IL-12 family members: differential kinetics of their TLR4-mediated induction by *Salmonella* Enteritidis and the impact of IL-10 in bone marrow-derived macrophages

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Abstract

The members of the IL-12 family of heterodimeric cytokines play a pivotal role in initiation and regulation of cell-mediated immunity. Best known is IL-12p70, which promotes an immune response towards Th1 bias. Other members of this family (IL-23, IL-27) are less well characterized in terms of induction and function. Using either heat-killed or viable *Salmonella* Enteritidis or LPS as a stimulus, the kinetics of mRNA production of each member of the IL-12 family (p19, p28, p35, p40, Ebstein-Barr-Virus-induced gene 3 (EBI-3)) were determined in BMDM originating from wild-type, Toll-like receptor (TLR)2- and/or TLR4-deficient mice. It was found that following either type of stimulation, a characteristic mRNA expression pattern was observed for each cytokine subunit. Whereas p19 was induced early and transiently, p40 and p35 were up-regulated later and then continuously, but the secretion of IL-23 and IL-12p70 was significantly reduced by IL-10. The up-regulation of p28 mRNA occurred also delayed and declined afterwards, whereas the initial high-level expression of EBI-3 remained almost unchanged in BMDM. Furthermore, a splice variant of the EBI-3 mRNA was discovered. In this context, the cytokine mRNA up-regulation by whole *Salmonella* Enteritidis is mediated chiefly by TLR4, but depends on additional pattern recognition receptors other than TLR2 expressed by macrophages.

Introduction

Antigen-presenting cells (APCs) play a central role in the initiation of an immune response, since they recognize very efficiently certain highly conserved microbial constituents with pattern recognition receptors (PRRs) and translate the received stimuli into appropriate cytokine signals (1). The APC-derived cytokine IL-12, also known as IL-12p70, consists of two subunits p40 and p35, which are covalently linked (2). IL-12 represents the first heterodimeric protein in an expanding cytokine family, that includes the two new but so far not well-characterized members IL-23 and IL-27, recently discovered based on their structural similarity to IL-12 (3, 4).

IL-12p70 is essential for induction of the Th1 immune response (5, 6), that is important to protect against intracellular pathogens (7–10). Experimentally, IL-12-deficient mice show a dramatically impaired IFN-γ release and a marginal delayed-type hypersensitivity response, demonstrating the importance of this factor for the protection against intracellular bacterial infections (11). The gene loci and promoters of both subunits are quite different, implying a differential regulation of these genes (12, 13) and even functional differences can be observed between the p35- and p40-deficient mice. IL-12p35-deficient mice, however, still develop a marginal Th1 response to *Mycobacteria* spp. or *Salmonella* spp. infections. Additional p40-dependent cytokines that facilitate cellular immune responses are in place that help to protect against intracellular microbes (14–16).

The search for new members of the IL-12 cytokine family led to the discovery of IL-23 that contains the p40 subunit of IL-12p70 and the newly discovered p19 subunit (17). Transgenic mice for p19 show a multi-organ inflammation, neutrophilia and...
advances IFN-γ exposure to LPS (20), suggesting an as yet unknown response factor (G-CSF) characterized by a typical four-homology to p35, p19, IL-6 and granulocyte colony-stimulating receptor family (23), and p28, which exhibits Virus-induced gene 3 (EBI-3) subunit, which belongs to the a heterodimeric protein consisting of a p40-like Ebstein-Barr virus (21, 22).

Toxoplasma gondii However, demonstrated an uncontrolled inflammatory response after infection with Toxoplasma gondii or Trypanosoma cruzi, emphasizing an immunoregulatory rather than an inflammatory function of this cytokine (27, 28).

Recent reports have suggested that IL-12 family members are induced after APCs were exposed to Gram-positive or Gram-negative bacteria (29–31). The Gram-negative Salmonella Enteritidis belong to the group of facultative intracellular bacteria, which resist intracellular elimination (32). For a protective immunity against these pathogens an IL-12-mediated T1 response is necessary that in turn depends on IFN-γ-triggered intracellular host defense mechanisms (33, 34). The main immunostimulatory component within the cell wall of Gram-negative bacteria is LPS. Certain inbred mouse strains, such as the C57BL/10ScSn strain, are highly resistant to LPS and it was shown that a natural mutation in the lps gene locus rendered the Toll-like receptor (TLR) 4 non-functional (35). The introduction of the TLR4 transgene restored the ability to respond to LPS (36).

TLRs belong to the group of PRRs which bind in a specific manner to typical microbial products also known as pathogen-associated molecular patterns. In addition to LPS, Salmonellae produce other immunostimulatory factors. Cell wall components such as lipopolysaccharides and peptidoglycans are recognized by TLR2 in combination with TLR1 or TLR6 (37), flagellin by TLR5 (38, 39) as well as the non-methylated CpG motif of bacterial DNA recognized by TLR9 (40). Expressed by APCs, this group of receptors form the link between antigen recognition and induction of the initial immune responses (41).

