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Inherited IL-12 Unresponsiveness Contributes to the High LPS Resistance of the $Lps^d$ C57BL/10ScCr Mouse

Thomas Merlin, Andreas Sing, Peter J. Nielsen, Chris Galanos, and Marina A. Freudenberg

$Lps^d$ mouse strains are characterized by the presence of a defective $Lps/tlr4$ gene that make them refractory to the biological activity of LPS. One of the mouse strains commonly used to study LPS defects is the C57BL/10ScCr (Cr) strain. However, unlike other $Lps^d$ strains, the Cr strain also has a heavily impaired IFN-γ response to micro-organisms. As a consequence, unlike other $Lps^d$ mouse strains, they do not acquire a partial LPS susceptibility when treated with sensitizing bacteria. Because IL-12 is important for the microbial induction of IFN-γ, we investigated whether the production or function of IL-12 might be defective in Cr mice. IL-12 mRNA (p35 and p40) was present in the spleen of untreated Cr mice, IL-12p40 mRNA was inducible in mice injected with live or killed Salmonella typhimurium, and IL-12 (p70) was inducible in macrophages by bacteria. Thus, Cr mice exhibit normal IL-12 responses.

In functional tests, splenocytes of untreated or of S. typhimurium-infected mice failed to produce IFN-γ when stimulated with murine rIL-12 or with a combination of IL-12 and murine rIL-18 or Con A. Furthermore, Cr mice were identical with IL-12p35/p40 and IL-12 receptor β1 knockout mice in their impaired in vivo and in vitro IFN-γ responses to bacteria. Thus, Cr mice carry a second genetic defect unrelated to the $Lps/tlr4$ mutation that underlies the IL-12 unresponsiveness and contributes to the LPS resistance and impaired innate immune response in this strain. The Journal of Immunology, 2001, 166: 566–573.

The identification of LPS-resistant mouse strains led to the recognition that in mice, sensitivity to LPS is controlled by a gene locus on chromosome 4 called the $Lps$ gene (1). The terms $Lps^d$ and $Lps^a$ were then introduced to designate mice with normal and defective LPS responses, respectively. $Lps^d$ mice are highly resistant to the biological activity of LPS. Today, three $Lps^d$ mouse strains with natural mutations in the $Lps$ gene are known, C3H/HeJ (2), C57BL/10ScCr (Cr) (3), and its progenitor strain C57BL/10ScSn (Sn) (4). In addition, LPS-resistant BALB/c (BALB/cI) mice were produced independently in two laboratories by backcrossing the defective $Lps$ gene from C3H/HeJ mice into the BALB/c background (5, 6). Recently, the $Lps$ gene was shown to correspond to the Toll-like receptor-4 gene ($tlr4$) (7) and in the meantime this was confirmed (8). The mutation displayed by C3H/HeJ mice resulted in an exchange of proline for histidine at position 712 of the Tlr4 protein, while the Cr mice carry a null mutation of $tlr4$ (7). Since then, additional evidence that $tlr4$ and $Lps$ are identical was obtained by showing that transfection of a number of cell lines with Tlr4 results in the acquisition of LPS susceptibility (9, 10). Furthermore, the disruption of $tlr4$ by molecular targetting led to an LPS-resistant phenotype of the resulting knock-out mice (11).

The LPS susceptibility of mice may increase considerably during infection or after treatment with a variety of killed bacteria, such as Calmette-Guerin bacillus, Propionibacterium acnes, Coxella burnetti, and Salmonella typhimurium (12–16). The bacterial-induced sensitization toward LPS is mediated by endogenously produced IFN-γ (13, 17). Upon LPS challenge, sensitized $Lps^a$ mice produce enhanced amounts of proinflammatory cytokines, such as TNF-α, IL-6, or IFN-γ, and are hypersensitive to the lethal activity of LPS and TNF-α. Sensitization to LPS proceeds also in the $Lps^d$ C3H/HeJ and BALB/cI mice, leading to a change of phenotype from LPS resistant to partially LPS sensitive (15, 18, 19). However, a similar sensitization is lacking in Cr mice, which remain LPS resistant after treatment with sensitizing bacteria (20).

