Requirement for TLR9 in the Immunomodulatory Activity of Propionibacterium acnes

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Propionibacterium acnes (formerly Corynebacterium parvum) is part of the human flora and, as such, is associated with several human pathologies. It possesses strong immunomodulatory activities, which makes this bacterium interesting for prophylactic and therapeutic vaccination. The bacterial component(s) and the host receptor(s) involved in the induction of these activities are poorly understood. We show in this study that TLR9 is crucial in generating the characteristic effects of killed P. acnes priming in the spleen, such as extramedullary hemopoiesis and organ enlargement, and granuloma formation in the liver. Furthermore, the ability to overproduce TNF-α and IFN-γ in response to LPS, lipid A, synthetic lipopeptide Pam3CysK4, or whole killed bacteria was present in P. acnes-primed wild-type, but not TLR9−/−, mice. Finally, P. acnes priming failed to induce enhanced resistance to murine typhoid fever in TLR9−/− mice. Thus, TLR9 plays an essential role in the induction of immunomodulatory effects by P. acnes. Because IFN-γ is a key mediator of these effects, and enhanced IFN-γ mRNA expression was absent in spleen and liver of P. acnes-primed TLR9−/− mice, we conclude that TLR9 is required for the induction of IFN-γ by P. acnes. The Journal of Immunology, 2005, 174: 4295–4300.

Propionibacterium acnes (formerly Corynebacterium parvum) is a member of normal flora of skin and gastrointestinal tract. It is also a human pathogen associated with various inflammatory diseases (1, 2). Administration of killed P. acnes in mammals induces multiple immunomodulatory effects, including inhibition of tumor growth and increased resistance against various infections (3–5). Priming of mice with killed P. acnes represents a well-established experimental model that has been used to study these effects. P. acnes stimulates the reticuloendothelial system (6), whereby treated mice develop splenomegaly (7–9), extramedullary hemopoiesis, and intrahepatic granulomas (6, 10). The degree of splenomegaly was shown to parallel the antitumor activity of P. acnes (11). Furthermore, P. acnes-treated mice develop a state of hypersensitivity to bacterial LPS (12, 13). The maximum LPS sensiti-

ized treatment with P. acnes induces hypersensitivity not only to LPS, but also to bacterial lipopeptides and possibly other pathogen-associated molecular patterns (16–18). The hypersensitivity to microbial components allows enhanced recognition of pathogens and elicitation of an accelerated and enhanced inflammatory reaction. This forms the basis for the enhanced resistance of P. acnes-primed animals to infection. At the same time, it enhances the risk of developing septic shock, as is the case with Gram-negative pathogens containing LPS. The induction of microbial hypersensitivity is strictly IFN-γ dependent and does not proceed in mice with impaired IFN-γ production or function (13, 19, 20).

Although priming with P. acnes is widely used in immunological research and is of interest for the prevention and treatment of tumors and infectious diseases in man and animals, the host receptors involved in the immunostimulatory process remain unknown. Because TLRs on cells of the innate immune system recognize highly conserved microbial structures (21–24), they are potential candidates for the induction of immunostimulatory effects of P. acnes. The importance of TLR2 signaling in cytokine induction by P. acnes has been reported (25). Experiments with TLR2−/− and TLR2−/−/TLR4−/− mice, however, revealed no significant involvement of TLR2 in the induction of hypersensitivity to LPS and other bacterial components (18). Based on previous reports, however, a role for TLR9 in the immunostimulatory effects of P. acnes may be envisaged. TLR9 is an intracellular receptor, expressed in the endoplasmic reticulum and the lysosomal compartment (26), and bacteria causing hypersensitivity, including P. acnes are intracellular parasites. Furthermore, the TLR9 ligands, bacterial DNA and synthetic CpG oligodeoxynucleotide, were shown to induce splenomegaly as well as extramedullary hemopoiesis (27) and to sensitize mice to LPS (28). In this study using TLR9-deficient mice, we demonstrate the requirement for TLR9 in the induction of IFN-γ and in the resulting characteristic effects of P. acnes priming.

Materials and Methods

Animals

C57BL/6 and TLR9−/− mice (29) (backcrossed three times to the C57BL/6 background) used in this study were bred under specific pathogen-free conditions in the animal facilities of the Max Planck Institut für Immunobiologie. TLR9−/− mice backcrossed 10 times to the C57BL/6 background
were provided by H. Wagner (Institute of Medical Microbiology, Immunology, and Hygiene, Technische Universität, Munich, Germany).

