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*J Immunol* 2002; 168:3428-3436; doi: 10.4049/jimmunol.168.7.3428

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The IFN-Inducible Golgi- and Endoplasmic Reticulum-Associated 47-kDa GTPase IIGP Is Transiently Expressed During Listeriosi

Jens Zerrahn,†* Ulrich E. Schaible,* Volker Brinkmann,† Ute Guhlich,* and Stefan H. E. Kaufmann*

Members of the 47-kDa GTPase family are implicated in an IFN-γ-induced, as yet unclear, mechanism that confers innate resistance against infection with intracellular pathogens. Overt immunological parameters are apparently uncompromised in mice deficient for individual members and the prototype of this family, IGTP, localizes to the endoplasmic reticulum. This suggests that these GTPases are involved in intracellular defense. We analyzed the expression of the 47-kDa GTPase cognate, IIGP, in splenic sections from mice infected with the intracellular pathogen Listeria monocytogenes by immunohistochemistry. An early transient IIGP induction was observed revealing the IFN-γ responsiveness of cellular subcompartments within the spleen in early listeriosis. Marginal metallophilic macrophages and endothelial cells within the red and white pulp strongly expressed IIGP, while other splenocytes remained negative. In vitro analyses show that both type I and type II IFNs are prime stimuli for IIGP induction in various cells, including L. monocytogenes-infected or LPS-stimulated macrophages, endothelial cells, and activated T cells. Contrary to the subcellular localization of IGTP, IIGP was predominantly associated with the Golgi apparatus and also localizes to the endoplasmic reticulum. We conclude that IIGP exerts a distinct role in IFN-induced intracellular membrane trafficking or processing. The Journal of Immunology, 2002, 168: 3428–3436.

The pleiotropic cytokine IFN-γ is central to protective immunity against various microbial pathogens. It induces a large number of different cellular programs in both hematopoietic and nonhematopoietic cells that contribute to distinct aspects of innate and adaptive immunity (1–3). Recently, the analysis of IFN-γ-regulated genes led to the identification of the 47-kDa GTPase family, which to date comprises six known members, namely, IRG-47, LGIR-47, TGTP, GTPI, IGTP, and IIGP (4–9). In vitro, immune as well as nonimmune cells respond upon IFN-γ stimulation with a strong transcriptional induction of these 47-kDa GTPases, and evidence has been provided that these genes quantitatively dominate the IFN-γ-induced gene expression (4). Correspondingly, IFN-γR-deficient mice fail to up-regulate expression upon infection with Listeria monocytogenes (4).

The exact molecular functions as well as the overall mechanisms governed by these GTPases are unknown. IGTP localizes to the cytoplasmic surface of the endoplasmic reticulum (ER), suggesting an involvement in the processing of proteins or lipids traversing the ER compartment or more likely in the vesicular transport emanating from the ER (10). Recently, the analysis of IGTP-deficient mice provided first clues about the role of the 47-kDa GTPase family in immunity against intracellular pathogens. These knockout mice displayed significant susceptibility to acute Toxoplasma gondii infections (11). In contrast, normal resistance was maintained against other intracellular pathogens, like L. monocytogenes and murine CMV. Remarkably, no alterations in cytokine expression (IL-12, IFN-γ, TNF-α) or NO production were detected, and overt immune cell functions apparently were not compromised. LGIR-47- and IRG-47-deficient mice display a comparable but distinct phenotype, in that they show differential susceptibility against various intracellular pathogens (12). Although these results suggest that these 47-kDa GTPases contribute to innate immune mechanisms, their differential impact on different pathogens raises the question about distinct mechanistic aspects and effector functions mediated by these GTPases. Elucidation of these issues will provide insights into important cellular immune processes of host defense.

We have identified IIGP within a differential cDNA library, yielded by cDNA subtraction of splenocytes from Listeria-infected mice with reference to naive cells. To gain further insights into its function, an anti-IIGP mAb was raised. Immunohistological analysis of splenic sections of mice infected with L. monocytogenes revealed transient up-regulation of the IIGP protein predominantly in the early phase of infection. IIGP-expressing splenocytes were identified as marginal metallophilic macrophages and endothelial cells. Furthermore, analysis of the subcellular localization of IIGP provided evidence that a fraction of cellular IIGP is associated with the ER, while a predominant fraction colocalized with the Golgi apparatus. Stimuli capable of up-regulating IIGP mRNA expression in vitro were analyzed revealing type I and type II IFNs as the prime inducers. Accordingly, L. monocytogenes-infected macrophages produce type I IFN, leading to an induction of IIGP expression.

