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Toll-like receptor 4 mutation impairs the macrophage TNFα response to peptidoglycan

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Abstract

Macrophages produce TNFα when infected by bacteria, a response that follows recognition of microbial components by members of the Toll-like receptor (TLR) family. Cells that lack functional TLR4 are known to have markedly diminished responses to Gram-negative lipopolysaccharide. We demonstrate in the present work that peritoneal macrophages derived from strains of mice that carry a spontaneous, inactivating mutation in TLR4 also have impaired production of TNFα in response to peptidoglycan, a ligand for TLR2. This impairment is at a step of biosynthesis subsequent to the generation of mRNA. TLR4-activated signals act at this step to enhance peptidoglycan-induced TNFα production in wild-type mice. Based on these observations, we conclude that macrophages from wild-type mice are primed by chronically acting TLR4 signals, probably resulting from exposure to environmental lipopolysaccharide. These signals are required for optimal production of TNFα in response to TLR2 stimulation, and are absent in macrophages from TLR4 mutant animals.

Keywords: Macrophage; Inflammation; Lipopolysaccharide; Peptidoglycan; TNFα; Toll-like receptors

Members of the Toll-like receptor (TLR) family of cell surface receptors play a key role in activating innate immune responses to microbial infection by recognizing molecules that are expressed by broad groups of microorganisms but not by mammalian cells [1]. TLR2, in a heterodimeric complex with either TLR1 or TLR6, senses several different bacterial ligands, including peptidoglycan (PGN), an important component of the cell walls of both Gram-negative and Gram-positive bacteria. TLR4, on the other hand, responds exclusively to Gram-negative lipopolysaccharide (LPS). The TLRs are all connected to similar intracellular signaling pathways, and activate similar cellular responses, including the production of pro-inflammatory cytokines such as TNFα. However, it is becoming increasingly clear that there are aspects of signal transduction and induced response that are unique to individual TLRs [2–6]. Furthermore, signals activated by one type of TLR can influence the concurrent or subsequent response to activation of another type of TLR [7–10]. Most microorganisms express ligands for multiple TLRs. Therefore, the quantitative and qualitative nature of the response that results from an encounter between microbes and cells of the innate immune system will be influenced by the pattern of specific TLRs that are activated during the interaction, and by any previous exposure of the cells to TLR ligands.

The C3H/HeJ mouse strain carries a spontaneous point mutation in the *Tlr4* gene that leads to an inactivating amino acid substitution in the cytoplasmic domain of the receptor [11]. As a result, macrophages and other cells from these mutant mice display markedly diminished responses to LPS. These cells respond relatively normally to microbial components other than LPS, but we wondered, based on the interplay of TLR

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activated signals alluded to above, whether the lack of a functional TLR4 could result in alterations in the response to activation of other TLRs. We test this idea in the present work by examining the role of TLR4 signals in the TNFα response to PGN.

Materials and methods

Reagents. *Staphylococcus aureus* PGN was purchased from Fluka (Milwaukee, WI) and resuspended in sterile water according to the manufacturer's instructions. Highly purified LPS from *Salmonella* Minnesota R595 was obtained from List Biological Laboratories (Campbell, CA) and stock solutions were made in sterile water.

Muramidase treatment of PGN. PGN was treated overnight in PBS at 37 °C with 0.1 mg/ml mutanolysin (Sigma, St. Louis, MO) and then boiled for 10 min to inactivate the enzyme. After pelleting insoluble material, the supernatant was used to treat the macrophages. Mock treatment of PGN was carried out in parallel, except that the mutanolysin was replaced with PBS.

Murine peritoneal macrophages. All mouse strains were obtained from Jackson Laboratories, Bar Harbor, ME. The C3H/HeJ strain carries a spontaneous point mutation in the cytoplasmic domain of TLR4, resulting in cellular non-responsiveness to LPS [11]. The C3HeB/FeJ or C3H/HeOuJ strains were used, depending on availability, as the corresponding wild-type controls. The C.C3-Tlr4$^{Lps\text{-}d}$/J strain contains the *Tlr4* congenic interval from C3H/HeJ bred onto the Balb/c background. Balb/cJ mice were used as the corresponding wild-type strain. Thioglycolate-elicited peritoneal macrophages were prepared as previously described [12].

Treatment and infection of macrophages. Macrophages were incubated with PGN at a final concentration of 25 μg/ml for different times depending on the experiment. LPS pre-treatment was at 10 ng/ml overnight. The cells were then washed and rested for 1–2 h before the subsequent treatment with PGN.

TNFα ELISA. Triplicate wells of peritoneal macrophages were treated as described in the individual experiments, and supernatants were collected after 2 or 20 h. Fifty-microliter aliquots of the supernatants were analyzed in triplicate by ELISA following a standard protocol that we have described in detail earlier [12]. The total protein content of the macrophages was used as a way of correcting TNFα levels for any differences in cell number between mouse strains or between individual wells. After removing the supernatants, the macrophages were washed and lysed in 1% Triton X-100. The protein concentration of the lysates was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA) following the manufacturer's recommendations. Normalized TNFα was derived by dividing the amount of TNFα produced in each well by the total amount of protein in the cell lysate from that well.