Lipopolysaccharide

A highly pure preparation of Salmonella abortus equi LPS in the uniform triethylenamine salt form used in the present study was prepared as described previously (30). A sterile aqueous LPS stock solution (20 mg/ml) was prepared and stored at 4°C until use. For administration in mice, LPS was further diluted with PBS, pH 7.2, to the required concentration and injected i.v. into the lateral tail vein.

Bacteria

Heat-killed P. acnes was prepared as described previously (13). For priming, mice received P. acnes (25 μg/0.2 ml PBS/g mouse b.w.) i.v.

A highly virulent strain of Salmonella enterica serovar Typhimurium C55 was grown overnight at 37°C on Luria-Bertoni agar medium (Difco). Bacteria from a single colony were suspended in PBS, pH 7.2, and the desired concentration was adjusted turbidometrically. The exact numbers of bacteria were determined by plating the bacterial suspension on Luria-Bertoni agar plates and counting the CFU after overnight culture. For infection, 2 × 10^6 CFU/0.2 ml PBS/mouse was administered i.v. Determination of viable bacteria in infected mice was conducted as described previously (31). For induction of cytokines, killed serovar Typhimurium was prepared by heating 5 × 10^10 CFU/ml PBS at 60°C for 1 h.

Virulent Listeria monocytogenes (EGD strain) was obtained from an overnight culture grown in tryptose-soy broth (Difco) and washed with pyrogen-free PBS. For infection, 10^9 CFU/0.2 ml PBS/mouse was administered i.v. Determination of viable bacteria in infected mice was conducted as described previously (31). For induction of cytokines, killed bacteria were prepared by heating 5 × 10^10 CFU/ml PBS at 60°C for 1 h.

Killed serovar Typhimurium and L. monocytogenes were stored in aliquots at −80°C. Before use, the bacteria were additionally diluted with PBS to a concentration of 10^9 CFU/0.2 ml.

The Limulus amebocyte lysate test, conducted according to the instructions of the manufacturer (Pyrotell), revealed no detectable amounts of LPS in the Gram-positive P. acnes or L. monocytogenes preparations.

Infection of mice with LCMV

Mice were infected i.p. with 2 × 10^5 PFU of lymphocytic choriomeningitis virus (LCMV) strain WE according to established protocols (32, 33).

TNF-α and IFN-γ induction and estimation

Mice, either 7 days after P. acnes administration or untreated controls, received the test agent i.v. in 0.2 ml of PBS. One hour (TNF-α) or 4 h (IFN-γ) after challenge, the animals were exsanguinated under isoflurane anesthesia. The heparinized blood was centrifuged at 4°C, and the resulting plasma was stored in aliquots at −80°C.

TNF-α in plasma was measured in a cytotoxicity test using a TNF-sensitive L929 cell line of fibroblasts in the presence of actinomycin D as described previously (34). The detection limit of the assay was 32 pg of TNF-α/ml plasma. Rabbit anti-mouse TNF-α (Genzyme) was used as an inhibitor to test the specificity of the assay. IFN-γ in plasma was estimated by a previously described ELISA (35). The limit of IFN-γ detection was 70 pg/ml plasma.

Histopathology

Groups of control and P. acnes-primed mice were killed 7 days after priming. Liver and spleen were removed and fixed in 4% buffered formaldehyde. Horizontal organ slices (5 μm thick) were prepared and stained using H&E. Microscopic pictures were taken from representative areas of the organ.

RNA extraction

Total RNA was isolated from freshly removed organs or organs that were preserved with RNAlater solution (Ambion) by a guanidinium isothiocyanate-pHENOL-chloroform-isooamyl alcohol procedure (36), as described previously (37). For enhanced RNA purity, the method was modified as follows. After precipitation with isopropanol, the RNA pellet was resuspended in lysis buffer (Stratagene) and additionally purified over an RNA binding Spin Cup using the RNA RT-PCR MiniPrep kit (Stratagene) according to the manufacturer’s instructions, including a DNase digestion step. The RNA concentration was determined by absorbance at 260 nm.

