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

By Thomas Fehr,* Gabriele Schoedon,‡ Bernhard Odermatt,§ Thomas Holtschke,‖ Markus Schneemann,‡ Martin F. Bachmann,*¶ Tak W. Mak,¶ Ivan Horak,‖ and Rolf M. Zinkernagel*

*From the *Institute of Experimental Immunology, University Hospital, CH-8091 Zürich, Switzerland; ‡Department of Internal Medicine, University Hospital, CH-8091 Zürich, Switzerland; §Department of Pathology, University Hospital, CH-8091 Zürich, Switzerland; ‖Department of Molecular Genetics, Research Institute of Molecular Pharmacology, D-12207 Berlin, Germany; and ¶Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, M4X1K9, Canada*

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 grampositive 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)[1] (14–19) as well as reactive nitrogen

[1]*Abbreviations used in this paper:* ICSBP, interferon consensus sequence binding protein; i.f., into the footpad; iNOS, inducible nitric oxide synthase; IRF1/2, interferon regulatory factor 1/2; ISGF, interferon-stimulated gene factor; ISRE, interferon-stimulated response element; NO, nitric oxide; PEM, peritoneal macrophage; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; STAT, signal transducers and activators of transcription.

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Table 1. *Interferon Signal Transduction*

Interferons		
IFN type I (α/β)		IFN type II (γ)
Receptors		
Type I IFN-R		Type II IFN-R
Protein kinases		
Only Type I	Type I/II	Only Type II
Tyk-2	Jak-1, PKC	Jak-2
Transcription factors		
Only Type I	Type I/II	Only Type II
ISGF-3a (+)	STAT 1 (+)	ISGF-3γ (+)
(= *STAT1b/STAT2*)	(= *p 91 monomer*)	(= *p48*)
	IRF-1 (+)	GAF (+)
		(= *p91 dimer*)
		ICSBP (−)
IFN-stimulated genes		
Only type I	Type I/II	Only Type II
Mx-1	MHC class I	MHC class II
	IFN type I	
	iNOS	
	antimicrobicidal 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), IRF1 (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 IRF1 (37) and ISGF3 (29) are activating transcription of genes containing the interferon-stimulated response element (ISRE) in their promotor sequence, whereas ICSBP (38) and IRF2 (37) have repressor activity for ISRE-containing genes.

The generation of gene-targeted mice for the transcription factors IRF1 (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), IRF1(129Sv), IRF2 (C57BL/6), IFN I and II receptor (both 129Sv) were generated by gene targeting in embryonic stem cells as described (12, 26, 36, 39). IRF1-deficient mice were kindly provided by Prof. Charles Weissmann (Institute for Molecular Biology I, 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 I, 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 let to adhere to plastic to deplete them from macrophages. After 2 h 3×10^7 splenocytes were transferred into nonirradiated RAG2$^{-/-}$ recipients. On day 1 the recipients were infected with 2×10^5 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%; Merck, 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 stimulated with 200 ng/ml LPS, with 200 U/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-ethylene-diamine-dihydrochloride/0.5% sulfanilamide/2.5% phosphoric acid; all from Fluka, Buchs, Switzerland) as described (41). 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_2O_2 production by cultured PEM upon PMA (Sigma, Buchs, Switzerland) stimulation as described (18). In brief, H_2O_2 secretion of macrophages was quantified by chemiluminescence under presence of horseradish peroxidase type I (Sigma) and 5-amino-2,3-dihydro-1,4-phthalazinedione (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_2O_2 after calibration by the scopoletin method (42). The cells on the cover slips were counted, and values for NO and H_2O_2 calculated as nmol/10^5 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 eccess of substrate (luminol).

In Vitro Killing Assay. PEM cultures in antibiotic-free medium as described above were infected with 10^7 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 t_0, 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 t_0. For details, see reference 18.

Immunohistochemistry. 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 Microbiology, 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 peripherally 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	Surviving/infected d6	Surviving/infected d12
	CFU			
$ICSBP^{-/-}$	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
	5×10^1	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.

