Interleukin-11 Stimulates Multiple Phases of Erythropoiesis In Vitro

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INTERLEUKIN-11 (IL-11), a pleiotropic cytokine originally isolated from a primate bone marrow stromal cell line, has been shown to stimulate T-cell–dependent B-cell maturation, megakaryopoiesis, and various stages of myeloid differentiation, but to inhibit adipogenesis. Because stromal cells are essential for the maintenance of early hematopoietic progenitors, we investigated the effects of IL-11 on multipotential and erythroid precursors from murine bone marrow in vitro in suspension and semisolid cultures. Our results show that in the presence of IL-3 or c-kit ligand (KL), IL-11 has profound stimulatory effects on primitive multilineage hematopoietic progenitors, pre-CFC<sub>multi</sub>, as well as on precursors representing various stages of erythroid differentiation observable in vitro, including CFC<sub>multi</sub>, BFU-E, and CFU-E. In addition, the combination of KL with IL-11 also stimulated highly proliferative erythroid progenitors that yield remarkable macroscopic erythroblast colonies in culture. These results indicate that IL-11 is likely to play a pivotal role in early hematopoiesis and at multiple stages of erythropoiesis.

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RESULTS

IL-11 preferentially promotes multilineage and erythroid colonies in IL-3-containing cultures. Increasing concentrations of IL-11 significantly enhanced the IL-3-dependent colony formation measured at day 9 of incubation in the presence of Epo (Fig 1). This effect was most prominent on CFC<sub>multi</sub> and erythroid colonies whose numbers increased 200% and 175%, respectively, in the presence of IL-11 (maximal at 10 to 20 ng/mL) compared with cultures

MATERIALS AND METHODS

Cytokines. Recombinant mIL-3, hIL-18, and hIL-6 were produced by Sandoz Pharma Ltd (Basel, Switzerland). Recombinant hIL-11, expressed in COS-1 cells or in Escherichia coli (2 × 10<sup>6</sup> and 1.8 × 10<sup>9</sup> U/mg, respectively), was obtained from Genetics Institute (Cambridge, MA). Recombinant mKL was provided either as a COS cell supernatant (Sandoz Pharma Ltd) or as purified material by Dr S. Gillis (Immunex Inc, Seattle, WA).

Colony assays. Methylcellulose culture of normal murine bone marrow cells (5 × 10<sup>6</sup> cells/mL) were prepared in Iscove’s modified Dulbecco’s medium (IMDM) containing 4% fetal calf serum (FCS), bovine serum fraction V, transferrin, and lipids according to Iscove et al.16 BFU-E-derived erythroid bursts were counted on day 7. Macroscopic (>1 mm) multilineage colonies comprising erythroid, megakaryocytic, and other myeloid cells (CFC<sub>multi</sub>), erythroid colonies (either pure or mixed with megakaryocytes, E), and granulocyte-macrophage (GM) colonies were counted on day 9. The colonies were identified by their morphology and were periodically picked, spread, and stained (Wright/Giemsa) for confirmation.

CFU-E assays were performed with 12-day murine fetal liver (6 × 10<sup>6</sup>/mL) or adult murine bone marrow cells (10<sup>5</sup>/mL) in methylcellulose cultures containing IMDM, 4% FCS as previously described.17 CFU-E-derived colonies were counted after 2-day incubation at 37°C.

Replating experiments. Macroscopic compact erythroblast colonies were picked from methylcellulose cultures on day 8. The cells were then either spread on a slide for Wright/Giemsa staining or resuspended in IMDM, counted, and replated in secondary methylcellulose cultures. Replating was performed in the presence of either (1) IL-3, IL-1, Epo, and G-CSF; (2) Epo alone; (3) IL-11 alone; or (4) in the absence of growth factor. CFU-E–like colonies were counted after 2 days, and the plates were further observed up to 9 days.

Suspension cultures. Suspension cultures consisted of normal mouse bone marrow cells (10<sup>5</sup> cells/mL) cultured in 1 mL IMDM containing α-thioglycerol, 5% FCS, and 0.1% bovine serum albumin (BSA) in 35-mm plastic bacteriologic Petri plates, according to Iscove et al.16 IL-1, IL-3, IL-11, and KL were added to the cultures as indicated. After 4 days of incubation at 37°C, the cells were gently resuspended and harvested and the content of colony-forming cells was determined in methylcellulose cultures containing optimized concentrations of IL-3 (30 ng/mL), IL-1 (10 ng/mL), and Epo (0.08 U/mL).

