ICSBP/IRF-8 retrovirus transduction rescues dendritic cell development in vitro

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Dendritic cells (DCs) develop from bone marrow (BM) progenitor cells and mature in response to external signals to elicit functions important for innate and adaptive immunity. Interferon consensus sequence binding protein (ICSBP; also called interferon regulatory factor 8 [IRF-8]) is a hematopoietic cell–specific transcription factor expressed in BM progenitor cells that contribute to myeloid cell development. In light of our earlier observation that ICSBP−/− mice lack CD8α−DCs, we investigated the role of ICSBP in DC development in vitro in the presence of IL-13 ligand. Immature ICSBP+/-DCs developed from BM progenitor cells showed assorted defects, did not mature in response to activation signals, and failed to express CD8α and interleukin 12 (IL-12) p40, a feature consistent with ICSBP−/−DCs in vivo. We show that retroviral introduction of ICSBP restores the development of immature DCs that can fully mature on activation signals. All the defects seen with ICSBP−/−DCs were corrected after ICSBP transduction, including the expression of CD8α and IL-12 p40 as well as major histocompatibility complex class II and other costimulatory molecules. ICSBP is known to regulate gene expression by interacting with partner proteins PU.1 and IRFs, thereby binding to target elements ISRE and EICE. Analysis of a series of ICSBP mutants showed that the intact DNA-binding activity as well as the ability to interact with partner proteins are required for the restoration of DC development/maturation, pointing to the transcriptional function of ICSBP as a basis of restoration. Taken together, this study identifies ICSBP as a factor critical for both early differentiation and final maturation of DCs. (Blood. 2003; 101:961-969)

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Introduction

Dendritic cells (DCs) develop from bone marrow (BM) progenitor cells and mature in response to external signals.1,2 Previous efforts to dissect DC heterogeneity led to the classification of murine DCs into the CD8α+ and CD8α− subsets, which are associated with distinct functions. CD8α+ cells are proficient in interleukin 12 (IL-12) production and promote Th1 responses, whereas CD8α− cells are less efficient in IL-12 production, facilitating Th2 responses in some cases.3,4 It has been shown that CD8α is not a marker that defines the developmental origin of DCs as previously thought.7,8 Recent studies indicate that CD8α expression is plastic and may, in fact, be associated with the stage of DC maturation.7,9,10 On the other hand, evidence from other studies suggests that CD8α serves as a marker for separable pathways of DC differentiation.7,11

Despite much progress in understanding the biology of DCs, molecular events that specify DC development are still largely unknown. It is not clear what transcription factors are involved in multiple steps of DC development. Similarly, little is known regarding the mechanisms controlling the expression of genes important for DC function, including IL-12 and major histocompatibility complex (MHC) class II. Several transcription factors have been shown to contribute to DC development. For example, mice with a dominant-negative Ikaros gene lack both CD8α+ and CD8α−DCs, indicating that the Ikaros family of transcription factors is important for generating most of the DCs.11 Interestingly, mutant mice with the disrupted Rel-B or Ikaros gene are shown to retain CD8α− DCs, although they are devoid of CD8α+ DCs.11,12 The disruption of the PU.1 gene has also been reported to selectively deplete CD8α− DCs,13 although another report indicates that PU.1 affects both types of DCs.14 These reports suggest that CD8α+DCs can be generated in the absence of CD8α−DCs and without requiring the above factors, raising the possibility that some other transcription factors are involved in the development of CD8α+DCs. Although these studies shed light on the roles of these factors in cell autonomous DC development, the question of what transcription factors are involved in triggering DC maturation and by what mechanism remain unanswered.

