The Journal of Immunology

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J Immunol 2002; 169:1261-1269; ;
doi: 10.4049/jimmunol.169.3.1261
http://www.jimmunol.org/content/169/3/1261

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IFN Consensus Sequence Binding Protein/IFN Regulatory Factor-8 Guides Bone Marrow Progenitor Cells Toward the Macrophage Lineage[1]

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IFN consensus sequence binding protein (ICSBP; IFN regulatory factor-8) is a transcription factor of the IFN regulatory factor family. Disruption of this gene results in a leukemia-like disease in mice. To investigate the role of ICSBP in myeloid cell development, lineage marker-negative (Lin^-) bone marrow progenitor cells were purified from $ICSBP^{+/+}$ and $ICSBP^{-/-}$ mice and tested for gene expression and colony-forming ability. ICSBP was expressed in Lin^- progenitor cells, and its levels were markedly increased by IFN-γ. The colony-forming potential of $ICSBP^{-/-}$ progenitor cells was grossly abnormal, as they gave rise to a disproportionately high number of granulocyte colonies and many fewer macrophage colonies. IFN-γ inhibited colony formation, while promoting macrophage maturation in $ICSBP^{+/+}$ cells. In contrast, the effects of IFN-γ were completely absent in $ICSBP^{-/-}$ progenitors. By retrovirus transduction we tested whether reintroduction of ICSBP restores a normal colony-forming potential in −/− progenitor cells. The wild-type ICSBP, but not transcriptionally defective mutants, corrected abnormal colony formation by increasing macrophage colonies and decreasing granulocyte colonies. Taken together, ICSBP plays a critical role in myeloid cell development by controlling lineage selection and is indispensable for IFN-γ-dependent modulation of progenitor cell maturation. ***The Journal of Immunology,*** **2002, 169: 1261–1269.**

Hemopoietic stem cells progressively restrict their multipotency during their development (1). It is generally held that myeloid progenitor cells derived from the pluripotent stem cells are not yet committed to a single lineage and can give rise to either the granulocyte or monocyte-macrophage lineage. The development of myeloid progenitor cells involves a complex interplay of intrinsic and extrinsic factors (2). Growth factors such as G-CSF, M-CSF, and GM-CSF are important regulators of myeloid cell development. In addition, various transcription factors are shown to play important roles in the regulation of myeloid cell development (3, 4). For example, PU.1, a transcription factor of the Ets family, is a master regulator of both the lymphoid and myeloid cell lineages (5, 6). Moreover, transcription factors of the bZip family, C/EBPα and C/EBPϵ, are both central to granulocyte development. Genetic ablation of the C/EBPα gene results in a complete block in granulocyte formation, but not monocyte formation (7). The disruption of the C/EBPϵ gene prevents functional maturation of granulocytes, but not granulocyte formation per se (8).

IFN consensus sequence binding protein (ICSBP),[5] recently renamed IFN regulatory factor-8 (IRF-8; hereafter ICSBP) is a member of IRF family that is involved in IFN-α/IFN-β-dependent transcription (9). Unlike many other IRF members, ICSBP is expressed only in hemopoietic cells and is induced by IFN-γ (10, 11). Analyses of $ICSBP^{-/-}$ mice revealed a previously unsuspected role for ICSBP in myeloid cell development; there is a striking increase in granulocytes in various lymphoid organs in −/− mice, and as a result, −/− mice develop, with 100% penetrance, a syndrome similar to chronic myelogenous leukemia (12). Furthermore, $ICSBP^{-/-}$ macrophages are functionally defective. They do not produce sufficient amounts of reactive oxygen intermediates upon microbial challenges and fail to express IL-12, making these mice highly susceptible to infections (13–18). Scheller et al. (19) provided evidence that these abnormalities are traced to a defect in myeloid progenitor cells. Also, by studying myeloid clones established from $ICSBP^{-/-}$ bone marrow, we have recently shown that ICSBP directs their differentiation into macrophages in vitro (20). Additionally, recent reports from several laboratories documenting the involvement of ICSBP in the pathogenesis of chronic myelogenous leukemia further strengthen the possible role for ICSBP in myeloid cell development (21–24).

Despite the growing evidence that ICSBP has a pivotal role in the development of myeloid progenitor cells, a critical question as to whether normal progenitor cells express ICSBP has remained unresolved. With this uncertainty there remain two possible mechanisms by which ICSBP could contribute to myeloid cell development. If ICSBP expression is absent in the progenitor cells, its role in regulating the development must be indirect, presumably mediated through regulation of the cytokine milieu. However, if ICSBP is expressed in progenitor cells, it would support a more

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Received for publication February 27, 2002. Accepted for publication May 16, 2002.

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[1] H.T. was supported by the JSPS Research Fellowship for Japanese Biomedical and Behavioral Researchers at National Institutes of Health.

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[5] Abbreviations used in this paper: ICSBP, IFN consensus sequence binding protein; CSFR, CSF receptor; GFP, green fluorescence protein; IRF, IFN regulatory factor; IAD, IRF association domain; Lin^-, lineage marker negative; SCF, stem cell factor; EGFP, enhanced GFP; SR, scavenger receptor.

0022-1767/02/$02.00

direct, intrinsic role for ICSBP in controlling myeloid cell development. Furthermore, if the latter proves to be the case, it would be possible for us to further assess the function of ICSBP by reintroducing the gene into −/− progenitor cells.

In addition to these issues we have been interested in the effect of IFN-γ on myeloid cell development. IFN-γ is an immunoregulatory cytokine produced by activated T cells and other cells in response to various stimuli (25). IFN-γ elicits vital effects on myeloid cells; it stimulates maturation of the monocyte-macrophage lineage and activates macrophage functions by enhancing various immunological activities (25). The contribution of IFN-γ to hemopoiesis, however, may be complex. Although IFN-γ is generally thought to suppress hemopoietic cell growth with an implication for aplastic anemia (26, 27), its effects on early progenitor cells appear to be mixed (28).

Here we show that ICSBP is expressed in normal bone marrow progenitor cells and that the colony-forming potential of −/− progenitor cells is severely distorted. Furthermore, although IFN-γ promoted macrophage maturation in +/+ cells, it was unable to exert these effects on −/− cells. Finally, reintroduction of an ICSBP retrovirus vector into −/− progenitor cells fully restored the colony-forming potential of +/+ cells. These results indicate that ICSBP contributes to myeloid cell development by an intrinsic mechanism.

Materials and Methods

Mice

Experiments were performed with 6- to 10-wk-old homozygous ICSBP$^{-/-}$ and ICSBP$^{+/+}$ mice on a C57BL/6 background housed under specific pathogen-free conditions. All animal work conformed to the guidelines of the animal care and use committee of National Institute of Child Health and Human Development.

Cytokines and cell cultures

Recombinant murine GM-CSF, stem cell factor (SCF), and M-CSF were purchased from BioSource International (Camarillo, CA), and G-CSF, IL-3, and IL-6 were obtained from PeproTech (Rocky Hill, NJ). Murine IFN-γ was provided by Genentech (South San Francisco, CA). Murine cells were cultured in RPMI 1640 (Quality Biologicals, Gaithersburg, MD) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD) with 20% FBS (Atlantic Biologicals, Norcross, GA). BOSC23 obtained through American Type Culture Collection (Manassas, VA) were maintained in DMEM (Quality Biologicals) containing 2 mM L-glutamine, 10% FBS, and penicillin/streptomycin (29).

