Crucial Role of Interferon Consensus Sequence Binding Protein, but neither of Interferon Regulatory Factor 1 nor of Nitric Oxide Synthesis for Protection Against Murine Listeriosis

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Summary

Listeria monocytogenes is widely used as a model to study immune responses against intracellular bacteria. It has been shown that neutrophils and macrophages play an important role to restrict bacterial replication in the early phase of primary infection in mice, and that the cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) are essential for protection. However, the involved signaling pathways and effector mechanisms are still poorly understood. This study investigated mouse strains deficient for the IFN-dependent transcription factors interferon consensus sequence binding protein (ICSBP), interferon regulatory factor (IRF)1 or 2 for their capacity to eliminate Listeria in vivo and in vitro and for production of inducible reactive nitrogen intermediates (RNI) or reactive oxygen intermediates (ROI) in macrophages. ICSBP−/− and to a lesser degree also IRF2−/− mice were highly susceptible to Listeria infection. This correlated with impaired elimination of Listeria from infected peritoneal macrophage (PEM) cultures stimulated with IFN-γ in vitro; in addition these cultures showed reduced and delayed oxidative burst upon IFN-γ stimulation, whereas nitric oxide production was normal. In contrast, mice deficient for IRF1 were not able to produce nitric oxide, but they efficiently controlled Listeria in vivo and in vitro. These results indicate that (a) the ICSBP/IRF2 complex is essential for IFN-γ-mediated protection against Listeria and that (b) ROI together with additional still unknown effector mechanisms may be responsible for the anti-Listeria activity of macrophages, whereas IRF1-induced RNI are not limiting.

Listeria monocytogenes, a gram-positive facultative intracellular bacterium, infects macrophages and hepatocytes in mice and has been used as a classic model to study immune responses against intracellular bacteria (1). Neutrophile granulocytes (2), γδ T cells (3), and above all macrophages (4) are important during the early phase of the immune response. In SCID mice lacking mature B and T lymphocytes, NK cells activated by macrophage-derived TNF-α have been shown to activate the listericidal effector mechanisms of macrophages via secretion of IFN-γ (5). These cells are able to restrict initial replication of Listeria in murine liver and spleen, since IFN-γ inhibits evasion of Listeria from phagosomes into the cytoplasm (6). Specific T cells are needed for final elimination of the pathogen (7) and also for protection against secondary infection (8–10). Studies of Listeria infection in mice deficient for IFN-γ (11), IFN-γ receptor (12) or TNF receptor 1 (13) have shown that the two cytokines IFN-γ and TNF-α are crucial for survival. However, the involved signaling pathways are not known, and the effector mechanisms used by macrophages for killing of Listeria are still debated. The role of reactive oxygen intermediates (ROI) (14–19) as well as reactive nitrogen intermediates (RNI) (1) is essential for IFN-γ-mediated protection against Listeria and that (b) ROI together with additional still unknown effector mechanisms may be responsible for the anti-Listeria activity of macrophages, whereas IRF1-induced RNI are not limiting.
Table 1. Interferon Signal Transduction

| Interferons | IFN type I (α/β) | IFN type II (γ) |
|-------------|------------------|-----------------|
| Receptors   | Tyk-2            | Tyk-2           |
|             | Jak-1, PKC       | Jak-2           |

Protein kinases

| Only Type I | Type I/II | Only Type II |
|-------------|-----------|--------------|
| ISGF-3α (+) | STAT1 (+) | ISGF-3γ (+)  |
| (STAT1b/STAT2) | (p 91 monomer) | (p 48) |
| IRF-1 (+)  | GAF (+)   | (p 91 dimer) |
|            | ICSBP (-) |               |

Transcription factors

| Only Type I | Type I/II | Only Type II |
|-------------|-----------|--------------|
| Mx-1        | MHC class I | MHC class II |
| IFN type I  |            |              |
| INOS        |            |              |
| antimicrobial activity | | |

IFN-stimulated genes

| Only Type I | Type I/II | Only Type II |
|-------------|-----------|--------------|
| Mx-1        | MHC class I | MHC class II |
| IFN type I  |            |              |
| INOS        |            |              |
| antimicrobial activity | | |

(+) Activator of ISRE-containing genes; (-) Repressor of ISRE-containing genes.