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IL-11 supports CFU-E maturation. To further investigate the effects of IL-11 on the late stages of erythroid development, its ability to stimulate CFU-E-derived colonies was determined next. Figure 3 shows that IL-11 alone does indeed stimulate, dose dependently, the development of colonies from these late-stage erythroid precursors obtained from either fetal liver (6,000 cells from 12-day gestation per mL, Fig 3A) or adult bone marrow (10^5 cells/mL, Fig 3B). The number of colonies obtained was approximately 30% of the maximum number found in cultures stimulated with Epo alone. The stimulation of CFU-E-derived colony formation by IL-11 was additive at suboptimal Epo levels (0.02 U/mL) but combination of IL-11 with optimal concentrations of Epo was not more effective than Epo alone (data not shown). These findings further indicate that IL-11 has an effect on relatively late stages of erythroid development.

A novel type of macroscopic erythroblast colony is shown by combinations of IL-11, KL, and Epo. To further analyze the effects of IL-11 on primitive colony-forming progeni-

![Fig 1. IL-11 stimulation of IL-3-dependent colony formation. Methylocellulose cultures of normal murine bone marrow cells (5 x 10^4 cells/mL) contained optimized concentrations of IL-3 (10 ng/mL) and Epo (0.08 U/mL) plus increasing concentrations of IL-11. Macroscopic (>1 mm) multilineage colonies comprising erythroid, megakaryocytic, and other myeloid cells (CFU-multi) [C3], erythroid colonies (either pure or mixed with megakaryocytes, E [Δ]) and granulocyte-macrophage (GM; [□]) colonies were counted on day 9. The results are expressed as the increase in colony number compared with cultures containing no IL-11 (3 ± 1 CFU-multi, 33 ± 4 erythroid, and 40 ± 5 GM colonies per 10^6 cells) and are the mean ± SEM of four independent experiments each with 3 to 4 plates per point. In the absence of IL-3, IL-11 stimulated <4 E and no GM colonies/10^6 cells.

![Fig 3. Stimulation of CFU-E maturation by IL-11. CFU-E-derived colonies were counted in methylcellulose cultures containing increasing concentrations of IL-11 plus either 12-day fetal liver cells (A) or adult bone marrow cells (B). Results are expressed as a percentage of the maximal stimulation obtained with 0.1 U/mL Epo (1,300 CFU-E/10^6 fetal liver cells and 880 CFU-E/10^6 bone marrow cells, four and three experiments, respectively).]
Fig 2. Stimulation of BFU-E-derived colony formation by IL-11 plus IL-3. Methyccellulose cultures of murine bone marrow cells contained IL-11 (50 ng/mL) and IL-3 (10 ng/mL) either alone or in combination, in the presence (A) or in the absence (B) of 0.08 U/mL Epo. Erythroblast bursts were counted on day 7. The results are the mean ± SEM from three independent experiments with 3 to 4 plates per point. Epo alone stimulated no BFU-E. The insets show typical bursts obtained in response to IL-11 plus IL-3 with (A) or without (B) Epo.

Fig 4. Stimulation of macroscopic erythroblast colonies by a combination of IL-11, KL, and Epo. Methyccellulose cultures of murine bone marrow cells contained 0.08 U/mL Epo plus the following factors, used either alone or in combination: KL (10 µL/mL of COS cell supernatant), IL-11 (50 ng/mL), IL-3 (10 ng/mL), and IL-1β (10 ng/mL). The day 7 macroscopic erythroblast colonies (inset A) developed into very large (>1 mm diameter) compact hemoglobinized erythroid colonies by day 11 (inset B) when they were counted. The results are the mean ± SEM of seven to eight observations from three independent experiments.

Fig 5. Increase of pluripotential precursors CFC multiline in response to IL-11 after 4 days in suspension culture. Normal murine bone marrow cells were incubated in liquid cultures containing IL-1 (10 ng/mL), IL-3 (10 ng/mL), and IL-11 (50 ng/mL), alone or in combination, (A) without or (B) with KL (100 ng/mL). After 4 days, the liquid cultures were harvested and the content of colony-forming cells was determined. Macroscopic CFC multiline colonies (inset) were counted on day 9. The input number of CFC multiline was 11/10^6 fresh bone marrow cells. Results are mean ± SEM from three independent experiments (four plates each).
tors, IL-11 was tested in combination with KL, a stromal cell-derived cytokine found to act synergistically with many different growth factors in supporting hematopoietic colony formation.11,13,16 Most interestingly, IL-11 plus KL and Epo stimulated the development of large colonies with a distinct morphology that consist predominantly of erythroblasts (Fig 4). These colonies are round and compact, with no evidence of the multicentric morphology typical of erythroid bursts (Fig 2), and within 8 days of culture contain between 3 and 16 x 10^4 cells of early erythroblast morphology. By day 12 of culture, these colonies comprise almost exclusively mature hemoglobinized erythroid cells (Fig 4, inset).

