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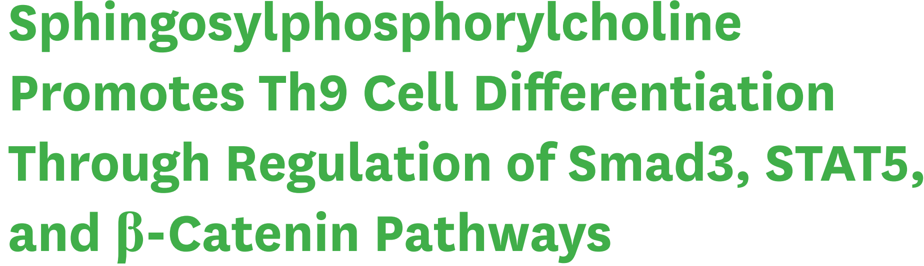
Original Article



Sphingosylphosphorylcholine Promotes Th9 Cell Differentiation Through Regulation of Smad3, STAT5, and β-Catenin Pathways

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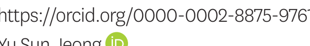

ABSTRACT

Sphingosylphosphorylcholine (SPC) is one of sphingomyelin-derived sphingolipids. SPC levels are increased in ascitic fluids of ovarian cancer patients and stratum corneum of atopic dermatitis (AD) patients. SPC has antitumor activity against several cancer cells by reducing proliferation and migration and increasing apoptosis *in vitro*. SPC can also cause scratching, potentially exacerbating symptoms of AD. However, the role of SPC in modulating immune responses, particularly in the differentiation of Th9 cells, which carry the most powerful antitumor activity among CD4$^+$ T cells, has yet to be investigated. In this study, we found that SPC is another inducer of Th9 cell differentiation by replicating TGF-β. SPC upregulated Smad3, STAT5, and β-catenin signaling pathways. Increased Smad3 and STAT5 signaling pathways by SPC promoted the differentiation of Th9 cells and increased β-catenin signaling pathway resulted in a less-exhausted, memory-like phenotype of Th9 cells. Increased Smad3, STAT5 and β-catenin signaling pathways by SPC were mediated by increased mitochondrial ROS. These results suggest that SPC is an important endogenous inducer of Th9 cell differentiation and may be one of the targets for treating Th9-related diseases, and that enhancing Th9 differentiation by SPC may be helpful in adoptive T cell therapy for cancer treatment.

Keywords: Sphingosylphosphorylcholine; Interleukin-9; Smad3 protein; STAT5 transcription factor; Beta-catenin; Reactive oxygen species

INTRODUCTION

During antigen stimulation, naïve CD4$^+$ T cells differentiate into various Th subsets (e.g., Th1, Th17, Th9) depending on the specific cytokine environment. In the presence of IL-4 and TGF-β, Th9 cells can be generated from naïve CD4$^+$ T cells *in vitro* (1-3). Th9 cells can also be generated by *in vitro* culture with activin A (a member of TGF-β superfamily), or IL-1β in the presence of IL-4 (4,5). In addition, type I interferon, IL-1β, IL-25, TSLP, and several TNF

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Abbreviations

Acvr2a, activin receptor type 2A; AD, atopic dermatitis; CAR, chimeric antigen receptor; GSEA, gene set enrichment analysis; IRF4, interferon regulatory factor 4; RT, room temperature; S1P, sphingosine-1-phosphate; SIS3, Smad3 inhibitor; SPC, sphingosylphosphorylcholine; T9 CAR-T, chimeric antigen receptor T cells expanded under Th9 polarizing conditions.

Conflict of Interest

Ji Cheol Kim and Yoe-Sik Bae have pending patents related to this discovery. The other authors have no competing interests to disclose.

Data Availability Statement

The datasets presented in this study can be found in online repositories. RNA sequencing data is available in the Gene Expression Omnibus (GEO) database accession number with GSE237940 for Th9 cells differentiated with SPC.

Author Contributions

Conceptualization: Kim JC, Lee M, Park B, Park JS, Zabel BA, Bae YS[1], Bae YS[2]; Data curation: Kim JC, Park JY; Formal analysis: Kim JC; Funding acquisition: Bae YS[2]; Investigation: Kim JC, Hu W, Lee M, Bae GH, Park JY, Lee SY, Jeong YS, Park B; Methodology: Hu W, Lee M, Bae GH, Park JY, Lee SY, Jeong YS, Park B; Project administration: Bae YS[2]; Resources: Park JS, Bae YS[2]; Supervision: Bae YS[1], Bae YS[2]; Visualization: Kim JC; Writing - original draft: Kim JC, Zabel BA, Bae YS[2]; Writing - review & editing: Kim JC, Zabel BA, Bae YS[1], Bae YS[2].

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family members, including TNF-α, OX40L, TL1A and GITRL, are known to enhance Th9 differentiation (5-14). Although several transcription factors such as Smad, PU.1, interferon regulatory factor 4 (IRF4), STAT5, STAT6, and forkhead box protein O1 are involved in Th9 differentiation, transcriptional regulation of Th9 differentiation has not been fully elucidated (15-18). Because IL-9 and IL-21 produced by Th9 are considered type 2 cytokines, Th9 cells have been associated with defense against certain parasites (2,19) and allergic inflammation (8,9). Th9 cells also have potent IL-9- and IL-21-dependent antitumor activity in a mouse melanoma model (7,20). Th9 cells have superior antitumor activity compared to other Th cells because of their less-exhausted phenotype, enhanced cytolysis, and hyperproliferative activities (21).

