Interferon (IFN) Consensus Sequence-binding Protein, a Transcription Factor of the IFN Regulatory Factor Family, Regulates Immune Responses In Vivo through Control of Interleukin 12 Expression

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Summary

Mice with a null mutation of the gene encoding interferon consensus sequence-binding protein (ICSBP) develop a chronic myelogenous leukemia-like syndrome and mount impaired responses to certain viral and bacterial infections. To gain a mechanistic understanding of the contributions of ICSBP to humoral and cellular immunity, we characterized the responses of control and ICSBP$^{-/-}$ mice to infection with influenza A (flu) and *Leishmania major* (*L. major*). Mice of both genotypes survived infections with flu, but differed markedly in the isotype distribution of antiflu antibodies. In sera of normal mice, immunoglobulin (Ig)G2a antibodies were dominant over IgG1 antibodies, a pattern indicative of a T helper cell type 1 (Th1)-driven response. In sera of ICSBP$^{-/-}$ mice, however, IgG1 antibodies dominated over IgG2a antibodies, a pattern indicative of a Th2-driven response. The dominance of IgG1 and IgE over IgG2a was detected in the sera of uninfected mice as well. A seeming Th2 bias of ICSBP-deficient mice was also uncovered in their inability to control infection with *L. major*, where resistance is known to be dependent on IL-12 and IFN-γ as components of a Th1 response. Infected ICSBP-deficient mice developed fulminant, disseminated leishmaniasis as a result of failure to mount a Th1-mediated curative response, although T cells remained capable of secreting IFN-γ and macrophages of producing nitric oxide. Compromised Th1 differentiation in ICSBP$^{-/-}$ mice could not be attributed to hyporesponsiveness of CD4^{+} T cells to interleukin (IL)-12; however, the ability of uninfected and infected ICSBP-deficient mice to produce IL-12 was markedly impaired. This indicates that ICSBP is a deciding factor in Th responses governing humoral and cellular immunity through its role in regulating IL-12 expression.

The IFN system is a vital part of innate and adaptive immunity. IFN signaling involves a variety of *trans*- and *cis*-acting factors and is mediated through DNA motifs, designated the IFN-stimulated response element (ISRE) and the IFN-γ–activated sequence, found in promoters of IFN-inducible genes. These genes play prominent roles in the control of growth, differentiation, and activation of cells of the immune system (1–5).

The interferon consensus sequence-binding protein (ICSBP) belongs to the IFN regulatory factor (IRF) family of transcription factors that have in common the ability to bind to ISRE. ICSBP is constitutively expressed in the nuclei of many hematopoietic cells (6–8). The tyrosine-phosphorylated form of ICSBP does not bind to DNA independently (9), but does bind when complexed with other members of the IRF (IRF-1 and IRF-2) or Ets (PU.1) families as well as with other elements of transcriptional machinery (10–13). Since the discovery of ICSBP, most attempts to establish its exact role in hematopoiesis, immunity, and IFN signaling were constrained to experiments with cell lines and led to the designation of ICSBP as a transcriptional repressor (14–16). Recent studies indicated a more complex role in

[1]*Abbreviations used in this paper:* CD40L, CD40 ligand; DLN, LN draining the site of infection; flu, influenza A; ICSBP, interferon consensus sequence-binding protein; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; NO, nitric oxide; ODN, oligodeoxynucleotide; RT, reverse transcriptase; SLA, soluble leishmanial antigen.

both immunity and cell cycle regulation (17–19). The first insights into the in vivo role of ICSBP came from analysis of recently generated ICSBP knockout mice (ICSBP$^{-/-}$). These mice develop a chronic myelogenous leukemia–like syndrome, but also exhibit impaired resistance to infections with vaccinia virus, lymphocytic choriomeningitis virus, and *Listeria*, but not vesicular stomatitis virus (20, 21). Although it was suggested that enhanced susceptibility to selected infections may be linked to reduced expression of IFN-γ, the exact mechanisms have remained elusive. Of note, increased susceptibility to infections is one of the major causes of death in patients with myeloid leukemias (22–24). The basis for impaired immunity in these conditions is uncertain, and there is currently no effective treatment.

To further understand the role of ICSBP in immunity to infections, we studied the responses of ICSBP mutant mice to exposure to influenza A (flu) as a model for antibody-dependent immune responses and *Leishmania major* as a model for innate and adaptive immunity. ICSBP$^{-/-}$ mice were resistant to flu, but were highly sensitive to infection with *L. major*. An analysis of changes in the immune system of ICSBP-deficient mice revealed a failure to develop Th-1–driven immune responses, which correlated with a defect in IL-12 production by cells of myeloid origin.

Materials and Methods

Mice. ICSBP mutant mice were generated as described (21). Homozygous mutant (−/−), heterozygous (+/−), and wild-type mice (+/+) on a (C57BL/6 × 129/Sv) F_2 background were bred and maintained under specific pathogen-free conditions.

Cell Cultures. Single-cell suspensions were prepared from spleen, lymph nodes, bone marrow, or whole blood lysed with ammonium chloride lysing buffer and resuspended in complete 1640 medium: 10% fetal calf serum, 1.5 mM l-glutamine, 100 U/ml penicillin/streptomycin, nonessential amino acids (GIBCO BRL, Gaithersburg, MD, or Biofluids, Inc., Rockville, MD), and 50 μM 2-mercaptoethanol. Peritoneal washout cells were harvested from untreated mice or mice injected 5 d previously with thioglycollate medium. To obtain macrophage-enriched populations, cell suspensions were subjected to 2–4 h of adherence on plastic and nonadherent cells removed before further use. For in vitro studies, cells were routinely incubated as 200 μl triplicate aliquots in 96-well plates at 37°C in 5% CO_2 in air.

In Vitro Cell Treatments. The panel of in vitro stimuli used included *Escherichia coli* LPS (Sigma Chemical Co., St. Louis, MO), *E. coli* DNA (Sigma Chemical Co.), murine recombinant IFN-γ (Genzyme, Cambridge, MA), IL-2 and IL-4 (Biosource Int., Camarillo, CA), IL-12 (a gift from Dr. S. Wolf, Genetics Institute, Cambridge, MA), Con A (Sigma Chemical Co.), and soluble leishmanial antigens (SLAs) prepared from freeze-thawed *L. major* as described (25) at concentrations indicated in the text or in the legends to the figures. CD40 ligand (CD40L) in the form of plasma membrane vesicles prepared from Sf9 insect cells infected with baculovirus recombinant for full-length mouse CD40L was a gift from Dr. M. Kehry (Boehringer Ingelheim, Ridgefield, CT). Oligodeoxynucleotides (ODNs) were synthesized, purified, and used as described (26, 27). Anti–mouse IL-4 and anti–IFN-γ mAb were purchased from PharMingen (San Diego, CA).

