Fas signaling-mediated T_H9 cell differentiation favors bowel inflammation and antitumor functions

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Fas induces apoptosis in activated T cell to maintain immune homeostasis, but the effects of non-apoptotic Fas signaling on T cells remain unclear. Here we show that Fas promotes T_H9 cell differentiation by activating NF-κB via Ca\textsuperscript{2+}-dependent PKC-β activation. In addition, PKC-β also phosphorylates p38 to inactivate NFAT1 and reduce NFAT1-NF-κB synergy to promote the Fas-induced T_H9 transcription program. Fas ligation exacerbates inflammatory bowel disease by increasing T_H9 cell differentiation, and promotes antitumor activity in p38 inhibitor-treated T_H9 cells. Furthermore, low-dose p38 inhibitor suppresses tumor growth without inducing systemic adverse effects. In patients with tumor, relatively high T_H9 cell numbers are associated with good prognosis. Our study thus implicates Fas in CD4\textsuperscript{+} T cells as a target for inflammatory bowel disease therapy. Furthermore, simultaneous Fas ligation and low-dose p38 inhibition may be an effective approach for T_H9 cell induction and cancer therapy.
Receiving specific patterns of cytokine signaling during activation, CD4+ T cells will differentiate into different effector T cell subsets. Interleukin-12 (IL-12) promotes T helper type 1 (Th1) cell differentiation. IL-4 promotes Th2 cell differentiation. Transforming growth factor-β1 (TGF-β1) and IL-6 stimulate Th17 cell differentiation, while TGF-β1 alone drives T regulatory cell (Treg) differentiation. IL-9-producing Th9 cells were first identified by reprogramming Th2 cells with TGF-β1 or Foxp3+ Tregs with IL-4. Currently, it is generally accepted that IL-4 and TGF-β1 can induce Th9 cell differentiation. Th9 cells are reported to exacerbate autoimmune and allergic diseases.

They also exhibit robust antitumor activity superior to that of Th1 and Th17 cells. A recent publication demonstrated that one transfer of Th9 cells is sufficient to eradicate advanced tumors, indicating the promise of Th9 cells in adoptive cancer therapy. Therefore, it is necessary to further elucidate the underlying mechanism of Th9 cell differentiation.

Accumulating evidence has unveiled the role of Th9 cell differentiation at the molecular level. CD4+ T cells with interferon-regulatory factor 4 (IRF4), GATA-3, or STAT6 deficiency fail to develop into Th1, Th9, and Th2 cells. The cooperative signaling among the TGF-β1-activated kinase TAK1, STAT5, Notch, Smad, and RBP-Jκ participates in Th9 cell differentiation.OX40 and glucocorticoid-induced tumor necrosis factor receptor (TNF-R)-related protein, two members of the TNF-R superfamily, induce Th9 cells by activating the nuclear factor-κB (NF-κB) pathway. Moreover, DR3, another member of the TNF-R family, enhances Th9 cell differentiation through a STAT5-dependent mechanism. Whether other TNF-R family molecules regulate Th9 cell differentiation has yet to be explored.

Fas, a member of the TNF-R family, plays a critical role in programmed cell death. Activation-induced cell death (AICD), mediated by the interaction of Fas and Fas ligand (FasL), is important in maintaining T cell homeostasis. Fas is also necessary for T cell proliferation and activation. A recent publication demonstrated that Fas promotes Th17 cell differentiation and inhibits Th1 cell development. However, whether non-apoptotic Fas signaling participates in regulating Th9 cell differentiation remains unknown.

Here, we demonstrate that Fas activates protein kinase Cβ (PKCβ) in a Ca2+-dependent manner. PKCβ then induces NF-κB activation and, in cooperation with NFAT1, NF-κB-mediated Th9 cell differentiation. By contrast, PKCβ-activated p38 inactivates NFAT1, thereby limiting Fas-mediated Th17 cell differentiation. Fas ligation-induced Th9 cells (FasL-Th9) exacerbates inflammatory bowel disease (IBD). In parallel, low-dose p38 inhibitor restores Fas-mediated Th17 cell induction in vitro and in vivo, and greatly suppresses tumor progression via IL-9 without inducing systemic adverse effects. Thus, our study reveals crucial functions of Fas-induced non-apoptotic signaling in Th9 cell induction, and may have important clinical implications in autoimmune disease and cancer therapy.

**Results**

Fas signaling promotes Th9 cell differentiation in vitro. To determine the role of Fas signaling in Th9 cell differentiation, we differentiated naive CD4+ T cells from wild-type (WT) and Fas−/− mice (termed WT or Fas−/− CD4+ T cells, respectively) into Th1, Th2, Th9, and Th17 cells and Tregs in vitro. The Fas−/− CD4+ T cells generated substantially lower levels of IL-9-producing cells and IL-9 protein than did the WT CD4+ T cells (Fig. 1a, b). Consistent with a previous publication, increased Th1 and decreased Th2 and Th17 cell differentiation could be observed with the Fas−/− CD4+ T cells (Fig. 1a, b). However, Treg differentiation was significantly inhibited in our polarization systems (Fig. 1a). Changes in signature cytokines or TF messenger RNA (mRNA) levels between the WT and Fas−/− Th9 cells also confirmed these results (Fig. 1c). Th9 cells differentiated from the WT or Fas−/− CD4+ T cells (termed WT-Th9 and Fas−/−-Th9, respectively) expressed high levels of the genes encoding the Th9-related TFs PU.1 (Spu1) and IRF4 (Irfa) (Supplementary Fig. 1a). Nevertheless, the gene levels of the Th9-related TFs Tbet (Tbx21), GATA-3 (Gata3), RORγt (Rorc), and Foxp3 (Foxp3), respectively, and the corresponding signature cytokines IFN-γ (Ifng), IL-4 (Il4), and IL-17A (Il17a) were very low in these cells (Supplementary Fig. 1a). These results demonstrated that Fas signaling enhanced, but did not skew, Th9 cell differentiation. Th9 differentiation in WT CD4+ T cells with Fas knockdown was also blunted (Supplementary Fig. 1b–d), excluding the possibility that the decreased Th9 cell-polarizing ability resulted from developmental defect in the Fas−/− CD4+ T cells. To further confirm the role of Fas signaling in Th9 cell differentiation, we ligated Fas on WT CD4+ T cells with anti-Fas (Jo2) in vitro and found that Jo2 markedly increased the frequency of IL-9-producing Th cells and the IL-9 protein and mRNA levels (Fig. 1d–f). As Fas signaling mediates T cell apoptosis, we detected the apoptosis of Th9 cells with or without Jo2 stimulation. We found that Jo2 did not affect Th9 cell apoptosis (Supplementary Fig. 1e). Moreover, Jo2 did not alter Th9 cell proliferation (Supplementary Fig. 1f).

Fas signaling-induced AICD is the essential mechanism that maintains T cell homeostasis. To assess AICD in FasL-Th9, Th9 cells induced by conventional or Fas ligation methods were restimulated with anti-CD3 and anti-CD28. Compared with the conventionally differentiated Th9 cells (cTh9), the FasL-Th9 showed increased AICD (Supplementary Fig. 1g). However, the FasL-Th9 secreted more IL-9 than the cTh9 (Supplementary Fig. 1h). These results further proved that Fas signaling promotes induction of IL-9-producing T cells.

To test whether autoactivated Fas signaling can increase IL-9-producing T cell numbers, we detected Fas and FasL mRNA levels in Th9 cell subsets and found that Th9 cells had lower Fas but higher FasL gene expression than the other Th9 subsets (Supplementary Fig. 1i, j). The ratio of the Fas gene level to the Fasl gene level was highest in the Th9 cells (Supplementary Fig. 1k), suggesting that autoactivated Fas signaling may play an important role in the induction of IL-9-producing T cells. Then, we differentiated naive CD4+ T cells from WT and Fasl−/− mice into Th9 cells in vitro. Fasl deficiency greatly inhibited the induction of IL-9-producing Th cells. Then, we differentiated naive CD4+ T cells from WT and Fasl−/− mice into Th9 cells in vitro. Fasl deficiency greatly inhibited the induction of IL-9-producing Th cells. Then, we differentiated naive CD4+ T cells from WT and Fasl−/− mice into Th9 cells in vitro. Fasl deficiency greatly inhibited the induction of IL-9-producing Th cells. Then, we differentiated naive CD4+ T cells from WT and Fasl−/− mice into Th9 cells in vitro. Fasl deficiency greatly inhibited the induction of IL-9-producing Th cells.

