Cytokine-like molecule CCDC134 contributes to CD8+ T Cell effector functions in cancer immunotherapy

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Abstract

CCDC134 is a poorly characterized secreted protein that may act as an immune cytokine. Here we show that CCDC134 is differentially expressed on resting and activated immune cells and that it promotes CD8<sup>+</sup> T cell activation, proliferation and cytotoxicity by augmenting expression of the T cell effector molecules IFN-γ, TNF-α, granzyme B and perforin. CCDC134 facilitated infiltration of CD8<sup>+</sup> T cells with enhanced cytolytic activity into tumors, demonstrating strong antitumor effects in a CD8<sup>+</sup> T cell-dependent manner. Mechanistically, in CD8<sup>+</sup> T cells exposure to CCDC134 promoted cell proliferation through the JAK3-STAT5 pathway, a classic feature of many cytokines of the common γ-chain (γ<sub>c</sub>) cytokine receptor family. Overall, our results provide evidence that CCDC134 may serve as a member of the γ<sub>c</sub> cytokine family, and they show illustrate its potent antitumor effects by augmenting CD8<sup>+</sup> T cell-mediated immunity.

Precis

Findings offer strong evidence for a new member of the γ<sub>c</sub> cytokine family that mediates powerful support for CD8<sup>+</sup> T cell-mediated immunity, with potential implications for therapeutic applications.
Introduction

The eradication of tumors by the immune system depends on the generation of antigen-specific T cells that can migrate to tumor sites and perform effector functions. CD8+ T cells, also called cytotoxic T lymphocytes (CTLs), play a critical role in the antitumor immune response (1, 2). Upon encountering antigens, CD8+ T cells undergo a program of activation, clonal expansion and differentiation into effector cells (3, 4). These CTLs are capable of killing tumor cells via the granule exocytosis pathway, which involves the release of effector molecules, such as granzyme B and perforin, and the secretion of proinflammatory cytokines, such as IFN-γ and TNF-α (5). It is believed that the perforin/granzyme-mediated pathway of cytotoxicity is the primary mechanism for tumor cell killing by CD8+ T cells (6), although many recent studies have shown that IFN-γ appears to be the most critical molecule for CD8+ T-cell-mediated tumor immunity (7-12). Furthermore, tumor cells themselves are major targets of IFN-γ via the anti-proliferative and anti-metabolic effects of this cytokine as well as the ability of this cytokine to inhibit angiogenesis (13-15).

For processes ranging from activation by antigen to differentiation into effector cells, CD8+ T cells require support from cytokines produced by CD8+ T cells themselves or other immune cells such as CD4+ T cells, B cells or dendritic cells. Many studies have demonstrated that certain cytokines exert overlapping and distinct regulation over CD8+ T cell responses to tumor antigens. The cytokines belonging to the γc cytokine receptor family are particularly important for these CD8+ T cell responses. This cytokine family consists of interleukin-2 (IL-2), IL-4, IL-7, IL-9,
IL-15 and IL-21 and is so named because the receptors for these cytokines share the $\gamma_c$ receptor chain. The cytokines of the $\gamma_c$ family all signal through JAK-STAT pathway. Interestingly, all of these cytokines activate JAK1 and JAK3, and it is believed that JAK3 selectively associates with $\gamma_c$ but not with other cytokine receptors (16-19). Furthermore, IL-2, IL-7, IL-9 and IL-15 primarily activate STAT5, whereas IL-4 primarily activates STAT6 and IL-21 primarily activates STAT3. Among these cytokines, IL-2, the first cytokine to be extensively tested in the clinic for immunotherapy, is a potent growth factor for T lymphocytes and has mainly displayed antitumor effects in the generation and maintenance of CTL responses to tumors. IL-15 is the primary cytokine known to stimulate the maintenance of memory CD8$^+$ T cells, and IL-7 can act in synergy with IL-15 to support the survival and expansion of memory CD8$^+$ T cells. Many studies have demonstrated that some $\gamma_c$ cytokines, including IL-2, IL-7, IL-15 and IL-21, have the potential to expand and prolong CTL responses at different stages of the immune response by promoting the activation, proliferation and effector function of CTLs (18, 20). Therefore, these cytokines have potential roles in adoptive transfer therapy using CTLs (21, 22).

However, the molecular and cellular nature of the tumor immune microenvironment influences the antitumor function of CD8$^+$ T cells and the disease outcome by altering the balance of suppressive and cytotoxic responses in the vicinity of the tumor. To inhibit the antitumor immune response, local tumor cells often produce inhibitory factors, such as TGF-β, IL-10 and galectins (23, 24), or induce a population of suppressive cells, such as regulatory T cells, tumor-associated
macrophages or tolerogenic dendritic cells, to inhibit the activation and cytotoxic responses of CD8\(^{+}\) T cells (25, 26). Therefore, the naturally occurring T cell responses against malignancies are often not sufficient to cause the regression of tumors. However, in recent years, there has been increasing evidence indicating that adoptive cell transfer (ACT) of cells cultured in the presence of a proinflammatory cytokines, particularly \(\gamma_c\) family-cultured tumor-infiltrating CD8\(^{+}\) T cells isolated from tumor tissues, has the potential to expand antitumor CD8\(^{+}\) T cells and enhance CTL killing activity \textit{in vitro} and \textit{in vivo} (27, 28).

