Interleukin-27 Inhibits the Growth of Pediatric Acute Myeloid Leukemia in NOD/SCID/Il2rg−/− Mice

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Abstract

Purpose: Acute myeloid leukemia (AML) accounts for more than half of fatal cases in all pediatric leukemia patients; this observation highlights the need of more effective therapies. Thus, we investigated whether interleukin (IL)-27, an immunomodulatory cytokine, functions as an antitumor agent against pediatric AML cells.

Experimental Design: Expression of WSX-1 and gp130 on AML cells from 16 pediatric patients was studied by flow cytometry. Modulation of leukemia cell proliferation or apoptosis upon IL-27 treatment in vitro was tested by bromodeoxyuridine/propidium iodide (PI) and Ki67, or Annexin V/PI staining and flow cytometric analysis. The angiogenic potential of AML cells treated or not with IL-27 was studied by chorioallantoic membrane assay and PCR array. In vivo studies were carried out using nonobese diabetic/severe combined immunodeficient (NOD/SCID)/Il2rg−/− mice injected intravenously with five pediatric AML cell samples. Leukemic cells engrafted in PBS and IL-27–treated animals were studied by immunohistochemical/morphologic analysis and by PCR array for expression angiogenic/dissemination-related genes.

Results: We provided the first demonstration that (i) AML cells injected into NOD/SCID/Il2rg−/− mice gave rise to leukemia dissemination that was severely hampered by IL-27, (ii) compared with controls, leukemia cells harvested from IL-27–treated mice showed significant reduction of their angiogenic and spreading related genes, and (iii) similarly to what was observed in vivo, IL-27 reduced in vitro AML cell proliferation and modulated the expression of different genes involved in the angiogenic/spreading process.

Conclusion: These results provide an experimental rationale for the development of future clinical trials aimed at evaluating the toxicity and efficacy of IL-27.

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Introduction

Interleukin (IL)-27 is a heterodimeric cytokine belonging to the IL-12 family (1). It is composed of the EBV-induced 3 (EBI3) and p28 subunits that are homologs of the p40 and p35 chains of IL-12, respectively (2). IL-27 is produced by antigen-presenting cells, which protect their local microenvironment from host pathogens, and it acts on different cell types expressing the full receptor (R) complex (3, 4). The IL-27R contains the unique receptor subunit WSX-1, also known as IL-27Ra/TCCR, paired with the gp130 chain (5, 6). Although IL-27R was found to be expressed on different human hematologic malignancies (7, 8), no information is available on the role played by IL-27 on pediatric AML cells.

IL-27 exerts antitumor activity against different tumors through indirect mechanisms, such as induction of natural killer (NK) and CTL response or inhibition of angiogenesis primarily due to induction of CXCL10 and CXCL9 (3, 9–14). It has also been reported that IL-27 can directly inhibit the proliferation of human melanoma cell lines (15), suppresses tumorigenicity and invasiveness of human lung adenocarcinoma cell lines (16), and dampens multiple myeloma cell growth in vivo, inhibiting osteoclast differentiation and function and inducing osteoblast proliferation (8). In addition, we recently reported that IL-27 inhibits B-acute lymphoblastic leukemia (ALL) cell spreading in animal models (7).

AML includes a heterogeneous group of hematologic tumors deriving from malignant transformation of myeloid...
progenitors that proliferate and accumulate in the bone marrow (BM; refs. 17, 18) that accounts for more than half of fatal cases in all pediatric leukemia patients (19, 20). The role of cytokines in the treatment of acute leukemias including AML has been investigated in several studies because of their immunologic and/or cycling properties (21). Cytokines such as IL-2, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-CSF (GM-CSF) have been mainly used as adjuvants of chemotherapy, as they may stimulate immune cells (22) or limit the duration of neutropenia and promote cell cycling of quiescent leukemia cells, making them more sensitive to chemotherapeutic drugs (21).

In this study, we investigated the ability of IL-27 to restrain pediatric AML cell growth both in vitro and in vivo, unraveling the potential mechanisms involved.