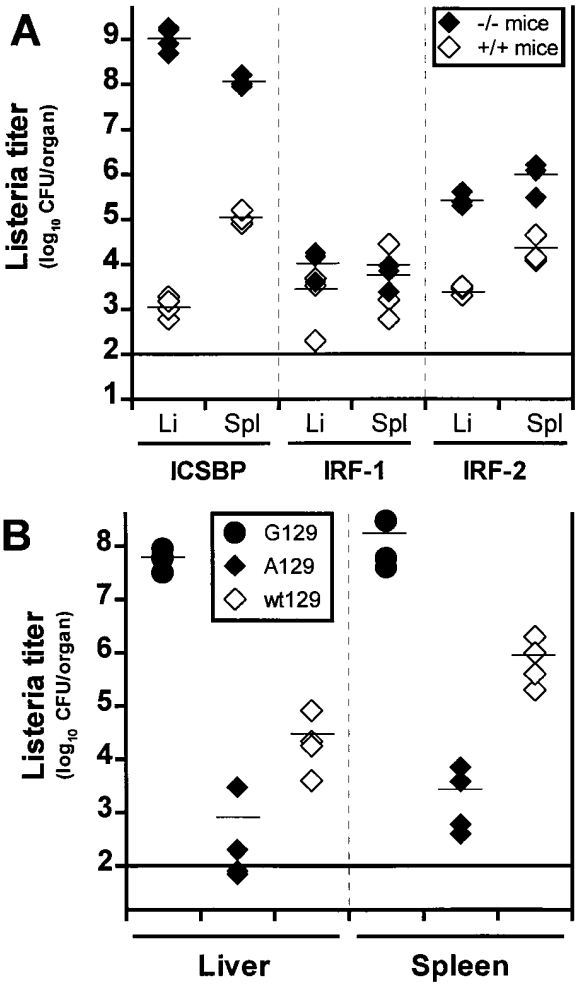

Figure 1. *Listeria* titers in organs of different mouse strains deficient for molecules in the IFN signaling pathway. (*A*) Mice deficient for IFN-related transcription factors (ICSBP, IRF1, IRF2; *filled diamonds*) or (*B*) mice lacking functional IFN type I (A129; *filled diamonds*) or type II (G129; *filled circles*) receptors and control littermates (*open diamonds*) were infected with 5×10^3 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.

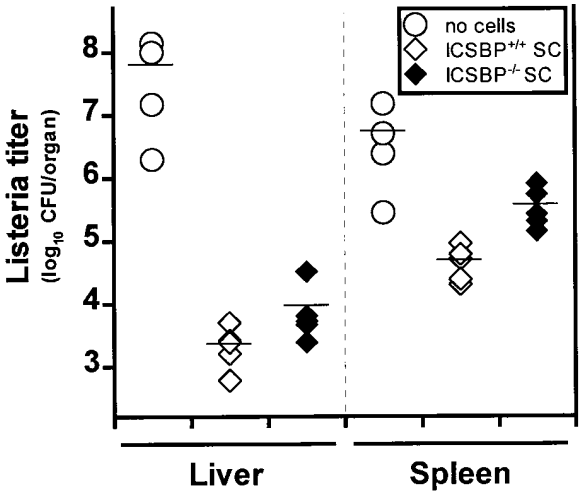

Figure 2. Capacity of ICSBP$^{-/-}$ spleen cells to transfer *Listeria* resistance to RAG2$^{-/-}$ mice. Non adherent spleen cells of ICSBP$^{+/+}$ (*open diamonds*) or ICSBP$^{-/-}$ (*filled diamonds*) mice were transferred to RAG2$^{-/-}$ mice. After 24 h the recipients as well as untreated RAG2$^{-/-}$ mice (*open circles*) were infected with 2×10^5 CFU of *Listeria*, and bacterial titers were determined on day 9 after infection to assess overall protection. Groups of four to five recipient mice were used. Each symbol represents one mouse. One of three comparable experiments is shown.

