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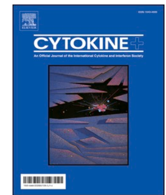

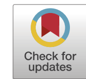

CLCF1 promotes IL12Rβ2 proteolysis and limits Th1 differentiation

Véronique Laplante [a], Marine Rousseau [b,c], Sarah Pasquin [b,c], Capucine Bourel [b,c], Maxime Uriarte [c], El Bachir Affar [c,d], Rafael Najmanovich [a], Sylvie Lesage [b,c], Jean-François Gauchat [a,*]

[a] Département de pharmacologie et physiologie, Université de Montréal, Montréal, Québec H3T 1J4, Canada
[b] Département de microbiologie, infectiologie et immunologie, Université de Montréal, Montréal, Québec H3T 1J4, Canada
[c] Centre de recherche de l'Hôpital Maisonneuve-Rosemont, Université de Montréal, Montréal, Québec H1T 4B3, Canada
[d] Département de médecine, Université de Montréal, Montréal, Québec H3T 1J4, Canada

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ABSTRACT

Cardiotrophin-like cytokine factor 1 (CLCF1) is a cytokine of the IL6/IL12 family with immune-modulating functions, mainly on B cells and myeloid cells. CLCF1 also plays a crucial role in the embryonic development of motor neurons, such that *Clcf1*-knock out mice are not viable. In order to further study the immune activities of CLCF1, we used a mouse model with a knock-out of *Clcf1* in hematopoietic cells under the Vav promoter. While characterizing this model, we observed that CD4$^+$ T cells from *Clcf1*$^{-/-}$ mice produced more IFNγ than those from *Clcf1*$^{+/+}$ mice when activated in the presence of IL12. We also observed that CLCF1 induces a downregulation of IL12Rβ2 expression levels. We further demonstrated that CLCF1 interacts with IL12Rβ2 and promotes its degradation through the proteasome in a manner independent of ubiquitination. Altogether, these results suggest that CLCF1 can act as a negative regulator of IL12 activity, a role which could be exploited therapeutically to dampen the inflammatory response driven by Th1 cells. Our observations may also hint at a new role for CLCF1 as a mediator of protein degradation.

1. Introduction

Cardiotrophin-like cytokine factor 1 (CLCF1) is a member of the IL6/IL12 cytokine family [1]. CLCF1 is a ligand for the ciliary neurotrophic receptor (CNTFR) which is composed of three chains, CNTFRα, LIFRβ and gp130 [2]. CLCF1 is highly conserved between vertebrate species, with 97 % identity between the human and mouse proteins [3]. CLCF1 has important neurotrophic functions for the embryonic development of motor neurons, such that *Clcf1*-knock out mice cannot suckle and die at P1 [4]. Like other members of the IL6/IL12 family, CLCF1 is secreted in complex with a subunit that is structurally related to a cytokine receptor [5], the cytokine receptor-like factor 1 (CRLF1) [2]. Immune-modulating functions have been identified for CLCF1, mainly on B cells and myeloid cells. Indeed, injections or overexpression of CLCF1 in mice lead to B cell hyperplasia in secondary lymphoid organs [6,7], hyperglobulinemia [6,7], and increased numbers of circulating CD11b$^+$ cells [8]. Administration of CLCF1 in a mouse model of idiopathic pulmonary fibrosis also leads to a decrease in pulmonary fibrosis and an accumulation of CD4$^+$ T cells in lung tissues [9]. Still, the mechanism by which CLCF1 modulates immune responses remains to be defined.

Phylogenetic studies show a very close relationship between the IL6 and IL12 cytokine families [10]. The prototypical member of the IL12 family, IL12, is composed of two subunits, p40 and p35 [11–13], and is mainly secreted by professional antigen-presenting cells and phagocytes following exposure to pathogens [14–18]. It plays a pivotal role in the differentiation of pro-inflammatory type 1 T helper (Th1) cells [16,19], which are characterized by the production of IFNγ [16,19] and the upregulation of the transcription factor T-bet [20,21]. IL12 is also known to promote the cytotoxic activity of NK cells and CD8$^+$ T cells [11,22,23]. IL12 activates a receptor comprising the IL12Rβ1 and IL12Rβ2 chains, which bind p40 and p35 respectively [24,25]. While IL12Rβ1 is shared with IL23 (p40-p19) [25,26] and is more ubiquitously expressed, IL12Rβ2 expression is primarily restricted to IL12-responding cells, such as T and NK cells, and is upregulated following activation [27–29].

We have recently developed a flow cytometry detection

* Corresponding author at: Université de Montréal, Département de pharmacologie et physiologie, Pavillon Roger-Gaudry, S-455 Case postale #28, 2900 Bld Édouard-Montpetit, Montréal, Québec H3T 1J4, Canada.
E-mail address: jf.gauchat@umontreal.ca (J.-F. Gauchat).

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methodology for CLCF1, and detected the presence of CLCF1 protein in all main immune cell populations of mouse splenocytes, as well as differentiated Th1 cells [30]. We therefore hypothesized that CLCF1 might play a role in the differentiation of Th1 cells.

Using Vav-iCre *Clcf1*$^{fl/fl}$ mice, which have a deletion of *Clcf1* specifically in hematopoietic cells, we observed that *Clcf1*$^{-/-}$ CD4$^+$ T cells activated in the presence of IL12 produced more IFNγ than CD4$^+$ T cells isolated from *Clcf1*$^{+/+}$ mice. This result suggests that CLCF1 could be a negative regulator of IL12-induced Th1 cell differentiation. Moreover, we observed that CLCF1 downregulates the expression of IL12Rβ2. Our data further indicate that CLCF1 can interact with IL12Rβ2, and promote its degradation through the proteasome pathway in a manner independent of ubiquitination. Overall, these results suggest that targeted overexpression of CLCF1 in CD4$^+$ T cells could be used to dampen Th1 cell-driven inflammation, and may indicate a new role for CLCF1 as a mediator of protein degradation.

2. Methods

2.1. Experimental animals

For the detection of CLCF1, CRLF1 and IFNγ mRNA, female C57BL/6 mice (Strain #027) were purchased from Charles River Laboratories (Wilmington MA). For the overexpression of CLCF1 in Th1 cells, GREAT IFNγ-IRES-eYFP female reporter mice [31] (Stock #017581) and control C57BL/6 mice (Stock #000664) were purchased from The Jackson Laboratory (Bar Harbor ME). All animals were used at 8–12 weeks of age.

The *Clcf1* floxed C57BL/6 mouse model with LoxP sites inserted in 5′ and 3′ of the *Clcf1* third exon has been previously reported [32]. These mice were crossbred to wild-type C57BL/6 mice (Strain #000664; The Jackson Laboratories) for 6 generations. *Clcf1*$^{fl/fl}$ mice were then bred to C57BL/6 Vav-iCre (Strain #008610; The Jackson Laboratories) [33]. The experimental animals thus produced were used at 6–12 weeks of age. Age and sex-matched *Clcf1*$^{fl/fl}$ Cre$^-$ or *Clcf1*$^{fl/wt}$ Cre$^-$ mice were used as controls.

All procedures were performed in accordance with the ARRIVE guidelines, as well as the Canadian Council on Animal Care guidelines, and were approved by the Comité de déontologie de l'expérimentation sur les animaux (CDEA) de l'Université de Montréal and the Comité de protection des animaux du CIUSS de l'Est-de-l'Île-de-Montréal (CPA-CEMTL).