Sphingosylphosphorylcholine (SPC) is a naturally occurring bioactive sphingolipid produced from sphingomyelin by sphingomyelin deacylase (22). Increased SPC levels have been found in various diseases, such as Niemann Pick disease (23), metabolic syndrome (24), ovarian cancer (25), and atopic dermatitis (AD) (22,26). SPC levels are increased in the stratum corneum of AD patients due to increased activity of sphingomyelin deacylase, resulting in a decrease in ceramides that make up the skin barrier (22,26). SPC may also contribute to accelerated skin barrier breakdown because intradermal injection of SPC induces scratching behavior of mice (27,28). AD is a well-known type 2 inflammatory disease. Type 2 cytokines such as IL-4, IL-5 and IL-13 are increased in AD patients (29). Recently, it has been reported that IL-9 and IL-21 are increased in AD patients (30,31). Interestingly, there is an inverse association between AD and malignant melanoma (32,33). SPC shows an antitumor activity against multiple types of cancer by inhibiting tumor cell proliferation and migration and promoting apoptosis *in vitro* (34). However, the effect of SPC on Th9 differentiation and subsequent immunological roles in antitumor activity remains to be determined.

In this study, we found that SPC could promote Th9 cell differentiation that leads to a less-exhausted, memory-like phenotype. SPC mediates these effects by upregulating Smad3, STAT5 and β-catenin signaling pathways through increased mitochondrial ROS. Our data suggest that SPC is an important factor for driving Th9 cell differentiation and contributes to understanding and treating Th9-related diseases.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from Orient Bio (Seongnam, Korea). All experimental mice were housed in a clean animal facility of Sungkyunkwan University (Suwon, Korea). Six-to-eight-week old mice were used for experiments. All experiments complied with protocols (SKKUIACUC-2020-10-07-1, SKKUIACUC-2021-10-60-1, and SKKUIACUC-2022-12-50-1) approved by the Institutional Review Committee for Animal Care and Use at Sungkyunkwan University (Suwon, Korea).

Reagents

Anti-mouse CD3e (clone 145-2C11) and CD28 (clone 37.51) antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant mouse IL-6, IL-10, IL-12 and TGF-β1 also were purchased from Invitrogen. Recombinant mouse IL-2 and IL-4 were purchased from Peprotech (Rocky Hill, NJ, USA). Human IL-4 and TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA). Anti-mouse IL-2 (clone JES6-1A12) and anti-mouse TGF-β1, 2, 3 (clone 1D11.16.8)-neutralizing antibodies were purchased from Invitrogen. Anti-mouse IL-4

(clone 11B11) and anti-mouse IFN-γ (clone R4-6A2)-neutralizing antibodies were purchased from Bio X Cell (Lebanon, NH, USA). SPC (d18:1), sphingosine-1-phosphate (S1P, d18:1) and MitoTEMPO were purchased from Sigma-Aldrich (St. Louis, MO, USA). Activin receptor type 2A (Acvr2a) was purchased from R&D Systems. LF3 was purchased from Selleckchem (Houston, TX, USA).

In vitro Th cell differentiation

Naïve $CD4^+$ T cells were purified from spleens of mice with a Magnisort™ mouse CD4 naïve T cell enrichment kit (Invitrogen) according to the manufacturer's protocol. Culture medium was RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% antibiotics-antimycotic and 2-mercaptoethanol (50 μM, Sigma-Aldrich). Isolated naïve $CD4^+$ T cells were cultured in flat-bottom 96 well plates and stimulated with plate-bound anti-CD3e (1 μg/ml) and soluble anti-CD28 (1 μg/ml) together with different cytokine combinations for 3–5 days: IL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml) were added for Th1 cells; IL-4 (10 ng/ml) and anti-IFN-γ (10 μg/ml) were added for Th2 cells; IL-4 (10 ng/ml), TGF-β1 (2 ng/ml), and anti-IFN-γ (10 μg/ml) were added for Th9 cells. Th0 cells were cultured without cytokines. For Th17 differentiation, naïve $CD4^+$ T cells were cultured in round-bottom 96 well plates with soluble anti-CD3e and anti-CD28, IL-6 (20 ng/ml), and TGF-β1 (2 ng/ml) for 3–5 days. In some experiments, enzyme inhibitors, receptor antagonists or antibodies against cytokines were added at the beginning of T cell culture.

Human T cell culture

Peripheral blood samples were collected from healthy donors. Experiments with human peripheral blood were approved by Ajou University Hospital's Institutional Review Board for ethics (AJOUIRB-SMP-2018-431). Naïve $CD4^+$ T cells were isolated from peripheral blood mononuclear cells with a Magnisort™ human CD4 naïve T cell enrichment kit (Invitrogen) according to the manufacturer's protocol. Isolated cells were cultured in flat-bottom 96 well plates and stimulated with human T-activator CD3/CD28 Dynabeads (Invitrogen) together with different cytokine combinations for 5 days. For Th9 cells, human IL-4 (10 ng/ml), human TGF-β1 (2 ng/ml) and anti-IFN-γ (10 μg/ml) were added. Th0 cells were cultured without cytokines.