Antibodies

FITC-conjugated Abs against Mac-1 (CD11b, M1/70), Gr-1 (Ly6-G), c-Kit (CD117, 2B8), PE-conjugated anti-Sca-1 (Ly-6A/E, E13-161.7), and B220 (CD45R, RA3-6B2) were purchased from BD PharMingen (San Diego, CA). Anti-F4/80 (Ly-71) Ab was obtained from Caltag Laboratories (Burlingame, CA).

Cell preparations

Lineage marker-negative (Lin$^-$) cells were prepared from bone marrow cell suspensions using the Enrichment of Murine Hemopoietic Progenitor Cells (StemCell Technologies, Vancouver, Canada). Briefly, lineage marker-positive cells were depleted with a biotinylated Ab mixture for CD5 (Ly-1), CD45R (B220), CD11b (Mac-1), Gr-1, TER 119, and 7/4, followed by immunomagnetic depletion on a StemSep column. Sca-1$^+$ or c-Kit$^+$ cells (30, 31) were purified from bone marrow cells based on the MACS system (Miltenyi Biotec, Auburn, CA) using Sca-1 MultiSort Kit and anti-FITC MicroBeads, respectively. Gr-1$^+$ granulocytes (32) and F4/80$^+$ macrophages (33) were purified from spleen cell suspensions with anti-FITC MicroBeads. Granulocytes were collected from the pellets following density gradient centrifugation of cells on Lympholyte-M (Cedarlane Laboratories, Hornby, Ontario, Canada). The yields of Lin$^-$ cells are summarized in Table I. Although the average numbers of unfractionated bone marrow cells were 2-fold greater in ICSBP$^{-/-}$ mice than in ICSBP$^{+/+}$ animals, there was no significant difference in the yield of Lin$^-$ cells between −/− and +/+ mice. B cell-enriched preparations were obtained from spleen suspensions by density gradient centrifugation on Lympholyte-M.

Table I. *Yields of Lin$^-$ cells from ICSBP$^{+/+}$ and ICSBP$^{-/-}$ mice*[a]

ICSBP	Unfractionated Bone Marrow Cells/Mouse	Yields of Lin$^-$ Cells/Mouse (%)	Purity of Lin$^-$ Cells (%)
+/+	$3.7 \pm 1.8 \times 10^7$	$3.4 \pm 1.4 \times 10^5$ (1.0 ± 0.5)	99.4 ± 0.3
−/−	$7.0 \pm 2.3 \times 10^7$	$3.2 \pm 1.4 \times 10^5$ (0.5 ± 0.2)	99.1 ± 0.2

[a] Lin$^-$ cells were purified by the two-step immunomagnetic procedure (see *Materials and Methods*). Bone marrow cells were pooled from three to six mice in each experiment. The purity of Lin$^-$ cells was confirmed by flow cytometry. Values represent the average of six experiments for ICSBP$^{+/+}$ mice and nine experiments for ICSBP$^{-/-}$ mice ± SD.

Colony forming assay

Cells were mixed in Methocult M3100 (StemCell Technologies), 30% FBS, 1% BSA (Sigma-Aldrich, St. Louis, MO), 10^{-4} M 2-ME, and the indicated cytokines. The evaluation of colony formation was performed on days 7–9. Colonies containing 50 or more cells were scored. Colonies were classified microscopically into three types (CFU-M, CFU-G, and CFU-GM) according to the size, cell distribution patterns, and morphology as previously described (34, 35). Statistical analysis was performed with Student's *t* test. Colonies placed on cytospin slides (Thermo Shandon, Pittsburgh, PA) were routinely inspected for morphological verification of colony types.

Retroviral transduction

Retrovirus vectors harboring the wild type and mutants have been previously described (20). A vector harboring ICSBP/enhanced green fluorescence protein (EGFP)-producing chimeric ICSBP fused to green fluorescence protein (GFP) at the C terminus was constructed as follows: open reading frames of ICSBP or K79E (20) with the stop codon removed were inserted into pEGFPN3 (Clontech Laboratories, Palo Alto, CA) to construct ICSBP/EGFP and K79E/EGFP, which were then transferred into pMSCV-puro (Clontech Laboratories). Transfection of these viruses was conducted as previously described (36) with some modifications (20). BOSC23 ecotropic retroviral packaging cells (29) were transiently transfected with retroviral vectors using Lipofectamine 2000 (Life Technologies). Retroviral supernatants were collected at 48 h. Virus titers measured on 32D cells were $>1 \times 10^6$ infectious particles/ml. Before viral transduction, Lin$^-$ bone marrow cells (5×10^5/ml) were incubated in 24-well plates for 1 day in the presence of 6 ng/ml IL-3, 10 ng/ml IL-6, and 50 ng/ml SCF. Cells were transduced by two spinoculations (first, 2500 rpm for 1 h at 33°C; second, 2500 rpm for 45 min at 33°C) in a retrovirus supernatant supplemented with 4 μg/ml polybrene for 2 days. Twenty-four hours after spinoculation, cells were washed twice and plated in methylcellulose medium or cultured in suspension. Transduced cells were selected by 0.5 μg/ml puromycin, which produced no colonies from nontransduced cells, or were identified by the expressing of GFP.

Flow cytometric analysis

For detection of surface markers, cells were blocked with anti-mouse FcγR Ab (CD16/CD32. 2.4G2; BD PharMingen), followed by incubation with either specific Abs or isotype controls at 4°C for 30 min. Flow cytometry was performed on FACSCalibur, and data were analyzed using the FlowJo (Tree Star) or CellQuest software (BD Biosciences, Mountain View, CA).

Semiquantitative RT-PCR

This was performed for ICSBP, β-actin, the M-CSF receptor gene (c-*fms*), scavenger receptor (SR), FcγRI, the G-CSF receptor gene (G-CSFR), C/EBPα, C/EBPε, and PU.1 transcripts as previously described (20). Fifty nanograms of cDNA was used for reaction of SR, FcγRI, C/EBPα, C/EBPε, and PU.1; 5 ng cDNA was used for ICSBP, c-*fms*, and G-CSFR; and 1 ng cDNA was used for β-actin. Reactions were performed at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 45 s for 30 cycles in the presence of 0.2 mM dNTPs, 2.5 μM $MgCl_2$, and 500 nM 5′ and 3′ primers. RT-PCR products were quantified by NIH Image software and were normalized by β-actin.