2.2. Detection of IFNγ, T-bet and IL12Rβ2 expression in Th1 differentiated cells

All mouse primary cells were grown in RPMI 1640 medium supplemented with fetal bovine serum (FBS; 10 %) (Cat# 090–150; Wisent, Saint-Jean-Baptiste QC), L-glutamine (4 mM; Wisent), penicillin (100 U/ml; Wisent), streptomycin (100 μg/ml; Wisent), HEPES pH 7.4 (10 mM; Wisent) and β-mercaptoethanol (0.05 mM; Sigma-Aldrich, Oakville ON) ("complete RPMI medium").

Mouse mononucleated splenocytes were isolated with histopaque (Sigma-Aldrich) density gradient centrifugation, and CD4$^+$ T cells were purified using the EasySep mouse CD4$^+$ T cells isolation kit (Cat# 19852; STEMCELL Technologies, Vancouver BC) according to manufacturer's instructions. Cells were stimulated for 5 d with coated anti-TCR (5 μg/ml, clone H57–597; homemade) and soluble anti-CD28 (1 μg/ml; BD Biosciences, Franklin Lakes NJ) alone (*i.e.* Th0), or supplemented with anti-mouse IL4 (10 μg/ml, clone BVD4-1D11; BD Biosciences) and murine recombinant IL12 (10 or 1 ng/ml; PeproTech, Cranbury NJ) (*i.e.* Th1). Cell culture medium was changed and cytokines were replenished on day 3.

For the detection of IFNγ and T-bet expression by flow cytometry, cells were stimulated on day 5 with the eBioscience™ cell stimulation cocktail plus protein transport inhibitors (Thermo Fisher Scientific, Burlington ON) in complete RPMI medium for 4 h. Stimulated cells were stained with eFluor 506 Fixable Viability Dye (Thermo Fisher Scientific) on ice for 15 min, and fixed and permeabilized using the FoxP3/transcription factor staining buffer set (Thermo Fisher Scientific) according to manufacturer's instructions. Cells were incubated with 2 % normal rat serum (STEMCELL Technologies) at room temperature for 15 min, and stained with Alexa Fluor 488-conjugated anti-IFNγ (Clone XMG1.2) and PerCP-Cyanine5.5-conjugated anti-T-bet (Clone eBio4B10) (both from Thermo Fisher Scientific) at room temperature for 45 min. Samples were analysed by flow cytometry as described below.

IFNγ concentration in the cell supernatant of Th0 or Th1 differentiated cells was determined on day 5 using an ELISA kit (Cat# 88–7314-22; Thermo Fisher Scientific) according to manufacturer's instructions.

For the detection of IL12Rβ2 expression by flow cytometry, Th0 or Th1 differentiated cells on day 5 were stained with unlabelled anti-mouse IL12Rβ2 (Clone #918102; Novus Biologicals, Minneapolis MN) for 2 h, followed by eFluor 506 Fixable Viability Dye and PE-conjugated anti-rat IgG (BioLegend, San Diego CA) for 1 h, all in PBS containing 0.1 % BSA at 4 °C. Samples were analysed by flow cytometry as described below.

2.3. Overexpression of CLCF1 in Th1 differentiated cells

Codon optimized synthetic cDNA coding for human CLCF1 and tdTomato fluorescent protein were generated by GeneArt (Thermo Fisher Scientific), and subcloned into the pMX retroviral expression vector [34], with tdTomato replacing the puromycin resistance gene. HEK-293 PlatE cells were maintained in DMEM medium supplemented with FBS (10 %), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (4 mM) ("complete DMEM medium"). HEK-293 PlatE cells were transfected for 24 h in Opti-MEM medium (Thermo Fisher Scientific) with pMX derivatives (4 μg) and polyethylenimine. Transfected cells were then incubated in complete DMEM medium for two 24 h cycles. Recombinant retroviruses harvested at 24 and 48 h post-transfection were concentrated with 10 % (*w/v*) polyethylene glycol [35].

CD4$^+$ T cells from GREAT IFNγ-IRES-eYFP reporter mice [31] were isolated as described above, and stimulated with coated anti-TCR and soluble anti-CD28 in complete RPMI medium for 48 h. Cells were mixed with the retroviruses and polybrene (8 μg/ml), centrifuged at 30 °C for 90 min, and incubated for 4 h. Following transduction, cells were stimulated with coated anti-TCR and soluble anti-CD28 for 72 h, after which Th1 differentiation was induced as described above with 10 ng/ml of mouse IL12 for 5 d. Differentiated cells were stimulated with the eBioscience™ cell stimulation cocktail plus protein transport inhibitors for 4 h, and stained with eFluor 506 Fixable Viability Dye on ice for 15 min. Samples were analysed by flow cytometry as described below.

2.4. Detection of CLCF1, IFNγ and CRLF1 mRNA

For the detection of CRLF1 and CLCF1 mRNA, spleen mononucleated cells were isolated by histopaque density gradient centrifugation. For the detection of IFNγ and CLCF1 mRNA, purified CD4$^+$ T cells were activated in Th1 polarizing conditions for 5 d with 10 ng/ml of IL12 as described above. Aliquots of cells were treated upon isolation and every day during the differentiation process with the eBioscience™ cell stimulation cocktail plus protein transport inhibitors in complete RPMI medium for 4 h. Cells were lysed in TRIzol (Thermo Fisher Scientific), chloroform was added, and total RNA was isolated from the aqueous phase using the RNeasy kit (Qiagen, Toronto ON) according to manufacturer's instructions. TaqMan real-time quantitative RT-PCR analysis was conducted by the Genomics platform at the Institute for Research in Immunology and Cancer (Université de Montréal, Montréal QC).

Gene	Oligo FWD	Oligo REV	RefSeq

(*continued on next page*)

(*continued*)

Gene	Oligo FWD	Oligo REV	RefSeq
Clcf1	gccctgggcagagtttct	cgctacctggagcatcaact	NM_019952.3
Crlf1	cctaggctcagcaagatctga	acactcagctggtcctcca	NM_018827
Ifng	atctggaggaactggcaaaa	ttcaagacttcaaagagtctgaggta	NM_008337.3
Gapdh	tgtccgtcgtggatctgac	cctgcttcaccaccttcttg	NM_008084.2
Hprt	tcctcctcagaccgctttt	cctggttcatcatcgctaatc	NM_013556.2

2.5. Transient transfection of IL12Rβ2 and CLCF1 cDNA in HEK-293T cells

Codon optimized synthetic cDNA coding for protein C epitope (EDQVDPRLIDGK)-tagged human CLCF1 ($CLCF1_{ProtC}$), protein C epitope-tagged human p40 ($p40_{ProtC}$) (both tagged in carboxyl), and human CRLF1 (CRLF1) were generated by GeneArt. cDNA comprising the IL12Rβ1 and IL12Rβ2 open reading frames were obtained from OriGene (Rockville MD) and DNAFORM (Yokohama City, Japan) respectively, and subcloned in the pcDNA3.4 expression vector (GeneArt). A FLAG epitope (DYKDDDDK)-tagged full length human IL12Rβ2 ($IL12R\beta2_{FLAG}$) pcDNA3.4 derivative was generated by inserting the FLAG epitope sequence 3′ of the IL12Rβ2 signal peptide using standard molecular biology techniques. FLAG-tagged cDNA derivatives coding for the extracellular domains of IL12Rβ1 ($sIL12R\beta1_{FLAG}$) and IL12Rβ2 ($sIL12R\beta2_{FLAG}$) were generated using standard molecular biology techniques and subcloned in pcDNA3.4.

