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The Composite Cytokine p28/Cytokine-Like Factor 1 Sustains B Cell Proliferation and Promotes Plasma Cell Differentiation

Aurélie Jeanne Tormo,* Yasmine Meliani,* Linda Ann Beaupré,* Mukut Sharma, ‡ Jörg H. Fritz, ‡ Greg Elson, § Sandrine Crabé,*† and Jean-François Gauchat*†

Interleukin-27 is composed of the association of the four-helix bundle cytokine p28 with the soluble cytokine receptor EBV-induced gene 3 (EBI3) (1). IL-27 activates a receptor formed by gp130, the common receptor chain for the IL-6 family cytokines, IL-27Rα (WSX-1 or TCCR), which defines ligand specificity for the receptor complex (2). IL-27 was initially identified as a cytokine produced by APCs promoting CD4 T cell proliferation and Th1 differentiation (1). It has pleiotropic effects on multiple immune cells (3, 4). Besides its effects on Th1 differentiation, IL-27 inhibits Th17 differentiation (5, 6), induces the production of anti-inflammatory cytokines such as IL-10 (7, 8) and limits IL-2 production (9, 10), thus regulating regulatory T cell generation (11) (12).

p28 and EBI3 can be secreted independently and are thought to have IL-27–independent functions (6, 12, 13). p28 was reported to bind gp130 with low affinity and to act as an IL-6 antagonist (1, 12). We observed that p28 can form an alternative complex with the EBI3 homolog cytokine-like factor 1 (CLF; CRLF1), p28/CLF modulates NK cell activity and CD4 T cell cytokine production in vitro. In this study we used IL-6–dependent plasmacytoma cell line B9 and CD4 T cells from IL-27Rα-deficient mice to demonstrate that p28/CLF activates IL-27–unresponsive cells, indicating that p28/CLF and IL-27 signal through different receptors. The observation that p28/CLF, unlike IL-27, sustains B9 plasmacytoma cell proliferation prompted us to investigate the effects of p28/CLF on mouse B cells. We observed that p28/CLF induces IgM, IgG2c, and IgG1 production and plasma cell differentiation. p28/CLF therefore has the potential to contribute to B and plasma cell function, differentiation, and proliferation in normal and pathological conditions such as Castelman’s disease and multiple myeloma. The Journal of Immunology, 2013, 191: 1657–1665.

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*Département de Pharmacologie, Université de Montréal, Montréal, Québec H3T 1J4, Canada; ‡ Renal Division, Kansas City Veterans Affairs Medical Center, Kansas City, MO 64128; Complex Traits Group, Department of Microbiology and Immunology, McGill University, Montréal, Québec H3G 0B1, Canada; and §NovImmune SA, 1228 Plan-les-Ouates, Geneva, Switzerland

†Current address: WittyCell, Evry, France.

Materials and Methods

Experimental animals

All procedures conformed to the Canadian Council on Animal Care Guidelines and were approved by the Animal Ethics Committee of the
Production of mouse p28/CLF

The mouse p28 cDNA used (clone I830047N17) was obtained from the FANTOM Consortium, Institute of Physical and Chemical Research (Saitama, Japan) Genome Exploration Research Group (provided by K.K. DNAFORM, Ibaraki, Japan). It was modified in 3’ with a sequence encoding for a protein C epitope (EDQVDPRLIDGK) and 6 His. To generate a bicistronic vector, the CLF cDNA was fused at the C-terminal to a sequence encoding for a FLAG-epitope (DYKDDDDK) and the T2A “self-cleaving” (EGRGSILTCGVDVEINFPG) peptide followed by the coding sequence of the modified p28 cDNA.

The p28/CLF cDNA was recloned in the insect expression vector pIB/V5-His (Invitrogen, Burlington, ON, Canada) and verified by DNA sequencing. Recombinant proteins were expressed in High Five insect cells and purified under LPS-free conditions as described previously (13). Recombinant proteins were dialyzed and quantified by Western blot analysis using protein C and 6 His-tagged mouse ciliary neurotrophic factor (24) as standard and anti-penta His mAb (Qiagen, Toronto, ON, Canada) or anti-protein C (HPC4; Roche Applied Sciences). C57BL/6 mice were provided by Genentech (San Francisco, CA).