CFU injected intravenously. However, IRF1$^{-/-}$ 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 (2×10^5 CFU) and 5 d after an intermediate dose (5×10^3 CFU) of *Listeria* injected intravenously. In the first 24 h, when neutrophils seem to play an important role (2), there was almost no titer difference between the three strains and only a 10-fold difference compared to control mice (data not shown). However, after 5 d when activated macrophages are essential for control of *Listeria* infection, ICSBP$^{-/-}$ and IRF2$^{-/-}$ showed between 10^2- and 10^6-fold higher titers in liver and spleen, whereas IRF1$^{-/-}$ mice controlled *Listeria* replication comparable to controls (Fig. 1 *A*). 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 5×10^3 CFU of *Listeria* and determined bacterial titers in liver and spleen 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

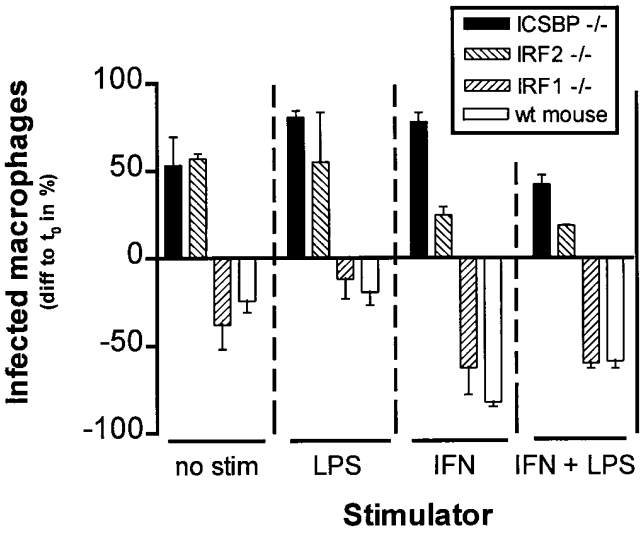

Figure 3. Elimination of *Listeria* from a PEM culture stimulated with LPS, IFN-γ or both. PEM of mice deficient for different IFN-related transcription factors were elicited with starch solution, harvested after 5 d and put into culture on cover slips under presence of the indicated stimulators. After 42 h (at time point t_0) they were infected with *Listeria* and allowed to digest the bacteria during 7 h. Then, cover slips were taken out, dried and May-Grünwald-Giemsa stained. The number of infected macrophages was counted under the microscope, compared to the infection rate at t_0 and expressed as difference in percentage of infected cells. Error bars represent standard deviation. Examples for absolute numbers: the number of infected macrophages at t_0 was between 80 and 106/200 for all strains; the number of infected cells in IFN-γ-stimulated cultures after 7 h of digestion was 19/200 for wild-type and 168/200 for ICSBP$^{-/-}$ mice.