HEK-293T cells were maintained in complete DMEM medium. HEK-293T cells were transfected for 5 h in Opti-MEM medium (Thermo Fisher Scientific) with $sIL12R\beta1_{FLAG}$, $sIL12R\beta2_{FLAG}$, $CLCF1_{ProtC}$, CRLF1 or $p40_{ProtC}$ pcDNA3.4 derivatives (4 μg) and polyethylenimine. A pcDNA3.4 derivative coding for eGFP was used as control. Transfected cells were incubated in complete DMEM medium overnight, and in serum-free Opti-MEM medium for 3 d. Cell supernatant was collected, and cells were lysed in cell lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 % Nonidet P-40, 0.5 % sodium deoxycholate) supplemented with 1× cOmplete™ protease inhibitors cocktail (Roche Sigma-Aldrich). Samples were analysed by Western blot as described below.

2.6. Isolation of the membrane fraction

HEK-293T cells were transfected for 5 h in Opti-MEM medium with $IL12R\beta2_{FLAG}$, $CLCF1_{ProtC}$ or $p40_{ProtC}$ pcDNA3.4 derivatives (4 μg) and polyethylenimine. A pcDNA3.4 derivative coding for eGFP was used as control. Transfected cells were cultivated in complete DMEM medium overnight, and in Opti-MEM medium for 3 d. Cells were harvested in PBS containing 1× cOmplete™ protease inhibitors cocktail, and lysed using a 28G syringe. The membrane fraction was isolated by ultracentrifugation at 100000 *g*, and resuspended in TGH buffer (50 mM HEPES pH 7.3, 50 mM NaCl, 5 mM EDTA, 10 % glycerol and 1 % Triton X-100) supplemented with 1× cOmplete™ protease inhibitors cocktail. Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. Samples were analysed by Western blot as described below.

2.7. Inhibition of protein degradation

HEK-293T cells were transfected for 5 h in Opti-MEM medium with $IL12R\beta2_{FLAG}$, $CLCF1_{ProtC}$ or $p40_{ProtC}$ pcDNA3.4 derivatives (4 μg) and polyethylenimine. A pcDNA3.4 derivative coding for eGFP was used as control. Transfected cells were cultivated in complete DMEM medium overnight, and in Opti-MEM medium for 3 d. Cells were then incubated in complete DMEM medium supplemented with MG-132 (20 μM; Sigma-Aldrich), Chloroquine (50 μM; Sigma-Aldrich), TAK-243 (1 μM; MedChemExpress, Monmouth Junction NJ), or appropriate vehicles for 12 h. Cells were lysed in cell lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 % Nonidet P-40, 0.5 % sodium deoxycholate) supplemented with 1× cOmplete™ protease inhibitors cocktail, and protein concentration was determined using the Pierce BCA protein assay according to manufacturer's instructions. Samples were analysed by Western blot as described below.

2.8. Co-immunoprecipitation

HEK-293T cells were transfected for 5 h in Opti-MEM medium with $sIL12R\beta2_{FLAG}$ or $sIL6R\alpha_{FLAG}$ pcDNA3.4 derivatives (4 μg) and polyethylenimine. A pcDNA3.4 derivative coding for eGFP was used as control. Transfected cells were cultivated in complete DMEM medium overnight, and in Opti-MEM medium for 3 d.

Cell supernatant from transfected HEK-293T cells was mixed with recombinant biotinylated human CLCF1 produced in *E. coli* BL21 [36] and triton X-100 (1 % final concentration). $sIL12R\beta2_{FLAG}$ was immunoprecipitated using anti-FLAG (Clone M2) affinity gel (Sigma-Aldrich) according to manufacturer's instructions, and eluted using SDS-PAGE sample loading buffer without β-mercaptoethanol. β-mercaptoethanol was added to the samples after elution (100 mM final concentration), and samples were analysed by Western blot as described below.

Alternatively, cell supernatant from transfected HEK-293T cells was mixed with recombinant protein C-tagged human CLCF1 ($CLCF1_{ProtC}$), $CaCl_2$ (1 mM final concentration) and Nonidet P40 (1 % final concentration). $CLCF1_{ProtC}$ was immunoprecipitated using anti-ProtC affinity matrix (Roche) according to manufacturer's instructions, and eluted with the suggested elution buffer (20 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA). Samples were analysed by Western blot as described below.

2.9. Western blot analysis

All samples were subjected to 12 % ($CLCF1_{ProtC}$, $p40_{ProtC}$, $sIL12R\beta2_{FLAG}$, $sIL12R\beta1_{FLAG}$, GAPDH and β-catenin) or 7,5 % ($IL12R\beta2_{FLAG}$) polyacrylamide SDS-PAGE, and electrotransferred to PVDF membranes. Membranes were probed with HRP-conjugated streptavidin (GE Healthcare, Chicago IL); anti-protein C (Clone HPC4; GenScript, Piscataway NJ), anti-GAPDH (Thermo Fisher Scientific) or anti-β-catenin (Cell Signaling Technology, Danvers MA) followed by HRP-conjugated anti-rabbit IgG; or anti-FLAG (Clone M2; Sigma-Aldrich) or anti-CLCF1 (Clone #138815; R&D systems, Minneapolis MN) followed by HRP-conjugated anti-mouse IgG. Chemiluminescence signal was detected using an ImageQuant LAS 4000 (GE Healthcare, Chicago IL) or autoradiography films.

2.10. Detection of IL12Rβ2 and IFNγ expression in NK cells

For the detection of IFNγ expression, mononucleated splenocytes were isolated with histopaque density gradient centrifugation and stimulated in complete RPMI medium with murine IL15 (100 ng/ml; BioLegend) and murine IL12 (20 ng/ml) for 24 h. Cells were restimulated with the eBioscience™ cell stimulation cocktail plus protein transport inhibitors in complete RPMI medium for 4 h. Cells were stained with BV711-conjugated anti-CD19 (Clone 6D5; BioLegend), Alexa Fluor 700-conjugated anti-CD3 (Clone 17A2), PE-Cy7-conjugated anti-NK1.1 (Clone PK136), eFluor 450-conjugated anti-CD49b and eFluor 506 Fixable Viability Dye (all from Thermo Fisher Scientific) for 45 min at 4 °C. Cells were then fixed and permeabilized with the Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences) according to manufacturer's instructions, and stained with Alexa Fluor 488-conjugated anti-IFNγ for 45 min at room temperature. Samples were analysed by flow cytometry as described below.

For the detection of IL12Rβ2 expression, mononuclear splenocytes were isolated with histopaque density gradient centrifugation and stimulated in complete RPMI medium with murine IL15 for 72 h. Cells were stained with unlabelled anti-mouse IL12Rβ2 for 2 h at 4 °C,

followed by PE-conjugated anti-rat IgG for 1 h at 4 °C. Cells were then stained with BV711-conjugated anti-CD19, Alexa Fluor 700-conjugated anti-CD3, PE-Cy7-conjugated anti-NK1.1, eFluor 450-conjugated anti-CD49b and eFluor 506 Fixable Viability Dye for 45 min at 4 °C. Samples were analysed by flow cytometry as described below.

