IL-7Rα glutamylation and activation of transcription factor Sall3 promote group 3 ILC development

Benyu Liu1,2, Buqing Ye1, Xiaoxiao Zhu3, Guanling Huang1,2, Liuliu Yang1,2, Pingping Zhu1, Ying Du1, Jiayi Wu1,2, Shu Meng3, Yong Tian2,3 & Zusen Fan1,2

Group 3 innate lymphoid cells (ILC3) promote lymphoid organogenesis and potentiate immune responses against bacterial infection. However, how ILC3 cells are developed and maintained is still unclear. Here, we show that carboxypeptidase CCP2 is highly expressed in common helper-like innate lymphoid progenitors, the progenitor of innate lymphoid cells, and CCP2 deficiency increases ILC3 numbers. Interleukin-7 receptor subunit alpha (IL-7Rα) is identified as a substrate of CCP2 for deglutamylation, and IL-7Rα polyglutamylation is catalyzed by polyglutamylases TTLL4 and TTLL13 in common helper-like innate lymphoid progenitors. IL-7Rα polyglutamylation triggers STAT5 activation to initiate transcription factor Sall3 expression in common helper-like innate lymphoid progenitors, which drives ILC3 cell differentiation. Moreover, Ttll4−/− or Ttll13−/− mice have reduced IL-7Rα polyglutamylation and Sall3 expression in common helper-like innate lymphoid progenitors. Importantly, mice with IL-7Rα E446A mutation have reduced Sall3 expression and ILC3 population. Thus, polyglutamylation and deglutamylation of IL-7Rα tightly controls the development and effector functions of ILC3s.
Innate lymphoid cells (ILCs) are part of the innate immune system1–3. ILCs reside in the mucosal tissues and respond rapidly to pathogen infection or tissue damage via germ line-encoded receptors4–5. ILCs can be categorized into three groups based on their signature effector cytokines analogous to the classification of CD4+ helper T-cell subsets6. Group 1 innate lymphoid cells (ILC1), including natural killer (NK) cells and ILC1s, function in the immune response to intracellular pathogens via secreting interferon-γ (IFN-γ)7. Group 2 innate lymphoid cells (ILC2), including natural helper cells, nuocytes and innate helper 2 cells, enhance the resistance to helminth infection through secreting type 2 T helper (Th2) cytokines8,9. Group 3 innate lymphoid cells (ILC3), including lymphoid tissue inducer (LTI) cells, natural cytotoxicity receptor positive (NCR+) and NCR tissue inducer (LTi) cells, natural cytotoxicity receptor positive (CCPs)26. Misregulations of glutamylation contribute to several physiological abnormalities. CCP1 deactivation causes a complete loss of ILC3s but not ILC1s or ILC2s. Of note, the cytokine receptor chain IL-7R (Lin−CD45+RORγt−) receptors, such as CD4+ ILC3s, NKp46+ ILC3s, and CD4−NKP46− ILC3s (DN ILC3s)30. We then determined changes of NKP46+ ILC3s (Lin−CD45+RORγt−NKP46+) and NKP46− ILC3s (Lin−CD45+RORγt−NKP46−) in C. rodentium−/− mice. We observed that both of NKP46+ ILC3s and NKP46− ILC3s were markedly increased in C. rodentium−/− mice, but not in other CCR-deficient mouse strains (Fig. 1b and Supplementary Fig. 1d). These observations were further verified by immunofluorescence staining (Fig. 1c). By contrast, CCP2-deficient mice displayed reduced numbers of ILC1s and ILC2s (Supplementary Fig. 1e, f).

NKp46+ ILC3s substantially secrete IL-2213, 31, which has a crucial function in the early host defense against Citrobacter (C.) rodentium infection. As expected, IL-22 secreting (IL-22+ILC3s) were three times increased in the small intestine of C. rodentium−/− mice compared to that of littermate wild-type (WT) mice (Fig. 1d and Supplementary Fig. 1g). However, these IL-22+ ILC3s were unchangeable in other CCR-deficient mouse strains (hereafter we used Ccp6 KO mice as a negative control) (Fig. 1d). We next infected Ccp2−/− or Ccp6−/− mice with C. rodentium. We noticed that Ccp2−/− mice were more resistant to C. rodentium infection compared with their littermate WT mice (Fig. 1e–g). By contrast, Ccp6−/− mice had comparable bacterial loads compared to their littermate WT mice. Furthermore, Ccp2−/− mice displayed increased numbers of IL-22+ ILC3s in the small intestine after C. rodentium infection (Fig. 1h). In addition, higher expression of Il22 messenger RNA (mRNAs) in Ccp2−/− ILC3s was further confirmed with C. rodentium challenge (Fig. 1i). Consistently, with IL-23 stimulation, Ccp2−/− ILC3s produced much higher levels of IL-22 protein than WT ILC3s (Fig. 1j). By contrast, Ccp6−/− mice had no such effect. Taken together, CCP2 deficiency causes an increased number of ILC3s that enhance clearance of C. rodentium.

**Results**

CCP2 deficiency potentiates ILC3 differentiation from CHILPs. We next analyzed expression patterns of CCP members in the mouse hematopoietic system. We found that Ccp2 displayed distinct expression profiles in different hematopoietic cell populations and their progenitors (Fig. 2a). Of note, Ccp2 was highly expressed in the CHILPs and ILC3s (Fig. 2a). Intriguingly, CCP2 deficiency led to reduced numbers of CHILPs, whereas more ILCPs in BM (Fig. 2b and Supplementary Fig. 1h), suggesting CCP2 was involved in the development of ILC3s from the stage of CHILPs. We then conducted in vitro differentiation assays. We isolated CHILPs from Ccp2+/+ and Ccp2−/− mice and cultured them with OP9 feeder cells in the presence of murine IL-7 (25 ng/ml, Peprotech) and SCF (25 ng/ml, Peprotech). We noticed that Ccp2−/− CHILPs generated more ILC3s compared to Ccp2+/− CHILPs (Fig. 2c, d and Supplementary Fig. 1i). Moreover, overexpression of CCP2 dramatically reduced the formation of ILC3s, indicating that CCP2 was implicated in the development of ILC3. CoCl2 is an agonist for CCP family proteins32, and phenanthroline (Phen) is their pan inhibitor26.