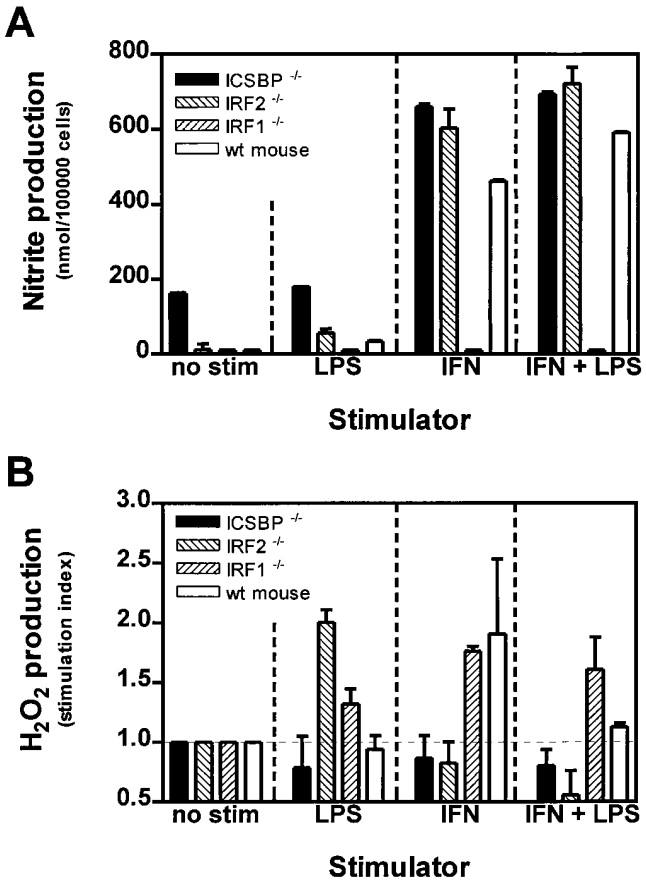

Figure 4. NO production and respiratory burst in PEM cultures. PEMs of mice deficient for different IFN-related transcription factors were cultured as described in Fig. 3. (*A*) After 42 h NO production was measured by determination of nitrite accumulation in the culture supernatants by using Griess reagent. Values are calculated as nmol nitrite per 10^5 cells. One of three independent and comparable experiments is shown. (*B*) Respiratory burst capacity of the same PEMs was determined by measuring H_2O_2 production by chemiluminescence after stimulation with PMA. Values were first calculated as nmol H_2O_2 per 10^5 cells, and then a stimulation index of stimulated versus unstimulated cultures was determined (stimulation index of unstimulated culture = 1). Error bars indicate standard deviation.

into circulation), this may be an additional factor explaining the results in A129 mice and also the difference between IRF1$^{-/-}$ mice held under conventional versus SPF conditions.

Capacity of Adoptively Transferred ICSBP$^{-/-}$ Spleen Cells to Correct Immunodeficiency of RAG2$^{-/-}$ Mice. Our results of anti-*Listeria* immune response in ICSBP$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ spleen cells into RAG2$^{-/-}$ mice, challenged them with a high dose of *Listeria* (2×10^5 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 and as a negative control no spleen cells were transferred. The result (Fig. 2) revealed no difference of *Listeria* counts between recipients of ICSBP$^{-/-}$ and ICSBP$^{+/+}$ spleen cells; in contrast RAG2$^{-/-}$ mice that did not receive spleen cells exhibited 100- (spleen) to 1,000-fold (liver) higher bacterial counts. This result indicates that ICSBP$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$, IRF1$^{-/-}$, and IRF2$^{-/-}$ PEM were elicited by starch injection intraperitoneally, plated onto cover slips and cultured as described in Materials and Methods. After 42 h the cultures were infected with *Listeria* in vitro, and the number of infected cells determined at time point t_0 and after 7 h of infection. The results (Fig. 3) show that

