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The IL-27 p28 Subunit Binds Cytokine-Like Factor 1 to Form a Cytokine Regulating NK and T Cell Activities Requiring IL-6R for Signaling

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The IL-27 p28 subunit binds cytokine-like factor 1 to form a cytokine regulating NK and T cell activities requiring IL-6R for signaling. IL-27 is formed by the association of a cytokine subunit, p28, with the soluble cytokine receptor EBV-induced gene 3 (EBI3). The IL-27R comprises gp130 and WSX-1. The marked difference between EBI3−/− and WSX-1−/− mice suggests that p28 has functions independent of EBI3. We have identified an alternative secreted complex formed by p28 and the soluble cytokine receptor cytokine-like factor 1 (CLF). Like IL-27, p28/CLF is produced by dendritic cells and is biologically active on human NK cells, increasing IL-12- and IL-2-induced IFN-γ production and activation marker expression. Experiments with Ba/F3 transfectants indicate that p28/CLF activates cells expressing IL-6Rα in addition to the IL-27R subunits. When tested on CD4 and CD8 T cells, p28/CLF induces IL-6Rα-dependent STAT1 and STAT3 phosphorylation. Furthermore, p28/CLF inhibits CD4 T cell proliferation and induces IL-17 and IL-10 secretion. These results indicate that p28/CLF may participate in the regulation of NK and T cell functions by dendritic cells. The p28/CLF complex engages IL-6R and may therefore be useful for therapeutic applications targeting cells expressing this receptor. Blocking IL-6R using humanized mAbs such as tocilizumab has been shown to be beneficial in pathologies like rheumatoid arthritis and juvenile idiopathic arthritis. The identification of a new IL-6R ligand is therefore important for a complete understanding of the mechanism of action of this emerging class of immunosuppressors.

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Interleukin 27 is a composite cytokine formed by the association of a four helix bundle cytokine, p28, with a soluble receptor, Epstein-Barr virus-induced gene 3 (EBI3) (1). IL-27 is produced by activated APCs (1) and signals through a receptor formed by WSX-1 and gp130 (2). Initial characterization of IL-27 indicated multiple activities, such as the capacity to sustain naive CD4 T cell proliferation, to induce NK cell coactivation, to induce mast cell activation and to modulate B cell Ig class switching, and to induce Th1 differentiation induced by IL-6 and TGF-β (21). This suggests that p28 exerts biological activities independently of EBI3. Substantiating this hypothesis, p28 can inhibit Th1 differentiation induced by IL-6 and TGF-β in vitro (11).

IL-27 functions have also been studied using EBI3 knockout. Marked differences have been observed between mice deficient in EBI3 and WSX-1. EBI3−/− mice are protected from Con A-induced liver injury, whereas WSX-1−/− mice showed exacerbated hepatitis (19, 20). Similarly, naive T cells from WSX-1−/−/EBI3−/− mice showed opposite phenotypes with respect to IFN-γ production (5, 21). This suggests that p28 exerts biological activities independently of EBI3. The IL-27 p28 subunit shares closest homology with IL-11 and cardiotrophin-like cytokine (CLC) (1). Like p28, CLC requires a soluble cytokine receptor, cytokine-like factor (CLF), to be secreted (22). This prompted us to investigate whether CLF could form a secreted, biologically active complex with p28.

Materials and Methods

Generation of stable HEK-293 transfectants expressing human p28, CLF, p28/CLF, or GFP

The human p28 cDNA (IRAU969H1198D; Deutsches Ressourcenzentrum für Genomforschung) was modified by introducing the sequence coding for a carboxyl-terminal protein C epitope (EQVDPRLIDGK). The
CLF cDNA (23) was modified by introducing a sequence coding for a FLAG epitope (DYKDDDDK) at the COOH terminus containing the protein. The p28-protC and CLF-FLAG cDNAs were cloned in the vector pcDNA5 (Invitrogen) alone or as a bicistronic insert containing an internal ribosome entry site and used to generate stable Flp-In-293 (Invitrogen) transfectants as previously described (24).

**Purification of human p28/CLF complex**

Human p28/CLF was produced using Flp-In-293 cells stably transfected with the p28-protC/CLF-FLAG bicistronic construct. Transfectants were expanded and recombinant p28/CLF was isolated from the culture medium following the procedure described previously for CLC/CLF (24) using LPS-free reagents and glassware. Purified protein batches were dialyzed against RPMI 1640 medium (Invitrogen). LPS contamination was determined using the Limulus amebocyte lysate test (QCL-1000; Cambrex) and was always <0.1 ng/μg (1 endotoxin unit/μg). Protein concentrations were determined by SDS-PAGE and Coomassie blue staining using BSA as standard.