T Helper Differentiation. We used an in vitro assay (28) to assess Th differentiation. CD4$^+$ T cells were purified from pooled lymph nodes of wild-type or knockout mice and incubated with mAb to B220 and FcRγ before two rounds of panning on goat anti–rat Ig-coated plates followed by positive FACS® (Becton Dickinson, Mountain View, CA) selection. The purity of sorted CD4$^+$ T cells was 97–99%. To initiate Th development, 10^6/ml cells were plated onto 24-well plates coated with anti-TCR mAb (H57-597; 3 μg/ml). IL-2 at 50 U/ml was added to all duplicate cultures. Addition of 1 ng/ml IL-12 and 10 μg/ml anti–IL-4 served to provide Th1-promoting conditions; 1,000 U/ml IL-4 and 10 μg/ml of anti–IFN-γ provided Th2-promoting conditions. After 5 d of incubation, cells were washed and restimulated with immobilized anti-TCR mAb in the presence of IL-2 for 24 h. The concentration of IL-4 and IFN-γ in supernatants was then determined by ELISA.

Reverse Transcriptase PCR Analysis. Total RNA was prepared from individually frozen tissues or single-cell suspensions using RNAzol B (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. The sequences for primers and probes, as well as reverse transcriptase (RT)-PCR protocols, were as described previously (29–33) and as given below for myeloperoxidase (MPO): probe GGGGTGTACGGCAGCGAGGA; *c-fms*: probe CCAGCTGCCCATTGGACCATT; CD40L: 5′-GTCTGTTCACTTGGGCGGAG-3′; 3′-TTATTCCAGCTCTATGTGCCTTG-5′; probe CCTGCCCTGTGTTGAACTGCC; IFN-α1: 5′-TGTCTGATGCAGCAGGTGG-3′; 3′-AAGACAGGGCTCTCCAGAC-5′; probe: CAGGAATTTCCCCTGACC; IFN-β: 5′-CCATCCAAGAGATGCTCCAG-3′; 3′-GTGGAGAGCAGTTGAGGACA-5′; probe: GTACGTCTCCTGGATGAACT. Enhanced chemiluminescence reagents (Amersham Life Science Division, Arlington Heights, IL) were used for visualization of the RNA transcripts after electrophoresis of the samples and Southern blot hybridization. Hypoxanthine phosphoribosyltransferase (HPRT RNA) expression was used as a reference for equalization of cDNA input for PCR.

ELISA. ELISAs were used to determine cytokine and immunoglobulin concentrations in biological fluids. Amounts of IL-4 and IFN-γ in the supernatants from cultured cells were determined using reagents and protocols from PharMingen. mAbs for IL-12p40 protein detection were purified from C15.1 and C15.6 hybridomas (a gift from Dr. G. Trinchieri, The Wistar Institute, Philadelphia, PA). Serum Igs of different isotypes, excluding IgG2a, were determined using pairs of corresponding capture and peroxidase-conjugated detecting antibodies from SBA, Inc. (Birmingham, AL). IgG2a reagents were obtained from PharMingen.

ELISpot Assay. Modification of an ELISA method was used to determine frequencies of IL-12–producing cells in the spleen and lymph nodes of mice (26, 34). Serial dilutions of single-cell suspensions were incubated on 96-well microtiter plates coated with anti–IL-12 mAb (17.8; a gift from Dr. G. Trinchieri). After 4–6 h of incubation, plates were overlaid with a secondary biotinylated anticytokine antibody, washed, and treated with avidin-conjugated alkaline phosphatase (Vector Laboratories, Inc., Burlingame, CA). Individual producers were visualized and quantitated.

Virus Experiments. Live flu A/Philippines/2/82/X-79 (H3N2 type) virus was administered intranasally to mice anesthetized with methoxyflurane. Sublethal doses for immunization had been determined previously by in vivo titration in normal mice. 3 wk after primary infection with 10^2 tissue culture infectious dose 50% ($TCID_{50}$) U/mouse, animals were bled from the tail. Sera were analyzed for the presence of flu-specific antibodies by ELISA as described elsewhere (35). In brief, sera were titrated on plates

coated with formalin-inactivated flu vaccine and specific antibody isotypes were visualized with alkaline phosphatase-conjugated anti-IgG2a or anti-IgG1 reagents (Southern Biotechnology Associates, Inc., Birmingham, AL) to specify Ig isotype. Statistical analysis is described in the footnote to Table 1.

L. major Experiments. *L. major* (WHOM/IL/80/Friedlin) was provided by Dr. D. Sacks (National Institute of Allergy and Infectious Diseases, National Institutes of Health). Stationary phase promastigotes (10^6) in 50 μl of PBS were injected into the right hind footpads of mice. Disease progression was monitored by appearance of the infected footpads and frequent measurements of their thickness with a metric caliper (Fisher Scientific, Pittsburgh, PA). At defined time points, groups of mice were killed, and parasite growth and dissemination were determined by limiting-dilution assay (25, 33, 36, 37). In brief, serial dilutions of macerated organs or single-cell suspensions in complete M199 medium were plated onto 96-well plates containing an underlay of NNN blood agar (for assays done at 6 and 19 d after infection) or 30% fetal calf serum in M199 medium (for assays done at 49 and 53 d after infection). After 14 d of incubation at 28°C, the plates were microscopically inspected, and the parasite burden was scored as the titer of the last positive (parasite-containing) well.

For histopathologic analysis, spleens, livers, lymph nodes draining the site of infection (DLNs), and footpads from infected mice were fixed, sectioned, and stained with hematoxylin and eosin. For RNA expression analysis, DLNs were snap frozen into RNAzol and kept at −70°C until further RT-PCR processing, as specified above. Analysis of cytokine responses and nitric oxide (NO) production by spleen and DLN cells after 7 wk of infection was performed by stimulating serially diluted 8×10^6 cells/ml with 5 μg/ml Con A or 25 μg/ml SLA. An ELISA was used for determination of IL-4 or IFN-γ content in supernatants harvested after 24 or 72 h of incubation. Production of NO was assesed by measurement of $NaNO_2$ concentration using Greiss reagent.

Results

Responses to Infection with Flu Virus. Although previous studies showed ICSBP to be crucial for survival after challenge with lymphocytic choriomeningitis virus and *Listeria* (20, 21), the signaling pathways and effector mechanisms mediating normal resistance are incompletely known. The present studies were therefore designed to generate a deeper understanding of the relative role of ICSBP in humoral and cellular immunity. Resistance of mice to infection with flu involves secretion of antiviral antibodies, providing an opportunity to evaluate Th-dependent antibody responses in $ICSBP^{-/-}$ mice. Infection with *L. major* was chosen for analyzing aspects of innate and cellular immunity.

We first examined the responses of $ICSBP^{+/+}$, $ICSBP^{+/-}$, and $ICSBP^{-/-}$ mice to infection with flu using infectious virus at priming doses chosen as sublethal in normal mice. Mice of all three genotypes handled the infection without significant morbidity and no mortality. When challenged 8 wk later with a higher virus dose (10^4 $TCID_{50}$) that is lethal to unimmunized mice, they survived as well.