**Fas signaling activates genes related to Th9 cell functions.** To better understand the effects of Fas signaling on the Th9 cell program, we performed RNA-sequencing (RNA-seq) analysis of WT-Th9 and Fas−/− Th9. There were 204 differentially expressed genes (DEGs) between the WT-Th9 and Fas−/− Th9 (Supplementary Data 1). Among these DEGs, 84 genes had upregulated expression, and 120 genes had downregulated expression in the Fas−/− Th9 (Fig. 2a). Il9 was one of the most highly downregulated genes in the Fas−/− Th9 (Fig. 2a). Gzm genes have been
reported to have increased expression in T<sub>H</sub>9 cells and contribute to the antitumor activity of T<sub>H</sub>9 cells. We found that Gzma, Gzm, Gzmδ, Gzem, and Gzemγ were downregulated, but the master regulator of granzyme Eomes<sup>27</sup> and Gzmδ were upregulated in the Fasl<sup>p<sub>r</sub></sup>-T<sub>H</sub>9 (Fig. 2a), which were confirmed by real-time PCR (Fig. 2b), suggesting Eomes-independent regulation of granzyme in T<sub>H</sub>9 cells. A heat map obtained by unsupervised hierarchical clustering showed that the WT-T<sub>H</sub>9 and Fasl<sup>p<sub>r</sub></sup>-T<sub>H</sub>9 converged in the same cluster and showed overlapping gene expression (Fig. 2c). Moreover, T<sub>H</sub>1 cell-, T<sub>H</sub>2 cell-, T<sub>H</sub>17 cell-, and Treg-related genes showed no obvious differences between the WT-T<sub>H</sub>9 and Fasl<sup>p<sub>r</sub></sup>-T<sub>H</sub>9 (Fig. 2d). These results indicated that Fas deficiency does not globally affect T<sub>H</sub>9 cell differentiation. T<sub>H</sub>9 cells play a key role in the progression of autoimmune diseases<sup>28</sup>. DEG results from an analysis of the most highly enriched pathways revealed that genes related to autoimmune thyroid disease, type I diabetes mellitus, and systemic lupus erythematosus were downregulated in the Fasl<sup>p<sub>r</sub></sup>-T<sub>H</sub>9 (Fig. 2e). Therefore, RNA-seq analysis demonstrated that Fas signaling contributes to the gene activation, and is responsible for T<sub>H</sub>9 cell-specific functions.

Fas signaling induces T<sub>H</sub>9 cells by activating NF-κB. To examine how Fas signaling induces T<sub>H</sub>9 cells, we first investigated the differentiation of FasL-T<sub>H</sub>9 in the presence of the pancaspase inhibitor z-VAD-fmk<sup>29</sup> and found that z-VAD-fmk did not alter the Jo2-mediated increase in IL-9-producing T cells (Supplementary Fig. 2a), indicating caspase-independent induction of T<sub>H</sub>9 cells. Since Fas defects inhibit T<sub>H</sub>9 cell and Treg differentiation, and IL-2 is critical for both cell differentiation<sup>30,31</sup>, we next tested the role of IL-2 in Fas-mediated T<sub>H</sub>9 cell differentiation. We found that I<sub>L2</sub> mRNA levels were not different between WT-T<sub>H</sub>9 and Fasl<sup>p<sub>r</sub></sup>-T<sub>H</sub>9 (Supplementary Fig. 2b). Moreover, neither the addition of exogenous IL-2 nor neutralization of endogenous IL-2 rescued differentiation inferiority of Fasl<sup>p<sub>r</sub></sup>-T<sub>H</sub>9 (Supplementary Fig. 2c, d), indicating IL-2-independent increase of T<sub>H</sub>9 cell differentiation by Fas. STAT1,
STAT3, STAT5, STAT6, IRF4, PU.1, Gata3, NF-κB, and Akt are all involved in T17 cell differentiation. Mitogen-activated protein kinases also participate in IL-9 expression. We detected the activation and total protein levels of these TFs and kinases in WT and Faslpr CD4+ T cells cultured under T17-skewing conditions for 15 min or 24 h and found that the activation of p65 and p38 was obviously inhibited in the Faslpr CD4+ T cells (Supplementary Fig. 2e, f). In contrast, Fas ligation markedly induced the activation of p65 and p38 (Supplementary Fig. 2e, f). Furthermore, the activation of IKKa, IKKβ, IκBa, p65, and p38 markedly decreased in the Faslpr CD4+ T cells at different time points (Fig. 3a), indicating Fas signaling-dependent activation of the NF-κB and p38 pathways in differentiating T17 cells. To test the role of the NF-κB pathways in Fas-mediated T17 cell differentiation, we treated CD4+ T cells with the IκB phosphorylation inhibitor BAY 11-7082 or the IKK2 inhibitor LY2409881 before inducing polarization. Both BAY 11-7082 and LY2409881 completely abolished the differentiation superiority of FasL-T17 (Fig. 3b), indicating that Fas-induced IL-9-producing T cell generation is NF-κB dependent.

PKC has been reported to participate in the activation of NF-κB. There are three major groups of PKC isoforms. These groups include classic PKCs (PKCsα, PKCsβ1, PKCsβ2, and PKCsγ), novel PKCs (PKCsδ, PKCsε, PKCsζ, PKCsθ, and PKCsβ3), and atypical PKCs (PKCsδ and PKCsδ). Fas is known to play a role in PKCsβ2 activation. Next, we examined whether PKC is responsible for Fas-induced NF-κB activation. We found that Fas ligation obviously increased the recruitment of PKCsβ2 to the plasma membrane and its colocalization with Fas (Fig. 3c). Increased PKCsβ1 recruitment to the plasma membrane and colocalization with Fas were also observed after Fas ligation (Fig. 3c). The selective PKCsβ inhibitor enzastaurin and the pan-PKC inhibitor Go 6983 both abrogated the Fas ligation-induced phosphorylation of p65 and increase in IL-9-producing T cells (Fig. 3d, e). These results demonstrated that PKCsβ behaves as an activator of NF-κB after Fas ligation.

The activation of classic PKCs is Ca2+ dependent. J02 stimulation evoked Ca2+ flux in WT CD4+ T cells under T17-skewing conditions (Supplementary Fig. 2g). To define the function of Ca2+ in PKCsβ activation, we pretreated cells with 2-aminoethoxydiphenyl borate (2-APB) or xestospongin C (XC), which are inhibitors of inositol 1,4,5-trisphosphate-induced Ca2+ release, and found that both prevented PKCsβ2 and PKCsβ1 recruitment to the plasma membrane and colocalization with Fas in response to Fas ligation (Fig. 3f). Consistently, both inhibitors abolish the activation of p65 and enhancement of IL-9-producing T cell generation (Fig. 3g, h), suggesting that PKCsβ activation by Fas ligation is Ca2+ dependent.

Fas can trigger Ca2+ signaling by activating phospholipase Cγ1 (PLCγ1). We observed reduced phosphorylated PLCγ1 level in Faslpr CD4+ T cells under T17-skewing conditions (Supplementary Fig. 2h). Both U73122, a potent PLC inhibitor, and mananolide, an irreversible PLC inhibitor, abolished Fas ligation-induced increases in Ca2+ flux and IL-9-producing T cells (Supplementary Fig. 2i, j), suggesting that the increased Ca2+ response by Fas signaling is PLCγ1-dependent.
Tyrr224 and Tyr274 in Fas mediate PLCγ1 activation. Given that Fas has no enzymatic activity, we questioned how PLCγ1 was activated. Zap-70 can interact with Fas and participate in the phosphorylation of PLCγ1 in T cells. Fas ligation obviously increased the colocalization of Fas and Zap-70 and phosphorylated Zap-70 level under Th9-skewing conditions (Supplementary Fig. 3a, b). Knocking down Zap-70 expression markedly decreased the Fas ligation-induced phosphorylation of PLCγ1 and generation of IL-9-producing T cell (Supplementary Fig. 3c–f), indicating the Zap-70-dependent phosphorylation of PLCγ1.