**Production of mouse p28/CLF and mouse “hyper-IL-6”**

To generate a bicistronic vector encoding mouse p28 and mouse CLF, the mouse p28 cDNA (RIKEN clone B80047N17) was obtained from the FANTOM Consortium, Institute of Physical and Chemical Research (RIKEN, Saitama, Japan) Genome Expression Research Group (provided by K. K. DNAFORM, Ibaraki, Japan). The cDNAs were modified by PCR to generate bicistronic protein encoding for mouse CLF (25) fused at the carboxy-terminal to the FLAG epitope and the T2A “self-cleaving” peptide followed by mouse p28 tagged at the CHOO terminus by a protein C epitope and 6 His. For the production of a mouse hyper IL-6 (25), the cDNAs coding for mouse IL-6α and IL-6 (IMAGE ID 5135770 and 40130735; Open Biosystems) were modified by PCR to generate a single-chain fusion protein encoding for the extracellular domain of IL-6α fused to the mature form of IL-6 by a GGKGGGGG/GSHHHHHH linker. The inserts were recombined in the insect expression vector pIB/V5-His (Invitrogen). Stable High Five (Invitrogen) transfectants were generated and expanded in Express Five Medium (Invitrogen) according to the supplier’s recommended procedure. The p28/CLF complex and hyper-IL-6 were purified by immobilized metal-affinity chromatography on Ni-NTA agarose (Sigma-Aldrich). Following extensive washing, the precipitates were concentrated by ultrafiltration and incubated overnight at 4°C (excitation wavelength, 530 nm) with a Wallac Victor2 1420 Multilabel Microplate Reader (PerkinElmer Life and Analytical Sciences). Throughout the study, the purity of the NK cell populations used was >99%, NK cells at 1 × 105 cells/ml were stimulated with 50 ng/ml human p28/CLF in the absence or presence of either 100 or 500 U/ml IL-2 and either 5 or 25 ng/ml IL-12 (both from R&D Systems) for 48 h in 48-well plates. IFN-γ was then quantified in the cell-free supernatants using capture and detection Abs from Mabtech; sensitivity 4 pg/ml. Membrane CD54 and CD69 expression was evaluated by flow cytometry with FITC-labeled anti-CD54 and CD69 mAbs (Serotec). Results are expressed as mean fluorescence intensity (MFI) after subtracting the MFI obtained with the control Ab.

**Cytokine-binding assays**

Mouse IL-13R, WSX-1, or IL-6α cDNA were cloned in the vector pcDNA5 and used to generate Flp-In-293 stable transfectants. IL-13R-, WSX-1-, or IL-6α expressing Flp-In-293 transfectants were incubated for 1 h on ice with p28-protC/CLF-FLAG (at 1 μg/ml). For competition assays, binding was performed in the presence of IL-27 (10 ng/ml) or mouse ciliary neurotrophic factor (10 ng/ml). Binding was then evaluated with mouse anti-protein C Ab (5 μg/ml) and Alexa Fluor 633-labeled goat anti-mouse IgG Ab. Dead cells were excluded by propidium iodide staining (1 μg/ml). Fluorescence was assessed by flow cytometry.

**Generation of stable Ba/F3 transfectants**

The cDNA coding for mouse gpl30 (IMAGE ID 6834623; Open Biosys) was cloned in the expression vector pMiG (InvivoGen/Medigen). The cDNAs encoding mouse IL-6α (IMAGE ID 40130735) and IL-13R (IMAGE ID 3585897) were cloned in the expression vector pSFFV-Neo (27). Linearized plasmids were transfected in Ba/F3. Stable transfectants were selected using hygromycin (1 mg/ml) and/or genetin (1 mg/ml). Clones were expanded using mouse IL-6 (25 ng/ml), IL-11 (25 ng/ml), or in-house produced hyper-IL-6 (5 ng/ml).

**Measurement of STAT1 and STAT3 activation by flow cytometry**

Ba/F3 cells were serum- and cytokine-starved for 4 h. Lymph node cells or Ba/F3 cells were activated for 15 min at 37°C with mouse IL-27 (50 ng/ml; R&D Systems), p28/CLF (50 ng/ml), IL-11 (50 ng/ml), IL-6 (50 ng/ml), or hyper-IL-6 (10 ng/ml). Cells were fixed with 2% formaldehyde for 10 min at 37°C, washed with cold PBS, resuspended in ice-cold Perm-III (BD Biosciences), and incubated on ice for 30 min. After two washes in PBS, cells were stained in PBS/2% FBS, stained with anti-STAT1 and anti-STAT3 Abs (R&D Systems) and analyzed by flow cytometry.

**Proliferation assay**

Ba/F3 transfectants (5 × 104 cells/well in 96-well plates) were incubated in triplicates with indicated dilutions of recombinant proteins for 72 h in the presence of 10 ng/ml IL-2. Proliferation was measured using a fluorometric assay (Alamar blue; Serotech). Fluorescence was measured at 590 nm (excitation wavelength, 530 nm) with a Wallac Victor2 1420 Multilabel Microplate Reader (PerkinElmer Life and Analytical Sciences).
CD4 T cell differentiation assays

Spleen and lymph node CD4 T cells were enriched by magnetic bead depletion of CD8 and NK cells according to the manufacturer’s instructions (StemCell Technologies). Cells were plated in 96-well round-bottom plates (Corning) at a density of 5 x 10^4 cells/ml. Cells were stimulated with plate-coated anti-CD3 and soluble anti-CD28 (both at 1 µg/ml; BD Biosciences). Cultures were supplemented with anti-IFN-γ and anti-IL-4 (both at 10 ng/ml; eBioscience). IL-17 production was induced using human TGF-β (1 ng/ml; Sigma-Aldrich) and IL-6 (10 ng/ml; PeproTech). When added, IL-27 (R&D Systems) and p28/CLF were used at 50 ng/ml. CD4 T cells were supplemented with fresh medium and reagents at day 3. After 4 days of culture, enriched CD4 T cells were restimulated with PMA (50 ng/ml) and ionomycin (1 µg/ml) for 4 h. During the last 2 h, cytokine secretion was blocked with brefeldin A (5 µg/ml). Cells were collected and fixed with formaldehyde (2%) for 30 min at room temperature. Cells were permeabilized with saponin (0.5%) and stained with FITC-labeled anti-CD4 and PE-labeled anti-IL-17 (eBioscience) for 1 h at room temperature. Fluorescence was measured using a FACS Calibur (BD Biosciences) and data were analyzed with the FlowJo software (Tree Star).