Materials and Methods

Patients

The study was approved by the Institutional Review Board of IRCCS G. Gaslini Institute, Genoa and IRCCS Fondazione Policlinico San Matteo, Pavia, Italy. This investigation complied with the Principles of Helsinki Declaration, and parents/legal guardians of the patients gave written informed consent to study participation. The initial diagnosis of AML, together with subtype identification, was established according to the WHO and the French–American–British classification (18). Clinical characteristic of the children enrolled in the study are detailed in Table 1. In the study, we employed aliquots of patient BM samples collected at disease onset for diagnostic purposes, which contained at least 87% leukemia cells (median 92.5%, range: 87%–98%). Mononuclear cells were isolated from BM by Ficoll-Hypaque density gradient (Sigma Chemical Co.), cells were collected and cryopreserved for later use.

Antibodies, reagents, and flow cytometry

The following antibodies (Ab) were used for flow cytometric analyses: fluorescein isothiocyanate (FITC)-, PE-Tricolor-, APC-conjugated CD45, CD33, and anti-gp130 monoclonal Abs (mAb) obtained from Invitrogen; PE- and FITC-conjugated anti-CXCR4 mAb, PE-conjugated anti-WSX1 mAb purchased from R&D System, Inc.; FITC-conjugated CD3 (Immunotools GmbH) and PC7-conjugated CD19 (Beckman Coulter). All of the above mAbs reacted only with human cells. FITC-conjugated anti-human Ki67, a nuclear cell-cycle molecule marker of dividing cells at a given time, and anti-bromodeoxyuridine (BrdUrd) mAbs were obtained from Dako and BD Biosciences, respectively. Cells were scored using a FACSCalibur analyzer (BD Biosciences) and data were processed using CellQuest software.

<table>
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<th>Sex</th>
<th>Age at diagnosis</th>
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</table>

Abbreviations: t-AML, therapy-related AML; M, male; F, female.
5% CO₂, and then processed following instructions of the manufacturer. Cells were scored by Gallios flow cytometry, 10⁴ events were acquired and analyzed using Kaluza software (Beckman Coulter). Data were expressed as percentage of phospho-STAT–positive cells and as mean relative fluorescence intensity (MRFI), calculated as fluorescence intensity obtained with specific mAb/fluorescence intensity obtained with irrelevant isotype-matched mAb.

**Signaling pathway**

IL-27 signaling pathway was investigated in purified AML cells from patients no. 3, 14, and 16 by flow cytometry. Signal transduction was investigated using the following: Alexa 488 fluorochrome–conjugated mAbs: anti–phospho-STAT1 (clone 58D6), anti-STAT3 (clone D3A7), anti-STAT5 (clone C71E5; Cell Signaling Technology, Inc.), and fluorochrome-matched isotype antibodies (Beckman Coulter). Cells were resuspended in RPMI 10% AB serum and incubated with medium alone or hrIL-27 for 30 minutes at 37°C, 5% CO₂, and then processed following instructions of the manufacturer. Cells were scored by Gallios flow cytometry, 10⁴ events were acquired and analyzed using Kaluza software (Beckman Coulter). Data were expressed as percentage of phospho-STAT–positive cells and as mean relative fluorescence intensity (MRFI), calculated as fluorescence intensity obtained with specific mAb/fluorescence intensity obtained with irrelevant isotype-matched mAb.

**Cell proliferation and apoptosis assay**

Nine pediatric AML cell samples (patients no. 1–9) were cultured for 24, 48, and 60 hours, either in the presence or absence of 50 ng/ml IL-27 (concentration selected according to previous studies; refs. 7, 8), and were tested for cell proliferation. All 16 AML cell samples were tested for apoptosis following the same treatment schedule used for proliferation assay. AML cell proliferation was studied by intracellular staining with anti-Ki67 mAb using the Cytofix/Cytoperm Kit (BD Biosciences) and by BrdUrd staining. AML cells were pulse-labeled with 10 μmol/L BrdUrd (Sigma) for 30 minutes at 37°C temperature. Then, cells were washed and BrdUrd taken up was stained with FITC-conjugated anti-BrdUrd mAb and 5 μg/ml propidium iodide (PI; Sigma), according to the manufacturer protocol. Finally, flow cytometric analysis was carried out. Apoptosis was assessed using the rhAnnexin V/FITC Kit from Immunostep and apoptotic cells were identified as Annexin V⁻/PI⁺ cells.