Interferon consensus sequence binding protein (ICSBP; also called interferon regulatory factor 8 [IRF-8]) is a DNA-specific transcription factor that belongs to the IRF family.15 It is expressed only in the hematopoietic cells including lineage-negative BM cells, as well as macrophages and lymphocytes.16,17 ICSBP interacts with partner proteins to bind to well-studied target elements, interferon-stimulated response element (ISRE) and Ets-IRF composite element (EICE), and regulates gene expression in the immune system.16,17 ISRE is present in many interferon-inducible genes and is the target element for all IRF proteins. EICE is a composite element for ICSBP and PU.1 and is found in many genes active in the immune cells.16 ICSBP-null mice develop a leukemialike disease18 and are susceptible to infection by a variety of pathogens.19,20 The high susceptibility to pathogens is accounted for by the absence of ICSBP, which is known to play a role in the development of DCs.
for by the inability of ICSBP−/− mice to produce IL-12 rapidly and in sufficient quantity.18,21,22 These abnormalities are traced to a developmental defect that originates in the BM stem cells.24,25 In line with a role for ICSBP in hematopoietic cell development, we showed that introduction of ICSBP into ICSBP−/− myeloid progenitors stimulates their differentiation toward macrophages.17,25 More recently we have found that ICSBP−/− mice possess few CD8α+ DCs.26 Supporting a cell autonomous role for ICSBP in the development of CD8α+ DCs, BM chimera experiments indicated that the failure to generate CD8α+ DCs was not due to an external environment generated by the leukemialike syndrome in which ICSBP−/− DCs develop.

The present study investigates the development of ICSBP−/− DCs in an Flt3 ligand (Flt3L)–based culture system that supports the generation of mature DCs capable of expressing CD8α in response to lipopolysaccharide (LPS).27 Immature DCs developed from ICSBP−/− BM cells displayed multiple defects in expressing DC-specific cell surface molecules. Moreover, they were unable to fully mature in response to multiple maturation signals. We show that most, if not all, of these defects were rescued on ICSBP retrovirus transduction, including expression of CD8α and production of IL-12. Taken together, these results demonstrate that ICSBP plays a critical role in DC development and maturation and is essential for expression of CD8α and IL-12 p40.

Materials and methods

Mice

Experiments were performed with 6- to 10-week-old homozygous ICSBP−/− and ICSBP+/+ mice on a C57BL/6 background.

DC preparation and culture

The method with Flt3L27 was used with a small modification. Briefly, BM mononuclear cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, other ingredients, and recombinant human Flt3L (100 ng/mL, Peprotech, Rocky Hill, NJ) for 9 days. During the final 24 hours of culture, cells were stimulated with 1 μg/mL Escherichia coli–derived LPS (Sigma, St Louis, MO), 5 μg/mL CpG, or 2 μg/mL of the soluble extract of the parasite Toxoplasma gondii (STAg). Nonadherent DCs were harvested by gentle pipetting leaving adherent cells behind. In some experiments, CD11c+ cells were separated on a magnetic activated cell sorter (MACS; Miltenyi Biotec, Auburn, CA).

Retroviral transduction

Full-length ICSBP cDNA was ligated into the EcoRI site of pMSCV-EGFP28 to construct pMSCV-ICSBP-EGFP. Retroviral pMSCV vectors harboring the wild-type ICSBP and mutants 1-390, 1-356, and Lys79Glu (K79E) were described.25 Mutants Ser258Ala (S258A) and Arg289Glu (R289E) were generated by site-directed mutagenesis using QuikChange vectors containing the wild-type and mutant ICSBP were selected by 0.5 μg/mL puromycin for 5 days starting 2 days after spinoculation. Twenty-four hours after the second spinoculation, cells were harvested with 0.5% saponin (Sigma), and stained with allophycocyanin (APC)–conjugated antirat ICSBP IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Nonadherent DCs were harvested in a Flt3 ligand (Flt3L)–based culture system that supports mice to produce IL-12 rapidly and in sufficient quantity.18,21,22 These abnormalities are traced to a developmental defect that originates in the BM stem cells.24,25

Flow cytometry

Specific antibodies used for flow cytometry (all purchased from BD Pharmingen, San Diego, CA) include fluorescein isothiocyanate (FITC)–conjugated antibodies against CD11c (HL3), phycoerythrin (PE)–conjugated antimonique CD8α (Ly-2), I-Aα (Aeα), CD80 (B7-1), CD40 (3/23), or Flt3 (Ly-72). For IL-12 intracellular staining, cells pretreated with 10 μg/mL of brefeldin A (Sigma) for 2 hours were stained for CD11c and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized with 0.5% saponin (Sigma), and stained with allophycocyanin (APC)–conjugated antimonique IL-12 (p40/p70). Stained cells were collected on FACSCaliber (Becton Dickinson, San Jose, CA) and data were analyzed by FlowJo software (Tree Star, San Carlos, CA).

