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A Polyglutamic Acid Motif Confers IL-27 Hydroxyapatite and Bone-Binding Properties

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The p28 subunit of the composite cytokine IL-27 comprises a polyglutamatic acid domain, which is unique among type I cytokines. This domain is very similar to the acidic domain known to confer hydroxyapatite (HA)-binding properties and bone tropism to bone sialoprotein. We observed IL-27 binding to HA, in accordance with previous studies reporting successful p28 HA chromatography. The IL-27 polyglutamic acid domain is located in a flexible inter-α helix loop, and HA-bound IL-27 retained biological activity. Using IL-27 alanine mutants, we observed that the p28 polyglutamatic acid domain confers HA- and bone-binding properties to IL-27 in vitro and bone tropism in vivo. Because IL-27 is a potent regulator of cells residing in endosteal bone marrow niches such as osteoclasts, T regulatory, memory T, plasma, and stem cells, this specific property could be beneficial for therapeutic applications. IL-27 has potent antifungal and antiosteoclastogenic activities. It could therefore also be useful for therapies targeting hematologic cancer or solid tumors metastasis with bone tropism. Furthermore, these observations suggest that polyglutamatic motifs could be grafted onto other type I cytokine inter-α helix loops to modify their pharmacological properties. The Journal of Immunology, 2013, 190: 2931–2937.

Interleukin-27 is an IL-12-like type I cytokine known to regulate the function and differentiation of multiple immune and nonimmune cell lineages such as T cells, B cells, NK cells, mast cells, osteoblasts, and stem cells (1–4). IL-27 plays an important role in the resolution of inflammation in different models of infection and has marked beneficial effects in the experimental autoimmune encephalitis model of multiple sclerosis (5–10). IL-27 has also been shown to have potent beneficial antitumor effects in leukemia and solid cancer models in mice (11–19). Moreover, IL-27 has been described to inhibit osteoclastogenesis and to protect against bone destructive disorders (20). The therapeutic potential of IL-27 might, however, be compromised by the large range of cells it regulates, as previously observed for other type I cytokines with limited target specificity (1–4).

The IL-27 complex comprises p28, a four α helix-bundle cytokine subunit, and EBV-induced gene 3 (EBI3), a subunit structurally similar to soluble type I cytokine receptors (1). The polypeptide loop connecting the p28 C and D α helices contains a stretch of polyglutamatic acids (poly-E) unique among helical cytokines and highly conserved between species (1). This poly-E stretch is very similar to the acidic domain known to confer hydroxyapatite (HA)-binding properties and bone tropism to bone sialoprotein. We observed IL-27 binding to HA, in accordance with previous studies reporting successful p28 HA chromatography. This domain is very similar to the acidic domain known to confer hydroxyapatite (HA)-binding properties and bone tropism to bone sialoprotein. We observed IL-27 binding to HA, in accordance with previous studies reporting successful p28 HA chromatography. More generally, acidic amino acid tags are used to direct recombinant proteins to bones (27, 28). We therefore compared the properties of wild-type (WT) p28 and IL-27 with those of mutants lacking the poly-E acidic motif. We observed that the poly-E motif confers HA- and bone-binding properties to IL-27 and that IL-27 immobilized on HA retains its biological activity. The C-D loop acidic motif could therefore confer IL-27–specific pharmacological properties beneficial for therapeutic applications targeting osteoclasts, stem or immune cells residing at the endosteal surface of bone marrow. Similarly, it may serve to enhance the antitumor activity of IL-27 on leukemic or cancer metastatic cells with bone tropisms.

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 Université de Montréal. Six to 8 wk-old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Generation of stable Ba/F3 cells expressing gp130

The cDNA coding for mouse gp130 (Image ID 6834623; Open Biosystems) was recloned in the expression vector pMG (InvivoGen, Cedarlane, Burlington, ON, Canada). Linearized plasmids were transfected by electroporation in Ba/F3 cells. Stable transfectants were selected using hygromycin (1 mg/ml). Clones were expanded using in-house—produced mouse hyper–IL-6 (5 ng/ml) and selected for proliferation in response to mouse IL-27 (10 ng/ml; R&D Systems, Minneapolis, MN).