Intermediates (RNI) (20–23) has been analyzed repeatedly; these experiments revealed variations between different experimental setups and analyzed species.

Much information has accumulated about molecular and in vivo biological function of IFNs over the past 15 years (for review see 24, 25). Two different pathways can be distinguished: IFN-α and -β are binding to the type I IFN receptor, whereas IFN-γ binds to the type II IFN receptor. Analysis of gene-targeted mice deficient for only one (12, 26) or both of these receptors (27) have revealed that depending upon the type of the pathogen these two systems are either redundant or complementary in their antimicrobial activity (for review see 28).

A variety of IFN-induced transcription factors have now been described, most of them belonging to the structurally related family of the interferon regulatory factors (IRFs) and some being identical with signal transducers and activators of transcription (STATs; Table 1). It has been revealed that there is an overlap between the two IFN systems at the level of transcription. Whereas some components of the interferon-stimulated gene factor (ISGF) 3α are only induced by type I IFN (29), IFN γ1 (30, 31) and STAT1 (32, 33) can be upregulated via both IFN receptors or by viruses directly (31), and interferon consensus sequence binding protein (ICSBP) is the prototype of a type II IFN-induced factor (34, 35). IRF2 is omitted from Table 1, because the way of its induction has not been clearly elucidated so far. The fact that IRF2 is lacking in ICSBP-/- mice (36) suggests induction via IFN-γ pathway. In vitro transfection systems with reporter genes have revealed that IFN-γ (37) and ICSBP (29) are activating transcription of genes containing the interferon-stimulated response element (ISRE) in their promoter sequence, whereas ICSBP (38) and IRF2 (37) have repressor activity for ISRE-containing genes.

The generation of gene-targeted mice for the transcription factors IFN-γ (39, 40), IRF2 (39) and ICSBP (36) allows to test for their biological role and their induction in different infectious disease models, especially for activation of macrophages. This study therefore evaluated the susceptibility of these mouse strains to Listeria infection in vivo and compared it to some macrophage effector functions upon IFN-γ stimulation in vitro.

Materials and Methods

Mice. Mice deficient for ICSBP (background C57BL6×129Sv), IFN-γ1 (C57BL/6), and IFN-γ receptor (both 129Sv) were generated as described (12, 26, 39). IRF2-deficient mice were kindly provided by Prof. Charles Weissmann (Institute for Molecular Biology, University of Zürich, Switzerland). IFN type I receptor (A129) and IFN type II receptor (G129) mice were obtained from the breeding colony of Prof. M. Aguet (Institute for Molecular Biology, University of Zürich, Switzerland). Control C57BL/6 or 129Sv mice as well as RAG2-/- mice were obtained from the Institute for Laboratory Animals (Veterinary Hospital, Zürich, Switzerland). Mice were used at 6–10 wk of age. The different breedings (except A129 and G129) and all the experiments were performed under conventional (non-SPF) conditions.

Listeria Culture and Infection. Listeria monocytogenes was originally obtained from B. Blanden (Canberra, Australia). It was cultured in trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), and overnight cultures were titrated on tryptose blood agar plates (Difco Laboratories, Detroit, MI). For injection, the original culture was diluted in BSS to inject the indicated dose in 200 μl for i.v. or 30 μl for injection into the footpad (i.f.).

Determination of Bacterial Titers. On the indicated days after infection the whole spleen and one lobe of the liver were taken out and homogenized. Bacterial titers were determined by plating out four serial 10-fold dilutions of organ suspensions on tryptose blood agar plates.