ELISA and MRls

DCs (2 × 105 cells in 200μL) generated in vitro were stimulated with or without LPS, CpG, or STAg for 24 hours. IL-12 p40 in supernatants was measured by an enzyme-linked immunosorbent assay (ELISA) using a kit (BD Pharmingen).

For mixed leukocyte reactions (MLRs), increasing numbers of in vitro–generated and irradiated CD11c+ DCs (0.3 × 104 to 1 × 105) were incubated with 1 × 106 BALB/c splenic lymphocytes in 100 μL media for 3 days and pulsed with 0.5 μCi (0.0185 MBq) [3H]thymidine (TdR; Amersham, Piscataway, NJ) for 8 hours. 3H-TdR incorporation was measured on a β-plate counter.

RT-PCR

Semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed for indicated transcripts as described.25 For real-time PCR, amplification of sample cDNA was monitored with the fluorescent DNA-binding dye SYBR Green (DNA Master SYBR Green I kit, Roche, Indianapolis, IN) in combination with the LightCycler system (Roche), according to the manufacturer’s instructions. Transcript levels were normalized by hypoxanthine-guanine phosphoribosyltransferase (HPRT) levels. Primer sequences used for PCR are available on request.

Immunofluorescent staining

CD11c+ DCs were placed on coverslips (Becton Dickinson) and fixed with 4% paraformaldehyde and permeabilized by 0.5% saponin. Cells were incubated with goat anti-ICSBP IgG (Santa Cruz Biotechnology, Santa Cruz, CA) followed by FITC goat IgG (Jackson Immuno Research, West Grove, PA). Stained cells were viewed with a confocal microscope (Leica).

EMSA

In vitro transcription/translation (IVT) of ICSBP and partners as well as an electrophoretic mobility shift assay (EMSA) were performed as described.25

Results

Defects of ICSBP−/− DCs developed in vitro

ICSBP−/− mice are deficient in CD8α+ DCs, although they have normal numbers of CD8α− DCs.26 We observed that ICSBP−/− splenic DCs (CD8α−) in vivo do not induce CD40, CD80, and MHC class II molecules after injection of LPS or CpG, indicating that the defects of ICSBP−/− DCs are not restricted to CD8α expression, but extend to functions and even to a maturation step. In an effort to better define the defects in ICSBP−/− DCs and elucidate the role of ICSBP in DC development, we used an Flt3L-based culture system.27 In this system, BM progenitor cells differentiate into immature DCs in about 9 days and on maturation, about 25% of cells are induced to express CD8α. Prior to this study we confirmed that ICSBP−/− lineage-negative BM progenitor cells express Flt3 at levels comparable to ICSBP+/+ cells (not shown), making this culture system suitable for studying ICSBP−/− DC development. Following 9 days of culture in the presence of Flt3L, ICSBP−/− and ICSBP+/+ BM cells gave rise to a similar number of
cells (1-3 x 10^6 cells/plate). About 90% of cells derived from ICSBP^-/- BM cultures were CD11c+ and showed typical DC morphology as described,22 whereas about 60% of cells from ICSBP^-/- BM cultures were CD11c+, which appeared monocyte-like with fewer dendrites. On LPS stimulation, ICSBP^-/- cells attained features characteristic of mature DCs. However, ICSBP^-/- cells did not show changes indicative of DC maturation.

Figure 1A depicts flow cytometry analysis of CD8α expression on in vitro–generated ICSBP^-/- and ICSBP^+/+ DCs. Although not expressed prior to stimulation, CD8α was induced in about 25% of ICSBP^-/- cells on LPS addition, consistent with the previous report.22 In contrast, less than 1.5% of ICSBP^-/- cells were positive for CD8α expression on addition of LPS, in keeping with the lack of CD8α^+ DCs in ICSBP^-/- mice. CD8α was also induced in ICSBP^-/- DCs on CpG and STAg stimulation, but not in ICSBP^-/- DCs (Figure 2A). Expression of MHC class II, CD80, and CD40 on in vitro–generated CD11c+ DCs is shown in Figure 1B. Constitutive MHC class II expression was significantly lower in ICSBP^-/- DCs than ICSBP^+/+ cells, in agreement with the data for in vivo DCs.20 Further, whereas LPS treatment markedly increased MHC class II levels in ICSBP^+/+ cells, it led to a meager increase in ICSBP^-/- cells (note the difference in mean fluorescence intensity [MFI]). Levels of CD80 and CD40 were also markedly increased in ICSBP^+/+ cells after LPS stimulation, but the increase was very modest in ICSBP^-/- cells. A similar outcome was observed on stimulation with CpG or STAg (not shown). These results indicate that ICSBP^-/- BM cells are defective in developing immature DCs and that ICSBP^-/- DCs fail to mature properly in response to maturation signals to express CD8α and other DC markers. The inability of ICSBP^-/- DCs to respond to LPS and other signals is not due to a defect in toll-like receptor (TLR) signaling.