In experiments in which intracellular TNFα was measured, cells were lysed in 0.2% Triton X-100, 10 mM Tris–HCl, pH 8.0, and 150 mM NaCl, containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation at 13,000*g* for 10 min at 4 °C, and aliquots of the cleared lysate were used for ELISA and determination of protein concentration.

Quantitative real-time PCR. Total RNA was prepared from control or stimulated cells with the Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's directions. The RNA was reverse transcribed with random hexamers using the GeneAmp kit (Roche, Nutley, NJ). One-quarter of each reaction was then used for PCR amplification with primers specific for either TNFα or GAPDH (R&D Systems, Minneapolis, MN) and the SYBR Green master mix (Applied Biosystems, Foster City, CA) as described earlier [12]. The threshold cycle (C_t), the cycle number at which the fluorescence crosses a specific threshold value in the exponential phase of amplification, was determined. The relative amount of TNFα transcript was normalized to the amount of GAPDH transcript by subtracting the mean C_t value of the latter from the mean C_t value of the former for each experimental condition. The difference between the normalized TNFα C_t values of the treated cells and the control cells is a measure of the change in TNFα mRNA expression. Because of the exponential nature of the PCR, a difference of n in C_t values represents a 2^n-fold difference in transcript levels.

Statistical analysis. Statistical significance was determined by the Student's t test, with a P value of <0.05 taken to be significant.

Results and discussion

Peritoneal macrophages from TLR4 mutant mice produce less TNFα in response to PGN and Gram-positive bacteria than wild-type macrophages

Our previously published findings showed that macrophages from the TLR4 mutant C3H/HeJ mice have dramatically impaired production of TNFα in response to *Salmonella* infection [12], an observation that has been made by others [13–15]. Since *Salmonella* expresses molecules such as PGN and lipopeptides that are capable of inducing TNFα production via activation of TLR2, we wondered whether the diminished *Salmonella*-induced TNFα response of C3H/HeJ macrophages might reflect the influence of TLR4 signals on TLR2-dependent functions. To test this idea, we examined the amount of TNFα produced by either C3H/HeJ or the wild-type C3HeB/FeJ macrophages in response to treatment with 25 μg/ml of purified *Staphylococcal* PGN. As shown in Fig. 1A, the amount of TNFα produced by the C3H/HeJ macrophages was significantly less than that produced by the wild-type C3HeB/FeJ macrophages, particularly at the 20 h time point of stimulation. Similar results were obtained when the wild-type C3H/HeOuJ strain was used for comparison or when the dose of PGN was increased to 100 μg/ml (data not shown). Based on observations in multiple experiments, the mean difference between the wild-type and mutant strains at 2 h of stimulation was 2.3 ± 0.9-fold ($n = 9$) and at 20 h it was 3.6 ± 0.9-fold ($n = 3$). The difference between the C3H/HeJ and wild-type macrophages was not confined to the response to PGN. It was also seen when macrophages were treated with another TLR2 ligand, lipoteichoic acid (data not shown), or when they were infected with the Gram-positive bacterium *Bacillus subtilis* (Fig. 1B, mean difference 2.2 ± 0.9-fold, $n = 3$).

To determine whether the difference in the TNFα response was related to the genetic background of the animals, we examined another TLR4 mutant strain, C.C3-Tlr4^{Lpsd}/J, that contains the *Tlr4* congenic interval from the C3H/HeJ strain bred onto the Balb/c background. As shown in Fig. 1C, a clear and significant difference in the amount of PGN-induced TNFα was observed be-