CCP2 deficiency increases ILC3 numbers. We previously demonstrated that deficiency in CCP5 or CCP6 leads to susceptibility to virus infection29. CCP5 and CCP6 are required for the activation of TF IRF5 and IFN induction. We, therefore, sought to explore whether glutamylation was involved in the development of ILCs and their defense against bacterial infection. We used previously established Ccp1−/− knockout (KO) mice and further validated deletion of these genes in mouse bone marrow (BM) (Supplementary Fig. 1a). We analyzed ILC3s (Lin−CD45+RORγt+) in the small intestine lamina propria in all six deficient mouse strains and found that the number of ILC3 cells was significantly increased in Ccp2−/− mice, but not in other CCR KO mouse strains (Fig. 1a and Supplementary Fig. 1b, c). ILC3 cells can be divided into a set of subpopulations according to their expression of CD4 and NKp46 (encoded by Ncr1) receptors, such as CD4+ ILC3s, NKp46+ ILC3s, and CD4−NKP46− ILC3s (DN ILC3s)30. We then determined changes of genes via secreting interferon-γ (NCR+) and NCR tissue inducer (LTi) cells, natural cytotoxicity receptor positive (CCPs)26. Misregulations of glutamylation contribute to several physiological abnormalities. CCP1 deactivation causes a complete loss of ILC3s but not ILC1s or ILC2s. Of note, the cytokine receptor chain IL-7R (Lin−CD45+RORγt−) receptors, such as CD4+ ILC3s, NKp46+ ILC3s, and CD4−NKP46− ILC3s (DN ILC3s)30. We then determined changes of CCP6 deficiency in CHILPs and all ILCs, and forms a heterodimer with the common γ-chain of IL-2R or thymic stromal lymphopoietin (TSLP) receptor to detect IL-7 and TSLP, respectively14. However, how IL-7R signaling regulates the ILC development and/or maintenance still remains elusive.

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**Fig. 1** CCP2 deficiency increases ILC3 numbers. 

**a** Percentages of ILC3s (Lin<sup>−</sup>CD45<sup>−</sup>ROR<sup>γ</sup><sup>+</sup>) in small intestine lamina propria from wild-type (WT), 

C<sup>cp2<sup>/−</sup></sup>, C<sup>cp2<sup>/−</sup></sup>, C<sup>cp3<sup>/−</sup></sup>, C<sup>cp4<sup>/−</sup></sup>, C<sup>cp5<sup>/−</sup></sup> and C<sup>cp6<sup>/−</sup></sup> mice were analyzed by flow cytometry. 

**b** Analysis of NKp46<sup>+</sup> ILC3s (Lin<sup>−</sup>CD45<sup>−</sup>ROR<sup>γ</sup><sup>+</sup>NKp46<sup>+</sup>) and NKp46<sup>−</sup> ILC3s (Lin<sup>−</sup>CD45<sup>−</sup>ROR<sup>γ</sup><sup>+</sup>NKp46<sup>−</sup>) from C<sup>cp2<sup>/−</sup></sup> and C<sup>cp2<sup>/−</sup></sup> mice by flow cytometry. 

**c** Analysis of ILC3s in C<sup>cp2<sup>/−</sup></sup> and C<sup>cp2<sup>/−</sup></sup> small intestines by immunofluorescence staining. 

Arrowhead denotes ILC3 cells. Scale bars, 50 μm. 

**d** Analysis of IL-22<sup>+</sup> ILC3 in WT, C<sup>cp2<sup>/−</sup></sup> and C<sup>cp6<sup>/−</sup></sup> small intestines after IL-23 stimulation. Cells were gated on Lin<sup>−</sup>IL-22<sup>+</sup>. 

**e-g** C. rodentium titers in spleen, liver, and fecal for each group. 

**h** Analysis of IL-22<sup>+</sup> ILC3 in WT, C<sup>cp2<sup>/−</sup></sup> and C<sup>cp6<sup>/−</sup></sup> small intestines after C. rodentium infection. 

**i-j** IL-22 expression was detected by real-time qPCR after C. rodentium infection. 

1 x 10<sup>4</sup> ILC3s (Lin<sup>−</sup>CD45<sup>−</sup>CD90<sup>+</sup>) isolated from WT, C<sup>cp2<sup>/−</sup></sup> and C<sup>cp6<sup>/−</sup></sup> intestines were cultured at 37 °C in vitro for 24 h in the presence of IL-23. 

IL-22 was examined by ELISA. 

n = 6 per group. *P < 0.05, **P < 0.01 (Student’s t-test). NS, no significant. Data are representative of three independent experiments. Error bars in **a**, **b**, and **d-j** indicate s.d.
Fig. 2 CCP2 deficiency potentiates ILC3 differentiation from CHILPs. a Total RNAs were extracted from hematopoietic populations. Indicated gene expression levels of Ccp1, Ccp2, Ccp3, Ccp4, Ccp5, and Ccp6 were examined by real-time qPCR. b Gating strategies and flow cytometry analysis of CLP (Lin−IL-7Rxα−Sca-1−c-Kit−), dILP (Lin−IL-7Rxα−Sca-1−c-Kit+αβ+), CHILP (Lin−IL-7Rxα−Flt3−CD25−αβ+), and ILCP (Lin−IL-7Rxα−αβ+PLZF+) in BM from Ccp2+/- and Ccp2−/− mice. n = 6 per group. Numbers of indicated cells in Ccp2+/- and Ccp2−/− mice were calculated as means±s.d. c, d CHILPs were isolated from Ccp2+/- or Ccp2−/− mice and cultured on OP9 cells in the presence of SCF and IL-7 for 12 days. Percentages and total numbers of ILC3s were examined by flow cytometry, gated on CD45−Lin−RORγt+. The CCP agonist CoCl2 (10 μM) and antagonist Phen (2 μM) were added for in vitro differentiation assays. e Apoptosis of CHILPs from Ccp2+/- and Ccp2−/− mice was analyzed with Annexin V/PI. Apoptotic cells were calculated and shown as means±s.d. f Active caspase 3 in CHILPs from Ccp2+/- and Ccp2−/− mice was detected by flow cytometry. g Schematic representation for BM transplantation assays. h i 5 × 10⁴ CD45.2− LSK from Ccp2+/- or Ccp2−/− mice with 5 × 10⁶ CD45.1− helper cells were transplanted into lethally irradiated CD45.1− recipients. After 8 weeks, percentages of CHILPs, ILCP, and ILC3s in chimeras were checked by FACS. n = 6 for each group. k A 50/50 mixture of CD45.1− wild-type and CD45.2− Ccp2+/- or Ccp2−/− bone marrow was transplanted into lethally irradiated CD45.1− recipients. The ratio of CD45.1− to CD45.2− ILC3s in chimeras (n = 6) were analyzed by gating on CD45.2−Lin−RORγt− (Ccp2+/- or Ccp2−/−) and CD45.1−Lin−RORγt− (WT). *P < 0.05, **P < 0.01 (Student’s t-test). Data are representative of three independent experiments. Error bars in a-f and h-k indicate s.d.
As expected, CoCl₂ treatment suppressed the generation of ILC3s, whereas Phen treatment increased the formation of ILC3s (Fig. 2c, d). Finally, Ccp2⁻/⁻ CHILPs did not undergo apparent apoptosis (Fig. 2e, f). Altogether, polyglutamylation is required for the differentiation of ILC3s from their progenitor CHILPs.