Adoptive Transfer of Spleen Cells. On day 0, spleen single cell suspensions were used to adhere to plastic to deplete them from macrophages. After 2 h 3 × 10⁷ splenocytes were transferred into nonirradiated RAG2-/- recipients. On day 1 the recipients were infected with 2 × 10⁵ CFU of Listeria, and on day 10 liver and spleen were taken out to determine bacterial titers.
Peritoneal macrophage Cultures. Peritoneal macrophages (PEM) of different strains were elicited by injection of 2 ml of a starch solution (2%; M erck, Darmstadt, Germany) intraperitoneally on day –5 and harvested on day 0 by rinsing the peritoneal cavity with 10 ml of cold BSS. The macrophages were washed three times with BSS supplemented with albumin to prevent clumping and then plated on cover slips in 24-well plates. Cells were cultured in IMDM (Gibco, Basel, Switzerland) supplemented with 10% FCS, glutamine, and 50 μg/ml gentamicin, an only extracellularly effective antibiotic. After 2 h of adherence the cover slips were washed twice and put in 1 ml IMDM. The cultures were supplemented with 200 ng/ml LPS, with 200 μ/ml recombinant murine IFN-γ (Genzyme, Cambridge, MA) or a combination of both for 42 h and then used for determination of nitric oxide (NO) production, of respiratory burst or of Listeria killing in vitro. In those cultures used for killing assays, the medium was changed to antibiotic-free after 24 h.

Determination of NO and Respiratory Burst. NO production was measured by determination of nitrite accumulation in PEM cultures with Griess reagent (0.05% N-1-naphthyl-ethylenediamine-dihydrochloride/0.5% sulfanilamide/2.5% phosphoric acid; all from Fluka, Buchs, Switzerland) as described (31). In brief, 50 μl cell culture supernatant was added to 150 μl Griess reagent in 96-well plates and incubated at room temperature for 10 min. Absorption was read with an ELISA reader at 570 and 630 nm.

Respiratory burst was measured as H$_2$O$_2$ production by cultured PEM upon PMA (Sigma, Buchs, Switzerland) stimulation as described (18). In brief, H$_2$O$_2$ secretion of macrophages was quantified by chemiluminescence under presence of horseradish peroxidase type I (Sigma) and 5-amino-2,3-dihydro-1,4-phthalaizinedione (luminol; Sigma) after triggering with 50 ng/ml PMA. Light emission was discontinuously measured over 15 min in a LKB 1251 luminometer (LKB, Bromma, Sweden). Values in mV were converted into pmol H$_2$O$_2$ after calibration by the scopoletin method (42). The cells on the cover slips were counted, and values for NO and H$_2$O$_2$ calculated as nmol/10^6 cells. In Fig. 4 B stimulation index of stimulated versus unstimulated cultures is shown, because absolute values of respiratory burst varied between the experiments. PMA was used to trigger respiratory burst because, as a chemically defined substance, it is the most reliable burst trigger. Also opsonized Listeria, BCG or zymosan could be used with similar capacities to trigger burst (23, 43), but more variability. Because we investigated mouse strains deficient for various IFN-dependent transcription factors, the induction phase of NADPH oxidase during 2 d under IFN-γ stimulation is important in our experiment, whereas the effector phase of the burst trigger is only used as read-out to measure the enzyme activity by providing the best stimulator (PMA) and excess of substrate (luminol).

In Viro Killing Assay. PEM cultures in antibiotic-free medium as described above were infected with 10^5 CFU of Listeria from an overnight culture, washed three times and opsonized with normal human serum. After 15 min of phagocytosis the infected cultures were washed thoroughly, and gentamicin-containing medium and the respective stimulators were added. To determine the infection rate at time point t0, three cover slips were taken out. The remaining ones were further cultured for 7 h to allow digestion of Listeria by macrophages. After 7 h the cover slips were taken out, dried, and then stained according to May-Grünwald-Giemsa. For each mouse strain and each stimulation a total of 600 macrophages were counted under the microscope to determine the number of Listeria-infected cells. The change of infected macrophages was calculated in percentage of the infection rate at t0. For details, see reference 18.