This study focuses on the kinetics of mRNA expression pattern of all IL-12 family cytokines induced by the Gram-negative Salmonella Enteritidis in dependence of TLR4 and TLR2 to elucidate the central steps that lead to the induction of protective immunity. Using BMDMφ we also investigated the production of IL-23 and IL-12p70 proteins and describe the influence of IL-10 on their secretion.

**Methods**

**Mice**

C57BL/10ScSn (wild-type) mice, TLR4<sup>Δ/Δ</sup> (ScN), TLR2<sup>Δ/Δ</sup> (ScSn-TLR2<sup>Δ/Δ</sup>) or TLR4<sup>Δ/Δ</sup>/TLR2<sup>Δ/Δ</sup> littermates were used as described elsewhere (42), and were provided by the animal facility of the Max Planck Institute of Immunobiology (Freiburg, Germany). C57BL/6 mice were bred in our animal facility (College of Veterinary Medicine, University of Leipzig, Germany). C57BL/6 IL-10<sup>Δ/Δ</sup> mice were kindly provided by the animal facility of the Max Planck Institute for Infection Biology (Berlin, Germany). All animals were housed under specific-pathogen-face (SPF) conditions.

**Reagents and bacteria**

LPS from Salmonella abortus equi S-form and Lipid A from Salmonella Minnesota R595 were purchased from Alexis (Grüenberg, Germany). Viable Salmonella Enteritidis (vSE) and heat-killed Salmonella Enteritidis (hkSE; ade<sup>+</sup>, his<sup>+</sup>, SALMOVAC SE), an attenuated vaccination strain, were kindly provided by the Impfstoffwerke Dessau-Tornau (Rosslau, Germany) (43, 44). Salmonella Enteritidis were heat-killed by incubation of vSE at 60°C for 60 min. Murine IL-10 was purchased from R&D Systems (Wiesbaden, Germany). Murine IL-23 was kindly provided by DNAX Research Institute (Palo Alto, CA, USA).

**BMDMφ**

Bone marrow cells recovered from femurs of 6- to 10-week old sacrificed mice were flushed out from the bone cavities. Bone marrow cells (1 x 10<sup>6</sup> cells ml<sup>−1</sup>) were cultivated for 10 days at 37°C in the presence of 30% L-conditioned medium derived from the cell line L929 (45). After a 10-day differentiation period at 37°C in humidified atmosphere with 8% CO<sub>2</sub>, macrophages were washed twice with a serum-free, high-glucose formulation of Dulbecco’s modified Eagle medium and incubated overnight (5 x 10<sup>5</sup> per well) in a 24-well plate until stimulation. BMDMφ were stimulated with Lipid A (5 µg ml<sup>−1</sup>), LPS (5 µg ml<sup>−1</sup>) and hkSE or vSE (5 x 10<sup>6</sup> c.f.u. ml<sup>−1</sup>). After 1, 6, 12, 24 and 48 h of stimulation, supernatants were collected and frozen at −80°C.

**RNA preparation and real-time reverse transcription–PCR**

Cell lysates of stimulated and non-stimulated BMDMφ were prepared with RLT lysis buffer (Qiagen, Hilden, Germany) and stored at −80°C. RNA was extracted with RNaseasy<sup>®</sup> Mini Kit (Qiagen). To avoid contamination with genomic DNA, RNA samples were treated with DNase I (Promega, Mannheim, Germany) for 30 min at 37°C. DNase was inactivated using the provided stop solution and incubated at 65°C for 10 min. Oligo d(T)-primer (2.5 µM; New England Lab, Frankfurt, Germany) was supplemented to the reaction mix. The addition of RNAsin<sup>®</sup> RNase-Inhibitor (0.4 U µl<sup>−1</sup>; Promega) and reverse transcription (RT) with Moloney Murine Leukemia Virus (MMLV)
reverse transcriptase (1.25 U μl⁻¹; Amersham Pharmacia Biotech, Freiburg, Germany) followed immediately. According to the standard protocol, the RT reaction was performed at 37°C for 60 min. The enzyme was finally heat-inactivated at 95°C for 5 min.