**Cell-intrinsic modulations of ILC3 differentiation by CCP2.**

We next sought to determine whether Ccp2 deficiency-mediated ILC3 development was intrinsic or extrinsic. We transfected CD45.2⁺ Ccp2⁻/⁻ or Ccp2⁺/⁺ BM cells into lethally irradiated CD45.1⁺ recipients (Fig. 2g). Eight weeks after transplantation, Ccp2⁻/⁻ BM transferred recipients displayed reduced number of CHILPs but increased counts of ILCPs compared with those of Ccp2⁺/⁺ BM transplantation (Fig. 2h, i). ILC3s were consequently increased in Ccp2⁻/⁻ BM transferred recipients (Fig. 2j). We also performed competitive BM transplantation assays. We transfected a 1:1 mixture of CD45.1⁺ WT and CD45.2⁺ Ccp2⁻/⁻ or Ccp2⁺/⁺ BM into lethally irradiated recipient mice (Fig. 2k). Eight weeks after transplantation, reconstituted recipients showed increased numbers of ILC3s (Fig. 2l). Collectively, Ccp2 is an intrinsic factor in the regulation of ILC3 development.

**IL-7Rα is a substrate of CCP2 in CHILPs.**

To further explore the molecular mechanism of Ccp2-mediated ILC3 differentiation, we analyzed lysates of Ccp2⁺/+ and Ccp2⁻/⁻ BM by immunoblotting with a glutamylation-specific antibody GT335. The antibody GT335 specifically recognizes the branch points of glutamate side chains and detects all glutamylation forms of target proteins. After immunoblot analysis, one band around 60 kD appeared in the lane of Ccp2-deficient BM lysates (Fig. 3a). This band was undetectable in the corresponding lane from the littermate control BM lysates. Thus, this band could be a potential candidate substrate for Ccp2. To identify the candidate substrates of Ccp2, we generated an enzymatically inactive mutant of Ccp2 (Ccp2-mut) through H425S and E428Q mutations as previously described. WT Ccp2 (Ccp2-wt) and Ccp2-mut were immobilized with Anti-Myc antibody by western blotting. Arrowhead denotes the differential band. Recombinant Ccp2-wt and enzymatic inactive Ccp2 mutant (Ccp2-mut) were immobilized with Affi-gel10 resin and assessed by addition of Ccp2⁻/⁻ BM lysates. The eluted fractions were resolved by SDS-PAGE, followed by silver staining. M: molecular weight marker. A differential band of ~60 kD appeared in Ccp2-mut lane and was cut for mass spectrometry. The peptide sequences of the band were analyzed by LC-LTQ MS/MS are shown in the bottom graph.
IL-7Rα) (Fig. 3c). By contrast, Flag-CCP2-mut failed to precipitate Myc-tagged extracellular segment of IL-7Rα (Fig. 3c). Consistently, glutamylated GST-tagged intracellular segment of IL-7Rα protein could pull down MBP-CCP2-mut by a pulldown assay (Fig. 3d), suggesting the intracellular segment of IL-7Rα was deglutamylated by CCP2. Moreover, MBP-tagged mutant CCP2 (MBP-CCP2-mut) was able to pull down native IL-7Rα from BM lysates, whereas the enzymatic active CCP2 (MBP-CCP2-wt) could not precipitate IL-7Rα (Fig. 3e). These observations suggest that the intracellular segment of IL-7Rα undergoes deglutamylation by CCP2. With immunofluorescence staining, hyperglutamylation of IL-7Rα appeared in primary CCP2-deficient CHILPs (Fig. 3f). In parallel, IL-7Rα was highly polyglutamylated in BM lysates of Ccp2−/− mice (Fig. 3g). Finally, BM cells treated with the CCP antagonist Phen increased substantial amounts of polyglutamylated IL-7Rα, whereas treatment with the CCP
agonist CoCl₂ abrogated the glutamylation of IL-7Rα (Fig. 3h). Collectively, we conclude that IL-7Rα is a novel substrate for CCP2.

**IL-7Rα is polyglutamylated at Glu446 by TTLL4 and TTLL13.** Nine polyglutamylases have been reported to catalyze protein glutamylation. To determine the physiological polyglutamylases catalyzing IL-7Rα glutamylation, we examined expression patterns of all nine polyglutamylases in αLPs and CHILPs of mouse BM through quantitative real-time PCR. We observed that Ttll4 and Ttll3 were highly expressed in αLPs and CHILPs, with peak expression in CHILPs (Fig. 4a). Additionally, Ttll4 and Ttll3 were highest expressed in CHILPs among all the hematopoietic progenitor cells (Fig. 4b). We next incubated recombinant intracellular segment rGST-IL-7Rα with Flag-tagged TTLL4 or TTLL13 in vitro. We noticed that Flag-tagged TTLL4 and TTLL13 were able to precipitate rGST-IL-7Rα (Fig. 4c). Their interactions were further verified by co-transfection assays (Fig. 4d). Moreover, IL-7Rα was co-localized with TTLL4 and TTLL13 in CHILPs (Fig. 4e). We then conducted in vitro glutamylation assays by incubation of rGST-IL-7Rα with Flag-TTLL4 or Flag-TTLL13. We found that rGST-IL-7Rα was polyglutamylated by TTLL4 and TTLL13 (Fig. 4f). Importantly, TTLL4- and TTLL13-mediated polyglutamylation of rGST-IL-7Rα was successfully removed by enzymatic active CCP2 (Fig. 4g). These data indicate that TTLL4 and TTLL13 are two polyglutamylases for IL-7Rα polyglutamylation.

Glutamate-rich stretches and acidic environment at the acceptor sites have been reported to be important for glutamylation. Based on the conservative amino acid sequence analysis, only Glu446 and Glu447 were two conserved identical glutamic acid residues located on the loop region of intracellular domain of IL-7Rα (Supplementary Fig. 2b), which might be potential acceptor site candidates for glutamylation. We then mutated Glu446 to Ala of IL-7Rα (E446A-IL-7Rα) and incubated recombinant intracellular E446A-IL-7Rα protein with Flag-TTLL4 or Flag-TTLL13 in vitro. We observed that E446A-IL-7Rα mutant abolished TTLL4- or TTLL13-mediated glutamylation (Fig. 4h), suggesting IL-7Rα is catalyzed by TTLL4 and TTLL13 at Glu446.