Flow cytometry

Flow cytometry analysis was performed following the protocol outlined in previous reports (35,36). Briefly, for intracellular cytokine staining, cultured cells were restimulated for 4 h with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of protein transport inhibitor cocktail (1:500). These cells were then stained with a fixable viability dye (1:1,000, Invitrogen) and blocked with anti-CD16/32 (clone 2.4G2, Bio X Cell) in FACS buffer (0.5% BSA in PBS). Cell surface markers were stained in FASC buffer for 30 min at 4°C. After surface staining, cells were fixed with IC fixation buffer (Invitrogen) for 1 h at room temperature (RT) or overnight at 4°C. For transcription factor detection, cells were fixed with Foxp3/Transcription Factor Fixation/Permeabilization buffer (Invitrogen) for 1 h at RT or overnight at 4°C after surface staining. Fixed cells were incubated with permeabilization buffer (Invitrogen) for 30 min at RT and then stained with indicated antibodies of cytokines or transcription factors for an additional 30 min. For intracellular phospho-protein detection, cells fixed with IC fixation buffer were incubated with methanol for 30 min at 4°C. Cells were then washed by FACS buffer and stained with indicated antibodies of proteins. Cells were analyzed with FACSCanto™ II (BD Bioscience, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo software. Antibodies used for flow cytometry were as follows: FITC-conjugated anti-CD3e (clone 145-2C11, Invitrogen), PE-conjugated anti-CTLA-4 (clone

UC10-4B9, Invitrogen), anti-IL-4 (clone 11B11, Invitrogen), anti-IL-17A (clone, eBio17B7, Invitrogen), anti-IFN-γ (clone XMG1.2, Invitrogen), anti-phospho-Smad2/3 (clone O72-670, BD Bioscience), anti-phospho-STAT5 (clone SRBCZX, Invitrogen), and anti-human IL-9 (clone MH9D1, Invitrogen), PerCP-Cy5.5-conjugated anti-CD4 (clone RM4-5, Invitrogen), APC-conjugated anti-LAG-3 (clone eBioC9B7W, Invitrogen) and anti-human IL-4 (clone 8D4-8, Invitrogen), and eFluor 660-conjugated anti-IL-9 (clone RM9A4, Invitrogen), anti-IRF4 (clone 3E4, Invitrogen) and anti-β-catenin (clone 15B8, Invitrogen), and anti-TCF1 (Cell Signaling Technology, Danvers, MA, USA), and Alexa Fluor™ 647-conjugated goat anti-Rabbit IgG (H+L) (Invitrogen).

ELISA

Cytokines in culture supernatant were measured with ELISA kits for mouse IL-2, IL-9, IL-10, TGF-β1 (Invitrogen), and activin A (R&D Systems) according to each manufacturer's protocol.

Real-time PCR

Total RNA was extracted from cultured T cells using TRIzol (Invitrogen) and then used for cDNA synthesis with a Maxime™ RT PreMix (Oligo dT primer; iNtRON Biotechnology, Seongnam, Korea) kit according to the manufacturer's protocol. Quantitative PCR was performed using a Rotor-Gene SYBR Green PCR kit (Qiagen, Hilden, Germany). Sequences of the primer used include *Il2*: forward, 5'-TCAGCAACTGTGGTGGACTT-3', reverse, 5'-GAGGGCTTGTTGAGATGATGCT-3', *Il9*: forward, 5'-AAGGATGATCCACCGTCAAA-3', reverse, 5'-AACAGTCCCTCCCTGTAGCA-3', *Il10*: forward, 5'-CAGAGCCACATGCTCCTAGA, reverse, 5'-GTCCAGCTGGTCCTTTGTTT-3', *Irf4*: forward, 5'-CTCTTCAAGGCTTGGGCATT-3', reverse, 5'-TGCTCCTTTTTTGGCTCCCT-3', *Tcf7*: forward, 5'-AGGTGGCATGCACTATCTCG-3', reverse, 5'-CCGCCTCTTCTTCTTTCCGT-3' and *Gapdh*: forward, 5'-AATGTGTCCGTCGTGGAT CT-3', reverse, 5'-CCACCACCCTGTTGCTGTA-3'. Data were analyzed using the delta-delta CT method with rotor-gene Q series software. Expression was normalized to the expression of mouse *Gapdh*.

RNA sequencing

Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without SPC for 3 days. Cells were then harvested for RNA extraction. Total RNA was extracted using TRIzol. Library construction was performed using NEBNext Ultra™ II Directional RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). RNA sequencing was then performed as paired-end 100 sequencing using Novaseq 6000 (Illumina, San Diego, CA, USA). RNA sequencing and data analysis were performed by Ebiogen (Seoul, Korea). Clustering heat map was created using MeV software (version 4.9). For a better understanding about signaling pathway or gene ontology, gene set enrichment analysis (GSEA) was performed.

Tumor model and adoptive transfer

B16-OVA melanoma cells were provided by Prof. Y. T. Im (Sungkyunkwan University, Suwon, Korea). Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics-antimycotic (Gibco, Waltham, MA, USA) at 37°C with 5% CO_2. Mice were injected intravenously with 3×10^5 B16-OVA cells. For adoptive transfer experiments, naïve $CD4^+$ T cells isolated from OT-II mice were cultured under Th9 polarizing condition with or without SPC. On day 4 after tumor inoculation, mice were injected intravenously with 2×10^6 OT-II Th9 or SPC-treated Th9 cells. At day 14 or 15 after tumor injection, mice were sacrificed for measuring metastatic lung foci.