The absence of sensitization in Cr mice is due to a defect in IFN-γ production in response to bacteria and parasites (13, 20–22). The impaired IFN-γ production is not due to a general defect in IFN-γ response, because Cr mice are capable of producing IFN-γ when stimulated with the T cell mitogen Con A and CD3 mAbs (20–22). Different cell types and soluble factors are involved in the induction of IFN-γ. Thus, in addition to IFN-γ-producing cells (T cells, NK cells, and macrophages) and accessory cells (macrophages and dendritic cells) a number of cytokines are known to participate directly or indirectly in the induction of IFN-γ (22–32). It has been shown that IFN-β is not produced in bacteria-treated Cr mice (22). However, it was recently shown that this lack of IFN-β production is due to their LPS unresponsiveness and cannot explain the IFN-γ defect of Cr mice (33). Furthermore, we demonstrated that the production of IL-1, IL-10, and TNF-α (33), as well as of IL-2, IL-4, IL-15, IL-18, TGF-β, and the membrane-associated proteins B7-1, B7-2, and CD40 (our unpublished observations), which are all known to be involved in IFN-γ production (34, 35), are normal in Cr mice. Therefore, the IFN-γ defect of Cr mice does not seem to be related to a defective production of one of the above factors.

Among the soluble factors, IL-12 is of special interest, being capable of directly inducing IFN-γ in T and NK cells and promoting T cell differentiation in the IFN-γ-producing Th1 subset (reviewed in Ref. 36). Its IFN-γ-inducing activity can be enhanced further by a synergistic action with accessory cytokines, such as...

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2 Abbreviations used in this paper: Cr, C57BL/10ScCr; ScN, C57BL/10ScN; BALB/cI, LPS-resistant BALB/c; mrIL, murine recombinant IL; Sn, C57BL/10ScSn; RPA, RNase protection assay.

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IL-18, or with other stimuli, such as Con A (37). Early in vitro studies showed that bacteria, bacterial products (including LPS), and intracellular parasites were among the most powerful inducers of IL-12 in accessory cells (38, 39). Several in vivo studies have shown that IL-12 is the main factor responsible for the IFN-γ production during various bacterial, parasitic, and viral infections (reviewed in Ref. 40). In the present study it is shown that the IFN-γ defect of Cr mice is caused by their inability to respond to IL-12.

Materials and Methods

Materials

Bacteria. S. typhimurium (C5), Staphylococcus aureus, Listeria monocytogenes, and P. acnes (strain ATCC 12930; American Type Culture Collection, Manassas, VA) were grown and killed as described previously (33). For use, the bacteria were suspended in pyrogen-free PBS, pH 7.2. LPS of Salmonella abortus equi in its uniform triethylamine salt form was obtained as described previously (41). A sterile aqueous stock solution (10 mg/ml) was prepared and stored at 4°C. Before use, the LPS was diluted further with pyrogen-free PBS to the desired concentration. Murine rIL-12 (mIL-12) was purchased from Pharmingen (San Diego, CA), and mIL-18 was obtained from PANSYSTEMS (Aidenbach, Germany). Con A was purchased from Pharmacia (Freiburg, Germany).

Animals

Lpsd mice, C57BL/10ScSn (Sn), BALB/c, 129 SvPas, IL-12β2/−/− 129 Sv background, and IL-12p53/p40−/− on BALB/c background, and Lpsd mice, Cr and BALB/c mice, were bred under specific pathogen-free conditions in the animal facilities of the Max Planck Institut für Immunbiologie. Breeding pairs of IL-12β2/− and IL-12p53/p40−/− were provided by Dr. M. K. Gately (Hoffmann-La Roche, Nutley, NJ). Lpsd ScN breeding pairs were purchased from the Animal Production Program (San Diego, CA) and kept at conventional clean conditions in the animal facilities of the Max Planck Institute. Five- to 10-wk-old animals of both sexes were used in this study.

P. acnes sensitization and TNF-α induction

Groups of mice received 625 µg of P. acnes in 0.2 ml of PBS/25 g body weight i.v. Seven days later, the animals received an i.v. injection of LPS (0.2 ml of PBS/25 g). One hour after challenge, the animals were exsanguinated under ether anesthesia. The blood was collected in heparinized tubes and centrifuged at 4°C. The resulting plasma was aliquoted and stored at −80°C.

TNF-α bioassay

TNF-α in plasma was measured in a cytotoxicity test using a TNF-α-sensitive L929 cell line in the presence of actinomycin D as described previously (42, 43). The detection limit of the assay was 60 pg of TNF/ml plasma. Rabbit anti-mouse TNF-α (Genzyme, Boston, MA) was used as an inhibitor to test the specificity of the assay.

Infection

For infection, S. typhimurium (C5), previously passed through Sn mouse spleens to warrant its virulence, was grown overnight at 37°C on Luria Bertoni agar (Difco, Detroit, MI). Bacteria were suspended in PBS to a concentration of 107/ml, and placed (2 × 106/well) in 96-well plates (Nunc, Roskilde, Denmark) and cultured at 37°C in a humidified atmosphere containing 8% CO2 for 24 h. Culture supernatants were hybridized overnight with an excess of labeled probes. After hybridization, the filters were washed three times in 2× saline sodium citrate, once in 0.5× saline sodium citrate, and once in 2× SSC containing 0.1% SDS. The filters were then exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) for 1–7 days at −70°C.