Generation of stable B9 transfectants

The cDNA coding for mouse IL-27Rα (IMAGE ID 1244998; Open Biosystems) was recloned in the expression vector pME3 (InvivoGen/Cedarlane Laboratories, Burlington, ON, Canada). Linearized plasmids were transfected by electroporation in B9 cells (25). Stable transfectants were selected using hygromycin (1 mg/ml). Clones were expanded using mouse IL-6 (1 mg/ml; R&D Systems, Minneapolis, MN) and selected for proliferation in response to mouse IL-27 (10 ng/ml; R&D Systems).

Analysis of transcription factor expression by quantitative RT-PCR

B220+ B cells were isolated from mice splenocytes by magnetic bead separation according to the manufacturer’s instructions (StemCell Technologies, Vancouver, BC, Canada). Cells were plated in 96-well round-bottom plates (BD Biosciences, Mississauga, ON, Canada) at a density of 1 × 10^5 cells/ml in RPMI 1640 cell culture medium (Invitrogen) supplemented with 10% FBS, 2 mM l-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. When added, IL-6, IL-27 (both from R&D Systems), and p28/CLF were used at 50 ng/ml whereas the LPS (026:B6; Sigma-Aldrich, St. Louis, ON, Canada) was used at 50 μg/ml as a positive control.

Plasma cell differentiation assays

Purified B220+ cells were plated in 96-well round-bottom plates (BD Biosciences) at a density of 1 × 10^3 cells/ml. Plasma cell differentiation was induced using LPS (100 ng/ml) and IL-6 (50 ng/ml). When added, p28/CLF was used at 50 ng/ml. After 72 h of culture, cells and cell culture media were collected. Cells were stained with PE-labeled anti-B220 and allophycocyanin-labeled anti-CD138 for 30 min on ice. Viability was assessed by staining with 7-aminoactinomycin D (BD Biosciences). Fluorescence was analyzed by flow cytometry.

Th2 differentiation

C57BL/6 spleen and lymph node CD4+ T cells were enriched by depleting CD8 and NK cells by negative magnetic bead selection (StemCell Technologies) following the manufacturer’s instructions. Cells were stimulated using plate-coated anti-CD3 and soluble anti-CD28 (both at 1 μg/ml; BD Biosciences). Cultures were supplemented with anti–IFN-γ, anti–IL-12 alone (IL-4–stimulated cells), or in combination with anti–IL-4 (all at 10 μg/ml; BD Biosciences). Sera samples were taken at days 16 and 23.

Analysis of Ig secretion

Levels of IgM, IgG2c, and IgG1 in the B cell culture media and mice sera were measured by ELISA using anti-IgM, anti-IgG1 (BD Biosciences), or anti-IgG2c (SouthernBiotech, Birmingham, AL) capture and detection Abs, HRP-conjugated streptavidin (BD Biosciences), and tetramethylbenzidine. Absorbance at 450 nm was analyzed with a Wallac 1420 Victor2 multilabel microplate reader (PerkinElmer Life and Analytical Sciences, Gallatin, MA). The sera levels of anti-OVA Ab were assessed by ELISA using OVA-coated plates and isotype-specific biotinylated detection Abs.

Statistics

Statistical analysis was performed using one-way ANOVA tests with Bonferroni correction where appropriate. A p value < 0.05 was considered statistically significant. Error bars in the figures represent SD.

Results

The p28/CLF complex activates the IL-6–dependent, IL-27–unresponsive mouse plasmacytoma cell line B9