Results

Expression of ICSBP in bone marrow progenitor cells

Growing evidence indicates that ICSBP plays a role in myeloid cell development (12, 19, 20). However, it has not been clear whether ICSBP is indeed expressed in hemopoietic progenitor cells. To study this question, Lin^- cells were isolated from $ICSBP^{+/+}$ bone marrow and tested for ICSBP transcript expression by semiquantitative RT-PCR. Lin^- cells contain both primitive and mature progenitors that can differentiate into cells of every hemopoietic lineage, including the myeloid lineage (30, 37, 38). As shown in Fig. 1*A*, ICSBP transcripts were detected in Lin^- progenitor cells. Levels of the transcripts in the progenitor cells were about half those in splenic macrophages and B cell-enriched splenocytes, both known to express high levels of ICSBP (10). The transcript levels in the progenitor cells were much higher than those in splenic granulocytes. Interestingly, ICSBP expression in the progenitor cells was increased by ~2.5-fold upon IFN-γ treatment, similar to other cells (Fig. 1*A*). To further confirm ICSBP expression in progenitor cells, Sca-1^+ cells and c-Kit^+ cells were isolated separately and tested for the transcripts. As depicted in Fig. 1*B*, the transcripts were expressed in these cells at similar levels as Lin^- cells. These results indicate that ICSBP is expressed in hemopoietic progenitor cells, and the expression is augmented by IFN-γ.

Abnormal increase in granulocyte colony formation and the lack of IFN-γ inhibition in $ICSBP^{-/-}$ Lin^- progenitor cells

To assess whether +/+ and −/− progenitors can properly give rise to the cells of the macrophage and granulocyte lineages, methylcellulose-based colony forming assays were performed with Lin^- cells. Lin^- progenitor cells rather than total bone marrow cells (19) were chosen for this test, because we wanted to gain accurate information on the developmental potential of immature cells. The recovery of Lin^- cell is summarized in Table I, which indicated that $ICSBP^{+/+}$ and $ICSBP^{-/-}$ mice had a similar number of progenitor cells in bone marrow, enabling us to compare their colony-forming potential. Lin^- cells were allowed to form colonies in the presence of SCF (39) plus three separate growth factors, M-CSF, GM-CSF, and G-CSF, for 7–9 days. Colonies were classified into three distinct types, granulocyte, macrophage, and granulocyte/macrophage colonies according to the standard morphological criteria (34). As shown in Fig. 2, several features of colony formation were strikingly different between +/+ and −/− cells. First, the number of total colonies produced from $ICSBP^{-/-}$ cells was greater than that from $ICSBP^{+/+}$ cells in all three growth factors. In the presence of M-CSF, $ICSBP^{-/-}$ cells generated 60% more colonies than $ICSBP^{+/+}$ cells. The difference was less pronounced in the presence of G-CSF and GM-CSF. Second, $ICSBP^{-/-}$ cells gave rise to a disproportionately large number of granulocyte colonies relative to $ICSBP^{+/+}$ cells in all three growth factor conditions tested. Conversely, macrophage colonies produced from −/− progenitor were many fewer than those from +/+ cells. This was most noticeable in the presence of M-CSF, where macrophage colonies were <5% of the total −/− colonies, but >30% of the +/+ colonies were macrophages (Table II).

We also sought to determine the effects of IFN-γ on colony formation, since this cytokine enhanced ICSBP expression in Lin^-

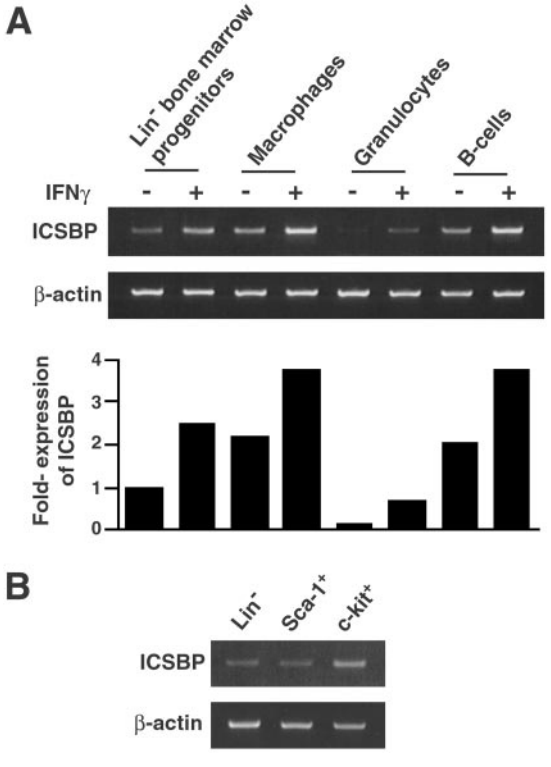

FIGURE 1. Differential expression of ICSBP in hemopoietic cells. *A*, Lin^- progenitor cells, macrophages ($F4/80^+$), granulocytes (Gr-1^+), and B cell-enriched splenocytes were prepared as described in *Materials and Methods*, with purities of 98, 85, 93, and 83%, respectively. Semiquantitative RT-PCR was performed with fresh samples and those treated with IFN-γ (100 U/ml) for 6 h. The *lower panel* represents relative ICSBP transcript levels normalized by β-actin transcripts. *B*, Semiquantitative RT-PCR was performed for ICSBP with Sca-1^+ and c-Kit^+ cells.

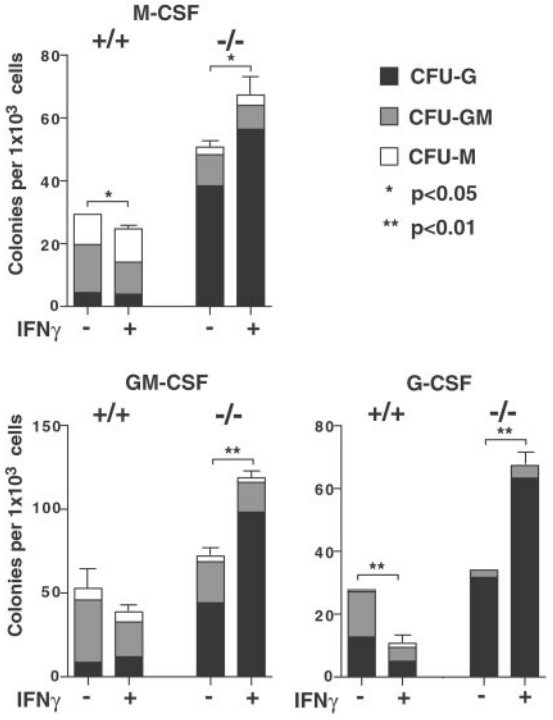

FIGURE 2. Colony-forming ability of $ICSBP^{+/+}$ and $ICSBP^{-/-}$ progenitor cells. Lin^- cells were cultured in the methylcellulose medium in the presence of M-CSF (10 ng/ml) or G-CSF (10 ng/ml) at 1×10^3 cells/ml or in the presence of GM-CSF (10 ng/ml) at 5×10^2 cells/ml for 7 days. IFN-γ was added at 100 U/ml at the initiation of culture. Colonies were classified into granulocyte (CFU-G), granulocyte/macrophage (CFU-GM), and macrophage (CFU-M) colonies according to the standard morphological criteria. Values are the mean ± SD of triplicate cultures. * and **, Statistically significant difference in colony number between IFN-γ-treated and untreated cells.