IL-10 secretion assays

For IL-10 secretion assays with purified CD4 T cells, cells were isolated by FACS sorting and stimulated with anti-CD3 and anti-CD28 as described in the previous section. IL-6, IL-27, and p28/CLF were used at 33 ng/ml. For secretion assays with unfractionated splenocytes, cells were activated with plate-coated anti-CD3 and soluble anti-CD28 (both at 1 µg/ml) with concentrations of IL-6, IL-27, and p28/CLF ranging from 0 to 600 ng/ml. After 4 days of culture, IL-10 secretion was measured by ELISA (BD Biosciences) and data were analyzed with the FlowJo software (Tree Star).

Proliferation measurements using CFSE

CD4 T cells purified by FACS sorting were stained with CFSE (Molecular Probes) following the manufacturer’s instructions. Cells were stimulated with plate-coated anti-CD3 and anti-CD28 mAbs (both at 1 µg/ml; BD Biosciences) at 2 x 10^6 cells/ml in 96 round-bottom well plates. For certain experiments, CD4 T cells were mixed with APCs at 1:3 ratios. APCs were obtained by FACS sorting and were negative for CD8, CD4, and NK1.1 markers. Cultures were supplemented with anti-IFN-γ and anti-IL-4 (both at 10 µg/ml; eBioscience). IL-27, IL-6 and p28/CLF were used at 50 ng/ml. Proliferation was assessed by flow cytometry at day 3.

Results

The IL-27 p28 subunit and CLF form a secreted complex

To study whether p28 can be secreted as a complex with CLF, we generated HEK-293 transfectants expressing epitope-tagged derivatives of p28 and CLF alone or in combination. GFP cDNA transfectants were used as a control. The culture medium were subjected to immunoprecipitation with tag-specific mAbs and the isolated fractions were analyzed by Western blot following the manufacturer’s instructions. Cells were stimulated with plate-coated anti-CD3 and anti-CD28 mAbs (both at 1 µg/ml; BD Biosciences) at 2 x 10^6 cells/ml in 96 round-bottom well plates. For certain experiments, CD4 T cells were mixed with APCs at 1:3 ratios. APCs were obtained by FACS sorting and were negative for CD8, CD4, and NK1.1 markers. Cultures were supplemented with anti-IFN-γ and anti-IL-4 (both at 10 µg/ml; eBioscience). IL-27, IL-6 and p28/CLF were used at 50 ng/ml. Proliferation was assessed by flow cytometry at day 3.

The p28/CLF complex is produced by DC

As experiments with HEK-293 transfectants indicated that p28 and CLF can be secreted as a complex, we investigated whether p28/CLF could be produced by immune cells. Activated APCs, such as DC express p28 mRNA (1, 28). As expected (1), LPS-induced DC maturation up-regulated p28 mRNA levels in both human and mouse DC (Fig. 1, B and C). Human monocyte (B)-derived and mouse BM-derived DC (C) were left unstimulated (iDC) or stimulated (mDC) with LPS (20 ng/ml or 1 µg/ml, respectively). RNA was isolated at 48 h (human DC) or 18 h (mouse DC). Expression of p28 and CLF mRNA was assessed by RT-PCR. The control without reverse transcriptase is indicated as “−RT”. D, BM-derived DC were left unstimulated (iDC) or stimulated for 18 h with LPS (mDC). After lysis, the p28/CLF complex was immunoprecipitated with anti-CLF Ab (lanes +) or isotype control (lanes −) and analyzed by Western blot with anti-p28 mAb. Mouse 3T3 fibroblasts were used as negative control.

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We next investigated whether p28 and CLF form a complex in DC. As the commercial anti-mouse p28 mAb suitable for Western blot analysis was more sensitive than the anti-human p28 (supplementary Fig. S1A and data not shown), experiments were performed with mouse BM-derived DC. When immature or mature DC were lysed and subjected to immunoprecipitation with an anti-CLF Ab, a p28 signal was detected by Western blot in the purified

\footnote{The online version of this manuscript contains supplemental material.}
The p28/CLF complex stimulates human NK cells

The observation that DC produce the p28/CLF complex (Fig. 1D) prompted us to test whether p28/CLF was biologically active. As NK cells respond to DC signals (29) and are activated by IL-27 (1), we tested whether p28/CLF could affect NK cell function. NK cells are negative for EBI3 mRNA even after activation, allowing us to exclude a contribution of EBI3 secreted by target cells (1).

Highly purified human NK cells were incubated with human p28/CLF alone or in combination with IL-2 or IL-12. The production of IFN-γ was measured by ELISA. Incubation with p28/CLF up-regulated IFN-γ production by NK cells stimulated with sub-optimal concentrations of IL-2 or IL-12 in a dose-dependent manner (Fig. 2, A and B). We analyzed the effect of p28/CLF on the expression of the NK cell activation markers CD54 and CD69. Expression of both CD54 and CD69 was up-regulated by p28/CLF in NK cells stimulated with IL-2 or IL-12 (Fig. 2C). Incubation with p28/CLF did not modulate NK cell proliferation, perforin/granzyme B expression, and cytotoxic activity on K562 target cells (Fig. 2D and data not shown).