Analysis of Ig isotypes for titers of anti flu serum antibodies revealed striking differences among the three groups of mice (Table 1). The IgG2a responses of +/+ mice were much higher than the IgG1 responses, whereas the opposite was true for the responses of −/− mice. The IgG2a and IgG1 responses of +/− mice fell between those of the +/+ and −/− mice, indicative of a gene dosage effect.

It is well established that Ths play a prominent role in regulating Ig isotype switching in response to T cell–dependent antigens. Th1s expressing IFN-γ strongly promote class switching to IgG2a and IgG3, whereas Th2s producing IL-4 strongly bias switching toward IgG1 and IgE (38). The isotype distributions of antiflu antibodies thus suggest a Th1-driven response by +/+ mice and a Th2-driven response by ICSBP-deficient mice. To determine whether similar biases were evident in the responses of these mice to environmental antigens, we examined the distribution of Ig isotypes in sera of uninfected wild-type and knockout mice (Fig. 1 *A*). The observations that the levels of IgG1 and IgE were markedly higher and the levels of IgG2a reduced in the sera of −/− mice compared with that of +/+ mice were consistent with the hypothesis of Th2 dominance in $ICSBP^{-/-}$ mice.

To determine if the patterns of cytokines expressed by uninfected $ICSBP^{-/-}$ mice would show a bias toward a

Table 1. *Titers of Virus-specific IgG1 and IgG2a Antibodies in the Sera of ICSBP Mutant Mice Infected with Flu**

ICSBP genotype	IgG1	IgG2a	Ratio IgG1/IgG2a
+/+	54 (28–104)	1280 (1280–1280)	0.04
+/−	218 (88–526)	98 (32–296)	2.22
−/−	2827 (2136–3740)	20 (10–39)	141.35
Pairwise comparisons‡			
+/+ vs. −/−	$P < 0.05$	$P < 0.05$	–
+/− vs. −/−	$P < 0.05$	$P > 0.05$	–
+/+ vs. +/−	$P > 0.05$	$P < 0.05$	–

*The numbers in the table are geometric means for sera from seven infected mice/group tested individually. Statistical analysis was performed by one way analysis of variance. The titers of virus-specific IgG1 and IgG2a in the serum of uninfected mice were all <20.
‡The significance of difference for IgG1 was $P = 0.002$ and for IgG2a was $P = 0.004$, based on comparison of three groups.

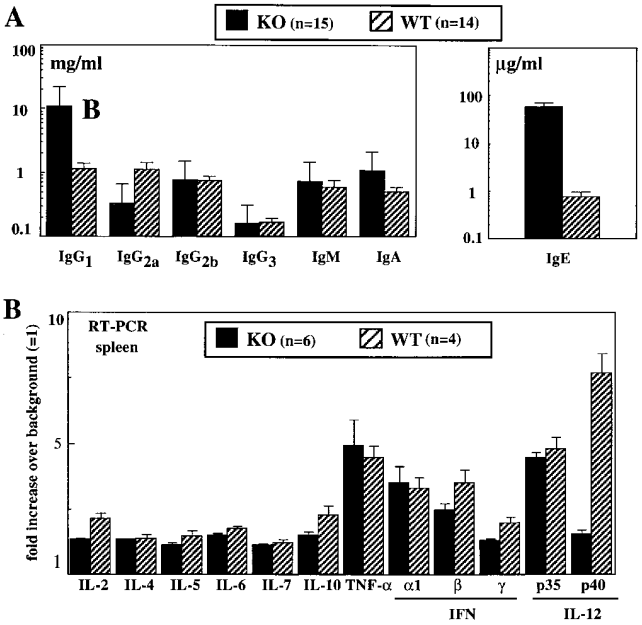


Figure 1. Distribution of serum Ig isotypes with the pattern of constitutive cytokine expression by spleen cells of ICSBP$^{-/-}$ mice. (*A*) Concentration of Ig isotypes in serum of intact ICSBP$^{-/-}$ (*KO*) and ICSBP$^{+/+}$ (*WT*) mice as determined by ELISA. (*B*) RT-PCR analysis of cytokine RNA expression in spleen of intact KO and WT mice. The mean ± SEM for each group of mice is shown.

Th2 profile, we compared the levels of cytokine transcripts in spleens of wild-type and knockout mice (Fig. 1 *B*). Transcripts for the Th2 cytokines (IL-4 and IL-5) were unchanged; transcripts for IL-10 and the Th1 cytokines (IL-2, IFN-γ) were slightly reduced in the spleens of ICSBP$^{-/-}$ mice. In contrast, IL-12p40 transcripts, as we reported previously (21), were markedly reduced in the knockout mice. Since IL-12 is required for differentiation of Th1 from Th0 cells, these findings suggest that in ICSBP$^{-/-}$ mice, Th2 cells may develop in a default pathway that exists in the knockout mice due to chronically impaired IL-12 expression. Alternatively, Th cells from ICSBP$^{-/-}$ mice may be unable to respond to stimuli that normally drive Th1 responses.

CD4$^+$ T Cells from ICSBP$^{-/-}$ Mice Are Able to Differentiate into Th1s and Th2s. To evaluate the capacity of CD4$^+$ T cells from ICSBP$^{-/-}$ mice to differentiate, we stimulated purified CD4$^+$ T cells with immobilized anti–TCR-α/β mAb under conditions conducive to polarized differentiation to Th1s or Th2s. Parallel cultures were activated with medium containing IL-12 and anti–IL-4 mAb to drive Th1 differentiation or IL-4 and anti–IFN-γ mAb to drive Th2 differentiation. After 5 d, the cells were washed and restimulated with anti–TCR-α/β and IL-2.

Analyses of cytokines in the supernatants of these cultures showed that CD4$^+$ T cells from wild-type or homozygous mutant mice generated highly polarized responses to either regimen (Table 2). In relation to production by cells of knockout mice, CD4$^+$ T cells from ICSBP$^{+/+}$ mice made more IFN-γ under Th1 conditions and less IL-4 under Th2 conditions. These results demonstrated that ICSBP-deficient CD4$^+$ T cells were capable of responding to differentiative effects of exogenous IL-12 and IL-4. Responsiveness to Th1-inductive stimuli was well maintained in ICSBP$^{-/-}$ mice; this suggested that decreased expression of Th1 cytokines in these animals and the Th2-like pattern of Ig isotype distribution were due to a deficiency of Th1-promoting signals. The demonstration that IL-12 is a key inducer of Th1 differentiation (39, 40) prompted us to examine the characteristics of IL-12 expression in ICSBP$^{-/-}$ mice.

