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CUTTING EDGE

Cutting Edge: IFN Consensus Sequence Binding Protein/IFN Regulatory Factor 8 Drives the Development of Type I IFN-Producing Plasmacytoid Dendritic Cells[1]

Hideki Tsujimura, Tomohiko Tamura, and Keiko Ozato[2]*

IFN consensus sequence binding protein (ICSBP/IFN regulatory factor 8) is a hematopoietic cell-specific transcription factor essential for the generation of CD8α$^+$ dendritic cells (DCs). We found that ICSBP$^{-/-}$ mice lack B220$^+$CD11b$^-$ plasmacytoid DCs (pDCs) in addition to CD8α$^+$ DCs. Although ICSBP$^{-/-}$ mice have B220$^-$CD11b$^+$ myeloid DCs (mDCs), they fail to mature upon Toll-like receptor signaling. Accordingly, ICSBP$^{-/-}$ bone marrow progenitor cells were defective in generating pDCs in the fms-like tyrosine kinase 3 ligand-based culture system and mDCs generated in this system were defective in maturation. We demonstrate that introduction of ICSBP rescues the development of pDCs from −/− bone marrow progenitors. ICSBP also restored the ability of both pDCs and mDCs to mature after Toll-like receptor signals. ICSBP-restored DCs produced IFN-α and IL-12p40 in a DC subset-selective manner with the amounts comparable to those by +/+ DCs. Together, ICSBP is essential for early pDC development and final maturation of both pDCs and mDCs. The Journal of Immunology,* 2003, 170: 1131–1135.

Dendritic cells (DCs)[3] play an important role in both innate and adaptive immunity. They are composed of heterogeneous cell populations and are classified into distinct subsets based on surface phenotypes, functional potentials, and localizations (1). In humans, an immature DC subset with the plasmacytoid morphology (pDCs) has been described. Human pDCs are capable of producing type I IFNs in response to viral infection or CpG DNA at high levels and are thought to be long-missing major IFN producers (2, 3). These DCs are critical for the promotion of Th1 immune responses (3, 4). Recently, a subset equivalent to the human pDCs has been identified in the mouse (5–7). Mouse pDCs also produce type I IFN at high levels upon virus or CpG stimulation. In addition, they produce IL-12 in response to these stimuli (6, 7). Mouse pDCs display a unique surface marker profile, CD11c^{int}B220$^+$CD11b$^-$Gr-1$^+$ and express MHC class II and costimulatory molecules at lower levels than CD11c^{high}B220$^-$CD11b$^+$Gr-1$^-$ DCs.

ICSBP/IFN regulatory factor (IRF) 8 (hereafter ICSBP) is a DNA-specific transcription factor that belongs to the IRF family. Among other cell types, it is expressed in bone marrow (BM) progenitor cells and regulates the development of myeloid cells (8). We have recently shown that ICSBP$^{-/-}$ mice lack the CD8α$^+$ DC subset, although they have CD11c$^+$CD8α$^-$ DCs (9). The defect in ICSBP$^{-/-}$ mice is not limited to the lack of the CD8α subset, in that the residual ICSBP$^{-/-}$ DCs do not mature in response to Toll-like receptor (TLR) signals and fail to produce IL-12p40 (10). The present article further examines the role of ICSBP in the development and maturation of DCs by focusing on the pDC subset in ICSBP$^{-/-}$ mice and studying −/− DCs complemented with the ICSBP expression vector.