**Chorioallantoic membrane assay**

Chorioallantoic membrane (CAM) assay was carried out as previously reported (23). Sponges were loaded with 1 μL PBS (negative control), 1 μL PBS with 250 ng VEGF (R&D Systems, positive control), 1 μL conditioned medium from unfractionated AML cell samples (patients no. 1, 9, 13, 14, and 16) cultured for 36 hours with or without IL-27 (50 ng/ml); IL-27 diluted in medium at the same final concentration used to treat tumor cells; 250 ng VEGF+IL-27 diluted in medium at the same final concentration used to treat tumor cells. All conditioned medium were tested in triplicate and means ± SD were calculated. CAM were examined daily until day 12 and photographed in vivo with a stereomicroscope equipped with a camera and image analyzer system (Olympus). On day 12, the angiogenic response was evaluated by the image analyzer system as the number of vessels converging toward the sponges.

In additional experiments, CAM were treated on day 8 with sponges loaded with 1 μL of medium from AML cells treated with IL-27 (patients no. 1, 9, 13, 14, and 16) alone or containing 0.5 μg/ml of an anti-human IFN-γ mAb (EBiosciences). Appropriate isotype-matched mAbs were used as controls.

Differences in the number of vessels formed in CAM assay and differences in percentage and absolute numbers were evaluated by Student t test with 99% confidence interval (99% CI, GraphPad Prism 3). All statistical tests were 2-tailed. A P value lower than 0.05 was considered to be statistically significant.

**Mice studies**

Five/6-week-old nonobese diabetic/severe combined immunodeficient/IL2rzy-deficient (NSG) mice (Jackson Laboratory, Bar Harbor, ME), including both females and males, were housed under specific pathogen-free conditions. All procedures involving animals were done in accordance with national and international current regulations (Italian D.L 27/01/1992, n.116, European Economic Community Council Directive 86/609, OIL 358, December 1, 1987). A total of 5 × 10⁶ AML cells from each patient (nos. 1, 2, 3, 9, and 13) were injected intravenously in 2 groups of 3 to 6 animals each. These AML samples were selected because they were highly enriched (>95%) in neoplastic cells. One group of mice was treated intravenously with 2 doses per week of hrIL-27 (1 μg/mouse/dose) starting from 3 days after tumor cell injection. The other group of animals was treated with PBS (controls) according to the same time schedule. Ten to 12 weeks after tumor cell inoculation, mice were sacrificed and the following tissues were collected from each animal: spleen, peripheral blood (PB) from the retro-orbital vein, and BM obtained by femur flushing. An engraftment criterion of more than 1% of human CD45⁺/CD33⁺ cells in mononuclear cells from murine tissues assessed by flow cytometry was used as biologically significant cut-off. Human leukemic cells were identified as CD45⁺/CD33⁺ cells.