RT-PCR and real-time RT-PCR

Total RNA (1 μg) from liver and spleen was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers (Expand reverse transcriptase kit; Roche) according to the manufacturer’s instructions. PCR was performed using FastStart Taq DNA polymerase (Roche) according to the manufacturer’s instructions. Primers were chosen for the amplification of murine TNF-α and murine IFN-γ: TNF-α: sense, 5'-TCTTACATGTTCTATGGCCC; antisense, 5'-GGGATAGACAGGTAACACCC212 bp (product size); and IFN-γ: sense, 5'-CTCTGTAAGACAAAGTGAACGC; antisense, 5'-AACGGATAATCTGTGCTCTGC-272 bp (product size). β-Actin expression was analyzed using the primers: sense, 5'-TGGAAATCTGGGCACTGATCAGAAC; and antisense, 5'-TAAAACGCGACTCAAGACGTCGG-348 bp (product size). An annealing temperature of 56°C was used for all primer pairs.

Real-time hot-start PCR was performed with the LC FastStart DNA Master SYBR Green I Kit (Roche) in a LightCycler instrument (Roche) according to the manufacturer’s instructions. Murine TNF-α, IFN-γ, and β-actin were amplified using the primer pairs described above at an annealing temperature of 58°C. β-Actin expression was used to normalize cDNA levels. The PCR products were analyzed by melting curve and agarose gel electrophoresis assays to ascertain the specificity of the amplification. RT reactions, in which reverse transcriptase was replaced with water as well as with genomic mouse DNA, were used as controls to exclude products derived from contaminating genomic DNA. All PCR products were resolved by 1.6% agarose gel electrophoresis and were visualized by ethidium bromide staining.

Results

Absence of splenomegaly and of histological alterations in the spleen and liver of P. acnes-primed, TLR9-deficient mice

To investigate the requirement for TLR9 in the induction of splenomegaly, we compared the spleen weights of P. acnes-treated, wild-type (C57BL6) and TLR9-deficient mice and untreated controls. As shown in Fig. 1 the characteristic enlargement of the spleen present in wild-type mice was absent in TLR9-deficient mice 7 days after P. acnes treatment.

Histological examination of the spleen revealed P. acnes-induced extramedullary hemopoiesis in wild-type, but not in TLR9-deficient, mice (not shown). Furthermore, in the liver of wild-type mice, multiple large granulomas were present, consisting mainly of mononuclear cells (Fig. 2). In contrast, these histological alterations were practically absent from the liver of TLR9-deficient mice, and only mild vacuolar degeneration (ballooning) of the hepatocytes was observed 7 days after P. acnes priming. Almost identical results (absence of P. acnes-induced splenomegaly, extramedullary hemopoiesis, and multiple granuloma formation) were obtained in TLR9−/− mice backcrossed 10 times to the C57BL6 background (not shown). The results make it evident that...

4 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; b.w., body weight; PGN, peptidoglycan.
wild type

TLR9 -/-

FIGURE 3. TLR9−/− mice fail to produce enhanced amounts of IFN-γ and TNF-α after P. acnes priming. Groups of four mice were treated i.v. with heat-killed P. acnes (25 μg/g b.w.) or remained untreated (day 0). On the indicated days after treatment, mice were challenged with LPS (0.2 μg in 0.2 ml of PBS) i.v. Plasma for TNF-α and IFN-γ determinations was collected 1 and 4 h after challenge, respectively. Before challenge (at all time points), neither TNF-α nor IFN-γ was detectable. One representative experiment of two is shown.

FIGURE 2. TLR9−/− mice fail to develop extensive granulomas in the liver after P. acnes pretreatment. Wild-type and TLR9−/− mice (four animals per group) were primed with heat-killed P. acnes (25 μg/g b.w.) i.v., and livers were removed 7 days after pretreatment. Left panel, Liver histology of a primed wild-type mouse, showing granulomas of mononuclear cells (arrows). Right panel, Liver histology of a primed TLR9−/− mouse showing moderate ballooning of hepatocytes (arrowheads) and a small accumulation of mononuclear cells (m). H&E staining; primary magnification, ×40.

The typical P. acnes-induced alterations in the spleen and liver do not develop in mice lacking a functional TLR9 receptor.