CCDC134 was first identified in our lab. CCDC134 is conserved across different species, including human, rat, mouse and dog, but shares no obvious homology with any known proteins. We previously reported that CCDC134 plays an important role in Elk1 transcription regulation and MAPK signal transduction through the Raf-1/MEK/Erk and JNK/SAPK pathways (29).

In the present study, we report that CCDC134 demonstrates functions similar to those of the \(\gamma_c\) cytokine IL-2 by directly promoting activation, proliferation and antigen-specific cytotoxicity of CD8\(^{+}\) T cells \textit{via} the JAK3-STAT5 signaling pathway \textit{in vitro}. Moreover, CCDC134 displayed a strong antitumor effect in a CD8\(^{+}\) T-cell-dependent manner \textit{in vivo}, which was correlated with increased lymphocyte infiltration into the tumor and enhanced cytolytic activity of CD8\(^{+}\) T cells. These results clearly indicate that CCDC134 is a novel stimulator of CD8\(^{+}\) T cells, with the potential to be applied therapeutically during cancer immunotherapy.
Materials and Methods

Mice

Six week-old female C57BL/6 and SCID-beige mice from Vital River Laboratories were used for in vivo experiments. TCR transgenic OT-I mice (H-2b) recognize SIINFEKL from chicken ovalbumin (OVA257-264) and IFN-γ−/− mice (B6.129S7-Iifngtm1Ts/J) were kindly provided by Dr. Zhang MH (Tsinghua University) and Dr. Yin YH (Peking University) respectively.

For generation of CCDC134 transgenic mice, the pcDB-CCDC134 construct was linearized to release the human CCDC134 gene with a CMV promoter, and then the CMV-CCDC134 was injected into B6 mouse zygotes using standard techniques. The zygotes were implanted of into pseudopregnant recipient mice and genotyping founder mice were gotten. After potential founder mice were screen by PCR, the human CCDC134 expression was further identified by in situ hybridization and ELISA. After repetitious passage and identification, two inbred strains of CCDC134 transgenic mice were obtained.

Recombinant CCDC134 Protein Production

The cDNA for human or mouse CCDC134 was subcloned into the pMH expression vector with C-terminal 6xHis tag. Proteins were produced via transient expression in HEK293E cells. For experiments requiring pure proteins, purification was performed by affinity chromatography using with a Ni-Sepharose™ 6 Fast Flow column (GE Healthcare, USA). Peak fractions of >95% pure CCDC134 were
quantified by HPLC, SDS-PAGE and Coomassie blue staining with BSA as a standard. Endotoxin levels (0.023 eu/mg protein) were determined (BioWhittaker Limulus Amebocyte Lysate QCL-1000 pyrogen testing).

**Immunostaining for FACS analysis**

For intracellular cytokine staining, CD8^+^ T cells were maintained in media with brefeldin A (10μg/ml, Sigma-Aldrich) for 5 h. Cells were first stained with surface markers, fixed with 4% formaldehyde for 20 min, and permeabilized with 0.1% triton X-100 (eBiosciences). Intracellular cytokine staining was performed using specific antibodies. Flow cytometry analysis was performed using FACSCalibur and analyzed with FlowJo software (BD Biosciences).

**CFSE proliferation assay**

PBL or isolated CD8^+^ T cells at a concentration of 0.5~50×10^6^/ml were labeled with 2.5 μM CFSE (Invitrogen) in 5% FBS/PBS at 37°C for 5 min. Cells were washed twice, CFSE-labeled cells were incubated in RPMI 1640 with 10% FBS and cell division was detected.

**In vitro CTL assay**

OT-I CD8^+^ T cells were stimulated with anti-CD3/anti-CD28 mAbs *in vitro* for 4 d as effector cells. The EG7 target cells (1×10^4^ cells/200 μl/well) and serial dilutions of effector cells were incubated in a 96-well U-bottomed plate at 37°C for 8 h. Reactions were performed in triplicate at the indicated effector target ratios. The target cells or effector cells alone were used as spontaneous lactate dehydrogenase (LDH)
release control. For the maximum target LDH release, lysis solution was added to the target cells. The supernatants were assayed for LDH release using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). The percentage of cell-mediated cytotoxicity was determined by the following equation: cytotoxicity (%) = (experimental−effector spontaneous−target spontaneous)/(target maximum−target spontaneous )×100.