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Statistical analysis

All statistical analyses were performed using GraphPad prism software. Results were subjected to Student's *t*-test or one-wasy ANOVA. All results are expressed as mean ± standard deviation. A p-value <0.05 was considered statistically significant.

RESULTS

SPC enhances Th9 cell differentiation *in vitro*

To investigate whether SPC affects Th9 cell differentiation, mouse naïve CD4⁺ T cells were cultured under Th9 polarizing condition with various concentrations of SPC for 3 days. SPC treatment increased the percentage of IL-9 producing CD4⁺ T cells in a concentration dependent manner (**Fig. 1A**). IL-9 secretion and mRNA expression were also increased by

Figure 1. SPC enhances Th9 cell differentiation. (A-E) Mouse naïve CD4⁺ T cells were cultured under Th9 polarizing condition with various concentration of SPC for 3 days. (A) Flow cytometry analysis of IL-9⁺CD4⁺ T cells. (B) IL-9 production was measured by ELISA in the culture medium. (C) Expression levels of *Il9* and (D) *Irf4* were measured by real-time quantitative PCR. (E) Flow cytometry analysis of IRF4. (F) Mouse naïve CD4⁺ T cells were cultured under Th9 polarizing condition with or without SPC for 5 days. Expression level of *Il9* was measured by quantitative PCR. (G) Human naïve CD4⁺ T cells were cultured under Th9 polarizing condition with or without SPC for 5 days. Flow cytometry analysis of IL-9⁺ CD4⁺ T cells. The results shown are representative of 3 independent experiments (A left and G left). Data are expressed as mean ± SD (n=3 for A right, B, E and G right, n=4 for C, D and F).
*p<0.05, ***p<0.001, and ****p<0.0001 (Student's t-test).

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SPC treatment (**Fig. 1B and C**). We also investigated the expression of IRF4, which plays a crucial role in Th9 generation (15,16), and found that mRNA and protein levels of IRF4 were increased by SPC treatment (**Fig. 1D and E**). To analyze time kinetics of *Il9* expression, we assessed *Il9* expression in Th9 cells with or without SPC for 5 days. In the absence of SPC, *Il9* expression peaked on day 2. It then declined continuously, disappearing completely on day 4 (**Fig. 1F**), which may be due to transient expression of IL-9 in Th9 cells both *in vitro* (37,38) and *in vivo* (19). However, in the presence of SPC, *Il9* expression peaked on day 3 and remained higher levels than in the vehicle group for 5 days (**Fig. 1F**). We also examined effect of SPC on Th9 generation in human $CD4^+$ T cells. SPC treatment elicited similar results, promoting IL-9 production under Th9 polarizing conditions (**Fig. 1G**). Although a SPC-specific receptor has not been clearly defined, there are several promiscuous cognate receptors for SPC such as S1P receptors, G protein-coupled receptor 68 and G2A (39). Among them, S1P receptors are common receptor of S1P and SPC (39). However, S1P treatment had no effect on Th9 generation (**Supplementary Fig. 1A**). Taken together, these results demonstrate that SPC can enhance Th9 cell differentiation *in vitro*.

SPC promotes IL-9 production by upregulating Smad3 signaling in a TGF-β independent manner

To investigate candidate targets of SPC, we analyzed bulk mRNAseq during Th9 differentiation and which gene sets were enriched among the experimental groups. Among the enriched signaling pathways with GSEA, TGF-β signaling gene set was significantly upregulated in SPC-treated Th9 cells compared to that in vehicle-treated Th9 cells (**Fig. 2A**). SPC treatment also enhanced phosphorylation of Smad2/3 under Th9 polarizing conditions (**Fig. 2B**).

Based on these results, we checked whether SPC affected the polarization of other types of Th cells. SPC treatment inhibited Th1 and Th17 cell differentiation (**Supplementary Fig. 1B and C**). However, SPC treatment did not induce IL-9 from $CD4^+$ T cells under Th1 or Th17 polarizing conditions (**Supplementary Fig. 1B and C**). Interestingly, in contrast to Th1 or Th17 polarizing conditions, under Th2 polarizing conditions SPC treatment not only induced IL-9 production, but also promoted IL-4 production (**Supplementary Fig. 1D**). Moreover, administration of SPC alone in the presence of IL-4 was enough to phosphorylate Smad2/3 in the absence of TGF-β (**Fig. 2C**). Th9 cell differentiation requires IL-4 and TGF-β (1-3). It has been reported that SPC can induce differentiation of human mesenchymal stem cells into smooth-muscle-like cells by inducing TGF-β production (40). We therefore asked if SPC induced TGF-β production but found that SPC treatment failed to induce TGF-β under Th2 polarizing conditions (**Fig. 2D**). TGF-β is produced in a latent form, and its activation can also induce TGF-β signaling regardless of its production (41). We examined whether SPC-induced IL-9 production was inhibited by treatment with TGF-β neutralizing antibody known to block TGF-β activity. Neutralizing antibody treatment failed to inhibit SPC-induced IL-9 production under Th2 polarizing conditions (**Fig. 2E**). To determine whether Smad2/3 signaling mediated IL-9 production in response to SPC, we tested effects of a TGF-β receptor kinase inhibitor (ALK4, 5 and 7 inhibitor, SB431542) and a Smad3 inhibitor (SIS3) on IL-9 production induced by SPC. SB431542 and SIS3 treatment dramatically inhibited SPC-induced IL-9 producing $CD4^+$ T cell populations under Th2 polarizing conditions (**Fig. 2F**). SPC-induced IL-9 secretion and mRNA expression were also significantly inhibited by SB431542 and SIS3 (**Fig. 2G and H**).