Together, these observations indicate that p28/CLF is biologically active: it increases IFN-γ production by activated NK cells.

The p28/CLF complex activates cells expressing IL-6R and IL-27R

IL-27R is made up of gp130 and WSX-1 (2). WSX-1 alone is sufficient for IL-27 binding (1). The formation of a complex between p28 and EBI3 is required for receptor recognition, as neither p28 nor EBI3 alone bind to IL-27R (2). We examined whether CLF could substitute for EBI3 in forming an alternative complex activating IL-27R. Experiments with mouse HEK-293 transfectants expressing WSX-1 indicated that p28/CLF binds WSX-1 and that the binding could be prevented by competition with IL-27, suggesting that WSX-1 was involved in the p28/CLF receptor (supplementary Fig. S1B). We next used the IL-3-dependent mouse pro-B cell line Ba/F3 to study functional responses to p28/CLF. Ba/F3 cells can be rendered responsive to cytokines by transfection with receptor chain cDNAs and have been used to characterize both the IL-6R and CLC/CLF receptors (1, 22). Ba/F3 cells express endogenous WSX-1 and can be rendered IL-27 responsive by introducing gp130 cDNA (2). In the presence of IL-2, both Ba/F3 cells with mouse gp130 cDNA and tested the effect of purified mouse recombinant p28/CLF on STAT1 and STAT3 phosphorylation. Incubation of Ba/F3-gp130 transfectants with p28/CLF led to a detectable induction of STAT3 phosphorylation but no STAT1 activation, suggesting that p28/CLF receptor activation was incomplete in these cells (Fig. 3A, left panels). The Ba/F3-gp130 cells responded robustly to both mouse IL-27 and the p28 ligand hyper-IL-6 (25), indicating that the lack of response was not due to insufficient expression of WSX-1 or gp130 (supplementary Fig. S2A and Fig. 3A).

IL-6 family members like p28 have three potential receptor binding sites (30, 31). According to the current IL-27/IL-27R complex model, p28 binds EBI3 through site I, WSX-1 through site II, while gp130 recruitment is mediated by site III (30). EBI3 functions as a soluble α-chain, required for receptor recognition and activation (2, 30). Whereas CLF was initially considered a soluble α-chain, it does not play this role in the CLC/CLF complex which requires the nonsignaling CNTFRα chain in addition to two signal-transducing subunits for functional responses (22). Mutagenesis analysis indicated that, whereas CNTFRα binds CLC site I (the site interacting with α-chains in the IL-6 family (31)), CLF binds the CLC site III (32). Therefore, if p28/CLF is structurally similar to CLC/CLF, its receptor requires an α-chain in addition to WSX-1 and gp130. To investigate this hypothesis, we introduced cDNAs encoding mouse IL-6Rα or IL-11Rα into gp130-transfected Ba/F3 cells. These transfectants express functional receptors for either IL-27 and IL-6 (i.e., WSX-1, gp130, and IL-6Rα) or IL-27 and IL-11 (i.e., WSX-1, gp130, and IL-11Rα). As expected, introduction of IL-6Rα or IL-11Rα rendered the Ba/F3-gp130 cells responsive to IL-6 or IL-11, respectively. When analyzed for STAT1 and STAT3 phosphorylation (Fig. 3A). Strikingly, introduction of the IL-6Rα chain strongly increased p28/CLF-induced STAT3 phosphorylation (Fig. 3A). In the presence of IL-6Rα, STAT1

\[ \text{FIGURE 2.} \quad \text{The p28/CLF complex enhances NK cell activation.} \]

A. NK cells purified from three independent donors were stimulated with the indicated concentrations of IL-2 or IL-12 in the presence or absence of purified p28/CLF (100 ng/ml) for 48 h and IFN-γ production was measured by ELISA. B. Purified NK cells were incubated for 48 h with medium alone, IL-2 (100 U/ml), or IL-12 (5 ng/ml) and various concentrations of p28/CLF (2–50 ng/ml). Errors bars indicate the SEM of triplicate cultures. C. NK cells were incubated for 24 or 48 h with medium alone, IL-2 (100 U/ml), p28/CLF (100 ng/ml), or a combination of the two cytokines. Expression of CD64 and CD69 was assessed by flow cytometry. D. NK cells stimulated with IL-2 (100 U/ml), IL-2 and p28/CLF (100 ng/ml), or IL-2 and IL-27 (100 ng/ml) for 48 h were incubated for 4 h at 37°C with K562 target cells at the indicated E:T ratios and the fraction of specific lysis was determined. Errors bars indicate the SEM of triplicate cultures.
**FIGURE 3.** Both p28 and the p28/CLF complex induce STAT1 and STAT3 phosphorylation in Ba/F3 cells transfected with gp130 and IL-6Rα. A, Ba/F3 transfectants expressing endogenous WSX-1 in combination with either gp130, gp130, and IL-11Rα or gp130 and IL-6Rα were stimulated with the cytokines indicated for 15 min, then fixed and permeabilized. STAT phosphorylation was assessed by flow cytometry. B and C, Ba/F3-IL-6Rα cells were preincubated with blocking anti-IL-6Rα mAb (0.5 µg/ml; B) or anti-gp130 mAb (1 µg/ml; C) before stimulation. MFI values are indicated in the histograms. Data are representative of three independent experiments. D, Representation of the putative p28/CLF receptor based on the current models of the IL-27-IL-27R and CLC/CLF-CNTFR complexes (2, 30, 32). According to this model, the CLF Ig-like domain would engage the p28 binding site III. The p28/CLF complex would bind IL-6Rα and WSX-1 through cytokine-binding homology regions (CHR)-p28 site I and site II interactions, respectively. Recruitment of the gp130 subunit would require an exchange between the Ig-like domains of CLF and gp130. Fn III, Fibronectin III domains.
phosphorylation was comparable to the one induced by IL-6 (Fig. 3A). The effect of IL-6Rα cDNA transfection was specific, as no difference in the response induced by p28/CLF was observed between Ba/F3 transfected with gp130 alone or with gp130 plus IL-11Rα (Fig. 3A). These results suggest that the receptor for p28/CLF is tripartite and that the α-chain required for functional activity is IL-6Rα (Fig. 3D). In accordance with this model, we could detect p28/CLF binding on HEK-293 transfected expressing mouse IL-6Rα (supplemental Fig. S1C).