**Tumor challenge, isolation of tumor-infiltrating lymphocytes and flow cytometry**

Mice were injected s.c. with 2×10^5 B16 cells in the axilla. And rhCCDC134 or PBS as control were injected intraperitoneally (i.p.) once each day when tumor are already established (mean diameter is about 3 mm or average volume of tumors to 16mm^3, 6~10 d). Tumor volumes were measured along two orthogonal axes (a=length, b=width) and calculated as tumor volume = ab^2/2. Tumors were excised and weighed after sacrificing animals.

The individual tumors were homogenized through 200 mesh strainers to obtain single cell suspensions. RBCs were lysed using an ammonium chloride lysis buffer. Tumor-infiltrating lymphocytes (TILs) were isolated from tumor suspensions using Percoll (GE Healthcare). The collected cells were stained with different antibodies and flow cytometry analysis was performed as described previously. The total number of different immune cells per 10^7 of tumor cells was calculated by multiplying the percentage of positive cell population.

**CD8^+ and CD4^+ T cell depletion**
In vivo depletion of CD8+ and CD4+ T cell was performed using blocking mAbs. Each mouse was injected i.p. with 0.5 mg rat anti-CD8 (clone 2.43), anti-CD4 (clone GK1.5) mAbs, or normal rat IgG (Beijing Zsbio. Co.) as control antibody in 200 μl PBS on day 1 prior to rhCCDC134 administration (twice per week). And the depletion of CD4+ and CD8+ T cells was consistently greater than 95% as determined by flow cytometry.

Statistical analysis

Statistical significance of the tumor-free survival plots was determined using the log rank test. A Student's t test and one way ANOVA was applied to compare statistical significance between treatment groups. All analysis was performed using Prism 4 software (GraphPad Software).

Results

CCDC134 is expressed and secreted in activated T cells

We first evaluated whether CCDC134 is expressed in human primary immune cells using RT-PCR. We found that CCDC134 transcripts were present in T cells and dendritic cells (Fig. 1A). Moreover, CCDC134 expression and secretion was induced following the activation of T cells with anti-CD3/anti-CD28 mAbs or PHA (Fig. 1B and C). To further compare the expression levels of CCDC134 mRNA during the activation of CD4+ and CD8+ T cells, we sorted CD4+ and CD8+ T cells from PBMCs of healthy donors and then stimulated these cells with anti-CD3/anti-CD28 mAbs. Increased expression of CCDC134 was detected both in activated CD8+ and CD4+ T cells.
cells. Notably, the expression of CCDC134 was more than five-fold higher in CD8$^+$ T cells than CD4$^+$ T cells. These results indicate that CCDC134 expression may be related to the activation of T cells, especially CD8$^+$ T cells (Fig. 1D).

**CCDC134 directly promotes the activation of CD8$^+$ but not CD4$^+$ T cells**

To address the effects of CCDC134 on T cells, we obtained recombinant human CCDC134 protein (rhCCDC134) from engineered HEK293E cells. We isolated PBLs from human healthy donors, and added rhCCDC134 to these cells stimulated with anti-CD3/anti-CD28 mAbs, and monitored the proliferation of T cells. The results revealed that CCDC134 promoted the proliferation of CD8$^+$ and CD4$^+$ T cells in response to anti-CD3/anti-CD28 mAb stimulation (Fig. 1E).

To further investigate whether CCDC134 directly promotes the proliferation of CD4$^+$ or CD8$^+$ T cells, we isolated CD8$^+$ and CD4$^+$ T cells from human PBL and stimulated them with anti-CD3/anti-CD28 mAbs in the presence or absence of rhCCDC134 (1, 10 or 100 ng/ml). We found that CCDC134 significantly induced the up-regulation of CD25 and CD69, two early and transient T-cell activation markers (30-31), in activated CD8$^+$ T cells (Fig. 1F), but not activated CD4$^+$ T cells (data not shown). Furthermore, the expression of effector molecules including IFN-$\gamma$, perforin and TNF-$\alpha$, was enhanced in these activated CD8$^+$ T cells based on FACS and ELISA analysis. Maximal stimulation was observed with 10 ng/ml of rhCCDC134 (Fig. 1F and G).

In addition, the CCDC134 cDNA sequences from both human and mouse encode
229-amino-acid-long polypeptides, and the homology between their CCDC134 amino acid sequences without a signal peptide is as high as 90%, suggesting that mouse CCDC134 may have the same function as human CCDC134 (Supplementary Fig. S1). To address whether mouse CCDC134 mediates the specific functions in CD8\(^{+}\) T cells, we purified recombinant mouse CCDC134 protein (rmCCDC134). As expected, we found that rmCCDC134 augmented the expression of CD69, CD25 and IFN-\(\gamma\) in mouse CD8\(^{+}\) but not CD4\(^{+}\) T cells, following co-stimulation with anti-CD3/anti-CD28 mAbs (data not shown). Furthermore, rhCCDC134 demonstrated similar effects on mouse splenic CD8\(^{+}\) T cells. The rhCCDC134 stimulation resulted in increase of CD25 and CD69, as well as TNF-\(\alpha\), granzyme B, IFN-\(\gamma\) and perforin expression in CD8\(^{+}\) but not CD4\(^{+}\) T cells of mouse spleen (Fig. 2 and Supplemental Fig. S2A and B). Maximal effects were also observed with 10 ng/ml of rhCCDC134. These results suggest that CCDC134 directly promotes the activation of CD8\(^{+}\) but not CD4\(^{+}\) T cells co-stimulated with anti-CD3/anti-CD28 mAbs in both humans and mice.