In addition to TGF-β, activin A, a TGF-β superfamily, can also induces IL-9 production (4). The level of activin A is significantly higher in activated Th2 cells than in Th1 cells (42). Because SPC enhanced Th2 differentiation (**Supplementary Fig. 1D**) and induced

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Figure 2. SPC enhances Smad3 signaling in a TGF-β independent manner. (A, B) Mouse naïve CD4⁺ T cells were cultured under Th9 polarizing condition with or without SPC. (A) GSEA of TGF-β signaling in SPC-treated Th9 cells compared with vehicle-treated Th9 cells. (B) Smad2/3 phosphorylation was measured by flowcytometry at 48 h. (C, D) Mouse naïve CD4⁺ T cells were cultured under Th2 polarizing condition with or without SPC. (C) Smad2/3 phosphorylation was measured by flowcytometry at 120 h. (D) Production of TGF-β was measured by ELISA in the cell culture medium. (E) Mouse naïve CD4⁺ T cells were cultured under Th2 polarizing condition with or without SPC in the presence of vehicle (D.W) or anti-TGF-β (10 μg/ml) for 5 days. IL-9 expression was detected by flow cytometry. (F-H) Mouse naïve CD4⁺ T cells were cultured under Th2 polarizing condition with or without SPC in the presence of vehicle (0.05% DMSO), SB431542 (10 μM) or SIS3 (3 μM) for 5 days. (F) Flow cytometry analysis of IL-9⁺ CD4⁺ T cells. (G) IL-9 production was measured by ELISA in the supernatant. (H) Expression level of *Il9* was determined by quantitative PCR. The results shown are representative of 3 independent experiments (E and F left). Data are expressed as mean ± SD (n=3 for B, C, D, F right and G, n=4 for H).
D.W, distilled water.
*p<0.05, ***p<0.001, and ****p<0.0001 (Student's t-test).

Smad2/3 phosphorylation (**Fig. 2C**), we checked whether SPC induced activin A production. Activin A production was also significantly increased by SPC treatment (**Supplementary Fig. 2A**). Recombinant Acvr2a treatment, which can neutralize activin A, slightly inhibited IL-9 production under Th2 polarizing conditions (**Supplementary Fig. 2B**). However, in the presence of TGF-β, neutralizing of activin A could not block SPC-mediated Th9 differentiation (**Supplementary Fig. 2C**). These results suggest that SPC regulates IL-9 production differently under Th2 and Th9 polarizing conditions. Collectively, our results demonstrate that SPC can promote IL-9 production by enhancing Smad3 signaling in a TGF-β independent manner.

SPC enhances Th9 cell differentiation through IL-2 production

IL-2 is known to enhance Th9 cell differentiation (1). Through GSEA, we found that SPC enhanced IL-2-STAT5 signaling pathway (**Fig. 3A**). To ascertain whether SPC could increase IL-2 signaling, we measured STAT5 phosphorylation in response to SPC. As expected, SPC increased phosphorylation of STAT5 (**Fig. 3B**). Next, we investigated whether SPC could promote IL-2 production. SPC treatment significantly increased the IL-2 producing $CD4^+$ T cell population (**Fig. 3C**). IL-2 secretion and mRNA expression were also enhanced by SPC treatment and maintained at high levels for 5 days (**Fig. 3D and E**). To investigate the role of IL-2 in Th9 generation by SPC, we examined effect of IL-2 neutralizing antibody on SPC-induced response. We found that IL-2 neutralizing antibody treatment completely inhibited SPC-enhanced Th9 cell differentiation (**Fig. 3F and G**).

It has been reported that IL-10 can impair the stability of Th9 cells by activating STAT3 (43). SPC treatment dramatically inhibited IL-10 production (**Supplementary Fig. 3A and B**). However, the addition of IL-10 did not inhibit the effect of SPC on Th9 generation (**Supplementary Fig.**

Figure 3. SPC enhances STAT5 signaling by promoting IL-2 production. (A-C) Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without SPC for 3 days. (A) GSEA of IL-2-STAT5 signaling pathway in SPC-treated Th9 cells compared with vehicle-treated Th9 cells. (B) STAT5 phosphorylation was measured by flow cytometry at 48 h. (C) Flow cytometry analysis of $IL\text{-}2^+$ $CD4^+$ T cells. (D, E) Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without SPC for 5 days. (D) Expression level of *Il2* was measured by quantitative PCR. (E) IL-2 production was measured by ELISA in the culture medium. (F, G) Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without SPC in the presence of vehicle (D.W) or anti-IL-2 (20 μg/ml) for 3 days. (F) Flow cytometry analysis of $IL\text{-}9^+$ $CD4^+$ T cells. (G) IL-9 production was measured by ELISA in the supernatant. The results shown are representative of 3 independent experiments (C left and F left). Data are expressed as mean ± SD (n=3 for B, C right, F right and G, n=4 for D and E).
D.W, distilled water.
$^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$ (Student's t-test).