Expression and isolation of recombinant proteins

The construct for the expression of mouse ciliary neurotrophic factor (CNTF) was described previously (29). The p28 cDNA (RIKEN clone number I830047N17) was obtained from the FANTOM Consortium (The Institute of Physical and Chemical Research, Saitama, Japan) Genome Exploration Research Group (provided by K.K. DNAFORM, Ibaraki, Japan). The sequence coding for the mature form of p28 was modified in 3’ with a sequence coding for c-Myc (EQKLISEEDL) and 12× His epitopes and cloned in the expression vector pET24d (30). To generate a vector coding for mouse p28-poly-A, the codons coding for the aa 163–177 were
mutated to code for alanine. Recombinant proteins were expressed in BL21 Star (DE3) bacteria (Life Technologies) under denaturing condition and purified by immobilized metal affinity chromatography (IMAC) as described previously (29). For renaturation, WT and mutant p28 were dialyzed against PBS supplemented with 2 mM reduced and 0.2 mM oxidized glutathione. Denatured and renatured proteins were quantified by Western blotting using 6X His-tagged mouse CNTF as a standard and anti-pana His mAb (Qiagen, Toronto, ON, Canada).

For the production of WT and mutant IL-27, a construct coding for the sequences of EBI3, a flexible 3X (SGGGG) linker and WT or poly-A p28 were generated using a synthetic EBI3 cDNA (Geneart, Life Technologies Burlington, ON) and cloned in the insect cell expression vector pB/V5-His (Life Technologies) using standard molecular biology techniques (31). cDNAs coding for WT and mutant IL-27 were transfected in the High Five insect cell line (Life Technologies). Supernatants of transfected cells were collected and concentrated as described previously (32). Recombinant proteins were purified by IMAC and quantified by Western blotting using 6X His-tagged mouse CNTF as a standard.

**Binding to HA beads**

For binding assays under denaturing conditions, 2 mg HA beads (Bio-Rad, Mississauga, ON, Canada) were washed, equilibrated with 6 M urea, 0.05 M phosphate (pH 7.3), and 2 mM 2-ME in 10 mM polyproline columns (Bio-Rad), and incubated with 10 µg denatured WT or mutant p28 in the same buffer. The columns were washed with equilibration buffer, and proteins bound to HA were eluted with increasing concentrations of phosphate (50–400 mM) in 6 M urea, according to the manufacturer’s instructions. For binding assays under native conditions, the HA chromatography was performed with renatured WT or mutant p28 in the absence of urea. The flow-through and elution fractions were analyzed by Western blot as described above.

**Binding to secreted bone matrix in vitro**

Multipotent stromal cells (MSC) (a gift from Dr. C. Beauséjour, Université de Montréal) were isolated from bone marrow using polystyrene adherence (33). MSCs were induced towards osteoblast differentiation, cells were cultured in 6-well plates in DMEM containing 100 nM dexamethasone, 50 µM vitamin C, 50 µM vitamin D3, 10 mM β-glycerophosphate for 10 d. Osteogenic differentiation was confirmed using alizarin red S (Sigma-Aldrich, St. Louis, MO) staining. For binding assays, the cell cultures were incubated with 1 µg WT or mutant IL-27 purified from High Five cell supernatant in 0.5 ml DMEM culture medium for 1 h in ice. After extensive washing with ice-cold DMEM, the cultures were lysed in 8 M urea and 400 mM phosphate (pH 7.3) complemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, Rockford, IL). Lysate protein contents were quantified by bicinchoninic acid after dilution to reduce urea concentration (Thermo Fisher Scientific). Aliquots of 50 µg total protein were analyzed by Western blotting. IL-27 was detected using biotinylated goat anti-mouse IgG (Thermo Fisher Scientific) and HRP-labeled streptavidin (Thermo Fisher Scientific).