**CCDC134 directly promotes CD8\(^{+}\) T cell proliferation and enhances the cytotoxicity of tumor antigen-specific CD8\(^{+}\) T cells in vitro**

To investigate whether CCDC134 directly promote the expansion of CD8\(^{+}\) T cells in a manner independent of other immune cells, purified CD8\(^{+}\) or CD4\(^{+}\) T cells from mouse spleen were labeled with CFSE and then stimulated with anti-CD3/anti-CD28 mAbs plus rhCCDC134. Like the IL-2 stimulation, the CCDC134 significantly promoted the proliferation of CD8\(^{+}\) T cells in a dose-dependent manner (Fig. 3A), but
not CD4$^+$ T cells (Supplemental Fig. S2C).

In addition, we isolated CD8$^+$ T cells from OT-1 mice and stimulated these cells with anti-CD3/anti-CD28 mAbs plus rhCCDC134. Our results revealed that CCDC134 significantly promoted the killing activity of CTLs against EG7 targets and increased the cytotoxicity to levels well above the limit of detection at E:T ratios of 5:1 or higher in a dose-dependent and effector-target ratio-dependent manner (Fig. 3B). Moreover, CCDC134 significantly enhanced the levels of TNF-$\alpha$, granzyme B and IFN-$\gamma$ (Fig. 3C). These results suggested that CCDC134 directly promotes the proliferation and the cytotoxicity of CD8$^+$ T cells.

CCDC134 shows antitumor activity that depends on lymphocytes in vivo

To further understand the activity of CCDC134 in vivo, we established three tumor graft models using the B16 mouse melanoma. Initially, CCDC134-transgenic mice were established, in which human CCDC134 expression was identified by in situ hybridization and ELISA assay (Supplemental Fig.3A and B). Then B16 tumor cells were subcutaneously (s.c.) inoculated into CCDC134-transgenic mice, and the tumor cell growth and survival rates of tumor-bearing mice were evaluated. Significant retardation of established B16 tumor growth was detected and the survival time was markedly prolonged in CCDC134-transgenic mice (Fig. 4A and B).

Next we established B16 graft tumors in C57BL/6 mice. Treatment with rhCCDC134 resulted in a significant suppression of tumor growth in a
dose-dependent manner (Fig. 4C). In addition, CCDC134 also demonstrated strong antitumor activity against EG7 thymoma and Lewis lung adenocarcinoma (LLC-1) cells (Supplemental Fig. S4A-C). To further examine whether the antitumor activity of CCDC134 was dependent on lymphocytes, we established B16 graft tumors in C.B.17 SCID-beige mice. We found that CCDC134 was unable to suppress tumor growth in SCID-beige mice (Fig. 4D), suggesting that the antitumor activity of CCDC134 was dependent on lymphocytes.

**Potent antitumor activity of CCDC134 in vivo is dependent on CD8⁺ T cells**

We first analyzed the absolute number of immune cells, particularly CD8⁺ T cells, at tumor sites after CCDC134 treatment. As expected, CCDC134 administration significantly enhanced migration of CD8⁺ T cells into tumors. Interestingly, greater numbers of CD4⁺ T and B cells, but not NK cells, were also recruited into the tumors after treatment with CCDC134 (Fig. 5A). Enhanced expression of the IFN-γ, granzyme B, perforin and TNF-α was also observed in CD8⁺ T cells from CCDC134-treated mice (Fig. 5B and C). Similarly, we also found that CCDC134 could promote the expression of granzyme B and perforin, but not IFN-γ, in CD4⁺ T cells (Supplemental Fig. S5A and B).

Then we treated C57BL/6 mice with depleting anti-CD8 or anti-CD4 mAbs and monitored CCDC134-induced growth suppression to B16 tumors. Our results significantly revealed that anti-CD8 mAbs completely blocked the antitumor activity of CCDC134. However, much less efficient residual tumor growth was observed in
mice treated by anti-CD4 mAbs (Fig. 5D and E). Surprisingly, after treatment with the anti-CD8 mAb, not only the CCDC134-mediated recruitment of CD8$^+$ T cells, but also the CD4$^+$ T cells and B cells, to the tumors was also diminished (Fig. 5F). However, after CD4$^+$ T cells were depleted, there was no decrease in the CCDC134-recruited CD8$^+$ T cells or B cells to the tumor sites, suggesting that the infiltration of CCDC134-recruited CD8$^+$, but not the CD4$^+$ T cells, into the tumor is essential for the antitumor activity of CCDC134.