Real-time PCR for different members of the IL-12 family were conducted with primers (1.0 μM) and fluorogenic probes (0.2 μM) listed in Table 1. For the investigation of the EBI-3 splice variant primers termed EBI-3_V were used. PCR was performed with Taq polymerase from Biostar (45 U ml⁻¹; Cologne, Germany) in an iCycler (Biorad, Munich, Germany). To assess the x-fold up/down-regulation, Ct values of the housekeeping gene β-actin were recorded for all cDNA samples. The signals for cytokine mRNA were normalized by calculating the differences (ΔCt) of Ctβ-actin and Ct cytokine. Subsequently, the x-fold up/down-regulation was calculated by comparing stimulated with non-stimulated macropages (ΔΔCt) and using the formula, x-fold up/down-regulation = 2−ΔΔCt.

mRNA copy number quantification

Freshly prepared PCR products were inserted into pCR®2.1-TOPO vector from the TOPO TA Cloning® Kit (Invitrogen, Karlsruhe, Germany) and cloned in 10F Escherichia coli according to the instructions. Plasmids of transformed E. coli were isolated with QIAfilter™ Plasmid Midi Kit (Qiagen) and were completely linearized with XhoI (Invitrogen) according to the manufacturer’s instructions. Linear plasmid DNA was purified with a QIAquick® PCR Purification Kit (Qiagen). Absolute quantification was performed by gel calibration (Roboscreen, Leipzig, Germany) and by measuring the optical density at 260 nm with GeneQuant II (Amersham Pharmacia). Absolute quantification was performed by gel calibration (Roboscreen, Leipzig, Germany) and by measuring the optical density at 260 nm with GeneQuant II (Amersham Pharmacia). To determine the copy number of cDNA in the samples of cultured BMDM, the calibrated PCR standards ranging from 10⁵ to 10¹ molecules μl⁻¹ (diluted in canine genomic DNA; 2 μg ml⁻¹) were used in each single PCR test.

Cytokine and nitric oxide measurements

Cytokine concentrations were measured by sandwich ELISA. TNF-α and IL-17 were analyzed with Duo-Set® (R&D Systems) according to recommended standard protocols. IL-10 was assessed with the mAb JESS-2AS (0.2 μg ml⁻¹; Pharmingen, Heidelberg, Germany) used as a capture antibody and for detection the biotinylated mAb SXC-1 (2.5 μg ml⁻¹; Pharmingen) were applied. IL-12p40, IL-12p70 and IL-23 were measured using the mAbs 5C3 (25 μg ml⁻¹), 48110 (2 μg ml⁻¹; R&D Systems) and 20C10 (1 μg ml⁻¹; DNAx Research Institute), respectively, as capture antibodies, and biotinylated goat anti-mouse IL-12p40-purified IgG (1:1000; both provided by H. Gallati, F. Hoffmann-La Roche Ltd, Basel, Switzerland) as the detection antibody. For the development with ABTS (Sigma-Aldrich, Taufkirchen, Germany) the biotin-labeled detection antibodies were enzyme linked with streptavidin–HRP (1:3000; Southern Biotech, Birmingham, AL, USA). The measurements of ELISA at 415 nm were performed with a Spectra-max 340 ELISA reader (Molecular Devices, Munich, Germany). Nitric oxide (NO) from BMDM supernatants were quantified with Griess reagent as described elsewhere (46).

FACS

In vitro-differentiated BMDMΦ were harvested at day 10, pre-treated with anti-CD16/CD32 Fc block (clone 2.4G2; BD, Heidelberg, Germany) and subsequently stained with rat IgG-isotype control–FITC/PE (BD), anti-mouse F4/80–FITC (Caltag Laboratories, Hamburg, Germany) or anti-mouse CD11c (clone HL3; BD). Cells were washed twice with FACS buffer (PBS/3% FCS/0.1% Na azide), fixed with PBS/1% (v/v) formaldehyde and subjected to FACS analysis (FACSCalibur; BD). Analyses were performed with the software CellQuest™ (BD).

IL-23 bioassay

Splenocytes were recovered from female C57BL/6 p35-def/40-def mice and adjusted to 4 × 10⁶ cells ml⁻¹. After the addition of Con A (final concentration 5 μg ml⁻¹), the cell suspension was dispensed in 96-well culture plates at a final concentration of 4 × 10⁵ cells per well. Thereafter, 100 μl of supernatants (derived from LPS- or hkSE-stimulated BMDM) to be tested, recombinant murine IL-23 at varying concentrations (positive control) and LPS or hkSE (negative controls) were added to appropriate wells and incubated at 37°C for

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**Table 1. List of primers and probes used**

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Hybridization probe (FAM-TAMRA)</th>
<th>Amplicon (bp)</th>
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<tr>
<td>β-actin****</td>
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<tr>
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<td>ACCAGCAGAGAGAGCCG</td>
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<td>TAACGAGAAGAGGAGG</td>
<td>TATGCGCTGTCGCGAGCTC</td>
<td>121</td>
</tr>
<tr>
<td>p28****</td>
<td>AAACATGAGAGAAGAGAGAAGAAGAGT</td>
<td>TGGCGCTGTCGCGAGCTC</td>
<td>84</td>
</tr>
<tr>
<td>IL-23R***</td>
<td>TTGACAGAAGGAGGAGG</td>
<td>ACCAGCAGAGAGAGAGG</td>
<td>80</td>
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<tr>
<td>EBI-3_V</td>
<td>TACACTAATCTCGGAGGTAGGAGG</td>
<td>GCTGACACCCCTGAGATGACTC</td>
<td>643/513</td>
</tr>
</tbody>
</table>

Specific oligonucleotide sequences were designed with Primer Express™ 1.5 software (Applied Biosystems, Darmstadt, Germany) or taken from publications.