The lack of IL-12 production by ICSBP^-/- DCs

IL-12 is a cytokine critical for DC function.29 In view of our previous observations that ICSBP^-/- macrophages do not express IL-12 p40,22 we were interested in determining whether ICSBP^-/- DCs also fail to express the gene. BM-derived DCs were stimulated with LPS, CpG, or STAg for 24 hours and expression of IL-12 p40 mRNA was examined by semiquantitative RT-PCR. Before stimulation, ICSBP^+/+ DCs expressed IL-12 p40 mRNA at a low level, followed by a marked increase on stimulation by all 3 agents. Similarly, CD8α transcripts were increased after stimulation in ICSBP^+/+ cells. In contrast, neither IL-12 p40 nor CD8α transcripts were detectable in ICSBP^-/- DCs before or after stimulation.

To establish that IL-12 p40 transcript induction results in the production of IL-12 protein in ICSBP^-/- DCs, LPS-stimulated DCs were stained for intracellular IL-12 p40. As shown in Figure 2B, about 25% of ICSBP^+/+ DCs were positive for IL-12 protein on LPS treatment, although few cells expressed the protein before treatment. In contrast, less than 2% of ICSBP^-/- cells were positive for IL-12 protein before and after stimulation. To verify that cells producing IL-12 actually secrete the cytokine into the medium, ELISA analysis was performed with supernatants from DCs generated in vitro. As shown in Figure 2C, LPS-stimulated ICSBP^+/+ DCs produced a large amount of IL-12 p40. In contrast, ICSBP^-/- DCs did not secrete a measurable amount of protein before or after stimulation. Together, these results show that ICSBP^-/- DCs are essentially devoid of IL-12 p40 gene expression resulting in the lack of IL-12 protein. Underscoring these in vitro observations, splenic ICSBP^-/- DCs also failed to express IL-12 p40 on injection with LPS, CpG, and STAg (not shown).

Impaired allogeneic MLR by ICSBP^-/- DCs

A hallmark of mature DCs is their strong capacity to stimulate T cells, which can be assessed by allogeneic MLRs.3 To evaluate the ability of in vitro–generated DCs to stimulate MLRs, ICSBP^+/+ and ICSBP^-/- DCs (both H-2k) were cocultured with BALB/c (H-2k) spleen lymphocytes for 3 days and the proliferative responses were measured by [3H]-Tdr uptake. As shown in Figure 2D, ICSBP^-/- cells treated with LPS exhibited the highest amount of [3H]-Tdr incorporation. Although about 3 times less efficient,
untreated ICSBP−/− cells also led to significant levels of 3H-TdR incorporation. However, ICSBP−/− cells, even after LPS treatment, led to a modest 3H-TdR uptake. These results indicate that ICSBP−/− DCs are defective in stimulating MLRs.