Table 2. *Th Development of Lymph Node CD4$^+$ T Cells from ICSBP Mutant Mice**

	Th1 (IL-12 + anti–IL-4)		Th2 (IL-4 + anti–IFN-γ)	
Secretion	IFN-γ	IL-4	IFN-γ	IL-4
	ng/ml	*ng/ml*	*ng/ml*	*ng/ml*
Genotype				
ICSBP$^{+/+}$	225 ± 10	0	3.4	20 ± 4
ICSBP$^{-/-}$	112 ± 56	0.15	3.3	83 ± 19

*CD4$^+$ T cell purification and cultures were performed as described in Materials and Methods. Data are representative of four experiments.

IL-12 Secretion Is Impaired in ICSBP$^{-/-}$ Mice. Although transcripts of IL-12p35 were comparable in ICSBP$^{-/-}$ and ICSBP$^{+/+}$ mice, transcripts for IL-12p40 were markedly lower in ICSBP-deficient mice (Fig. 1 *B*; reference 21). IL-12p35 expression is constitutive in many cell types, whereas IL-12p40 is produced after activation of monocytes, macrophages, neutrophils, dendritic cells, and B cells by various stimuli (for review see reference 41). In the first set of experiments, we examined the ability of cells from spleen, peritoneum, or bone marrow, either unseparated or enriched by adherence for macrophages, to secrete IL-12. The cultures were treated with IFN-γ and either LPS or CD40L (Fig. 2 *A*). Cells from wild-type mice produced consistently higher levels of IL-12p40 than did cells from ICSBP-deficient mice, with the responses from the latter often being at or below the limits of detection.

The combinations of IFN-γ with LPS or CD40L are very effective at stimulating IL-12 production by macrophages, granulocytes, and dendritic cells (42–46). Recent studies, however, demonstrated the greater effectiveness of unmethylated CpG motifs present in bacterial DNA at stimulating B cells to produce IL-12 (26). In a second set of experiments, we used an ELISpot assay to determine the frequencies of lymph node and spleen cells producing IL-12 after stimulation with synthetic CpG-containing ODNs or bacterial DNA from *E. coli* (Fig. 2 *B*). Although ODNs and *E. coli* DNA induced IL-12 production in mice of either genotype with ODNs being more potent, the proportion of ICSBP$^{-/-}$ spleen cells that could be triggered to secrete IL-12 was much lower than that from wild-type spleen cells; however, the fold increase in the number of IL-12–secret-

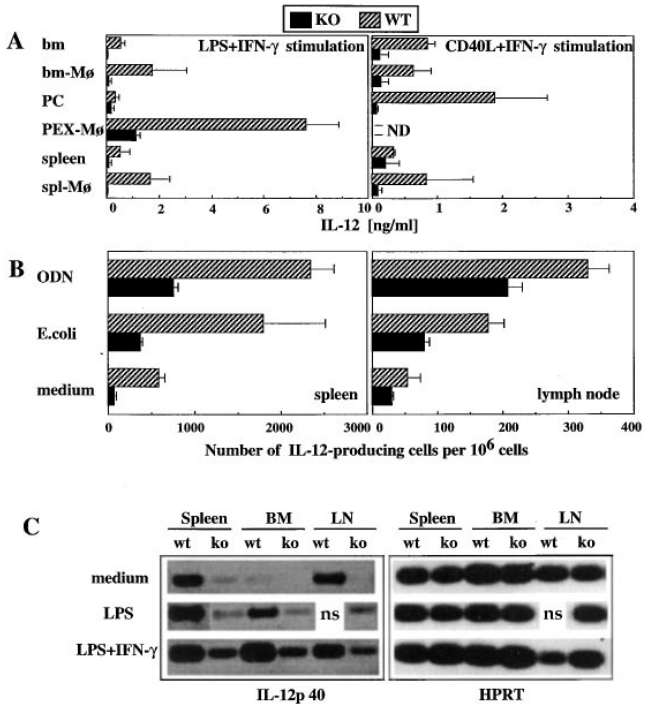


Figure 2. Impaired IL-12 expression in ICSBP$^{-/-}$ mice. (*A*) Concentration of IL-12p40 in supernatants obtained from indicated cell populations after 18–24 h of incubation with 200 U/ml IFN-γ in combination with 100 ng/ml LPS (*left*) or 1:1,000 diluted preparation of CD40L (*right*). Spleen (*spl*) and bone marrow (*bm*) cells were plated at 2×10^6/well; normal peritoneal washout (*PC*) or thioglycollate-elicited exudate (*PEX*) cells were used at 6×10^5/well. In a number of experiments, serial dilution analysis was performed. Data indicate the mean ± SEM of IL-12p40 concentration in supernatants from the indicated cell populations as well as from macrophages (*MØ*) enriched by adherence. The results summarize data from two to six experiments with two to three mice in each experiment. (*B*) ELISpot analysis of the ability of ODN or *E. coli* DNA to trigger IL-12p40 secretion by cells from spleen (*left*) or lymph nodes (*right*) of ICSBP$^{-/-}$ (*KO*) and ICSBP$^{+/+}$ (*WT*) mice. The frequency of IL-12–producing cells (mean ± SEM) for three individual mice is shown. (*C*) RT-PCR analysis of IL-12p40 RNA expression after 6 h of stimulation of 10^7 hematopoietic cells with 100 ng/ml LPS ± 100 U/ml IFN-γ. Data shown are representative of three independent studies. *ns*, no sample.