Table II. *Formation of myeloid colonies from ICSBP$^{+/+}$ and ICSBP$^{-/-}$ progenitors*[a]

Cytokine	ICSBP$^{+/+}$ (%)			ICSBP$^{-/-}$ (%)		
	M	GM	G	M	GM	G
M-CSF	9.6 ± 1.8 (33.4)	15.3 ± 2.8 (53.3)	4.4 ± 0.6 (15.3)	2.4 ± 1.0[b] (4.7)	9.9 ± 2.2 (19.6)	38.4 ± 2.8[b] (75.7)
M-CSF + IFN-γ	10.6 ± 1.3 (42.9)	10.2 ± 1.6[c] (41.4)	3.9 ± 0.8 (15.7)	3.3 ± 2.2[b] (4.9)	7.6 ± 1.6 (11.3)	56.4 ± 3.2[b,d] (83.8)
GM-CSF	6.9 ± 4.0 (13.1)	37.2 ± 5.3 (70.5)	8.6 ± 4.3 (16.4)	3.4 ± 1.9 (4.7)	24.5 ± 2.5[e] (34.0)	44.1 ± 1.5[b] (61.3)
GM-CSF + IFN-γ	6.0 ± 1.2 (15.4)	20.8 ± 2.1[d] (53.8)	11.9 ± 1.5 (30.8)	2.7 ± 1.7 (2.3)	17.8 ± 4.1 (15.0)	98.2 ± 7.9[b,d] (82.7)
G-CSF	0.6 ± 0.1 (2.2)	14.4 ± 1.2 (52.1)	12.7 ± 0.9 (45.7)	0 (0)	2.3 ± 0.7[b] (6.9)	31.7 ± 1.0[b] (93.1)
G-CSF + IFN-γ	1.3 ± 0.2[d] (12.2)	4.4 ± 0.3[d] (41.5)	5.0 ± 0.3[d] (46.3)	0 (0)	4.0 ± 2.8 (5.9)	63.3 ± 2.8[b,d] (94.1)

[a] Colonies were classified into M (macrophage colonies), GM (granulocyte-macrophage colonies), or G (granulocyte colonies) as described previously (38, 39). The number of colony types (per 1×10^3 Lin$^-$ bone marrow cells) was determined from the experiments in Fig. 2 and represents the mean of triplicate samples ± SD.
[b] $p < 0.01$, ICSBP$^{+/+}$ vs ICSBP$^{-/-}$ cells.
[c] $p < 0.05$, IFN-γ-treated vs untreated samples.
[d] $p < 0.01$, IFN-γ-treated vs untreated samples.
[e] $p < 0.05$, ICSBP$^{+/+}$ vs ICSBP$^{-/-}$ cells.

progenitor cells (Fig. 1*A*). IFN-γ has been shown to inhibit bone marrow stem cell growth and colony-forming ability (26, 27). As shown in Fig. 2, IFN-γ treatment caused markedly different effects on +/+ and −/− cells. IFN-γ inhibited colony formation in +/+ cells in the presence of M-CSF and G-CSF. This inhibition was mostly due to the inhibition of GM colonies and, to a lesser degree, that of granulocyte colonies (see Table II also). In contrast, colony formation was not inhibited in −/− cells by IFN-γ. Rather, IFN-γ markedly increased the total number of colonies from −/− cells, resulting in at least twice as many colonies as those from +/+ colonies. This was due to a large increase in the number of granulocyte colonies formed from −/− cells after IFN-γ addition. Granulocyte colonies formed in the presence of IFN-γ were, in general, smaller and more tightly aggregated than those formed in its absence both in +/+ and −/− cells. These results indicate that the colony-forming potential of −/− progenitor cells is markedly different from that of the normal progenitor cells, and that in the absence of ICSBP, IFN-γ fails to inhibit colony formation.

Impaired macrophage development in ICSBP$^{-/-}$ progenitors

It was remarkable that ICSBP$^{-/-}$ progenitor cells gave rise to few macrophage colonies (<5% of total colonies) even in the presence of M-CSF, where macrophage colonies generated by +/+ progenitors were >33% of all colonies (Table II). To further investigate progenitor differentiation in the presence of M-CSF, cells were cultured under the same conditions as described above, but in suspension without methylcellulose for 7 days. Within a few days following culture, many +/+ cells adhered to plastic plates, and the adherence was intensified by IFN-γ. In contrast, few ICSBP$^{-/-}$ cells adhered to plastic, even in the presence of IFN-γ. Wright-Giemsa staining of cytospin slides shown in Fig. 3*A* revealed that following 7 days of culture, ~60% of ICSBP$^{+/+}$ cells were macrophages in morphology, while the majority of ICSBP$^{-/-}$ cells were typical granulocytes. IFN-γ facilitated morphological maturation of macrophages in +/+ cells, but not in −/− cells. Upon IFN-γ treatment, −/− cells transformed into large cells with abnormal morphology. Flow cytometric analysis of day 7 cells shown in Fig. 3*B* indicate that ~50% of +/+ cells expressed F4/80, a marker specific for macrophages (33). In the presence of IFN-γ, F4/80-positive cells were increased to 75%. In contrast, only 5% of −/− cells expressed F4/80, and this was at a low level. Even in the presence of IFN-γ, F4/80-positive cells were <15% in the −/− cell culture. In addition, we tested the expression of Mac-1, a myeloid cell marker normally expressed at higher levels in macrophages than granulocytes (40). Most +/+ cells expressed high levels of Mac-1, but the levels in −/− cells were, on the average, ~3- to 4-fold lower than those in +/+ cells. These results are consistent with the data in Fig. 2 and indicate that ICSBP$^{-/-}$ progenitor cells tend to generate more granulocytes and fewer macrophages than +/+ cells in the presence of M-CSF.

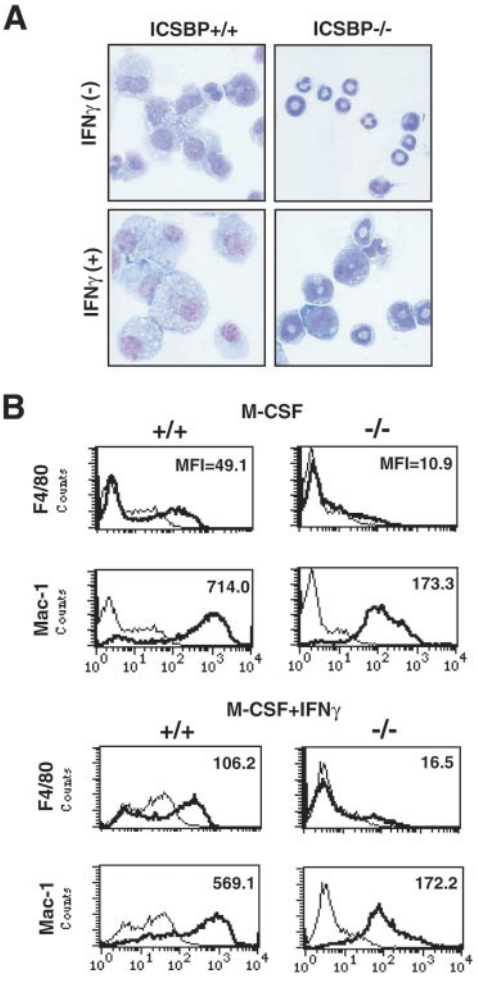

FIGURE 3. Morphology and cell surface markers. Lin$^-$ cells were cultured in suspension (5×10^4 cells/ml) in the presence of M-CSF (10 ng/ml) with or without IFN-γ (100 U/ml) for 7 days. *A*, Wright-Giemsa staining. *B*, Flow cytometric analysis of F4/80 and Mac-1 expression. The thick lines represent fluorescent signals by specific Ab, and thin lines represent background fluorescence. The numbers in each panel indicate the mean fluorescence intensity (MFI).