2.11. Flow cytometry analysis

Fluorescence was quantified using a FACSymphony A1 flow cytometer (BD Biosciences). After exclusion of doublets and non-viable cells (*i. e.* "live cells" gate), 10,000 to 30,000 events were recorded for every sample. Data were analysed using the FlowJo software (BD Biosciences). The gating strategy is shown in Supplementary Fig. 5.

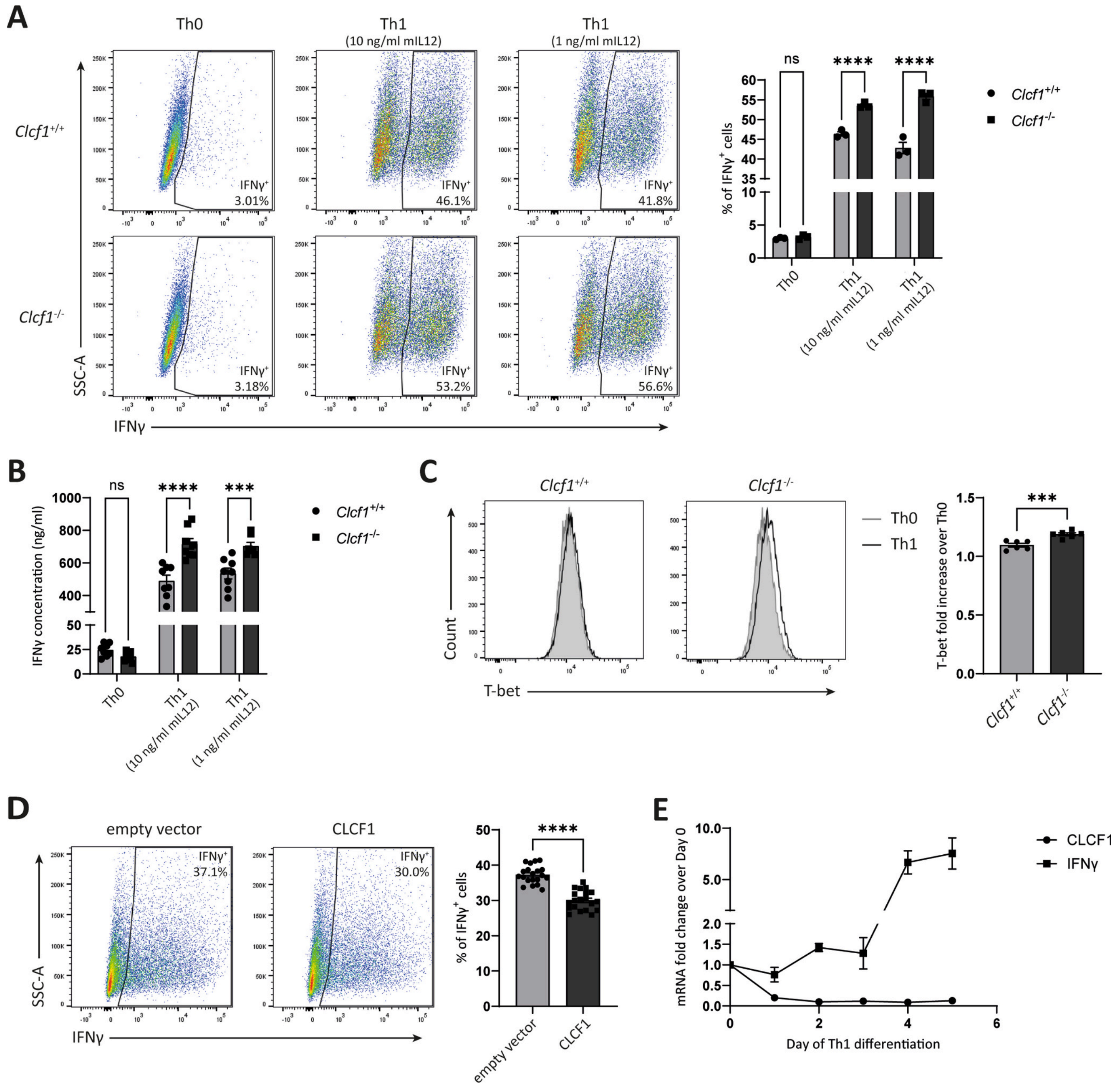

Fig. 1. CLCF1 negatively regulates the differentiation of mouse Th1 cells. (A-C) CD4$^+$ T cells from *Clcf1*$^{+/+}$ or *Clcf1*$^{-/-}$ mice were stimulated with anti-TCR and anti-CD28 alone (Th0) or supplemented with mouse IL12 (Th1) for 5 d. (A) Representative flow cytometry profiles and bar graph showing the percentage (%) of IFNγ$^+$ cells ± SEM. Experiment was performed in 1–3 replicate culture wells, and replicated in four independent experiments. (B) Quantification of IFNγ production by ELISA in ng/ml ± SEM. Experiment was performed in 4 replicate culture wells, and replicated in two independent experiments. (C) Representative flow cytometry profiles of T-bet expression in Th0 (grey filled histogram) and Th1 cells (10 ng/ml mIL12) (black line histogram). Bar graph shows fold increase of T-bet mean fluorescence intensity (MFI) over Th0 cells ± SEM. Experiment was performed in 3 replicate culture wells, and replicated in two independent experiments. (D) Representative flow cytometry profiles of IFNγ$^+$ cells in IFNγ-eYFP reporter mice overexpressing CLCF1. Bar graph shows the % of IFNγ$^+$ cells ± SEM. Experiment was performed in 5–20 replicate culture wells, and replicated in three independent experiments. (E) Quantification of CLCF1 and IFNγ mRNA expression by Taqman real-time quantitative RT-PCR in CD4$^+$ T cells upon isolation and throughout Th1 differentiation. Graph shows mRNA fold change over Day 0 for CLCF1 and IFNγ ± SEM. Experiment was performed in technical duplicates for three experimental animals. (A-D) Statistical significance was assessed using (A-B) a two-way ANOVA with a Šídák correction, or (C–D) Student's *t*-test, with ***p < 0,001 and ****p < 0,0001.

2.12. In silico docking analysis

Putative IL12Rβ2-CLCF1 complex structures were modelled using the AlphaFold3 [37] server (alphafoldserver.com). For each of the top 5 results returned by the server, we utilized the Surfaces method [38] for the quantification of molecular interactions. Among the five AlphaFold models, model 4 was predicted to be the most stable. That model was subjected to a molecular dynamics energy minimization using the Yasara server [39]. Gibbs free energy change (ΔG) values were converted into dissociation constant (K_D) values using the following equation $\Delta G = R \bullet T \bullet \ln(K_D)$, where R represents the gas constant (1.9872 cal/mol·K) and T represents the temperature in Kelvin (K).

2.13. Statistical analysis

Unpaired Student's *t*-test, or two-way ANOVA with a Šídák correction were used where appropriate. The results presented in the figures are marked using asterisks, with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. Data were analysed using the GraphPad prism software (GraphPad, La Jolla CA).