Materials and Methods

Cell preparation and culture

DC-enriched cell fractions and lineage marker negative (Lin$^-$) BM cells were prepared as described elsewhere (9, 11). An fms-like tyrosine kinase 3 ligand (Flt3L)-based DC culture was described previously (10). DCs were allowed to develop from Lin$^-$ progenitor cells using the Cell Culture Inserts system (0.45-μm filter; BD Biosciences, Mountain View, CA). Lin$^-$ cells (2×10^5 cells/ml) were cocultured in the presence of Flt3L with BM mononuclear cells as feeders (1×10^6 cells/ml in the upper chamber) that were separated by the microporous membranes for 10 days. During the final 24 h of culture, cells were stimulated with 100 ng/ml *Salmonella minnesota*-derived LPS (Sigma-Aldrich, St. Louis, CA) or 1 μM CpG oligomer DNA D19 (12). For some experiments, B220$^+$ pDCs and B220$^-$CD11b$^+$ myeloid DCs (mDCs) were purified on the MACS system (Miltenyi Biotec, Auburn, CA). The purity of each fraction was ~90%.

Retroviral transduction

Retroviral pMSCV-puro and pMSCV-enhanced green fluorescent protein (EGFP) harboring full-length ICSBP cDNA and transduction into Lin$^-$ BM cells have been described elsewhere (10).

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[3] Abbreviations used in this paper: DC, dendritic cell; pDC, plasmacytoid DC; mDC, myeloid DC; ICSBP, IFN consensus sequence binding protein; IRF, IFN regulatory factor; TLR, Toll-like receptor; BM, bone marrow; Flt3L, fms-like tyrosine kinase 3 ligand; GFP, green fluorescent protein; EGFP, enhanced GFP; MyD88, myeloid differentiation factor 88.

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Flow cytometry

Abs used for flow cytometry were all purchased from BD PharMingen (San Diego, CA). Stained cells were collected on a FACSCalibur (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

ELISA and RT-PCR

In vitro-generated DCs were stimulated with LPS or CpG for 24 h. Supernatants were analyzed for IFN-α and IL-12p40 by ELISA using commercially available kits (PBL, Piscataway, NJ and BD PharMingen). Semiquantitative RT-PCR was performed as described previously (11). Primer sequences used for PCR are available on request.

Results and Discussion

ICSBP$^{-/-}$ mice lack the pDC subset

Flow cytometry analysis of the DC-enriched low-density fraction from ICSBP$^{+/+}$ spleen confirmed that ~29% of cells were pDCs carrying CD11c^{int}B220^{+}CD11b^{-} on the surface. Consistent with the pDC profile (5, 6), these cells were also Gr-1^{+}MHC II^{low}CD80^{low}(data not shown). In contrast, only 1–4% of ICSBP$^{-/-}$ cells showed the typical pDC profile (Fig. 1*A*). Hence, the total number of pDCs in an ICSBP$^{-/-}$ spleen was <15% of normal +/+ spleen (Fig. 1*B*). In addition to the deficiency in spleens, the pDC subset was markedly reduced in ICSBP$^{-/-}$ thymi and lymph nodes compared with those in their +/+ counterparts (data not shown). During the preparation of this article, Schiavoni et al. (13) reported a similar deficiency in ICSBP$^{-/-}$ mice.

The absence of pDCs in −/− mice raised the possibility that ICSBP is differentially expressed in DC subsets. In Fig. 1*C*, ICSBP expression was examined for low-density spleen cells by intracellular Ab staining, and the levels were quantified by flow cytometry. CD11c^{int}B220^{+} pDCs showed a single peak of intense ICSBP expression. On the other hand, CD11c^{high}B220^{-} DCs exhibited two peaks, one large peak to which the majority of cells belonged showed only weak ICSBP expression. The other minor peak containing ~25% of cells showed high ICSBP expression. Three-color staining of the two populations identified ICSBP^{high} cells to be CD11c^{high}CD8α^{+}, whereas ICSBP^{low} cells were CD11c^{high}CD8α^{-} (data not shown). The levels of ICSBP expressed in the pDCs and CD8α^{+} cells were similar and more than four times higher than those of B cells. CD3^{+} T cells did not show significant ICSBP expression, as expected (11). Thus, pDCs and CD8α^{+} DCs, the subsets selectively missing in −/− mice, constitutively express ICSBP at high levels.