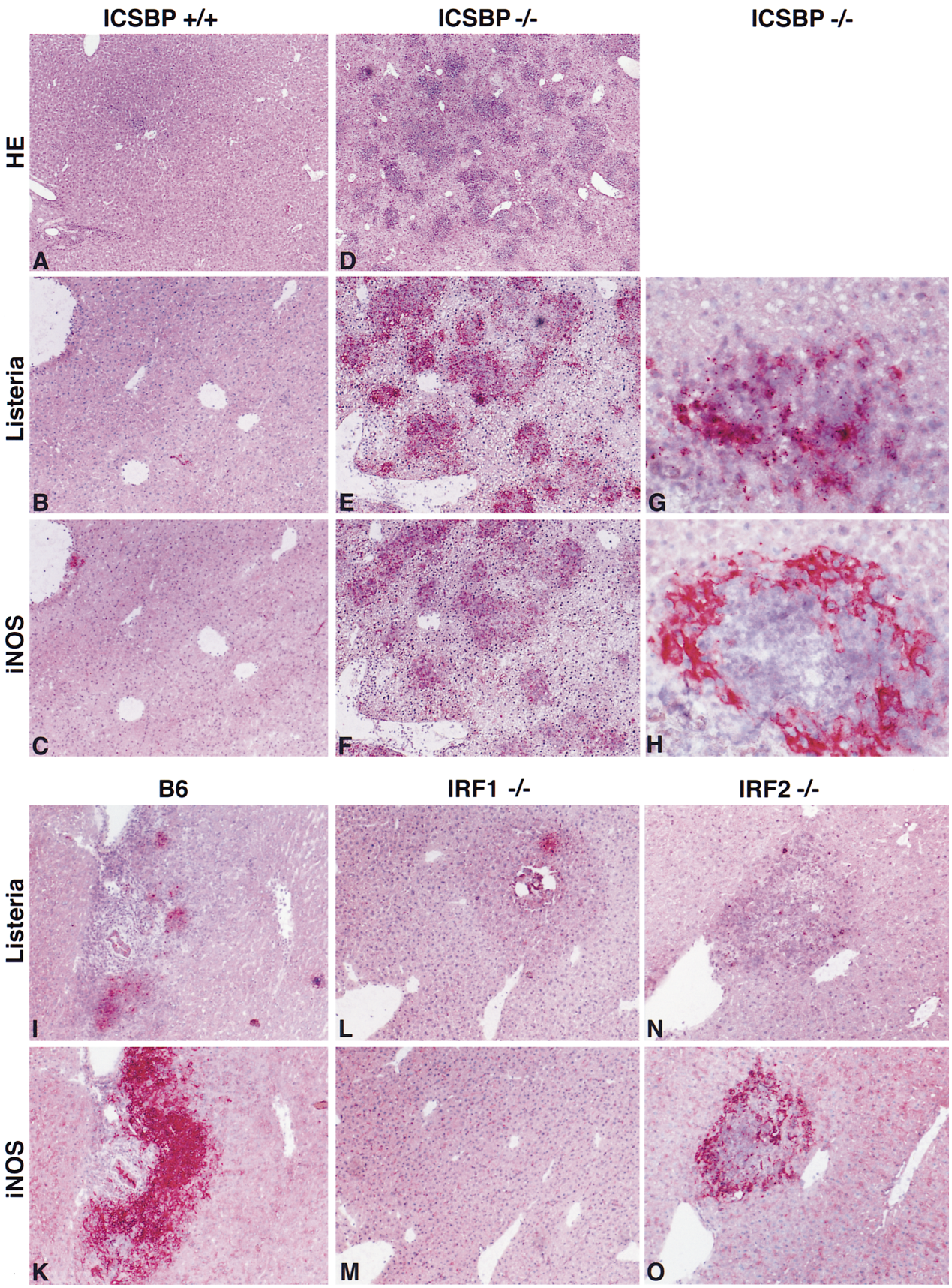

Figure 5. *Listeria* replication and iNOS expression in the liver after infection with *Listeria.* ICSBP$^{-/-}$ (*D–H*), IRF1$^{-/-}$ (*L*, *M*), IRF2$^{-/-}$ (*N*, *O*), and control mice (*A–C*, *I*, *K*) were infected with 5×10^3 CFU of *Listeria*. After 5 or 6 d liver and spleen were taken out. Conventional HE staining (*A*, *D*) and immunohistology for *Listeria* (*B*, *E*, *G*, *I*, *L*, *N*) and iNOS (*C*, *F*, *H*, *K*, *M*, *O*) was performed using polyclonal primary antibodies. Magnifications: (*A*, *D*), ×35, (*B*, *C*, *E*, *F*, *I–O*), ×60 (*G*, *H*), ×220.

PEM of ICSBP$^{-/-}$ and, to a lesser degree, IRF2$^{-/-}$ mice allowed enhanced replication of *Listeria*, whereas PEM of IRF1$^{-/-}$ and normal mice were able to reduce the bacterial load in these macrophage cultures. Also the number of bacteria per macrophage was higher in ICSBP$^{-/-}$ mice (mostly more than 10 bacteria/cell) compared to their controls (0–4 bacteria/cell), revealing some macrophages with plenty of *Listeria* and typical comet tails (18). This finding confirms the defect in macrophage effector function, which correlates with the in vivo susceptibility of these mouse strains to *Listeria* (ICSBP$^{-/-}$ >IRF2$^{-/-}$ >IRF1$^{-/-}$).