**Morphologic and immunohistochemical analyses**

Tissue samples were fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E). For immunohistochemistry, formalin-fixed, paraffin-embedded sections were immunostained with rabbit anti-human/mouse laminin Ab (Biogenex). After washing, sections were overlaid with goat anti-rabbit immunoglobulin (Ig) conjugated with peroxidase-labeled dextran (EnVision⁺ Peroxidase, rabbit/mouse; Dako) for 30 minutes. Unbound Ig was removed by washing and slides were incubated with ABC (avidin–biotin complex)/alkaline phosphatase (Dako) for 30 minutes; then, sections were counterstained with H&E. Silver impregnation was done with Silver Impregnation For Reticulum Staining 04-040801 Kit (Bio-Optica Milano S.p.A.), on paraffin-embedded sections according to manufacturer’s instructions.
PCR array
Total RNA was extracted using the RNeasy Micro Kit (Qiagen, GmbH) from the following cell samples: (i) spleen and BM from 1 control and 1 IL-27–treated mouse injected with AML cells from patient no. 1, (ii) spleen and BM from 1 control and 1 IL-27–treated animal infected with AML cells from patient no. 13, and (iii) AML cells from patients no. 1 and 13 at diagnosis cultured 36 hours in the presence or absence of hrIL-27. AML harvested from murine tissues were purified using human CD45 MicroBeads (Miltenyi Biotec). The purity of isolated CD45+ cells was more than 95%, as assessed by flow cytometry.

Contaminant genomic DNA was removed by Dnase treatment (Qiagen GmbH). RNA was retrotranscribed with the RT2 First Strand cDNA Synthesis Kit (SABioscience). Human Angiogenesis (code #PAHS-024A) and Metastasis (code #PAHS-028A) RT2 PCR Array and RT2 Real-Time SyBR Green/ROX PCR Mix were from SABioscience. PCR was done on the ABI Prism 7700 Sequence Detector (Applied Biosystems). Gene expression of IL-27–treated and control samples were analyzed separately in different PCR array plates. For each plate, results were normalized on the median value of a set of housekeeping genes. Then, changes in gene expression between IL-27–treated and control samples were calculated using the ΔΔCt formula. Results from the different IL-27–treated and control samples, done in duplicate, were pooled and analyzed by the software provided by the manufacturer. A significant threshold of 4-fold change in gene expression corresponded to P < 0.001.

Chemotaxis
Chemotaxis of human CD45+ cells purified from BM (n = 2 from controls and n = 2 from IL-27–treated mice) and spleens (n = 2 from controls and n = 2 from IL-27–treated mice) was tested using transwell plates (5-μm pore size; Costar). A total of 2 × 10^5 human cells were dispensed in the upper chamber and CXCL12 (300 ng/mL; R&D System) or medium alone was added to lower chamber. Plates were incubated 2 to 5 hours at 37°C. Cells from the lower compartments were collected and counted.

Statistical analysis
Differences in the number of vessels formed in CAM assay were evaluated by Student t test; differences in STAT phosphorylation, percentage, and absolute numbers were evaluated by Mann–Whitney test with 99% CI (GraphPad Prism 3). All statistical tests were 2-tailed. A P value lower than 0.05 was considered to be statistically significant.

Results
Expression and function of IL-27 receptor in pediatric AML cells
We initially investigated the expression of both chains of IL-27R, named WSX-1 and gp130, in primary leukemia cells from 16 pediatric patients with a diagnosis of either de novo or therapy-related AML. To this end, 3 color staining of BM mononuclear cells from AML patients for CD45, CD33, and WSX-1 or gp130 was done and cells were analyzed by flow cytometry. WSX-1 and gp130 expression was analyzed on gated CD45+CD33+ AML cells.

Such analyses revealed that AML cells constitutively expressed both chains of IL-27R (mean percentage of gp130+ cells being 67%, range: 58%–81%; mean percentage of WSX-1+ cells being 43%, range: 6%–80%), thus representing a target of IL-27. Three representative experiments showing WSX-1 and gp130 expression are reported in Fig. 1A, top and bottom panels, respectively. Data on MRFI and percentages of WSX-1 expression in blasts from each patient is reported in Table 1.