To evaluate the role of ICSBP in the development of the pDC subset, we used an Flt3L-based culture system that supports the generation of pDCs from BM cells (14). We cultured Lin^{-} progenitor cells on BM feeders that supplemented soluble factors important for DC development. Following 10 days of culture, ICSBP$^{+/+}$Lin^{-} progenitors gave rise to both B220^{+}CD11b^{-} and B220^{-}CD11b^{+} populations (Fig. 2*A*, *left*). The former expressed MHC II and CD80 at low levels, compatible with the pDC phenotype. The latter correspond to the mDC phenotype (14). Consistent with the absence of pDCs in −/− mice, ICSBP$^{-/-}$Lin^{-} BM cells failed to give rise to B220^{+}CD11b^{-} pDCs, although they generated B220^{-}CD11b^{+} mDCs (Fig. 2*A*, *right*). These results indicate that ICSBP is important for the development of pDCs from BM progenitors. We found that the feeder cells from ICSBP$^{+/+}$ and −/− BM cells were equally effective in supporting pDC development (data not shown), suggesting that ICSBP acts as an intrinsic factor rather than as an extrinsic factor affecting the environment in which pDCs develop.

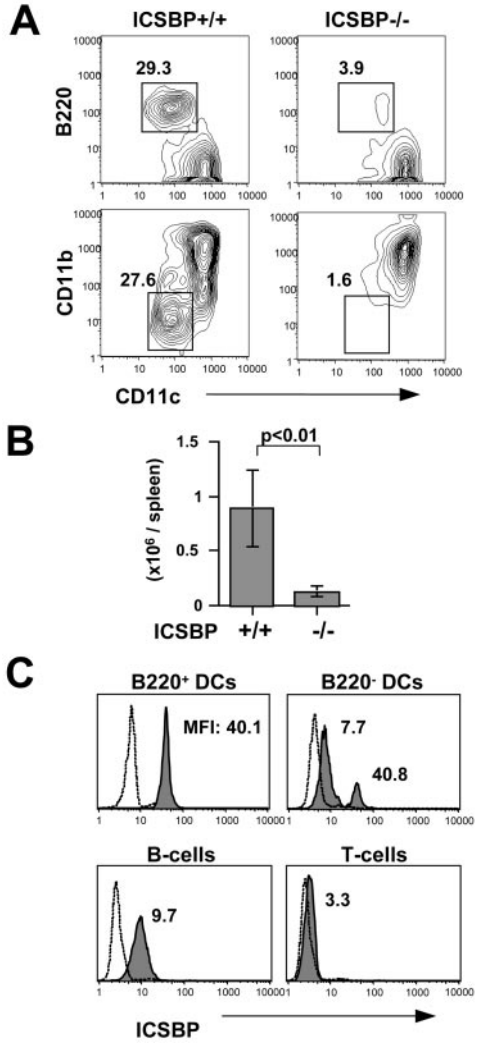

FIGURE 1. The absence of pDCs in ICSBP$^{-/-}$ spleens. *A*, Flow cytometry detection of B220^{+} and CD11b^{-} cells in the gated CD11c^{+} cells of the low-density fraction of ICSBP$^{+/+}$ and −/− spleens. Numbers indicate the percentage of CD11c^{int} pDC population. *B*, The total number of CD11c^{int}B220^{+} pDCs in an ICSBP$^{+/+}$ and −/− spleen. Values represent the mean of five spleens ± SD. *C*, Low-density cells from ICSBP$^{+/+}$ spleens were stained for the surface markers, fixed, permeabilized, and stained with anti-ICSBP IgG followed by FITC-conjugated second Ab. The filled histogram represents cells with ICSBP staining. The open histogram indicates control staining without primary Ab. Numbers indicate the mean fluorescence intensity (MFI) of each peak.