We previously observed that p28/CLF activates Ba/F3 cells co-expressing IL-6R and IL-27R (i.e., IL-27Rα, gp130, and IL-6Rα) and induces IL-6–like effects on CD4+ T cell cytokine production in vitro (13). As an initial step for investigating the capacity of p28/CLF to activate cells of the B cell lineage, we tested its effects on the IL-6–dependent mouse plasmacytoma cell line B9 (25). As expected, activation of B9 cells with IL-6 led to both STAT1 and STAT3 phosphorylation and sustained proliferation (Fig. 1A, 1C). Neither STAT phosphorylation nor proliferation...
FIGURE 1. The p28/CLF complex activates IL-6–dependent B9 plasmacytoma cells. Untransfected B9 cells (A) or B9 cells stably transfected with mouse IL-27Rα cDNA (B) were stimulated with the cytokines indicated for 30 min. Cells were fixed, permeabilized, and phosphorylation of STAT1 and STAT3 was assessed by flow cytometry using PE-labeled anti-pSTAT1 or anti-pSTAT3 mAbs. Filled histograms indicate (Figure legend continues)
was detected when B9 cells were incubated with IL-27 (Fig. 1A, 1C). The B9 cell line was therefore used to discriminate between the roles of IL-6R and IL-27R in p28/CLF signaling. Incubation of B9 cells with p28/CLF led to a detectable induction of STAT3 phosphorylation, indicating that IL-27R is dispensable for cell activation by this complex (Fig. 1A). When compared with IL-6, higher cytokine concentrations of p28/CLF were required for equivalent proliferation (Fig. 1C). To investigate whether IL-27R contributes to the signaling induced by p28/CLF, we generated IL-27R−/− mice (20). As expected, stimulation of these cells with either IL-6 or IL-27 resulted in proliferation (Fig. 1B, 1D). The expression of IL-27Rα did not modify the proliferation in response to p28/CLF (Fig. 1D). Taken together, these results indicate that 1) p28/CLF can substitute for IL-6 in activating STAT1 and STAT3 and supporting B9 cell proliferation; 2) the receptors for p28/CLF and IL-27 can be dissociated using this system; and 3) transfection with IL-27Rα renders B9 cells sensitive to IL-27 without affecting their proliferation in response to p28/CLF.

**p28/CLF activates STAT1 and STAT3 phosphorylation in IL-27Ra−/−-deficient splenocytes**

To investigate the contribution of IL-27Ra to the activation of primary mouse cells by p28/CLF, we compared the response of CD4 T cells isolated from wild-type (WT) and IL-27Ra−/− mice (20). As expected, STAT1 and STAT3 phosphorylation induced by IL-27 stimulation was completely abolished in IL-27Ra−/− CD4 T cells (Fig. 2). Incubation with either p28/CLF or IL-6 led to STAT1 and STAT3 phosphorylation in CD4 T cells isolated from either WT or IL-27Ra−/− (Fig. 2), showing that IL-27Ra is not required for the activation of primary mouse immune cells by p28/CLF. Overall, these results indicate that p28/CLF differs from IL-27 in that it can activate cells independently of IL-27Ra.

*The p28/CLF complex increases B cell proliferation*

Because p28/CLF induces the proliferation of B9 cells, we investigated whether p28/CLF had effects similar to IL-6 on primary mouse B cells. We compared the proliferation of purified spleen B220+ cells stimulated with suboptimal concentrations of LPS or with anti-CD40 in the presence or absence of either IL-6 or p28/CLF. We observed that p28/CLF and IL-6 increased cell proliferation to comparable extents, showing that p28/CLF is active on primary B cells (Fig. 3A). In line with this observation, stimulation of B cells with p28/CLF induced STAT1 and STAT3 phosphorylation in a significant fraction of spleen B220+ B cells (Fig. 3B).

*The p28/CLF complex increases LPS-induced Ig production*

We next investigated whether p28/CLF could modulate the production of Ig by B cells in vitro by analyzing the production of IgM, IgG2c, and IgG1. These isotypes were selected because C57BL/6 mice have the IgG2a C region replaced by IgG2c (30), and IgG2a and IgG1 are Ig isotypes that require class switching known to be affected by IL-6 (31). Interestingly, p28/CLF alone or in combination with suboptimal concentrations of LPS increased the levels of IgM, IgG2c, and IgG1 measured in the cell culture medium of B220+ cells at day 3 (Fig. 4). The increase in IgG1 titer was greater in the cell culture medium of B cells incubated with p28/CLF than in the culture medium of cells incubated in the presence of IL-6, suggesting that p28/CLF acts preferentially on the class switching to the γ1 C region and/or on IgG1-producing cells.

