Role for CD14, TLR2, and TLR4 in bacterial product-induced anorexia


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MAMMALS RESPOND TO MICROBIAL infections with both innate and adaptive immune responses. Microorganisms trigger the production of immune system mediators (e.g., cytokines and chemokines) that induce multiple physiological and behavioral changes, including lethargy, hypermetabolism, fever, anorexia, and body weight loss. These changes are part of the host’s “acute phase response,” which is also observed after parenteral administration of bacterial lipopolysaccharide (LPS; see Refs. 6 and 43) or muramyl dipeptide (MDP; see Ref. 24). LPS is a purified component of the outer membrane of most gram-negative bacteria, and MDP is the minimal immunologically active structure of the gram-positive bacterial cell wall.

Myeloid cells use several proteins to mount an immune response to LPS and MDP. LPS-binding protein (LBP), an acute-phase protein, the myeloid differentiation protein 2 (MD-2), the glycoprotein CD14, which exists in soluble (sCD14) or membrane (mCD14)-bound form, the toll-like receptors 2 and 4 (TLR2 and TLR4; see Refs. 1, 3, 14, and 17), and the myeloid differentiation factor 88 (MyD88; see Ref. 39). LPS increases the plasma levels of LBP and CD14. LBP catalyzes the binding of LPS to mCD14, which then transfers the microbial products in cooperation with the adapter molecule MD-2 (33, 45) to TLR4. MDP binds directly to the mCD14, which then interacts with TLR2 (39).

The intracellular signaling pathways downstream of TLR2 and TLR4 are similar and eventually result in activation of the transcription factor nuclear factor-κB (NF-κB). NF-κB then triggers the release of several cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α; see Ref. 31), which orchestrate nonspecific and specific immune reactions.

CD14, TLR2, and TLR4 are important in mediating immune effects of bacterial products in the host, but it is presently unknown whether these cell-surface molecules are also essential for the anorexia during bacterial infection. We therefore used mice genetically deficient in CD14, TLR2, and TLR4 to examine the role of these components in the anorectic effects of LPS and MDP. LPS and MDP increase IL-1β synthesis and release, and IL-1β is, despite some limitations, believed to be one possible mediator of LPS- and MDP-induced anorexia (see, e.g., Ref. 20). IL-1β was therefore included as a positive control because its effects are mediated by the IL-1 receptor (27) and should not depend on the presence of CD14 and/or TLRs.

GENERAL METHODS

Animals and housing. The following three groups of male mice with different genotypes were used: 1) 16-week-old C57/BL6 wild-type (CD14-WT) and CD14 knockout (CD14-KO) mice from the University Hospital in Basel, Switzerland. CD14-WT controls were homozygotes, and the CD14 knockout (KO) were transgenic homozygotes with an insert that disrupts the CD14 gene, as described previously (25, 35, 42); 2) 12-week-old C57/BL6 TLR2 wild-type (TLR2-WT) and TLR2-KO male mice were also obtained from the University Hospital in Basel, Switzerland. TLR2-WT controls were homozygotes, and the TLR2 knockout (TLR2-KO) were transgenic homozygotes with an insert that inactivates the TLR2 gene, as described previously (39); and 3) 10-week-old WT and TLR4-deficient (TLR4-DEF) mice of BALB/C background. Breeding pairs of these mice were kindly provided by Dr.
Marina Freudenberg (Max-Plank Institute, Freiburg, Germany) and were maintained as a breeding colony under specified pathogen-free conditions in our animal facility in Schwerzenbach, Switzerland. Wild-type controls (TLR4-WT) were homozygotes, and the TLR4-DEF mice were homozygotes with a null mutation of the TLR4 gene.

The mice were housed individually in stainless steel hanging cages with wire mesh bottoms equipped with plastic food cups and hanging food cups to sit in. Animal rooms were maintained at 22 ± 0.5°C on a 12:12-h dark-light cycle with lights out at 0900. Standard powdered laboratory chow (Nafag, Gossau, Switzerland) and water were available ad libitum. All procedures were approved by the Canton of Zürich’s Animal Use and Care Committee.

PCR verification of genotype. The correct genotypes of the CD14 and TLR2-KO as well as the corresponding WT mice were confirmed by PCR analysis of genomic DNA isolated from plasma samples.