To further assess the involvement of IL-6Rα and gp130 in the p28/CLF receptor complex, we preincubated Ba/F3-gp130-IL-6Rα cells with blocking Abs specific for these two receptor chains (Fig. 3, B and C). Incubation with either anti-IL-6Rα (Fig. 3B) or anti-gp130 (Fig. 3C) Abs resulted in a clearly decreased level of STAT3 and STAT1 phosphorylation in response to p28/CLF, confirming the contributions of these two chains. No inhibition was observed when Ba/F3-gp130-IL-6Rα cells were stimulated with IL-27 in the presence of an anti-IL-6Rα Ab, indicating that the effect observed was specific for p28/CLF (supplemental Fig. S2B). The involvement of WSX-1 could not be directly tested due to the lack of availability of suitable anti-WSX-1 Abs and the inefficiency of soluble mouse WSX-1-Fc in blocking IL-27-induced responses in Ba/F3 cells transfected with gp130 at the concentrations of IL-27 required to induce a detectable STAT3 and STAT1 phosphorylation or cellular proliferation (data not shown). Together, these results indicate that p28/CLF can activate JAK/STAT signaling pathways in cells expressing IL-6Rα, gp130, and WSX-1. A putative model of the p28/CLF receptor is depicted in Fig. 3D.

To study the functionality of the STAT signaling in response to p28/CLF in the Ba/F3 transfecants, we measured cell proliferation. A clear proliferation was observed when Ba/F3 cells transfected with gp130 and IL-6Rα were incubated with p28/CLF (Fig. 4B). This proliferation could be inhibited by blocking anti-IL-6Rα or anti-gp130 Abs (Fig. 4C). The blocking Abs had no effect on IL-3-induced Ba/F3 proliferation, indicating that the effect on the p28/CLF was specific (data not shown). Whereas Ba/F3-gp130 cells proliferated strongly when cultured with IL-27, their proliferation in response to p28/CLF was very limited (Fig. 4A). As expected, almost no proliferation was induced by p28/CLF in Ba/F3-gp130-IL-11Rα cells (data not shown). This indicates that the STAT3 phosphorylation induced by the p28/CLF complex in the absence of IL-6Rα (Fig. 3A) triggers minimal proliferation and that the STAT3 and STAT1 phosphorylation induced in Ba/F3 transfecants expressing WSX-1, gp130, and IL-6Rα is followed by a robust proliferation.

**CLF is not required for the activation of cells expressing IL-6Rα**

In the case of CLC/CLF, CLF is required for CLC secretion but is dispensable for CNTFR activation (22). It was previously reported that p28 is biologically active (11). We therefore compared p28 and p28/CLF using Ba/F3 transfecants expressing IL-27R or IL-27R plus IL-6Rα. Interestingly, unlike p28/CLF, p28 neither induced detectable STAT3 or STAT1 phosphorylation nor proliferation in Ba/F3 transfecants expressing IL-27R (Figs. 3A and 4A). This indicates that CLF is involved in the activation leading to low-level STAT3 phosphorylation in the absence of IL-6Rα (Fig. 3A). In Ba/F3-gp130-IL-6Rα cells (expressing IL-27R and IL-6Rα), p28 induced STAT1 and STAT3 phosphorylation followed by a proliferation comparable to that induced by the p28/CLF complex (Figs. 3A and 4B). STAT activation and proliferation could be inhibited using blocking anti-IL-6Rα and anti-gp130 Abs (Figs. 3B and 4C). These results indicate that although p28 and p28/CLF have comparable activities on transfecants expressing WSX-1, gp130, and IL-6Rα, p28/CLF might have additional functions linked to its ability to induce STAT3 activation in cells expressing only IL-27R.

**The p28/CLF complex induces STAT1 and STAT3 phosphorylation in CD4 and CD8 T cells and cytokine production in CD4 T cells**

To evaluate whether p28/CLF could induce signaling in T cells, we assessed the effect of p28/CLF stimulation on STAT1 and STAT3 phosphorylation in lymph node cells. Stimulation with p28/CLF induced an increase in STAT1 and STAT3 phosphorylation in both CD8 and CD4 T cells (Fig. 5A). Interestingly, no effect could be detected on the cells negative for CD4 or CD8 (Fig. 5A). This suggests that the requirement for the IL-6Rα chain may render p28/CLF more specific for T cells than IL-27. To verify that the IL-6Rα chain was required for p28/CLF activity on T cells, we preincubated lymph node cells with an anti-IL-6Rα mAb (Fig. 5B). The blocking mAb markedly reduced the response induced by IL-6 or p28/CLF (Fig. 5B). As expected, no effect was observed on the induction of STAT1 and STAT3 phosphorylation by IL-27.
This indicates that IL-6Rα constitutes an essential part of the p28/CLF receptor in primary immune cells.