Retroviral ICSBP transduction restores DC development from ICSBP−/− BM cells

The above data indicated that the absence of ICSBP causes broad defects in DC development, which extend from the immature stage to the maturation phase. It was of interest to study whether these defects could be ameliorated by exogenous introduction of ICSBP. If the defects were corrected by reintroduction of ICSBP, it would indicate a direct role for ICSBP. However, if the defects were not corrected by exogenous ICSBP, they are likely to be due to an indirect, secondary change brought about by the absence of ICSBP. To reintroduce ICSBP into ICSBP−/− BM progenitor cells, we first used an MSCV-based retrovirus vector expressing ICSBP and green fluorescent protein (GFP; ICSBP-EGFP in Figure 3). As a control, a vector that expresses GFP only (EGFP in Figure 3) was tested. Fresh ICSBP−/− BM cells were transduced with the vectors in the presence of Flt3L. Flow cytometry analysis in Figure 3A depicts expression of surface markers on GFP+ and GFP− cells. The former represented transduced cells, whereas the latter represented untransduced cells. When cells were transduced with control vector, the percentage of CD11c+ cells remained unchanged from untransfected cells. These cells also did not express CD8α before and after LPS, as expected. In contrast, when cells were transduced with the ICSBP-EGFP vector, the percentage of CD11c+ cells markedly increased both before and after LPS. Significantly, about 12% of these cells expressed CD8α after LPS stimulation. Further confirming CD8α induction, the MFI for CD8α was increased by more than 3-fold in ICSBP-EGFP–transduced cells (Figure 3B). Both constitutive and LPS-inducible expression of MHC class II molecules, defective in ICSBP−/− DCs, was restored following ICSBP-EGFP transduction to a level comparable to ICSBP+/+ cells. Likewise, CD80 expression was increased on ICSBP-EGFP transduction to ICSBP+/+ DC levels before and after LPS stimulation. In contrast, cells expressing GFP only did not restore the expression of any of these molecules. Transduction of ICSBP-EGFP vector into ICSBP−/− BM cells led to a slight increase in CD8α, without affecting MHC class II and CD80 levels, which were already very high before transduction (not shown). Thus, simple reintroduction of ICSBP into ICSBP−/− BM progenitors restores expression of CD8α and other surface molecules on DCs generated in vitro, indicating that ICSBP has an important role in promoting DC development/maturation.

Exogenously expressed ICSBP restores IL-12 p40 production

To further investigate the effect of ICSBP reintroduction on DC development, we used another retroviral vector that harbored a puromycin-resistant gene. The use of this vector allowed us to select transduced cells, eliminating untransduced cells from the culture. Immunofluorescent staining in Figure 4A shows that ICSBP−/− DCs transduced with the ICSBP virus expressed the ICSBP protein in the nucleus, although the level of expression appeared lower than that of untransduced ICSBP+/+ DCs. Cells transduced with control vector did not show a detectable ICSBP staining, as expected. Moreover, cells transduced with the ICSBP vector underwent morphologic transformation consistent with proper DC differentiation (Figure 4B); these cells developed many long dendrites on LPS stimulation, similar to ICSBP+/+ DCs. However, cells transduced with control vector developed fewer and shorter dendrites.

We then investigated whether ICSBP can restore IL-12 p40 expression in ICSBP−/− DCs. To this end, cells transduced with ICSBP or control vector were stimulated with LPS, CpG, or STA and the IL-12 protein secreted into the media was measured by ELISA. As shown in Figure 4C, cells transduced with ICSBP produced IL-12 p40 at high levels in response to all agents tested, whereas cells transduced with control vector did not produce the cytokine at a detectable level, demonstrating that ICSBP rescues IL-12 protein production.