IgG1 class switching is preferentially induced by cytokines involved in Th2 response such as IL-4 (32). We therefore examined the effect of p28/CLF on the production of IL-4 by CD4 T cells (Supplemental Fig. 1). Results indicated that the frequency of CD4 T cells producing IL-4 was increased in cultures activated in unstimulated cells. Right panels show average mean fluorescence intensity (MFI) ± SD of three independent Phosflow assays. Statistical significance was assessed using the ANOVA test. 

(C and D) Untransfected B9 cells or B9 cells stably transfected with mouse IL-27Ra cDNA (B9-IL-27Ra) were incubated with the indicated cytokines for 72 h and the proliferation was measured by fluorescence using the Alamar blue test. Data are representative of three independent experiments. *p < 0.05.

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**FIGURE 2.** p28/CLF induces STAT1 and STAT3 phosphorylation in IL-27Ra−/− CD4 T cells. CD4 T cells isolated from WT (IL-27Ra+/+) or IL-27Ra−/− mouse spleen were stimulated with the indicated cytokines for 15 min. Cells were fixed, permeabilized, and phosphorylation of STAT1 and STAT3 was assessed by flow cytometry using PE-labeled anti-pSTAT1 or anti-pSTAT3 mAbs. Filled histograms indicate unstimulated cells. Mean fluorescence intensity (MFI) values of the unstimulated (left numbers) and stimulated cells (right numbers) are indicated. Data are representative of three independent experiments.
the presence of p28/CLF. These results suggest that, similar to IL-6 (33, 34), the composite cytokine promotes Th2 differentiation.

**p28/CLF induces plasma cell differentiation in vitro**

The observation that the production of Ig was increased in B cells cultured in the presence of p28/CLF and that p28/CLF enhanced the proliferation of B9 plasmacytoma cells prompted us to investigate the effects of this cytokine on plasma cell differentiation in vitro. When purified B cells were maintained in culture with p28/CLF, either in the presence or absence of LPS, a marked increase in the fraction of cells positive for syndecan-1 (CD138) with an intermediate expression of B220 was observed. This expression pattern is characteristic of mouse Ab-secreting cells (35) (Fig. 5A).

The increase in CD138^B220^int was comparable to that induced by the combination of LPS and IL-6 or LPS and IL-27 (Fig. 5A). Induction of plasma cell differentiation in response to p28/CLF could also be observed with B cells isolated from IL-27R^−/−^ mice (Fig. 5B), confirming that p28/CLF can mediate its effect on B cells independently of IL-27R. It indicates that the effect observed is not due to IL-27 complexes that would form between the p28 subunit of the p28/CLF added to the cell culture medium and EBI3 released by the cultured cells.

Plasma cell differentiation depends on the coordinated regulation of transcription factor levels, notably requiring the downregulation of Pax5 and the induction of Sdc-1, Xbp-1, and IRF-4 expression (35). As expected, incubation of B cells with IL-6 in conjunction with LPS induced a decrease of Pax5 combined with an increase of Xbp-1 and Sdc-1 mRNA levels (Fig. 5C). A similar decrease of Pax5 and increase of Sdc-1 and Xbp-1 mRNA was observed when B cells were cultivated with p28/CLF and LPS (Fig. 5C). Interestingly, p28/CLF alone induced a significant increase of Sdc-1 and Xbp-1 mRNA. Collectively, these results indicate that p28/CLF can elicit plasma cell differentiation in vitro.

**The p28/CLF complex induces JAK/STAT signaling in plasma cells**

We observed that p28/CLF induces STAT3 phosphorylation in B9 plasmacytoma cells and B cells (Figs. 1, 3B). To determine whether p28/CLF stimulation triggers JAK/STAT signaling in plasma cells, we isolated B220^+^ bone marrow cells and investigated the effect of p28/CLF on STAT phosphorylation in CD138^+^ cells. Stimulation with p28/CLF induced an increase in STAT1 and STAT3 phosphorylation in a large fraction of CD138^+^ cells (Fig. 5D). The effect of p28/CLF on STAT1 and STAT3 phosphorylation was very similar to that of IL-6 (Fig. 5D).