*Overbergh et al. (59).
*bLehmann et al. (16).
*cPflanz et al. (24).
*dParham et al. (20).
3 days. The IL-17 concentrations in the supernatants of the bioassay were analyzed by ELISA as outlined above.

Results

Induction of IL-12 family members is characterized by differential kinetics

It has been previously shown that LPS is able to induce the production of p40 molecules in APCs (47, 48). In this study we were interested in the chronological sequence Salmonella Enteritidis induces the subunits p40 and p35 of IL-12p70, and how especially the other new members of the IL-12 cytokine family are regulated upon stimulation. To this end we used an in vitro culture system with bone marrow cells derived from C57BL/10ScSn mice, which differentiated into BMDM under the influence of macrophage colony-stimulating factor (M-CSF)-containing, L929-conditioned medium. The generated cells represented a homogenous population of F4/80+ macrophages. No CD11c+ cells were detectable in this culture (Fig. 1A).

BMDM were exposed to hkSE, vSE or LPS derived from Salmonella. By real-time RT–PCR the x-fold up-regulation of mRNA expression in comparison to untreated macrophages was determined for p40, p35, p19, p28 and EBI-3.

After each of the three stimuli was applied, an up-regulation of mRNA was observed with a characteristic pattern. The IL-12p70 subunits p40 and p35 start a striking up-regulation at 6 h post-stimulation, that increases up to 24 h, whereas the expression of the IL-23 subunit p19 reaches the peak of induction very early (within 1 h after stimulation) and decreases afterwards. The IL-27 subunit p28 is induced with a peak at 6 h post-stimulation, whereas a minimal up-regulation of EBI-3 is apparent after LPS or Salmonella Enteritidis exposure (Fig. 1B). This indicates that a de novo mRNA synthesis of the cytokine subunits is induced in response to LPS or Salmonella starting with a characteristic early up-regulation of p19 (IL-23) at 1 h and followed by p35, p40 (IL-12p70) and p28 (IL-27) at 6 h after stimulation. In order to investigate the capability of macrophages to respond to IL-23 directly, we determined the induction of the IL-23R by real-time PCR. Six hours post-stimulation with LPS or Salmonella Enteritidis we found an up-regulation of the specific IL-23R mRNA (Fig. 1B).

Starting at different initial expression levels in resting cells, the induction of p40, p35, p19 and p28 mRNA by Salmonella Enteritidis is mediated by TLR4 but not TLR2

Since we could demonstrate an mRNA up-regulation of the IL-12 family members by Salmonella Enteritidis in BMDM, we next addressed the question which role TLR4 and TLR2 play during their induction. Therefore mRNA of stimulated BMDM from TLR4def, TLR2def or TLR4def/TLR2def mice were compared with wild-type BMDM.

As shown in Fig. 2(A) TLR4def macrophages do not respond to the highly purified Lipid A and LPS. After stimulation with Salmonella Enteritidis, however, TLR4def cells respond with

![Fig. 1.](image-url) mRNA of the IL-12 family members is up-regulated by Salmonella Enteritidis as well as by LPS in F4/80-positive BMDM. (A) FACS analysis of the expression levels of F4/80 and CD11c on BMDM cultured in the presence of L-conditioned medium produced by L929 cells. (B) BMDM were treated with LPS (5 μg ml⁻¹), hkSE or vSE (BMDM : SE = 1 : 10). The x-fold up-regulation of mRNA post-stimulation was calculated by normalizing the amount of cytokine mRNA with the housekeeping gene β-actin, and comparing results from stimulated to resting BMDM as described in Methods [means ± SEM (n = 3) of one from three independent experiments are shown]. Kinetic analysis of cytokine subunits of the IL-12 family and the IL-23R are shown.
Fig. 2. mRNA expression of IL-12 family members in BMDM after exposure to Salmonella Enteritidis is mediated by TLR4 but not by TLR2. (A) Wild-type, TLR2<sup>−/−</sup>, TLR4<sup>−/−</sup> and TLR4<sup>−/−</sup>/TLR2<sup>−/−</sup> BMDMs were treated with LPS (5 μg ml<sup>−1</sup>), Lipid A (5 μg ml<sup>−1</sup>), hkSE or vSE (BMDM/S E = 1:10) in comparison to medium control (contr). Absolute molecule copy number per microliter of cDNA was calculated by comparing results from cDNA with PCR standards with known molecule content and normalizing the amount of cytokine mRNA with the housekeeping gene β-actin. Real-time PCR was performed on iCycler (Biorad) with 5’Fam-3’TAMRA molecular probes. Representative results of one from three independent experiments are shown (nd = not done). (B) XhoI-linearized pCR<sup>®</sup>2.1 TOPO<sup>®</sup>; vector with specific inserts, which are representative for the PCR-amplified fragments for following cytokine subunits: p40 (40), EBI-3 (E), p35 (35), p19 (19) and p28 (28). Comparable molecule numbers of used PCR standards are shown in a 2-fold dilution series on a 1.0% agarose gel.
a low to moderate mRNA synthesis, and the mRNA production of p35, p19 and p28 is dramatically reduced in comparison to wild-type macrophages. The p40 mRNA production in TLR4<sup>del</sup> cells is also reduced, although the decrease is more subtle (Fig. 2A). Unexpectedly, TLR4<sup>del</sup>/TLR2<sup>del</sup> macrophages, that lack both TLRs, react stronger than the TLR4<sup>del</sup> macrophages. Unlike in TLR4<sup>del</sup> cells, the absence of TLR2 has no effect on mRNA induction of the IL-12 family members, since TLR2<sup>del</sup> cells respond similarly to wild-type cells in terms of mRNA expression of IL-12 family members.