3. Results

3.1. CLCF1 is a negative regulator of the Th1 differentiation in mouse $CD4^+$ T cells

The IL6/IL12 family of cytokines comprises key regulators of $CD4^+$ Th differentiation [40]. To investigate the role of CLCF1 in the control of Th differentiation, we used mice with a floxed *Clcf1* gene crossed with Vav-iCre transgenic mice to induce a constitutive knock-out (KO) of *Clcf1* in hematopoietic cells [32], hereafter referred to as *Clcf1*$^{-/-}$. $CD4^+$ T cells isolated from *Clcf1*$^{-/-}$ mice and activated *in vitro* in the presence of IL12 (*i.e.* under Th1 polarizing conditions) showed a higher percentage of IFNγ-producing cells than $CD4^+$ T cells from control *Clcf1*$^{+/+}$ mice (Fig. 1A). Accordingly, we measured a higher concentration of IFNγ in the cell culture medium of *Clcf1*$^{-/-}$ Th1 cells (Fig. 1B). We also observed an increase in the expression of the transcription factor T-bet in *Clcf1*$^{-/-}$ Th1 cells (Fig. 1C). Furthermore, induction of CLCF1 overexpression in $CD4^+$ T cells through transduction with recombinant retroviruses led to a decrease in the fraction of IFNγ-producing cells (Fig. 1D). Taken together, these results suggest that CLCF1 is a negative regulator of the IL12-induced Th1 differentiation.

We next investigated the expression pattern of CLCF1 during the Th1 differentiation process. Using RT-qPCR, we observed that CLCF1 mRNA is drastically downregulated early on during the differentiation of Th1 cells (Fig. 1E), supporting the hypothesis that CLCF1 is a negative regulator of Th1 differentiation.

3.2. CLCF1 downregulates the expression of IL12Rβ2 in Th1 cells

We previously showed that CLCF1 is expressed by mouse splenocytes using flow cytometry [30]. CLCF1 is usually secreted as a complex with the soluble cytokine receptor-like CRLF1 subunit [2]. However, CRLF1 mRNA does not appear to be significantly expressed by mouse immune cells, as CRLF1 mRNA levels were under the detection limit of RT-qPCR in mouse splenocytes (Supplementary Fig. 1). Therefore, although CLCF1 is expressed by mouse splenocytes, the absence of CRLF1 mRNA suggests that CLCF1 may not be secreted by these cells. Thus, we hypothesize that CLCF1 may influence Th1 differentiation by acting as an intracellular factor.

In an attempt to elucidate a putative intracrine mechanism by which CLCF1 represses the IL12-induced differentiation of Th1 cells, we investigated possible interactions between CLCF1 and the IL12 signaling pathway. The IL12 receptor comprises two chains, IL12Rβ1 and IL12Rβ2, that bind the p40 and p35 subunits of IL12 respectively [24]. IL12Rβ2 expression is upregulated during Th1 differentiation [27,28]. Interestingly, we observed a larger upregulation of IL12Rβ2 expression in Th1 cells isolated from *Clcf1*$^{-/-}$ mice (Fig. 2A), suggesting that CLCF1 can impede the increase of IL12Rβ2 expression in Th1 cells.

Conversely, we observed no effect of CLCF1 on the expression of IL12Rβ2 at the surface of NK cells (Supplementary Fig. 2A), which are also known to respond to IL12 stimulation. Accordingly, the production of IFNγ by NK cells was not affected in *Clcf1*$^{-/-}$ mice (Supplementary Fig. 2B). These results suggest that the effect of CLCF1 on IL12Rβ2 expression could be cell type-specific.

3.3. CLCF1 can form a complex with IL12Rβ2 and promote its degradation

To investigate whether the effect of CLCF1 on IL12-induced Th1 differentiation and IL12Rβ2 expression could be mediated by a physical interaction between the cytokine and the cytokine receptor, we expressed a FLAG-tagged soluble derivative of IL12Rβ2 comprising the extracellular portion of the receptor ($sIL12R\beta2_{FLAG}$) in HEK-293T cells, and mixed the cell culture medium with biotinylated recombinant CLCF1. Immunoaffinity chromatography with anti-FLAG beads, followed by Western blot analysis showed that CLCF1 co-immunoprecipitates with the extracellular domain of IL12Rβ2 (Fig. 2B), suggesting a cytokine-cytokine receptor interaction between CLCF1 and IL12Rβ2. This interaction was further confirmed with a second immunoaffinity chromatography assay using recombinant protein C-tagged CLCF1 as bait protein (Fig. 2C). Moreover, the assay's specificity was confirmed using a soluble derivative of IL6Rα (sIL6Rα) as a negative interaction control (Fig. 2C).

To better understand how CLCF1 and IL12Rβ2 interact, we performed an *in silico* docking analysis (Fig. 3A). The resulting minimized IL12Rβ2-CLCF1 complex is predicted to have a ΔG of −14.27 kcal/mol (*i.e.* $K_D \approx 34$ pM). Thus, the CLCF1-IL12Rβ2 interaction is comparable to the interaction of IL12 with the heterodimeric IL12 receptor ($K_D \approx 50$ pM) [41] and IL12Rβ2 alone ($K_D = 5$ nM) [24]. Furthermore, the most important interactions in the IL12RB2-CLCF1 interface appear to be TYR177-HIS52, HIS152-SER139, GLN112-ALA152 and ASP150-HIS135. Interestingly, previously identified CLCF1 site 1 and 3 residues [42] do not seem to be implicated in the predicted interaction interface. Instead, the CLCF1 residues which are predicted to be involved (LYS42, ASP45, ARG48, TYR49, HIS52, HIS135, SER139, LEU144 and ALA152; Fig. 3B) correspond to CLCF1 site 2, and are the same residues that were recently shown to interact with CRLF1 [43]. On the other hand, the IL12Rβ2 residues predicted to be involved (PHE73, LEU98, GLN112, ASP150, HIS152, TYR154, ILE176 and TYR177; Fig. 3B) correspond to the immunoglobulin-like (Ig-like) domain (D1) and the first fibronectin type III (FNIII) domain (D2) of IL12Rβ2. These IL12Rβ2 domains have also been implicated in the interaction of IL12Rβ2 with p35 [44,45].

Next, we used co-expression of CLCF1 and IL12Rβ2 in HEK-293T cells as a model to analyse the mechanism by which CLCF1 regulates the expression of IL12Rβ2 in $CD4^+$ T cells. Firstly, we co-transfected the FLAG-tagged soluble form of IL12Rβ2 with ProtC-tagged CLCF1. We used a soluble form of IL12Rβ1 as a control receptor chain, and the β subunit of IL12 (p40) as a control cytokine subunit to test the specificity of the effect of CLCF1 on IL12Rβ2. Western blot analysis showed a striking decrease of sIL12Rβ2 expression in both the cell lysate and the cell supernatant when the cytokine receptor was co-expressed with CLCF1 (Fig. 2D). Conversely, we observed no downregulation of sIL12Rβ2 expression when co-transfected with p40 cDNA (Fig. 2D), thus showing that this effect is specific to CLCF1. We also discerned no effect of CLCF1 on the expression of sIL12Rβ1, the shared receptor chain between IL12 and IL23 (Fig. 2D), which suggests that CLCF1 could specifically inhibit the IL12 pathway without affecting IL23 signaling. Furthermore, inducing CLCF1 secretion by co-transfecting the cDNA for its secretion partner CRLF1 did not abrogate the cytokine's effect on sIL12Rβ2 expression (Fig. 2D).