To elucidate the downstream pathway induced by IL-27, we analyzed the ability of the cytokine to induce activation of specific STAT molecules in AML cells, as reported for human tonsil B cells (24) and plasma cells (7). To this end, 3 purified AML cell suspensions were treated with medium or IL-27 and tested for intracellular phosphorylation (p) of STAT1, 3 and 5, by flow cytometry. These experiments revealed that IL-27 induced a significant phosphorylation of STAT1 (IL-27 vs. medium: MRFI 2.15 ± 0.07 vs. 1.68 ± 0.25, P = 0.0357; percentage of p-STAT1+ cells 35 ± 11.31 vs. 13 ± 8.48, P = 0.0027) and of STAT3 (IL-27 vs. medium: MRFI 2.25 ± 0.35 vs. 1.895 ± 0.27, P = 0.0079; percentage of p-STAT3+ cells 31.5 ± 9.19 vs. 17 ± 11.31, P = 0.0035), but not of STAT5 in purified AML cells (Fig. 1B).

IL-27 inhibits pediatric AML cell growth in NSG mice
The antitumor activity of IL-27 against pediatric AML cells was tested in vivo in NSG mice, which represent the most suitable model for efficient engraftment of primary neoplastic cells (25). Thus, each AML cell suspension obtained from 5 patients (patients no. 1, 2, 3, 9, and 13) was injected intravenously in 2 groups of 3 to 6 animals each (total animals injected = 44); mice were sacrificed 10 to 12 weeks later when signs of poor health became evident in controls. PB, spleen, and BM from each animal were collected and subjected to immunophenotypic evaluation and flow cytometric analysis. Human cells were identified using the human CD45 surface marker, in association with CD33. Such analyses revealed that AML cells efficiently engrafted in all control and treated mice, as witnessed by the presence of at least 1% human CD45+ cells, that consistently coexpressed the CD33 marker, in PB, BM, and spleens (mean percentage of CD45+CD33+ cells = 94.5%, range: 89%–97% without significant differences between PB, BM, and spleen). Low levels of CD3+ or CD19+ cells within CD45+ compartment from the same samples were observed (mean percentage of CD45+CD3+ cells: 3%, range: 1%–5%; mean percentage of CD45+CD19+ cells: 2.5%, range: 1%–4%; not shown).

Notably, IL-27 significantly reduced the presence of human CD45+CD33+ leukemia cells in PB (P < 0.0001), BM (P = 0.0015), and spleen (P < 0.0001) as compared with control mice (Fig. 1C). Representative CD45 stainings of BM, spleen, and PB obtained from animals injected with AML cells from patient no 9 and treated with either PBS or hrIL-27 are shown in Fig. 1D. Cells obtained from patients no. 3 and 9 and recovered from mice carried the same
cytogenetic anomaly detected at diagnosis, namely monosomy 7 and inv (16), thus proving that leukemia cells engrafted in the animal (not shown). This finding was in accordance with previous results (26). Summary of human CD45\(^{+}\)CD33\(^{+}\) cells present in each animal is reported in Table 2.

**IL-27 modulated genes involved in angiogenesis and progression of AML in NSG mice**

The data from *in vivo* experiments prompted us to investigate the potential mechanisms underlying the effects of IL-27 on leukemia cell growth in *vivo*. Morphologic and immunohistochemical analyses of spleens collected from controls (*n* = 5) and IL-27–treated mice (*n* = 5) revealed that human AML cells gave rise to multiple and wide neoplastic infiltrates (Fig. 2A, panel a). These infiltrates were supplied with a well-developed microvascularization (Fig. 2A, panel b) and sustained by a rich stromal reticulin fiber network that seemed to be shaped to intratumoral microvascularization (Fig. 2A, panel c). By contrast, the spleen of IL-27–treated mice showed smaller and less frequent neoplastic infiltrates (Fig. 2A, panel d) and an almost absent microvascularization and reticulin fiber support, as revealed by both laminin staining (Fig. 2A, panel e) and silver impregnation (Fig. 2A, panel f; refs. 27, 28).