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

To assess WT and mutant IL-27 biological activity by phosflow, 106 mouse splenocytes were activated for 15 min at 37˚C with mouse IL-6 (50 ng/ml), commercial IL-27 (50 ng/ml) (both from R&D Systems), or in house High Five cell-produced WT IL-27 and IL-27-poly-A (50 ng/ml) in a final volume of 200 µl RPMI 1640 medium supplemented with 20 mM HEPES (pH 7.4) and 10% fetal bovine serum. Cells were fixed with 4% paraformaldehyde and stained with a combination of PE-labeled anti-CD8 and biotinylated anti-CD4 (BD Biosciences). Cells were stained with 0.5 ml DMEM culture medium for 1 h in ice. After extensive washing with RPMI 1640 medium supplemented with 20 mM HEPES (pH 7.4) for 15 h at 4˚C. After extensive washing with RPMI 1640 medium, the beads were resuspended to 2 mg/ml in RPMI 1640 medium supplemented with 20 mM HEPES (pH 7.4) and incubated with 105 splenocytes for 15 min at 37˚C. For the assessments of cytokine leakage, cytokine-loaded beads were washed and incubated in RPMI 1640 medium supplemented with 20 mM HEPES (pH 7.4) and incubated for 15 min at 37˚C. The incubation medium was separated from the beads using 40-μm cell strainers (BD Falcon) and incubated with splenocytes for 15 min at 37˚C. STAT activation was measured as described above.

**Proliferation assays**

The proliferation of Ba/F3-gp130 transfectants was measured using an Alamar blue fluorometric assay as described previously (32).

**Hydrodynamic transfection with IL-27 and IL-27-poly-A cDNAs**

Chimeric cDNA coding for the sequences of EBI3, a flexible 3X (SGGGG) linker and the WT or poly-A p28 tagged in carboxy with an Avitag (34) and 8X His were generated using standard molecular biology techniques (31). These chimeric cDNAs were fused with sequences coding for a T2A autocleavable peptide (35) and a secreted derivative of the biotin ligase BirA (36, 37). The WT or mutant IL-27-BirA bicistronic cDNA as well as the secreted BirA cDNA were recloned in the mammalian expression vector pcDNA5-His (Life technologies). LPS-free plasmid DNA was purified using an endotoxin-free maxi-prep plasmid isolation kit (Qiagen). To test the constructs, the plasmids were transfected in HEK-293 Flp-In cells (Life Technologies) (32), and the cell culture medium was subjected to Western blot analysis with HRP-labeled anti-p28 and HRP-labeled streptavidin. For hydrodynamic transfection experiments, groups of two to three C57BL/6 mice were injected in the tail vein with 50 µg of the pcDNA5-His derivatives containing WT IL-27-T2A-BirA cDNA, mutant IL-27-T2A-BirA cDNA, or BirA cDNA under hydrodynamic transfection conditions (38). Briefly, the plasmids were diluted in Ringer’s solution supplemented with 150 mM NaCl to a final volume corresponding to 10% of the weight of the mice. Injections were performed by a blinded operator in 5 s in mice subjected to gaseous anesthesia (isoflurane 2–3% in oxygen). Mice were sacrificed after 48 h, and serum, liver, and bone were isolated. Femur bone marrows were flushed, and the bones were cavitated with repetitive washing 15 min with 1 ml 8 M urea and 400 mM phosphate. Livers were homogenized in 50 mM Tris (pH 7.4), 100 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA, 1% Triton X-100, and 10% glycerol. The bone and liver extracts were subjected to IMAC on Ni-NTA agarose (Qiagen), according to the manufacturer’s instructions. The proteins isolated by IMAC were analyzed by Western blotting using “high sensitivity” HRP-conjugated streptavidin (Thermo Fisher Scientific).

**Measurement of bone marrow cell STAT1, STAT3, and STAT5 activation in mice subjected to hydrodynamic transfection with IL-27 and IL-27-poly-A cDNAs**

Forty-eight hours posthydrodynamic transfection, mice were sacrificed. Bone marrow mononuclear cells were purified by centrifugation on a Histopaque-1083 cushion (Sigma-Aldrich). Cells were fixed with 2% formaldehyde and stained with a combination of PE-labeled anti-CD8 and either anti-phospho-STAT1, STAT3, or STAT5 mAbs (all from BD Biosciences). Blood samples were collected by cardiac puncture. Sera were diluted 20-fold in 50 mM Tris (pH 7.4), 100 mM NaCl, and 20 mM imidazole and subjected to IMAC with 15 µl Ni-NTA agarose (Qiagen). The IMAC eluates were analyzed by Western blotting using “high sensitivity” HRP-conjugated streptavidin.