Comparing the initial mRNA expression levels in untreated BMDM<sup>Φ</sup>, there are obviously strong differences between the subunits of the IL-12 family members. To determine the copy number of mRNA molecules in the test samples, PCR standards were adjusted to the same molecule number (Fig. 2B) and were used in the appropriate PCR assays. Interestingly, the EBI-3 subunit of IL-27 shows a relatively minor mRNA up-regulation after stimulation with LPS or Salmonella Enteritidis when compared with control cells, although Ct values obtained by real-time PCR revealed a high expression level of the EBI-3 mRNA in all cells including the non-stimulated macrophages. By determining the exact mRNA copy numbers it became evident that the IL-27 subunits p28 and especially EBI-3 are expressed on a high level. In contrast, p35 is not detectable, and p19 is only barely expressed in untreated macrophages. The mRNA of p40 is detectable in non-stimulated BMDM<sup>Φ</sup>, but at a lower level than EBI-3 or p28 (Fig. 2A, contr).

In stimulated macrophages there are also differences in the copy numbers between the IL-12 family members. Wild-type and TLR2<sup>del</sup> macrophages stimulated with Salmonella Enteritidis or LPS show an evident induction of p19 and p35 mRNA, but the mRNA copy numbers are much lower than those of p40 and of IL-27 (EBI-3 and p28) (Fig. 2A).

**Secretion of IL-12p40, IL-10, TNF-α and NO by BMDM<sup>Φ</sup> is chiefly mediated by TLR4**

Next we investigated the secretion of cytokines, such as IL-12p40, IL-12p70, IL-23, IL-10, TNF-α and the mediator molecule NO, by wild-type versus TLR4<sup>del</sup> and TLR2<sup>del</sup> BMDM<sup>Φ</sup>. Comparing the secretion of the p40 proteins using a p40-specific assay, there is a strong difference between TLR4<sup>del</sup> and wild-type or TLR2<sup>del</sup> cells, that is more striking than on the mRNA expression level of p40 (Fig. 3A). During the stimulation period there is a continuous increase of p40 protein level in the supernatant of the stimulated wild-type and TLR2<sup>del</sup>-macrophages, reflecting the enduring mRNA up-regulation of that subunit. Analogous to the mRNA expression levels of p40 after stimulation with Salmonella Enteritidis, the protein secretion by TLR4<sup>del</sup>/TLR2<sup>del</sup> macrophages is also stronger than by macrophages with a single TLR4 deficiency (Fig. 3A). Using specific antibodies for the heterodimeric IL-12p70 and IL-23, both cytokines were not detectable in the supernatants of stimulated and non-stimulated BMDM<sup>Φ</sup> of all genotypes used in the experiment, whereby the detection limit of both assays was 40 pg ml<sup>−1</sup> (data not shown). Since it was recently shown that IL-10-producing, M-CSF-differentiated macrophages, designated as macrophage type 2, are unable to secrete IL-12p70 and IL-23 (49), we determined the ability of these BMDM<sup>Φ</sup> to produce IL-10. The stimulated BMDM<sup>Φ</sup> secreted IL-10 in a TLR4-mediated manner (Fig. 3A).

We further studied the secretion of TNF-α and NO that are important for the defense against intracellular pathogens by autocrine activation of macrophages (50, 51). TNF-α and NO were secreted by BMDM<sup>Φ</sup> after stimulation with LPS and Salmonella Enteritidis in a TLR4-mediated but TLR2-independent manner, shown by the dramatically reduced secretion in TLR4<sup>del</sup> macrophages but not in TLR2<sup>del</sup> cells (Fig. 3B).