TLR9 signaling is essential for the induction of hypersensitivity to bacterial components by P. acnes

To investigate whether TLR9 is also required in the P. acnes–induced hypersensitivity to bacterial components, we first compared the LPS sensitivity of wild-type and TLR9-deficient mice after P. acnes treatment. As a measure of sensitivity, we used the levels of TNF-α and IFN-γ induced in response to LPS. Groups of wild-type and TLR9-deficient mice received an i.v. LPS challenge at different time points after P. acnes priming, and the levels of circulating cytokines were determined 1 h (TNF-α) and 4 h (IFN-γ) later. Before challenge with LPS or other bacterial components or after challenge with PBS alone, no detectable TNF-α or IFN-γ was found in P. acnes-treated mice (not shown). The cytokine responses in wild-type mice increased with time from day 3 after P. acnes treatment and thereafter (Fig. 3). They reached maximum values on day 7, by which time the plasma levels of TNF-α and IFN-γ were 100- and >1000-fold higher, respectively, compared with untreated controls. In contrast, P. acnes priming did not augment the cytokine responses of TLR9−/− mice to LPS. Primed and unprimed TLR9−/− mice exhibited comparable TNF-α and IFN-γ responses at all time points investigated, with only minor differences between the two groups (Fig. 3). Almost identical results were obtained when TLR9−/− mice backcrossed 10 times to the C57BL/6 background were used (not shown), indicating that the lack of LPS sensitization in the knockouts was due to the absence of TLR9. Results similar to these were obtained in additional experiments in which, instead of LPS, a number of other agents (lipid A, synthetic lipopeptide Pam3CysK4, or whole killed serovar Typhimurium or L. monocytogenes) were used. Again, only P. acnes-primed, wild-type mice exhibited enhanced TNF-α responses, whereas the responsiveness of TLR9−/− mice remained unchanged after priming (Fig. 4). The results indicate a complete absence of P. acnes-induced hypersensitivity to different bacterial constituents in mice lacking TLR9.

TLR9 is not required in the development of LPS hypersensitivity induced by serovar Typhimurium, L. monocytogenes, or LCMV

In this study we investigated whether the requirement for TLR9 signaling seen in the induction of hypersensitivity by P. acnes is true for the development of hypersensitivity induced by sensitizing pathogens in general. For this purpose, we primed wild-type and TLR9−/− mice with three pathogens (S. enterica serovar Typhimurium, L. monocytogenes, and LCMV), which are known to strongly sensitize mice to LPS, and 4, 5, or 7 days later, respectively, measured the TNF-α response to LPS (Fig. 5). In contrast to P. acnes, all three pathogens induced strong LPS hypersensitivity in all mice regardless of the presence or absence of TLR9. This finding indicates that there is no general requirement for TLR9 in the different microbial sensitization models, and that various pathways are involved in the induction of hypersensitivity by different pathogens.

Induction of IFN-γ mRNA, but not TNF-α mRNA, by P. acnes is TLR9 dependent

The development of P. acnes-induced hypersensitivity is strictly dependent on IFN-γ production. RT-PCR analysis of total liver RNA revealed that in unsensitized mice (both strains), the levels of IFN-γ mRNA were below the detection limit. On days 3 and 7 after P. acnes administration, readily detectable IFN-γ mRNA expression was determined by RT-PCR in liver of wild-type, but not TLR9-deficient, mice (not shown). In contrast to that in liver, a detectable amount of IFN-γ mRNA was present in the spleen of unsensitized mice of both strains. In this organ, an increase in the relative IFN-γ mRNA levels was observed in wild-type mice on day 3, and the level was even higher on day 7 after P. acnes administration (Fig. 6a). However, there was no similar P. acnes-induced increase in the expression of IFN-γ mRNA in the spleen of TLR9-deficient mice (Fig. 6a). Interestingly, we found no difference between the two mouse strains in the induction of enhanced TNF-α mRNA expression in the spleen after P. acnes administration (Fig. 6b). P. acnes strongly induced the levels of TNF-α mRNA within 1 h after administration, which were still elevated after 4 h and returned nearly to normal after 24 h (Fig. 6b). Thus, the early induction of TNF-α by P. acnes in mice, unlike the later induction of IFN-γ, is TLR9 independent.