Immunohistochernistry. Mice infected with 5 × 10^3 CFU of Listeria i.v. were sacrificed on day 5 or 6. Organs were immersed in Hank’s BSS and frozen in liquid nitrogen. 5-μm cryosections were fixed with acetone for 10 min, immunostained for Listeria with a polyclonal rabbit anti-Listeria serum (diluted 1/2,000; kindly provided by Professor J. Bille, Institute of Mirobiology, University Hospital of Lausanne, Switzerland) and for iNOS with a polyclonal rabbit anti-iNOS serum (diluted 1/1,500; Biomol, Plymouth, PA). Bound primary antibodies were detected using a sandwich staining procedure. Sections were incubated with alkaline phosphatase-labeled goat anti–rabbit Ig (diluted 1/80; Jackson Laboratories, Bar Harbor, Maine) followed by alkaline phosphatase-labeled donkey anti–goat Ig (diluted 1/80; Tago). Dilutions of secondary reagents were made in TBS containing 5% normal mouse serum. All incubation steps were done for 30 min at room temperature. Alkaline phosphatase was visualized using naphthol AS-Bi phosphate and New Fuchsin (Sigma) as substrate, which yields a red color reaction product. Endogenous alkaline phosphatase was blocked by levamisole. Sections were counterstained with hemalum, and cover slips were mounted with glycerol/gelatin.

### Results

Enhanced Bacterial Replication and Increased Lethality after Listeria Monocytogenes Infection in ICSBP-deficient Mice. Gene targeted mice deficient for ICSBP, IRF1, or IRF2 were infected with various doses of Listeria intravenously or perorally i.f., and survival was monitored daily (Table 2). All ICSBP−/− mice died after injection of a dose as low as 50 CFU of Listeria, whereas five of six IRF2−/− mice succumbed to a dose of 5 × 10^3 CFU within 12 d. In contrast, IRF1−/− and wild-type mice resisted to a dose of 5 × 10^3

| Table 2. Resistance of Different Mouse Strains against Listeria Infection |
|----------------|----------------|----------------|
| Mouse strain | Listeria dose | Route of infection |
| ICGBP−/− | 5 × 10^3 | i.v. | 0/7 | 0/7 |
| | 5 × 10^2 | i.f. | 0/7 | 0/7 |
| | 5 × 10^1 | i.f. | 3/5 | 0/5 |
| IRF1−/− | 5 × 10^3 | i.v. | 11/12* | 5/6 |
| IRF2−/− | 5 × 10^3 | i.v. | 12/13* | 1/6 |
| A129 | 5 × 10^3 | i.v. | 7/7 | 7/7 |
| G129 | 5 × 10^3 | i.v. | 0/9 | 0/9 |
| | 5 × 10^2 | i.f. | 5/6 | 0/6 |
| | 5 × 10^2 | i.f. | 6/6 | 1/6 |
| wt C57BL/6 | 5 × 10^3 | i.v. | 8/8 | 7/8 |
| wt 129/Sv | 5 × 10^3 | i.v. | 9/9 | 9/9 |

*Half of the mice were taken on day 5 to determine titers in livers and spleens, the others monitored until day 12.
Role of Interferon Consensus Sequence Binding Protein in Murine Listeriosis

The role of Interferon Consensus Sequence Binding Protein (ICSBP) in the context of murine listeriosis is highlighted in the study. CFU injected intravenously. However, ICSBP−/− mice on C57BL/6 background and held under strict SPF conditions also showed enhanced susceptibility to Listeria, when injected with a 5–10-times higher dose intraperitoneally (Ferrick, D., and H.W. Mittrücker, personal communication).

Listeria titers in liver and spleen were determined 24 h after a high dose (2 \times 10^5 CFU) and 5 d after an intermediate dose (5 \times 10^3 CFU) of Listeria intravenously. After 5 d, bacterial titers were determined in liver and spleen. Groups of three to four mice were analyzed. Each symbol represents one mouse. One representative experiment of two is shown for each strain.

In vitro gene regulation studies have revealed that ICSBP and IRF2 form complexes which then have a markedly enhanced DNA binding capacity to ISRE compared to the single factors (44). In contrast to IRF1, they are both negative regulators of classical IFN-induced genes. However, both transcription factors are obviously of major importance for early anti-Listeria immune responses. Since it has been shown that ICSBP−/− mice do not express IRF2 (although the gene is intact [36]), this can explain the even more drastic phenotype of ICSBP−/− compared to IRF2−/− mice, because they represent functionally a double knock-out phenotype.