**Results**

The IL-27 poly-E motif confers HA binding properties to the IL-27 p28 subunit

Both the human and mouse IL-27 amino acid sequences contain an acidic poly-E motif (1). The p28 poly-E stretch is highly similar to the acidic domain, which mediates bone sialoprotein binding to HA (Fig. 1) (21–23), and HA chromatography was used successfully to purify Escherichia coli–produced p28 (39). To investigate whether the poly-E stretch of p28 contributes to HA binding, we produced E. coli–derived recombinant WT p28 or a mutant p28 derivative with the glutamic acids of the poly-E stretch replaced by neutral alamines, using the approach previously used to map the bone sialoprotein HA-binding domain (24). Although the WT p28 bound HA beads under both denaturing (Fig. 2A) and non-denaturing (Fig. 2B) conditions, this property was lost in the p28 mutant (referred to as p28-poly-A) (Fig. 2). These results indicate that the stretch of polyglutamic acids of the polypeptide loop connecting the C and D helices of p28 confers HA binding properties to p28. Because this connecting loop is outside the regions known to be directly involved in receptor
recognition (1, 40, 41), we investigated whether IL-27 immobilized on HA remained biologically active.

**IL-27 immobilized on HA activates STAT signaling in immune cells**

To investigate whether IL-27 bound to HA retains biological activity or represents a latent form of the cytokine, we tested the effect of HA beads loaded with single-chain commercial IL-27 on the activation of STAT1 and STAT3 using unfractionated mouse splenocytes as targets. This biological activity assay was chosen because it could be performed under cell culture conditions and in a timeframe (15 min) unlikely to result in a leakage of the cytokine from the beads. When splenocytes were incubated with HA beads preincubated with IL-27, a strong upregulation of both STAT1 and STAT3 phosphorylation could be detected by flow cytometry (Fig. 3A). No induction was observed when splenocytes were stimulated with beads preincubated with culture medium only, indicating that the effect observed was not due to a nonspecific activation of the splenocytes by HA (Fig. 3A). To exclude effects mediated by free IL-27 released from HA during the test, we incubated IL-27-loaded HA beads with cell culture medium for 15 min and analyzed the capacity of the conditioned media to stimulate splenocytes once the beads had been removed. No induction of STAT activation could be detected (Fig. 3B). This indicates that the activation of STAT1 and STAT3 observed is mediated by immobilized IL-27 rather than IL-27 leaking from the HA beads during the phosflow assays.

**The IL-27 poly-E motif confers bone-binding properties to the IL-27 p28 subunit**

To investigate whether the HA-binding properties of the IL-27 p28 subunit resulted in bone tropism in vitro, we expressed single-chain WT or mutant IL-27 in which the poly-E motif was replaced by alanine in the High Five insect cell line (IL-27 poly-A). The two recombinant proteins, which were tagged in carboxy with a 12-His motif, were concentrated and purified by IMAC. The two recombinant cytokines were biologically active when tested for their capacity to induce the proliferation of mouse Ba/F3 cells transfected with gp130 cDNA or to induce STAT phosphorylation of murine splenocytes (Fig. 4). To compare the bone-binding properties of the WT IL-27 and the poly-alanine IL-27 mutant, we induced the differentiation of MSC (33) in osteocytes in vitro. Osteocyte differentiation was paralleled by bone synthesis, detected by staining with a calcium-specific alizarin red S (Fig. 5A). MSC-derived osteocyte cell cultures were incubated with WT IL-27 or the poly-alanine IL-27 mutant produced in insect cells and

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**FIGURE 1.** The sequence of the p28 subunit of IL-27 contains a polyglutamic acidic motif. The sequences of mouse (m) and human (h) bone sialoprotein (BSP) and IL-27 are indicated. The poly-E motifs are boxed.