Enhanced resistance to serovar Typhimurium infection in P. acnes-primed mice requires TLR9

Priming with heat-killed P. acnes is known to enhance the natural resistance of mice to subsequent infection with various pathogens, including serovar Typhimurium (31). To investigate the possible requirement for TLR9 in the induction of increased resistance to infection, we infected control and P. acnes-treated, wild-type and TLR9−/− mice with serovar Typhimurium and compared their
susceptibilities to this pathogen. Unprimed wild-type and TLR9<sup>−/−</sup> controls showed no recognizable difference in susceptibility to murine typhoid. They exhibited comparable numbers of bacteria in the liver 4 days after infection (Fig. 7). By day 6, all infected controls showed severe signs of illness and had to be killed. As shown in Fig. 7, wild-type mice primed with <i>P. acnes</i> showed strongly reduced numbers of viable bacteria in liver on day 4 after infection compared with nonprimed controls. Furthermore, priming of wild-type mice with <i>P. acnes</i> led to 100% survival (not shown), demonstrating the protective effect of the sensitization. In contrast, no similar reduction of bacterial counts was observed in liver of <i>P. acnes</i>-treated TLR9<sup>−/−</sup> mice. All mice in this group developed severe signs of illness and had to be killed on day 6. The results indicate that TLR9 signaling plays a decisive role in the development of enhanced resistance to infection in <i>P. acnes</i>-primed mice.

**Discussion**

This study is the first report demonstrating a functional requirement for TLR9 in the ability of a bacterial pathogen to stimulate the mammalian immune system. Using wild-type mice and mice deficient in TLR9, we demonstrate that this intracellular receptor is essential for the induction of the immunomodulatory effects by <i>P. acnes</i>. These effects encompass induction of splenomegaly, extramedullary hemopoiesis in the spleen, histological alterations in liver, as well as induction of hypersensitivity to bacterial components and enhanced resistance to infection. The absence of <i>P. acnes</i>-induced effects in TLR9<sup>−/−</sup> mice was paralleled by the absence of induction of IFN-γ mRNA in spleen and liver. Because splenomegaly and hypersensitivity to bacterial components are proven IFN-γ effects, we conclude that the absence of these effects in TLR9<sup>−/−</sup> mice is the direct consequence of the impaired IFN-γ induction.

The above immunomodulatory activity of <i>P. acnes</i> in mice correlates directly with the ability of this bacterium to escape in vivo intracellular degradation and to persist for many days in the reticuloendothelial system (38, 39). The resistance to degradation was attributed to the unique structure of the peptidoglycan (PGN) component, which has been identified as the active principle of cell wall skeleton preparations of <i>P. acnes</i> (40, 41). At present it is not known whether the unique structure of <i>P. acnes</i> PGN is responsible for the TLR9-mediated effects. Our study revealed, however, that in addition to the essential TLR9-dependent induction of IFN-γ, there is a TLR9-independent induction of TNF-α in <i>P. acnes</i>-primed mice. TNF-α is an additional mediator, which participates in the development of <i>P. acnes</i>-induced immunological responses.

**FIGURE 4.** TLR9<sup>−/−</sup> mice fail to develop hypersensitivity to various bacterial components or whole heat-killed bacteria. For sensitization, mice received killed <i>P. acnes</i> (25 μg/g b.w.) administered i.v. or remained untreated (controls). Seven days later, groups of four or five animals were challenged with 0.2 μg of LPS, 2.0 μg of lipid A, 2 μg of lipoprotein (LP), 5 μg/g b.w. heat-killed serovar Typhimurium, or 15 μg/g b.w. heat-killed <i>L. monocytogenes</i> i.v. Plasma for TNF-α determination was collected 1 h after challenge. Before challenge, neither TNF-α nor IFN-γ was detectable. One representative experiment of three is shown.

**FIGURE 5.** TLR9 is not involved in the development of LPS hypersensitivity induced by serovar Typhimurium, <i>L. monocytogenes</i>, or LCMV. Groups of six mice were infected with serovar Typhimurium (2 × 10<sup>7</sup> CFU i.v.), <i>L. monocytogenes</i> (10<sup>5</sup> CFU i.v.), or LCMV (2 × 10<sup>5</sup> PFU i.p.) or remained uninfected (control). Serovar Typhimurium-infected mice were challenged i.v. with 10 μg of LPS on day 4. <i>L. monocytogenes</i>-infected mice were challenged with 0.2 μg of LPS on day 5, and those infected with LCMV were challenged with 0.2 μg of LPS on day 7 after infection. Plasma for TNF-α determination was collected 1 h after challenge. One representative experiment of two is shown.