Evaluation of NO Synthesis and Respiratory Burst as Anti-Listeria Effector Functions of Macrophages. The effector mechansim responsible for listericidal properties of macrophages is widely studied and still not clearly defined. ROI (14–19) as well as RNI (20–23) have been proposed to be of major importance. Therefore NO production (measured as nitrite accumulation in culture medium) and respiratory burst upon PMA stimulation (H_2O_2 production measured by chemiluminescence) were tested in PEM cultures stimulated with LPS and/or IFN-γ as described in Materials and Methods. NO production (Fig. 4 *A*) was absent in IRF1$^{-/-}$ mice confirming earlier results that iNOS cannot be induced by IFN type I or II combined with LPS and/or TNF in the absence of IRF1 (48, 49). In contrast, iNOS activity was normal in ICSBP$^{-/-}$ and in IRF2$^{-/-}$ mice as well as in G129 mice (50). This finding in IRF2$^{-/-}$ mice differs from recently published results (51). The high susceptibility of the ICSBP$^{-/-}$ and G129 strains to *Listeria* infection in vivo and in vitro indicates that the NO effector mechanism does not play a limiting role in *Listeria* clearance. The phenotype of IRF1$^{-/-}$ mice found here is compatible to the published results of *Listeria* infection in iNOS-deficient mice (23). These mice showed also a slightly enhanced bacterial replication (10–100-fold) and a higher lethality to *Listeria* infection, but only after injection of 6×10^4 CFU i.v. The LD_{50} of iNOS-deficient mice was only a factor 10 lower compared to their normal littermates, whereas in the case of ICSBP$^{-/-}$ mice this difference is more than 10,000-fold (Table 1; LD_{50} for C57BL/6 mice is $\sim 3 \times 10^5$ CFU [52]). The fact that iNOS induction in the susceptible strains (ICSBP$^{-/-}$, IRF2$^{-/-}$) was higher than in controls (Figs. 4 *A*, and 5) could even indicate that NO may have a toxic effect on infected cells during murine listeriosis.

With respect to respiratory burst upon PMA challenge, all mouse strains showed comparable basal activity of unstimulated cultures; however, in ICSBP$^{-/-}$ and IRF2$^{-/-}$ mice ROI production could not be stimulated by IFN-γ (Fig. 4 *B*) and was 3–5 min delayed compared to control mice (data not shown). This finding suggests that deficient ROI production might be partially responsible for the high susceptibility of ICSBP$^{-/-}$ mice to *Listeria*, but it cannot fully explain the drastic phenotype of these mice. At least a third effector pathway not yet known may have to be evoked to explain this phenotype (see Discussion). In addition, the fact that LPS-induced respiratory burst was enhanced in IRF2$^{-/-}$ mice correlates inversely with a recently published finding of high IRF2 levels in LPS-hyporesponsive mouse strains (53). Thus, IRF2 may mediate the macrophage deactivating effect of LPS (42).

Expression of iNOS in Liver and Spleen after Listeria Infection of ICSBP$^{-/-}$ and IRF2$^{-/-}$ Mice, but not of IRF1$^{-/-}$ Mice. Apart from macrophages hepatocytes are a major target cell in murine listeriosis. They are infected by direct cell-to-cell spread of the pathogen that is able to associate with actin filaments of the cytoskeleton (54). Hepatocytes can produce NO. Therefore, to see whether the phenotype of ICSBP$^{-/-}$ and IRF2$^{-/-}$ mice is due to a localized inability of iNOS expression in the liver, immunohistological analysis of iNOS expression in liver (Fig. 5) and spleen (not shown) after *Listeria* infection was performed. Mice of the three gene-targeted and control strains were infected with *Listeria* (5×10^3 CFU i.v.). On day 5 or 6 liver and spleen were taken, cryosectioned, and then immunostained for *Listeria* and for iNOS with an appropriate polyclonal rabbit antiserum. Induction of iNOS comparable to wild-type mice could be demonstrated in all strains except IRF1$^{-/-}$ (Fig. 5, *C*, *F*, *K*, *M*, *O*). It was abundant in regions where *Listeria* and abcesses were found (detail shown in Fig. 5, *G* and *H*). In addition fulminant *Listeria* proliferation in liver (Fig. 5 *E*) and spleen of ICSBP$^{-/-}$ mice was found with accompanying tissue destruction (Fig. 5 *D*) correlating with the high bacterial titers (Fig 1). This analysis also shows that the susceptibility of ICSBP$^{-/-}$ and IRF2$^{-/-}$ mice to *Listeria* is not due to inefficient NO production in the liver. In contrast, IRF1$^{-/-}$ mice were well protected and did not express iNOS in the liver. This cannot be explained by earlier decline of the bacterial load because these mice had equal *Listeria* titers in the liver as wild-type mice 5 d after infection (Fig. 1 *A*). The same analysis was performed on spleen sections with comparable results (not shown).