Genomic DNA was extracted using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI). DNA was then amplified by PCR using a primer set that selectively amplifies specific isoforms of CD14 or TLR2. The primers for the PCR amplification of the CD14-KO mice were as follows: 362 forward, GAT GTG GAA TGT GTG CGA G and 656 reverse, GCC AAG GAT ACA TAG CCA GAC (362 forward and 656 reverse give a 424-bp product) and CD14 WT no. 19, CCA AGT TTT GTC CAG TAC AGG G and no. 78 KOAS, GCC CCA GCT CAT TCC TTC CAC TCA T, which give a 334-bp product. The primers for the PCR amplification of the TLR2-KO were as follows: no. 76 S, CCT CCT GAA TTT GTC CAG TAC AGG G and no. 78 KOAS, GCC CCA GCT CAT TCC TTC CAC TCA T, which give a 334-bp product. The primers for the PCR amplification of the TLR2-KO were as follows: no. 76 S, CCT CCT GAA TTT GTC CAG TAC AGG G and no. 77 AS, CTA CGA GCA AGA TCA ACA GGA G, giving a 499-bp product. PCR was performed in a 50-μl reaction mixture containing 100 ng DNA, 15 pmol of each primer, and PCR Supermix (Invitrogen, Basel, Switzerland). The CD14-PCR amplification conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 7 min at 72°C. The TLR2-PCR amplification conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1.30 min, followed by 7 min at 72°C.

Injection protocol. In all experiments, genetically deficient (CD14-KO, TLR2-KO, and TLR4-DEF) and corresponding WT mice were distributed in two groups according to baseline food intake. Food cups were removed from the cages 1 h before lights out, filled with food, and weighed. LPS (from Escherichia coli, serotype 011:B4, no. L-2630; Sigma), MDP (serotype 011K1388, no. L-9519; Sigma), or recombinant human IL-1β (R&D Systems) and the corresponding vehicle solutions were intraperitoneally injected 15 min before lights out. Results from previous studies (21, 22) indicate that there are no carryover effects that might obscure the results when these substances are injected sequentially. Except when otherwise noted, food intake (corrected for spillage) was then measured at 6, 12, and 24 h after injections. The mice were left undisturbed for 1 wk between each trial.

Experiment 1: LPS, MDP, and IL-1β injections in CD14-KO and CD14-WT mice. We used 18 CD14-KO (30 ± 2 (SE) g body wt) and 18 CD14-WT (32 ± 2 g body wt) mice. Each animal was used in four trials. In the first trial, nine CD14-KO and nine CD14-WT mice were injected (ip) with LPS (2 μg/mouse) dissolved in saline (0.3 ml), and food intake was measured at 6, 12, and 24 h; the other animals (9 KO and 9 WT mice) received an equivalent volume of vehicle. In the second trial, MDP (10 mg/kg body wt), also dissolved in saline (0.3 ml), or vehicle was injected intraperitoneally, in the way that mice treated with LPS in the first trial received vehicle. In the third trial, mice received IL-1β (150 ng/mouse), diluted in PBS/BSA (0.3 ml), or vehicle. In an additional trial 10 days after the IL-1β trial, we injected a much higher dose of LPS (40 μg/mouse) under otherwise identical conditions to test whether the nonresponsiveness of the CD14-KO mice to the low LPS dose reflected an absolute refractoriness to LPS under our conditions or a marked shift in the dose-response curve. In this trial, the “vehicle” mice of the first LPS trial received LPS to ensure that no mouse received LPS two times. Food intake was measured daily until 5 days after injection.

Experiment 2: LPS, MDP, and IL-1β injections in TLR2-KO and TLR2-WT mice. Following the same procedure as in experiment 1, LPS, MDP, and IL-1β or vehicle were injected in 20 TLR2-KO (30 ± 2 g body wt) and 20 TLR2-WT (30 ± 2 g body wt) mice. In the MDP trial, the number of animals/treatment was increased by crossing the mice over between trials, i.e., treatment to vehicle/vehicle to treatment. In this manner, no mouse received MDP two times.

Experiment 3: LPS, MDP, and IL-1β injections in TLR4-DEF and TLR4-WT mice. This experiment tested the effects of LPS, MDP, and IL-1β on food intake in 20 TLR4-DEF (30 ± 2 g body wt) and 20 TLR4-WT (30 ± 2 g body wt) mice. Again we followed the same procedure as in experiment 1, including injection of the high LPS dose (40 μg/mouse) 10 days after the IL-1β trial.