ELISAs

IFN-γ in supernatants of splenocyte cultures and in murine plasma was estimated by a previously described ELISA (44). The limit of IFN-γ detection was 60 pg/ml. IL-12 (p70) in supernatants of macrophage cultures was estimated by ELISA using anti-IL-12 mAb (C17.8) and biotin-labeled anti-IL-12 mAb (C15.6; Pharmingen, Hamburg, Germany) as described previously (45). The limit of detection was 15 pg/ml.

RNA extraction

Total RNA was isolated from freshly removed spleens or from cultured splenocytes by a guanidinium isothiocyanate-phenol-chloroform-isooamyl alcohol procedure (46) as described previously (33). The RNA concentration in RNase-free H2O was determined by absorbance at 260 nm.

RT-PCR

Expression of IL-12p35, IL-12p40, and β2-microglobulin mRNA was determined by RT-PCR. One microgram of total RNA was reverse transcribed using the Moloney leukemia virus reverse transcriptase from Life Technologies (Gaithersburg, MD) as described in the provider’s protocol. The RNA samples were hybridized overnight with random primed 32P-labeled cDNA probes as described earlier (22). The IFN-γ probe was a 247-bp cDNA fragment of mouse IFN-γ (EcoRI/EcoRV digest of mIL-12 cDNA) with the 5′ flanking region and U3 promoter as described previously (33). The IL-12p40 probe was a 340-bp cDNA fragment from a cDNA library from murine splenocytes. The IL-12p40 group of primers anneal across the IL-12p40 gene body and amplify a 334-bp DNA fragment. The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG). The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GTGGTACACCGACCCAGT; antisense, 5′-GTGGTCCACCGACCCAGT; antisense, 5′-GTGGTACACCGACCCAGT; antisense, 5′-GTGGTACACCGACCCAGT). RNAs were hybridized overnight at 65°C with random primed 32P-labeled cDNA probes as described earlier (22). The IL-12p40 probe was a 340-bp cDNA fragment from a cDNA library from murine splenocytes. The IL-12p40 group of primers anneal across the IL-12p40 gene body and amplify a 334-bp DNA fragment. The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG). The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG). The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG).

Northern blot analysis

RNA samples were fractionated on 1.2% denaturing agarose-formaldehyde gels and transferred to Nytran filters as described previously (47). RNAs were hybridized overnight at 65°C with random primed 32P-labeled cDNA probes as described earlier (22). The IFN-γ probe was a 440-bp cDNA fragment of mouse IFN-γ (EcoRI/EcoRV digest of mIL-12 cDNA) with the 5′ flanking region and U3 promoter as described previously (33). The IL-12p40 probe was a 340-bp cDNA fragment from a cDNA library from murine splenocytes. The IL-12p40 group of primers anneal across the IL-12p40 gene body and amplify a 334-bp DNA fragment. The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG). The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG). The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG). The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG). The 3′ flanking fragments were amplified using primers specific for IL-12p35 (sense, 5′-GATGACATGTTGAAGCCG; antisense, 5′-GGAGTTTTCGCGCAGAAGG; antisense, 5′-GGAGTTTTCGCGCAGAAGG).

RNase protection assay (RPA)

IL-12β2 and β2-mRNA were detected by RPA using a RiboQuant MultiProbe RPA system for the detection of cytokine receptors mRNA (Pharmingen, San Diego, CA), as described in the provider’s protocol. Briefly, the template set (mCR-3) was used for a T7 RNA polymerase-dependent synthesis of 32P-labeled antisense RNA probes. The RNA samples were hybridized overnight with an excess of labeled probes. After treatment with RNase A and T1 and with proteinase K, the samples were loaded on a 6% polyacrylamide-Tris-borate-EDTA-urea gel and run at 1900 V with 1× Tris-borate-EDTA electrophoresis buffer, pH 8.3. The gel was dried and exposed on film (Biomax MS; Eastman Kodak, Rochester, NY) using a cassette with an intensifying screen (Cronex Lightning Plus; DuPont, Wilmington, DE) and kept at −70°C until film development.
IL-12 UNRESPONSIVENESS OF THE C57BL/10ScCr MICE

Results

Presence of IL-12p35 and IL-12p40 mRNA in the spleen of Cr and Sn mice

IL-12 is a heterodimer of two polypeptide chains, IL-12p35 (p35) and IL-12p40 (p40). The presence of p35 and p40 mRNA in IFN-γ-defective, Cr and IFN-γ-normal, Sn mice was tested for by analysis of the total spleen RNA using RT-PCR. As shown in Fig. 1, p35 and p40 mRNA were present in unstimulated splenocytes of both mouse strains. Thus, both IL-12 genes can be transcribed in Cr mice.