Identification of ICSBP domains required for restoration of DC development

To address the mechanism by which ICSBP rescues DC development and confers the ability to mature, we examined several ICSBP mutants (Figure 5A). ICSBP carries the DNA-binding domain (DBD) in the N-terminal region involved in the binding to target DNA elements, the ISRE and EICE.16 It also has the IRF association domain (IAD) in the C-terminal region involved in the interaction with partner proteins, including IRF-1, IRF-2, and PU.1.16,25 Binding of ICSBP to target DNA is dependent not only on the intact DBD but an interaction with a specific partner, in that it can bind to the ISRE, if it interacts with IRF-1 or IRF-2, whereas it can bind to the EICE when interacting with PU.1.16 Mutant 1-390
is a truncation lacking the C-terminal 34 amino acids. This mutant retains the DNA-binding activity, interacts with partners, and similar to the wild-type ICSBP, is capable of stimulating transcription and macrophage differentiation. However, Lys79Glu, having a point mutation in the DBD, does not bind to target elements and is defective in transcription and in stimulating macrophage differentiation. Similarly, 1-356, lacking the critical region in the IAD, does not interact with partners, fails to bind to either target DNA, and thus is functionally defective.25 Besides these mutants, 2 additional mutants, Ser258Ala and Arg289Glu, were constructed and tested in this work. Both Ser258Ala and Arg289Glu harbor a point mutation in the IAD. Serine at 258, equivalent to the serine at 260 of the human ICSBP, is thought to be functionally important because it is phosphorylated through the association with the CSN2 in the COP9/signalosome complex.30 Arginine at 289, located in an α-helix of the IAD, is highly conserved at equivalent positions in several IRF IADs and is believed to be indispensable for interaction with PU.1.31 These residues are replaced by alanine and glutamic acid, respectively. By EMSAs, we first examined whether new mutants Ser258Ala and Arg289Glu can form a complex with a partner and bind to the ISRE and EICE. Figure 5B depicts IVT products indicating proteins of expected size. In Figure 5C, EMSAs were performed and tested for the 2 target elements. ISRE binding was examined with wild-type or mutant ICSBP along with IRF-2, whereas EICE binding was tested along with PU.1.25 These mutants were cloned in the puromycin-resistant vector and introduced into ICSBP-/- BM cells, and DC development was monitored. The ability of these mutants to restore DC development completely coincided with the ability to bind to DNA and to interact with partners; whereas the wild-type ICSBP, 1-390, and Ser258Ala fully restored DC development, mutants 1-356, Lys79Glu, and Ser258 did not. Cells transduced with the latter mutants did not show even a sign of partial restoration and were indistinguishable from control cells before and after LPS stimulation. Further confirming these results, data in Figure 5D show that CD8α and IL-12 p40 mRNA expression was restored only with wild-type ICSBP and the mutants retaining partner interaction and DNA-binding activities. The mutants without partner/DNA binding elements and is defective in transcription and in stimulating macrophage differentiation.
activities were totally inactive in expressing these genes. Figure 5 also shows that all mutants were expressed at equivalent levels as wild-type ICSBP, indicating that the inability of restoring DC development is not due to low expression. These results indicate that restoration of DC development depends on the ability of ICSBP to interact with partners and to bind to target DNA, a primary requirement for transcriptional function.

ICSBP stimulates MHC class II gene expression through a DC-specific CIITA promoter

The observation that the ICSBP-EGFP vector increased MHC class II surface expression (Figure 4) implied that ICSBP regulates MHC class II transcript expression. This possibility was interesting because it has previously been shown that MHC class II expression induced by interferon-γ (IFN-γ) is normal in ICSBP−/− peritoneal macrophages.18 In the left panel of Figure 6A, levels of MHC class II transcripts (I-Aα) were tested by real-time PCR for ICSBP+/+ and ICSBP−/− DCs generated in vitro. Constitutive levels of class II transcripts were about 10-fold higher in ICSBP+/+ cells than ICSBP−/− cells. Stimulation by LPS or IFN-γ did not significantly change transcript levels. As shown in the right panel of Figure 6A, transduction of the wild-type ICSBP or 1-390 vector led to an approximate 4-fold increase in the constitutive expression of MHC class II transcripts compared with cells transduced with the control vector. In contrast, no increase in MHC class II mRNA levels was seen with the mutants 1-356 and Lys79Glu. These results indicate that ICSBP plays an important role in the expression of MHC class II genes in DCs.

Constitutive and IFN-γ-inducible transcription of MHC class II genes is governed by the class II–specific transactivator CIITA, whose expression is differentially regulated by cell type–specific promoters.32 It has been shown that promoter I is selectively used in DCs.33 We examined whether expression of promoter I–specific CIITA mRNA is impaired in ICSBP−/− DCs, and if so, whether the CIITA expression is restored by ICSBP transduction. Data in Figure 6B (left panel) showed that the levels of promoter I CIITA transcripts were about 8-fold lower in ICSBP−/− DCs than ICSBP+/+ cells, indicating a defect in CIITA transcription in ICSBP−/− DCs. In line with the previous report, the transcript levels in ICSBP+/+ cells were reduced after addition of LPS or IFN-γ,33 but this was not seen in ICSBP−/− cells. In the right panel of Figure 6B, transduction of wild-type ICSBP and 1-390 vectors increased constitutive CIITA transcript levels 3- to 4-fold over those by mutants 1-356 and Lys79Glu and control vector. These results indicate that ICSBP regulates CIITA transcription through promoter I, thereby enhancing MHC class II expression in DCs.