Myeloid-specific gene expression in Lin$^-$ progenitor cells

To assess gene expression patterns in +/+ and −/− progenitor cells developing in the presence of M-CSF, semiquantitative RT-PCR was conducted for the FcγRI and SR genes that are specifically expressed in macrophages (41, 42). In addition, the G-CSFR gene; the C/EBPα and C/EBPε genes, encoding transcription factors involved in granulocytic differentiation (7, 8, 43); and c-*fms*, known to stimulate the growth and differentiation of monocyte-macrophages (44) were tested. The expression of PU.1, a transcription factor critical for myeloid cell development that interacts with ICSBP (4, 6, 22, 45, 46), was also examined. The results are shown in Fig. 4*A*, where expression levels of each transcript are compared with those in ICSBP$^{+/+}$ cells on day 0. Expression of c-*fms* was detected in −/− cells at levels slightly higher than or comparable to those in +/+ cells throughout the culture period (see *Discussion*). G-CSFR transcripts were expressed in ICSBP$^{-/-}$ progenitor cells at higher levels than in +/+ cells on days 0 and 2, although the levels became comparable to those in +/+ cells upon subsequent periods of culture. Expression patterns for C/EBPα, C/EBPε, and PU.1 transcripts were similar, in that they were expressed at higher levels in −/− cells than in +/+ cells in early stages, but the levels became comparable in later stages. Fig. 4*B* shows RT-PCR analysis of macrophage-specific genes, FcγRI and SR, tested in the presence or the absence of IFN-γ. FcγRI was expressed constitutively in +/+ cells on day 7, and the expression levels were augmented by IFN-γ. However, in −/− cells this transcript was not expressed constitutively and was only induced following IFN-γ treatment. SR transcripts were constitutively expressed on day 2 in +/+ cells, but not in −/− cells, and were only weakly expressed on day 7. These results indicate that the expression of genes linked to granulocyte development is higher in −/− cells than in +/+ cells, and conversely, the expression of genes linked to macrophage development is lower in −/− cells.

ICSBP retrovirus transduction rescues macrophage colony formation in −/− progenitors

To assess whether reintroduction of ICSBP reverses the distorted colony-forming potential of −/− cells, ICSBP$^{-/-}$ Lin$^-$ cells were transduced with ICSBP retroviruses and tested for colony-forming ability. The wild-type ICSBP and three mutant ICSBP constructs were tested (Fig. 5*A* for a diagram of mutants) (20). ICSBP binds two target DNA elements: the ISRE, a target sequence of all IRF family proteins, and the EICE, a composite element to which ICSBP binds by interacting with PU.1 (45). Binding of ICSBP to both elements is dependent on the intact DNA binding domain in

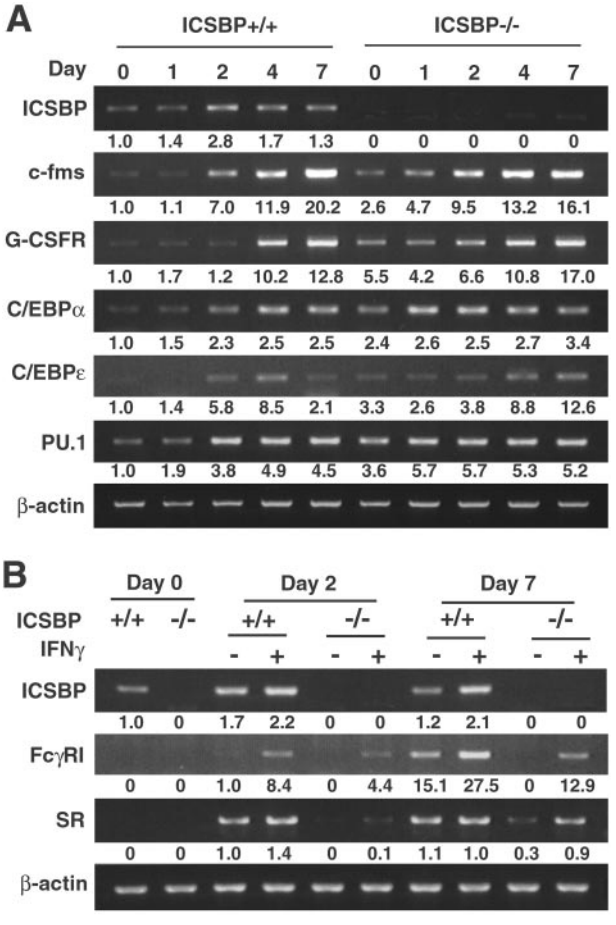

FIGURE 4. Expression of myeloid-specific genes. *A*, Lin$^-$ cells were cultured in suspension in the presence of M-CSF (10 ng/ml), with or without IFN-γ (100 U/ml). *B*, Semiquantitative RT-PCR was performed on each gene on the indicated days. Transcript levels were quantified by normalizing by β-actin levels and are expressed relative to transcript levels of ICSBP$^{+/+}$ cells on day 0.

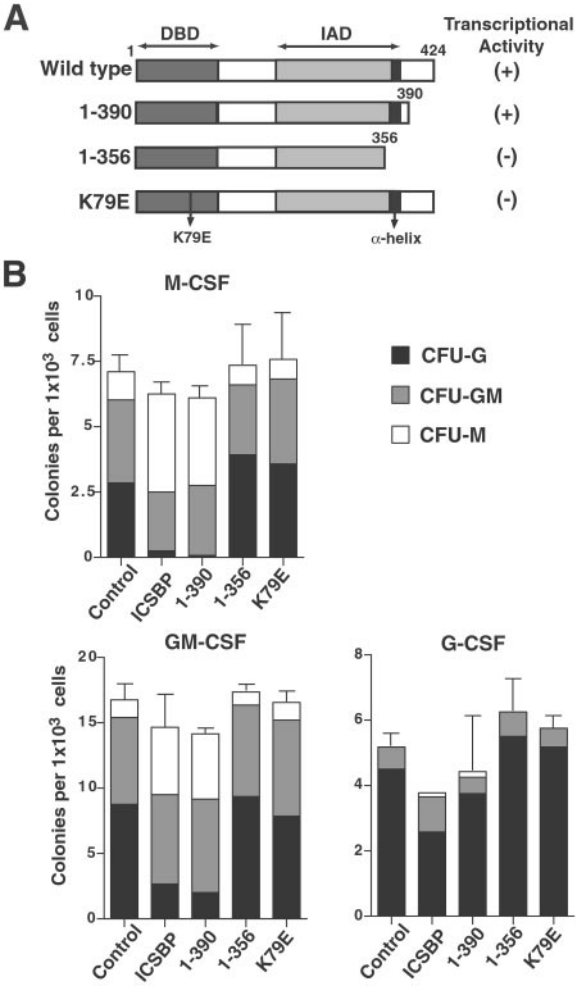

FIGURE 5. Colony-forming ability of ICSBP retrovirus-transduced −/− progenitors. *A*, Diagram of wild-type and mutant ICSBP. *B*, ICSBP$^{-/-}$ Lin$^-$ cells were transduced with the indicated retroviruses. Cells were cultured in methylcellulose medium in the presence of puromycin and the indicated growth factors (10 ng/ml each) as described in Fig. 2. Values are the mean ± SD of triplicate cultures.