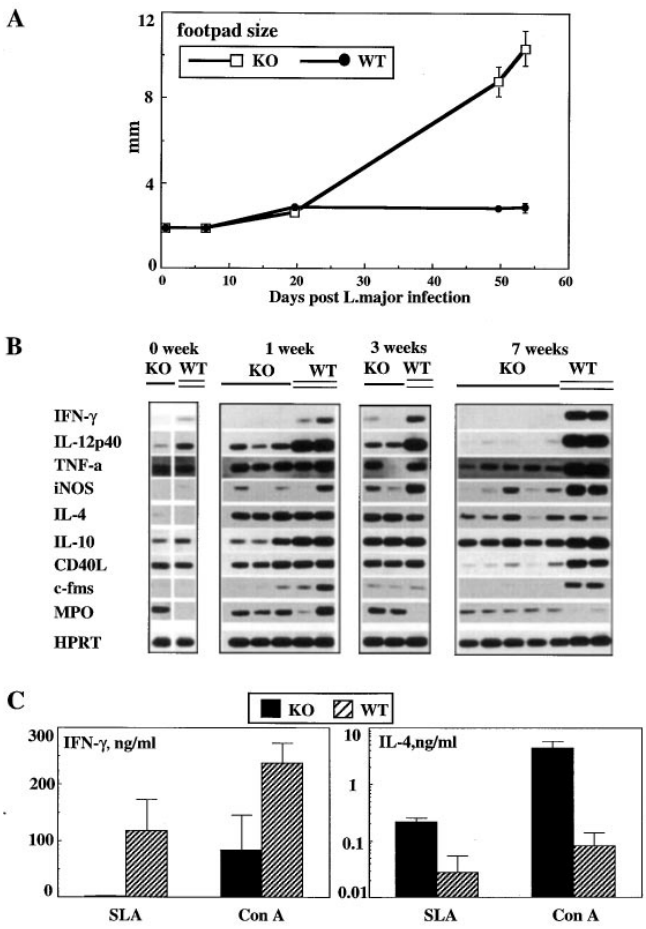


Figure 3. Course of *L. major* infection in ICSBP mutant mice. (*A*) Swelling of infected footpads of ICSBP$^{-/-}$ (*KO*; $n = 15$) and ICSBP$^{+/+}$ (*WT*; $n = 10$) mice was assessed as described in Material and Methods. (*B*) Expression of genes involved in control of the resistance to *L. major* by ICSBP$^{-/-}$ mice. RT-PCR analysis was performed on DLN cells. 7-wk data represent RNA expression in DLNs obtained on day 49 after infection. Identical results were obtained for DLNs on day 53. (*C*) Impairment of IFN-γ but not IL-4 production by DLN cells obtained from infected ICSBP$^{-/-}$ mice after restimulation with SLA and Con A in vitro. Data indicate the mean ± SEM in supernatants of 8×10^6 cells/ml derived from 7-wk–infected ICSBP$^{-/-}$ (*KO*) and ICSBP$^{+/+}$ (*WT*) mice. A summary of two separate experiments conducted on day 49 and day 53 after infection is shown.

ing spleen cells stimulated with ODNs was 11 for the knockout mice versus 4 for wild-type mice (Fig. 2 *B*). The difference in the absolute numbers of IL-12–secreting cells, as well as the degree of activation, was less dramatic for lymph node cells. *E. coli* DNA triggered a threefold increase and ODNs a six- to sevenfold increase in the quantity of the IL-12 producers, regardless of genotype. Of interest, the frequencies of IL-12–secreting cells among unstimulated spleen and lymph node cells of wild-type mice were substantially higher than those in cultures from ICSBP-deficient mice, an observation in keeping with the relative levels of IL-12p40 transcripts (Figs. 1 *B*, 2 *B*, and 3 *B*).

Further studies were undertaken to determine whether the deficit in IL-12 production in ICSBP$^{-/-}$ mice was indicative of a global change in macrophage and neutrophil functions or in their responsiveness to LPS, IFN-γ, or CD40 crosslinking. It has been reported that thioglycollate-elicited peritoneal macrophages from ICSBP$^{-/-}$ mice produced high levels of NO, but were impaired for H_2O_2 production and anti-*Listeria* cytotoxicity after in vitro stimulation with LPS and IFN-γ (20). In the current study, we screened selective responses of blood, spleen, bone marrow, or peritoneal cavity cells from ICSBP knockouts and littermate controls to stimulation with IFN-γ, IL-4, LPS, CD40 crosslinking, PMA, and FMLP in vitro, as well as to thioglycollate in vivo. We found that after appropriate stimulation, cells from knockout and normal mice were comparable for (*a*) production of NO and H_2O_2, (*b*) ability to upregulate MHC class II expression, exhibit a Ca^{2+} influx, and undergo chemotaxis; and (*c*) transcriptional elevations of TNF-α, iNOS, IL-6, KC, and MIP-2 (data not shown).

For cells from either normal or knockout mice, we also

Table 3. *Parasite Burdens in Tissues of ICSBP Mutant Mice during L. major Infection*

Tissues	Days after infection			
	6	19	49	53
Foot (log_2 titer per mg of tissue)				
ICSBP$^{-/-}$	10 ± 1 ($n = 3$)	>15 ($n = 3$)	13 ± 1 ($n = 3$)	8 ± 4 ($n = 3$)
ICSBP$^{+/+}$	1 ($n = 2$)	>15 ($n = 2$)	nd† ($n = 3$)	2 ± 2 ($n = 2$)
Spleen (log_2 titer per 10^6 of nucleated cells)				
ICSBP$^{-/-}$	–*	–	14 ± 2 ($n = 5$)	12 ± 1 ($n = 4$)
ICSBP$^{+/+}$	–	–	nd ($n = 3$)	nd ($n = 3$)
Draining LN (log_2 titer per 10^6 of nucleated cells)				
ICSBP$^{-/-}$	–	–	17 ± 1 ($n = 5$)	14 ± 1 ($n = 4$)
ICSBP$^{+/+}$	–	–	2 ± 1 ($n = 3$)	1 ($n = 3$)

*–, not done.
†nd, not detectable.