The levels of STAT1 and STAT3 phosphorylation in T cells were lower than those observed following IL-27 stimulation. However, the rIL-27 used might have a higher potency than native IL-27 because it is a fusion protein structurally analogous to hyper-IL-6, which is two orders of magnitude more potent than the IL-6-soluble IL-6Rα complex (25).

Because p28/CLF induced STAT signaling in CD4 T cells, we investigated whether p28/CLF had a functional effect on these cells. Purified CD4 T cells stained with CFSE were stimulated with anti-CD3 and anti-CD28 in the presence or absence of APCs and proliferation was assessed by flow cytometry (Fig. 5C). Under both culture conditions, CD4 T cell proliferation was inhibited by p28/CLF. Growth inhibition was stronger in the absence of APCs, indicating a direct effect of p28/CLF on CD4 T cells. We next investigated whether the inhibition of CD4 T cell proliferation was paralleled by an effect on differentiation. Since Th17 differentiation has been shown to be induced by IL-6 plus TGF-β and inhibited by IL-27 (10, 11, 33–35), we assessed IL-17 and IFN-γ expression in CD4 T cells. Under both culture conditions, CD4 T cell proliferation was inhibited by p28/CLF. Growth inhibition was stronger in the absence of APCs, indicating a direct effect of p28/CLF on CD4 T cells. We next investigated whether the inhibition of CD4 T cell proliferation was paralleled by an effect on differentiation. Since Th17 differentiation has been shown to be induced by IL-6 plus TGF-β and inhibited by IL-27 (10, 11, 33–35), we assessed IL-17 and IFN-γ expression in CD4 T cells (Fig. 6A). Interestingly, both IL-6- and p28/CLF-driven Th17 differentiation was suppressed by IL-27 (Fig. 6A, lower panels).

Both IL-27 and IL-6 have been shown to induce the production of the anti-inflammatory cytokine IL-10 (15–17). This production is involved in the beneficial effect of IL-27 administration in the experimental autoimmune encephalomyelitis model of multiple sclerosis (16). We therefore investigated whether p28/CLF could also induce IL-10 production by activated CD4 T cells (Fig. 6B). When tested on purified CD4 T cells, p28/CLF, IL-6, and IL-27 induced similar peak levels of IL-10 (Fig. 6B). We compared the potency of p28/CLF, IL-6, and IL-27 in inducing IL-10 production. When tested on splenocytes, the potency of p28/CLF was similar to that of IL-6 but markedly lower than that of IL-27 (Fig. 6C).

Discussion

The difference between the phenotype of EBI3−/− and WSX-1−/− mice suggests that WSX-1 has additional ligands or that p28 forms complexes capable of activating IL-27R with alternative cytokine receptors (5, 19–21). We observed that tagged derivatives of p28 and CLF coexpressed in HEK-293 transfectants form a stable, secreted complex. As activated APCs have been shown to synthesize IL-27 p28 (1, 28, 36), we used mouse BM-derived DC to show that a p28/CLF complex could also be formed between the endogenous cytokine and the soluble cytokine receptor.
besides gp130 and IL-6R,

contrast to IL-6, the p28/CLF receptor is tripartite and comprises, p28/CLF binds to WSX-1 transfectants. This suggests that, in contrast to IL-6R/H9251, it has already been shown that IL-6R binds CNTFR-A and can substitute for CNTFR/H9251. Therefore, these results bring to three the number of IL-6R ligands. We observed that p28/CLF binds to WSX-1 transfectants. This suggests that, in contrast to IL-6, the p28/CLF receptor is tripartite and comprises, besides gp130 and IL-6R, WSX-1 (Fig. 3D). However, the implication of this IL-27R signaling chain remains to be directly established using cells from WSX-1 knockout mice. An alternative model in which p28/CLF would signal by recruiting two gp130 chains cannot be excluded.

We examined whether CLF could substitute for EBI3 in forming an alternative complex activating IL-27R. To investigate the activation of cells expressing IL-27R by p28/CLF, we used the mouse pro-B cell line Ba/F3. This cell line expresses WSX-1 and can be rendered IL-27 responsive by solely introducing gp130 cDNA (2). Ba/F3 transfected with gp130 showed a partial response to p28/CLF: incubation with the complex led to STAT3 phosphorylation and proliferation. This response could be blocked using anti-IL-6R Ab. This indicates that, like in the CLC/CLF complex, CLF is not required for receptor recognition (22, 39). However, unlike p28, p28/CLF induced a STAT3 phosphorylation in transfectants expressing only IL-27R. We confirmed this observation using HEK-293 cells transfected with gp130 and WSX-1 (data not shown). These observations suggest that in cells expressing IL-27R but not IL-6R, CLF could confer specific properties to p28. These p28/CLF-specific effects are likely related to those induced by IL-6 family members activating preferentially STAT3, such as IL-6 (40). The mechanism by which CLF is modifying p28 activity remains to be determined. CLF could potentially increase the affinity of p28 for the WSX-1/gp130 receptor signaling chains in the absence of the IL-6Rα chain.