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3C and D). Taken together, these results demonstrate that SPC can enhance differentiation of Th9 cells by promoting IL-2 production.

SPC downregulates expression of exhaustion markers and upregulates expression of memory T cell-related genes through β-catenin signaling pathway

It has been reported that Th9 cells are less-exhausted effector cells (21). With analysis of GSEA, we found that exhaustion associated gene sets were significantly downregulated in SPC-treated Th9 cells compared to those in vehicle-treated Th9 cells (**Fig. 4A**). Transcriptome analysis revealed that SPC-treated Th9 cells showed low expression levels of exhaustion marker genes (*Ctla4, Havr2, Lag3*) compared to vehicle-treated Th9 cells (**Fig. 4B**). Surface expression levels of CTLA4 and LAG3 were also reduced by SPC treatment (**Fig. 4C**).

Figure 4. SPC-treated Th9 cells have less-exhausted features by enhanced β-catenin signaling pathway and exhibit increased antitumor activity *in vivo*. (A-G) Mouse naïve CD4+ T cells were cultured under Th9 polarizing condition with or without SPC for 3 days. (A) GSEA of exhaustion associated gene sets in SPC-treated Th9 cells compared with vehicle-treated Th9 cells. (B) Heatmap of indicated gene expressions analyzed by RNA-seq. (C) Flow cytometry analysis of CTLA4 and LAG3. (D) GSEA of memory T cell associated gene sets in SPC-treated Th9 cells compared with vehicle-treated Th9 cells. (E) Expression level of *Tcf7* was measured by quantitative PCR. (F) Flow cytometry analysis of TCF1. (G) Flow cytometry analysis of β-catenin at 48 h. (H) Mouse naïve CD4+ T cells were cultured under Th9 polarizing condition with or without SPC in the presence of vehicle (0.05% DMSO) or LF3 (20 μM) for 3 days. Flow cytometry analysis of LAG3. (I) C57BL/6 mice were injected intravenously with 3×10^5 B16-OVA tumor cells. Mice were injected intravenously with PBS, OT-II Th9 cells or SPC-treated Th9 cells (2×10^6) 4 days after tumor cell injection. On day 15, tumor foci were measured. Data are expressed as mean ± SD (n=3 for C and F to H, n=4 for E). Data are pooled from 3 independent experiments with 5–6 mice per group (I).
*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 (Student's t-test for C, E-H, one-way ANOVA for I).

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In addition to exhaustion-related genes, we found that SPC treatment upregulated memory T cell associated gene sets (**Fig. 4D**) and increased *Il2* expression (**Fig. 3D**), which is also associated with memory cells (21, 44). TCF1 is a well-known transcription factor for memory T cell. It is associated with β-catenin signaling pathway (45). Wnt/β-catenin signaling can inhibit effector T cell differentiation and generate memory stem cells (45). SPC treatment significantly upregulated expression of *Tcf7* gene encoding TCF1 (**Fig. 4E**). TCF1 expression was also increased by SPC treatment (**Fig. 4F**). To identify the upstream pathway of TCF1, we checked β-catenin expression. β-catenin expression was also increased by SPC treatment (**Fig. 4G**). We next treated the cells with β-catenin inhibitor LF3 to determine whether the decreased expression of CTLA4 and LAG3 was due to β-catenin signaling. We found that LF3 restored the reduced expression of LAG3 (**Fig. 4H**). These data suggest that enhanced β-catenin signaling pathway by SPC makes Th9 cells show a less-exhausted phenotype.

To test their respective antitumor activity *in vivo*, we transferred Th9 cells or SPC-treated Th9 cells obtained from OT-II mice into C57BL/6 mice with established B16-OVA lung metastatic murine melanoma on day 4. Lung metastasis was analyzed on day 15 after tumor inoculation. Transfer of Th9 cells reduced the number of foci metastasizing to lungs compared to PBS control (**Fig. 4I**). Interestingly transfer of SPC-treated Th9 cells significantly decreased the number of lung metastatic foci compared to Th9 cells (**Fig. 4I**). These data suggest that enhanced β-catenin signaling pathway by SPC makes Th9 cells show a less-exhausted phenotype, which could increase the antitumor activity *in vivo*.

Enhanced Th9 cell differentiation by SPC is mitochondrial ROS-dependent

Mitochondrial ROS are essential for T cell activation and proliferation (46,47). Mitochondrial ROS are required for production of IL-2 and IL-4 (46,47). In addition to IL-2 regulation, mitochondrial ROS are also involved in the regulation of TGF-β and Wnt/β-catenin pathways (48,49). Because SPC treatment increased Smad3, STAT5 and β-catenin signaling pathways (**Figs. 2-4**), we investigated whether SPC treatment increased mitochondrial ROS generation. Results showed that SPC treatment significantly increased mitochondrial ROS generation (**Fig. 5A**). To determine the functional role of mitochondrial ROS by SPC in Th9 generation, we added MitoTEMPO, which is a mitochondrially targeted antioxidant, to Th9 polarizing conditions with or without SPC. MitoTEMPO treatment dramatically inhibited increases of β-catenin and phosphorylation of Smad2/3 and STAT5 induced by SPC (**Fig. 5B**). As a result, increased expression levels of IRF4 and TCF1 by SPC were also significantly inhibited by MitoTEMPO treatment (**Fig. 5C**). Next, we investigated whether MitoTEMPO treatment inhibited SPC-dependent Th9 cell differentiation and restored the reduced expression of LAG3 by SPC. As expected, increased production of IL-9 and IL-2 by SPC was inhibited by MitoTEMPO treatment (**Fig. 5D and E**). The reduced expression of LAG3 by SPC was also dramatically restored by MitoTEMPO treatment (**Fig. 5F**). Taken together, our data suggests that increased mitochondrial ROS production by SPC enhances Th9 differentiation.