If the cells from day 8 colonies are replated into secondary cultures containing a variety of growth factors (Epo plus IL-3, IL-1, and G-CSF), only typical CFU-E-derived erythroid colonies are obtained. The plating efficiency in Epo alone is approximately 36% ± 5% (n = 7), suggesting that a large majority of the cells within these colonies are at a comparable stage of development. When replated into IL-11 alone, up to 23% of the number of colonies stimulated by Epo were observed (mean 13% ± 3%, n = 7, with only 3% ± 1% in the absence of factor). Cell densities as low as 1,800 cells/mL (mean 3,035 ± 455, n = 7) were used in the replating experiments.

The frequency of the BFU-E-derived colonies was not diminished in these cultures compared with cultures supported by IL-3 plus Epo, or IL-3 and KL plus Epo (12 ± 3 BFU-E per 5 x 10^4 cells with KL, IL-11 plus Epo v 10 ± 3 with IL-3 and Epo, and 19 ± 4 with KL and IL-3 plus Epo, four experiments), suggesting that the progenitors yielding the macroscopic erythroblast colonies are different from BFU-E. The formation of total erythroid and myeloid colonies supported by KL and Epo was enhanced by IL-11 in these cultures (2.9 ± 1.0- and 2.2- ± 0.2-fold, respectively). Multilineage colonies that are normally poorly stimulated by KL and Epo alone were also observed in cultures containing IL-11 plus KL and Epo, indicating that this combination of growth factors can sustain the growth of all hematopoietic lineages.

**Early pluripotential progenitors proliferate in response to IL-11 plus IL-3 or KL.** The effect of IL-11 on more primitive pluripotential progenitors (pre-CFC<sub>multi</sub>) was then examined in a two-step culture assay described by Issac et al.16 The pre-CFC<sub>multi</sub> has been characterized by its ability to divide during 4 days in liquid culture and yield daughter cells that generate macroscopic CFC<sub>multi</sub> in secondary methylcellulose cultures. The proliferative response of pre-CFC<sub>multi</sub> (8- to 10-fold expansion) was previously reported to depend uniquely on the combination of IL-1 and IL-3<sup>19</sup> (Fig 5A). We show here that IL-11 could effectively replace IL-1 and, in combination with IL-3, stimulated a 10-fold increase of the number of CFC<sub>multi</sub> recovered after liquid culture (Fig 5A). This effect was maximal at 10 to 50 ng/mL IL-11. IL-11 had no effect on its own. In addition, IL-11 also proved effective in combination with KL in the same system. Although neither KL alone (up to 100 ng/mL) nor KL in combination with IL-1 significantly stimulated the expansion of CFC<sub>multi</sub> during 4 days in culture, combination of KL with either IL-11 or IL-3 resulted in a 10-fold increase of this cell population. Interestingly, the combination of the two stromal cell-derived factors IL-11 and KL in the absence of IL-3 yielded optimal results that were not substantially improved by addition of IL-3.

**DISCUSSION**

In this study, IL-11 was shown to have a stimulatory effect on a variety of murine bone marrow and fetal liver derived progenitor cells at different stages of differentiation. The effects of IL-11 on committed progenitors were most pronounced within the erythroid lineage. Early in erythroid development, IL-11 proved capable of stimulating BFU-E when combined with IL-3, even in the absence of Epo. The Epo independence distinguished the activity of IL-11 from KL, which always required the addition of Epo for promoting BFU-E-derived colonies, indicating a different activity of these two stromal factors on terminal erythroid differentiation. Later in erythroid differentiation, IL-11 alone supported the maturation of BFU-E from fetal liver or bone marrow cells and from a more homogenous population of cells from the day 8 macroscopic erythroblast colonies replated at low density. This latter result suggests that IL-11 acts directly on the terminal differentiation of the erythroid precursors.