**FIGURE 2.** The p28 poly-E motif mediates HA binding. WT (WT p28) or mutant p28 (p28-poly-A) produced in bacteria were subjected to HA chromatography under denaturing (A) or native (B) conditions. Input samples, flow-through, and elution fractions were analyzed by Western blot using an anti-His tag Ab. Six His-tagged CNTF was used as control.
the protein bound to the cultures was analyzed by Western blot with an Ab specific for p28 (Fig. 5B). The amount of mutated IL-27 cytokine recovered was much lower than that of WT IL-27, indicating that the polyglutamic acids contributed to the binding of this cytokine to the osteocytes cell cultures in vitro (Fig. 5B).

The poly-E motif modifies IL-27 pharmacology in vivo

To analyze the effect of replacing polyglutamic acids by alanine in vivo, we generated derivatives of the mammalian expression vector pcDNA5-His coding for WT and poly-A IL-27 tagged in carboxy with an Avitag biotin ligase peptide substrate (34) and 8 His. The modified cDNA inserted in the mammalian expression vector were multicistronic, comprising the sequences coding for a T2A autocleavable peptide (35), followed by a secreted derivative of biotin ligase to catalyze IL-27 biotinylation (34, 36, 37). The constructs containing the cDNA coding for WT and poly-A IL-27 were compared for expression and biotinylation by transfection in HEK-293 Flp-In cells. Similar signals were observed in the cell culture medium of HEK-293 transfected with vectors coding for either WT or mutant IL-27 using anti-p28 or streptavidin, indicating that the expression, secretion and biotinylation of WT and IL-27 were identical (data not shown).

To compare the properties of the two IL-27 derivatives in vivo, we subjected mice to hydrodynamic transfection (38) with the cDNA coding for WT IL-27 or IL-27 poly-A. This transfection procedure is known to result in transient liver production and systemic release of the recombinant proteins (38). It has been used extensively to analyze the effect of IL-27 in vivo (16, 19, 25). When livers from mice subjected to hydrodynamic transfection were analyzed for IL-27 expression by IMAC, followed by Western blot analysis for biotinylated protein, both WT and poly-A mutants IL-27 could be detected (Fig. 5C). As expected, no biotinylated IL-27 could be detected in mice transfected with the control plasmid coding only for the secreted biotin ligase (Fig. 5C). To evaluate whether IL-27 binding to bone could be detected in vivo, we flushed the mice femurs from the bone marrow and then extensively washed the bone lumens with a urea–phosphate buffer solution to recover bound IL-27. The extracts were subjected to IMAC and Western blot analysis. Interestingly, we could only detect IL-27 in the eluates of bones isolated from the mice transfected with WT IL-27 cDNA and not poly-A IL-27 cDNA (Fig. 5D).
The poly-E motif is required for STAT activation in bone marrow CD8 cells

We analyzed whether the IL-27 polyglutamic acid motif affects its capacity to activate immune cells in the bone. Bone marrow cells from groups of three mice subjected to hydrodynamic transfection with control plasmid, IL-6, WT IL-27, or poly-A mutant IL-27 produced in High Five insect cells for 1 h on ice. Lane commercial IL-27, 100 ng IL-27 from R&D Systems. After extensive washing, cell cultures were lysed and analyzed for p28 binding by Western blot using a biotinylated anti-p28 mAb. Data are representative of three independent experiments. (C and D) Mice (two for each conditions) were tail vein-injected with LPS-free mammalian expression vector DNA coding for biotin ligase (control) IL-6, IL-27, or poly-A. Mice were sacrificed at 48 h, and proteins from liver homogenates (C) or eluted from bone lumen using urea and phosphate (D) were analyzed by IMAC and Western blot with HRP-labeled, high-sensitivity streptavidin. Data are representative of two independent experiments.