**Involvement of IFN-$\gamma$ in the induction of antitumor activity mediated by CCDC134**

IFN-$\gamma$ is the most critical molecule for CD8$^+$ T cell-mediated tumor immunity (8, 9, 15). Therefore, we investigate the role of IFN-$\gamma$ in the CCDC134-induced antitumor effect. Surprisingly, we found that the therapeutic benefit of CCDC134 was absent in IFN-$\gamma^{-/-}$ mice (Fig. 6A). However, we found that IFN-$\gamma$ was not responsible for the CCDC134-induced recruitment of CD8$^+$ T, CD4$^+$ T or B cells to the tumor (Fig. 6B), although depletion of IFN-$\gamma$ abrogated the CCDC134-induced augmentation of lytic activity in CD8$^+$ T cells via blocking the production of granzyme B, perforin and TNF-$\alpha$ (Fig. 6C and D). In addition, the absence of IFN-$\gamma$ also blocked the CCDC134-induced production of granzyme B and perforin in CD4$^+$ T cells (Supplemental Fig. S5C and D). These results suggest that IFN-$\gamma$ is essential for CCDC134-induced CD8$^+$ T cell-mediated tumor immunity.
CCDC134 might be a novel member of the γ_c family

As described above, we found that CCDC134 has similar activity to CD8^+ T cells with IL-2, so we surmise whether CCDC134 shares a co-receptor with IL-2. We first confirmed the fact that CCDC134 can significantly bind to activated CD8^+ T cells by reacting ^125_I-CCDC134 (Fig. 7A), and the binding could be competitively inhibited by increasing concentrations of unlabeled CCDC134, IL-2 or IL-15 (both IL-2 and IL-15 can bind IL-2R), but not the IL-12 as negative control (Fig. 7B).

To investigate whether CCDC134 activate the intracellular signals of IL-2R, we determined the phosphorylation of JAK3, STAT3 and STAT5 in CCDC134-activated CD8^+ T cells. We observed that like IL-2, CCDC134 significantly promoted STAT5 phosphorylation. In contrast, unlike IL-6 (a positive control for p-STAT3), there was no significant change in the STAT3 phosphorylation after CCDC134 treatment. Furthermore, CCDC134 selectively induced an increase in the phosphorylation of JAK3 (Fig. 7C).

Moreover, JAK3 inhibitor (JAK3-I) completely blocked the CCDC134-mediated increase of p-STAT5 but not p-STAT3 (Fig. 7D). However, we found that JAK3-I blocked the IL-2-mediated increase in both p-STAT5 and p-STAT3, as it is believed that IL-2-induced activation of CD8^+ T cells is dependent on either the JAK3-STAT5 or JAK3-STAT3 pathway (32).

Next, we assessed the role of JAK3-STAT5 signaling in mediating the proliferative effects of CCDC134 on CD8^+ T cells. As expected, the inhibitors of
JAK3 (JAK3-I) or STAT5, but not the inhibitor of STAT3, completely blocked CCDC134-induced proliferation of CD8⁺ T cells (Fig. 7E), suggesting that activation of JAK3-STAT5 is required to stimulate the proliferation of CD8⁺ T cells mediated by CCDC134.

**Discussion**

In the present study, we identified a previously unknown γc-related protein, CCDC134, which significantly augments the activation, proliferation and cytotoxicity of CD8⁺ T cells *in vitro* and exerts the ability to suppress tumor growth in a CD8⁺ T-dependent manner *in vivo*.

So far, the physiological bioactivity of CCDC134 as a classical secreted protein remains unclear. We previously identified that CCDC134 could inhibit MAPK-mediated Elk1 transcriptional activity in HeLa cells (29). CCDC134 also serve as a regulator of hADA2a (human alteration/deficiency in activation 2a) in acetyltransferase activity, DNA damage-induced apoptosis and cell cycle arrest (33). Recently, it was found that CCDC134 inhibit the migration and invasion of tumor cells via the MAPK pathway (34).

As CCDC134 is expressed by many lineages of immune cells and can particularly be upregulated and secreted by activated T cells, we hypothesized that the function of CCDC134 might involve T cells. Therefore, we selectively addressed whether CCDC134 was involved in the function of CD4⁺ or CD8⁺ T cells. Initially, our results
clearly demonstrated that CCDC134 was capable of promoting TCR signaling-induced proliferation of CD4$^+$ and CD8$^+$ T cells in a PBMC culture system. Notably, the effect of CCDC134 on promoting the proliferation of CD8$^+$ T cells was stronger than the effect on CD4$^+$ T cells. Subsequently, we isolated CD4$^+$ or CD8$^+$ T cell populations and investigated the direct bioactivity of CCDC134 on them. These results revealed that CCDC134 significantly promoted activation and proliferation of CD8$^+$ T cells but not CD4$^+$ T cells. More importantly, we showed that CCDC134 directly promoted the expansion and antigen-specific cytotoxicity of CD8$^+$ T cells by augmenting the expression of IFN-γ, TNF-α, granzyme B and perforin in vitro.