Materials and Methods

Mice

IFNα/βR−/− mice, kindly provided by Dr. R. M. Zinkernagel (University Hospital, Zurich, Switzerland), have been described previously (13). C57BL/6

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Received for publication October 10, 2001. Accepted for publication January 30, 2002.

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2 Abbreviations used in this paper: ER, endoplasmic reticulum; BMM, bone-marrow derived macrophage; DC, dendritic cell; MZ, marginal zone; p.i., postinfection; PALS, periarteriolar lymphatic sheath; IDC, interdigitating DC; Lamp, lysosome-associated membrane protein.
(B6) and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions at our animal facilities at the Federal Institute for Health Protection of Consumers and Veterinary Medicine. Mice were infected i.v. with $5 \times 10^7 L. monocytogenes strain EGD.

**Cells, tissue culture, and stimulation**

Bone marrow-derived macrophages (BMM) from B6 and IFN-α/βR−/− mice were prepared as described elsewhere (14) and BMM at days 7–9 were used for all experiments. The BMM were cultured in DMEM (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, 5% horse serum, 20% L cell-conditioned medium, 5 $\times 10^{-3} M$ 2-ME, 200 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics. The same medium, but without horse serum and L cell-conditioned medium, was used for all other cells in culture. T cells were purified from lymph nodes of B6 mice by nylon wool passage followed by depletion of residual MHC class II cells with magnetic beads (Miltenyi Biotec, Auburn, CA). The endothelium cell line Ender 1 has been described elsewhere (15). Reagents used for stimulation were: 500 ng/ml LPS from Escherichia coli J5-Re mutant (Sigma-Aldrich, St. Louis, MO), 500 U/ml recombinant murine IFN-γ (kindly provided by Dr. G. Adolf, Bender, Vienna, Austria), 1000 U/ml recombinant murine IFN-α (kindly provided by Dr. C. Weissmann, University of Zurich, Zurich, Switzerland), 10 ng/ml recombinant murine IL-1β (Genzyme, Cambridge, MA), 10 ng/ml recombinant murine IL-1α (Chiron, Emeryville, CA), 0.5 ng/ml recombinant TNF-α (Peprotech, London, UK), 2 $\times 10^7$ CFU/ml heat-killed listeriae. Cells were infected with viable *L. monocytogenes* strain EGD at a 10:1 bacteriaceel ratio ($3 \times 10^6$ CFU/ml) for 30 min. Extracellular bacteria were removed by repeated washes with medium and further incubation in 10 $\mu$g/ml gentamicin-containing medium for 60 min. Subsequently, infected cells were incubated in medium containing 5 $\mu$g/ml gentamicin for the time periods indicated. Purification of infected cells was conducted in culture wells ($3 \times 10^6$ cells/well) of six-well plates (Nunc, Roskilde, Denmark) that had been coated with 10 $\mu$g/ml anti-CD3 (145-2C11) and 10 $\mu$g/ml anti-CD28 (37.51) for 1 or 2 days. Preparation of Nonidet P-40 lysis buffer and of whole-cell lysates, SDS-PAGE, Western blotting, and immunoprecipitation were performed according to standard protocols (16).

**Antibodies**

mAbs used were: anti-TCRβ (H57-597, ATCC HB-218; American Type Culture Collection (ATCC), Manassas, VA), anti-TCRγ (GL.3), anti-Mac-1 (M1/70), anti-Mac-3 (M3/84, ATCC TIB-168), anti-F4/80 (ATCC HB-198), anti-CD11c (HL3; BD Pharmingen, San Diego, CA), anti-LY-6G (RB6-8C5), anti-LY-6C (AL-21, BD Pharmingen), MOMA-1 (BMA, Augst, Switzerland), ER-TR9 (BMA), BMDM-1 (BMA), anti-endothelial cells (B76, kindly provided by Drs. A. Hamann and S. Sybre, Deutsche Rheumatics Association, Berlin, Germany) (17), anti-B220 (RA3-6B2), anti-MHC cl.II (M5/114, ATCC TIB-120), and anti-Fcγ (2.4G2, ATCC HB-197). For generation of the anti-IIGP mAb SD9, a full-length cDNA was expressed in the prokaryotic expression vector pSET (Invitrogen, San Diego, CA). Soluble His-tagged IIGP protein expressed in *E. coli* BL21(D3) was affinity purified, emulsified in ABM/SN adjuvants (Linaris, Bettingen, Germany), and used for immunization of BALB/c mice according to the recommendation of the manufacturer. Immune splenocytes were fused with SP2/0 cells (ATCC CRL 1646) and single-cell cloned hybridomas were established according to standard protocols (16).