DISCUSSION

Th9 cells are known to be generated by IL-4 and TGF-β (1-3). IL-9 production can be enhanced by additional cytokines such as type I interferon, IL-1β, IL-25, TSLP and several TNF family cytokines (TNF-α, OX40L, TL1A and GITRL) (6-14). Recently, it has been shown that activin A, a member of TGF-β superfamily, and IL-1β can replace TGF-β in Th9 cell differentiation (4,5). In the present study, we found that SPC not only enhanced Th9 cell differentiation

Figure 5. SPC-induced Th9 cell differentiation is mediated by mitochondrial ROS. (A) Mouse naïve CD4+ T cells were cultured under Th9 polarizing condition with or without SPC. Mitochondrial ROS was measured by flow cytometry at 1 h after vehicle (D.W) or SPC treatment. (B-F) Mouse naïve CD4+ T cells were cultured under Th9 polarizing condition with or without SPC in the presence of vehicle (D.W) or MitoTEMPO (200 μM) for 3 days. (B) Flow cytometry analysis of phosphor-Smad2/3, phosphor-STAT5 and β-catenin at 48–72 h. (C) Flow cytometry analysis of IRF4 and TCF1 (D) Flow cytometry analysis of IL-9+ CD4+ T cells. (E) IL-9 and IL-2 production were measured by ELISA in the culture medium. (F) Flow cytometry analysis of LAG3. Data are expressed as mean ± SD (n=3).
D.W, distilled water.
*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 (Student's t-test).

by promoting Smad3, STAT5, and β-catenin signaling pathways (**Figs. 2-4**), but also could replace TGF-β in Th9 cell differentiation (**Supplementary Fig. 1D**). We investigated whether the activation of these signaling molecules by SPC involves a shared upstream regulator, a common downstream target, or crosstalk. LF3, a β-catenin inhibitor, did not affect SPC-induced p-Smad2/3 signaling. However, the addition of anti-IL-2 antibodies blocked the elevation of p-Smad2/3 signaling induced by SPC, indicating that the increase in p-Smad2/3 signaling was mediated, at least in part, by p-STAT5 activation through the IL-2 pathway (**Supplementary Fig. 4A**). The SIS3 or LF3 had no effect on SPC-mediated increases in p-STAT5 signaling (**Supplementary Fig. 4B**). Anti-IL-2 or SIS3 did not inhibit SPC-induced upregulation of β-catenin levels (**Supplementary Fig. 4C**). The differentiation of Th9 cells is dependent on a network of interconnected signaling pathways. Our results demonstrate that inhibiting any one of these pathways can significantly reduce Th9 differentiation.

Using GSEA, we demonstrated that SPC enhanced TGF-β signaling pathway. Interestingly, SPC promoted phosphorylation of Smad2/3 in the absence of TGF-β. As a result, SPC

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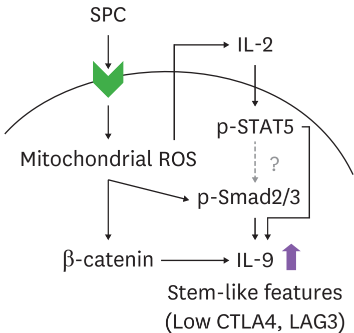

Figure 6. SPC enhances Th9 cell differentiation and function by upregulating Smad3, STAT5, and β-catenin signaling pathways, mediated by increased mitochondrial ROS.

induced IL-9 production by CD4⁺ T cells under Th2 polarizing conditions. This effect was dramatically blocked by SB431542 and SIS3 treatment (**Fig. 2F-H**). According to a previous report, human mesenchymal stem cells can be differentiated into smooth-muscle-like cells by SPC, which occurs through TGF-β production (40). However, SPC did not induce TGF-β production by CD4⁺ T cells (**Fig. 2D**). Furthermore, addition of TGF-β neutralizing antibody under Th2 polarizing condition did not affect SPC induced IL-9 production (**Fig. 2E**). These results indicate that SPC might induce Smad signaling by a TGF-β-independent mechanism. Previous studies have demonstrated that mitochondrial ROS play a critical role in TGF-β signaling. They are essential for normal TGF-β-mediated gene expression and can influence Smad2 signaling (48,50,51). Additionally, ROS can activate MAPK pathways and enhance NF-κB transcriptional activity, indicating a collaborative role between ROS and TGF-β receptors in downstream signaling (52). Given these mechanisms, we propose that SPC-induced mitochondrial ROS contributed to enhanced TGF-β signaling via Smad3, STAT5, and β-catenin, leading to Th9 cell differentiation (**Fig. 6**).