Statistical analysis. Data from the three experiments were analyzed separately. Within each experiment, results from LPS/MDP/IL-1β × genotype (WT vs. KO or DEF) interaction trials were analyzed using general linear model procedures appropriate for a 2 × 2 factorial arrangement of LPS/MDP/IL-1β and genotype in a randomized complete block design. In the MDP trial of experiment 2, in which mice were switched within main effects and injected a second time, each mouse’s data for both trials and day of trial were included in the analysis. In all experiments, blocks consisted of mice with similar control food intake. The ability of the CD14-KO, TLR2-KO, and TLR4-DEF mice to resist to LPS-, MDP-, or IL-1β-induced anorexia was noted by a significant (P < 0.05) interaction between main effects (genotype × TRT). To characterize treatment effects within genotype, we conducted Tukey’s test.

RESULTS

Experiment 1: Effects of intraperitoneal LPS, MDP, and IL-1β in CD14-KO and CD14-WT mice. LPS (2 μg/mouse) and MDP (10 mg/kg) reduced 6-h food intake significantly more in CD14-WT mice than in CD14-KO mice [LPS0.05: genotype × treatment interaction: F(1,34) = 4.7, P < 0.04;
Food intake reduction in CD14WT/KO, TLR2WT/KO, and TLR4WT/KO mice injected ip with either LPS, MDP, or IL-1β

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Values are mean ± SE % reductions (control = 100%) of 9–10 or 20 [muramyl dipeptide (MDP) in toll-like receptor-2 (TLR2) wild-type (WT)/knockout (KO) mice/group. *Significant food intake reduction resulting from the treatment.

Reduced food intake at all time points, regardless of genotype (all P < 0.01). The IL-1β effect was, however, weaker in CD14-KO than in CD14-WT mice [IL-1βgenotype × treatment interaction: F(1,32) = 13.96, P < 0.01; Fig. 1]. Furthermore, the high LPS dose (40 μg/mouse) reduced food intake significantly more in CD14-WT than in CD14-KO mice at all time points (all P < 0.005). Zero to 24-h food intake was 0.1 ± 0.02 g (LPS) vs. 3.3 ± 0.4 g [control; genotype × treatment interaction: F(1,31) = 9.04, P < 0.005 (Fig. 2); for further results, see Table 1].

Experiment 2: Effect of intraperitoneal LPS, MDP, and IL-1β on food intake in TLR2-KO and TLR2-WT mice. LPS (2 μg/mouse) reduced 6-h food intake similarly in both TLR2-KO and WT mice [LPSgenotype × treatment interaction: F(1,36) = 2.00, P < 0.2; Fig. 3]. This was also true for 12 and 24 h (Table 1). In contrast, MDP reduced 6-h food intake only in TLR2-WT but not in TLR2-KO mice [MDPgenotype × treatment interaction: F(1,71) = 5.94, P < 0.02; Fig. 3]. After injection (12 h), MDP had only a significant effect on TLR2-WT mice (P < 0.03), but the genotype × treatment interaction was not significant [F(1,71) = 1.05, P < 0.3; Table 1]. IL-1β again reduced food intake regardless of genotype [0–6 h, genotype × treatment interaction: F(1,36) = 0.36, P < 0.6 (Fig. 3); 12- and 24-h data, see Table 1].

Experiment 3: Effect of intraperitoneal LPS, MDP, or IL-1β on food intake in TLR4-DEF and TLR4-WT mice. LPS (2 μg/mouse) reduced 6-h food intake in TLR4-WT mice significantly more than in TLR4-DEF mice [Fig. 4; LPSgenotype × treatment interaction: F(1,36) = 6.26, P < 0.02]. This was true for all time points (12- and 24-h data,
see Table 1). In contrast, MDP and IL-1β both reduced food intake to the same degree in all mice tested, regardless of genotype [F_{MDP,0-6h}(1,35) = 0.02, P < 0.9, F_{IL-1β,0-6h}(1,36) = 0.46, P < 0.5 (Fig. 4); 12- and 24-h values, see Table 1]. Furthermore, the high LPS dose (40 µg/mouse) reduced food intake in TLR4-WT mice significantly more than in TLR4-DEF at all time points [genotype × treatment interaction: all P < 0.01 (Fig. 5 and Table 1)].
Fig. 4. LPS (2 μg/mouse) reduced food intake in TLR4-WT mice significantly more than in TLR4-KO mice. MDP (10 mg/kg body wt) and IL-1β (150 ng/mouse) reduced food intake significantly in all mice, regardless of genotype. Injections were given shortly before lights out. TLR4-DEF, TLR4 deficient. Each bar represents the mean ± SE of 9 mice. *Significant treatment effect and significant (P < 0.05) treatment × genotype interaction.