Retroviral ICSBP transduction restores pDC development in vitro

To begin to address the mechanism by which ICSBP regulates pDC development, we considered the following questions. Does ICSBP regulate DC development by an indirect mechanism, such as that affecting environment, or by a cell autonomous mechanism at the Lin^{-} cell level? To this end, we introduced an ICSBP-green fluorescent protein (GFP) retroviral vector (10) into ICSBP$^{-/-}$Lin^{-} progenitor cells and tested for the development of B220^{+}CD11b^{-} pDCs. As a control, a vector with GFP only was tested in parallel. Transduced cells were monitored by the expression of GFP by flow cytometry. Fig. 2*B* shows the expression of B220 and CD11b on gated GFP^{+} cells.

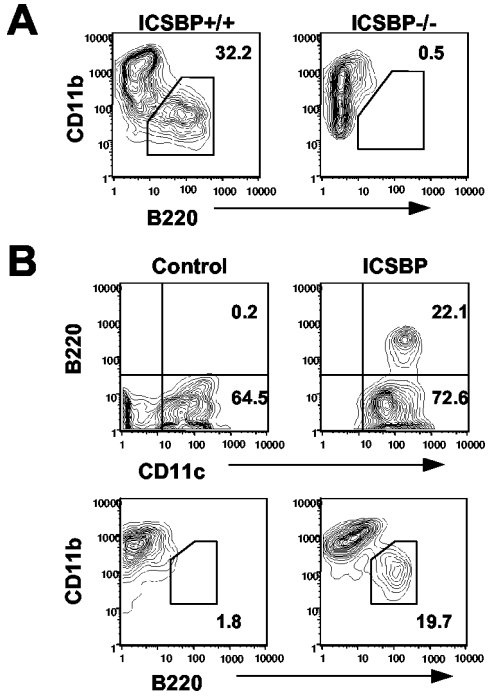

FIGURE 2. The absence of pDC development in vitro from ICSBP$^{-/-}$ progenitors and the restoration after ICSBP transduction. *A,* ICSBP$^{+/+}$ or $-/-$ Lin$^-$ cells were placed on BM feeder cells and cultured in the presence of Flt3L for 10 days. CD11c$^+$B220$^+$CD11b$^-$ cells were detected by flow cytometry. Numbers indicate the frequency of B220$^+$CD11b$^-$ pDC population. *B,* ICSBP$^{-/-}$Lin$^-$ cells were transduced with control-EGFT or ICSBP-EGFP vector and cultured as above. Cells with GFP signal were analyzed for the expression of indicated markers. Numbers indicate the frequency of cells in each quadrant (*upper panels*) or the percentage of B220$^+$CD11b$^-$ pDCs (*lower panels*).