**Subcellular location**

Cells were fixed with 4% (v/v) paraformaldehyde in PBS for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked with 10% FCS, 0.05% BSA, and 0.05% Tween 20 in PBS for 60 min. Blocking solution also contained the anti-FcR mAb 2.4G2 if no primary detection Ab of rat origin was used. The cells were then incubated for 30 min with anti-IIGP mAb SD9, rabbit anti-calcinein (Stressgene, Victoria, Canada), rat anti-mouse transferrin receptor mAb R17 217.1.3 (ATCC TIB-219), anti-lysozyme-associated membrane protein (LAMP) 1 mAbAb4D (kindly provided by Dr. J. T. August, Developmental Hybridoma Bank), anti-LAMP-2 mAb HRP108 (kindly provided by Dr. M. C. Skipper, Developmental Hybridoma Bank), anti-α-mannosidase (both kindly provided by Dr. A. Haas, University of Würzburg, Würzburg, Germany) in blocking solution. Afterward, the cells were incubated with Cy2- or Cy3-conjugated secondary Abs (Dianova, Hamburg, Germany), goat anti-mouse, goat anti-rabbit, or goat anti-rat IgG/Fc, respectively, in blocking solution. The cells were washed several times with HBSS. Tween 20 in PBS between the individual steps. After mounting in mowiol, the cells were analyzed with a Leica TCS-SP confocal laser scanner (Leica, Deerfield, IL) equipped with a DMRB microscope (Leica). Individual scans were analyzed using the TCS-NT software and Adobe Photoshop (Adobe Systems, Mountain View, CA). For electron microscopic analysis, spleens from infected mice were fixed in freshly prepared periodate-lysine-paraformaldehyde solution (19). Fixed cells were embedded in 4% low melting agarose. Briefly, 10-μl droplets were allowed to gel and then cut into pieces smaller than 1 mm$^3$ and infiltrated in 1.6 M saccharose with 25% (w/v) polyvinylpyrrolidone (RVP 10000) in PBS for 6 h. Specimens were mounted on metal stubs, frozen in liquid nitrogen, and 60-nm sections were cut on a RMC 7000 ultramicrotome equipped with an RMC cryochamber. Sections were collected on formvar- or carbon grids and incubated on Ab solutions (anti-IIGP mAb SD9 and anti-α-mannosidase). After washing, bound primary Abs were detected using goat anti-mouse and goat anti-rabbit secondary Abs coupled to 5- and 12-nm gold clusters, respectively (Jackson ImmunoResearch Laboratories, West Grove, PA). The grids were washed, embedded in a methyl cellulose/uranyl acetate solution, dried, and analyzed using a Leo 906E (Leo, Oberkochen, Germany) transmission electron microscope equipped with a TECNA II slow scan charge-coupled device camera (SIS, Muenster, Germany).

**Northern blot analysis**

Total RNA was isolated from cells using TRIzol (Life Technologies, Grand Island, NY) as recommended by the manufacturer. For Northern blot analysis, 10 μg of total RNA was separated on 1.2% agarose/formaldehyde gels and transferred onto Hybond-N$^+$ membrane (Amersham Biosciences, Uppsala, Sweden) by standard procedures. The blots were probed with randomly primed [γ$^32$P]dCTP-labeled IIGP cDNA or murine β-actin cDNA. Hybridization was performed overnight at 62–65°C in a solution containing 0.5 M NaPO$_4$ (pH 7.2), 7% SDS, 1 mM EDTA, 1% BSA, and 100 μg/ml salmon sperm DNA. The blots were washed at 62–65°C with 2× SSC, 0.5% SDS (twice for 30 min), and subsequently with 0.2× SSC/0.5% SDS for 30 min. Autoradiography was done using Hyperfilm-MP film (Amersham Biosciences). Stripping of IIGP cDNA-probed membranes was done as recommended by the manufacturer, and equal loading of RNA was verified by reprobing the blots with β-actin cDNA.