Fig. 5. LPS (40 μg/mouse) reduced food intake in TLR4-WT mice significantly more than in TLR4-KO mice. Injections were given shortly before lights out. Each bar represents the mean ± SE of 9 mice. *Significant treatment effect and significant (P < 0.05) treatment × genotype interaction.
The present experiments were conducted to determine whether the glycoprotein CD14 or TLR2 and TLR4 are involved in the signaling pathways of LPS- and MDP-induced anorexia. IL-1β was injected to test whether the presence or absence of any of these cell-surface molecules alters cytokine-induced anorexia. A low and a very high dose of LPS reduced food intake significantly less in CD14-KO and TLR4-DEF mice than in WT mice, whereas TLR2-KO mice fully responded to LPS. Furthermore, MDP reduced food intake in the TLR4-DEF mice but not in the two other genetically deficient genotypes tested. These results indicate that CD14 and TLR4 are important in the mediation of LPS-induced anorexia, whereas CD14 and TLR2 are involved in the anorectic response to MDP. Our results are consistent with previous findings suggesting that TLR4 is the primary signal transducer for LPS (3, 14) and that TLR2 acts as a receptor for gram-positive bacterial products (17, 38, 39). Our results also correspond to previous findings showing that the “pattern recognition receptor” CD14 is involved in the signaling pathway of both gram-negative (31, 44, 48) and gram-positive bacterial cell wall components (8, 28, 46). Although MDP and LPS both failed to significantly reduce food intake in CD14-KO mice, it appeared as if LPS had some residual effect in this genotype. Because TLRs are widely distributed (3, 26) and because sCD14, in concert with TLRs, can confer responsiveness to microbial products to cells that do not express CD14 (19), the findings do not suggest a particular site of action (i.e., peripheral vs. central) for bacterial products to inhibit feeding.

IL-1β reduced food intake similarly in all genotypes tested, suggesting that mice genetically deficient in either CD14, TLR2, or TLR4 still have a normal signaling pathway downstream of the cytokine release, at least for IL-1β. Previous studies have shown that CD14, TLR2, and TLR4 are involved in bacterial product-induced release of cytokines (40, 41). A blunted cytokine production may therefore be involved in the failure of LPS and MDP to reduce food intake in CD14-KO mice, as it appeared as if LPS had some residual effect in this genotype. Because TLRs are widely distributed (3, 26) and because sCD14, in concert with TLRs, can confer responsiveness to microbial products to cells that do not express CD14 (19), the findings do not suggest a particular site of action (i.e., peripheral vs. central) for bacterial products to inhibit feeding.

At first glance, contamination of our IL-1β preparations has been shown to eliminate LPS signaling through both human and murine TLR2 (13).

Perspectives

We present here evidence for an important role of the glycoprotein CD14 and the TLR4 in peripheral LPS-induced anorexia. The TLR2 seems to be important in the mediation of the MDP-induced anorexia. This results from previous studies showing that CD14 and TLRs are important components of the innate immune system, mediating the stimulation of cytokine production by bacterial products. The postreceptor signaling pathways of TLRs involve the adapter protein MyD88 and the Ser/Thr kinase IRAK, which interacts with TRAF6 and leads to the activation of the transcription factor NF-κB and activating protein-1 (26). These transcription factors stimulate the transcription of genes for prostanoids, proinflammatory cytokines, and other downstream mediators of LPS effects. The present results therefore demonstrate that anorexia occurs due to activation of cellular pathways involved in innate immune responses. It should be possible to design drugs that target
specific TLRs, CD14, or the downstream signaling mediators and are thus able to mitigate the worst consequences of microbial infections, including anorexia and cachexia. Interestingly, some evidence indicates that LBP and sCD14 not only mediate LPS effects but that administration of exogenous LBP and sCD14 can also mitigate excessive responses to LPS (31, 18) and might therefore hold some therapeutic potential.

ACKNOWLEDGMENTS

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