There are four Tyr sites in the intracellular domain of Fas (Tyr189, Tyr224, Tyr274, and Tyr284). Tyr284, which is located in the YXXL motif of Fas, is necessary for the binding of Fas with Zap-70. However, a Y284A mutation in Fas did not reduce the colocalization of Fas and Zap-70 (Supplementary Fig. 3g). However, the mutations Y224A and Y274A, but not the mutation Y189A, greatly decreased the colocalization of Fas and Zap-70 (Supplementary Fig. 3g). Consistently, the overexpression of unmutated Fas (Fas-WT) or Fas with the Y189A or Y284A mutation (Fas-Y189A, Fas-Y284A) but not Fas with the Y224A or Y274A mutation (Fas-Y224A, Fas-Y274A) significantly promoted the generation of IL-9-producing T cells from naive Fas<sup>b</sup> CD4<sup>+</sup> T cells (Supplementary Fig. 3h). These results demonstrated that both Tyr224 and Tyr274 in Fas are necessary for the Fas ligation-induced generation of IL-9-producing T cells.

p38 inhibits Th9 cell generation by mediated Fas signaling. Since Fas ligation obviously induced p38 activation (Supplementary Fig. 2e), we examined the role of p38 in the differentiation of FasL-Th9. Strikingly, Fas ligation induced a much higher frequency of IL-9-producing T cells in cells treated with the p38 inhibitor SB203580 than in untreated cells (Fig. 4a). However, SB203580 alone could not increase cTh9 (Fig. 4a). Additionally, knocking down p38a expression promoted the differentiation of Fasl-Th9 but not cTh9 (Supplementary Fig. 4a, b). PKCβ can mediate p38 activation. To elucidate whether PKCβ is involved in Fas ligation-induced p38 activation, we treated WT CD4<sup>+</sup> T cells with enzastaurin and found no increased activation of p38 due to Fas ligation (Fig. 4b). These results demonstrated that PKCβ-activated p38 provides negative feedback in the differentiation of Fasl-Th9.

NFAT1 cooperates with NF-kB to induce IL-9 transcription in CD4<sup>+</sup> T cells. p38 can inhibit NFAT signaling by mediating NFAT phosphorylation. We hypothesized that p38 might...
inhibit the differentiation of FasL-T$_{H9}$ by regulating NFAT1 phosphorylation. We first determined the role of NFAT in Fas signaling-induced IL-9-producing T cell generation and found that both FK506, an inhibitor of NFAT calcineurin inactivation, and INCA-6, a potent and selective inhibitor of calcineurin-NFAT signaling, partially inhibited the differentiation of FasL-T$_{H9}$ (Fig. 4c). Neither inhibitor could restrain the Fas ligation-induced activation of NF-κB (Supplementary Fig. 4c). These results indicated that NFAT1 synergistically enhanced the NF-κB-mediated induction of IL-9-producing T cells by Fas ligation. NFAT1 phosphorylation was markedly reduced in Faslpr CD4$^+$ T cells under T$_{H9}$-skewing conditions (Fig. 4d), indicating NFAT1 activation. In contrast, Fas ligation increased NFAT1 phosphorylation in WT CD4$^+$ T cells (Fig. 4e). Then, we examined the effect of p38 on NFAT1 phosphorylation and found that SB203580 treatment abolished the Fas ligation-induced increase in NFAT1 phosphorylation under T$_{H9}$-skewing conditions (Fig. 4f). SB203580 treatment also resulted in no difference in phosphorylated NFAT1 levels between Faslpr and WT CD4$^+$ T cells under T$_{H9}$-skewing conditions (Supplementary Fig. 4d). In the presence of INCA-6, SB203580 could no longer enhance the differentiation of FasL-T$_{H9}$ (Fig. 4g). These results indicated that p38 limits the differentiation of FasL-T$_{H9}$ by affecting NFAT1 phosphorylation.

**FasL-T$_{H9}$ exacerbate murine IBD via IL-9.** IL-9 determines the pathogenesis of ulcerative colitis. We tested the in vivo relevance of our observations in a murine IBD model. The transfer of either cTH9 or FasL-TH9 significantly increased weight loss and shortened colonic length in IBD mice (Fig. 5a, b). However, the ability of the FasL-TH9 to exacerbate IBD was stronger than that of the cTH9 (Fig. 5a, b). Histological analysis also revealed more leukocyte infiltration and more severe damage to glandular structures in the colonic tissue in the mice that received the transfer of FasL-TH9 (Fig. 5c). Moreover, FasL-T$_{H9}$ caused colonic tissue to produce more IL-6, TNF, IL-1β, IL-10, and IL-22 (Fig. 5d). In contrast, compared with WT-T$_{H9}$-treated mice, mice that received transfer of Faslpr-T$_{H9}$ showed reduced weight loss and colonic length shortening (Supplementary Fig. 5a, b). Histological assessment exhibited a similar trend (Supplementary Fig. 5c). These results indicated that Fas ligation exacerbated...
murine IBD. To test the effects of Fas signaling on $T_{H9}$ cell apoptosis and proliferation in vivo, we transferred carboxyfluorescein succinimidyl ester (CFSE)-labeled WT-$T_{H9}$ or $FasL^{-}T_{H9}$ into IBD mice and evaluated apoptosis and proliferation in the CFSE-positive cells in the mesenteric lymph nodes. We found that there were no obvious differences in apoptosis or proliferation between the WT-$T_{H9}$ and $FasL^{-}T_{H9}$ (Supplementary Fig. 5d). These findings excluded the possibility that the apoptosis and proliferation of $T_{H9}$ cells with Fas defects affect IBD pathogenicity.

To define the effect of IL-9 on $T_{H9}$ cell-mediated IBD progression, we neutralized IL-9 with anti-IL-9 when cTH9 or FasL-$T_{H9}$ was transferred. Both types of $T_{H9}$ cells barely exacerbated murine IBD after IL-9 neutralization (Fig. 5e–g). We also detected similar effects of both types of $T_{H9}$ cells on IBD $I_{H9^{-/-}}$ mice (Supplementary Fig. 5e–g). These results demonstrated that the aggravation of murine IBD by FasL-$T_{H9}$ depends on IL-9.

Published data suggest that in an inflammatory state in vivo, $T_{H9}$ cells are unstable and begin to secrete IFN-γ54. Therefore, we examined the stability of $T_{H9}$ cells in IBD mice in vivo by transferring Fas ligation-induced CD45.1 $T_{H9}$ cells into CD45.2 $T_{H9}$ mice. We found that the CD45.1 $T_{H9}$ cells within the lamina propria lymphocytes (LPLs) maintained their expression of $T_{H9}$-related genes ($I_{H9}$, $I_{Rf}$, and $I_{Spiil}$) and did not exhibit expression of $T_{H11}$-related genes ($I_{Fng}$ and $T_{Bx}21$) (Fig. 5h). These data indicated that FasL-$T_{H9}$ are stable in IBD mice in vivo.

Fas signaling relates to the antitumor activity of $T_{H9}$ cells. $T_{H9}$ cells are present in mice bearing melanoma tumors and exhibit prominent antitumor activity11,55. To dissect whether Fas signaling is related to $T_{H9}$ cells in tumor-bearing mice, we injected B16F10 cells intravenously into WT mice and analyzed $I_{H9}$ mRNA levels in Fas-positive and Fas-negative CD4+ T cells. We found that the $I_{H9}$ mRNA levels in the Fas+CD4+ T cells were significantly higher than those in the Fas−CD4+ T cells (Supplementary Fig. 6a). In addition, we detected higher $I_{Spil}$ and $I_{Rf}$ mRna levels in the Fas+CD4+ T cells than in the Fas−CD4+ T cells and similar $T_{Bx}21$, $Gata3$, $Ror$, and $Fop$ mRNA levels between the two cell subsets (Supplementary Fig. 6a), further supporting the idea that Fas signaling dictates $T_{H9}$ cell differentiation.