We have explored the antitumor role of CCDC134 in vivo. The results revealed that CCDC134 administration led to strong inhibition of tumor growth and prolonged survival in CCDC134-transgenic mice and C57BL/6 mice, but not in SCID mice. These results suggested that the antitumor activity of CCDC134 is immune system-dependent. To explore the possible antitumor mechanism of CCDC134, we evaluated the number and activation state of tumor-infiltrating CD8$^+$ T, CD4$^+$ T, B and NK cells. Our results showed that CCDC134 not only significantly promoted the infiltration of activated CD8$^+$ T, but also promoted infiltration of CD4$^+$ T and B, but not NK cells, into the tumor. However, only the depletion of CD8$^+$ T cell, but not the CD4$^+$ T cell, could completely block the antitumor activity of CCDC134. These data suggest that the antitumor activity of CCDC134 is dependent on CD8$^+$ but not CD4$^+$ T cells. Surprisingly, the intratumoral accumulation of CD4$^+$ T and B cells following CCDC134 treatment was also reduced with anti-CD8 mAb treatment,
suggesting that recruitment of CD4$^+$ T and B cells induced by CCDC134 may be
dependent on CD8$^+$ T cells. Richard et al. previously demonstrated that CD8$^+$ T cells
are capable of recruiting other immune cells into a tumor and proposed the theory that
in addition to the contact-mediated lytic mechanism, the control of tumor growth by
CD8$^+$ T lymphocytes is partially mediated by the recruitment of other host effector
cells to the tumor site (35-36).

IFN-γ is a cytokine known to play an essential role in coordinating tumor immune
responses by regulating the migration of T cells into tumor tissue (37). The IFN-γ
produced by tumor-infiltrating T cells plays two distinct roles in antitumor activity:
the activation of antitumor T cells and the generation of direct tumoricidal activity via
the production of inducible nitric oxide synthetase (15, 38). In this study, we found
that CCDC134 significantly promoted the expression of IFN-γ in tumor-infiltrating
CD8$^+$ but not CD4$^+$ T cells. Furthermore, upregulation of granzyme B, perforin and
TNF-α in tumor-infiltrating CD8$^+$ or CD4$^+$ T cells by CCDC134 was abolished, and
CCDC134 antitumor activity was also blocked in IFN-γ$^{-/-}$ mice. Based on these
findings, it is likely that the CCDC134-driven antitumor activity of CD8$^+$ T cells is
mainly mediated by IFN-γ.

In addition, we used the IL-2, a member of the γc family reported to function as a
CD8$^+$ T cell growth factor (39), as positive control, our results clearly revealed that
many similar activities were showed between CCDC134 and IL-2, such as they
directly promoted the activation and proliferation of CD8$^+$ T cells, and enhanced the
cytotoxicity of tumor antigen-specific CD8$^+$ T cells in vitro and in vivo. Moreover, it
has been shown that both CCDC134 and IL-2 could activate CD8\(^+\) T cells through the JAK3-STAT5 signaling pathway, the downstream signaling pathway of IL-2R \(\gamma_c\) (40). Importantly, two members of the \(\gamma_c\) cytokine family, IL-2 and IL-15 could significantly block the CCDC134 binding to activated CD8\(^+\) T cells. Based on these data, CCDC134 may signal through the \(\gamma_c\) chain as a potential member of the \(\gamma_c\) cytokine family. However, the receptor for CCDC134 remains to be confirmed.

In summary, our results suggest that CCDC134 represents a potential member of the \(\gamma_c\) cytokine family, as it demonstrated critical effects on the activation, expansion and effector functions of CD8\(^+\) T lymphocytes. Moreover, CCDC134 may serve as an attractive candidate to promote antitumor activity in cancer immunotherapy.