We next explored the physiological relevance of IL-7Rα glutamylation in ILC3 differentiation. We silenced IL-7Rα by LMP retrovirus-carried short hairpin RNA (shRNA) infection in CHILPs and then rescued expression of WT-IL-7Rα or E446A-IL-7Rα, followed by BM transplantation assays. Eight weeks after transplantation, IL-7Rα knockdown with empty vector infection remarkably decreased ILC3 numbers (Supplementary Fig. 2c). By contrast, WT-IL-7Rα restoration could rescue the normal number of ILC3s in recipient mice, whereas E446A-IL-7Rα mutant overexpression had no such effect (Supplementary Fig. 2c). Parallely, these observations were further validated by in vitro differentiation assays (Supplementary Fig. 2d, e).

Collectively, IL-7Rα glutamylation is required for ILC3 development.

To further validate the authentic role of IL-7Rα glutamylation in the regulation of ILC3 development, we generated E446A-IL-7Rα mutation (Il7rE446A) mice through CRISPR/Cas9 technology. We noticed that IL-7Rα really did not undergo glutamylation in CHILPs of Il7rE446A mice (Fig. 4i). Consistently, Il7rE446A mice displayed reduced numbers of ILC3s (Fig. 4j) and Supplementary Fig. 2d) and more severe intestinal injury post C. rodentium infection (Fig. 4k). Consequently, Il7rE446A mice died rapidly with C. rodentium challenge (Fig. 4l). Finally, we transplanted CD45.2⁺ WT or Il7rE446A BM cells into lethally irradiated CD45.1⁺ recipients. Eight weeks after transplantation, Il7rE446A BM transferred recipients displayed a reduced number of ILC3s compared to those of WT BM engraftment (Supplementary Fig. 2g). Collectively, we conclude that IL-7Rα glutamylation is required for ILC3 development from CHILPs.

**IL-7Rα glutamylation promotes Sall3 expression by STAT5.** IL-7Rα pairs with the common γ-chain of IL-2R or TSLP receptor to detect IL-7 and TSLP, respectively, for the activation of STAT proteins in DCs, CD4⁺ T as well as B cells. However, how IL-7Rα glutamylation regulates the development of ILCs remains unclear. We then analyzed all STAT protein phosphorylation signals in Ccp2⁺/⁺ and Ccp2⁻/⁻ CHILPs. We found that only STAT5 was hyperphosphorylated in Ccp2⁺/⁻ CHILPs compared to Ccp2⁺/⁺ CHILPs with IL-7 stimulation (Fig. 5a). However, other STAT proteins were not activated (Fig. 5a). We thus used STAT3 as a negative control in the following experiments. These observations were further validated by flow cytometry and immunofluorescence staining (Fig. 5b, c). These results indicate that IL-7Rα glutamylation leads to STAT5 activation in CHILPs after IL-7 stimulation.

To further determine which TFs regulated IL-7Rα glutamylation-mediated ILC3 development, we performed transcriptome microarray analysis of Ccp2⁺/⁺ vs. Ccp2⁻/⁻ CHILPs. Among top 10 upregulated TFs in Ccp2⁻/⁻ CHILPs (Fig. 5d), we focused on Spalt-like transcription factor 3 (Sall3), which was a highest differentially expressed TF in Ccp2⁻/⁻ CHILPs (Fig. 5e). Sall3, a member of the SAL family, is implicated in embryonic development and oncogenesis. However, how Sall3 regulates the development of ILC3s is unknown. Intriguingly, the promoter region of Sall3 gene contained the STAT5-binding motif (TTTNNNGAA) (Supplementary Fig. 2h). Of note, anti-STAT5 antibody could immunoprecipitate Sall3 promoter by chromatin immunoprecipitation (ChIP) assay, whereas the anti-STAT5 antibody failed to immunoprecipitate other TF promoters (Fig. 5f). Consistently, the Sall3 promoter of Ccp2⁻/⁻ CHILPs bound to substantial STAT5 proteins compared to that of Ccp2⁺/⁺ CHILPs (Fig. 5g). The binding of STAT5 to Sall3 promoter was further verified by an EMSA assay (Fig. 5h). Consequently, STAT5 was able to activate Sall3 transcription.
Fig. 5 IL-7Ra glutamylation promotes Sall3 expression by STATs. a-c Phosphorylation of STAT3 and STAT5 was tested by western blotting a, flow cytometry b and immunofluorescence staining c in CHILPs from WT and Ccp2−/− mice after IL-7 stimulation. p-STAT5, green; p-STAT3, red; nucleus, blue. d Heat map of representative gene expression values from microarray data. In all, 1 × 10^6 CHILPs (Lin−Flk1−CD150−IL-7RaαCbl−) from Ccp2+/+ or Ccp2−/− mice were sorted for microarray. e Analysis of indicated gene expression levels in Ccp2+/+ and Ccp2−/− CHILPs by quantitative reverse transcription PCR (RT-qPCR). Relative fold changes of gene expression values were normalized to endogenous Actb. f-g Enrichment assessment of STAT5 on Sall3 promoter in CHILPs from Ccp2+/+ and Ccp2−/− mice. h The association of STAT5 with Sall3 promoter was examined by EMSA. Sall3 promoter probe was biotin-labeled. i Flag-STAT5, pT, and pGL3- Sall3 promoter were transfected into 293T cells for luciferase assay. j DNaseI accessibility of Sall3 promoter in CHILPs from Ccp2+/+ and Ccp2−/− mice was assessed. k H3K4me3 enrichment on Sall3 promoter was determined. CHILPs were isolated from Ccp2+/+ and Ccp2−/− mice, followed by CHIP assay. l Sall3 mRNA levels were detected in WT, Stat3−/−, and Stat5−/− CHILPs. m CHILPs were isolated from WT mice and cultured with mitomycin C-treated OP9 cells for in vitro differentiation assay. Stats−/− CHILPs were transfected with STAT5 or Sall3 overexpression plasmid. Percentages of ILC3s were analyzed by flow cytometry. n Relative expression of indicated genes was analyzed by RT-qPCR. o Histology of colons from Sall3+/+ and Sall3−/− mice 8 days after C. rodentium infection. Scale bars, 50 μm. p IL-7Ra glutamylation and STATS phosphorylation in CHILPs in the absence or presence of IL-7. CHILPs from WT or Il7E446A BM were incubated with IL-7 and vehicle, followed by immunoblotting. β-actin was probed as loading controls. q Sall3 expression levels were tested in WT and Il7E446A CHILPs by RT-qPCR. Relative fold change of Sall3 expression values were normalized to endogenous Actb. **P < 0.01, ***P < 0.001 (Student’s t-test). Data represent three independent experiments. Error bars in e-g, i-n, and q indicate s.d.
Fig. 6 Deletion of Ttll4 or Ttll13 impairs ILC3 development. a Detection of IL-7Rα glutamylation in CHILPs by gating on Lin−IL-7Rα+Flt3−CD25−αβt−/− from WT, Ccp2−/−, Ttll4−/−, Ttll13−/−, and Ttll4−/−Ttll13−/− mice. b Examination of STATS phosphorylation in CHILPs from WT, Ccp2−/−, Ttll4−/−, Ttll13−/−, and Ttll4−/−Ttll13−/− mice. c Sall3 expression levels were tested in WT, Ttll4−/−, Ttll13−/−, and Ttll4−/−Ttll13−/− CHILPs by quantitative reverse transcription PCR (RT-qPCR). Relative fold change of Sall3 expression values were normalized to endogenous β-actin. d Flow cytometry analysis of ILC3s in small intestine lamina propria from WT, Ttll4−/−, Ttll13−/−, and Ttll4−/−Ttll13−/− mice. Percentages of indicated cells were calculated and shown as means±s.d. (right panel). n = 6 for each group. e Analysis of ILC3s in small intestines by immunofluorescence staining in situ. Arrowhead denotes ILC3 cells. Scale bars, 50 μm. f Analysis of IL-22+ ILC3s after IL-23 stimulation by flow cytometry. n = 6 for each group. g Body weight changes of WT, Ttll4−/−, Ttll13−/−, and Ttll4−/−Ttll13−/− mice post C. rodentium infection. n = 10 for each group. h Colon length from WT, Ttll4−/−, Ttll13−/−, and Ttll4−/−Ttll13−/− mice after C. rodentium infection. n = 6 per genotype group. i Histology of colons from WT, Ttll4−/−, Ttll13−/−, and Ttll4−/−Ttll13−/− mice 8 days after infection with C. rodentium. Scale bars, 50 μm. j Survival curves of WT, Ttll4−/−, Ttll13−/−, and Ttll4−/−Ttll13−/− mice after C. rodentium infection. n = 10 for each group. In all, 2 × 10^9 C. rodentium was used by oral inoculation. k A working model represents glutamylation-mediated IL-7 signaling in the regulation of ILC3 development. Pro promoter. *P < 0.05, **P < 0.01, and ***P < 0.001 (Student’s t-test). Data represent three independent experiments. Error bars in c, d, and f-h indicate s.d.
through in vitro luciferase assays (Fig. 5i). Moreover, Sall3 promoter in Ccp2−/− CHILPs was more accessible to DNase I digestion (Fig. 5j) and enriched more H3K4me3 (Fig. 5k), suggesting CCP2 deficiency promotes Sall3 expression.

