological effects through the activation of JAK-STAT pathways. To characterize the inhibition of JAK2 activity in melanocytes, we examined the cellular phosphorylation levels of JAK1, JAK2, STAT1 and STAT3 in human melanocytes after treatment with interferon (IFN)-γ and EGCG. It was shown that while IFN-γ increased the phosphorylation of JAK2, STAT1 and STAT3, EGCG suppressed the upregulation of protein phosphorylation in response to IFN-γ in a dose-dependent manner (Fig. 2). In terms of JAK1, its phosphorylation level wasn’t changed upon IFN-γ stimulation, but slightly increased after EGCG treatment. It is then suggested that EGCG specifically targets JAK2 in JAK-STAT pathway.

Various studies have supported a critical role of IFN-γ in vitiligo development. In vitiligo patients and animal models, IFN-γ level is significantly elevated in lesional skin. IFN-γ signaling regulates the expression of cytokine as well as adhesion molecules facilitating the lymphocyte migration. So that, local IFN-γ release promotes further recruitment of melanocyte-specific CD8+ T cells into the vitiligo lesion. Moreover, our previous study proved that IFN-γ directly caused apoptosis and senescence in melanocytes, and induced melanocyte to secret autoimmune enhancers including interleukin (IL)-6 and heat shock protein 70 (HSP70). Since IFN-γ mainly engages JAK-STAT pathways to achieve its biological effects, blocking IFN-γ signaling via the inhibition of JAK2 activity could be a promising therapeutic approach to prevent vitiligo development.

**EGCG Inhibited the Production of Chemoattractants** Increased level of chemoattractants including intracellular adhesion molecule (ICAM)-1 and CXCL10 has been found in the skin lesions of vitiligo patients. To further address the effects of EGCG on the expression of chemoattractants, we measured the expression levels of ICAM-1 and CXCL10, as well as monocyte chemotactic protein (MCP)-1 which is
also implicated in the pathogenesis of certain autoimmune diseases.\textsuperscript{25,26} The results showed that IFN-\(\gamma\) stimulation significantly increased the mRNA expression and protein secretion of ICAM-1, CXCL10, and MCP-1. Compared with the IFN-\(\gamma\) stimulation group, EGCG pretreatment significantly suppressed the IFN-\(\gamma\)-induced production of ICAM-1, CXCL10, and MCP-1 in a dose-dependent manner. As a positive control, the JAK2-specific kinase inhibitor AG490 also inhibited the induction of ICAM-1, CXCL10 and MCP-1 by IFN-\(\gamma\) (Fig. 3).

Chemoattractants are involved in the attraction and activation of leukocytes during the inflammatory process. ICAM-1, which can be expressed on the cellular membrane or secreted into extracellular matrix, is a ligand for leukocyte adhesion protein LFA-1. ICAM-1 promotes the leukocyte trans-endothelial migration as well as the localization of leukocyte to target cells.\textsuperscript{21,23} MCP-1 was also suggested to mediate lymphocyte migration.\textsuperscript{27} CXCL10 engagement is responsible for the stimulation of monocytes and the migration of NK and T-lymphocytes. CXCL10 was elevated in both vitiligo patient skin and serum. Due to the critical roles of these molecules in the skin homing of leukocytes, it is reasonable to presume them as targets for vitiligo treatment. In fact, application of CXCL10-neutralizing antibody has been proven to remarkably decrease the expression of CXCR3 and CCR2 in vitiligo.\textsuperscript{32}

It is notable that the expression of T cell chemoattractants is also controlled by other cytokines. For example, IL-6 could create a MCP-1-gradient, and increase the expression of ICAM-1, functional to inflammatory cells chemotaxis.\textsuperscript{28–31} As we have mentioned above, many cytokines elicit their biological effects through the activation of JAK-STAT pathways including IL-6.\textsuperscript{28} JAK2 inhibition by EGCG treatment simultaneously blocks the signaling from various cytokines and maximizes the effect on the blockage of chemoattractant expression. On the other side, the inhibition of chemoattractant expression by EGCG was more potent than that by AG490 treatment, suggesting that EGCG may work via other mechanism besides targeting JAK2 to achieve that effect.

EGCG Inhibited the Production of Chemoattractant Receptors in T Lymphocytes CXCR3 and CCR2 are the receptors to CXCL10 and MCP-1, respectively. CD11a is one of the molecules that form LFA-1 which is the receptor of ICAM-1. Levels of CXCR3 and CD11a were elevated on autoreactive lymphocytes in both human vitiligo patients and vitiligo models. In addition, CXCR3 was demonstrated to be essential for autoreactive T cell accumulation in the skin and subsequent depigmentation in a mouse model, implicating this chemokine pathway as functionally required for vitiligo.\textsuperscript{21,32}

We then evaluated the effect of EGCG on the expression of CD11a, CXCR3, and CCR2 in activated human T cells. Western blot analysis revealed that EGCG treatment for 24h decreased the expression of CD11a, CXCR3, and CCR2 in Jurkat, a CD4\(^+\) T leukemia cell line, in a dose-dependent manner (Fig. 4A). In purified CD8\(^+\) T cells from peripheral blood mononuclear cell (PBMC), EGCG inhibited the expression of those receptors as well (Fig. 4B). Comparatively, AG490 also inhibited the expression of CD11a, CXCR3, and CCR2, suggesting that the inhibiting effect of EGCG on chemoattractant receptor induction was at least partially through the inhibition of JAK2 activity.