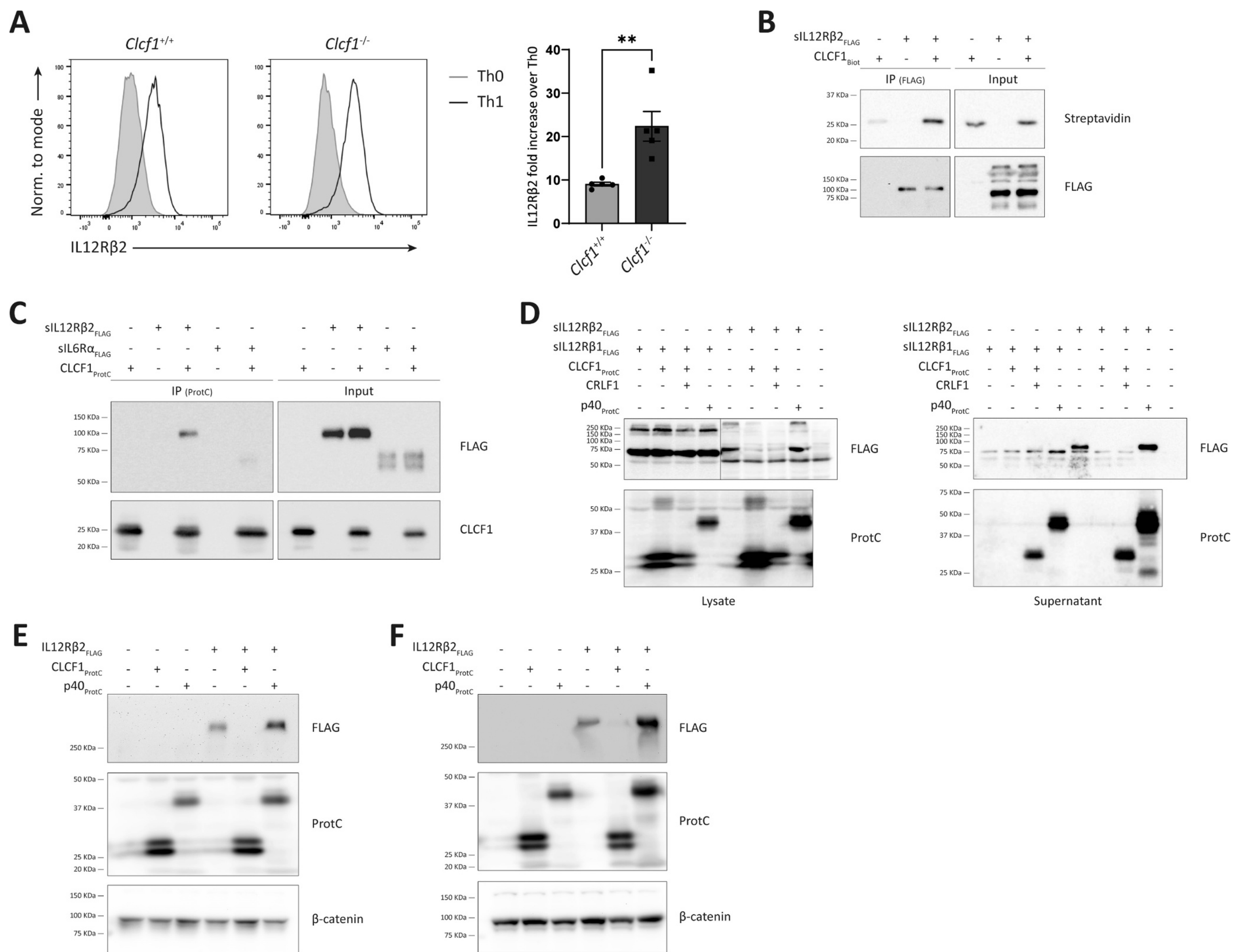

Fig. 2. CLCF1 downregulates the expression of IL12Rβ2. (A) CD4$^+$ T cells from *Clcf1*$^{+/+}$ or *Clcf1*$^{-/-}$ mice were stimulated with anti-TCR/anti-CD28 alone (Th0; grey filled histograms) or supplemented with 10 ng/ml mouse IL12 (Th1; black line histogram) for 5 d. Representative flow cytometry profiles show IL12Rβ2 expression, and bar graph shows fold increase of IL12Rβ2 MFI over Th0 cells ± SEM. Statistical significance was assessed using Student's *t*-test, with $^{**}p < 0.01$. Experiment was performed in 4–5 replicate culture wells, and replicated in two independent experiments. (B) Recombinant biotinylated CLCF1 (CLCF1$_{Biot}$) or (C) recombinant protein C-tagged CLCF1 (CLCF1$_{ProtC}$) were mixed with cell supernatant from HEK-293T cells transfected with eGFP control vector, sIL12Rβ2$_{FLAG}$ or sIL6Rα$_{FLAG}$. (B) sIL12Rβ2$_{FLAG}$ was immunoprecipitated using anti-FLAG affinity gel, or (C) CLCF1$_{ProtC}$ was immunoprecipitated using anti-ProtC affinity matrix. (B–C) Membranes were probed with streptavidin, anti-CLCF1 or anti-FLAG. Blots show eluted proteins (IP) or proteins prior to immunoaffinity chromatography (Input). (D) Western blot analysis of cell lysates (left panel) and supernatants (right panel) from HEK-293T cells transfected with indicated combinations of pcDNA3.4 derivatives coding for sIL12Rβ2$_{FLAG}$, sIL12Rβ1$_{FLAG}$, CLCF1$_{ProtC}$, p40$_{ProtC}$, CRLF1 or eGFP (used as negative control). Membranes were probed with anti-FLAG or anti-ProtC. (E–F) Western blot analysis of (E) total cell lysates or (F) cell membrane fractions from HEK-293T cells transfected with indicated combinations of pcDNA3.4 derivatives coding for IL12Rβ2$_{FLAG}$, CLCF1$_{ProtC}$, p40$_{ProtC}$ or eGFP (used as negative control). Membranes were probed with anti-FLAG or anti-ProtC. β-catenin was used as a loading control. (B–F) Western blot images were cropped, and original blots are presented in Supplementary Fig. 3. Figures are representative of 3–6 separate experiments.

After initially investigating the effect of CLCF1 on IL12R using a soluble derivative of IL12Rβ2, we next confirmed our results using a cDNA coding for a FLAG-tagged full-length membrane-bound form of IL12Rβ2 in the HEK-293T cell line. Western blot analysis of cell lysates from co-transfected cells showed that CLCF1 leads to a marked reduction of IL12Rβ2 expression (Fig. 2E). Isolation and analysis of cell membrane fractions indicated that IL12Rβ2 expression is also downregulated at the cell surface (Fig. 2F).

3.4. *CLCF1 can induce IL12Rβ2 proteolysis through the proteasome protein degradation pathway*

In eukaryotic cells, proteins are degraded through two main pathways, the ubiquitin-proteasome pathway, and the lysosomal proteolysis pathway [46–48]. To analyse which pathway is used by CLCF1 to induce the proteolysis of IL12Rβ2, we treated transfected HEK-293T cells with either chloroquine [49–51], a lysosomal inhibitor, MG-132 [52,53], a proteasome inhibitor, or TAK-243 [54], an inhibitor of the ubiquitin-activating enzyme.