**Histology and immunohistochemistry**

Spleens from mice, infected with *L. monocytogenes* or not, were immersed in HBSS or Tissue-Tek OCT (Miles, Elkhart, IN) and snap frozen in liquid nitrogen. Tissue sections of 5 μm were cut, placed on siliconized glass slides, air dried, and stored at −70°C. Thawed and dried tissue sections were fixed in acetone, dried, rehydrated in PBS, and blocked with rat anti-mouse FcR mAb 2.4G2 and goat serum in TBS. FITC-conjugated anti-mouse mAb 6D5 was used for detection of IIEP and revealed with rabbit anti-FITC (DAKO, Glostrup, Denmark) followed by goat-anti-rabbit-conjugated alkaline phosphates (Jackson ImmunoResearch Laboratories). Alkaline phosphatase was visualized by using naphthol-AS-BI phosphate (Sigma-Alrich, St. Louis, MO) and New Fuchsin (Merck, Darmstadt, Germany) as substrate. Endogenous alkaline phosphatase activity was blocked with levamisole (Sigma-Aldrich). Sections were counterstained with hemalum (Merck).

For immunofluorescence analysis, fixed, rehydrated, and blocked tissue sections were incubated with Cy3.5-conjugated anti-mouse mAb 5D9 in PBS/0.05% Tween 20. Staining of cellular marker Ags was performed with FITC- or Cy2-conjugated mAbs or unconjugated mAbs, subsequently revealed with (4-(4-dichloro-3-triazin-2-yl)amino)-fluorescin)- or Cy2-conjugated anti-rat, anti-rabbit, or anti-armenian hamster Abs (Dianova). Stained sections were thoroughly washed in PBS/0.05% Tween 20 and coverslips were mounted with mowiol. Analysis was performed with a Leica DMRB fluorescence microscope equipped with a HV-204 Hitachi video camera (Hitachi, Tokyo, Japan), DISKUS software (Mikrovid, Arnheim, Germany), and Adobe Photoshop.

**Results**

Anti-IIGP-specific mAb

In an attempt to identify new genes involved in innate immunity, we generated by suppression-subtractive hybridization a differential cDNA library derived from T and B cell-depleted spleenocytes from *L. monocytogenes*-infected mice 2 days postinfection (p.i.) with reference to cells from uninfected animals. Within this library we identified, besides a variety of clones representing previously unknown genes, partial cDNAs encoding the 47-kDa GTPase IIGP and subsequently cloned the full-length IIGP cDNA (GenBank accession number AF194871). For further analyses, a panel of mAbs against IIGP was generated upon immunization of BALB/c mice with affinity-purified His-tagged, recombinant full-length gene. For further analyses, a panel of mAbs against IIGP was generated upon immunization of BALB/c mice with affinity-purified His-tagged, recombinant full-length gene.
IIGP protein produced in E. coli BL21(D3). One of those IIGP-specific mAbs, 5D9 (IgGlk), was chosen for all further studies. Western blot analysis of total cellular lysates from either IFN-γ-stimulated or untreated NIH 3T3 fibroblasts developed with the mAb 5D9 revealed a single predominant band in the range of 47 kDa in lysates from IFN-γ-stimulated cells (Fig. 1A). Similarly, the mAb 5D9 immunoprecipitated a single protein in the same size range from total cellular lysates of labeled BMM with mAb 5D9 upon preclearing of the lysate with an irrelevant mouse IgG1 Ab.

Expression of IIGP during listeriosis

Previous in vitro analysis of various hematopoietic and nonhematopoietic cells and cell lines indicated the ubiquitous inducibility of expression of various members of the 47-kDa GTPase family upon IFN-γ stimulation (4–9, 20). However, the kinetics of induction and identity of cells up-regulating cognates of the 47-kDa GTPase family in situ during infection has so far not been analyzed. To gain deeper insights into this issue, we analyzed splenic sections from mice infected with L. monocytogenes for IIGP protein expression immunohistochemically. No IIGP protein could be detected in the spleen from uninfected mice (Fig. 2). One day p.i., weak but significant staining was observed, indicating the onset of IIGP expression. The expression pattern did not change on day 3 p.i., but was more pronounced. Strong expression of IIGP was obvious in regions of the marginal zone (MZ) and on scattered cells in the splenic cords of the red pulp. Furthermore, a number of cells, evenly distributed in the periairiotiolar lymphatic sheaths (PALS), were strongly positive for IIGP. Remarkably, the overall expression of IIGP analyzed 6 days p.i. significantly decreased and was indistinguishable from that on day 1 p.i. (Fig. 2). Taken together, IIGP expression was transiently up-regulated in the early phase of listeriosis.