We next generated Stat5 KO CHILPs via Cas9 knockin mice as described39 and Stat3 conditional KO mice by crossing Stat3lox/lox mice to Mx1-Cre mice. STAT5 and STAT3 were successfully deleted in CHILPs (Fig. 5l). We noticed that Stat3−/− CHILPs abrogated Sall3 expression, while Stat3−/− CHILPs did not impact the expression of Sall3 (Fig. 5l). We then cultured CHILPs with OP9 cells in the presence of SCF and IL-7 in vitro. We found that Stat5 KO drastically reduced ILC3 numbers (Fig. 5m). However, rescue expression of STAT5 or Sall3 in Stat5−/− CHILPs was able to restore ILC3 numbers comparable to WT mice (Fig. 5m), suggesting Sall3 was a downstream target for STAT5 activation during ILC3 differentiation. We also generated Stat3 KO mice via Cas9 knockin mice. We observed that Sall3 KO mice also displayed decreased ILC3s and more susceptibility to C. rodentium infection compared with WT control mice (Fig. 5n, o and Supplementary Fig. 2i). We next engrafted 5 × 10^5 CD45.2+ C. rodentium−/− WT or Sall3−/− mice with 5 × 10^6 CD45.1+ helper cells into lethally irradiated CD45.1+ recipients for 8 weeks, followed by analysis of mixed chimeras. We found that Sall3−/− BM transferred recipients displayed a reduced number of ILC3s compared to those of WT BM engraftment (Supplementary Fig. 2j). More importantly, IL-7Rα polyglutamylation indeed occurred in WT CHILPs with IL-7 stimulation, and STAT5 phosphorylation appeared as well (Fig. 5p). However, in the absence of IL-7, IL-7Rα did not undergo polyglutamylation and no STAT5 phosphorylation appeared in WT CHILPs (Fig. 5p). By contrast, in the presence of IL-7, CHILPs from Il7rE446A mice did not undergo polyglutamylation and no STAT5 phosphorylation appeared either (Fig. 5p). Finally, Il7rE446A CHILPs did not activate the expression of Sall3 (Fig. 5q). Taken together, we conclude that IL-7Rα glutamylation-mediated STAT5 activation initiates Sall3 expression that drives the development of ILC3s from CHILPs.

Deletion of Ttll4 or Ttll13 impairs ILC3 development. We next generated Ttll4 and Ttll13 KO mice via CRISPR/Cas9 technology (Supplementary Fig. 3a, b). We noticed that deletion of Ttll4 or Ttll13 abrogated IL-7Rα glutamylation in CHILPs (Fig. 6a). Importantly, Ttll4−/− or Ttll13−/− CHILPs impaired STAT5 phosphorylation and blocked Sall3 expression in CHILPs (Fig. 6b, c). Consistently, Ttll4 or Ttll13 KO abolished ILC3 differentiation (Fig. 6d and Supplementary Fig. 3c). Ttll4 and Ttll13 double KO (DKO) almost suppressed the ILC3 formation in small intestines (Fig. 6d). These results were further confirmed by immunofluorescence staining (Fig. 6e). Additionally, Ttll4 or Ttll13 KO also dramatically reduced numbers of IL-22+ ILC3s (Fig. 6f). As expected, Ttll4 or Ttll13 KO markedly declined ILC3 population (Supplementary Fig. 3d). These data suggest that Ttll4- and Ttll13-mediated IL-7Rα glutamylation is required for the activation of Sall3 in CHILPs that drives the differentiation of ILC3s.