We observed that treating transfected HEK-293T cells with chloroquine did not restore IL12Rβ2 expression levels when the receptor was co-expressed with CLCF1 (Fig. 4A), suggesting that CLCF1 does not induce the degradation of IL12Rβ2 through the lysosomal proteolysis pathway. Conversely, treating transfected cells with MG-132 to inhibit the proteasome almost completely restored IL12Rβ2 expression in the presence of CLCF1 (Fig. 4B). However, inhibiting protein ubiquitination with TAK-243 did not prevent CLCF1-induced IL12Rβ2 proteolysis (Fig. 4C). Altogether, these results suggest that CLCF1 targets IL12Rβ2 to the proteasome in a manner that is independent of ubiquitination.

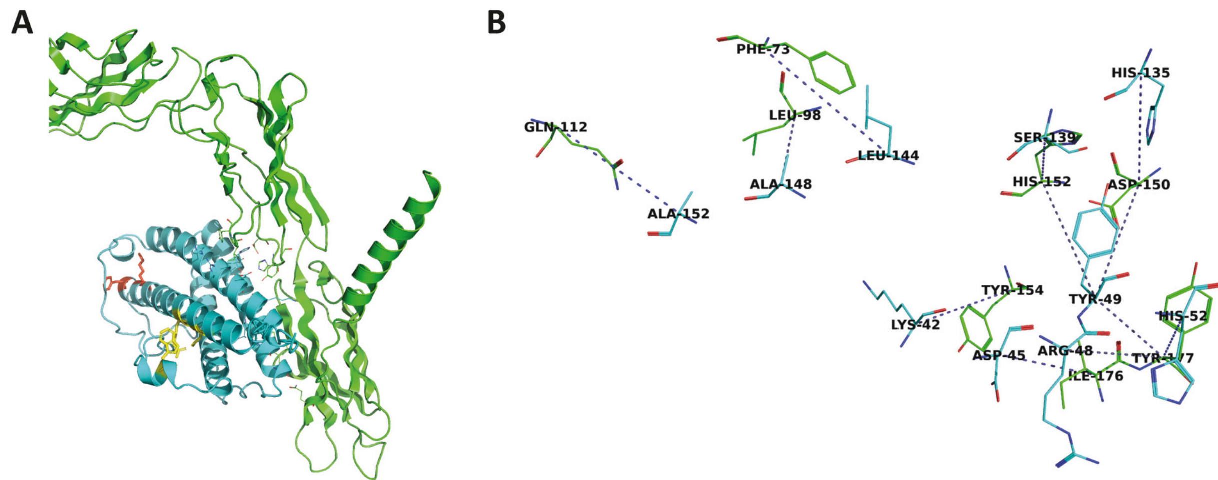

Fig. 3. Predicted IL12Rβ2-CLCF1 complex structure and interacting interface. (A) IL12Rβ2 (green) is predicted to interact with CLCF1 (cyan) with a ΔG of −14.27 kcal/mol. The interaction interface comprises CLCF1 site 2 residues. CLCF1 sites 1 and 3 are shown in yellow and red respectively. (B) Residues in green and cyan represent IL12Rβ2 and CLCF1 respectively. Pairwise interactions are denoted by dashed lines connecting the Ca atoms of the respective residues and represent the sum of all pairwise atomic interactions between the atoms in the main or side chain of the two residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

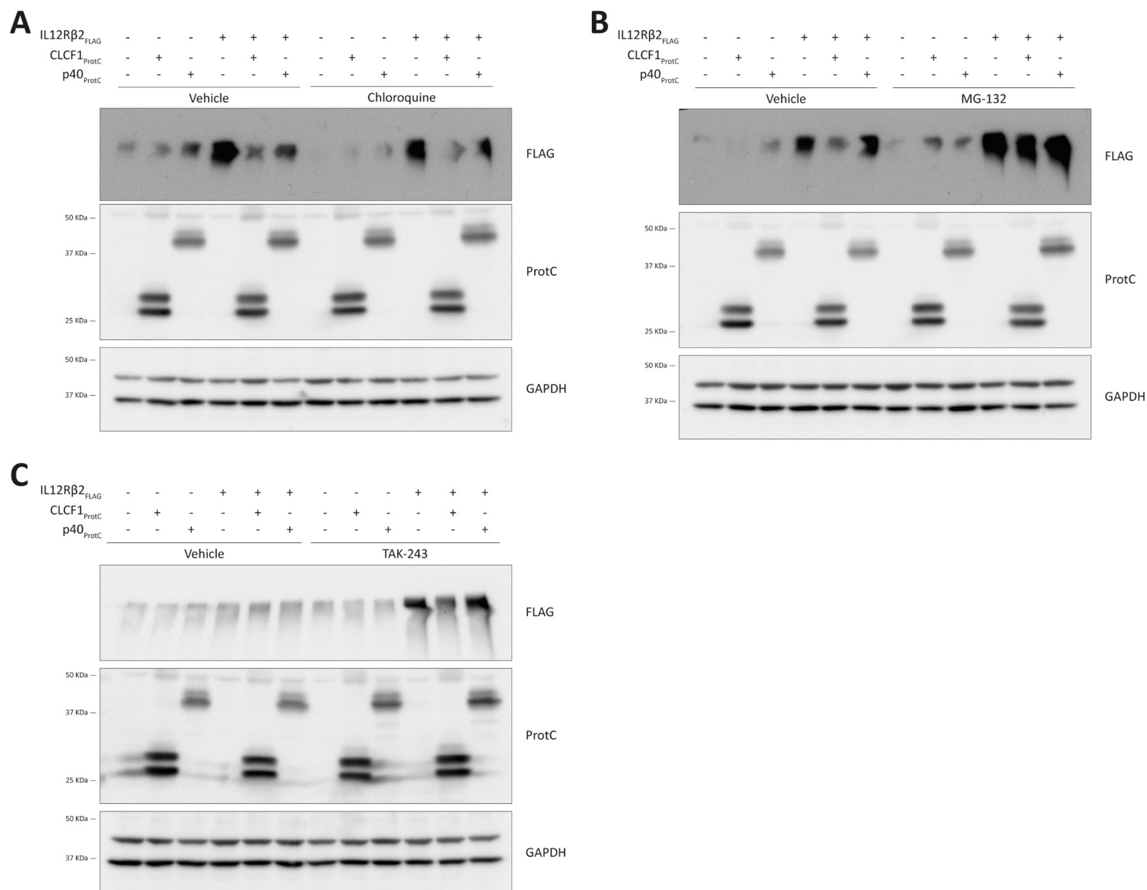

Fig. 4. CLCF1 induces IL12Rβ2 proteolysis through the proteasome. (A-C) Western blot analysis of cell lysates from HEK-293T cells transfected with indicated combinations of pcDNA3.4 derivatives coding for IL12Rβ2$_{FLAG}$, CLCF1$_{ProtC}$, p40$_{ProtC}$ or eGFP (used as negative control). Cells were treated 3 d post-transfection with (A) 50 μM chloroquine, (B) 20 μM MG-132, (C) 1 μM TAK-243, or appropriate vehicles. Membranes were probed with anti-FLAG or anti-ProtC. GAPDH was used as a loading control. Western blot images were cropped, and original blots are presented in Supplementary Fig. 4. Figures are representative of 4–7 separate experiments.