**IL-10 limits the secretion of IL-23 and IL-12p70 by BMDM<sup>Φ</sup>**

To investigate the influence of IL-10 on the secretion of IL-23 and IL-12p70, we used BMDM<sup>Φ</sup> derived from IL-10<sup>del</sup> C57BL/6 mice and compared these cells with wild-type macrophages. In contrast to macrophages with the C57BL/10 genetic background, C57BL/6 wild-type macrophages secreted measurable amounts of IL-23 and IL-12p70 after stimulation with LPS, hkSE or vSE. Nevertheless, wild-type cells differed from IL-10<sup>del</sup> macrophages in terms of a significantly reduced IL-23 and IL-12p70 secretion (Fig. 4A). The mRNA expression pattern of the p19 subunit in the IL-10<sup>del</sup> macrophages was similar to that observed in wild-type cells (data not shown). Both, IL-12p70 and IL-23, were detectable between 12 and 24 h post-stimulation. While IL-23 peaked at 24 h and started to decrease thereafter, IL-12p70 accumulated continuously. To characterize the impact of IL-10 on the synthesis of IL-23 and IL-12p70, murine IL-10 at varying concentrations was added to stimulated BMDM<sup>Φ</sup> derived from C57BL/6 IL-10<sup>del</sup> and wild-type mice. The addition of exogenous IL-10 resulted in a dose-dependent inhibition of the IL-23 and IL-12p70 production (Fig. 4B).

Recent work has shown that IL-23 is able to induce IL-17 in T cells, especially memory T cells (21). In order to examine whether supernatants from stimulated BMDM<sup>Φ</sup> contained bioactive IL-23 we developed a bioassay. Therefore, we used Con A-stimulated splenocytes from C57BL/6 p35<sup>del</sup>/40<sup>del</sup> mice that are unable to produce endogenous IL-23. Exogenous IL-23 caused Con A-stimulated splenocytes to release IL-17 into the culture supernatant in a dose-dependent manner (Fig. 4C). Supernatants from LPS-stimulated BMDM<sup>Φ</sup> (C57BL/6 wild-type) induced an enhanced IL-17 release, while supernatants from non-stimulated macrophages did not. Similarly, supernatants from hkSE- or vSE-stimulated BMDM<sup>Φ</sup> caused an elevated IL-17 release by splenocytes, which was clearly higher than the IL-17 output of splenocytes exposed to hSE or LPS only (Fig. 4C).

**BMDM<sup>Φ</sup> express an EBI-3 mRNA splice variant**

When EBI-3-specific conventional PCR assays were performed, an additional truncated amplicon (130 bp) was found. Analysis of organ tissues in different mouse strains (129 Sv/Ev, C57BL/6 and BALB/c) revealed that the additional fragment was present in spleen, liver and kidney of all mouse strains (data not shown). Sequence analysis of the truncated amplicon showed that the discovered fragment is a splice variant of EBI-3 mRNA, characterized by the absence of exon 2 of the full-length mRNA. Software-aided translation of the mRNA sequence into the corresponding amino acid sequence
revealed an early stop codon, which probably results in a non-functional protein. Conventional PCR was used since the primers of the real-time PCR do anneal to exon 2 of EBI-3 and therefore do not detect the short splice variant. We recorded the expression pattern of the EBI-3 transcripts by analysis of equal amounts of cDNA (adjusted by β-actin PCR) derived from LPS-, hkSE- or vSE-exposed BMDM_U from C57BL/10 mice. Both, the full-length transcript and the splice variant were detectable by conventional PCR before and after stimulation (Fig. 5). To investigate the expression of this EBI-3 splice variant in other APCs, we generated bone marrow-derived dendritic cells using granulocyte macrophage colony-stimulating factor (GM-CSF)-containing medium, as described elsewhere (52). Dendritic cells expressed both the full-length transcript and the splice variant before and after stimulation (data not shown) similar to BMDM_U.

**Discussion**

The studies presented here focus on the detailed expression patterns of the IL-12 family cytokines induced by the Gram-negative *Salmonella Enteritidis*. We demonstrate that a pure population of BMDM_U responds to the exposure to both LPS and *Salmonella Enteritidis* identically with the production of IL-12 family cytokine mRNAs, but the kinetics of mRNA expression vary considerably among the family members. Furthermore, we demonstrated for the first time that the up-regulation of mRNA expression of all IL-12 family members by *Salmonella Enteritidis* is mediated by TLR4 and other PRRs, but not by TLR2. Finally, we are able to show that the secretion of IL-23 in BMDM_U is inhibited by IL-10 as has been demonstrated for IL-12p70 (53). The heterodimeric IL-12 family members are IL-12p70 and the two new less well-defined members IL-23 and IL-27. The latter cytokines are assumed to play a crucial role in
cell-mediated immune responses (54). Even if it was shown previously that a broad spectrum of bacteria is able to provoke the up-regulation and expression of various subunits within the IL-12 family (29–31), a detailed kinetic analysis regarding the expression pattern of these factors has not been performed so far. The data presented here show that all members of the IL-12 family are induced by Salmonella Enteritidis albeit with varying kinetics and at different expression levels. Particularly, our studies illustrate that a quick up-regulation of p19 mRNA occurs in BMDM upon stimulation with LPS, Lipid A and Salmonella Enteritidis, whereas the mRNA syntheses for subunits that are necessary to produce IL-12p70 and IL-27 followed by a 6-h delay. This is supported by data published by Li et al., who have shown that during the