Identification of IIGP-expressing splenocytes

Given the observed compartmentalization of the IIGP protein expression in the spleen of L. monocytogenes-infected mice, we subsequently identified these IIGP-positive cells by means of double-immunofluorescence staining of splenic sections at day 3 p.i. A variety of distinct cell types reside in the MZ, including marginal metallophilic macrophages, MZ B cells, subpopulations of dendritic cells (DCs), small numbers of T cells, and endothelial cells lining sinuses (21). The majority of cells showing strong IIGP expression in the regions of the MZ were positive for MOMA-1, identifying marginal metallophilic macrophages (Fig. 3A) (22). In contrast, ER-TR9-positive MZ macrophages (23) and ER-BMDM1 (aminopeptidase N)-positive myeloid cells, which include MZ DCs and interdigitating DCs (IDCs) in the T cell areas of PALS (24), were negative (data not shown). Similarly, T cells in the PALS (Fig. 3B) and throughout the red pulp were negative for strong IIGP expression. This is surprising, since T cells respond to IFN-γ with the transcriptional up-regulation of the IIGP gene (see below). The T cell area depicted in Fig. 3B reveals a central arteriole showing marked IIGP expression. This observation is further substantiated by specific detection of endothelial cells lining the central arterioli (Fig. 3C), which strongly express IIGP. In accordance with this finding, endothelial cells throughout the red pulp also revealed significant IIGP expression (Figs. 2C and 3D). Remarkably, F4/80-positive resident macrophages in the red pulp were absolutely negative for IIGP (Fig. 3E). This is in line with the finding that the majority of Mac-1-positive cells, which include red pulp resident macrophages, but also monocytes (F4/80 negative) and activated neutrophils (25, 26), were IIGP negative (Fig. 3F). However, a small fraction of strong Mac-1-positive cells in the red pulp stained also intensively for IIGP (Fig. 3G). As to the identity of these cells, we rule out those cells to be activated neutrophils, since specific detection of neutrophils with the antibody 6G mAb RB6 did not reveal any obvious IIGP expression (data not shown). Previous studies have shown that transcriptional up-regulation of the IIGP gene upon infection with L. monocytogenes exclusively depends on IFN-γ (4). Our results illustrate that the inflammatory stimuli induced upon listerial infection, with IFN-γ being the most relevant, cause a distinct splenic cellular activation pattern as exemplified by IIGP expression.

Taken together, we have identified endothelial cells and marginal metallophilic macrophages as IIGP-expressing cells at day 3 p.i. At this time point we did not detect significant numbers of αβT cells, γδT cells, B cells, neutrophils, CD11c+ DCs (data not shown), and F4/80-positive red pulp resident macrophages (Fig. 3E) as IIGP-expressing cells. We cannot exclude that some IIGP-expressing cells were not detected by immunofluorescence such as certain subpopulations of DCs, splenic stroma cells, like reticuloctyes, or NK/NKT cells. However, many IIGP-positive cells in the red pulp were positive for the Mac-3 Ag (Fig. 3H). Although often used as a macrophage-specific surface marker, it has been reported that the Mac-3 Ag detecting mAb M3/84 also reveals endothelial cells and presumably also DCs in splenic sections (26, 27). The discrete and granular staining that we observed in our analysis has been noted previously (26) and most likely reflects the endosomal/lysosomal distribution of Mac-3 identified as the LAMP2 protein (18).

Induction of IIGP mRNA expression in vitro

Previous analyses have indicated that other stimuli besides IFN-γ can induce transcriptional up-regulation of members of the 47-kDa GTPase family. In macrophages, IGTP and LRG-47 can also be induced by LPS, and IFNαβ is an inducer of LRG-47 and TGTP (8, 9, 20). Because of the differential IIGP expression within subpopulations of splenic macrophages and of endothelial cells upon listerial infection, the stimuli driving IIGP induction were analyzed in more detail in BMM and the endothelioma cell line sEnd.1.

**FIGURE 1.** Recognition of IIGP by mAb 5D9. **A**, NIH 3T3 fibroblasts were stimulated with 1000 U/ml IFN-γ for 20 h or left untreated. Whole-cell lysates were separated by SDS-PAGE, blotted, and developed with the mAb 5D9. **B**, IIGP was immunoprecipitated from Nonidet P-40 cellular lysates of IFN-γ-stimulated and 35S metabolically labeled BMM with mAb 5D9 upon preclearing of the lysate with an irrelevant mouse IgG1 Ab.
Stimulation of BMM with IFN-γ, LPS, IFN-α, or infection with viable *L. monocytogenes* induced significantly IIGP mRNA expression within 20 h (Fig. 4A). In contrast, stimulation of BMM with heat-killed listeria, IL-1α, or IL-1β had no obvious effects, although a very faint signal of IIGP mRNA could be detected upon TNF-α stimulation. The analysis of the kinetics of IIGP mRNA induction in BMM revealed detectable IIGP mRNA within 2 h of stimulation with IFN-γ, IFN-α, or LPS (Fig. 4B). Maximal IIGP expression by these inducers was evident following 4–8 h of treatment. The endothelial cell line sEnd.1 is responsive to LPS, IL-1, and TNF-α (15). However, neither of these stimuli, nor viable or heat-killed listeria, elicited transcriptional IIGP induction (Fig. 4C). This was only observed upon stimulation with IFN-γ or IFN-α. In contrast to BMM, stimulation of endothelial sEnd.1 cells with viable *L. monocytogenes* failed to up-regulate IIGP mRNA expression. This observation most likely reflects the relatively low efficacy of listerial invasion of endothelial cells or disparate configurations with regard to available signal transduction pathways.