We next infected WT, Ttll4−/−, Ttll13−/−, or Ttll4−/−Ttll13−/− mice with C. rodentium. We observed that Ttll4−/− and Ttll13−/− mice had higher bacterial loads in fecal, spleen, liver, and blood on day 8 post infection compared with their littermate WT control mice (Supplementary Fig. 3e–h). Additionally, Ttll4−/− and Ttll13−/− mice lost their weights over C. rodentium infection (Fig. 6g), accompanied with shrinking length of colons (Fig. 6h). Following infection with C. rodentium, Ttll4−/− and Ttll13−/− mice displayed much more persistent intestinal damage, encompassing greater epithelial injury, crypt hyperplasia, and more infiltration of inflammatory cells, than those of their littermate WT control mice (Fig. 6i). Consistently, Ttll4−/− and Ttll13−/− mice succumbed to bacterial infection (Fig. 6i). Expectedly, Ttll4 and Ttll13 DKO displayed much higher susceptibility to C. rodentium infection (Fig. 6g–j). In sum, Ttll14- and Ttll13-mediated IL-7Rα glutamylation has a critical function in the differentiation and effector functions of ILC3s.

Discussion

ILCs are a distinct arm of the innate immune system, which can directly communicate with other hematopoietic and non-hematopoietic cells to regulate immunity, inflammation and tissue homeostasis1. However, how these ILC lineages develop and/or maintain remains unclear. In this study, we show that CCP2 deficiency causes increased numbers of ILC3s. With IL-7 engagement, IL-7Rα undergoes polyglutamylation in CHILPs. IL-7Rα polyglutamylation specifically activates STAT5 phosphorylation to initiate Sall3 expression for ILC3 development (Fig. 6k). In addition, Ttll4−/− and Ttll13−/− mice abrogate IL-7Rα polyglutamylation and Sall3 expression in CHILPs, leading to impaired ILC3 differentiation and more susceptibility to C. rodentium infection. Finally, E446A-IL-7Rα mutation mice indeed abrogates Sall3 expression and ILC3 development.

The earliest progenitor cells specific to ILCs are CXCR6+ integrin αβ+ expressing CLPs, referred to as α-lymphoid precursor (αLP) cells, which give rise to ILC1, ILC2, ILC3, and conventional NK cells (cNK)40. The common progenitor to all ILC lineages (CHILP) is identified as its Lin−IL-7Rα+ Id2+CD25+αβ+ phenotype and differentiates to all ILC subsets, but not cNK41. The common precursor to ILCs (ILCP) is defined by expression of TF PLZF and generates ILC1, ILC2, and ILC3 subpopulations15. In this study, we show that CCP2 is highly constitutively expressed in CHILPs and ILC3s, which blocks the deglutamylation of IL-7Rα to drive ILC3 development. CCP2 deficiency causes increased numbers of ILC3s, but reduced numbers of ILC1s and ILC2s, which augments clearance of C. rodentium. Given that CCP2 is also moderately expressed in other cells such as CD3+ T cells, we thus cannot exclude the potential involvement of other cells in the bacterial clearance of CCP2 deficiency. Of note, CCP2 deficiency does not impact cell death of CHILPs and all ILC lineages. A recent study showed that different ILC subsets are defined by distinct gene-expression patterns41. Of note, cytokines such as IL-7, IL-15, and IL-2 play major roles in the regulation of ILC development. However, how CCP2-mediated IL-7 signaling regulates the switch balance of ILC development still needs to be further investigated. We notice that CCP members are differentially expressed in the hematopoietic progenitors and lineages we checked. We previously demonstrated that CCP6 is mostly highly expressed in BM and megakaryocytes, and also exhibits different expression profiles in different tissues and cell types25. Our findings suggest that different tissue and cell type distributions of CCPs may exert unique roles in the modulation of different physiological and pathological processes.

Protein polyglutamylation is catalyzed by a family of polyglutamylases, also called Ttlls24, 25. The well-known substrates of polyglutamylation are tubulins and nucleosome assembly proteins35. Through regulating the interaction of microtubules (MTs) and MT-associated proteins (MAPs), polyglutamylation may exert major effects on MT-related cellular processes, including stability of centrosomes32, motifility of cilia and flagella33, 44, neurite outgrowth45, as well as neurodegeneration26. A recent study delineates a structural MT recognition basis by catalysis with Ttll721. Ttlls have different expression patterns in diverse tissues and their functions are not entirely redundant43.
We recently reported that TL14 and TL16 are most highly expressed in megakaryocytes, both of whom catalyze polyglutamylation of Mad2 to modulate megakaryocyte maturation. Here we demonstrate that TL14 and TL16 are constitutively elevated in CHILPs, both of which can catalyze polyglutamylation of IL-7Rα to regulate the development of ILCs. Deletion of TL14 or TL16 impairs ILC3 differentiation and their effector functions. Thus, IL-7Rα polyglutamylation mediated by TL14 or TL16 has a critical function in the regulation of ILC3 development from the stage of CHILPs.

IL-7Rα (CD127), encoded by Il7r gene, forms a receptor complex with the common cytokine receptor γ-chain of IL-2R or TSLP receptor to sense IL-7 and TSLP, respectively. The IL-7Rα–TSLP ligand-receptor pair signaling is critical for proliferation and survival of T and B lymphocytes in a non-redundant fashion. Genetic aberrations of IL-7Rα signaling lead to immune deficiency syndromes and other immune diseases. It has been reported that all ILC lineages express high levels of IL-7Rα. Of note, the ILCP CHILPs also express IL-7Rα, which gives rise to all ILCs. However, the molecular mechanism by which IL-7Rα signaling regulates the development of ILCs remains elusive. In this study, we show that TL14 and TL16-mediated IL-7Rα polyglutamylation regulates the differentiation of ILC3s from CHILPs. Mechanistically, polyglutamylation of IL-7Rα is able to activate STAT5 and phosphorylated STAT5 can directly bind to Sall3 promoter to initiate its transcription, which drives the development of ILC3s from CHILPs.

A CHILP cell has been defined that lacks expression of Flt3 and CD25 but expresses IL-7Rα and αR1. CHILPs differ from αL-αL-IPs in that CHILPs express Id2. CHILPs generate all ILCs, including LTi cells, but they fail to give rise to conventional NK cells. Subsequently, their downstream precursor ILCPs (common precursor of ILCs), characterized by expression of the TF PLZF, lose the ability to generate LTi cells and produce all ILC1, ILC2, and ILC3 subsets. RORyt (encoded by Rorc) drives differentiation of ILC3s from their precursor ILCPs. RORyt deletion causes a complete loss of ILC3s but not ILC1s or ILC2s. Runx3 is also required for the development of ILC1s and ILC3s, but not for ILC2s. GATA3 is also involved in the development of ILC3s. It and continues to exert a critical role in mature ILC3s, but not for ILC2s. GATA3 is also involved in the development of ILC3s. It and continues to exert a critical role in mature ILC3s.