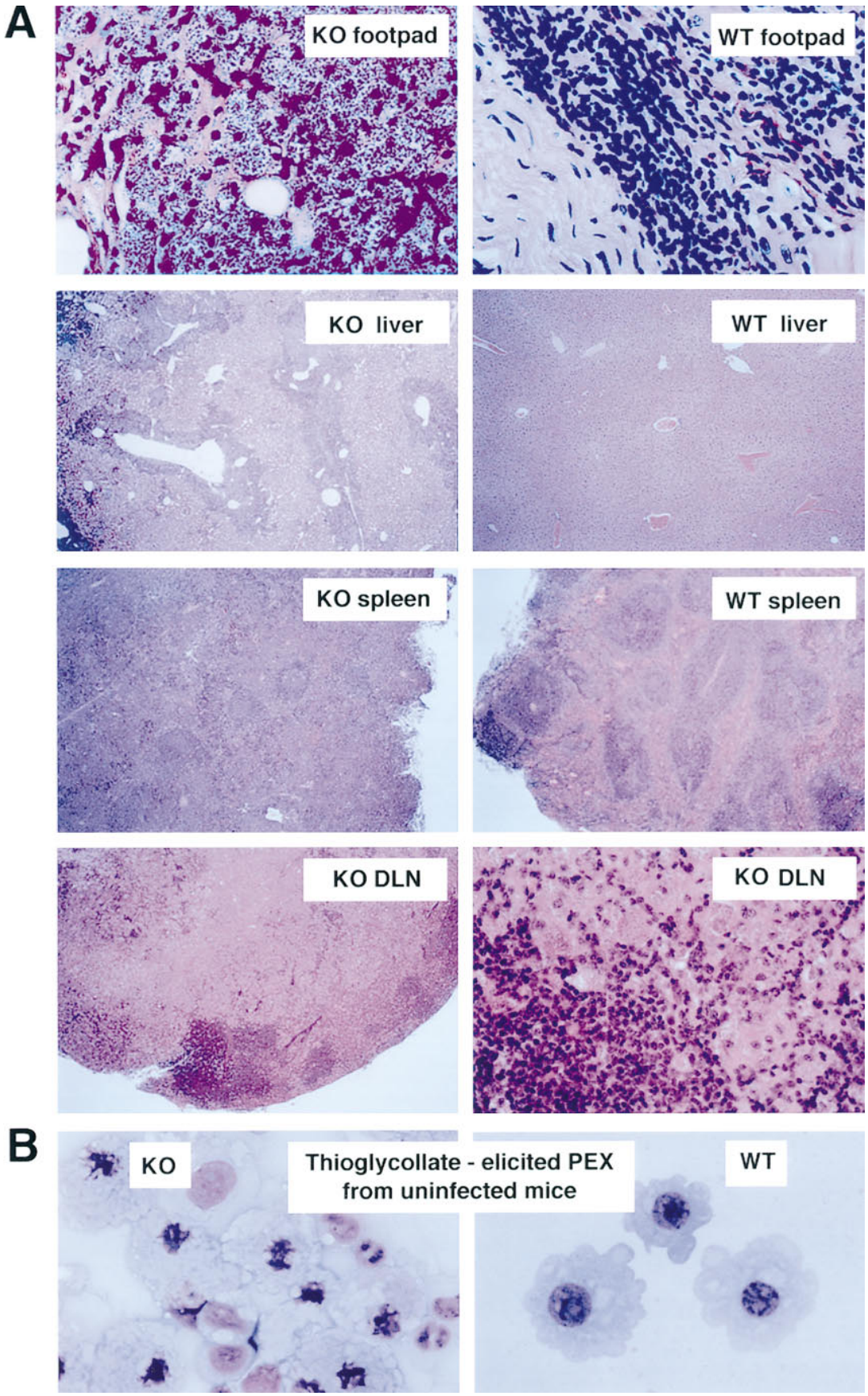
detected upregulation of IL-12p40 transcription after stimulation in vitro with LPS (Fig. 2 *C*). Transcript levels were further enhanced by the addition of IFN-γ to the induction regimen; however, the levels of induction seen with cells from knockout mice were considerably lower than those seen with cells from control animals. This suggests the existence of both ICSBP-dependent and -independent pathways for IL-12 induction, with the dependent pathway being more potent. Alternatively, ICSBP may be an important but nonessential component of IL-12 induction that amplifies the response when it is present. Together, the results of RT-PCR analyses and induction studies demonstrate that ICSBP$^{-/-}$ mice have greatly reduced steady-state levels of IL-12 expression and are markedly impaired in their ability to produce IL-12 in response to a variety of stimuli. Since IL-12 is a major stimulator of IFN-γ synthesis as well as of NK and CTL activity in vivo and appears to be indispensable for generation of protective Th1 immune responses against intracellular pathogens, we reasoned that T cell–mediated immunity should be severely altered in ICSBP$^{-/-}$ mice.

ICSBP-deficient Mice Are Highly Susceptible to Infection with L. major. ICSBP$^{-/-}$, ICSBP$^{+/-}$, and ICSBP$^{+/+}$ mice were inoculated in the footpads with 10^6 viable *L. major* promastigotes. Enlargement of the infected footpads and dissemination of the parasite to the DLNs and internal organs served as indicators of disease progression. The experiments were terminated at 53 d after infection when necrotic lesions developed in the footpads of ICSBP-deficient mice. These studies revealed that mice of a normally genetically resistant background [(B6 × 129) F_2], but deficient in ICSBP, were unable to control the infection. The response to infection of heterozygous mice was similar to that of −/− mice (data not shown). At day 6 after infection, the numbers of parasites in the footpads of ICSBP$^{-/-}$ mice were more than 250-fold higher than those of wild-type mice (Table 3), although footpad swelling was comparable (Fig. 3 *A*). Both footpad swelling and parasite numbers then decreased for wild-type mice, but progressed inexorably in the knockout mice, with elevated levels of parasites in the DLNs and dissemination to the spleen and liver (Table 3).

Histologic studies of infected wild-type mice showed that the inflamed footpads gradually healed and that there were no lesions in the spleens or livers (Fig. 4 *A*). In contrast, the footpads of ICSBP$^{-/-}$ mice exhibited marked swelling with both superficial and deep ulceration, necrosis, and striking accumulation of extremely heavily parasitized macrophages (⩾100 parasites/cell). Of interest, the lesions contained few neutrophils or lymphocytes, and signs of chronic inflammation or formation of granulation tissue were not observed. Excessive parasite burdens were also detected in the DLNs (Fig. 4 *A*), in which isolated foci of lymphocytes were shouldered to the periphery by sheets of ballooned, infested macrophages. The DLNs of infected ICSBP$^{-/-}$ mice were also significantly enlarged over those of wild-type animals, with weights of 98 ± 19 versus 17 ± 3 mg, respectively, at 53 d after infection. The cellularity of nodes from the knockout mice was increased only 1.5-fold over that of their wild-type counterparts, however, reflecting the differential contributions of parasitized macrophages versus responsive lymphocytes to nodal structure.

Sections from the spleens and livers of infected knockout mice and controls were strikingly different. Although wild-type livers appeared intact, the liver parenchyma of the knockout mice was modestly infiltrated with macrophages containing large numbers of parasites, and there was noticeable dilation of the sinusoids and marked accumulation of neutrophils and plasma cells in the periportal areas (Fig. 4 *A*). The spleens of infected ICSBP$^{-/-}$ mice showed alterations in the normal architecture due to sweeping infiltration of macrophages into the red pulp. The white pulp was small and nonreactive with periarteriolar lymphoid sheaths containing parasitized macrophages and lacking germinal centers (Fig. 4 *A*).

Figure 4. Microscopic examination of tissues from ICSBP mutant mice. (*A*) Histopathology of infected footpads, liver, spleen, and DLNs of ICSBP$^{-/-}$ (*KO*) or ICSBP$^{+/+}$ (*WT*) mice injected with *L. major* on day 49 after infection. Sections were stained with hematoxylin and eosin. The original magnification was 4 for liver, spleen, and left DLN panel; 40 for footpads and right DLN panel. Detailed description is given in the text. (*B*) Photomicrograph of cytospin preparations of thioglycollate-induced peritoneal washout cells obtained from uninfected ICSBP$^{-/-}$ (*KO*) and ICSBP$^{+/+}$ (*WT*) mice (Wright's-Giemsa stain; original magnification: 100).



The Susceptibility of ICSBP Knockout Mice to L. major Was Due to IL-12 Deficiency and Failure to Initiate Protective Th1 Responses. The resolution of experimental *L. major* infection has been ultimately correlated with IL-12–dependent development of IFN-γ–producing Th1s capable of activating the leishmanicidal potential of macrophages. To determine the basis for susceptibility to *L. major* in ICSBP-deficient mice of otherwise resistant background, we examined RNA expression of important effector molecules in the DLNs of infected animals. Infection of +/+ mice with *L. major* induced long-lasting increases in transcription of iNOS, IFN-γ, TNF-α, and IL-12p40 (Fig. 3 *B*). In contrast, DLNs of infected ICSBP$^{-/-}$ mice exhibited undetectable or very low levels of transcripts for IFN-γ and iNOS at any time after infection, with some mouse-to-mouse variability for the latter. The TNF-α response of the knockout mice was similar to that of wild-type mice at 1 and 3 wk after infection, but was substantially lower at 7 wk. In contrast, low levels of IL-12p40 were induced at 1 wk after infection and dropped sharply to barely detectable levels at 7 wk after infection. Thus, in contrast to control mice, ICSBP$^{-/-}$ mice failed to develop a curative Th1 immune response.