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Legends for all figures

**Figure 1.** Expression and function of CCDC134. A, RT-PCR analysis of CCDC134 mRNA expression in different types of primary immune cells. r, resting; a, activated; im, immature; m, mature; DC, dendritic cells. B, CCDC134 mRNA expression was analyzed by RT-PCR in human PBLs. GAPDH was used as an internal control, and IL-2 as a positive control. C, CCDC134 protein expression in culture supernatants of human PBLs was detected by immunoblotting and ELISA assays. Medium was used as negative control. D, Quantitative RT-PCR analysis of CCDC134 mRNA levels from purified human CD4$^+$ or CD8$^+$ T cells stimulated with anti-CD3/anti-CD28 mAbs for various times. E, Proliferation of CFSE-labeled human PBLs stimulated with anti-CD3/anti-CD28 mAbs in the presence or absence of various amounts of recombinant human CCDC134 protein (rhCC: 1 or 10 ng/ml) or positive control (rhIL-2: 10 ng/ml) for 3 d. F & G, The expression of activation molecules CD25 and CD69 (24 h) and the effector molecule IFN-γ (72 h) were analyzed in purified CD8$^+$ T cells stimulated with anti-CD3/anti-CD28 mAbs in the presence or absence of rhCC (1, 10 or 100 ng/ml) by FACS. Open peaks with the black dotted lines represent positive staining, while light gray peaks represent isotype controls (F). After 72 h, the supernatants were monitored for TNF-α, IFN-γ and perforin in triplicate by ELISA (G). * p<0.05, ** p<0.01, *** p<0.001 compared with the negative control (0h or 0 ng/ml rhCC).
**Figure 2.** CCDC134 promotes the expression of activation and effector molecules in mouse CD8$^+$ T cells. Purified CD8$^+$ T cells from the spleens of B6 mice were stimulated with anti-CD3/anti-CD28 mAbs in the presence or absence of rhCC (1, 10 or 100 ng/ml) or rhIL-2 (20 ng/ml). At the indicated times, stimulated cells were analyzed for the expression of CD25 and CD69 (24 h) (**A**) or TNF-α, IFN-γ, granzyme B and perforin (72 h) (**B**) by FACS. Open peaks with the black dotted line represent positive staining, and light gray peaks represent isotype controls. **C,** The mRNA expression of TNF-α, granzyme B and IFN-γ were analyzed by qRT-PCR. **D,** The levels of TNF-α, IFN-γ, granzyme B in the culture supernatant were measured by ELISA. * p<0.05, ** p<0.01, *** p<0.001 compared with the negative control (0 ng/ml rhCC). The experiment was carried out in triplicate.

**Figure 3.** CCDC134 induces proliferation and enhances the antigen-specific cytotoxicity of mouse CD8$^+$ T cells. **A,** Proliferation of CFSE-labeled mouse splenic CD8$^+$ T cells stimulated with anti-CD3/anti-CD28 mAbs in the presence or absence of rhCC (0.1, 1, 10 or 100 ng/ml) or rhIL-2 (1 ng/ml) for 48, 72 and 96h. **B,** A LDH-based cytotoxicity assay was done using CD8$^+$ T cells from the spleens of OT-I mice as effectors. Following treatment with anti-CD3/anti-CD28 mAbs in the presence or absence of rhCC (1, 10 or 100 ng/ml) or rhIL-2 (20 ng/ml) for 4 d, CD8$^+$ T cells were incubated for 8 h with EG7-OVA targets at various E:T ratios to determine antigen-specific CTL activity. **C,** The levels of TNF-α, granzyme B and IFN-γ in the co-culture supernatant were measured by ELISA. Data are shown as the mean±SD. *
p<0.05, ** p<0.01, *** p<0.001 compared with the negative control (PBS). One representative experiment of three is shown.

Figure 4. Potent antitumor activity of CCDC134 in vivo is dependent on lymphocytes. A&B, Inhibition of in vivo B16 tumor growth in the CCDC134-transgenic mice model. CCDC134-transgenic mice or syngeneic wild-type mice were injected s.c. with 2×10^5 B16 tumor cells, and tumor growth (A) and survival rate (B) were monitored. C&D, In vivo B16 tumor growth following treatment with rhCC or PBS in wild-type C57BL/6 mice (C) or SCID-beige mice (D). Recipient mice received 2×10^5 B16 tumor cells s.c. in axilla. Beginning on day 9, mice were injected i.p. with rhCC or PBS as a negative control once each day. The tumor growth curves are shown. Data are shown as the mean±SD. * p<0.05, ** p<0.01, *** p<0.001 compared with wild-type mice or PBS-treated mice. One representative experiment of three is displayed.

Figure 5. Involvement of CD8^+ T cells in the induction of antitumor activity by CCDC134. A-C, B16 tumor-bearing mice were treated as outlined in Figure 4C. On day 18 post-tumor inoculation, TILs were isolated from treated mice with rhCC or PBS, and the absolute number of immune cells in tumor site were calculated (A). And single-cell suspensions from spleens were restimulated with PMA and ionomycin for 6 h, and then stimulated cells were analyzed for the expression CD25, TNF-α, IFN-γ, granzyme B and perforin by FACS analysis of gated CD8^+ T cells. B depicts
representative results; C depicts statistical results. * p<0.05; ** p<0.01; *** p<0.001.

D-F. B16 tumor-bearing mice were treated as outlined in Figure 4C. On day 9 post-tumor inoculation, mice were injected i.p. with 0.5mg either rat anti-CD4, anti-CD8 mAbs or rat IgG. The tumor growth curve (D) and the established tumors (E) were shown. The absolute number of TILs was calculated (F). Data are shown as the mean±SD. *,# p<0.05; **,## p<0.01; ***,### p<0.001. *, comparison between PBS-treated and CCDC134-treated mice, #, comparison between CCDC134-treated mice with and without CD8+ T cell depletion. One representative experiment of two is shown.