The observation that the p28/CLF complex could be detected in DC prompted us to evaluate whether the composite cytokine could act on NK cells. The function of these cells is known to be modulated by DC in vitro and in vivo (41–46). NK cells respond to DC prompted us to evaluate whether the composite cytokine could act on NK cells. The function of these cells is known to be modulated by DC in vitro and in vivo (41–46). NK cells respond to DC prompted us to evaluate whether the composite cytokine could act on NK cells. The function of these cells is known to be modulated by DC in vitro and in vivo (41–46). NK cells respond to DC prompted us to evaluate whether the composite cytokine could act on NK cells. The function of these cells is known to be modulated by DC in vitro and in vivo (41–46). NK cells respond to DC prompted us to evaluate whether the composite cytokine could act on NK cells. The function of these cells is known to be modulated by DC in vitro and in vivo (41–46). NK cells respond to DC prompted us to evaluate whether the composite cytokine could act on NK cells. The function of these cells is known to be modulated by DC in vitro and in vivo (41–46). NK cells respond to

In the CLC/CLF complex, CLF is required for CLC secretion but dispensable for cytokine activity (22, 38). Furthermore, it was reported that p28 is biologically active (11). We therefore compared p28 and p28/CLF activities using Ba/F3 cells transfected with gp130 or gp130 plus IL-6Rα. The activity of p28 and p28/CLF was indistinguishable in transfectants expressing IL-27R and IL-6Rα, indicating that, like in the CLC/CLF complex, CLF is not required for receptor recognition (22, 39). However, unlike p28, p28/CLF induced a STAT3 phosphorylation in transfectants expressing only IL-27R. We confirmed this observation using HEK-293 cells transfected with gp130 and WSX-1 (data not shown). These observations suggest that in cells expressing IL-27R but not IL-6Rα, CLF could confer specific properties to p28. These p28/CLF-specific effects are likely related to those induced by IL-6 family members activating preferentially STAT3, such as IL-6 (40). The mechanism by which CLF is modifying p28 activity remains to be determined. CLF could potentially increase the affinity of p28 for the WSX-1/gp130 receptor signaling chains in the absence of the IL-6Rα chain.

The observation that the p28/CLF complex could be detected in DC prompted us to evaluate whether the composite cytokine could act on NK cells. The function of these cells is known to be modulated by DC in vitro and in vivo (41–46). NK cells respond to IL-27 and do not express EBI3 (2). This excludes the neo formation of an IL-27 complex comprising the p28 subunit of p28/CLF and EBI3 released by target cells. Whereas no p28/CLF-induced response was detected in unstimulated human NK cells, reproducible up-regulation of IL-2- or IL-12-induced production of IFN-γ was observed. Coincubation of NK cells with p28/CLF led to increased expression of the activation markers CD54 and CD69. p28/CLF did not modulate the NK cell cytotoxic activities assessed by measuring the killing of the cell line K562. These observations suggest that the perforin/granzyme B-based pathway, which has been reported to be the main pathway mediating K562 cytolysis (47), is not affected by the composite cytokine.
The increase in IFN-γ production detected indicates that the production of one of the key NK cell–DC cross-talk mediator is regulated by p28/CLF. The observation that NK cell activation and IFN-γ production is up-regulated by p28/CLF (whereas cytotoxic activity is not affected) is in line with previous reports analyzing the effects of DC-produced cytokines on NK cells, indicating that the regulation of cytotoxicity is mediated by type I IFNs, whereas production of IFN-γ is regulated by cytokines such as IL-12 and IL-18 (42, 48).

The role of cytokines produced by DC in the activation of NK cells is well established (42, 44, 45, 49, 50). This comprises secreted composite cytokines of the IL-12/IL-27 family and cytokine complexes expressed at the surface of the DC such as the IL-15/IL-15Ra chain complex (1, 48, 50–53). Our results indicate that p28/CLF could, in combination with the structurally related IL-12 or with IL-2, participate in the cross-talk between DC and NK cells. NK cells are important components of the innate immune response and, through their production of cytokines and lytic activity against infected or transformed cells, are crucial for both controlling infection and immune surveillance (51, 54).

IL-27 has key anti-inflammatory roles in different models of infection and autoimmune disease, inhibiting Th17 differentiation and promoting the induction of the anti-inflammatory cytokine IL-10 by CD4 and CD8 T cells (10, 11, 15, 16, 18). IL-27 directly antagonizes the effect of IL-6 on CD4 T cell proliferation and Th17 differentiation, whereas both cytokines have similar activities regarding induction of IL-10 production by T cells (10, 11, 15, 16). IL-27 induces activation of multiple STATs in T cells (2, 6, 55, 56). The anti-inflammatory effects of IL-27 on T cells linked to either the inhibition of IL-2 production, Th17 differentiation, or the induction of IL-10 production have been shown to be mediated by STAT1 and STAT3 (10, 11, 13, 16, 17). We therefore evaluated whether p28/CLF activates STAT1 and STAT3 in T cells (57). Stimulation with p28/CLF induced STAT1 and STAT3 phosphorylation in lymph node cells expressing either CD4 or CD8, whereas no effect could be detected on cells negative for these markers. As the cells negative for CD4 and CD8 were barely responsive to IL-6 and markedly less responsive to IL-27 than the CD4- or CD8-positive cells, this observation is likely to reflect a higher threshold of activation of the non-T cell populations present in the cultures by this group of cytokines under the experimental conditions used. This could be the result of a lower expression of components of the receptor, of the JAK/STAT signaling pathway, or an increased steady-state activation of JAK/STAT- negative regulators. We could verify using a blocking Ab that the IL-6Ra chain was required for p28/CLF-induced T cell activation.