**The p28/CLF complex increases Ig production in vivo**

To investigate whether p28/CLF could increase Ig production in vivo, we subjected mice to hydrodynamic transfection (28) with a bicistronic mammalian expression vector coding for the two cytokine subunits. Because hydrodynamic transfection, which has been extensively used to investigate the functions of IL-27 (12, 36, 37), results in a transient release of cytokines in the circulation peaking around day 2 (28), it was repeated at day 14. Mice were immunized at day 2 with OVA alone or OVA emulsified in CFA. Total and OVA-specific Ig levels were assessed at days 16 and 23. The maximum effect of p28/CLF on total and Ag-specific Ig production was observed at day 16, with statistically significant increases of the serum levels of IgM, IgG1, IgG2c, anti-OVA IgG1, and anti-OVA IgG2c (Fig. 6 and data not shown). Collectively, the results indicate that p28/CLF has effects on Ig production in vivo and in vitro.

**Discussion**

We observed previously that the cytokine subunit of IL-27, p28, can form a biologically active complex with the soluble cytokine receptor CLF (13). Our present study indicates that the p28/CLF complex induces STAT3 phosphorylation and supports the proliferation of the IL-6–dependent plasmacytoma cell line B9. This cell line is IL-27–unsensitive by transfection with IL-27Rα cDNA. Expression of IL-27Rα did not modify B9 cell proliferation in response to the p28/CLF complex, suggesting that the IL-27R–specific subunit IL-27Rα does not contribute to the p28/CLF receptor. That IL-27Rα is not required for p28/CLF-induced signaling was further supported by the observation that the complex could induce STAT1 and STAT3 phosphorylation in CD4 T cells isolated from IL-27Rα.
knockout mice. CD4 T cells rather than B cells were used to unambiguously demonstrate that p28/CLF could act independently of IL-27, because the composite cytokine activates STAT1 and STAT3 phosphorylation in a larger fraction of the cells and because the Phosflow signal was stronger in CD4 T cells than in B cells (data not shown). Our results suggest that p28/CLF signals through the IL-6 receptor. Whereas the results obtained with p28/CLF might appear at odds with recent reports indicating that p28 is an IL-6 antagonist (12, 38), they are in line with the demonstration that p28 produced in *Escherichia coli* can activate IL-6R, published by Scheller and colleagues (39) while this manuscript was in revision. Using blocking mAbs directed against different domains of gp130, they demonstrated that p28 forms with membrane or soluble IL-6Rα a complex recruiting and activating a gp130 homodimer, indicating that IL-6 and p28 recruit the very same signaling chains (39). The recruitment of an unidentified cytokine receptor signaling chain expressed by the Ba/F3 and B9 cell lines cannot, however, be ruled out.

The observation that p28/CLF can substitute for IL-6 in supporting the growth of the B9 mouse plasmacytoma prompted us to investigate its effect on B and plasma cells. We observed that, similar to IL-6, p28/CLF increased LPS and anti-CD40–induced B cell proliferation and Ig production in vitro and in vivo. Furthermore, p28/CLF induced the differentiation of cells expressing high levels of syndecan-1 (CD138) and intermediate levels of B220, a phenotype that is used to monitor mouse plasma cell differentiation by flow cytometry (35). Interestingly, p28/CLF could upregulate the activation of the JAK/STAT pathway in a large fraction of bone marrow B220 cells positive for CD138, a finding that correlates with the ability of the cytokine to support the growth of B9 cells and suggests that p28/CLF might be a plasma cell trophic factor. In accordance such a putative role, both CLF and p28 protein levels are markedly upregulated by LPS or polynosinic-polyribidylic acid stimulation of bone marrow cells in vitro (data not shown).

The observation that IgG1 production is upregulated in vitro by p28/CLF indicated that it could induce an Ig isotype associated with Th2 response (32) and incited us to examine its effect on Th2 differentiation in vitro. The fraction of CD4 T cells producing IL-4 was increased when CD4 T cells were activated in the presence of p28/CLF. This shows that, similar to IL-6 (33, 34), p28/CLF can promote Th2 differentiation, and, together with our previous finding that in combination with TGF-β it induces IL-17 (13), further supports a similarity of properties between p28/CLF and IL-6. IL-4 is a cytokine also associated with T follicular helper cells, a subset of CD4 T cells that plays a key role in B cell help (40, 41). IL-6, together with IL-21, is involved in T follicular helper cell differentiation (42, 43). It will therefore be of interest to examine whether p28/CLF, similar to IL-6, also regulates B cell function indirectly by promoting IL-21 expression by T follicular helper cells.