Thus, neoplastic cells collected from (i) spleen and BM from 1 control and 1 IL-27–treated mouse injected with AML cells from patient no. 1 and (ii) spleen and BM from 1 control and 1 IL-27–treated animal injected with AML cells from patient no. 13 were purified as human
CD45+ (>95%) cells by immunomagnetic beads manipulation and used for evaluation of expression of a 84 angiogenic and 84 metastatic related genes, by PCR array. As shown in Fig. 2B, AML cells infiltrating the spleens from IL-27–treated mice showed downregulation of different proangiogenic genes including angiopoietin (ANGPT)2 and 3, CXCL6 and VEGF-C (Fig. 2B, left histogram), as well as genes involved in the dissemination/spreading process (Fig. 2B, right histogram) such as CXCR4 and matrix metalloproteinase (MMP)7. However, it is of note that IL-27 also induced upregulation of the angiostatic molecule tissue inhibitor of metalloproteinase (TIMP)2, indicating that IL-27–mediated inhibition of angiogenesis may result from the regulation of both proangiogenic as well as angiostatic factors.

Similarly, the angiogenesis-related genes IL-6, CXCL1 and 5 (Fig. 2C, left panel) or genes involved in the spreading process (Fig. 2C, right panel), including CXCR4, MMP2, and 9 were found to be downregulated in human CD45+ cells purified from murine BM of IL-27–treated mice versus controls. The other genes included in the PCR array plates were not significantly modulated by IL-27 (not shown).

**IL-27 antitumor activity against pediatric AML cells in vitro**

The antitumor activity of IL-27 against pediatric AML cells was tested in vitro in terms of (i) modulation of genes involved in angiogenesis and leukemic progression, (ii) modulation of cell proliferation, and (iii) induction of apoptosis. First, the angiogenic activity of 5 AML cell suspensions (patients no. 1, 9, 13, 14, and 16) cultured 36 hours with or without 50 ng/mL of hrIL-27 was investigated using the in vivo CAM system. CAM treated with sponges loaded with VEGF (positive control) or with conditioned medium from AML cells were surrounded by allantoic vessels developing radially toward the implant in a "spoked-wheel" pattern. In the representative experiment shown in Fig. 3A, left panel, the mean number of vessels formed in the presence of conditioned medium from AML cells (patient no. 14) was 25 ± 4, whereas that formed in the presence of VEGF was 30 ± 5 (not shown). When we tested the conditioned medium from IL-27–treated cells from the same AML patient in the CAM assay, a significant (*P* < 0.001) reduction of the angiogenic response was appreciable (mean number of vessels 10 ± 3; Fig. 3A, right panel), as compared with the AML conditioned medium. No vascular reaction was detected around the sponges upon exposure to IL-27 diluted in medium at the same final concentration used to treat tumor cells (mean number of vessels 6 ± 2 in the presence or absence of IL-27, not shown). Finally, medium containing IL-27 did not inhibit angiogenesis induced by VEGF (not shown). Similar results with the same statistical significance (*P* < 0.001) were obtained when

### Table 2. Absolute number of human CD45+CD33+ cells engrafted in spleens, BM, and PB from each NSG animal injected intravenously with different AML cell samples

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conditioned medium from the 4 other pediatric AML cell suspensions were tested. We checked by trypan blue staining the viability of leukemic cells incubated with or without IL-27 before harvesting supernatants. There were no significant differences in the proportions of viable cells between cultures done with or without IL-27. AML cell suspensions from patients no. 1 and 13 were analyzed by PCR array for expression of genes involved in the angiogenic and metastatic processes. As shown in the left histogram of Fig. 3B, IL-27 upregulated significantly (P < 0.001) a set of anti-angiogenic genes including IFN-γ, CXCL10, and TIMP2. Furthermore, IL-27 downregulated genes involved in tumor spreading such as CDH6 (Fig. 3B, right histogram). The other genes included in the PCR array plates were not modulated by IL-27 (not shown).

Thus, to test whether IFN-γ, the gene that was found most upregulated in primary AML cells by PCR array, was a key mediator of the angiostatic activity exerted by IL-27, we analyzed the angiogenic activity of IL-27–treated AML cell supernatants (patients no. 1, 9, 13, 14, and 16) following incubation with neutralizing antibodies to human IFN-γ. These experiments revealed that neutralization of IFN-γ did not affect significantly the inhibition of angiogenesis observed in supernatants from IL-27–treated leukemic cells (not shown).