Inducibility of IL-12p40 mRNA and of IFN-γ by killed S. typhimurium in the spleen of Cr and Sn mice

The detection of mRNA for the highly regulated p40 gene is a good indicator for IL-12 production (40). We investigated the induction of p40 mRNA in the spleen of Cr and Sn mice, stimulated with heat-killed S. typhimurium, by Northern blot analysis. As shown in Fig. 2a, only very faint bands of p40 mRNA were detectable in the spleen of unstimulated mice of either mouse strain. Stimulation with bacteria led to a strong induction of p40 mRNA in animals of both strains. The levels were highest at 1 and 2 h of stimulation and decreased 4 h after stimulation (Fig. 2a). However, while S. typhimurium-treated mice of both strains exhibited a similar induction of p40 mRNA, only Sn mice exhibited a strong induction of IFN-γ mRNA (Fig. 2a). In accordance, circulating IFN-γ was detectable only in Sn, but not in Cr, mice (Fig. 2b).

Inducibility of IL-12p40, IL-18, and IFN-γ transcripts in Lpsn and Lpsd mice by S. typhimurium infection

Induction of p40 mRNA was investigated also in the spleen of Sn and Cr mice, during the first 4 days of infection with S. typhimurium. In addition, induction of mRNAs for IL-18 and IFN-γ was followed in the infected mice. Because S. typhimurium contains LPS, and the two mouse strains, Lpsd Cr and Lpsn Sn, differ in their responsiveness to LPS, the induction of the above cytokines was also investigated in infected Lpsn and Lpsd mice on BALB/c background (BALB/c and BALB/c/l). Control noninfected mice of all four strains used exhibited no IFN-γ mRNA and no, or only a very weak, expression of p40 mRNA in the spleen. However, the mice showed a constitutive expression of IL-18 mRNA in this organ. All mice, especially those of the Lpsd strains, exhibited a detectable induction of p40 mRNA and a significant up-regulation of IL-18 mRNA on day 4 of infection. Interestingly, p40 mRNA bands induced in the spleen of Cr and BALB/c/l mice were always visibly stronger than those in Sn or BALB/c mice, respectively. This finding correlates with the faster progression of S. typhimurium infection observed in Lpsd mice and may be explained by the higher bacterial load present in the spleen of these animals (Table I). However, induction of IFN-γ mRNA became detectable on day 2 only in Sn, BALB/c, and BALB/c/l, and increased further on days 3 and 4 of infection (in Fig. 3, only expression on day 4 is shown). In Cr mice, IFN-γ mRNA was completely absent on days 2 and 3 (data not shown), and only a weak expression appeared first on day 4 (Fig. 3). In agreement, plasma levels of IFN-γ were detectable in infected Sn, BALB/c, and BALB/c/l, but not in infected Cr mice (Table I).

Production of IL-12 by Lpsn and Lpsd macrophages

To test the capability of Cr mice to produce and secrete IL-12, macrophages of Cr, Sn, BALB/c, and BALB/c/l mice were prepared and stimulated with killed S. typhimurium and S. aureus and with LPS. IL-12 in macrophage supernatants was determined by a

FIGURE 1. Expression of IL-12p35 and IL-12p40 mRNA in splenocytes of Sn and Cr mice. Total RNA of splenocytes from Sn and Cr mice was analyzed by RT-PCR for p35 and p40 mRNA expression. Expression of β2-microglobulin (β2m) was used for standardization of RNA amounts. The sizes of the amplified fragments corresponded to the expected sizes (IL-12p35, 402 bp; IL-12p40, 384 bp; β2m, 222 bp). One representative experiment of two is shown. c, control (PCR without cDNA).

FIGURE 2. Induction of IL-12p40 mRNA and secretion of IFN-γ in Sn and Cr mice injected with S. typhimurium. Sn and Cr mice were injected i.v. with killed S. typhimurium (Stm; 15 μg/g body weight). Untreated mice served as controls (c). a, At different time intervals after treatment the mice were sacrificed, and total spleen RNA was extracted and used for detection of IL-12p40 and IFN-γ mRNA by Northern blot analysis as described in Materials and Methods. For each strain and time point, RNA from two individual animals was analyzed. RNA applied to the gel (~15 μg/lane) was visualized for each sample by the intensity of the ethidium bromide-stained 18S rRNA bands. b, IFN-γ in plasma of mice 6 h after injection of S. typhimurium was measured by a specific ELISA. The data represent mean values of four mice per group ± SD.
sacrificed, and total spleen RNA was extracted and used for detection of treated mice served as controls (c). Four days after infection, the mice were a dose-dependent production of IFN-\(g\) inability of Cr mice to produce IFN-\(g\) are known to synergize with IL-12 in the induction of IFN-\(g\).