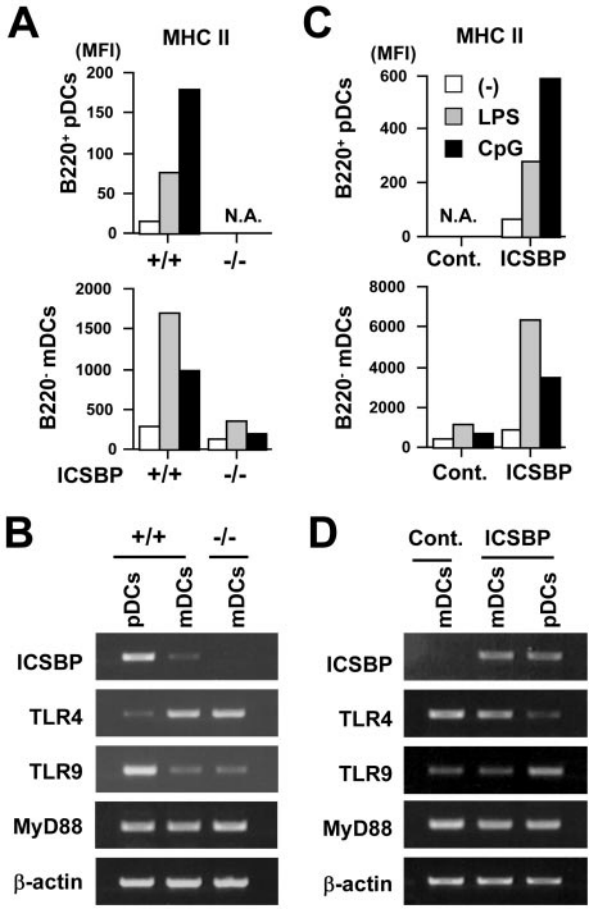

FIGURE 3. Restoration of MHC II and TLR9 expression in ICSBP-transduced pDCs. *A,* In vitro-generated DCs were stimulated with LPS (100 ng/ml) or CpG (1 μM) for the final 24 h and analyzed for MHC II expression on CD11c$^+$B220$^+$ pDCs (*upper panel*) and CD11c$^+$B220$^-$ mDCs (*lower panel*) by flow cytometry. The numbers indicate the mean fluorescent intensity (MFI). *B,* B220$^+$ pDCs (*upper panel*) and B220$^-$ mDCs (*lower panel*) were isolated by immunomagnetic separation, and expression of indicated mRNA was tested by semiquantitative RT-PCR. *C,* ICSBP$^{-/-}$Lin$^-$ BM were transduced with control (Cont.)-enhanced GFP or ICSBP-enhanced GFP vector for 10 days and were stimulated with LPS or CpG. MHC II expression on GFP$^+$ cells was detected as in *A. D,* ICSBP$^{-/-}$ Lin$^-$ BM were transduced with control or ICSBP vector and cultured in vitro in the presence of puromycin. pDCs and mDCs were isolated and tested for indicated mRNA as in *C.*

About 22% of cells transduced with the ICSBP vector expressed B220. Almost all of B220$^+$ cells were CD11b$^-$. Whereas, virtually no cells transduced with control vector expressed B220 and they remained CD11b$^+$. These data demonstrate that ICSBP introduction rescues the development of pDCs from $-/-$ progenitors.

ICSBP transduction rescues MHC II inducibility and subtype-selective TLR expression.

Human pDCs respond to CpG more efficiently than mDCs, but they respond to LPS less efficiently than mDCs (4). We next sought to examine whether ICSBP restores MHC II inducibility in pDCs and mDCs in a DC subset-selective manner. To study this question, it was first necessary to determine whether mouse pDCs and mDCs respond to CpG and LPS in a manner similar to the human subsets. Fig. 3*A* shows induction of MHC II in gated pDCs and mDCs following stimulation with LPS or CpG. ICSBP$^{+/+}$ pDCs responded to CpG more efficiently than LPS to enhance MHC II expression. Conversely, $+/+$ mDCs responded to LPS more efficiently than CpG, indicating the maintenance of subset selectivity in humans and the mouse. On the other hand, ICSBP$^{-/-}$ mDCs responded very poorly to either stimulus, resulting in meager MHC II enhancement. We next examined whether the subset-selective responsiveness is attributed to the expression pattern of TLR4 and TLR9, as reported for human DCs (4, 15). pDCs and mDCs generated in culture from $+/+$ and $-/-$ BM cells were isolated by magnetic separation and tested for TLR expression. Fig. 3*B* shows the results of semiquantitative RT-PCR. In agreement with TLR expression in human DCs, TLR9 was expressed predominantly in $+/+$ pDCs, whereas TLR4 was expressed in $+/+$ mDCs. Expression of myeloid differentiation factor 88 (MyD88), the adaptor critical for TLR signaling (16), was expressed at a comparable level among $+/+$ and $-/-$ DCs.