Fig. 4. Secretion of IL-23 and IL-12p70 by BMDM and the influence of IL-10. (A) IL-23 and IL-12 protein concentrations in culture supernatants from wild-type BMDM (C57BL/6) and IL-10-deficient littermates after stimulation with LPS (2 µg ml⁻¹), hkSE or vSE (Mφ : SE = 1 : 10). Representative results of one from three independent experiments are shown. For statistical analysis a Student’s t-test for each time was performed (**P < 0.01, ***P < 0.001 and *P < 0.05). (B) IL-23 and IL-12p70 secretion by hkSE-stimulated BMDM treated with increasing amounts of exogenous murine IL-10. The release of IL-12 and IL-23 is completely suppressed when IL-10 is added at a concentration of 1.0 ng ml⁻¹ or higher. (C) IL-23 bioactivity in culture supernatants from wild-type BMDM (C57BL/6) after they were exposed to LPS (2 µg ml⁻¹), hkSE or vSE (Mφ : SE = 1 : 10). For the bioassay, splenocytes from naive C57BL/6 p35def/40def mice were incubated with Con A (5 µg ml⁻¹) in the presence of the macrophage culture supernatant of interest. After a 3-day incubation period, the IL-17 output, a parameter that is directly linked to the IL-23 activity, was measured by ELISA. For statistical analysis a Kruskal–Wallis test and Dunn's Multiple Comparison test as post-test were performed for each time (*P < 0.05).
PCR quality was controlled with a negative control (H2O) and an EBI-3 estimate the size of amplicons a DNA mass standard (M) was used. PCR standard plasmid for the full-length transcript (+).

BMDM U

A moderate expression level in untreated BMDM IL-23, respectively. In contrast, the p40 subunit shows subunits is marginal, which limit the synthesis of IL-12p70 and high levels, whereas the expression of the p35 and p19 IL-27 subunits EBI-3 and p28 are expressed constitutively at

l with 5 μg ml⁻¹LPS (L), hkSE (hk) or infected with vSE (v) in a ratio of BMDMΦ : SE = 1 : 10 and compared with untreated BMDMΦ (c). To estimate the size of amplicons a DNA mass standard (M) was used. PCR quality was controlled with a negative control (H2O) and an EBI-3 PCR standard plasmid for the full-length transcript (+).

It is noteworthy that the mRNA data presented in this article are not limited to the sole calculation of mRNA up-regulation. We used calibrated PCR standards that allowed us to calculate the precise mRNA copy numbers in each test sample. This method provides information not only on the relative change in expression levels between treated and untreated cells but also on the precise expression levels of different factors in cells of interest. With this in mind it is remarkable that the initial expression levels of the various IL-12 family subunits vary tremendously in untreated BMDMΦ. The IL-27 subunits EBI-3 and p28 are expressed constitutively at high levels, whereas the expression of the p35 and p19 subunits is marginal, which limit the synthesis of IL-12p70 and IL-23, respectively. In contrast, the p40 subunit shows a moderate expression level in untreated BMDMΦ. Starting at this moderate expression level, p40 is early accessible for protein production (~6 h) and therefore no limiting factor for IL-12p70 or IL-23. Activated BMDMΦ also achieve a much higher mRNA expression level for the p40 subunit than for p19 and p35. As a consequence, p40 molecules were detected in large amounts, while IL-12p70 and IL-23 were secreted at a low level in C57BL/6 (wt) macrophages and were undetectable in C57BL/10 macrophages. Studies with human myeloid cells have shown that M-CSF-differentiated macrophages produce IL-10 and suppress the secretion of IL-12p70, whereas GM-CSF-differentiated macrophages are capable of secreting IL-12p70, and as recently described, are able to produce IL-23 (49, 56). In our studies we used BMDMΦ grown and differentiated with L-conditioned medium, derived from L929 cells, that mainly produce M-CSF and only small amounts of GM-CSF (45, 57). BMDMΦ used for our studies were phenotypically different from GM-CSF-driven bone marrow cells, which others and we propose to develop into dendritic cells (52). Since we could show that the stimulated BMDMΦ secrete IL-10, we were interested whether IL-10-deficient macrophages produce larger amounts of p40 cytokines. We found that IL-10 limits the secretion of IL-23, whereas the mRNA expression of the p19 subunit is unchanged. This suggests that IL-10 suppresses a post-transcriptional process that influences the secretion of IL-23, but does not act on the regulation of the p19 gene expression. Although the mRNA of the p19 subunit appeared prior to p35 mRNA, both cytokines, IL-23 and IL-12p70, were detectable after the same stimulation period in wild-type as well as in IL-10-deficient macrophages (~24 h). Since it was previously shown, that IL-23 causes T memory cells to release IL-17 (21), we consider that the enhanced IL-17 release in the IL-23 bioassay was caused by bioactive IL-23 from the culture supernatants of the stimulated macrophages.