Discussion

The type II IFN system has been shown to be of crucial importance for immunity against *Listeria*, because mice deficient for IFN-γ (11) or the type II IFN receptor (12) are highly susceptible to this bacterium. However, the intracellular signalling pathway and the final effector mechanisms involved in *Listeria* clearance are only incompletely understood. The presented analysis of three gene-targeted mouse strains deficient of ICSBP, IRF1, or IRF2 with respect to their capacity to survive and eliminate *Listeria* in vivo and in vitro and to the ability of their macrophages to respond with ROI or RNI production upon IFN-γ stimulation suggests three major conclusions: (*a*) ICSBP/IRF2 complex (but not IRF1) is of crucial importance for murine innate immunity to *Listeria* in vivo and in vitro, (*b*) iNOS induction and NO synthesis play no limiting role for anti-*Listeria* activity of macrophages upon IFN-γ stimulation, (*c*) stimulation of ROI production by IFN-γ together with a postulated third yet unknown effector pathway in macrophages may be responsible for protection in the early phase of primary *Listeria* infection. The role of NO for antimicrobial activity of macrophages has been tested in other

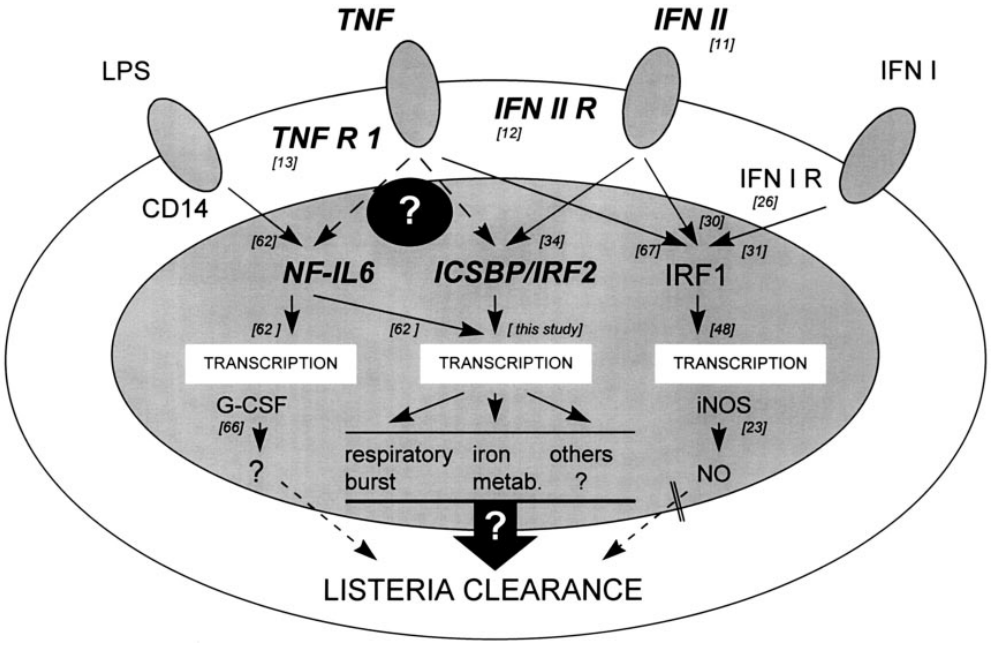

Figure 6. Proposed possible signaling events in macrophages after *Listeria* infection. IFN-γ–induced ICSBP is of crucial importance for protection in murine listeriosis, probably partly via ROI production. G-CSF (66) plays a minor and IRF1-induced RNI (23) no limiting role for bacterial resistance. 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.

infectious model systems. 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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Address correspondence to Thomas Fehr, Institute of Experimental Immunology, University Hospital, Schmelzbergstrasse 12, CH-8091 Zurich, Switzerland.

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