the N terminus (DBD in Fig. 5*A*) and the IRF association domain (IAD) in the C-terminal region (20, 45). Mutant 1–390 is a truncation lacking the C-terminal region, but retaining the IAD (45). This mutant is capable of binding to the both elements and is still functional (20). Mutant 1–356, on the other hand, lacks a portion of the IAD, does not bind to either element, and is without functional activity. K79E, carrying a point mutation in the DNA binding domain, similarly lacks DNA binding as well as transcriptional activities (20). Whereas untransduced cells produced no colonies in the presence of puromycin, cells transduced with retroviruses all produced many colonies. The efficiency of viral transduction was judged to be high, since the number of colonies produced in the presence of puromycin ranged from 73 to 98% of the colonies generated in its absence (not shown). Fig. 5*B* and Table III show the results of colony forming assays performed with virus-transduced cells in the presence of M-CSF, G-CSF, and GM-CSF. Cells transduced with the control vector gave rise to a disproportionately large number of granulocyte colonies and few macrophage colonies, similar to untransduced cells in Fig. 2. In contrast, cells transduced with the wild-type ICSBP yielded a dramatically higher number of macrophage colonies, concomitant with many fewer granulocyte colonies. The changes were especially evident with colony formation in M-CSF and GM-CSF where the number of macrophage colonies was increased by ~4-fold, while that of granulocyte colonies was decreased by 4- to 10-fold upon ICSBP transduction. Even in the presence of G-CSF, ICSBP transduction led to an ~45% reduction in granulocyte colonies. In all cultures the total number of colonies was reduced in ICSBP-transduced cells relative to that in cells transduced by the control vector. Among the mutants, only 1–390 rescued macrophage colony formation. Other ICSBP mutants, 1–356 and K79E lacking DNA binding activity and inactive in transcription, did not rescue macrophage colony formation, did not inhibit granulocyte colony formation, and did not reduce total colony numbers. Taken together, simple reintroduction of ICSBP corrected the distorted colony-forming potential of −/− cells, indicating that ICSBP has an intrinsic role in lineage selection during myeloid cell development. These results also indicate that the absence of ICSBP does not irreversibly fix the developmental fate of progenitor cells.

Morphology, cell surface markers, and gene expression in ICSBP-transduced cells

The morphology and surface makers were examined for ICSBP-transduced cells cultured in suspension for 7 days with M-CSF. As shown in Fig. 6*A*, cells transduced with the control vector differentiated mainly into granulocytes, similar to untransduced cells. In contrast, ~60% of ICSBP-transduced cells differentiated into macrophages, accompanied by a reduction in granulocytes. Consistent with the colony forming assay data in Fig. 5, cells transduced with the mutant 1–390 differentiated into mostly macrophages. On the other hand, mutants 1–356 and K79E produced mainly granulocytes, similar to the control vector. The results of flow cytometric analysis shown in Fig. 6*B* confirmed the morphological findings. While F4/80 expression remained negative in most cells transduced with the control vector, ~55% of ICSBP-transduced cells expressed the marker at high levels. Similarly, the expression of Mac-1, which was low in control cells, was increased in ICSBP-transduced cells. Levels of F4/80 and Mac-1 expressed in the cells transduced with mutant 1–390 were similar to those transduced by the wild-type ICSBP. In contrast, there was no increase in the expression of these markers when cells were transduced with mutants 1–356 or K79E. The results of semiquantitative RT-PCR performed with cells transduced with ICSBP and cultured as described above are shown in Fig. 6*C*. Macrophage-specific genes, FcγRI and SR, were both markedly induced in cells transduced with ICSBP, but not control vector. The expression of c-*fms* was, however, not significantly altered upon ICSBP transduction. Conversely, transcripts for C/EBPϵ and G-CSFR were reduced in ICSBP-transduced cells. The patterns seen by the mutant 1–390 was similar to those of the wild-type ICSBP, whereas mutants 1–365 and K79E displayed patterns similar to those of untransduced cells. These results indicate that ICSBP stimulates genes expressed along the macrophage lineage and represses those along the granulocyte lineage.

It was important to verify that ICSBP protein was expressed in retrovirus-transduced cells. To this end we constructed retrovirus vectors in which ICSBP was fused to GFP at the C terminus. The fusion of the GFP moiety to ICSBP at the C terminus was shown

Table III. *Formation of myeloid colonies from retrovirus-transduced ICSBP$^{-/-}$ progenitor cells*[a]

Cytokine	Retrovirus	No. of Colonies (%)		
		M	GM	G
M-CSF	Control	1.1 ± 0.3 (15.5)	3.2 ± 0.6 (45.1)	2.8 ± 0.8 (39.4)
	ICSBP	3.8 ± 0.7 (59.4)[b]	2.3 ± 0.9 (35.9)	0.3 ± 0.3 (4.7)[b]
	1–390	3.4 ± 0.3 (54.8)[b]	2.7 ± 0.1 (43.5)	0.1 ± 0.1 (1.6)[b]
	1–356	0.8 ± 0.3 (10.8)	2.7 ± 1.0 (36.5)	3.9 ± 1.2 (52.7)
	K79E	0.8 ± 0.5 (10.4)	3.3 ± 0.9 (42.9)	3.6 ± 1.3 (46.8)
GM-CSF	Control	1.3 ± 0.3 (7.8)	6.7 ± 0.3 (40.1)	8.7 ± 0.8 (52.1)
	ICSBP	5.2 ± 1.4 (35.4)[b]	6.8 ± 1.0 (46.3)	2.7 ± 0.8 (18.4)[b]
	1–390	5.0 ± 1.7 (37.0)[c]	7.2 ± 1.0 (53.3)	1.3 ± 2.0 (9.6)[b]
	1–356	1.0 ± 0.5 (5.8)	7.0 ± 0.1 (40.4)	9.3 ± 1.0 (53.8)
	K79E	1.3 ± 0.3 (7.9)	7.3 ± 2.0 (44.5)	7.8 ± 1.3 (47.6)
G-CSF	Control	0 (0)	0.7 ± 0.3 (13.5)	4.5 ± 0.9 (86.5)
	ICSBP	0.1 ± 0.1 (2.6)	1.1 ± 0.5 (28.9)	2.6 ± 0.6 (68.4)[c]
	1–390	0.2 ± 0.1 (4.4)	0.5 ± 0.3 (11.1)	3.8 ± 1.6 (84.4)
	1–356	0 (0)	0.8 ± 0.3 (12.7)	5.5 ± 0.8 (87.3)
	K79E	0 (0)	0.6 ± 0.8 (10.3)	5.2 ± 0.4 (89.7)

[a] Colonies were classified as described in Table II. Values represent the mean of triplicate cultures ± SD.
[b] $p < 0.01$ vs control retrovirus.
[c] $p < 0.05$ vs control retrovirus.