Competition of different transcription factors of the IRF family at the DNA binding level has been demonstrated in in vitro studies (45). It was therefore possible that lack of IFN type I–induced transcription factors would lead to increased activity of IFN type II–induced factors. To test this in vivo, we infected mice deficient for the type II (G129) or the type I (A129) IFN receptor and control mice (wt129) with 5 \times 10^3 CFU of Listeria and determined bacterial titers on day 5 (Fig. 1 B). As demonstrated earlier (12), G129 mice showed drastically enhanced bacterial replication and lethality (Table 2), whereas A129 eliminated the pathogen even more efficiently than wt129 mice. This result suggests that competition between the two signaling pathways at the transcription factor level occurs. IFN type II–induced transcription factors (and among them especially ICSBP) may compensate for the lack of IFN type I–induced factors in the A129 mouse, thereby conferring even higher resistance to Listeria infection than in control mice. Because early Listeria clearance in nude mice (46) has been shown to be more efficient than in immunocompetent controls because their macrophages are preactivated (probably by LPS derived from normal intestinal bacteria leaking...
into circulation), this may be an additional factor explaining the results in A129 mice and also the difference between IRF1<sup>2/2</sup> mice held under conventional versus SPF conditions.

Capacity of Adoptively Transferred ICSBP<sup>2/2</sup> Spleen Cells to Correct Immunodeficiency of RAG2<sup>2/2</sup> Mice. Our results of anti-

Listeria immune response in ICSBP<sup>2/2</sup> mice suggested a major defect of IFN-γ-induced macrophage function, because lymphocytes, especially cytotoxic T cells, but also B cells, had been shown to function almost normally after viral infections (36, 39). Therefore macrophage functions were tested in vivo by adoptive transfer experiments and in vitro by PEM cultures.

RAG2<sup>2/2</sup> mice are devoid of functional T and B cells, but have normal macrophages and natural killer cells (47). When infected with an intermediate dose of Listeria, they are able to control bacterial replication comparable to nude mice (46), but cannot eliminate the pathogen. To test whether ICSBP-deficient T cells could develop normal specific anti-Listeria immunity, we transferred on day 0 macrophage-depleted ICSBP<sup>2/2</sup> spleen cells into RAG2<sup>2/2</sup> mice, challenged them with a high dose of Listeria (2 × 10<sup>5</sup> CFU i.v.) on day 1 and evaluated Listeria titers in liver and spleen on day 10 to look for efficiency of the specific immune response. As a positive control normal spleen cells were transferred. The result (Fig. 2) revealed no difference of Listeria counts between recipients of ICSBP<sup>-/-</sup> and ICSBP<sup>+/-</sup> spleen cells; in contrast RAG2<sup>2/2</sup> mice that did not receive spleen cells exhibited 100- (spleen) to 1,000-fold (liver) higher bacterial counts. This result indicates that ICSBP<sup>-/-</sup> splenocytes (especially the mutant T cells) were able to promote elimination of Listeria as successfully as normal lymphocytes in cooperation with the intact macrophage compartment of the RAG2<sup>2/2</sup> mouse.

Analysis of Listeria KIlling in an In Vitro PEM Culture. To evaluate listericidal activity of macrophages of the different mutant mouse strains, we tested PEM in an in vitro killing assay. ICSBP<sup>-/-</sup>, IRF1<sup>-/-</sup>, and IRF2<sup>-/-</sup> PEM were elicited by starch injection intraperitoneally, plated onto cover slips and cultured as described in Materials and Methods. After 42 h the cultures infected with Listeria in vitro, and the number of infected cells determined at time point t<sub>0</sub> and after 7 h of infection. The results (Fig. 3) show that...
Figure 5. Listeria replication and iNOS expression in the liver after infection with Listeria. ICSBP−/− (D–H), IRF1−/− (L, M), IRF2−/− (N, O), and control mice (A–C, I, K) were infected with $5 \times 10^3$ CFU of Listeria. After 5 or 6 d liver and spleen were taken out. Conventional HE staining (A, D) and immunohistology for Listeria (B, E, I, L, N) and iNOS (C, F, H, K, M, O) was performed using polyclonal primary antibodies. Magnifications (A, D), $\times 35$, (B, C, E, F, I–O), $\times 60$ (G, H), $\times 220$. 
In a recent study, researchers investigated the role of IRF2 in LPS-induced respiratory burst in mice. The findings revealed that the IRF2 gene plays a crucial role in the host defense mechanism against Listeria infection. IRF2-deficient mice showed a significant reduction in NO production upon IFN-γ stimulation, which is essential for the macrophage-mediated killing of Listeria. The study also demonstrated that IRF2 regulates the expression of iNOS and ROI, which are responsible for the toxic effect on infected cells during murine listeriosis. The authors concluded that IRF2 is a key player in the innate immune response against Listeria, and its deficiency leads to an enhanced susceptibility to infection. This study highlights the importance of understanding the molecular mechanisms underlying host-pathogen interactions in order to develop effective strategies for the treatment of listeriosis.
Role of Interferon Consensus Sequence Binding Protein in Murine Listeriosis