We investigated whether the induction of STAT phosphorylation was paralleled by functional effects on CD4 T cells. We observed that p28/CLF inhibited the anti-CD3 plus anti-CD28-induced proliferation of CD4 T cells in the presence or absence of APCs, further indicating a direct effect of the complex on CD4 T cells. Interestingly, p28/CLF in combination with TGF-β induced the differentiation of CD4 T cells capable of producing IL-17 to a similar level than that of IL-6 plus TGF-β and this effect could be completely prevented by the addition of IL-27. The marked difference between the effects of IL-27 and those of p28/CLF under these culture conditions suggests that the type of α-chain involved (i.e., IL-6Ra or EB13) in the receptor complex changes the nature of the signal induced in the activated T cells. Marked differences between the responses induced by cytokines engaging different receptor α-chains but recruiting the same signaling chains have been reported previously (58). These results show that p28 and p28/CLF can have regulatory effects which differ from IL-27 regarding the production of IL-17, a cytokine which plays a pivotal role in several autoimmune diseases (59, 60).

Both IL-6 and IL-27 induce the production of IL-10 by T cells (10, 11, 15, 16). These cytokines play an important role in the control of inflammation and autoimmunity (61–64). We observed that the addition of p28/CLF to CD4 T cell cultures induced levels of IL-10 comparable to those induced by IL-6 or IL-27, indicating that p28/CLF could, like IL-27, have immunosuppressive effects through the induction of IL-10 production (15–17).

The initial characterization of p28/CLF using NK and T cells indicates that it could share functions with both IL-27 and IL-6. IL-6 is a key pleiotropic proinflammatory cytokine which drives the transition from the early innate immune response (involving neutrophil recruitment) to the mononuclear leukocyte-mediated adaptive immune response, promoting both humoral and cellular reactions (40). IL-27 is believed to be required for the resolution of adaptive responses and the prevention of chronic inflammation (4–12). A comparison between the phenotypes of mice deficient for IL-6 and IL-6Ra would provide information as to the roles of alternative IL-6Ra ligands such as p28/CLF in vivo.

Blocking IL-6Ra with mAbs is a promising therapeutic avenue that has been extensively investigated in preclinical models of inflammatory and autoimmune diseases (40, 65, 66). It led to the development of humanized blocking Abs such as tocilizumab (67), Tocilizumab has been tested in clinical trials for the treatment of Castelman’s disease (68), rheumatoid arthritis (69–71), juvenile idiopathic arthritis (72), and Crohn’s disease (73). It has been approved by Japanese and European regulatory agencies and is currently being reviewed by the U.S. Food and Drug Administration (74, 75). The observation that IL-6Ra is also engaged by p28/CLF may therefore have important implications for the full understanding of the mode of action of tocilizumab and for precise comparisons with other IL-6 inhibitors such as soluble gp130 derivatives (66, 76–78).

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Disclosures

The authors have no financial conflict of interest.

References


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ONLINE SUPPLEMENTAL MATERIAL

Supplementary figure legends

SUPPLEMENTARY FIGURE S1. BM cells expresses CLF and the p28/CLF complex binds on HEK 293 cells expressing WSX-1 or IL-6Rα. (A) BM cells and splenocytes were lysed and p28 was detected by SDS-PAGE and Western blot analysis. The positive control was purified rp28/CLF. (B) Transfectants were incubated with p28/CLF (1 μg/ml) for 1 h. Binding was then revealed by successive incubation with the anti-protein C epitope tag mAb HPC4 and Fluor 633-labelled anti-mouse IgG. In the left panel, the bold line indicates the fluorescence of HEK 293 cells expressing WSX-1 and the dashed line represents transfectants expressing IL-13Rα, used as negative control. Middle panel: the binding of p28/CLF (bold line) is lost when the cells were pre-incubated with an excess of IL-27, 10 μg/ml, dashed line). Left panel: the presence of an excess of the irrelevant cytokine CNTF (10 μg/ml) had no effect on p28/CLF binding. The filed grey histograms represent the negative control (non transfected cells or secondary Ab). (C) Transfectants were incubated with p28/CLF (1 μg/ml) for 1 h. Binding was revealed by successive incubation with the mAb HPC4 and Fluor 633-labelled anti-mouse IgG. Data are representative of 3 independent experiments.

SUPPLEMENTARY FIGURE S2. Ba/F3-gp130 transfectants are responsive to IL-27. (A) Ba/F3-gp130 were stimulated with IL-27 (bold line, 50 ng/ml) for 15 min, fixed and permeabilized. STAT phosphorylation was assed by flow cytometry. (B) Ba/F3-gp130-IL-6Rα cells were pre-incubated with (dashed line) or without (bold line) blocking anti-
IL-6Rα antibody (0.5 μg/ml) before IL-27 stimulation. Filed grey histograms, unstimulated cells. Data are representative of 3 independent experiments.
IL-27 stimulated Ba/F3-gp130

A

B

IL-27 stimulated Ba/F3-gp130

IL-27 stimulated Ba/F3-gp130-IL-6Rα

pSTAT1

pSTAT3

None + αIL-6Rα