**Figure 6.** The ability of CCDC134 to suppress tumor growth is abolished in IFN-γ−/− mice. B16 tumor-bearing IFN-γ−/− mice were treated as outlined in Figure 4C. A, The tumor growth curve is shown. B, On day 19 post-tumor inoculation, TILs were isolated and the absolute number of immune cells in the tumor site was calculated.

C&D. The expression CD25, TNF-α, IFN-γ, granzyme B and perforin was detected by FACS analysis of gated CD8+ T cells as outlined in Figure 5B. C depicts representative results; D depicts statistical results. Data are shown as the mean±SD. *, comparison between C57BL/6 mice treated and non-treated with CCDC134; #, comparison between CCDC134-treated C57BL/6 mice and IFN-γ−/− mice. *,# p<0.05; **,## p<0.01; ***,### p<0.001. WT, wild type C57BL/6 mice. One representative experiment of two is shown.
**Figure 7.** CCDC134 binds activated CD8+ T cells via IL-2R, and induces JAK3-STAT5 phosphorylation in CD8+ T cells. **A,** Binding curves was obtained with equivalent quantities of 125I-CCDC134 and various activated CD8+ T cells. The y-axis represents the radioactivity of the binding, and the x-axis represents the logarithmic form of activated CD8+ T cells number. **B,** For competitive binding assays, equivalent quantities of activated CD8+ T cells and 125I-CCDC134 were incubated with varying quantities of unlabeled CCDC134, IL-2, IL-15 or IL-12 as negative control. The y-axis represents the radioactivity of the specific binding complexes, and the x-axis represents the concentration of the competitors. **C,** Mouse CD8+ T cells were stimulated with anti-CD3/anti-CD28 mAbs for 24 h, and then rhCC (1, 10 or 100 ng/ml), rhIL-2 or rhIL-6 (20 ng/ml) were added. After 5 or 15 min, pY-STAT and pY-JAK were analyzed by western blot. For verification of the protein expression level, each blot was reprobed with anti-total STAT and anti-β-actin. **D,** Mouse CD8+ T cells were pre-treated with (gray filled) or without (opened) a JAK3-specific inhibitor (40 μM) for 2 h, followed by stimulation with anti-CD3/anti-CD28 mAbs for 24 h and the addition of rhCC or rhIL-2. After 5 or 15 min, the phosphorylation of STAT3 and STAT5 was examined by FACS. **E,** The CFSE-labeled mouse CD8+ T cells were pre-treated with or without specific inhibitors or STAT3 (40 μM), STAT5 (low dose: 40 μM, high dose: 100 μM) or JAK3 (40 μM) for 2 h. The cells were stimulated with anti-CD3/anti-CD28 mAbs in the presence or absence of rhCC or rhIL-2 for 72 or 96 h. Cell divisions were then analyzed by flow cytometry. Data shown represent the results at 72 h. One representative experiment of three is shown.
Figure 2

A

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<th>rhCC (ng/ml)</th>
<th>rhIL-2 (ng/ml)</th>
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Counts

CD69: 46.2%  55.2%  55.2%  42.7%  58.2%
CD25

B

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<tr>
<td>200</td>
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Counts

TNF-α: 31.8%  43.0%  43.6%  27.2%  46.8%
Granzyme B: 23.2%  47.3%  52.0%  34.9%  33.5%
INF-γ: 14.4%  35.1%  38.4%  24.5%  29.8%
Perforin

C

D
Figure 3

A

<table>
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48h

Counts

CFSE

72h

96h

Counts

CFSE

B

- PBS
- rhCC 100ng/ml
- rhIL-2 20ng/ml

Lysis (%)

E:T = 10:1

C

- TNF-α (pg/ml)
- GranB (pg/ml)
- IFN-γ (pg/ml)

rhCC: 0 1 10 100 (ng/ml)
rhIL-2: 0 0 0 20 (ng/ml)

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Figure 4

(A) tumor volume (mm$^3$) vs. days after B16 implantation for wild type (n=19), CCDC134$^{+/+}$ (F0&1) (n=8), and CCDC134$^{+/+}$ (F0&15) (n=13).

(B) percent survival vs. days after B16 implantation for wild type (n=19), CCDC134$^{+/+}$ (F0&1) (n=8), and CCDC134$^{+/+}$ (F0&15) (n=13).

(C) tumor volume (mm$^3$) vs. days after B16 implantation for PBS, rhCC 5µg, and rhCC 20µg (n=8~11).

(D) tumor volume (mm$^3$) vs. days after B16 implantation for PBS and rhCC 20µg (n=6~8).
Cytokine-like molecule CCDC134 contributes to CD8+ T Cell effector functions in cancer immunotherapy

Jing Huang, Lin Xiao, Xiaoting Gong, et al.

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