Since macrophages produce IFN-αβ upon LPS stimulation (28, 29), we investigated the impact of endogenously produced IFN-αβ on IIGP mRNA expression. Upon IFN-γ stimulation, BMM derived from either wild-type or IFN-αβR−/− mice expressed IIGP mRNA (Fig. 4D). However, upon stimulation with LPS or viable listeria, IIGP gene expression was completely negative in IFN-αβR−/− BMM. This suggests that endogenous IFN-αβ participates in the transcriptional IIGP induction under these conditions. In line with these results, inhibition of ongoing protein synthesis by cycloheximide abolished LPS mediated up-regulation of IIGP mRNA expression in BMM, while the up-regulation mediated by IFN-γ was not affected (data not shown). Furthermore, these findings suggest that sEnd.1 cells, in contrast to BMM, do not produce IFN-αβ upon stimulation with LPS or viable *L. monocytogenes*.

To further substantiate the requirements for IFNs for the transcriptional induction of members of the 47-kDa GTPase family in other cell types, we determined whether IIGP is expressed in T cells. So far, only TGTP has been shown to be up-regulated in T cells.
cells (5, 20). Purified lymph node-derived T cells from B6 mice were activated in vitro by immobilized anti-CD3 and anti-CD28 mAbs for 1–2 days. In parallel, we used purified T cells from IFN-γ/H9253R/H11002 mice to assess the possible IFN-γ dependency of IIGP mRNA induction. In TCR/CD28-cross-linked T cells from wild-type mice, increasing levels of IIGP mRNA expression were induced (Fig. 4E). In contrast, IIGP gene activation was not detected in IFN-γR-deficient T cells. Appropriate activation of IFN-γR−/− T cells was verified by analysis of expression of the sifn4 gene, which is up-regulated upon stimulation of peripheral T cells (30). Thus, IIGP is in fact strongly inducible in T cells upon TCR/CD28-mediated activation and relies on autocrine IFN-γ. These results indicate that type I and type II IFNs are the prime stimuli for the transcriptional induction of IIGP and, by implication, of the other members of the 47-kDa GTPase gene family in immune as well as in nonimmune cells.

**Subcellular distribution of IIGP**

To gain more insight into the putative function of IIGP and the other 47-kDa GTPases, their intracellular distribution was analyzed. So far only one member of this family has been studied, IGTP, revealing a predominant association with the ER (10). Two-color confocal laser scanning microscopy revealed IIGP in IFN-γ-activated macrophages, but not in untreated cells (data not shown), with a predominant localization on intracellular membranes extending into the cellular periphery and sparing the nucleus (Fig. 5, A and B). However, the immunostaining of IIGP showed perinuclear accumulation in globular structures, often in spatial proximity to the nucleus, and an occasional punctuate accumulation throughout the cytoplasm. Detection of the ER resident membrane protein calnexin revealed a considerable degree of colocalization with IIGP, but did not invariably include all calnexin-positive membranes (Fig. 5A). Abs to the Golgi resident membrane protein GM130 also stained IIGP, but to a lesser extent. Other early and transitional ER markers revealed a variable degree of colocalization with IIGP, supporting the notion that IIGP is a member of the ER/Golgi GTPase family (Fig. 5A). The subcellular localization of IIGP is consistent with a role in dynamic membrane trafficking, both in the secretory pathway and possibly in the endocytic pathway.
marker α-mannosidase identified the perinuclear regions, where accumulation of IIGP occurred, as the Golgi apparatus (Fig. 5B). Similar results were obtained with IFN-γ-stimulated NIH 3T3 fibroblasts (data not shown). This finding was further substantiated by electron microscopic examination of spleen cells from L. monocytogenes-infected mice 3 days p.i. Immunogold staining of IIGP and α-mannosidase revealed an association of IIGP with Golgi membranes (Fig. 5D). Further analysis indicated that IIGP localized neither to an early endosomal (transferrin receptor), late endosomal (mannose-6P receptor), nor lysosomal compartment (pulse/chase labeling for 16 h/2 h fluid phase marker) (data not shown). Hence, besides its association with the ER, IIGP preferentially accumulates in association with the Golgi apparatus.