Our results indicate that p28/CLF can exhibit IL-6–like properties on mouse T, B, and plasma cells in vitro. It will be of in-

![FIGURE 4](http://www.jimmunol.org/Download.png)

**FIGURE 4.** The p28/CLF complex stimulates IgM, IgG2c, and IgG1 secretion in vitro. Purified B220+ cells, isolated from mouse spleen, were incubated with the indicated cytokines for 3 d alone (at 50 ng/ml) or in combination with LPS (100 ng/ml). IgM (A), IgG2c (B), and IgG1 (C) levels were assessed by ELISA. Error bars indicate the SEM of triplicate cultures. Statistical significance was assessed using the ANOVA test. *p < 0.05. Data are representative of three independent experiments.
FIGURE 5. The p28/CLF complex induces plasma cells differentiation. (A and B) Purified B cells from WT (A) and IL-27R−/− (B) mouse spleen were incubated with indicated cytokines for 3 d and stained with anti-B220 and anti-CD138. Fluorescence was analyzed by flow cytometry. Dead cells were excluded by 7-aminoactinomycin D labeling. The percentages of cells gated as B220intCD138+ are indicated. Data are representative of three

(Figure legend continues)
interest to investigate whether p28/CLF contributes to the B cell functions retained in IL-6 knockout mice and whether IL-6 and IL-6R knockout mice differ in their B and plasma cell responses (31, 44). If confirmed in human B and plasma cells, these observations would suggest that the therapies based on the blockade of IL-6R (e.g., tocilizumab administration) or the inhibition of IL-6 (e.g., siltuximab administration) could differ in their effects. This might be particularly relevant for pathologies implying plasma

FIGURE 6. The p28/CLF complex stimulates IgM, IgG2c, and IgG1 production in vivo. Mice were subjected to hydrodynamic transfection with empty pcDNA5-His plasmid (empty vector histogram bars) or derivatives containing the indicted cytokine cDNA (IL-6, IL-27, or p28/CLF histogram bars). Forty-eight hours later, groups of six to eight transfected mice were kept unimmunized or immunized with OVA alone (OVA) or OVA in combination with CFA (OVA/CFA). Histograms show the mean serum levels of total IgM, IgG2c, and IgG1 (A–C) or OVA-specific IgM, IgG2c, and IgG1 (D–F) at day 16. OVA-specific Ab titers were below detection limits in unimmunized mice. Error bars indicate the SEM of individual mouse serum Ig levels quantified by ELISA. Statistical significance was assessed using the ANOVA test. Data are representative of two independent experiments. *p < 0.05.

independent experiments. (C) Purified B cells isolated from mouse spleen were incubated with LPS (100 ng/ml) with or without IL-6 (50 ng/ml), p28/CLF (50 ng/ml), or IL-27 (50 ng/ml) for 72 h. The levels of Pax-5, Xbp-1, Sdc-1, and IRF-4 mRNA were assessed by quantitative RT-PCR. Error bars indicate the SEM of triplicate cultures. Statistical significance was assessed using the ANOVA test. *p < 0.05. Data are representative of three independent experiments. (D) Purified B220+ bone marrow cells were stimulated with IL-6, IL-27, or p28/CLF (all at 50 ng/ml) for 15 min. Cells were stained with allophycocyanin-labeled anti-CD138 mAbs and either PE-labeled anti-pSTAT1 or anti-pSTAT3 mAbs. Fluorescence was analyzed by flow cytometry. The B200+ bone marrow cells analyzed were gated for CD138 expression. Filled histograms indicate unstimulated cells. Mean fluorescence intensity (MFI) values of unstimulated (left) and cytokine-stimulated (right) cells are indicated. Data are representative of three independent experiments.
cells such as Castleman’s disease for which tocilizumab was initially approved and siltuximab is currently being tested (16–18, 45).

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Disclosures

The authors have no financial conflicts of interest.

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