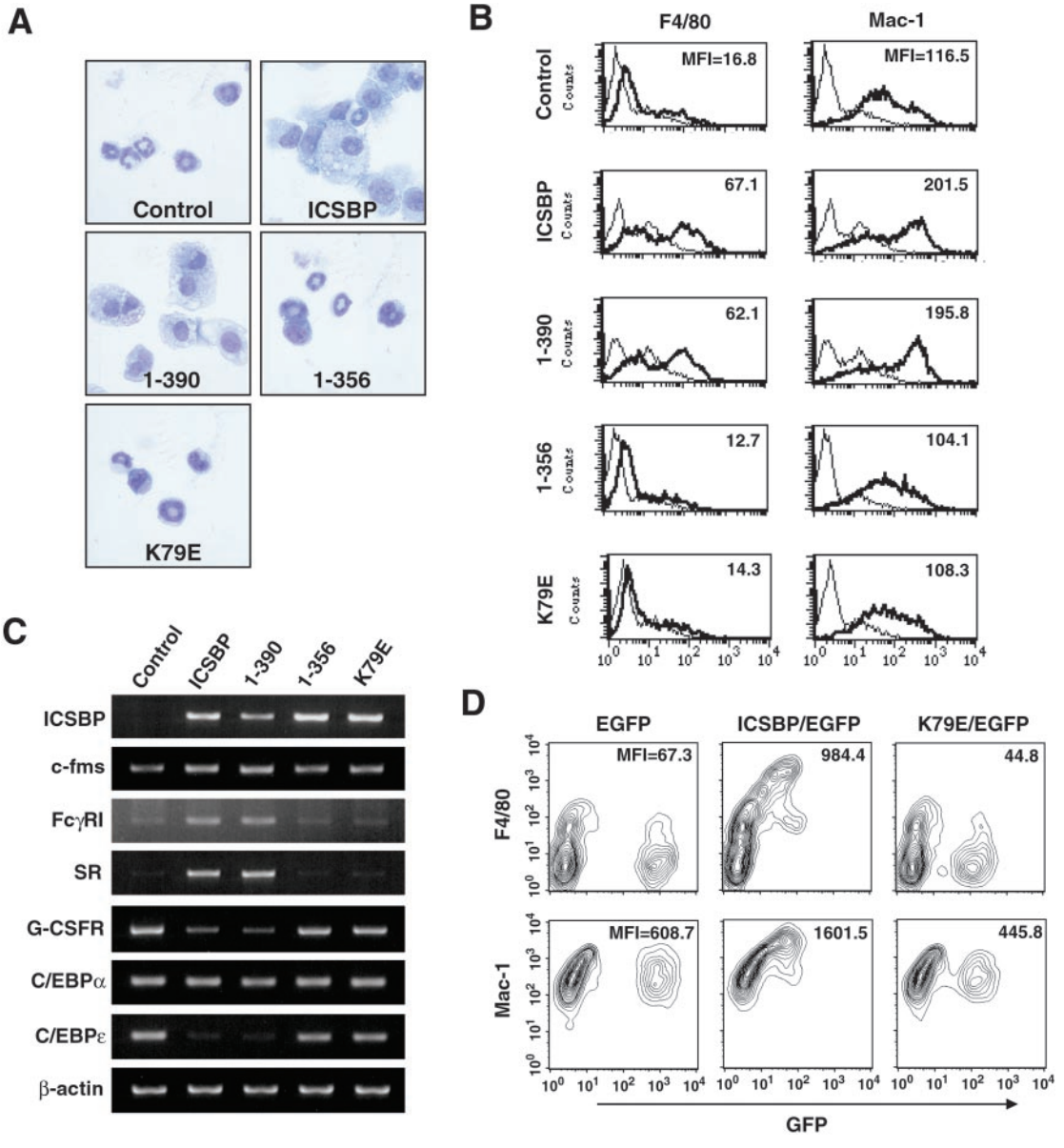

FIGURE 6. Morphology, surface markers, and gene expression of ICSBP-transduced cells. Retrovirus-transduced cells were cultured in suspension in the presence of M-CSF (10 ng/ml) for 7 days. *A*, Wright-Giemsa staining. *B*, Flow cytometric detection of F4/80 and Mac-1. Thick lines represent specific staining, while thin lines represent background staining. The numbers in each panel denote the mean fluorescent intensity (MFI). *C*, Semiquantitative RT-PCR analysis of indicated genes. *D*, Lin^- cells were transduced with the indicated retrovirus vectors harboring EGFP fusions. The expression of ICSBP/GFP fusion protein and F4/80 or Mac-1 was visualized by two-color flow cytometry. The numbers indicate the MFI of GFP^+ cells for F4/80 or Mac-1 in each culture.

to retain DNA binding activity and the ability to induce macrophage differentiation in a progenitor cell line (T. Tamura and K. Ozato, unpublished observation). The use of GFP fusions enabled us to visualize the expression of protein products in transduced cells. We tested two retrovirus vectors, the wild-type ICSBP (ICSBP/EGFP) and K79E/EGFP, the mutant ICSBP that failed to rescue macrophage colony formation in Fig. 5. $ICSBP^{-/-}$ Lin^- cells were transduced with these retroviruses and cultured for 7 days in the presence of M-CSF as described above. Flow cytometric analysis was performed to detect GFP signals and macrophage markers F4/80 and Mac-1. In these assays cells transduced with retroviruses were mixed with untransduced cells, providing an internal negative control. As shown in Fig. 6*D* (*left panel*), cells transduced with the vector for GFP only could be distinguished from untransduced cells, since the former exhibited positive GFP signals, while the latter did not. Both populations showed low F4/80 and Mac-1 expression, indicating that few macrophages were generated when transduced with GFP only. In contrast, when cells were transduced with ICSBP/EGFP vector (Fig. 6*D*, *middle panel*), the cell population that exhibited positive GFP signals, hence ICSBP, exhibited high levels of F4/80 and Mac1, much higher than those seen by cells with GFP only. On the other hand, cells transduced with the mutant K79E/EGFP, although exhibiting GFP signals at levels comparable with or greater than those of the wild-type ICSBP/EGFP, did not show a significant increase in the expression of F4/80 or Mac-1 (Fig. 6*D*, *right panel*). These results show that the ICSBP-GFP fusion protein is expressed in the progenitor cells upon viral transduction, and that only the wild-type ICSBP, but not the K79E mutant, enhances F4/80 and Mac-1 expression and stimulates macrophage development.

Discussion

The present study began with the observation that ICSBP was expressed in Lin^- progenitor cells as well as Sca-1^+ and c-Kit^+ cells and was induced by IFN-γ. In line with our findings, a recent report with purified stem cells indicates that ICSBP expression begins early in hemopoiesis (46). These results pointed to the possibility that ICSBP has a direct, intrinsic role in the regulation of myeloid cell development, rather than acting through an indirect mechanism such as regulation of cytokine environment.