Discussion

Bone sialoprotein is a major constituent of the bone. It comprises polyglutamic acid domains with HA-nucleating properties (23, 24). A highly homologous motif is present in the p28 C-D loop (1), and p28 purification by HA chromatography was reported previously (39). We confirmed that p28 binds HA under native conditions. Such binding could also be observed under more stringent denaturing conditions. Using mutants in which the acidic amino acids were replaced by alanines, we could show that the polyglutamic acid domain confers the HA-binding properties to the cytokine. IL-27 bound to HA beads retained the capacity to induce STAT1 and STAT3 phosphorylation in splenocytes. This biological activity assay was chosen because it can be performed under experimental conditions preventing leakage of HA-immobilized IL-27. These results indicate that HA-bound IL-27 is not a latent, inactive form of the cytokine and concurs with the current structural model of IL-27 according to which the C-D loop is not directly involved in receptor recruitment (1, 40, 41). We cannot, however, exclude that HA binding modifies the specific activity of the cytokine. Recently, mutations that reduce the flexibility of the IL-2 B-C loop have been shown to strongly increase the affinity of this cytokine for IL-2Rβ, indicating loops connecting the α helix can contribute to the biochemical properties of type I cytokines (43).

The IL-27 acidic motif resembles the poly-E stretch known to target the bone sialoprotein to growing bone in vivo (26). It is also similar to the acidic polypeptide tags used to confer bone targeting properties to therapeutic proteins (26–28). We therefore analyzed the bone-binding properties of IL-27 in vitro and in vivo. To assess this, we induced osteogenic differentiation of mouse multipotent stromal cell cultures. Binding to the mix of osteocytes and osteocyte-secreted bone matrix could be detected by Western blot analysis. No binding was observed using mutant IL-27 poly-A, indicating that the observed binding involved a polyglutamic
acid–HA interaction rather than binding to cell surface IL-27R expressed by osteocytes.

To analyze whether the pharmacological properties of IL-27 are influenced by the poly-acidic motif, we transfected mice with mammalian expression vector derivatives coding for WT and mutant IL-27. Both recombinant proteins were tagged in carboxy with 8-His and a biotin ligase epitope substrate (34). The expression vectors used coded for biotin ligase to catalyze the bio-
tinylation of IL-27 (34, 36, 37). Hydrodynamic transfection results predominantly in liver expression of the recombinant proteins (38). We could detect biotinylated WT and mutant IL-27 in transfected mouse liver. Interestingly, we could also isolate WT but not mutant IL-27 poly-A from the femur lumen of transfected mice. This suggests that the poly-E motif confers bone-binding properties to IL-27. The approach used was selected for its sensitivity and simplicity. It did not, however, allow the quantitation of the proportion of IL-27 immobilized in the bones, which remains to be determined. Analysis of STAT1, 3, and 5 phosphorylation in bone marrow CD8 cells in mice subjected to hydraulic transfection suggests that the poly-E motif can play a role in the targeting of bone marrow–resident cells.

It will be interesting to examine whether the poly-E motif influences other pharmacological properties of IL-27, such as its capacity to bind serum proteins, endothelial cells, or extracellular matrix.

The observation that IL-27 binds HA and bone suggests that the acidic motif confers to this cytokine a tropism for the bone endosteal surface. This location has been identified as a niche for hematopoietic stem cells (44–47) whose differentiation is known to involve CD8 T cells (11–19, 60). As bone marrow niches are also preferred sites for the metastasis of several solid tumors and for blood cell malignancies (61, 62), the conjunction of potent antitumoral activities and of bone binding properties of IL-27 could be beneficial therapeutically (11–19). This could be particularly relevant for multiple myeloma treatment as the IL-27 therapeutic activity on this neoplasia is accompanied by a capacity to inhibit osteoclastogenesis (17, 20, 63) and IL-17 production, which is known to promote myeloma growth (64). This property of IL-27 could also be promising for the treatment of bone metastatic prostate cancer, because IL-27 modifies the cross-talk between bone and immune cells (65).

The location of the IL-27 HA-binding motif in a flexible loop connecting the C-D α-helices suggests that this motif could be used to engineer type I cytokine derivatives with bone-binding properties. For example, this could be an attractive modification for G-CSF, which is used therapeutically for the mobilization of hematopoietic stem cells.

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Disclosures

The authors have no financial conflicts of interest.

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