**Materials and Methods.**

Materials and Methods.

plasma by a specific ELISA. The spleens were homogenized, and the bacteria in the organ enumerated as described in Materials and Methods. The data represent mean values of four to five animals per group \(\pm\) SD. One representative experiment of four is shown.

specific ELISA for IL-12p70. As shown in Fig. 4, unstimulated macrophages of all mouse strains used, including Cr, already exhibited a low constitutive production of IL-12, which increased by a factor of 10 or more upon addition of bacteria to the cultures. Addition of LPS, as expected, induced an IL-12 response only in macrophages of Lps\(a\) Sn and BALB/c, but not of Lps\(d\) Cr and BALB/c/l, mice.

**Inability of Cr splenocytes to produce IFN-\(g\) in response to mrIL-12.**

As shown in the foregoing experiments, Cr mice exhibit a normal production of IL-12 in response to bacteria. In view of the fact that IL-12 is a direct inducer of IFN-\(g\), we investigated whether the inability of Cr mice to produce IFN-\(g\) when treated with bacteria may be related to IL-12 unresponsiveness. Splenocytes of Cr, Sn, BALB/c, and BALB/c/l mice were stimulated in parallel with different amounts of mrIL-12, and IFN-\(g\) levels in culture supernatants were estimated by ELISA. As shown in Fig. 5, IL-12 induced a dose-dependent production of IFN-\(g\) in the Sn, BALB/c, and BALB/c/l cultures, but not in Cr cultures. Furthermore, in Sn splenocytes, the IFN-\(g\) responses to IL-12 were enhanced by addition of rIL-18 or suboptimal (low) amounts of Con A, both of which are known to synergize with IL-12 in the induction of IFN-\(g\) (Fig. 6, top). The IFN-\(g\) responses to IL-12 alone or in combina-

**FIGURE 4.** Levels of IL-12 in supernatants of Lps\(a\) and Lps\(d\) macrophages stimulated with different agents. Macrophages (10^6/ml) from Sn, Cr, BALB/c, and BALB/c/l mice were cultured with LPS (0.1 \(\mu\)g/ml), S. typhimurium (Stm; 100 \(\mu\)g/ml), and S. aureus (Sa; 100 \(\mu\)g/ml) for 24 h. IL-12p70 in cell-free supernatants was measured by a specific ELISA. The values are the means of duplicates. One representative experiment of four is shown. * \(\approx 4000 \text{pg/ml.}

**FIGURE 5.** IFN-\(g\) response of Lps\(a\) and Lps\(d\) splenocytes to rIL-12. Splenocytes (pooled cells from three animals) of Sn, Cr, BALB/c, and BALB/c/l mice were cultured with different amounts of mrIL-12 for 24 h. IFN-\(g\)-in cell-free supernatants was measured by a specific ELISA. Splenocytes cultured in the absence of stimuli produced no detectable IFN-\(g\). One representative experiment of three is shown.

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### Table I. Plasma IFN-\(g\) and number of bacteria (CFU) present in the spleens of mice infected with S. typhimurium

<table>
<thead>
<tr>
<th>Mouse</th>
<th>IFN-(g) (pg/ml)</th>
<th>CFU (\times 10^5)Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr</td>
<td>(\geq 60)</td>
<td>6.1 (\pm 2.0)</td>
</tr>
<tr>
<td>Sn</td>
<td>1994 (\pm 880)</td>
<td>0.43 (\pm 0.29)</td>
</tr>
<tr>
<td>BALB/c/l</td>
<td>4508 (\pm 1303)</td>
<td>35 (\pm 10)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>2156 (\pm 648)</td>
<td>1.3 (\pm 0.86)</td>
</tr>
</tbody>
</table>

* Mice were infected i.v. with 3 \(\times 10^5\) CFU of S. typhimurium. Four days after infection, mice were exsanguinated, and the IFN-\(g\) concentration was determined in plasma by a specific ELISA. The spleens were homogenized, and the bacteria in the spleens of mice infected with S. typhimurium (Stm; 100 \(\mu\)g/ml), S. aureus (Sa; 100 \(\mu\)g/ml) for 24 h. The IFN-\(g\) response of Cr mice was completely absent even when the amount of IL-12 used in this experiment was increased to 20 ng/ml.