To elucidate whether Fas signaling determines the antitumor effects of endogenous $T_{H9}$ cells, we reconstituted irradiated WT mice with $Fas^{pr}$ (Faspr→WT) or WT (WT→WT) bone marrow cells and established tumors with Lewis lung carcinoma cells expressing full-length ovalbumin (LLC-OVA) in these mice. We found that the tumor progression in the Faspr→WT mice...
Fig. 6 Fas signaling relates to the antitumor activity of T helper type 9 (Th9) cells. (a, b) Tumor growth (a) and survival (b) of irradiated WT mice reconstituted with bone marrow cells from wild-type (WT) or Faslpr mice for 2 months and then subcutaneously injected with LLC-OVA tumor cells with or without intravenous injection of 100 μg of anti-interleukin-9 (IL-9)-neutralizing antibodies every other day (n = 5). (c) Flow cytometric analysis of the frequencies of IL-9+, IFN-γ+, IL-17A+, and Foxp3+ cells among CD4+ T cells in the tumor-infiltrating lymphocytes (TILs) of the mice described in a 20 days after tumor inoculation (n = 3). (d) Enzyme-linked immunosorbent assay (ELISA) measurements of IL-9, IFN-γ, and IL-17A levels secreted by OVA323–339-stimulated TILs from the mice described in a 20 days after tumor inoculation (n = 3). (e, f) Representative lung appearance (e) and statistical analysis of the lung tumor foci (f) (n = 5) of WT mice 16 days after intravenous injection of B16F10-OVA melanoma cells with no transfer (NT) or transfer of OT-II cT,9 or Fasl-T,9 1 and 6 days later. (g, h) Tumor growth (g) and survival (h) of WT mice that received a subcutaneous injection of B16F10-OVA cells followed by NT or the intravenous injection of 2 × 106 OT-II cT,9 or Fasl-T,9 1 and 6 days later (n = 5). (i) Lung tumor foci of Il9r−/− mice 16 days after intravenous injection of B16F10-OVA melanoma cells with NT or transfer of OT-II cT,9 or Fasl-T,9 1 and 6 days later (n = 5). (j) Survival rate (%) of irradiated WT mice 16 days after intravenous injection of B16F10-OVA melanoma cells with NT or transfer of OT-II cT,9 or Fasl-T,9 1 and 6 days later (n = 5). (k) Tumor growth (j) and survival (k) of Il9r−/− mice that received a subcutaneous injection of B16F10-OVA cells followed by NT or intravenous injection of 2 × 106 OT-II cT,9 or Fasl-T,9 1 and 6 days later (n = 5). NS, not significant; *P < 0.05, **P < 0.01, and ***P < 0.001 (unpaired Student’s t test: a, c, d, f, g, i, j, and k, log-rank test: b, h, k). Compared with the Faslpr → WT mice in a, b, compared with cT,9 in g, h, j, and k. Representative results from three independent experiments are shown (mean ± s.d.).

was superior to that in the WT → WT mice, which was IL-9 dependent (Fig. 6a, b). The tumor-infiltrating lymphocytes (TILs) in the WT → WT mice demonstrated an enhanced percentage of IL-9-producing CD4+ T cells but no increased percentages of IFN-γ- or IL-17A-producing CD4+ T cells or Tregs (Fig. 6c). Restimulation of TILs with OVA323–339 in vitro resulted in an evident increase in IL-9 protein expression, but not in IFN-γ or IL-17A protein expression, in the CD4+ T cells within the TILs of WT → WT mice (Fig. 6d). IL-9 is reported to exert an antitumor effect by activating CD8+ T cells55. We detected an increased frequency of IFN-γ-producing CD8+ T cells within the TILs of the WT → WT mice (Supplementary Fig. 6b). Moreover, restimulation of TILs with OVA257–264 in vitro led to a notable increase in IFN-γ protein levels in the CD8+ T cells with the TILs of the WT → WT mice (Supplementary Fig. 6c). These results suggested that Fas defects probably restrain antitumor immunity by suppressing IL-9-producing T cell generation.

To directly demonstrate that Fas signaling influences the antitumor properties of T,9 cells, we differentiated naive OT-II CD4+ T cells into T,9 cells with or without Joz2 stimulation. OT-II CD4+ T cells transgenically express a TCR recognizing an epitope of OVA323–339 in the context of I-Ab12. We intravenously transferred differentiated OT-II T,9 cells with OVA-expressing B16F10 cells (B16F10-OVA) into WT mice on the same day and found that FasL-T,9 exhibited stronger antitumor effects than cT,9 (Fig. 6e, f). FasL-T,9 also had superior antitumor effects on a B16F10-OVA tumor model established by subcutaneous inoculation (Fig. 6g, h). By using LLC-OVA, we obtained similar results (Supplementary Fig. 6d–f). To assess the role of IL-9 in the antitumor effects of Fasl-T,9, we intravenously or subcutaneously challenged Il9r−/− mice with B16F10-OVA tumor cells and found that neither Fasl-T,9 nor cT,9 enhanced antitumor immunity (Fig. 6i–k). Collectively, these results demonstrated that Fasl-T,9 enhance antitumor immunity via IL-9.

p38 inhibitor exerts antitumor activity by inducing Th19. Given that a p38 inhibitor promoted Fas-induced IL-9–producing T cell generation, we investigated whether this p38 inhibitor could also exert antitumor effects dependent on T,9 cells. To test this, we first detected LLC-OVA tumor progression in WT mice with or without SB203580 treatment. Notable inhibition of tumor progression by SB203580 was observed, which was IL-9 dependent (Fig. 7a, b). The TILs from WT mice that received SB203580 treatment had a significantly higher frequency of IL-9–producing CD4+ T cells, but not the IFN-γ- or IL-17A–producing CD4+ T cells (Fig. 7c). Restimulation of the TILs with OVA323–339 in vitro induced increased production of the IL-9 protein, but not the IFN-γ or IL-17A protein, in the CD4+ T cells within the TILs of the WT mice that received SB203580 (Fig. 7d). Importantly, SB203580 could not inhibit tumor progression in Faslpr → WT mice, suggesting that SB203580 favored T,9 cell generation via...
Fas (Fig. 7e, f). Furthermore, SB203580 did not affect tumor growth in thymus-deficient nu/nu mice (Supplementary Fig. 7a), again highlighting the T cell-dependent antitumor effect. Usually, the dose of SB203580 used for in vivo treatment is 10 mg kg$^{-1}$ (high)$^{56,57}$ but the dose we used was 0.5 mg kg$^{-1}$ (low). We dynamically monitored the SB203580 concentration in the plasma of mice that received low- or high-dose SB203580 treatment. The plasma SB203580 concentration reached a peak of 0.15 μg ml$^{-1}$ at 1 h or of 19.46 μg ml$^{-1}$ at 0.5 h in the mice receiving low- or high-dose SB203580 treatment, respectively (Supplementary Fig. 7b). At a dose of 20 μg ml$^{-1}$, but not a dose of 0.15 μg ml$^{-1}$, SB203580 significantly inhibited LLC-OVA cell viability in vitro (Supplementary Fig. 7c). These data further supported the idea of a T$_{H}9$ cell-dependent antitumor effect of low-dose SB203580.

To evaluate the safety of systemic treatment with low- or high-dose SB203580, we compared the weights, and liver and renal functions of mice that received low-dose SB203580 treatment with those of mice that received high-dose SB203580 treatment. The two groups of mice showed no obvious differences in weight loss or impaired liver and renal functions (Supplementary Fig. 7d, e). Consistently, no pathological injury to the heart or other organs was found in either mouse group (Supplementary Fig. 7f). However, a decrease in the phosphorylated p38 level could be detected only in colonic tissues from the mice receiving high-dose SB203580 treatment (Fig. 7g), suggesting the possibility of systemic inhibition of the p3 pathway. Altogether, our data indicated that low-dose p38 inhibitor treatment restricts tumor progression by enhancing generation of IL-9-producing cells without systemic toxicity in vivo.

Since Fas ligation combined with the p38 inhibitor induced more IL-9-producing T cells, we hypothesized that Fas ligation plus a p38 inhibitor would induce a large number of T$_{H}9$ cells in the context of adoptive transfer treatment of tumors. To test this, we differentiated naive OT-II CD4$^{+}$ T cells into T$_{H}9$ cells with Jo2 stimulation or Jo2 stimulation plus SB203580 treatment (FasL + p38i-T$_{H}9$). In a B16F10-OVA tumor model established by subcutaneous inoculation, we found that FasL + p38i-T$_{H}9$ showed stronger antitumor effects than FasL-T$_{H}9$ (Fig. 7h, i). To elucidate the role of IL-9 in the antitumor effects of these cells, we subcutaneously challenged IL9r$^{-/-}$ mice with B16F10-OVA tumor cells and found that neither FasL + p38i-T$_{H}9$ nor FasL-T$_{H}9$ could inhibit tumor progression (Fig. 7j, k). These results indicated that FasL + p38i-T$_{H}9$ are promising for the adoptive transfer treatment of tumors.