**FIGURE 6.** <i>P. acnes</i> priming elevates TNF-α mRNA, but not IFN-γ mRNA, in the spleen of TLR9<sup>−/−</sup> mice. Mice were treated i.v. with heat-killed <i>P. acnes</i> (25 μg/g b.w.) or remained untreated (controls). The relative IFN-γ and TNF-α mRNA levels in the spleen at the indicated time points after pretreatment were analyzed by quantitative real-time PCR. Each value represents a mean from two to five animals. One representative experiment of three is shown.
activities, such as intrahepatic granuloma formation and sensitization to LPS (42). Because PGN is a known TNF-α inducer (43), its role in *P. acnes*-induced effects might be associated with a TLR9-independent induction of TNF-α. Previous studies showed that PGN is a ligand of TLR2 as well as of the intracellular receptors NOD1 and NOD2 for PGN of Gram-negative and -positive bacteria, respectively (44–46). A major involvement of TLR2, however, can be excluded, because *P. acnes*-induced hypersensitivity to bacterial components also develops in mice in the absence of TLR2 (18).

To date, DNA containing unmethylated CpG motifs are the only known bacterial ligands of TLR9. DNA is indeed a suitable candidate component responsible for the effects of *P. acnes*. There are intriguing similarities between the biological effects induced by *P. acnes* and CpG DNA in mice. Thus, Sparwasser et al. (27) demonstrated that administration of TNF-α in a macrophage-like cell line (47). Mice injected with bacterial DNA or synthetic CpG DNA exhibited enhanced resistance to *L. monocytogenes* infection. This effect required the presence of IFN-γ (48). Finally, treatment with CpG DNA, like treatment with *P. acnes*, exhibits antitumoral effects in mice (49). In our hands (results not shown), purified *P. acnes* DNA administered to mice at doses up to 25 μg/g b.w. i.v. exhibited no immunomodulatory activity. It should be mentioned, however, that 4 and 24 h after its administration, DNA was not detectable in the liver by *P. acnes*-specific PCR. In contrast, 4 and 24 h after the administration of 25 μg/g b.w. heat-killed *P. acnes* i.v., *P. acnes* DNA was easily detectable. We assume, therefore, that the concentration of *P. acnes* DNA in an adequately packed form in phagocytic cells of the liver and its gradual release (exposure) are probably necessary for the induction of TLR9-mediated *P. acnes* effects.

The immunomodulatory effects of killed *P. acnes* are also seen in man. For this reason, *P. acnes* has been used in therapeutic trials designed to combat malignant tumors (50–52). Because *P. acnes* is part of the normal human flora, a role of TLR9 in the normal resistance to infection and/or a preventive role in the development of malignant tumors may be envisaged. In this case, mutations in *tlr9* resulting in suppression or enhancement of *P. acnes* reactivity may impair natural resistance to infections and tumor development or may lower the threshold of incidence of oral and bowel inflammatory diseases, respectively. *P. acnes* is associated with various human pathologies, such as acne vulgaris (53), endophthalmitis (54), endocarditis (55), osteomyelitis (56), sarcoidosis (57), and prosthetic hip infections (58), often together with other bacteria. The identification of TLR9 as an essential factor of *P. acnes*-induced hypersensitivity to TLR4- and TLR2-dependent microbial components demonstrates that the signaling events through one TLR can lead to a drastic enhancement of the degree of responsiveness of other TLRs. Therefore, TLR9 may play an important role in the pathology of the above diseases and provide a potential target for the therapeutic intervention in *P. acnes*-associated pathologies.

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Disclosures

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FIGURE 7. Restriction of bacterial growth induced by *P. acnes* priming is impaired in TLR9−/− mice. Mice were treated i.v. with heat-killed *P. acnes* (25 μg/g b.w.) or remained untreated (controls). Seven days later, groups of five animals were infected with serovar Typhimurium (2 × 10^8 CFU, i.v.). The numbers of serovar Typhimurium (CFU) in the livers of mice 96 h after infection were determined as described in Materials and Methods. One representative experiment of two is shown.
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