Glutamylation is highly conserved in all metazoans and protists, exerting critical roles in many physiological and pathological processes. For example, TL17, the most abundantly expressed TTTTs in the mammalian nervous system, is conserved from acorn worm to primates, where it modulates neurite outgrowth and localization of dendritic MAPs. Glutamylation is abundantly expressed TTTLs in the mammalian nervous system, but not for ILC2s. GATA3 is also involved in the development of ILC3s. Therefore, we strongly believe that it is necessary to develop specific inhibitors or agonists for these related polyglutamylases and CCPs. Manipulating polyglutamylation profiles by using these compounds, we may potentially target ILC3s for future clinical applications. In sum, IL-7Rα polyglutamylation has a critical function in the regulation of ILC3 development and their effector function. Our findings provide new mechanistic insights into how polyglutamylation modulates ILC3 development.

Methods

Antibodies and reagents. Anti-CCP1 (LM-1A7), anti-CCP2 (S-13), anti-CCP3 (S-15), anti-CCP4 (T-17), anti-CCP5 (N-18), anti-CCP6 (N-14), anti-TTL14 (S-14), anti-TTLL7 (E-12), anti-TTLL9 (C-20), anti-TTLL13 (D-16), anti-STAT4 (C-4), anti-STAT4 (E-2), anti-STAT6 (M-20), anti-STAT6 (sc-11762), anti-Il7r (6G9C6), and anti-Myc (9E10) were from Santa Cruz Biotechnology; Anti-STAT1 (14994), anti-STAT1 (9167), anti-STAT2 (72604), anti-STAT3 (9193), anti-STAT3 (9174), anti-HK4 (9751), anti-STAT5 (9363), and anti-STAT5 (9351) were from Cell Signalling Technology (Danvers, USA). Anti-IL-7Rα (15004) was from Biorheology. The antibodies against P-STAT2 (SA4503836), Sall3 (SABZI0275), Flag-tag (M1), β-actin (SP124), and His-tag (6AT18) were from Sigma-Aldrich (St Louis, USA), GT3 antibody (AG-208-0020) was from AdipoGen. Antibodies against CD3 (17A2), CD19 (1D3), B202 (RA3-682), IL-7Rα (A7R34), c-kit (2B8), Sca-1 (D7), CD25 (PC61.5), CD11b (M170), CD11c (N418), Gr1 (RB6-8C5), F4/80 (BM8), Ter119 (TER-119), CD27 (LG729), CD96 (HIS11), CD45.2 (104), RORyt (AFKJS-9), Skp46 (29A1.4), CD244 (C9.1), Flt3 (AF210), qGAT (DKT32), CD54 (A20), NK1.1 (PK16), IL-22 (IL2IP0F), Thyl1.2 (30-H12), and PLZF (Mags.217F) were purchased from ebioscience (San Diego, USA). Active caspase 3 antibody (556914) was purchased from BD Bioscience. All primary antibodies were used in a 1:2000 dilution for western blotting, in a 1:500 dilution for immunofluorescence staining and in a 1:1000 dilution for flow cytometric staining. Paraformaldehyde (PFA, 185127), penanthrolone (Phe, 33510), CoCl2 (60818), and 4′,6-diamidino-2-phenylindole (DAPI; D9542) were from Sigma-Aldrich. IL-22 ELISA kit (BMS6022) was purchased from ebioscience.

Generation of knockout mice and Il7r E446A mice. Cpj1 and Cpj6 knockout mice were described previously. Cpj2−/−, Cpj3−/−, Cpj5−/−, Ttll4−/− and Ttll13−/− mice were generated through CRISPR-Cas9 approaches as described. Gt(Rosa26)ser1(MIC-Apcaq-caq,EGFP)Fpher, Ronc(rt+)Fpher and Id2−/−Fpher mice were purchased from the Jackson Laboratory. Stat3−/− mice was kindly provided by Dr. Akira (Osaka University, Japan). Stat3ox/ox, Mar2−/− mice were obtained by crossing Stat3−/− mice with Mar2−/− mice. To induce Stat3−/− deletion, 200 μg polynsine-polyacrylic acid (poly(lC)) was intraperitoneally injected to mice every other day for three times. Mouse experiments were performed according to the guidelines of the institutional animal care and use committees at the Institute of Biophysics, Chinese Academy of Sciences. For deletion of Sall3 in BM, B6.129-Gt(Rosa26)ser1(MIC-Apcaq-caq,EGFP)Fpher knockin mice were crossed with Vav-Cre transgenic mice to generate Rosa26-LSL-Cas9+/Vav-Cre mice. In all, 2 × 106 BM cells were infected with lentiviral-packing containing ssSall3 lentivirus. BM cells were then transferred into lethally irradiated recipient mice and analyzed 8 weeks after. Sall3 deletion was confirmed by immunoblotting. For generation of Id2GFPmice, the genome locus of Id2 gene was knocked in with in Il7r−/E446A mutation via a CRISPR-Cas9 approach. Mixture of Cas9 mRNA, single guide RNA (sgRNA), and IL-7Rα-E446A donor templates was microinjected into the cytoplasm of C57BL/6 fertilized eggs and transferred into the uterus of pseudogenetic ICR females. IL-7Rα-E446A mutations were identified by PCR screening and DNA sequencing. gRNA sequences are as follows: Cpj2: 5′-TTAGAAATTTTCGTTGTGTTG-3′; Cpj3: 5′-GGAGATGCTACGAGAAGATG-3′; Cpj4: 5′-AGCTCT-GAGCTGTTGCTCTCAGG-3′; Cpj5: 5′-GGCTACTCTGAGTGTGCGG-3′; Ttll4: 5′-TTTGGCTCTGACTTTGTGGCGG-3′; Ttll13: 5′-TTTGGCTCTGACTTCAACCGGAAGG-3′; Id2: 5′-TTTGGCTCTGACTTTGTGGCGG-3′; Sall3: 5′-CCGACATCTCAAGTCCGAGC-3′. Mice used in all experiments were 8-week old. And we performed three independent experiments from each mouse from at least three mice for each group. The background of mice was C57BL/6, and mice were housed under the same age and gender. Animal use and protocols were approved by the Institutional Animal Care and Use Committees at Institute of Biophysics, Chinese Academy of Sciences.
Histology analysis. Mouse colons after C. rodentium infection were fixed in 4% PFA (Sigma-Aldrich) for 24 h, washed twice with phosphate-buffered saline (PBS) and cleared through 70% ethanol before osmium tetroxide. Then colons in paraffin were sectioned and stained with hematoxylin and eosin (H&E) according to standard laboratory procedures.

Intestinal lymphocyte separation. Protocols for lymphocyte isolation from the intestine had been described[12]. With some modifications, intestines were dissected and cleaned, cut longitudinally, were rinsed and washed with Dulbecco’s Phosphate Buffered Saline (pPBS) five times. Then intestines were cut into pieces, and washed with solution I buffer (10 mM HEPES and 5 mM EDTA in Hank’s Balanced Salt Solution (HBSS)) five times. For FPL, isolation, the intestinal fragments were digested with solution II containing DnaseI, 5% FBS, 0.2 mg/ml collagenase II and collagenase III there times at 37 °C. Then the tissues were sifted through 70-μm strainers.