Several features of IL-12 regulation may be affected in ICSBP$^{-/-}$ mice. First, *L. major* infection caused a gradual increase in IL-10 expression in DLNs from ICSBP$^{-/-}$ mice, whereas IL-10 levels remained flat in ICSBP$^{+/+}$ mice. IL-10 may inhibit IL-12 production by macrophages, particularly in the absence of IFN-γ (43). Second, developmentally staged expression of specific functions by macrophages is a well-known phenomenon (see review in reference 47), and differences in IL-12p40 inducibility have been tied to specific stages of monocyte/macrophage differentiation/activation (48). Full activation of terminally differentiated macrophages is marked by increased RNA expression of *c-fms* and decreased expression of myeloperoxidase (32). This trend can be seen for transcripts from DLNs of wild-type mice, whereas expression of myeloperoxidase persisted in the infected knockout mice and only low levels of *c-fms* were seen early after infection (Fig. 3 *B*). This suggests that myeloid cells were not equally activated or differentiated in ICSBP$^{-/-}$ and ICSBP$^{+/+}$ mice. Finally, CD40L has been shown to be a crucial element in T cell–dependent pathways of macrophage activation, particularly in the regulation of IL-12 secretion (49–52). Levels of CD40L transcripts in lymph nodes were similar for mice of both genotypes before infection and up to 3 wk thereafter, but were markedly lower for the ICSBP$^{-/-}$ mice at 7 wk after infection (Fig. 3 *B*).

If IL-12 expression is reduced below a critical threshold, it would be expected that the mice would be able to develop only a Th2 response and would therefore be susceptible to infection. Remarkably, IL-4 transcripts in DLNs of ICSBP$^{-/-}$ mice did not differ significantly from those of wild-type mice at any time after infection, with IL-4 decreasing toward the late stages of disease (Fig. 3 *B*).

To examine the antigen-specific and polyclonal responses of cells from mice infected for 7 wk, spleen and DLN cells of wild-type and knockout mice were challenged in vitro with SLA or Con A, and the supernatants were tested for expression of IFN-γ, IL-4, and NO. After stimulation with SLA, DLN cells from ICSBP$^{-/-}$ mice were highly biased toward expression of IL-4, whereas cells from wild-type mice were biased toward IFN-γ (Fig. 3 *C*). Cells from mice of either genotype responded to stimulation with Con A by producing both IL-4 and IFN-γ, indicating that even after prolonged exposure to infection, T cells retained the capacity to be activated and to undergo either Th1 or Th2 differentiation, with preference for the latter in ICSBP$^{-/-}$ mice. Similar results were obtained for spleen cells (data not shown). Levels of NO in supernatants from DLNs and spleen cells stimulated with SLA were around the limits of detection (3 μM) for ICSBP$^{-/-}$ mice, as compared with 7.4 ± 2.9 μM (DLN) and 7.0 ± 0.9 (spleen) μM for ICSBP$^{+/+}$ mice; however, Con A triggered comparable release of NO by DLNs (5.8 ± 0.1 versus 6.5 ± 3.3 μM) and spleen cells (13.1 ± 2.6 versus 10.5 ± 2.3 μM) from infected knockout or wild-type mice, respectively. Additional experiments demonstrated that ICSBP$^{-/-}$ and ICSBP$^{+/+}$ thioglycollate-elicited macrophages infected with *L. major* in vitro and stimulated with LPS and IFN-γ produced similar amounts of NO (data not shown).

These observations suggest the following sequence of events leading to progressive infection with *L. major* in ICSBP$^{-/-}$ mice. Upon infection, the parasites quickly infest macrophages and multiply freely in the cytoplasm. This inefficient parasite containment and a deficiency in the early IFN-γ response may be due either to a yet-unknown defect in the NK cell compartment or to insufficient IL-12 production by macrophages and dendritic cells. As the disease advances, IL-12–deficient macrophages stimulated with suboptimal levels of IFN-γ and TNF-α fail to recruit and activate T cells in parasitized areas, further contributing to the IFN-γ deficiency. This closes the circuit by causing deficient iNOS expression by macrophages, leading to limited release of NO, the critical determinant of parasite killing. Therefore, the problems caused by early deficits in innate immunity, due to the lack of IL-12, are compounded by the failure to induce Th1 effectors of acquired immunity, resulting in disseminated, progressive disease.

Discussion

Previous studies established that mice deficient in ICSBP expression exhibit altered hemopoiesis and impaired immune responses to infections with vaccinia virus, lymphocytic choriomeningitis virus, and *Listeria* (20, 21). The failure of ICSBP$^{-/-}$ mice to cope with these infections was ascribed to an IFN-γ–related defect in immunity and, in the case of *Listeria*, impaired cytotoxicity due to poor production of reactive oxygen intermediates in macrophages.

The data presented in this study extend our understanding of ICSBP as a regulator of immune responses to infections in demonstrating that ICSBP$^{-/-}$ mice are markedly deficient in constitutive and induced expression of IL-12. This defect resulted in the failure of mutant mice infected

with *L. major* to mount primary or secondary Th1 responses and produce IFN-γ at high levels.

The defect in the ability of ICSBP$^{-/-}$ mice to express IL-12 was relative rather than absolute, with strong stimuli for phagocytic and dendritic cells being able to induce low levels of IL-12p40 messenger RNA but little if any protein. ODN and bacterial DNA, previously shown to activate IL-12 expression almost exclusively in B cells (26), were the most efficient inducers of IL-12 production from cells of both wild-type and knockout mice. Although further work is required to firmly establish this point, our results suggest that the defect in IL-12 expression is more profound in the macrophages and dendritic cells than in the B cells of ICSBP$^{-/-}$ mice.

The differential effects of ICSBP deficiency on IL-12 expression in B cells and macrophages may relate to cell lineage-specific variations in the constitutive and induced levels of other transcription factors that regulate IL-12 transcription. Current studies suggest that ICSBP may participate directly in the regulation of the IL-12 promoter (Wang, I.-M., and K. Ozato, personal communication), possibly as a heterodimer with IRF-1 (10). This view is strongly supported by the findings that the response of IRF-1$^{-/-}$ mice to infection with *L. major* is remarkably similar to that of ICSBP$^{-/-}$ mice (53). In IRF-1–deficient mice, the production of IFN-γ and IL-12 was greatly reduced, whereas levels of *L. major*-specific IgG1 and IgE antibodies were increased. Using different systems, it was concluded that the failure of Th1 responses to develop in IRF-1$^{-/-}$ mice either could, could in part, or could not (53, 54) be ascribed to Th precursors nonresponsive to IL-12.