Fig. 3*C* shows that introduction of the ICSBP vector restores MHC II induction by CpG and LPS both in pDCs and mDCs in a subset-selective manner. As expected, the control vector did not restore MHC II inducibility either by LPS or CpG. That ICSBP restored TLR responsiveness characteristic of DC subsets suggested that expression of TLR4 and TLR9 is also restored after ICSBP transduction. To test this, ICSBP$^{-/-}$Lin$^-$ cells were transduced with the pMSCV-ICSBP vector and selected by puromycin, and pDCs and mDCs were isolated as

above. RT-PCR analysis in Fig. 3*D* shows that indeed TLR9 and TLR4 were expressed in ICSBP-transduced pDCs and mDCs in a subset-selective manner similar to their +/+ counterparts.

ICSBP restores IFN-α and IL-12p40 production

Given that IFN-α production is a main feature of pDCs, we wished to ascertain whether ICSBP$^{-/-}$ DCs are deficient in the cytokine expression and, more importantly, whether ICSBP confers the ability to produce the cytokines. As seen in ELISA analysis in Fig. 4*A* (*upper panel*), a large amount of IFN-α was produced in +/+ pDCs following CpG but not LPS stimulation. A low level of IFN-α was found in the mDC population after CpG stimulation, which may come from a small number of pDCs in this population. This subtype-selective IFN-α production is analogous to that in human DCs (4). In contrast, ICSBP$^{-/-}$ DCs completely failed to produce IFN-α either by CpG or LPS. RT-PCR analysis indicated that −/− DCs failed to induce IFN-α mRNAs, indicating that *IFN-α* genes were not expressed in −/− DCs upon stimulation (data not shown). Data in Fig. 4*B* (*upper panel*) demonstrate that upon ICSBP transduction, −/− pDCs produced a large amount of IFN-α upon CpG (but not LPS) stimulation with the amount comparable to that by +/+ pDCs. As expected, −/− DCs transduced with control vector did not produce the cytokine.

Mouse pDCs are capable of inducing a high level of IL-12p40, a cytokine critical for Th1 responses (6, 7). We first examined whether ICSBP$^{+/+}$ DCs produce IL-12p40 in a subtype-selective manner (Fig. 4*A*, *lower panel*). Upon CpG stimulation, pDCs produced IL-12p40 in a quantity larger than mDCs. On the other hand, upon LPS stimulation, mDCs produced a larger amount of cytokine than pDCs, although LPS was less effective in the overall production of the cytokine than CpG, confirming subtype-selective cytokine induction. Consistent with our previous findings that ICSBP$^{-/-}$ mice are defective in IL-12p40 expression (8), ICSBP$^{-/-}$ DCs failed to produce the cytokine at a measurable level. Like IFN-α, the absence of IL-12p40 production was due to the lack of mRNA induction (data not shown). Experiments in Fig. 4*B* (*lower panel*) examined whether ICSBP rescues IL-12p40 expression in −/− DCs. Indeed, IL-12p40 production was completely restored in both −/− pDCs and mDCs after ICSBP transduction. Similar to +/+ pDCs, CpG stimulated IL-12p40 production more efficiently than LPS in ICSBP-transduced −/− pDCs where the amount of cytokine produced was equivalent to that in +/+ cells. Again reproducing the pattern in +/+ DCs, LPS moderately stimulated IL-12p40 in ICSBP-transduced mDCs, but less so in pDCs. As expected, transduction with control vector failed to rescue IL-12p40 expression. These results are in agreement with the previous observation that ICSBP is required for the activation of the IL-12p40 gene in macrophages and DCs (10, 17, 18).