Impaired lineage commitment in ICSBP$^{-/-}$ progenitor cells

The absence of ICSBP severely distorted the colony-forming potential of Lin^- cells, as evidenced by the disproportionately high number of granulocyte colonies generated from −/− progenitor cells, coinciding with a marked decrease in macrophage colonies. The distorted colony formation was seen in the presence of all three growth factors tested, although the reduction in macrophage colony formation was most pronounced in the presence of M-CSF. Thus, $ICSBP^{-/-}$ progenitor cells are deficient in the development of the macrophage lineage. This deficiency is apparently compensated by the increase in the granulocyte colony formation. Consistent with the impaired commitment to the macrophage lineage, cells developed from −/− progenitor cells in the presence of M-CSF expressed much lower levels of SR and FcγRI and only later in culture. Conversely, genes linked to granulocyte differentiation, C/EBPα and C/EBPε, were expressed at higher levels in −/− cells, indicating that the loss of ICSBP leads to preferential stimulation of granulocyte lineage and genes associated with the lineage. The expression of c-*fms*, encoding the receptor for M-CSF in $ICSBP^{-/-}$ cells was comparable to or higher than that in +/+ cells throughout the culture period, despite the fact that −/− cells were deficient in forming macrophage colonies in response to M-CSF. These results may have been unexpected, in light of the fact that c-*fms* confers responsiveness to M-CSF and, as such, stimulates the growth and maturation of cells in the monocyte-macrophage lineage (44). These results may imply that ICSBP does not directly control c-*fms* expression, a possibility consistent with the observation that retrovirus transduction of ICSBP did not significantly change c-*fms* expression in −/− cells (Fig. 6*C*). It is possible that ICSBP regulates a signaling event associated with the binding of M-CSF to the receptor or may affect a pathway downstream from ligand-receptor binding. Alternatively, however, it is possible that c-*fms* expression varies among different bone marrow cells, and ICSBP may contribute to the regulation of c-*fms* expression in some, but not all, cells. Supporting this possibility, Scheller et al. (19) reported that cells expressing M-CSFR were fewer in −/− than +/+ bone marrow and that −/− cells are hyporesponsive to M-CSF.

Requirement of ICSBP for IFN-γ-mediated promotion of macrophage development

IFN-γ elicited noticeable effects on colony formation by $ICSBP^{+/+}$ cells: it led to a reduction in total colony numbers, facilitated macrophage maturation, and stimulated the expression of SR and FcγRI genes. These effects suggested that IFN-γ can significantly alter the course of myeloid cell development by preferentially reinforcing the development of macrophage lineage. Our data on IFN-γ's effects on +/+ cells are in line with previous studies showing that IFN-γ stimulates macrophage differentiation in normal bone marrow cells (47) and in established myeloid cell lines (48). In addition, IFN-γ has been shown to inhibit the growth of bone marrow stem cells in various culture conditions (27). On the other hand, regulation of hemopoietic cell growth by IFN-γ appears to be complex and may be dependent on the developmental stages of cells, since other groups found that IFN-γ inhibits colony growth by normal bone marrow cells, but not by 5-fluorouracil-treated cells (28). Strikingly, the above-described effects of IFN-γ were completely absent in $ICSBP^{-/-}$ cells; instead of inhibiting colony formation, IFN-γ markedly increased the total numbers of colonies derived from −/− progenitors, which was attributed primarily to a large increase in the number of granulocyte colonies. Moreover, IFN-γ did not promote macrophage maturation in −/− cells, as judged by morphology and the lack of F4/80 induction. Furthermore, IFN-γ only weakly stimulated SR and FcγRI genes in −/− cells. Our data indicate that ICSBP is indispensable for the elicitation of the effects of IFN-γ on progenitor cells.

Intrinsic role of ICSBP

The distorted colony formation seen by −/− progenitor cells was fully corrected upon ICSBP-retrovirus transduction, resulting in a marked increase in macrophage colony formation and a sharp decrease in granulocyte colony formation. This was associated with the normalization of total colony numbers and the ratio of macrophage to granulocyte colonies and with the restoration of cellular morphology and gene expression patterns. Importantly, these changes were seen only with the wild-type ICSBP and mutant 1–390 that retained DNA binding and transcriptional activities. Other mutants inactive in these activities were unable to correct any aspects of abnormal colony formation associated with the absence of ICSBP. In the present study the expression of ICSBP protein in virus-transduced cells was verified by employing ICSBP-GFP fusions (Fig. 6*D*). The visualization of ICSBP-GFP allowed us to exclude the remote possibility that the rescue was due to an indirect mechanism, rather than to ICSBP protein expression. Taken together, these results provide compelling evidence that ICSBP intrinsically regulates the lineage fate of progenitor cells, presumably by regulating the transcription of various target genes.

It is interesting to note that the formation of granulocyte colonies and that of macrophage colonies was reciprocal in many criteria examined. Namely, when the former was increased, the latter was decreased and vice versa in our system. This reciprocity suggests that the macrophage and granulocyte lineages are inseparably coupled, and the development of one lineage is associated with the repression of the other. Supporting the coupling of two lineages, we previously observed that ectopic ICSBP expression stimulates macrophage differentiation, but inhibits granulocyte differentiation (20). Radmoska et al. (43) demonstrated another example for a possible lineage coupling by showing that ectopic expression of C/EBPα

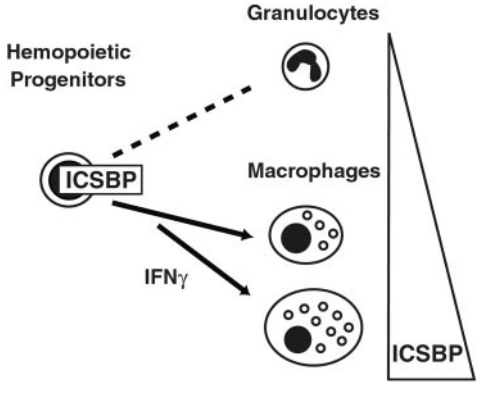

FIGURE 7. A model for the role of ICSBP. ICSBP is expressed in hemopoietic progenitor cells and promotes development along the macrophage lineage. Low ICSBP expression is associated with development of the granulocyte lineage. IFN-γ enhances ICSBP expression in progenitor cells and reinforces development of the macrophage lineage.

promoted granulocytic differentiation, while it blocked monocyte differentiation in U937 cells. In this context it is interesting to note that the expression of C/EBPα and C/EBPϵ is high in granulocytes and low in macrophages. Conversely, ICSBP expression is high in macrophages and very low in granulocytes (see Fig. 1*A*). These data suggest that expression levels of key transcription factors, such as C/EBPα and ICSBP, could influence the process of lineage selection and strengthen the idea that increased ICSBP expression favors the selection of macrophage lineage (see Fig. 7 for model).

In conclusion, ICSBP is active in myeloid progenitor cells and promotes their commitment along the macrophage lineage. IFN-γ reinforces this process by enhancing ICSBP expression.

Acknowledgments

We are grateful to Tanya L. Verney for superb technical support and Drs. L. Laricchia and A. Masumi for help in the construction of ICSBP/EGFP retroviruses and for reagents.

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