Several major differences between IRF-1– and ICSBP–deficient mice suggest, however, that the phenotype of ICSBP$^{-/-}$ mice cannot be understood as the lack of a suitable docking partner for IRF-1. These include (*a*) the distribution of Ig isotypes in IRF-1$^{-/-}$ mice differs from that of normal mice only after infection, (*b*) changes in resistance to infection with *L. major* are gene dose-dependent in mice bearing the IRF-1 (53) but not the ICSBP mutation (data not shown), (*c*) intact IRF-1$^{-/-}$ mice are highly impaired in their ability to produce NO (55), whereas this function is not affected in ICSBP-deficient macrophages (20), and (*d*) the defect in IL-12 expression in IRF-1$^{-/-}$ mice is clearly determined at the transcriptional level as documented by an absence of IL-12p40 transcripts in cells stimulated with LPS and IFN-γ (54). It should be pointed out that a model invoking IRF-1/ICSBP heterodimers in direct regulation of IL-12 expression through ISRE is inconsistent with analyses of the IL-12p40 promoter by Ma et al. (56). They reported that deletion of the IRF-1–binding element had no effect on responsiveness of the promoter in human cells stimulated with LPS and IFN-γ, whereas deletion of an Ets-binding site completely silenced the promoter. Recently, they described a novel Ets-binding complex which can activate the human IL-12p40 promoter (57). The ability of ICSBP to complex with IRF-1 and IRF-2 on one hand and with Ets family members on the other indicates a possible "recruiter–enhancer" function for ICSBP in regulation of the IL-12p40 promoter. The structures of murine and human IL-12p40 promoters were found to differ, and a classic ISRE was not identified in mice; however, a binding site for PU.1, an Ets family member, was found in close proximity to a NF-κB half site (58), making a "cooperative" hypothesis for the role of ICSBP in regulation of IL-12p40 expression more attractive.

It is also conceivable that other factors known to inhibit IL-12 production, IL-4, IL-6, IL-10, TGF-β, PGE_2, or type I IFN, may contribute to the low IL-12 phenotype of ICSBP$^{-/-}$ mice (41, 43, 59). For example, recent studies of dendritic cells deficient in IL-12 expression as the result of treatment with PGE_2 promoted the development of Th2s (60).

Perhaps the clearest results to come from this study are the repeated demonstrations that ICSBP$^{-/-}$ mice are functionally Th2 animals. This includes the following observations: (*a*) serum Ig isotypes in uninfected ICSBP-deficient mice were remarkable for high levels of IgG1 and IgE and lower than normal levels of IgG2a, (*b*) the ratio of IgG2a/IgG1 flu-specific antibodies in sera of ICSBP$^{+/+}$ mice was essentially reversed in flu-infected ICSBP$^{-/-}$ mice, (*c*) (B6 × 129) F_2 mice, normally resistant to *L. major*, were susceptible if homozygous for the ICSBP mutation, and (*d*) the secondary antigen-specific responses of *L. major*-infected ICSBP$^{-/-}$ mice were characterized by elevated production of IL-4 and the absence of IFN-γ. Since purified CD4$^+$ T cells from ICSBP$^{-/-}$ mice could be induced to differentiate into Th1s in vitro, and nonspecific activation of cells from *L. major*–infected ICSBP$^{-/-}$ mice with Con A induced IFN-γ secretion, the defect in vivo can be ascribed to an environment deficient in stimuli that promote Th1 differentiation. This result underscores the significance of IL-12 for development of antigen-specific Th1 responses in vivo.

The mechanisms mediating resistance to infection with *L. major* have become increasingly clear over the last few years (61–67). In resistant mice, the curative response is associated with early production of IL-12 and is crucially dependent on parasite containment mediated by activated NK cells and IFN-γ. Presentation of parasite antigen by infected macrophages and dendritic cells results in the antigen-specific priming and differentiation of chemokine-recruited T cells into Th1 effectors locally and in the DLNs. Induction of iNOS and production of NO affect parasite killing within macrophages. This pattern of resistance was found to be altered in ICSBP$^{-/-}$ mice at many steps along this path, beginning with low-level induction of IL-12. Subsequently, we documented impaired induction of IFN-γ, iNOS, and NO, with the end result being progressive, fulminant leishmaniasis.

IL-12 deficiency and its downstream repercussions, while likely the overriding influence in the failed response of ICSBP$^{-/-}$ mice to *L. major*, may not be the only features contributing to the severity of the infection. Although macrophages infiltrated the inoculated foot rapidly and in high numbers, the lesions were notably deficient in infiltrating lymphocytes, similar to the case of anergic cutaneous leishmaniasis in humans (68). The paucity of respond-

ing lymphocytes may be part of the reason for the limited cytokine responses by T cells as determined by RT-PCR. With disease advancement, the masses of infested macrophages aggressively displaced lymphocyte populations, drastically changing the architecture of involved tissues and organs.

It is also possible that responding macrophages are not at the same stage of maturity or activation in wild-type and knockout mice. Developmentally distinct macrophages are known to differ in their functional phenotypes (see reviews in references 47, 69). The findings of differential use of ISRE-positive promoters by the maturationally distinct WEHI-3 and RAW 264.7 cell lines (70, 71) and the dependency of the expression of the ISRE-carrying gene, crg-2 (IP-10), on the state of macrophage inflammatory activity in mice (72, 73), indicate the possibility of direct involvement of IRF family members in developmentally staged expression of macrophage functions. During our studies, we found that elicited peritoneal macrophages in uninfected mice of the two genotypes appeared to be equally phagocytic while differing in morphology (Fig. 4 *B*). In addition, macrophages in DLNs of infected mice differed in their expression of markers associated with maturational states. These variations between macrophages of normal and mutant mice may be associated with developmentally determined differences in IL-12 expression (41, 48) as well as with cytotoxic and antigen-presenting functions.

Since ICSBP is expressed in activated but not resting T cells (6), some of the abnormalities exhibited by T cells of infected ICSBP$^{-/-}$ mice may be attributable to altered expression of genes containing ISREs. We found that two ISRE elements are present in the promoter of the CD40L gene at positions −361 to −348 and −141 to −125 (74). Expression of CD40L transcripts was well maintained in uninfected, highly purified CD4^{+} T cells from ICSBP$^{-/-}$ mice (data not shown); however, transcripts for CD40L dropped markedly during the later stages of infection in knockout but not in wild-type mice.

Although molecular mechanisms remain to be defined in detail, it is clear from this work that defective expression of IL-12 and resulting enhanced susceptibility to certain infections are hallmarks of ICSBP deficiency.

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