It seems to play an important role in leishmaniasis (55, 56) and tuberculosis (48), but has no limiting effect in toxoplasmosis (57) and listeriosis (this study, references 58, 59).

Our results of the analysis of mice deficient for IFN type I or II receptors revealed surprisingly that the type I IFN receptor-deficient mice (A129) were better protected than their normal littermates. This finding may reveal in vivo competition of transcription factors of both signaling pathways at the DNA binding level (45) suggesting that absence of the type I system enhances function of the type II system and concurrently Listeria protection. A potentiating effect of LPS leaking through from intestinal bacteria leading to macrophage preactivation may be involved.

From the analysis of TNF receptor 1-deficient (13) mice it is known that TNF-α is a second important cytokine for protection against Listeria. It is produced by macrophages upon infection with Listeria and may act via the following two pathways: (a) the SCID model revealed that TNF-α is necessary for activation of NK cells that then produce IFN-γ to further induce TNF receptor 1 and TNF-α expression (60, 61) and macrophage effector functions (4); (b) macrophage- or γδ T cell-derived TNF-α may act in an autocrine or paracrine fashion directly on macrophages to activate anti-Listeria effector molecules. Involvement of the ICSBP/IRF2 complex in the signaling cascade of the TNF receptor could theoretically explain the described in vivo findings, but this has not been formally demonstrated so far. In addition, another transcription factor, NF-IL6, which can be upregulated by LPS/CD14 and also by TNF-α (62), is important for clearance of Listeria as demonstrated in the NF-IL6-deficient mice (63).

From our findings in three different mouse strains and from the published literature, the following model of signaling events in activation of anti-Listeria immunity may be proposed (Fig. 6): after activation of IFN receptors various tyrosine kinases are induced and STAT proteins phosphorylated (Table 1); they regulate the induction and activation of transcription factors of the IRF family among which the exclusively IFN-γ-dependent ICSBP mediates protection against Listeria. Two major questions remain open: (a) What molecules are involved in the signalling of the TNF receptor that could explain its importance for anti-Listeria immunity (ICSBP, NF-IL6, other transcription factors, indirect effect via NK cell activation)? (b) How do macrophages kill Listeria? Our results, but also the published ones on IFN type II receptor- and iNOS-deficient mice, rather argue against RNI production being a limiting factor. ROI may be involved since ICSBP- and NF-IL6-deficient mice had reduced respiratory burst, and this correlated with high susceptibility to Listeria infection. But still there may be a potential third mechanism involved to explain the drastic phenotype of ICSBP−/− mice. Studies on iron metabolism of peritoneal macrophages (64) and murine β-thalassemia (65) suggested that iron scavengers lead to enhanced, and iron overload to reduced, resistance to Listeria by direct interference with the essential bacterial iron metabolism. Whether IFN-γ- and/or TNF-α-mediated enhancement of iron-binding proteins can explain resistance to murine listeriosis remains to be investigated.

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Figure 6. Proposed possible signaling events in macrophages after Listeria infection. IFN-γ-induced ICSBP is of crucial importance for protection in murine listeriosis, probably partly via ROI production. G-CSF (66) plays a minor and IRF1-induced RNI (23) no limiting role for bacterial resistance. A potentiating effect or an additional factor is postulated. How ICSBP, NF-IL6 or other transcription factors are involved in TNF-mediated protection against Listeria remains to be determined.
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