TLR signaling in ICSBP−/− DCs

Given that LPS induction of CD8α and IL-12 p40 genes was defective in ICSBP−/− DCs, but rescued by ICSBP transduction, it seemed possible that this transcription factor is required for proper LPS signaling in DCs. LPS and other microbial products are recognized by a series of TLRs. Their signaling is mediated through the adaptor protein MyD88, resulting in the activation of transcription factor nuclear factor-κB (NF-κB).35,36 Although NF-κB is a major target of TLR-MyD88 mediated signaling, evidence indicates that MyD88 stimulates other transcription pathways as well.37 In addition, a recent study indicates that LPS triggers DC maturation through MyD88-dependent and -independent pathways.38 To gain insight into the role of ICSBP in LPS signaling, we examined TLR expression in ICSBP−/− DCs generated in vitro. Expression of TLR4 and TLR2 transcripts was tested because LPS signaling is shown to be largely dependent on TLR4,39,40 but TRL2 may also participate in LPS signaling.41 As shown in Figure 7A, TLR4 transcripts were constitutively expressed and down-regulated 3 and 8 hours after LPS stimulation both in ICSBP+/+ and ICSBP−/− cells. The down-regulation of TLR4 has been reported for LPS-treated macrophages and likely represents LPS tolerance.41 TLR2 transcripts were also constitutively expressed and slightly increased after LPS addition both in ICSBP+/+ and ICSBP−/− cells. MyD88 transcripts were also expressed in ICSBP−/− DCs at levels comparable to those in ICSBP+/+ DCs. Normal expression of TLRs and MyD88 as well as the down-regulation of TLR4 by LPS suggested that TLR signaling is intact in ICSBP−/− DCs. To further assess the functionality of TLR4 signaling in ICSBP−/− DCs, we examined IκBα transcript induction. IκB induction is an event that follows the degradation of IκB that is associated with the activation of NF-κB. It represents a feedback mechanism to restore IκB levels following NF-κB activation.42 In real-time PCR analysis shown in Figure 7B, IκBα transcripts were induced within 1 hour after LPS stimulation both in ICSBP+/+ and ICSBP−/− DCs at comparable levels, indicating that NF-κB activation is not impaired in ICSBP−/− DCs. These results indicate that the TLR-MyD88 signaling pathway is intact in ICSBP−/− DCs and is activated on LPS stimulation, suggesting that ICSBP functions along with the pathway, but acting separately from NF-κB.

Discussion

Disruption of the ICSBP gene causes specific defects in DC development in vivo; it eliminates CD8α+ cells and impairs the capacity of the remaining DCs to mature on activation.36 This paper shows that these defects were also seen with ICSBP−/− DCs developed in vitro in the presence of Flt3L. Most significantly this paper shows that reintroduction of ICSBP into BM progenitors completely corrects these defects, providing a definitive demonstration that this transcription factor controls multiple steps of DC development and maturation.

Figure 6. Rescue of MHC class II expression by ICSBP retrovirus transduction. (A) Expression of MHC class II mRNA in ICSBP+/+ or ICSBP−/− DCs (left graph) or ICSBP−/− DCs transduced with indicated vectors (right graph) was analyzed by real-time PCR. (B) Expression of promoter I–specific CIITA transcripts was analyzed by real-time PCR.
ICSBP confers CD8α expression

We have shown that ICSBP−/− DCs were devoid of both CD8α mRNA and the surface protein, and that both were induced on ICSBP transduction, indicating that ICSBP regulates CD8α gene expression in DCs, thereby contributing to the development of a CD8α+ subset. Given our previous results that ICSBP plays a role in lineage selection during myeloid cell development, it is possible that ICSBP acts in an early stage influencing the development of immature DCs. This transcription factor is critical for DC maturation and controls expression of IL-12 p40, CD8α, and MHC II in response to multiple activation signals. ICSBP directly regulates expression of genes marked in red, but not those in blue.