Finally, the ability of IL-27 to affect proliferation and apoptosis of primary AML cells was assessed by Ki67 or BrdUrd/PI (patients no. 1–9) and Annexin V/PI (patients no. 1–16) stainings, respectively. These analyses revealed that IL-27 significantly (P = 0.024) reduced the percentage of Ki67+ cells at 48 hours (Fig. 3C), but not at 24 hours (not shown). Cell-cycle analysis by BrdUrd/PI staining showed that 48 and 60 hours of IL-27 treatment caused an increase of percentage of AML cells in G1 and more marginally sub-G1 phase, paralleled by a decrease of cells in S and G2-M phases (1 representative experiment is shown in Fig. 3D). IL-27 treatment did not affect apoptosis at any time point tested (1 representative experiment is shown in Fig. 3E).

**IL-27 downregulated CXCR4 surface expression in vivo**

CXCR4 represents the receptor for CXCL12 which was found to be implicated in the growth and dissemination of solid and hematologic tumors (29, 30). Furthermore, CXCR4 antagonists inhibit ALL cell engraftment in the BM (30, 31). Because CXCR4 mRNA was consistently down-regulated by IL-27 treatment, we tested whether the corresponding protein was modulated at surface level in vivo. To this end, flow cytometric analyses was done on spleens, PB, and BM cell suspensions from NSG mice treated with PBS (spleens n = 15, PB = 15 and BM = 15) or IL-27 (spleens n = 15, PB = 15 and BM = 15).
n = 15, PB = 15 and BM = 15) injected with AML cells from patients no. 1, 3, 9, and 13. These experiments showed that IL-27 treatment in vivo decreased the presence of CD45^{-}CD33^{-}CXCR4^{+} cells homing in the different compartments in which leukemic cells engrafted. Absolute numbers of CXCR4 expressing leukemia cells are reported in Fig. 4A. One representative CXCR4 staining of human CD45^{-}CD33^{-} leukemia cells harvested from BM, PB, and spleen of PBS and IL-27–treated mice is shown in Fig. 4B and C, respectively.

Finally, human CD45^{-} cells purified from BM and spleens obtained from NSG mice injected with 2 AML cell samples (patients no. 1 and 3) were tested for their ability to respond to CXCL12, the ligand of CXCR4, by chemotactic assay. These experiments revealed that neoplastic cells isolated from the different murine compartments of both controls and IL-27–treated animals did not migrate in response to CXCL12 (not shown).

Discussion
AML is a clinically and genetically heterogeneous disease deriving from malignant transformation of hematopoietic progenitors that accumulate in the BM (17, 18, 20). Although advances in the treatment of childhood AML during the last 2 decades have significantly increased the expectancy of survival, AML still accounts for more than half of fatal cases of pediatric leukemia. In view of these considerations, novel, more effective, and specific therapies for high-risk and relapsed patients are desirable. With this background, we investigated whether IL-27, an immunomodulatory cytokine (1, 32) with antitumor properties...
against solid (3, 9, 33) and hematologic malignancies (7, 8), may function as an antileukemic agent against pediatric AML using a preclinical model. Here, using both in vivo and in vitro approaches, we showed for the first time that pediatric AML cells express functional IL-27R and that IL-27 represents an efficacious antitumor agent against AML. In this regard, it has to be mentioned that functional genetic screening of genes expressed in primary AML cells showed that the IL-27R is frequently expressed on the surface of these cells, suggesting that this gene may possess hematopoietic cell transforming properties (34). Although functional experiments on IL-27 activity on AML cells have not been carried out in a large cohort of patients, the latter work provided results that apparently differ from that reported here. In vivo experiments were conducted using NSG mice that represent an efficient animal model for engraftment of tumor cells and allowed us to evaluate the antitumor activity of human IL-27 on human AML cells in vivo in the absence of immune responses (25, 35, 36). We chose to use nonirradiated hosts and engrafted primary AML cells were detected in all NSG animals, consistent with previous results (26). This model allowed us to show that IL-27 strongly reduced the presence of leukemia cells in the PB and inhibited leukemia spreading in the BM and spleens. The unambiguous neoplastic nature of human cells engrafted in these mice upon injection with pediatric AML cells was shown here and already reported by us (26). In the latter study, it was clearly shown that human CD45+ cells engrafted in NSG mice retained the ability to growth in secondary recipients and showed the same phenotype and polymorphic short-tandem repeat loci detected at diagnosis in the original patient sample.