Fas-related T$_{H}9$ cells indicate a better prognosis. To extend our findings to humans, we investigated the effect of Fas ligation on human IL-9-producing T cell induction and found that Fas ligation significantly improved human IL-9-producing T cell generation (Fig. 8a–c). When Fas ligation was combined with a p38 inhibitor, this tendency was more evident (Fig. 8a–c). Then, we detected Fas and IL-9 expression in CD4$^{+}$ T cells from tumor tissues in a cohort of 36 cancer patients with non-small-cell lung carcinoma (NSCLC) (Supplementary Table 1). Immunofluorescence staining revealed that the patients with high numbers of Fas$^{+}$CD4$^{+}$ T cells often exhibited high numbers of IL-9$^{+}$CD4$^{+}$ T cells (Fig. 8d). Further analysis confirmed the positive correlation between the numbers of Fas$^{+}$CD4$^{+}$ T cells and IL-9$^{+}$CD4$^{+}$ T cells.
T cells in the tumor tissues (Fig. 8c). To confirm that T19 cells also have antitumor activity in humans, we divided the patients into high Fas+CD4+ T cell number (Fas+CD4+ T9) and low Fas+CD4+ T cell number (Fas+CD4+ Tlo) groups or high IL-9+CD4+ T cell number (IL-9+CD4+ T9) and low IL-9+CD4+ T cell number (IL-9+CD4+ Tlo) groups and found that the recurrence-free survival in both the Fas+CD4+ T9 and IL-9+CD4+ T9 patient groups was better than that in the Fas+CD4+ Tlo and IL-9+CD4+ Tlo patient groups, respectively (Fig. 8f). Collectively, these results demonstrated that Fas signaling also promotes human IL-9-producing T cell induction, which has a beneficial effect on the outcomes of patients with tumor.

Discussion

Fas signaling is important in the induction of apoptosis in activated T cells. However, there were no obvious differences in apoptosis or proliferation between WT-T19 and Fas−/−T19 after transfer into IBD mice. This lack of difference might have been caused by the short course of acute IBD, or the Fas levels in the WT-T19 might not have been sufficient to trigger apoptotic signaling when we evaluated them. However, Fas-mediated non-apoptotic signaling was observable because the mice receiving the transfer of WT CD4+ T cells differentiated under T19-skewing conditions showed IBD symptoms with increased severity dependent on IL-9. Therefore, non-apoptotic Fas signaling is critical to generate IL-9-producing T cells and induce IL-9-mediated physiological functions.

Under T19-skewing conditions with Fas ligation, TCR signaling activates PKCθ, mediating NF-kB activation. We demonstrated that Fas signaling activated PKCθ, which was necessary for increased NF-kB-dependent IL-9-producing cell induction. PKCθ was not implicated in Fas ligation-mediated IL-9-producing T cell induction because enzastaurin completely abolished this induction. Additionally, activated PKCθ could activate p38, which subsequently phosphorylated NFAT1, leading to NFAT1 inactivation. Since NFAT1 cooperates with NF-kB to promote T19 cell differentiation, Fas ligation initiates both positive and negative signaling for T19 cell differentiation. However, Fas signaling still markedly promoted T19 cell differentiation, indicating the decisive role of NF-kB.

p38 inhibitors have been demonstrated to exert antitumor effects58,39, but serious side effects resulting from the systemic administration of SB203580 restrain its application. Here, we showed that systemic administration of low-dose SB203580 did not cause obvious side effects. Interestingly, we elucidated that even at very low doses, SB203580 could effectively initiate a T19 cell-dependent antitumor effect. Moreover, T19 cells induced by Fas plus SB203580 showed relatively strong antitumor efficacy.

**Fig. 8** Fas-related T helper type 9 (T19) cells indicate a good prognosis. **a-c** Flow cytometric analysis of the frequency of IL-9+ (interleukin-9+) cells among CD4+ T cells (left) and the corresponding statistical analysis (right) (**a**), real-time PCR analysis of IL9 messenger RNA (mRNA) expression in T19 cells (**b**), and enzyme-linked immunosorbent assay (ELISA) measurement of the IL-9 level (**c**) in T19 cells after the stimulation of human naive CD4+CD45RA−CD45RO− T cells with 10 μg ml−1 ISO or antibodies against human Fas (anti-Fas) with or without the p38 inhibitor SB203580 (0.4 μM) under T19-skewing conditions for 4 days (**n** = 3). **d** Representative immunofluorescence staining of Fas+CD4+ and IL-9+CD4+ T cells in tumor tissues from non-small-cell lung carcinoma (NSCLC) patients. Arrows indicate Fas+CD4+ or IL-9+CD4+ T cells. **e** Pearson’s correlation between Fas+CD4+ T cells and IL-9+CD4+ T cells in tumor tissues from NSCLC patients. **f** The relationship between the recurrence-free survival rate of NSCLC patients and the corresponding Fas+CD4+ or IL-9+CD4+ T cell numbers in tumor tissues. Scale bar = 25 μm. **P < 0.01 and ***P < 0.001 (unpaired Student’s t test): **(right)***c, Spearman’s rank-order correlation test: **(e), or log-rank test: **(f). Representative results from three independent experiments are shown (mean and s.d.)
Given the important role of Th9 cells in antitumor immunity, SB203580 treatment is potential strategy for cancer therapy. After their localizing to the plasma membrane, classic PKCs can be activated by calcium. Both Fas and TCR signaling can initiate Ca²⁺ flux. It is difficult to discriminate between Ca²⁺ flux induced by Fas and that induced by TCR signaling. Fas signaling participated in PLCγ activation and the subsequent increase in Ca²⁺ flux. Fas also interacted with Zap-70, mediating PLCγ activation. The Fas mutations Y224A and Y274A abolished the interaction between Fas and Zap-70 and Th9 cell differentiation induced by Fas ligation. Therefore, Ca²⁺ flux induced by Fas signaling is indispensable for PKCβ activation and subsequent Th9 cell differentiation. The role of the Ca²⁺ flux induced by TCR signaling in the activation of PKCβ needs further study. Interestingly, Try284 is located in the YXXM motif of Fas, which is necessary for the binding between Fas and Zap-70. However, according to our results, the Y284A mutation in Fas had no effect on the interaction between Fas and Zap-70 or on the Fas ligation-induced induction of IL-9-producing T cells. This finding shows that Try224 and Try274, but not Try284, are responsible for Fas ligation-induced IL-9-producing Th9 generation. Thus, we revealed novel functional Tyr sites in Fas.

Fas has been reported to promote Th17 cell development, but inhibit Th1 and Th17 cell development by sequestering STAT1. We also detected decreased Th17 cell differentiation and increased Th1 cell differentiation in CD4⁺ T cells with Fas deficiency in vitro. However, in cells with a Fas defect, we detected reduced Th9 cell generation, but the generation of Th17 and Th1 cell remained unchanged in tumor-bearing mice. This result may be attributable to the different mice or different disease models we used. The same TF can play roles in the differentiation of different Th1 cell subsets. STAT1 is also involved in reinforcing Th9 cell development. However, Fas signaling-mediated Th9 cell differentiation is STAT1 independent. Therefore, the different environments that CD4⁺ T cells encounter determine their differentiation fates. Th9-skewing conditions are probably dominant in tumor-bearing mice.

Previous publications demonstrated IL-9- and IL-21-dependent antitumor effects of Th9 cells. In these papers, the authors also verified that Th9 cells inhibit tumor growth by activating CD8⁺ T cells. In another publication, the authors demonstrated that Th9 cells are directly cytotoxic to tumor cells and that the antitumor effects of Th9 cells are mast cell dependent. A recent report showed that IL-9 and CD8⁺ T cells only slightly affect the antitumor efficacy of Th9 cells, while the Eomes-dependent granzyme-mediated cytolytic activity and TRAF6-driven hyperproliferation of Th9 cells are responsible for their antitumor efficiency. Our data suggested that Fas signaling promoted IFN-γ production in CD8⁺ T cells. Fas signaling also upregulated granzyme expression, which appeared to be Eomes independent because a Fas defect induced rather than inhibited Eomes expression in Th9 cells. Overall, the Fas signaling-mediated antitumor efficacy of Th9 cells is IL-9 dependent. Although the antitumor effect of Th9 cells has been well established in mice, the antitumor effect of Th9 cells on humans is not well defined. Our results demonstrated that Fas ligation promoted human Th9 cell differentiation. The number of Fas⁺CD4⁺ T cells and IL-9⁺CD4⁺ T cells in tumor tissues was positively correlated, and higher Th9 cell numbers in patients with tumor indicated a better prognosis than lower Th9 cell numbers. Therefore, our findings provide evidence that Th9 cells also have antitumor effects on humans.