ICSBP confers IL-12 p40 expression

It was striking that ICSBP−/− DCs lacked IL-12 p40 expression under all conditions tested, before and after stimulation, but the defects were fully corrected after ICSBP transduction alone. Our findings suggest that ICSBP is a factor obligatory to the transcription of IL-12 p40 in DCs and that the restoration of mRNA expression suffices the production and secretion of the protein. An analogous situation has been observed with ICSBP−/− macrophages, in that IL-12 p40 transcripts are absent in ICSBP−/− macrophages and introduction of ICSBP vectors rescues the expression of endogenous IL-12 p40 mRNA and stimulates IL-12 p40 reporter activity. Thus, it appears that ICSBP is essential for IL-12 p40 expression both in DCs and macrophages, although this does not exclude the contribution of other transcription factors such as NF-κB. In any event, given the fact that IL-12 production is a critical aspect of DC function regulating the development of Th1 or Th2 cells, ICSBP seems to have a vital role in broadly influencing the nature of immune responses.

Mechanism of ICSBP action

By EMSA analysis, ICSBP mutants tested in this work were classified into 2 groups, ones that formed a complex with partners and bound to the ISRE and EICE targets, and the others that failed to do so. Whereas those in the former group fully restored DC development/maturation, those in the latter group completely failed to do so, showing perfect concordance between the ability to induce DC development/maturation and to act as a transcription factor. None of the mutants showed an intermediate phenotype in terms of both DNA/partner binding and restoration of DC development. These results indicate that ICSBP induces DC development/maturation by directly regulating target genes critical for DC development rather than acting indirectly along differentiation pathways. Target genes necessary for promoting DC development may carry either ISRE, EICE, or related sequences in the promoter. EICE and like elements are found in a series of genes important for macrophage and DC functions. The ISRE is also found in some genes important for innate immunity. Target genes activated by ICSBP may extend beyond genes carrying a classic ISRE or EICE, because ICSBP is shown to regulate gene expression through other elements. Our results also underscore the importance of partner proteins, without which ICSBP does not function. Consistent with this, PU.1, a partner for EICE binding has been shown to be involved in DC development as well as expression of genes important for innate immunity. It is interesting to note here that mutant Ser258Ala, lacking a CSN2 phosphorylation site, retained DNA/partner-binding activity and fully restored DC development, indicating that the CSN2-mediated phosphorylation is not essential for DC development/maturation.

Role for ICSBP in MHC class II expression

Among cell surface markers whose expression was defective in ICSBP−/− DCs, but rescued by the ICSBP reintroduction, MHC class II warrants some discussion, because unlike what was observed with ICSBP−/− DCs, ICSBP−/− macrophages express MHC class II antigens normally on stimulation with IFN-γ, indicating that the lack of ICSBP does not affect MHC class II expression in macrophages. Here we found that constitutive MHC class II expression is significantly lower in ICSBP−/− DCs than ICSBP+/+ cells, suggesting that ICSBP regulates class II genes in DCs, but not in macrophages. Pertinent to this issue, it has previously been shown that class II transactivator CIITA is differentially regulated in various cell types and that its transcription in DCs is specifically controlled by promoter I. We have shown that promoter I–driven CIITA transcript expression is
defective in ICSBP−/− DCs, but is rescued following ICSBP retrovirus transduction, with a concomitant restoration of MHC class II expression in these cells. Thus, ICSBP regulates MHC class II transcription in DCs by controlling promoter I–specific CIITA transcription.

TLR signaling and ICSBP

DC maturation is triggered by the engagement of TLRs and is mediated by the adaptor MyD88.35,36 Although NF-κB is a key downstream transcription factor activated by this signaling pathway, the recent report analyzing MyD88−/− mice indicates that the TLR-MyD88 signaling can act through pathways independent of NF-κB.37 In this context it is interesting to note that BM-derived DCs from MyD88−/− mice do not induce IL-12 p40 in response to LPS, suggesting that LPS induction of IL-12 p40 requires MyD88 signaling.38 We have shown that the TLR-MyD88 signaling pathway is functional in ICSBP−/− DCs, as evidenced by the expected down-regulation of TLR4 and induction of IκBα following LPS stimulation. In view of the shared defect between MyD88−/− and ICSBP−/− DCs in inducing IL-12 p40, it seems plausible that ICSBP works downstream of the MyD88 signaling pathway, presumably acting separately from NF-κB.

In conclusion, ICSBP is an integral part of the developmental program specifying the differentiation of both CD8α− and CD8α+ DCs and is necessary for triggering their final maturation, as shown in the model in Figure 7C.

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