Discussion

Mice deficient for individual members of the 47-kDa GTPase family display profound but distinct susceptibility against different intracellular pathogens (11, 12). The function of these GTPases is currently unknown, but available data indicate an involvement in defense against pathogens, most probably acting intracellularly (10–12). Therefore, the identification of the cellular distribution of 47-kDa GTPase cognates during infection and characterization of their intracellular location contributes to a better understanding of the role of this family in host defense. Expression of IIGP, taken here as a representative of this protein family, is transiently induced in spleens of mice infected with L. monocytogenes, reaching a maximum around day 3. Previous analyses described a strict dependency on IFN-γ for transcriptional expression of the 47-kDa GTPase genes in murine listeriosis (4). As shown here, the kinetics of splenic IIGP expression parallels transcriptional kinetics of splenic IFN-γ expression after low-dose i.v. infection, which is detectable within 24 h, remains stable between days 1 and 3–4 and decreases thereafter to baseline levels between days 5 and 6 (31, 32). Interestingly, 47-kDa GTPase-deficient mice succumb to Toxoplasma gondii (IGTP−/−; LRG-47−/−) or L. monocytogenes (LRG-47−/−) infection almost as rapidly as IFN-γ-deficient mice (11, 12). The differential susceptibility of these 47-kDa GTPase-deficient mice to distinct pathogens suggests nonredundant molecular defense functions. Taken together, these findings and the observed kinetics of IIGP expression suggest that IIGP mediates a protective mechanism which is effective during the early, innate immune response against distinct intracellular pathogens. However, depending on the pathogen and/or the route and dose of infection, the expression pattern for IIGP or for the other family members could vary. Therefore, protective mechanisms mediated by these 47-kDa GTPases could also gain importance at later stages of infection, especially when IFN-γ is secreted by Ag-specific T cells at sites of pathogen deposition.

Upon infection with L. monocytogenes, IIGP is most prominently expressed in the MZ, in an evenly distributed manner in the PALS, and more pronounced in a scattered fashion in the red pulp. IIGP expression was abundant in marginal metallophilic macrophages, endothelial cells in the red and white pulp, and in the majority of Mac-3-positive cells, but not in MZ macrophages. In splenic sections, the Mac-3 Ag can be detected in macrophages, endothelial cells, and DCs (26, 27). However, resident red pulp...
Macrophages, which are positive for the F4/80 or Mac-1 Ags, were almost exclusively negative for IIGP expression. Few Mac-1-positive cells in the red pulp showed strong IIGP expression. The exact identity of these cells remained elusive, since we found no evidence for IIGP expression by neutrophils, which strongly express the Mac-1 Ag in the activated state (26, 33). Similarly, we did not detect pronounced expression of IIGP in T cells, B cells, IDCs, MZ DCs, and CD11c+ DCs.

This cellular expression pattern in the spleen of L. monocytogenes-infected mice seems to reflect the dynamics of the invasion process in the spleen after i.v. infection. Listeria initially encounter endothelial cells upon vascular entry into the organ and become entrapped in the MZ, followed by invasion of the splenic cords in the red pulp (Ref. 34 and J. Zerrahn, unpublished data). This dynamic distribution of L. monocytogenes is accompanied by IFN-γ production. As to the precise nature of IFN-γ-producing splenocytes, NK cells are the predominant early IFN-γ producers (35, 36), but recent data suggest also a significant contribution by CD8+CD44+ T cells (37, 38) and by CD8α+ lymphoid DCs (39).