Cr and knockout mice, lacking endogenous IL-12 activity, exhibit similarly impaired IFN-\(g\) responses

From the above data we assumed that IL-12 unresponsiveness is the most likely reason for the defective IFN-\(g\) response of Cr mice to bacteria. To confirm this, we extended the studies to IL-12R \(\beta\) deficient (IL-12R \(\beta\) \(\beta\)) and IL-12p35/p40 \(2\) mice, both lacking endogenous IL-12 activity. A comparison of the IL-12R \(\beta\) \(\beta\), IL-12p35/p40 \(2\), and Cr mice revealed that all three types of mice were identical in all respects tested. Splenocytes from all types of mice exhibited no IFN-\(g\) response to killed bacteria (S. typhimurium, S. aureus, P. acnes, and L. monocytogenes in concentrations up to 100 \(\mu\)g/ml). Furthermore, no circulating IFN-\(g\) was detectable in plasma of mice infected with 3 \(\times 10^2\) or 3 \(\times 10^4\) CFU of S. typhimurium (measured up to day 5 after infection;
activity a
3
Sn and Cr mice, and from mice infected i.v. with 3 (pooled cells from four animals) were obtained from noninfected control
S. typhimurium
noninfected and
representative experiment of four is shown.

According to this reasoning, we compared the ability of mrIL-12 to induce IFN-γ in cell-free supernatants was measured by a specific ELISA. One representative experiment of three is shown.

Tables I and II). Finally, splenocytes of all three mouse strains exhibited easily detectable IFN-γ responses when stimulated in vitro with high amounts of Con A (5 μg/ml). However, these responses were often 2–3 times lower than the responses obtained in cultures of closely related mice with normal IL-12 activity (data not shown). From these results it is concluded that the inability of Cr mice to produce IFN-γ when stimulated with bacteria is due to IL-12 unresponsiveness.

Comparison of IL-12 responsiveness between Cr and their progenitor ScN mice

From the above results, it is evident that Cr mice exhibit, in addition to their known LPS response defect, a second phenotypic abnormality, namely IL-12 unresponsiveness. The question arose of whether the two defects were caused by a single mutational abnormality, namely IL-12 unresponsiveness. The question arose of whether the two defects were caused by a single mutational

Table II. Plasma IFN-γ and number of bacteria (CFU) present in spleens of S. typhimurium-infected mice lacking endogenous IL-12 activity.*

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>IFN-γ (pg/ml)</th>
<th>CFU × 10⁷/Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>1495 ± 341</td>
<td>1.2 ± 0.69</td>
</tr>
<tr>
<td>IL-12p35/p40−/−</td>
<td>≤60</td>
<td>10.4 ± 5.3</td>
</tr>
<tr>
<td>129 Sv background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>192 ± 92</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>IL-12p35/p40−/−</td>
<td>≤60</td>
<td>0.38 ± 0.33</td>
</tr>
<tr>
<td>IL-12β1−/−</td>
<td>≤60</td>
<td>0.42 ± 0.42</td>
</tr>
</tbody>
</table>

* Mice were infected i.v. with 3 × 10⁷ CFU (C57BL/6 background) or 3 × 10⁸ CFU (129 Sv background) of S. typhimurium. Four (C57BL/6 background) or 5 days (129 Sv background) after infection, mice were exsanguinated, and the IFN-γ concentration was determined in plasma by a specific ELISA. The spleens were homogenized, and the bacteria in the organ enumerated as described in Materials and Methods. The data represent mean values of four to six animals per group ± SD. One representative experiment of four is shown.

Pretreatment of mice with heat-killed P. acnes elicits an IFN-γ-dependent sensitization toward LPS (13, 48). In the following experiments we compared the LPS susceptibility of control and P. acnes-pretreated mice with either a functional (Sn, ScN, 129 SvPas, BALB/c, and BALB/c/f) or a defective (Cr, IL-12p35/p40−/−, and IL-12β1−/−) IL-12 system. An enhanced TNF-α response to LPS served as an indicator of sensitization. As shown in Fig. 8 pretreatment with P. acnes induced a strong sensitization toward LPS in all Lps+ mouse strains with functional IL-12. A sensitization toward LPS was also induced in both P. acnes-treated Lps− mice belonging to this group, as shown by a partial phenotype change from strictly LPS-nonresponsive to partially LPS-sensitive (Fig. 8). In contrast, sensitization was absent in Lps− IL-12p35/p40−/−, IL-12β1−/−, and Lps− Cr mice, showing that sensitization to LPS by P. acnes requires a functional IL-12 system. Thus, independently of the differences in the tlr4 gene, Cr mice share a similar phenotype with IL-12p35/p40−/− and IL-12β1−/− mice, being resistant to the sensitizing effect of P. acnes.