In cancer adoptive cell therapy, adoptive transfer T cells with a less-exhausted and memory-like phenotype leads to enhanced antitumor activity by enhanced persistence *in vivo* (45,53-56). Th9 cells also have a less-exhausted and hyperproliferative phenotype, which drives prolonged persistence *in vivo* (21). In addition to Th9 cells, Tc9 and chimeric antigen receptor (CAR) T cells expanded under Th9 polarizing conditions (T9 CAR-T) exhibit greater antitumor activity than Tc1 and T1 CAR-T respectively (57,58). This is attributed to Tc9 and T9 CAR-T cells having central memory, less-exhausted and hyperproliferative phenotypes like Th9 cells (57,58). Our results demonstrate that SPC treatment can upregulate memory-related gene sets and downregulate exhaustion markers in CD4⁺ T cells (**Fig. 4**). Moreover, SPC can enhance IL-2 production (**Fig. 3**), which is also associated with a memory-like phenotype and proliferation (21,44). Enhanced IL-2 production by SPC upregulated STAT5 signaling pathway and differentiation of Th9 cells. These phenotypes might contribute to enhancing the persistence of antitumor activity *in vivo*. In addition, SPC dramatically inhibited IL-10 production, which would further contribute to an enhanced antitumor activity.

SPC is upregulated in the stratum corneum of AD patients who have increased sphingomyelin deacylase activity (22,26). SPC regulates inflammatory processes in the epidermis through upregulation of intercellular adhesion molecule 1 and TNF-α (59) and increases scratching behavior (27,28), suggesting that it has a pathological effect on skin diseases. Recently, it has been reported that Th9 cells are increased in AD patients (30,31). From an immunological point of view, our study reveals that increased SPC might contribute to increased Th2 and Th9 cells

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in AD patients, suggesting that increased SPC might also have a deleterious role in allergic responses. In addition, there is an inverse correlation between AD and malignant melanoma (32,33). We suspect that Th9 cells increased by SPC might contribute to this phenomenon.

In summary, our results demonstrate that SPC can strongly promote Th9 cell differentiation, upregulate TCF1 expression and downregulate LAG3 expression by enhancing Smad3, STAT5 and β-catenin signaling pathways through increased mitochondrial ROS generation. Our findings suggest that SPC is a novel and potent inducer of Th9 cells and a target for treating allergic diseases. It could also be regarded as an effective additive for adoptive cell therapy in cancer therapy.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1

SPC induces IL-9 in the presence of IL-4. (A) Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without S1P (10 μM) for 3 days. (B-D) Mouse naïve $CD4^+$ T cells were cultured under indicated polarizing condition with or without SPC. (A) Flow cytometry analysis of $IL\text{-}9^+$ $CD4^+$ T cells and $IL\text{-}2^+$ $CD4^+$ T cells. (B) Flow cytometry analysis of $IL\text{-}9^+$ $CD4^+$ T cells and $IFN\text{-}\gamma^+$ $CD4^+$ T cells. (C) Flow cytometry analysis of $IL\text{-}9^+$ $CD4^+$ T cells and $IL\text{-}17A^+$ $CD4^+$ T cells. (D) Flow cytometry analysis of $IL\text{-}9^+$ $CD4^+$ T cells and $IL\text{-}4^+$ $CD4^+$ T cells. The results shown are representative of 3 independent experiments (A-C and D left). Data are expressed as mean ± SD (n=3 for C right).

Supplementary Figure 2

Enhanced IL-9 production by SPC is partially mediated by activin A under Th2 but not Th9 polarizing condition. (A, B) Mouse naïve $CD4^+$ T cells were cultured under Th2 polarizing condition with or without SPC in the presence of vehicle (D.W) or Acvr2a (2.5 μg/ml) for 5 days. (A) Activin A production was measured by ELISA in the culture medium. (B) Flow cytometry analysis of $IL\text{-}9^+$ $CD4^+$ T cells. (C) Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without SPC in the presence of vehicle (D.W) or Acvr2a (2.5 μg/ml) for 3 days. Flow cytometry analysis of $IL\text{-}9^+$ $CD4^+$ T cells. The results shown are representative of 3 independent experiments (B left and C). Data are expressed as mean ± SD (n=3 for A and B right).

Supplementary Figure 3

IL-10 is not involved in Th9 generation by SPC. (A, B) Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without SPC. (A) mRNA expression level of *Il10* was measured by quantitative PCR. (B) IL-10 production was measured by ELISA in the culture medium. (C, D) Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without SPC in the presence of vehicle (D.W) or IL-10 (10 μg/ml) for 3 days. (C) Flow cytometry analysis of $IL\text{-}9^+$ $CD4^+$ T cells. (D) IL-9 production was measured by ELISA in the culture medium. Data are expressed as mean ± SD (n=4 for A, n=3 for B to D).

Supplementary Figure 4

Independent regulation of Smad2/3, STAT5, and beta-catenin in response to SPC in $CD4^+$ T cells. (A, B) Mouse naïve $CD4^+$ T cells were cultured under Th9 polarizing condition with or without SPC in the presence of vehicle (0.05% DMSO), SB431542 (10 μM) or SIS3 (3 μM) or LF3 (20 μM) for 2 days. p-Smad2/3 phosphorylation (A), p-STAT5 phosphorylation (B), and the level of β-catenin (C) was measured by flow cytometry at 48 h. Data are expressed as mean ± SD (n=3 for A to C).

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