4. Discussion

We previously developed a mouse model with a Vav-iCre driven KO of *Clcf1* in hematopoietic cells to study the role of CLCF1 in immune cells [32,33]. While characterizing this model, we observed that, following IL12 stimulation, CD4^{+} T cells from *Clcf1*$^{-/-}$ mice produced more IFNγ than *Clcf1*$^{+/+}$ cells. We further showed that *Clcf1*$^{-/-}$ Th1 cells expressed higher levels of T-bet, the main transcription factor associated with the

Th1 cell differentiation [20]. These data suggest that CLCF1 could be a negative regulator of the Th1 differentiation and/or of IL12 activities. Supporting this hypothesis, we noted that *Clcf1*$^{-/-}$ Th1 cells expressed higher levels of IL12Rβ2, one of the two receptor chains that composes the IL12 receptor [24].

To investigate the mechanism by which CLCF1 can modulate IL12Rβ2 expression, we expressed IL12Rβ2 cDNA derivatives in the HEK-293T fibroblast cell line. In this model, we observed a protein-protein interaction between CLCF1 and IL12Rβ2. We also showed that co-transfection of CLCF1 and IL12Rβ2 cDNA led to a near-complete loss of IL12Rβ2 expression in HEK-293T cell lysate and at the cell surface. Furthermore, we observed no receptor proteolysis when IL12Rβ1 and CLCF1 cDNA, or IL12Rβ2 and IL12p40 cDNA were co-expressed, highlighting the specificity of CLCF1 effect on IL12Rβ2. As IL12Rβ2 is specific to IL12, unlike IL12Rβ1 which is shared by IL12 and IL23 receptors, our results thus suggest that CLCF1 could specifically inhibit IL12 activities without affecting IL23 biological functions. However, whether CLCF1 can regulate IL12Rβ2 expression in an exogenous manner remains to be investigated.

An *in silico* docking analysis further suggested that CLCF1 interacts with IL12Rβ2 through its site 2. CLCF1 site 2 mediates the interaction with the gp130 signaling chain in the CLCF1-CNTFR complex, as well as the interaction with CLCF1 secretion partner, CRLF1 [43]. Interestingly, while the presence of CRLF1 allows CLCF1 secretion, it does not abrogate the effect of CLCF1 on IL12Rβ2 expression in our transfected HEK-293T model. This could be explained by the fact that CRLF1 can also interact with CLCF1 site 3 [42,43], thus permitting the possibility of a CLCF1-CRLF1-IL12Rβ2 complex. Unfortunately, no mutations capable of inactivating CLCF1 site 2 have yet been identified. Therefore, the *in silico* docking analysis cannot be validated experimentally at this time. Furthermore, the *in silico* docking analysis suggests that CLCF1 interacts with D1 and D2 of IL12Rβ2. These domains are also responsible for the interaction of the receptor chain with p35. Thus, it would be interesting to study the impact of p35 expression on both the formation of the CLCF1-IL12Rβ2 complex and the effect of CLCF1 on IL12Rβ2 expression. Phylogenetic studies show that the IL6 and IL12 cytokine families are closely related [55–57], and some have even suggested that a clear distinction between these two families is impossible [58]. Indeed, the four α helix structure of IL6 family cytokines, such as CLCF1, is very similar to that of the small subunits of IL12 family cytokines like p35 [59]. Furthermore, many cytokines, like IL27 and IL35, are attributed to both the IL6 and IL12 cytokine families [60,61]. Thus, this new protein interaction between CLCF1, a cytokine of the IL6 family, and IL12Rβ2, a receptor of the IL12 family, further corroborates the close relationship between these two cytokine families.

Moreover, initial analysis of the degradation pathways involved in the effect of CLCF1 on IL12Rβ2 using pharmacological inhibitors point to a targeting of IL12Rβ2 by CLCF1 to the proteasome which is unaffected by ubiquitination inhibitors. Further studies are required to better understand the mechanism and the trafficking mediators that are implicated in CLCF1-driven proteolysis. Notably, CLCF1 can interact with sortilin [62], a member of the Vps10p-D family which can regulate protein sorting, trafficking and degradation [63,64]. Although sortilin has been mostly linked to the lysosomal degradation pathway, it would be interesting to study if sortilin is implicated in CLCF1-mediated proteolysis.

We have previously shown, using the same *Clcf1* conditional KO mice model, that CLCF1 expression can be detected in CD4^{+} T cells, as well as Th1 differentiated cells [30]. In the present study, we further showed that, although CLCF1 mRNA remains detectable throughout the activation timeline, its expression is strongly reduced early on during *in vitro* Th1 differentiation. This data supports a model in which CLCF1 is an IL12 pathway inhibitor which is downregulated during Th1 polarization. However, modulation of CLCF1 protein expression levels during Th1 polarization remains to be confirmed. Furthermore, as the mRNA for CRLF1, CLCF1 secretion partner, is undetectable in immune cells, CLCF1 likely exerts its function in T cells intracellularly, which is consistent with the effect of CLCF1 we observed on IL12Rβ2 expression. As CLCF1 possesses a conventional signal peptide [6], it is believed that CLCF1 enters the classical secretory pathway but remains trapped by an unknown mechanism [2]. Further studies are needed to determine in which cellular compartment CLCF1 and IL12Rβ2 co-localize and interact.

NK cells are also sensitive to IL12 stimulation [11]. Interestingly, we observed no effect of CLCF1 on IL12Rβ2 expression or IFNγ production in NK cells. This suggests that the mechanism by which CLCF1 can regulate IL12Rβ2 expression is not present in NK cells and might be specific to T cells. An in-depth comparison of the expression levels of the different proteasome components in CD4^{+} T cells and NK cells could help identify mediator candidates of CLCF1-driven proteolysis. Moreover, it would be interesting to study the effects of CLCF1 on IL12Rβ2 expression and IL12 activities in other types of IL12-responsive cells, such as CD8^{+} T cells.

In conclusion, we have uncovered a new role for CLCF1 as an intracellular ligand for IL12Rβ2, and a negative regulator of the Th1 differentiation. These data suggest that targeted overexpression of CLCF1 in CD4^{+} T cells could be used to modulate the Th1/Th2 balance and reduce Th1-driven inflammation. Further studies are necessary to better evaluate the *in vivo* impact of *Clcf1* KO on IL12-dependent activities and pathologies, such as the innate and adaptative response to pathogens. Our results also indicate a new biological function for CLCF1 in the regulation of IL12Rβ2 protein expression. It remains to be investigated whether CLCF1 could target the degradation of other receptor chains. Promising targeted protein degradation technologies, such as proteolysis targeting chimeras (PROTAC) [65] and cytokine receptor-targeting chimeras (KineTAC) [66], have recently been making their way to the clinic. Our work suggests that CLCF1-bait chimeras could be tested for the induction of specific cytokine receptor degradation in pathologies involving cytokine dysregulation.

CRediT authorship contribution statement

Véronique Laplante: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Marine Rousseau:** Writing – review & editing, Investigation. **Sarah Pasquin:** Writing – review & editing, Supervision. **Capucine Bourel:** Writing – review & editing, Methodology. **Maxime Uriarte:** Methodology. **El Bachir Affar:** Writing – review & editing, Methodology. **Rafael Najmanovich:** Writing – original draft, Visualization, Formal analysis. **Sylvie Lesage:** Writing – review & editing, Supervision, Conceptualization. **Jean-François Gauchat:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2025.156989.

Data availability

Data will be made available on request.

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