Induction of IIGP protein expression can be viewed as marker for an IFN-γ-mediated response. Hence, it is interesting, that we did not detect IIGP expression in MZ macrophages and also in resident red pulp macrophages (F4/80+), although expression of IIGP in splenic endothelial cells was revealed throughout the spleen. This could reflect the localized and paracrine effectiveness of IFN-γ produced in splenic subcompartments. Furthermore, functional differences between subpopulations of splenic macrophages may account for this observation. In fact, it has been reported that resident sinus lining macrophages (F4/80+) are only moderately responsive to IFN-γ compared with peritoneal macrophages and that the oxidative competence of listeria-infected splenocytes is associated with infiltrating monocytes rather than with resident macrophages (40). Alternatively, L. monocytogenes could down-modulate IIGP expression in infected cells because it interferes with IFN-γ-induced signaling (41–43). Nevertheless, it is tempting to speculate that cells which initially interact with blood-borne pathogens, like IFN-γ-activated macrophages in the MZ or endothelial cells, contribute to the control of pathogen replication and dissemination via mechanism(s) governed by members of the 47-kDa GTPase family. The precise identification of these individual cell populations and the significance of the processes controlled by this GTPase protein family for the fate of infection with different intracellular pathogens awaits future elucidation.

Our own findings confirm and extend previous data suggesting that type I and type II IFNs are the prime stimuli for expression of IIGP and, by implication, also for other members of the 47-kDa GTPase family (4). In contrast, IIGP induction is not compromised in IFN-γ-mediated type I IFN response. Hence, it seems reasonable to assume that the mechanisms mediated by these GTPases constitute a global cellular strategy for counteracting intracellular pathogens.

So far, the subcellular localization of the 47-kDa GTPase family has been described in detail only for one member, namely, IGTP (10). The majority of IGTP is associated with the cytoplasmic surface of ER membranes and with distinct globular structures.
located in the periphery of the ER (10). In contrast, we have found IIGP localizes to the ER, but additionally accumulates strikingly with the Golgi apparatus. Revelation of the exact way IIGP associates with these subcellular entities and the topography of the membrane association of IIGP remains to be addressed in further studies. However, sequence analysis of IIGP and its cognates as well as previous studies (10) suggest an association with these membranes on the cytoplasmic surface presumably via protein-protein interactions. The differential localization of two members of the 47-kDa GTPase along the endomembrane trafficking pathway provides evidence for their involvement in an IFN-inducible vesicular transport or processing pathway. Future studies will be directed at analyzing whether IIGP is involved in the ER to Golgi transport, as is suggested by the association of IIGP with both intimately connected cellular compartments. So far, our data are not consistent with an association of IIGP with early or late endosomes or lysosomes. We occasionally detected association of IIGP with Lamp1-positive vesicles (data not shown). Since Lamp1 has been found in a fraction of trans-Golgi vesicles (18), this observation would not contradict the notion of a nonlysosomal localization of IIGP. Nevertheless, given the possibility that IIGP is involved in intracellular vesicle transport processes, our findings do not exclude that IIGP-mediated trafficking intersects with endosomal or lysosomal compartments or the secretory pathway. Cellular activation mediated by IFNs leads to profound and distinct changes in vacuolar compartment dynamics (46) and in the composition of vesicular cargo targeted to distinct cellular sub-compartments (47–49) which contributes to increased antimicrobial potency. It is tempting to speculate that these 47-kDa GTPases participate in these processes. In analogy, a member of the GTP-binding Rab family, Rab5a, localizes to early endosomes and has been shown to be involved in listericidal activity in macrophages (50).

In conclusion, our findings provide the first analysis of in situ expression of a member of the 47-kDa GTPase family, IIGP, in the spleen during murine listeriosis. Our results add on previous notions that the up-regulation of these GTPases upon IFN stimulation constitutes a global potential of immune as well as nonimmune cells. However, during listeriosis the expression of IIGP proceeds transiently and is confined to certain splenic cell populations, a finding that presumably defines the effector cells in which the GTPase-mediated antimicrobial function(s) are operative. So far, 47-kDa GTPase-deficient mice have been reported to be susceptible against intracellular pathogens. Given the distinct subcellular localizations of IGTP and IIGP, being associated with the ER and ER/Golgi, respectively, an IFN-stimulated modulation of intracellular vesicular transport processes controlled by these 47-kDa GTPases seems likely. This might confer antimicrobial functions to intracellular compartments in which intracellular pathogens multiply. Future studies are aimed at the identification of protein-protein interactions with IIGP which will provide further insights into the mechanistic aspects of the molecular processes and thereby potentially also into the effector mechanisms against infectious agents.

Acknowledgments
We thank Dr. J. Allison for the anti-CD28 hybridoma and Dr. R. Zinkernagel and Dr. Weissmann for mice and helpful reagents. Many thanks to Dr. J. Mattow for matrix-associated laser desorption-mass spectrometry/mass spectrometry analysis, Dr. H. Collins and Frank Kaiser for help with the mAb generation, Beatrix Fauler for help with electron microscopy, and Lucia Lom-Terborg for reading this manuscript.

References