Flow cytometry. BM cells were flushed out from femurs in PBS buffer and sifted through 70-μm strainers for BM flow cytometric analysis. CLP (Lin-IL-7Ra-αSca-1-β Kit-)) were analyzed using qPCR. Primers used for ChIP were as follows: forward: 5'-AGGTGGGAGGG-3' and reverse: 5'-GAGCCTCGACTGGGGAGCTGAG-3'.

EMSA assay. EMSA experiments were conducted according to the manufacturer’s protocol with a Light Shift Chemiluminescent RNA EMSA Kit (Thermo Scientific). Briefly, Flag-STAT5 was incubated with or without unlabeled probe for competitive reaction and anti-STAT5 antibody for super shift at room temperature for 20 min in a reaction buffer. Then, Biotin-labeled probe was added into the reaction system and incubated for 20 min at room temperature. Samples were carried out in 4% polyacrylamide gel in 0.5 x TBE buffer. After transferred on a nylon membrane (Amersham Biosciences), the labeled DNA was cross-linked by ultraviolet, washed with streptavidin-HRP conjugate and then incubated with the detection substrate. The probe sequence for Sall3 was: 5’-GGAGGCCATAGAGCTCTGG-3'.

DNase I accessibility assay. DNasel digestion assay has been described previously[23]. In brief, nuclei were purified from CHILPs according to the manufacturer’s protocol with the Nuclei isolating Kit (Sigma-Aldrich). Then nuclei were resuspended with Dnase I digestion buffer and treated with indicated units of DNase I (Sigma, USA) at 37 °C for 5 min. In all, 2 x DNase I stop buffer (20 mM Tris Ph 8.0, 4 mM EDTA, 2 mM EGTA) was added to stop reactions. DNA was extracted and examined by qPCR.

BM transplantation. In all, 5 x 10^5 CD45.2^LSK from Cep21/^- or Cep21/- mice with 5 x 10^6 CD45.1^help cells were transplanted into lethally irradiated CD45.1^recipient mice. Eight weeks after transplantation, percentages of ILCs derived from donor cells were analyzed by FACS. For competitive transplantation, 1 x 10^6 CD45.2^BM cells and 1 x 10^6 CD45.1^BM cells were injected into lethally irradiated CD45.1^-recipient mice. Eight weeks after transplantation, ratios of CD45.2^-ILC3 to CD45.1^-ILC3 were analyzed. For IL-7Rα knockdown or over-expression, 1 x 10^6 CHILP cells were infected with retrovirus carrying shRNAs or overexpression sequences, followed by BM transplantation. Eight weeks after transplantation, chimeras were analyzed by FACS. Small interfering RNA sequences against IL-7Rα were cloned into LMP plasmid and IL-7Rα was cloned into pMY vector.

Gene deletion in CHILPs by CRISPR/Cas9 technology. Stat5α and Sall3 deletion in CHILPs were generated using Cas9 knockin mice according to the standard protocol provided by Zhang’s lab[31]. Briefly, sgRNA was generated by online CRISPR Design Tool (http://tools.genome-engineering.org) and cloned into lentICRISPRv2 for lentivirus production in 293 T cells. Then CHILPs were infected with lentivirus for Stat5α or Sall3 deletion, sgRNA sequences of Stat5α were: 5’-AGATGTCGCCAAGCCTGCTGAT-3’; Stat5β: 5’-AAATATGTTGCACCTGCGAT-3’.

RNA interference. Sequences for RNA interference were designed according to MSCV-LTRmiR30-PIG (LMP) system instructions. LMP vectors containing target sequences were constructed. The target sequence against IL-7Rα: 5’-GCGTAA TGCCACCACTGTCGAT-3’.

In vitro IL-7Rα silencing assay. In vitro IL-7Rα silencing assay was described previously[46]. In brief, CHILPs were sorted and cultured for 14 days on mitomycin C-treated OP9 feeder cells supplemented with IL-7 (25 mg/mL, Cat#: 217-17) and rSCF (25 mg/mL, Cat#: 250-03). Then cells were collected for flow cytometry.

C. rodentium infection. WT, Cep21^-/-, Cep21^-/-, Tll4/-; Tll13/^+ and Tll4^-/-Tll13^-/- mice were infected with 5 x 10^3 C. rodentium orally as described. C. rodentium was a gift from Dr Bauxee Ge (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). Mice were sacrificed by cervical dislocation to examine colon pathology and bacterial loads on day 8 post infection. Fecals, spleens, blood, small intestines, and colons were collected from infected mice on day 8 after infection. Fecals, spleens, and livers were weighed and homogenized, and homogenates were plated on MacConkey agar plates for analysis of bacterial counts. Lamina propria lymphocytes (LPs) were isolated from small intestines of infected mice, followed by analysis of ILCs and IL-22.

In vitro glutamylation assay. Detailed protocol for in vitro glutamylation assay was described previously[27]. In brief, CCP2 was cloned into H-MBP-3c for MBP-tagged protein expression vectors. IL-7Rα was cloned into pGEX-6p-1 plasmid for GST-tagged protein expression. Plasmids were transformed into E. coli strain BL21 (DE3), followed by induction with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) at 16 °C for 24 h. Cells were collected and lysed by sonipor, followed by purification through Amylose or GST resins.

Recombinant protein purification. CDs were cloned from a BM CD4+ T cell cDNA library. CDP2 was subcloned into H-MBP-3c for MBP-tagged protein expression vectors. IL-7Rα was cloned into pGEX-6p-1 plasmid for GST-tagged protein expression. Plasmids were transformed into E. coli strain BL21 (DE3), followed by induction with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) at 16 °C for 24 h. Cells were collected and lysed by sonipor, followed by purification through Amylose or GST resins.

Statistical analysis. An unpaired Student’s t-test was used as statistical analysis in this study. Statistical calculation was performed by using Microsoft Excel or SPSS 13.

Data availability. All data generated or analyzed during this study are included in this published article and its Supplementary Information Files. Microarray data, are deposited in the Genebank as GSE97487.
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Author contributions
B.L. designed and performed experiments, analyzed data, and wrote the paper; B.Y. performed experiments and analyzed data; G.H. performed some experiments; L.Y. crossed some mice; P.Z., J.W., and Y.D. analyzed data. X.Z., S.M., and T.Y. build up animal models and analyzed data; Z.F. initiated the study, organized, designed, and wrote the paper.

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