Compared with the other members of the IL-12 cytokine family, EBI-3 mRNA was detected in large amounts in resting culture macrophages. In addition, we discovered that the EBI-3 gene is transcribed in BMDMΦ as a full-length mRNA and as a truncated splice variant. Whether this additional splice variant influences the overall EBI-3 secretion is not known. The subunit p28 also shows a high level of expression in resting cells; nonetheless, the transcription of this subunit is well induced after stimulation with Salmonella Enteritidis. In analogy to IL-12p70 and IL-23 it is possible that the smaller subunit p28 dictates the secretion of the heterodimeric protein IL-27. Considering the elevated mRNA expression levels of p28 after exposure of BMDMΦ to LPS or Salmonella Enteritidis one would expect an enormous output of the corresponding protein, but to our current knowledge the detection of IL-27 is technically not possible.

Here we show that the expression of the IL-12 family subunits induced by stimulation with the Gram-negative bacterium Salmonella Enteritidis is chiefly mediated by TLR4. Using highly purified LPS or Lipid A we could confirm that the mRNA up-regulation of the IL-12 family subunits is exclusively mediated by TLR4. A TLR4-mediated induction of the p19 mRNA was also demonstrated in another infection model using the Gram-negative bacterium Klebsiella pneumoniae (58).

In contrast to TLR4-def macrophages, TLR2-def macrophages responded like wild-type cells in terms of cytokine up-regulation and expression. This implies that LPS is the major immunostimulatory component of Salmonella that induce the IL-12 family cytokines, whereas the TLR2-mediated recognition of lipoproteins and peptidoglycans is of negligible importance and apparently provokes no IL-12 family cytokine expression when cells are exposed to Salmonella Enteritidis. A recent study has shown that only Gram-negative, but not Gram-positive non-pathogenic intestinal bacteria induced elevated p19 and p28 levels in dendritic cells (31), which demonstrates the importance of LPS-TLR4-mediated induction of p19 and p28 mRNA. Nevertheless, a TLR4-independent

Fig. 5. BMDMΦ express a full-length and a splice variant mRNA of EBI-3. Semi-quantitative analysis of the amplified full-length EBI-3 mRNA fragment (643 bp) and its truncated splice variant (513 bp) is shown (1.0% agarose gel). Comparable amounts of cDNA (controlled by β-actin real-time PCR) were used. BMDMΦ were stimulated for 12 h with 5 μg ml⁻¹LPS (L), hkSE (hk) or infected with vSE (v) in a ratio of BMDMΦ : SE = 1 : 10 and compared with untreated BMDMΦ (c). To estimate the size of amplicons a DNA mass standard (M) was used. PCR quality was controlled with a negative control (H2O) and an EBI-3 PCR standard plasmid for the full-length transcript (+).
expression of the IL-12 cytokine family members after exposure to *Salmonella* Enteritidis, although at a much lesser extent, occurred in TLR4-deficient macrophages. These observations suggest that the induction of IL-12 cytokine members is chiefly mediated by TLR4, but other PRRs different from TLR2 are most likely involved in the *Salmonella* Enteritidis recognition and signal triggering.

Besides, we observed differences in the response of TLR4-def BMDM and macrophages that are deficient in both receptors (TLR4-def/TLR2-def). Double-deficient TLR4-def/TLR2-def macrophages always reacted stronger than the single-deficient TLR4-def cells. The reasons for that observation are not yet investigated and might be the consequence of a higher amount of accessible intracellular adaptor proteins in cells with two non-functional receptors or a compensatory up-regulation of other PRRs.

In summary, we have demonstrated a TLR4-mediated induction of mRNA of the IL-12 family members by *Salmonella* Enteritidis characterized by differing kinetics and varying expression levels in resting and stimulated BMDM. Macrophages show high-level production of mRNA for the IL-27 subunits, whereas the secretion of IL-23 and IL-12p70 is restricted by a moderate mRNA expression and is negatively regulated by IL-10. The quick and transient up-regulation of the p19 mRNA suggests a time-limited secretion of IL-23 cytokine after microbial activation of macrophages. Since the specific IL-23R was induced in macrophages after LPS stimulation (20), this implies that IL-23 released by macrophages probably unfolds an autocrine effect.

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### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow-derived macrophages</td>
</tr>
<tr>
<td>CSF</td>
<td>colony-stimulating factor</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBI-3</td>
<td>Ebstein-Barr-Virus-induced gene 3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony-stimulating factor</td>
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<tr>
<td>hKSE</td>
<td>heat-killed <em>Salmonella</em> Enteritidis</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>vSE</td>
<td>viable <em>Salmonella</em> Enteritidis</td>
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### References