The induction of transcripts of IL-12R chains in Cr mice is normal

The biological activity of IL-12 on cells is mediated via the IL-12R

Figure 6. IFN-γ response to different stimuli of splenocytes from noninfected and S. typhimurium-infected Sn and Cr mice. Splenocytes (pooled cells from four animals) were obtained from noninfected control Sn and Cr mice, and from mice infected i.v. with 3 (pooled cells from four animals) were obtained from noninfected control S. typhimurium noninfected and representative experiment of four is shown.

Figure 7. Differences in the IFN-γ response to rIL-12 by splenocytes of the closely related Cr, ScN, and Sn mice. Splenocytes from ScN, Sn, and Cr mice (pooled cells from four animals) were cultured for 24 h in the presence of rIL-12 (6.25 ng/ml) or Con A (5 μg/ml). IFN-γ in cell-free supernatants was measured by a specific ELISA. Splenocytes cultured in the absence of stimuli produced no detectable IFN-γ. One representative experiment of three is shown.
FIGURE 8. TNF-α response to LPS of control and P. acnes-sensitized Lpsn and Lpsd mice with a functional or defective IL-12 system. Lpsn and Lpsd mice with a functional IL-12 system (Sn, Scn, 129 SvPas, BALB/c, and BALB/c/l) or defective in IL-12 production (IL-12p35/p40−/−) or IL-12 signaling (Cr and IL-12Rβ2−/−) received P. acnes (25 μg/g mouse) i.v. or remained untreated. Seven days later all mice received LPS (Lpsn mice, 10 μg/mouse; Lpsd mice, 300 μg/mouse). Plasma for TNF-α determination was collected 1 h after LPS challenge. The bars represent the mean values of four mice per group ± SD. Differences between the control group and the P. acnes-treated group that are not significant (p > 0.05) are indicated (+).

FIGURE 9. Expression of IL-12Rβ2 and β2mRNA in Lpsn and Lpsd mice. a, Expression in Con A-stimulated splenocytes. Splenocytes (pooled cells from three animals) of Sn, Cr, BALB/c, and BALB/c/l mice were cultured as described in Materials and Methods and stimulated with Con A (5 μg/ml). At different time intervals after stimulation, total RNA was extracted. RNA (2.5 μg) from each sample was used for detection of IL-12Rβ2 and β2mRNA by RPA as described in Materials and Methods. b, Expression in the spleen of S. typhimurium-infected mice. Mice were infected i.v. with 3 × 10^7 CFU of S. typhimurium (inf.) or remained untreated (c). Four days after infection spleens of infected and uninfected mice were removed. Total spleen RNA was extracted separately for each animal and pooled for the four identically treated animals of each group. Ten micrograms of each RNA pool was used for detection of IL-12Rβ2 and β2mRNA in an RPA. A constitutive gene probe (GAPDH) was used for standardization of RNA amounts.

Discussion

In Cr mice the IFN-γ response to microorganisms is impaired, and this is responsible for the absence of sensitization to LPS in these mice during infection. Because the microbial induction of IFN-γ is predominantly IL-12 dependent, we investigated whether a defect in the production or function of IL-12 may be present in these mice. The present results provide evidence that bacterially induced IL-12 production in Cr mice is intact, being comparable to that of other mouse strains tested, especially to the responses of the Lpsd BALB/c/l mice.

The induction of IFN-γ in resting and activated splenocytes by rIL-12 was used as a measure of the IL-12 responsiveness in these cells. In complete contrast to splenocytes of Sn, BALB/c and BALB/c/l mice, neither splenocytes from healthy nor those from infected Cr mice responded to IL-12 alone or to a combination of IL-12 and IL-18 or Con A. Thus, the impaired IFN-γ response of Cr mice to bacteria and other micro-organisms investigated previously (13, 20–22) is explained by a lack of IL-12 responsiveness, and this is the underlying reason for the absence of sensitization of these mice to LPS by micro-organisms. The importance of IL-12 for the development of LPS hypersensitivity is evidenced further by the results obtained with IL-12β2−/− or IL-12Rβ2−/− knockout mice. These mice are phenotypically similar to Cr mice, exhibiting impaired IFN-γ responses to bacteria and no sensitization to LPS after P. acnes treatment. These results are in accordance with those of an earlier study that demonstrated that IL-12 plays an important role in LPS-induced pathogenicity in Calmette-Guérin bacillus-primed mice (49).