Suppression of T Lymphocyte Adhesion to Melanocytes by EGCG Treatment Given the importance of CXCL10/ CCR3 and ICAM-1/LFA-1 for lymphocyte migration and adhesion, we further examined the effects of EGCG on the adhesion of T lymphocytes to melanocytes. In our study, treatment of human melanocytes monolayers for 24h with 200U/mL IFN-\(\gamma\) resulted in a 5.7-fold (Jurkat cells, Fig. 5A) and 6.3-fold (CD8\(^+\) T cells, Fig. 5B) increase of T lymphocyte adhesion. In comparison, IFN-\(\gamma\)-induced T cell adhesion was strongly suppressed when both T cells and melanocytes were pre-treated with 40\(\mu\)M EGCG (Figs. 5A, B, the far right column). Furthermore, EGCG treatment of T cells or melanocytes alone also decreased the adhesion between T lymphocytes and melanocytes, which is consistent with the above results showing that the expression of chemoattractants and their receptors were all inhibited by EGCG.

Adhesion to target cells is the initial and necessary step for T lymphocytes to exert their functions. Adhesion molecules such as ICAM-1 and its receptors LFA-1 are crucial for immune synapse formation, antigen presentation\textsuperscript{33} and lymphocyte killing.\textsuperscript{34,35} It has been reported that adhesion defect in CD8\(^+\) T lymphocytes, which is caused by a combination of decreased cell surface levels of adhesion molecules, deficient LFA-1 Activation, and the failure to recruit essential adhesion receptors to the membrane contact site formed with cognate target cells, weakens their capacity of cytolysis.\textsuperscript{36} Therefore, EGCG-caused compromise of interaction between T lymphocytes and melanocytes will profoundly weaken the T lymphocyte activation and decrease the cytolysis of melanocytes by CD8\(^+\) CTL.

Collectively, EGCG inhibited the chemoattractant expression in melanocytes and the expression of the corresponding receptors in T lymphocytes via directly targeting JAK2 kinase, leading to the decrease of lymphocyte migration/
accumulation in vitiligo lesion as well as the weakening of T cell adhesion to melanocytes. The overall effects will be less activation of auto-reactive T lymphocytes and less destruction of melanocytes. Our study provides insights into the effects of EGCG on inflammation in vitiligo and elucidates JAK2 as its novel anti-inflammatory target. Further investigations should be carried out to further clarify the JAK2 as an independent anti-inflammatory drug target in vitiligo therapy.

MATERIALS AND METHODS

Reagents  EGCG, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), phorbol 12-myristate 13-acetate (PMA), ionomycin, human recombinant IFN-γ and IL-2 were obtained from Sigma (St. Louis, MO, U.S.A.). F12 medium, RPMI-1640 medium, penicillin, streptomycin, l-gultamine, and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Carlsbad, CA, U.S.A.). Ficoll–Paque was obtained from GE Healthcare (Little Chalfont, U.K.). Human CD8+ T Cell Isolation Kit was purchased from MiltenyiBiotec (Cologne, Germany). All antibodies for Western blotting were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.).

Computer Analysis of JAK2-EGCG Binding  The molecular docking was performed by using LigandFit module embedded in Discovery Studio 2.5.29. At first, the crystal structure of JAK2 was obtained from PDB bank (entry code: 4GMY), and then was removed water molecules and charged by CHARMm force field. The binding site was derived from the volume of co-crystal ligand. For generation of the ligands’ conformations, variable numbers of Monte Carlo simulations were employed. All the calculations during the docking steps were performed under the piecewise linear potential (PLP) energy grid. A short rigid body minimization was then performed and 50 preferable poses were saved according to their dock score. Based on the dock score and visual inspection, the most possible pose was selected for the further analysis.

In Vitro Kinase Assays  Inhibition of EGCG and peracetylated EGCG (AcEGCG) on JAK2 kinase activity was tested in Shanghai Chempartner Co., Ltd. (Shanghai, China). EGCG and AcEGCG were tested in duplicate for 10 concentrations. Samples were analyzed by Caliper mobility shift assay using LabChip 3000 (Caliper Life Sciences, MA, U.S.A.). Data was analyzed using HTS Well Analyzer Software from Caliper Life Sciences to determine IC₅₀.