In summary, we demonstrate that Fas signaling promotes Th9 cell differentiation through PKC-β-mediated activation of the NF-kB pathway. At the same time, PKC-β-activated p38 inactivates NFAT1 and abolishes the cooperative effect of NFAT1 on NF-kB, providing negative feedback to Fas-induced Th9 cell differentiation (Supplementary Fig. 8).

**Methods**

**Human samples.** Human blood from healthy volunteers and human lung cancer tissue samples were obtained from Zhejiang Cancer Hospital. The collection of human samples was approved by the local Ethical Committee and the Review Board of Zhejiang Cancer Hospital. All the patients were informed of the usage of their tissue samples, and signed consent forms were obtained.

**Mice and cell lines.** Female C57BL/6J (6- to 8-week-old) mice and BALB/c nu/nu mice were purchased from Joint Ventures Sipper BK Experimental Animal Co (Shanghai, China). Fas⁺ and Fas⁻ mice were purchased from the Jackson Laboratory (Farmington, CT, USA). Fas⁺ and Fas⁻ mice were kindly provided by Dr. Jean-Christophe Renauld (Université Catholique de Louvain, France). Mice were housed in a specific pathogen-free facility, and experimental protocols were approved by the Animal Care and Use Committee of the School of Medicine, Zhejiang University. Murine B16F10 tumor cells and HEK293 cells were obtained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (Lonza, Basel, Switzerland). ILC-OVA and HEK293 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. All cells were routinely tested for mycoplasma contamination using the Mycoplasma Detection Kit (Lonza) and were found to be negative.

**In vitro differentiation of T cells.** Naive CD4⁺CD62L⁻CD44⁻ T cells were obtained from the spleen and lymph nodes of mice. Sorted naive CD4⁺ T cells were routinely 98% pure. The sorted naive CD4⁺ T cells were stimulated with plate-bound anti-CD3 (145-2C11, 2 μg ml⁻¹, Bio X cell, West Lebanon, NH, USA) and anti-CD28 (PV-1, 2 μg ml⁻¹, Bio X cell) antibodies, and polarized into effector CD4⁺ T lymphocyte subsets without cytokines, and with anti-IFN-γ (BE0054, 10 μg ml⁻¹, Bio X cell) and anti-IL-4 (BE0045, 10 μg ml⁻¹, Bio X cell) antibodies. With IL-12 (130-096-707, 20 ng ml⁻¹, Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-IL-4 (10 μg ml⁻¹) for Th1 cell subsets with IL-12 (130-094-061, 20 ng ml⁻¹, Miltenyi Biotec) and anti-IFN-γ (10 μg ml⁻¹) for Th17 subsets with TGF-β1 (130-095-067, 2 ng ml⁻¹, Miltenyi Biotec). IL-4 (20 ng ml⁻¹) and anti-IFN-γ (10 μg ml⁻¹) for Th9 cells with TGF-β1 (2 ng ml⁻¹). IL-6 (130-094-065, 25 ng ml⁻¹, Miltenyi), anti-IFN-γ (10 μg ml⁻¹) and anti-IL-4 (10 μg ml⁻¹) for Th17 cells, or with TGF-β1 (2 ng ml⁻¹), anti-IFN-γ (10 μg ml⁻¹) and anti-IL-4 (10 μg ml⁻¹) for Treg cells. In some experiments, antibodies against murine Fas (Jo2, 10 μg ml⁻¹, Thermo Fisher Scientific, Wallharn, MA, USA), z-VAD-fmk (S9102, 1 μM, Selleck, Houston, TX, USA), BAY 11-7082 (S5003, 5 μM, Selleck), or INCA-6 (sc-203160, 50 nM, Santa Cruz, CA, USA) was added at the beginning of culture. Cells were classically harvested on day 3 for the detection of cytokines by enzyme-linked immunosorbent assay (ELISA) and real-time PCR analyses. In human for in vitro CD4⁺ T cell differentiation, naive CD4⁺CD45RA⁻CD45RO⁺ T cells were isolated from the peripheral blood mononuclear cells of healthy donors with the Human Naive CD4⁺ T Cell Isolation Kit II (STEMCELL, Vancouver, BC, V6A 1B6, Canada), stimulated with plate-bound anti-CD3 (5 μg ml⁻¹, Bio X cell) and anti-CD28 (5 μg ml⁻¹, BioLegend, San Diego, CA, USA) antibodies, and polarized into Th9 cells with human TGF-β1 (10 ng ml⁻¹) and IL-4 (5 ng ml⁻¹) (R&D Systems, Minneapolis, MN, USA).

**Immunoblotting analysis.** Purified naive CD4⁺ T cells were differentiated into Th9 cells for different times. Then, the cells were pelleted by centrifugation for 5 min at 2000 x g and lysed for 30 min at 4 °C in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate, 1 mM EDTA, and protease inhibitors). Subsequently, the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10–12%) and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% bovine serum albumin (BSA) in TBST buffer and then incubated with primary antibodies overnight at 4 °C. After incubating with the corresponding horse radish peroxidase-conjugated secondary antibodies for 1 h, the chemical signal were developed by NcmeCL Ultra (P10100A, P10100B, New Cell & Molecular Biotech Co. Ltd, Suzhou, Jiangsu, China), and then the membrane was scanned using the Tanon 4500 Gel Imaging System. Uncovered blotting scans were presented in the affili- ation for the methods file. The antibodies were diluted with 10% skim milk and a secondary antibody (WB500D, New Cell & Molecular Biotech Co. Ltd). The antibodies used and the corresponding dilutions are listed in Supplementary Table 2.
transmission of siRNA. Transient small interfering RNA (siRNA) transfection into naïve CD4+ T cells was performed in vitro using TransIT-TKO (Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, the CD4+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies, differentiated into Th9 cells as described above, and cultured for an additional 72 h after analysis with an siRNA specific for murine Foxp3, Zap70 (sc-29312 or sc-36687, respectively, Santa Cruz), or p38α (96417, Cell Signaling), or NC siRNA (sc-37007, Santa Cruz).

Real-time PCR. Total RNA was extracted from T cells using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's instructions. Complementary DNAs (cDNAs) was synthesized using a cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan) following the manufacturer's instructions. Real-time PCR was conducted using SYBR Green (TaKaRa). The following PCR conditions were used: 1 cycle at 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 34 s. Real-time PCR was performed with an Applied Biosystems 7500 real-time PCR system.

The primers used are listed in Supplementary Table 3.

Measurement of cytokine levels. After 72 h of polarization, cell culture supernatants were assayed by ELISA to measure the levels of mouse IFN-γ, IL-4, IL-9, IL-17A, and IL-17F (BioLegend). The following antibodies were used: anti-IFN-γ, anti-IL-4, anti-IL-9, anti-IL-17A, and anti-IL-17F. The ECL detection kit (Promega, Madison, WI, USA) was used for evaluation.

Retinoic acid induction of CD4+ T cells. Retinoic acid was produced by transfecting Plat-E cells with 7.5 μg of pMH-IRE5-GFP, pMH-Flas-WT IRES-GFP, pMFX-Fas-T189A IRES-GFP, pMFX-Fas-T224A IRES-GFP, pMFX-Fas-T244A IRES-GFP, or pMFX-Fas-T265A IRES-GFP. Cell culture medium was replaced with fresh medium after 10 h, and the retrovirus-containing supernatant was collected after an additional 72 h. To infect T cells, naïve CD4+ T cells were first stimulated with anti-CD3 and anti-CD28 antibodies. At 24- and 36-h time points, the activated T cells were infected for 1 h by centrifugation at 1500 × g with 500 μl of viral supernatant in the presence of 10 μg ml−1 polybrene and incubated at 37°C for an additional 1 h before the cells were removed from the viral supernatant and resuspended in the indicated T cell differentiation medium for 4 days.

Immunofluorescence and confocal microscopy. ISO- or Ij2-treated CD4+ T cells incubated with or without 2-APB or Xc for 15 min were fixed in prewarmed 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 5% BSA, the cells were incubated at 4°C overnight with anti-Fas (4C2, Cell Signaling) and anti-PCSK1 (A10-F, Abcam) or anti-PCKR2 (EPR18104, Abcam) primary antibodies. Primary antibodies were detected using DyLight 488- and DyLight 549-labeled secondary antibodies (Abcam). Nuclei were stained with 4',6-diamidino-2-phenylindole (Invitrogen). Human paraffin-embedded lung tumor sections were subjected to immunofluorescence staining. The stained tissue sections were viewed under a Nikon Fluoview FX100 confocal microscope, and images were acquired using an Olympus Fluoview version 1.4a viewer (Olympus Corp, Tokyo, Japan). Images of the cells and sections were captured, and positive areas were analyzed.