The inhibition of pediatric AML cell spreading is likely related to the ability of IL-27 to function directly against tumor cells through reduction of their angiogenic potential, modulation of genes involved in tumor spreading, and by inhibiting AML cell proliferation, as shown by our in vitro results. Morphologic and immunohistochemical analyses done on spleens explanted from control and treated animals revealed that IL-27 caused a strong inhibition of the leukemia homing, tumor vascularization, and stromal reticulin fiber network in the spleens. To gain more insights into this issue, we investigated the ability of IL-27 to modulate the expression of different genes involved in the angiogenic and dissemination processes. In leukemia cells purified from spleens and BM, we showed that IL-27 downregulated different proangiogenic genes, including angiopoietins (37, 38), CXCL chemokines (39, 40), and IL-6 (41, 42), as well as some genes involved in tumor progression such as cadherins, MMP, and CXCR4 (43–45). However, AML cells harvested from the 2 murine compartments showed a different pattern of genes modulated by IL-27, possibly because of the different microenvironments to which they have been exposed in vivo. The only exception was represented by CXCR4 that was consistently downregulated by IL-27, at mRNA and surface levels, in leukemic cells from each compartment. However, these cells failed to migrate in response to the CXCR4 specific ligand, that is, CXCL12. It is of note that, in acute leukemias, the CXCL12–CXCR4 axis represents much more than a traffic controller (29), as it is important for cell survival, proliferation, and release of soluble factors (29, 46–48). The potential involvement of this pathway has not here been investigated because the CXCR4 downregulation occurred in AML cells in vivo but not in vitro, thus suggesting that this finding may be related to microenvironmental stimuli mediated by the cytokine more than a specific direct activity of IL-27 on leukemic cells.

Similarly to observations in vivo, PCR array studies done on primary AML cells treated in vitro revealed that IL-27 modulated several genes involved in angiogenesis and leukemic dissemination. In particular, IL-27 strongly
upregulated the angiostatic cytokine IFN-γ and, although to a lesser extent, CXCL10 and TIMP2; moreover, it downregulated not only the proangiogenic angiopoietin-4 and CXCL3 but also cadherin-6. Although human IL-27 is not species specific (2), the in vivo antitumor mechanisms operated by IL-27 overlapped with those observed in vitro in leukemia cells, supporting the conclusion that the cytokine targets AML cells directly. In a translational perspective, we provided evidence that the cytokine exerts a strong antitumor activity against pediatric AML cells irrespective of specific leukemic subtypes. However, a large cohort of pediatric AML patients should be included in future studies to evaluate whether different AML subtypes may respond more or less efficiently to IL-27.

Overall, our results support the concept that IL-27 may represent a new antileukemic drug to be tested in future clinical trials, as this cytokine may target pediatric AML by inhibiting their angiogenic and dissemination potential directly. In this respect, it has been shown that IL-27 enhances proliferation and differentiation of nonleukemia human CD34+ cells (49), thus suggesting that this cytokine may have a favorable effect on normal hematopoietic stem cells, a subset often highly compromised in leukemia patients. An additional argument in favor of the hypothesis of using IL-27 is the low toxicity shown by the cytokine in animal models, likely in relation to the limited induction of IFN-γ in vivo (9). Finally, simultaneous activation of in vivo antitumor effector mechanisms mediated by CTL, NK, and CD8+ T cell may be promoted by IL-27 (1).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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