Cell Isolation and Culture  Methods for the isolation and
cultivation of primary normal melanocytes were described previously. Briefly, melanocytes were isolated from human foreskin specimens obtained after circumcision surgery. Cells were cultured in Hu16 medium (F12 supplemented with 10% fetal bovine serum (FBS), 20 ng/mL basic fibroblast growth factor (bFGF) and 20 mg/mL isobutyl methylxanthine (IBMX) and cultured at 37°C in an incubator with 5% CO₂ and 95% humidity. The cells were used between passages 2 and 5. Melanocytes were seeded at a density of $1 \times 10^4$ cells per well into 96-well plates, or at a density of $1 \times 10^5$ cells per well into 24-well plates, or at a density of $3 \times 10^5$ cells per well into 6-well plates and incubated overnight before experiments.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll–Paque density centrifugation. CD8⁺ T cells were then separated from PBMC using Human CD8⁺ T Cell Isolation Kit according to the protocol from manufacturer. Both purified CD8⁺ T cells and the human T lymphoma cell line Jurkat cells were cultured in RPMI-1640 medium supplemented with 100 units/mL streptomycin, 100 units/mL penicillin, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM nonessential amino acids, and 10% FBS. CD8⁺ T cells or Jurkat cells were stimulated for 48 h with PMA (25 ng/mL) and Ionomycin (1 µM) in the presence of IFN-γ (200 U/mL) before each experiment.

RNA Isolation and Quantitative Polymerase Chain Reaction (PCR) Total RNA was extracted from melanocytes with SV total RNA purification kit (Promega, Shanghai, China). Reverse transcript reaction was performed using QuantiTect Reverse Transcription Kit (QiaGen, Germany). Real time PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen). The expression levels of each gene was normalized against β-actin using the comparative Ct method, and expressed as percentage of IFN-γ group, with the IFN-γ group as 1. Primer sequences were as follows: CXCL10: forward primer 5’-GTG GCA TTG CGA GCT TTG AAT TT-3’/reverse primer 5’-CTG GCT GTA GCA TTA TTA TCT CC-3’; ICAM-1: forward primer 5’-TGTAATGGGCTCGATGGATT-3’; MCP-1: forward primer 5’-TAT GGCAACGACTCTTCTT-3’; reverse primer 5’-CATTCACCGTCACTTG-3’; MCP-1: forward primer 5’-TGGGCCCAGCAGTA GTC GTA TTT GT-3’; reverse primer 5’-TTCTGATCTACTTGGTTCTTCG-3’; β-actin: forward primer 5’-ATAGCAACGCTGGATAGCAAGCT-3’, reverse primer 5’-CACCTTTCTCAAACTGCTCGTGC-3’.

Enzyme-Linked Immunosorbant Assay (ELISA) Melanocytes were plated into six-well plates and incubated in 37°C in a humidified incubator at an atmosphere containing 5% CO₂. After being washed with warm phosphate buffered saline (PBS), melanocytes were pre-treated with increasing concentrations of EGCG (0, 10, 20, and 40 µM) and 25 µM AG-490 in Hu16 medium for 1 h, followed by IFN-γ (200 U/mL) stimulation for 48 h. The cell supernatants were then collected, and the expression levels intercellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1), and chemokines CXCL10 were analyzed by ELISA kits (R&D
Systems, Minneapolis, U.S.A.) according to the manufacturer’s instructions.

**Western Blot Analysis** Cells were collected and lysed in RIPA buffer (10 mM NaPO₄, pH 7.4, 300 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 1% deoxycholic acid and 2 mM EDTA) with protease inhibitors (Pierce, U.S.A.). Aliquots of 20–30 µg of protein from each sample (treated as indicated in the legends) were separated by 8–10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.). Aliquots of 20–30 µg of protein from each sample (treated as indicated in the legends) were separated by 8–10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.). After blocked with 5% BSA (Beyotime, China) for 1 h, membrane was incubated with specified antibodies overnight at 4°C followed by incubation with fluorescent dye-labeled secondary antibodies for 1 h at room temperature in the dark. The protein immuno-complex was visualized by an Odyssey Infrared Imaging System (LI-COR, U.S.A.). Quantification of band intensity was performed using the quantification software of the digital imaging system (ChemiDoc™ XRS+, Bio-Rad, U.S.A.).

**Adhesion Assay** Melanocytes were grown to confluence in six-well plates and incubated with or without IFN-γ (200 U/mL) for 24 h. Human T cells (Jurkat cells or purified CD8+ T cells) were labeled with 5 µM of DiO, a fluorescent dye, at 37°C, for 5 min, and subsequently washed twice by PBS. Labeled T cells (2×10⁶ cells/well) were then incubated with melanocytes in six-well plates at 37°C for 30 min. Non-adherent T cells were removed, and plates were gently washed twice with PBS. The numbers of adherent T cells were counted by four fields per 200× high-power field well using fluorescence microscope.

**Statistical Analysis** All experiments were performed at least three times in triplicate, with one representative experiment shown. All experimental data obtained from cultured cells were expressed as mean±standard deviation (S.D.). One-way ANOVA, followed by Student–Newman–Keuls multiple comparison tests, was used to analyze the statistical significance between groups. p values <0.05 were considered statistically significant.

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Conflict of Interest  The authors declare no conflict of interest.

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