RNA-seq analysis. Total RNA was isolated and reverse-transcribed into cDNA to generate an indexed Illumina library, followed by sequencing at the Beijing Genomics Institute (Shenzhen, China) using a BGISEQ platform. High-quality reads were aligned to a mouse reference genome (GRCm38) by Bowtie2. The expression levels of individual genes were normalized to the FKPM (fragments per kilobase million) reads by RNA-seq by an expectation maximization algorithm. Significant differential expression of a gene was defined as a >2-fold expression difference vs. the control with an adjusted P value <0.05. A heat map was analyzed by Gene Ontology using Cluster software and visualized with Java Treeview. DEGs were analyzed by Gene Ontology using the AMIGO and DAVID software. The enrichment degrees of DEGs were analyzed using Kyoto Encyclopedia of Genes and Genomes annotations.

Cell viability assay. LCL-OVA cell viability was measured using a CCK-8 assay according to the manufacturer's instructions. LCL-OVA cells were stimulated with dimethyl sulfoxide (DMSO) or 0.15, 10, 20, or 30 μg ml−1 SB203580 for 72 h in a 96-well plate, and 10 μl of CCK-8 (MultiSciences, Hangzhou, Zhejiang, China) was added per well and incubated for 2 h at 37°C. A multiplate reader was used to measure the absorbance at 450 nm. Cell viability was expressed as the absorbance at 450 nm.

Chromatographic conditions and data acquisition. Mice received an intraperitoneal injection of DMSO or 0.5 or 10 mg kg−1 SB203580. After 0.5, 1, 3, or 6 h, plasma was collected and centrifuged at 12,000 × g for 10 min, and the supernatants were subjected to chromatographic detection. High-performance liquid chromatography analyses were carried out using Agilent 1100 series LC equipment. The elution system was set up as follows: an Inertil ODS-4 column (4.6 × 250 mm, 5 μm) was eluted using a water and methanol gradient at 40°C with a constant flow rate of 1.0 ml min−1 (0–20 min, 50–100% methanol; 20–25 min, 100% methanol). The fluorescence detector was set at 325 nm (excitation wavelength) and 408 nm (emission wavelength). Under these chromatographic conditions, the retention time of SB203580 was 13.0 min. The acquired data were processed using the ChemStation for LC 3D software. To obtain chromatograms, data were extracted from the software and plotted in the Microsoft Excel 2010 program.

Transfer of Th9 cells into IBD mice. WT and Ij2−/− mice were randomized to produce groups with similar average body weights. Acute IBD was induced by administering 2.5% (w/v) DSS (MP Biomedicals, Solon, OH, USA) with a molecular weight of 36,000–50,000 in acidic drinking water for 11 days. The day that the mice started to drink the DSS solution was defined as day 0. Freshly isolated Th9 cells from WT mice were intravenously injected with 2 × 106 CD4+ Fluo4−/Th9, WT-Th9, or Fluo4−/Th9 cells on day 0. For in vivo blockade of IL-9 function, WT mice were intraperitoneally injected with 100 μg of anti-IL-9 (MM99C1, Bio X Cell) every other day. Mouse IgG2a (BD0085, Bio X Cell) was used as the isotype control antibody.

Colon tissue culture and isolation of LPLs. Colon sections were incubated longitudinally and cross-cut into 1 cm patches and washed with PBS. After 24 h, the supernatants were collected, and the production of IL-6, TNF, IL-1β, IL-10, and IL-22 was measured using ELISAs (BioLegend).

For the isolation of LPLs, small intestines were cut open longitudinally after removing Peyer's patches and washed with DMEM. Then, the open small intestines were cut into pieces approximately 5 mm in length, and these pieces were incubated in prewarmed DMEM containing 3% fetal calf serum, 0.2% Hank's solution, 1% RPMI EDTA, and 0.1% collagenase type I (Invitrogen). Collagenase digestion was stopped with Hank's solution and a 0.1% collagen concentration. Then, the small intestine samples were incubated in a solution of 3% DMEM, 0.2% fetal calf serum, 0.025% Hank’s solution, 50 mg ml−1 DNase, and 200 mg ml−1 collagenase II for 5 min, and then the dissociated cells were collected. Finally, the solution containing the digested tissue was passed through a 100-μm cell strainer, and LPLs were isolated with 80% Percoll (GE Healthcare, Uppsala, Sweden) gradient. The sorted LPLs were applied for the following experiments after washing with PBS.

Tumor growth experiments. Bone marrow cells were isolated from Flt3−/− or WT mice, and 1 × 107 cells were injected intravenously into C57BL/6j mice that had received sublethal irradiation (400 rad) 1 day before. The chimeric mice were used for tumor inoculation after 6–8 weeks. A total of 1 × 106 LLC-OVA cells were injected subcutaneously into the chimeric mice or WT mice. In vivo IL-9 neutralization was achieved by intraperitoneal injection of 100 μg of anti-IL-9-neutralizing antibodies (MM99C1, Bio X Cell) every other day following tumor implantation. To evaluate the safety of systematic treatment with low- or high-dose SB203580, mice received intraperitoneal injection of SB203580 (0.5 or 10 mg kg−1) every other day. Body weight was monitored, and the heart, liver, spleen, lungs, and kidneys were collected for histopathological analysis 20 days later. For p38 inhibition in vivo, mice received an intraperitoneal injection of SB203580 (0.5 mg kg−1) every other day following tumor implantation. Tumor size was monitored every other day by Vernier calipers. According to the criteria of the Animal Care and Use Committee of the School of Life Sciences, Uppsala University, when the tumor size was over 8000 mm3, the tumor-bearing mice were euthanized by an intraperitoneal injection of 50 mg kg−1 pentobarbital sodium. TILs were prepared by enzymatic digestion with 1 mg ml−1 collagenase (Sigma-Aldrich), 0.5 mg ml−1 Dnase I, and 25 μg ml−1 hyaluronidase (Sigma-Aldrich) at 37°C for 30 min, followed by Percoll (GE Healthcare) gradient purification. The isolated TILs were restimulated with the OVA323–339 or OVA323–326 peptides at a final concentration of 10 or 20 μg ml−1, respectively, in vitro.
To assess the antimurine effects of Fasl−, Tg9, 5 × 105 B16-OVA or LLC-OVA cells were injected intravenously into C57BL/6 mice on day 0. Then, on days 1 and 6, the mice received an intravenous injection of 2 × 106 effector OT-II Tg9 cells differentiated with or without Jo2. Alternatively, 1 × 106 B16-OVA cells were injected subcutaneously into C57BL/6 mice on day 0. Then, on days 1 and 6, the mice received an intravenous injection of 2 × 106 effector OT-II Tg9 cells differentiated with or without Jo2 or Jo2 combined with SB203580. Lung tumor foci were enumerated 16 days in a blinded manner. To assess the role of IL-9 in the antimurine effects of Fasl−, Tg9, 5 × 105 or 1 × 106 B16-OVA cells were injected intravenously or subcutaneously into B6 mice. Then, on days 1 and 6, the mice received an intravenous injection of 2 × 106 effector OT-II Tg9 cells differentiated with or without Jo2. The tumor-bearing mice with lung metastasis were euthanized by an intraperitoneal injection of 50 mg kg−1 pentobarbital sodium before the onset of delayed action.

**Histopathology.** Heart, liver, spleen, lungs, kidneys, and intestine were dissected from individual mice in groups and immediately fixed in 10% paraformaldehyde. The heart, liver, spleen, lung, kidney, and intestine samples were subjected to hematoxylin–eosin staining.

**Statistical analyses.** Data are expressed as the mean ± standard deviation. An unpaired Student’s t test was used for comparisons between two groups, the log-rank test was used for survival rate analysis, and the Spearman’s rank-order correlation test was used for Pearson’s correlation analysis using the GraphPad Prism 7.0 software. A difference was considered statistically significant if the P value was <0.05.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The data underlying all findings of this study are available from the corresponding author upon reasonable request and are provided as a separate Source Data file. The high-throughput RNA-seqing data have been deposited in the NCBI Sequence Read Archive under the BioProject accession number SRP159792.

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**References**

1. Murphy, K. M. & Reiner, S. L. The lineage decisions of helper T cells. *Nat. Rev. Immunol.* 2, 933–944 (2002).

2. Harrington, L. E., Mangan, P. R. & Weaver, C. T. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Curr. Opin. Immunol.* 18, 349–356 (2006).