The IL-12 unresponsiveness of Cr mice is not related to their tlr4 gene defect. As shown here, Scn mice, the progenitor strain of Cr, while carrying an identical tlr4 mutation (S. N. Vogel, unpublished observations; A. Poltorak and M. A. Freudenberg, unpublished observations), exhibit intact IL-12 responsiveness, indicating that this second mutation must have occurred after separation of Cr mice from the progenitor colony. As a result, Scn mice exhibit normal IFN-γ responses and, like Lpsd C3H/HeJ and BALB/c/l mice, become partially LPS responding, when pre-treated with P. acnes. Further evidence that in Cr mice the defect underlying IL-12 unresponsiveness is not related to the tlr4 mutation was obtained very recently in our laboratory. By intercrossing Cr and Sn mice, there was an independent assortment of the IL-12 unresponsiveness and tlr4 defect in the second filial generation (unpublished observations). Therefore, Cr mice carry two
independent mutations, both determining LPS resistance. In addition to the mutation of \textit{tlr4}, the defect in the IL-12 response exacerbates their LPS resistance. For this reason Cr mice are highly resistant to LPS under conditions (e.g., during infection) during which all other Lps\(^d\) mouse strains (C3H/HeJ, BALB/c, and SnC) become partially responsive to LPS.

However, it should be noted that Cr mice when treated with exogenous IFN-\(\gamma\) acquire partial LPS sensitivity. This raises the question of how a response to LPS, however low it may be, is possible in the complete absence of Tlr4. Two possibilities arise. One of these has been proposed by Vogel and coworkers, attributing the weak responses of sensitized Lps\(^d\) mice to bacterial contaminants (LPS-associated protein) present in the LPS preparations (50) that act synergistically with LPS (51). Although such an explanation is plausible, it is still not clear how the synergizing activity of LPS is expressed in Lps\(^d\) mice. Another explanation is that LPS may stimulate Lps\(^d\) mice via a Tlr4-independent pathway(s), however to a low degree that becomes clearly detectable only after sensitization with IFN-\(\gamma\). Such a pathway(s) may involve other members of the Tlr protein family, for example Tlr2 (52).

A large amount of information exists on IL-12 recognition and signaling in the mouse (40). We do not know yet whether the IL-12R itself or components of the signal transduction pathway are defective in Cr mice. However, because responses to other cytokines including TNF-\(\alpha\) (53), IFN-\(\alpha\), IFN-\(\beta\) (22), and IFN-\(\gamma\) (13) are intact in these mice, we assume that the defect concerns a component(s) involved more specifically in the IL-12 response. Components specific for recognition and signaling by IL-12, such as the two \(\beta\) subunits of IL-12R (\(\beta_1\) and \(\beta_2\)) (54, 55) or the transcription factor STAT4, have been demonstrated (56). We showed in this study that the induction of transcripts for both IL-12R \(\beta_1\) subunits does not differ from that seen in other IL-12 responder mouse strains investigated. However, this does not prove that functional receptor proteins are expressed on the surface of Cr cells.

In this study the indicator of IL-12 responsiveness in Cr mice was IFN-\(\gamma\) production. In further investigations other activities of IL-12, such as proliferative activity on T cells and NK cell cytotoxicity, should also be addressed. All these activities contribute to the natural resistance to infection. It should be mentioned in this connection that Cr mice were already shown to be highly susceptible to infection with \(L.\) \textit{major} (21), exhibiting a similar nonhealing phenotype as IL-12p40 and p35 knockout mice (57, 58).

Another important factor involved in antibacterial defense is the Lps\(^{tlr4}\) gene controlling LPS susceptibility, which is defective in BALB/c and Cr mice (7, 9–11). As shown in this study both mouse strains exhibited higher numbers of \(S.\) \textit{typhimurium} in their spleens and were more susceptible to infection than their related Lps\(^d\) BALB/c and Sn mice. The high number of bacteria in infected BALB/c mice is also a likely explanation for the high levels of IFN-\(\gamma\) appearing in the animals after the third day of infection. A similar observation was made earlier in \(S.\) \textit{typhimurium}-infected Lps\(^{tlr4}\) C3H/HeJ mice (20). Evidently the inability of Lps\(^d\) mice to sense LPS abrogates their ability to detect and react against invading Gram-negative bacteria at the early stages of infection.

Because both LPS and IL-12 responsiveness, which are necessary for the integrity of an antimicrobial defense, are impaired in Cr mice, it is postulated that Cr mice are highly sensitive to different pathogens.

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References


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