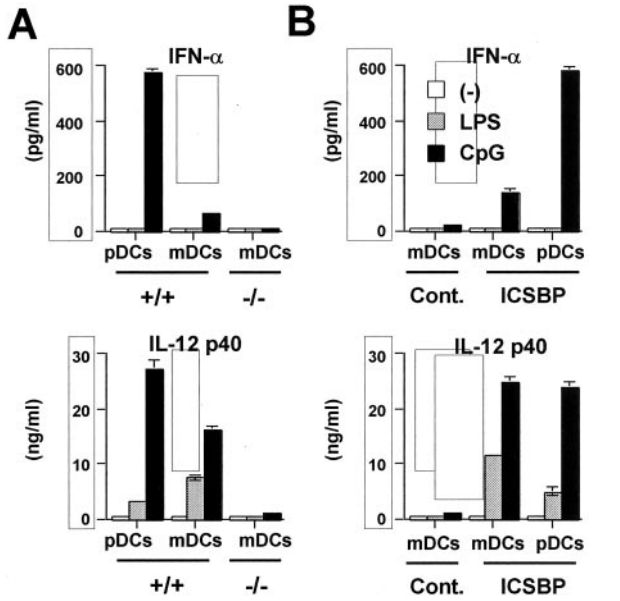

FIGURE 4. Production of IFN-α and IL-12p40 in ICSBP-transduced DCs. *A*, Supernatants from in vitro-generated DCs stimulated with LPS or CpG were tested for production of IFN-α (*upper panel*) and IL-12p40 (*lower panel*) by ELISA. *B*, ICSBP$^{-/-}$ pDCs and mDCs generated after transduction with control or ICSBP vector were stimulated with indicated agents, and levels of IFN-α and IL-12p40 in supernatants were measured as in *A*.

This study establishes that ICSBP$^{-/-}$ mice are deficient in pDCs (13) and that ICSBP$^{-/-}$ BM progenitors are incapable of developing pDCs in the Flt3L-based in vitro culture. The most significant aspect of the present work is that exogenous introduction of ICSBP into Lin$^-$ BM cells completely restored pDC development. That the rescue was possible in this system indicates that progenitors that can give rise to pDCs are present in ICSBP$^{-/-}$ mice and, when complemented with ICSBP at an appropriate stage, they can redirect the course of development to generate normal, functionally competent pDCs. Consistent with the idea that ICSBP acts during the progenitor stage, our previous work showed that ICSBP$^{+/+}$ and −/− BM cells contain similar numbers of common hematopoietic progenitors (9). Recently, the CD11c$^+$MHC II$^-$ DC precursors have been identified in murine peripheral blood (19). We found that this type of DC precursors is present in ICSBP$^{-/-}$ peripheral blood with a frequency equivalent to that of +/+ blood (data not shown), also supporting the role for ICSBP in the differentiation of DC precursors/progenitors.

Illustrating the role of ICSBP in final maturation, mDCs that remained in ICSBP$^{-/-}$ mice were defective in responding to TLR signaling to express MHC II and cytokines. That ICSBP corrected the generalized maturation failure and restored subtype-selective responsiveness demonstrates that TLR-mediated DC maturation is completely dependent on ICSBP. It is particularly significant that production of IFN-α and IL-12p40, essential for innate host defense and the Th1 immune responses, are both fully restored by ICSBP.

The mechanism by which ICSBP takes part in IFN-α gene induction in DCs is not clear at present. Viral induction of IFN genes is triggered by the activation of IRF-3, which leads to a cascade of gene activation resulting in the induction of IRF-7 and multiple IFN genes (20, 21). The dramatic restoration of IFN-α induction by exogenous ICSBP in −/− cells raises the possibility that ICSBP participates in IFN-α gene expression, perhaps indirectly, along with IRF-3 and IRF-7.

We have previously shown that ICSBP rescues IFN-γ/LPS induction of IL-12p40 in −/− macrophages and TLR triggered gene expression in −/− DCs at the level of transcription (10, 18). A recent article by Kaisho et al. (16) shows that IL-12p40 induction by LPS and CpG in DCs is dependent on the TLR adaptor MyD88, indicating that ICSBP is an activator of

the IL-12p40 gene that functions downstream of the MyD88 adaptor.

In conclusion, ICSBP is indispensable for the development of immature pDCs and for TLR-mediated maturation of both mDCs and pDCs.

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