In addition to the traditional types of BFU-E- and CFU-E-derived erythroid colonies, IL-11, when combined with KL and Epo, stimulated the growth of macroscopic erythroblast colonies containing erythroid progenitors able to form BFU-E-like colonies with a high frequency. KL has previously been documented to support the growth of macroscopic blast colonies containing macrophage and granulocyte progenitors.18 These colonies typically were found to contain 300 to 3,000 cells after 7 days in culture in KL alone or KL plus G-CSF.18 In contrast, the erythroid progenitors that were stimulated in the presence of IL-11, KL, and Epo yielded much larger colonies, typically containing 3 to 16 x 10^4 cells on day 8 of culture. These progenitors appear to undergo extensive proliferation without terminal maturation through day 7, a time when BFU-E-derived cells in the murine system are fully differentiated. They seem to be committed to the erythroid lineage because (1) they mature to large erythroid colonies by day 11 under culture conditions with IL-11, KL, and Epo, which also allowed the proliferation and full differentiation of granulocyte, macrophage, and multilineage precursors; and (2) their progeny consisted only of typical CFU-E-derived colonies in all culture conditions tested. On the other hand, that these erythroid progenitors are distinct from the typical BFU-E is suggested by the fact that (1) in the presence of IL-11, KL, and Epo, both macroscopic colonies of early erythroblasts and fully differentiated, hemoglobinized, BFU-E-derived colonies co-exist on day 7 in the same cultures, and (2) the frequency of the BFU-E colony formation was not diminished compared with cultures supported by IL-3 plus Epo, or IL-3 and KL plus Epo.

From the potent effect of IL-11 at different stages of erythropoiesis we would predict that this cytokine might affect the erythroid compartment in vivo. In normal mice,
the administration of IL-11 (3 μg/d subcutaneously for 7 to 10 days) largely resulted in increased levels of circulating neutrophils and platelets, with no apparent effect on erythrocytes. However, a striking effect on erythropoiesis was detected in lethally irradiated mice transplanted with bone marrow cells infected with a retroviral vector producing high levels of IL-11 (C. Wood, personal communication, March 1992). The drastically reduced hematocrits normally seen 8 to 14 days posttransplantation in animals reconstituted with bone marrow cells infected with a control, non-IL-11–producing virus was largely prevented when mice were transplanted with bone marrow cells infected with the IL-11–producing virus.

The multiplication of pre-CFCmUlti in suspension culture was previously reported to require both IL-3 and IL-1. We show here that IL-11 can effectively replace IL-1 in this system. In addition, the combination of IL-11 and KL, but not of IL-1 and KL, also yielded a 10-fold increase in the number of CFCmUlti obtained from these cultures, showing that even IL-3 could be omitted when the two stromal factors are combined. The physiologic role of IL-3 on early hematopoiesis has been questioned because neither IL-3 protein nor message could ever be detected in stromal-supported long-term bone marrow cultures or in the bone marrow itself in situ. We show here that an effect of IL-3 plus IL-1 on early pluripotential progenitors can be fully reproduced by two factors known to be produced by stromal cells, IL-11 and KL.

Many of the activities previously described for IL-11 overlap with activities ascribed to IL-6, including the ability to enhance IL-3–dependent colony formation by megakaryocytic progenitors and blast cell colony-forming cells and to inhibit adipogenesis. However, IL-11 and IL-6 do not compete for receptor binding on the plasmacytoma cell line T10, which responds to both factors. Our results further distinguish the activities of the two cytokines in hematopoiesis since IL-11, but not IL-6, promotes the growth of various erythroid colonies, including a progenitor of high proliferative capacity that yields macroscopic erythroblast colonies.

The growth and development of blood cells in the bone marrow microenvironment is believed to be regulated by interactions between stromal and hematopoietic cellular elements mediated, in part, by cytokines. Predominant among these is KL, a stromal cell-derived cytokine demonstrated by mouse genetics to have a central role in regulating hematopoiesis, especially primitive pluripotent progenitors and progenitors of the erythroid and mast cell lineages. Although equivalent genetic data do not exist for IL-11, our data differentiate it from KL and implicate it as a key player in the control of hematopoiesis. Like KL, IL-11 alone has little effect in vitro but, when combined with either KL or IL-3, it serves to promote the proliferation of primitive multipotent as well as more differentiated lineage-restricted progenitors. Our results show that IL-11 has a different spectrum of activities than other regulators known to act on pluripotent and erythroid progenitors and that these activities are consistent with this stromal cell-derived cytokine having a pivotal role in the regulation of early stages of hematopoiesis and many phases of erythropoiesis.

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