3. Sakaguchi, S., Miyara, M., Costantino, C. M. & Haller, D. A. FOXP3+ regulatory T cells in the human immune system. *Nat. Rev. Immunol.* 10, 490–500 (2010).

4. Veldhoen, M. et al. Transforming growth factor-beta ’reprograms’ the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat. Immunol.* 9, 1341–1346 (2008).

5. Dardalhon, V. et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and together with TGF-beta, generates IL-9+ IL-10+Foxp3− effector T cells. *Nat. Immunol.* 9, 1347–1355 (2008).

6. Kaplan, M. H. The transcription factor network in Th9 cells. *Semin. Immunopathol.* 39, 11–20 (2017).

7. Jager, A., Dardalhon, V., Sobel, R. A., Bettelli, E. & Kuchroo, V. K. Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J. Immunol.* 183, 7169–7177 (2009).

8. Staudt, L. et al. Interferon-regulatory factor 4 is essential for the differentiation and pathogenicity of IL-9-producing T cells. *Cell Death Differ.* 18, 619–631 (2011).

9. Meyer Zu Horste, G. et al. Fas promotes T helper 17 cell differentiation and inhibits T helper 1 cell development by binding and sequestering transcription factor STAT1. *Immunity* 35, 556–569 e557 (2018).

10. Steen, R. T. et al. Cell autonomous Fox (Cd95) Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybrids. *Nature* 373, 441–444 (1995).

11. Dhein, J., Walczak, H., Baumler, C., Debatin, K. M. & Krammer, P. H. Autocrine T-cell suicide mediated by Apo-1/Fas(Cd95). *Nature* 373, 438–441 (1995).

12. Pearce, E. L. et al. Control of effector CD4+ T cell function by the transcription factor Eomesoderm. *Science* 302, 1041–1043 (2003).

13. Yazdani, R. et al. Features and roles of T helper 9 cells and interleukin 9 in immunological diseases. *Allergol. Immunopathol. (Madr)* 47, 90–104 (2019).

14. Ilangovan, R., Marshall, W. L., Hua, Y. & Zhou, J. Inhibition of apoptosis by Z-VAD-fmk in SMN-depleted cells. *J. Biol. Chem.* 278, 30993–30999 (2003).

15. Horwitz, D. A., Zheng, S. G., Wang, J. & Gray, J. D. Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3+CD4+Treg. *Eur. J. Immunol.* 38, 912–915 (2008).

16. Xiao, Y. et al. Opposing actions of IL-2 and IL-21 on Th9 differentiation correlate with their differential regulation of BCL6 expression. *Proc. Natl. Acad. Sci. USA* 111, 3508–3513 (2014).

17. Chen, H. et al. mTORC2 controls Th9 polarization and allergic airway inflammation. *Allergy* 72, 1510–1520 (2017).

18. Ulrich, B. J., Verdan, F. F., McKenzie, A. N., Kaplan, M. H. & Olson, M. R. STAT3 activation impairs the stability of Th9 cells. *J. Immunol.* 198, 2302–2309 (2017).

19. Stassen, M. et al. p38 MAP kinase drives the expression of mast cell-derived IL-9 via activation of the transcription factor GATA-1. *Mol. Immunol.* 44, 926–933 (2007).

20. L Sullivan, P. et al. Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J. Biol. Chem.* 272, 21096–21103 (1997).

21. Deng, C. et al. The novel IKK2 inhibitor LY2409881 potently synergizes with histone deacetylase inhibitors in preclinical models of lymphoma through the downregulation of NF-kappaB. *Clin. Cancer Res.* 21, 134–145 (2015).

22. Su, T. et al. PKC-beta controls I kappa B kinase lipid raft recruitment and activation in response to BCR signaling. *Nat. Immunol.* 3, 780–786 (2002).

23. Khadra, N. et al. CD95 triggers Orai1-mediated localized Ca2+ entry, regulates recruitment of protein kinase C (PKC) beta2, and prevents death-inducing signaling complex formation. *Proc. Natl. Acad. Sci. USA* 108, 19072–19077 (2011).

24. Green, L. J. et al. Development and validation of a dual activity biomarker that shows target inhibition in cancer patients receiving enzastaurin, a novel protein kinase C-beta inhibitor. *Clin. Cancer Res.* 12, 3408–3415 (2006).

25. Redig, A. J. & Platania, L. C. The protein kinase C (PKC) family of proteins in cytokine signaling in hematopoiesis. *J. Interferon Cytokine Res.* 27, 623–636 (2007).

26. Dongm, M. et al. GABA uptake-dependent Ca2(+)-signaling in developing olfactory bulb astrocytes. *Proc. Natl. Acad. Sci. USA* 106, 17570–17575 (2009).

27. De Smet, P. et al. Xestospongin C is an equally potent inhibitor of the inositol 1,4,5-trisphosphate receptor and the endoplasmic-reticulum Ca2(+)-pumps. *Cell Calcium* 26, 9–13 (1999).

28. Prasolovier, A. et al. CD95-mediated calcium signaling promotes T helper 17 trafficking to inflamed organs in lupus-prone mice. *Immunity* 45, 209–223 (2016).
44. Bleasdale, J. E. et al. Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. J. Pharm. Exp. Ther. 255, 756–768 (1990).
45. Bennett, C. F. et al. Inhibition of phosphoinositide-specific phospholipase C by manolide. Mol. Pharmac. 32, 587–593 (1987).
46. Le Gallo, M., Poissonnier, A., Blanco, P. & Legembre, P. CD95/Fas, non-apoptotic signaling pathways, and kinases. Front. Immunol. 8, 1216 (2017).
47. Letellier, E. et al. CD95-ligand on peripheral myeloid cells activates Syk kinase to trigger their recruitment to the inflammatory site. Immunity 32, 240–252 (2010).
48. Williams, B. L. et al. Phosphorylation of Tyr319 in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C-gamma 1 and Ras activation. EMBO J. 18, 1832–1844 (1999).
49. Barry, O. P., Kazanietz, M. G., Pratico, D. & FitzGerald, G. A. Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase-dependent pathway. J. Biol. Chem. 274, 7545–7556 (1999).
50. Jash, A. et al. Nuclear factor of activated T cells 1 (NFAT1)-induced permissive chromatin modification facilitates nuclear factor-kappa B (NF-kappa B)-mediated interleukin-9 (IL-9) transactivation. J. Biol. Chem. 287, 15445–15457 (2012).
51. del Arco, P. G., Martinez-Martinez, S., Maldonado, J. L., Ortega-Perez, I. & Redondo, J. M. A role for the p38 MAP kinase pathway in the nuclear shuttling of NFATp. J. Biol. Chem. 275, 13872–13878 (2000).
52. Flanagan, W. M., Cortesky, B., Bram, R. J. & Crabtree, G. R. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352, 803–807 (1991).
53. Roehrl, M. H. et al. Selective inhibition of calcineurin-NFAT signaling by blocking protein-protein interaction with small organic molecules. Proc. Natl. Acad. Sci. USA 101, 7554–7559 (2004).
54. Jager, A. & Kuchroo, V. K. Effector and regulatory T-cell subsets in autoimmunity and tissue inflammation. Scand. J. Immunol. 72, 173–184 (2010).
55. Lu, Y. et al. Th9 cells promote antitumor immune responses in vivo. J. Clin. Invest. 122, 4160–4171 (2012).
56. Seeger, F. H. et al. Inhibition of the p38 MAP kinase in vivo improves number and functional activity of vasculogenic cells and reduces atherosclerotic disease progression. Basic Res. Cardiol. 105, 389–397 (2010).
57. Wang, X. Y., Tang, Q. Q., Zhang, J. L., Fang, M. Y. & Li, Y. X. Effect of SB203580 on pathologic change of pancreatic tissue and expression of TNF-alpha and IL-1-beta in rats with severe acute pancreatitis. Eur. Rev. Med. Pharmac. Sci. 18, 338–343 (2014).
58. Han, J. & Sun, P. The pathways to tumor suppression via route p38. Trends Biochem. Sci. 32, 364–371 (2007).
59. Leelahavanichkul, K. et al. A role for p38 MAPK in head and neck cancer cell growth and tumor-induced angiogenesis and lymphangiogenesis. Mol. Oncol. 8, 105–118 (2014).
60. Steenwinckel, V. et al. IL-13 mediates in vivo IL-9 activities on lung epithelial cells but not on hematopoietic cells. J. Immunol. 178, 3244–3251 (2007).