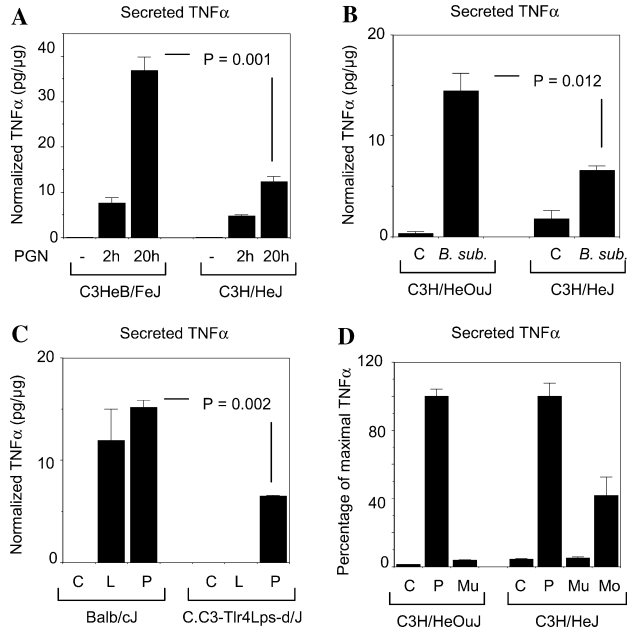

Fig. 1. (A) Comparison of PGN-induced TNFα responses in C3HeB/FeJ and C3H/HeJ macrophages. Peritoneal macrophages from either C3HeB/FeJ or C3H/HeJ mice were treated with 25 μg/ml PGN for either 2 or 20 h. Cell supernatants were collected and analyzed by ELISA for TNFα. Means and standard deviations of the supernatant TNFα from triplicate wells, normalized to cellular protein, are shown. (B) Comparison of the TNFα response of C3H/HeOuJ and C3H/HeJ macrophages to infection with *Bacillus subtilis*. Peritoneal macrophages from either C3H/HeOuJ or C3H/HeJ mice were infected with *B. subtilis* for 2 h, or were left untreated as controls (C). Cell supernatants were collected and analyzed by ELISA for TNFα. Means and standard deviations of the supernatant TNFα from triplicate wells, normalized to cellular protein, are shown. (C) Comparison of PGN-induced TNFα responses in C.C3-Tlr4$^{Lps\text{-}d}$/J and Balb/cJ macrophages. Peritoneal macrophages from either C.C3-Tlr4$^{Lps\text{-}d}$/J or Balb/cJ mice were treated with 25 μg/ml PGN (P) for 20 h or 100 ng/ml LPS (L) for 2 h, or were left untreated as controls (C). Supernatants were collected and analyzed for TNFα by ELISA. Means and standard deviations of the supernatant TNFα from triplicate wells, normalized to cellular protein, are shown. The results shown are representative of two separate experiments. (D) Effect of muramidase treatment on the TNFα response of C3H/HeOuJ and C3H/HeJ macrophages to PGN. Peritoneal macrophages from C3H/HeOuJ or C3H/HeJ mice were treated for 2 h with 25 μg/ml PGN (P) or an equivalent amount of PGN that had been treated overnight with mutanolysin (Mu) or mock (Mo) treated, as described in Materials and methods, or were left untreated as controls (C). Supernatants were collected and analyzed by ELISA for TNFα. Means and standard deviations of the supernatant TNFα from triplicate wells, normalized to cellular protein, are shown. The results shown are representative of two separate experiments.

tween the C.C3-Tlr4^{Lpsd}/J and wild-type Balb/cJ strains. Taken together, these results indicate that the lack of a functional TLR4 impairs the macrophage TNFα response to multiple TLR2 ligands, as well as to a Gram-positive bacterium, and that this effect is seen in at least two different genetic backgrounds. In agreement with our results, Weiss et al. [15] found that the amount of TNFα produced by TLR4−/− macrophages in response to synthetic bacterial lipopeptide, a TLR2 ligand, was found to be less than that produced by wild-type macrophages, both at 6 and at 24 h of treatment. Similarly, Takeuchi et al. [16] noted that macrophages from TLR4−/− mice produced significantly less TNFα than wild-type macrophages in response to cell wall preparations of the Gram-positive organism *S. aureus*. However, the response to purified *S. aureus* PGN was normal in this study, as were the responses to two other Gram-positive bacteria, *Corynebacterium diphtheriae* and *Nocardia coeliaca*, raising the possibility that the genetic background in which the TLR4 mutation occurs (129 × C57BL/6 for the TLR4 knockout versus C3H or Balb/c for the naturally occurring mutant strains) may determine how the mutation affects the response to TLR2 activation.

Small amounts of contaminants in the PGN preparation, LPS for instance, could account for the difference in the response of the TLR4 mutant and wild-type macrophages. To examine this possibility, we used the muramidase mutanolysin to hydrolyze the polysaccharide backbone of PGN. As can be seen in Fig. 1D, muramidase treatment of the PGN resulted in an approximately 95% reduction in its ability to induce TNFα in wild-type and C3H/HeJ macrophages. Mock treatment of the PGN resulted in approximately 50% reduction of activity, probably because of some losses in insoluble material. Similar results were obtained with wild-type Balb/cJ macrophages (data not shown). The specific effect of muramidase digestion makes it unlikely that contaminants in the PGN preparation contribute significantly to the different responses of the TLR4 mutant and wild-type strains.

Chronic TLR4 activation enhances subsequent TNFα production in response to PGN

The results described above are consistent with the possibility that signals activated by TLR4 regulate TLR2-dependent TNFα production. In further support of this idea, we found that pre-treatment of wild-type C3H/HeOuJ macrophages with a low dose (10 ng/ml) of LPS, that did not induce TNFα production on its own, significantly increased the amount of TNFα secreted in response to subsequent stimulation with PGN (4.6 ± 2.1-fold, $n = 3$). A representative result is shown in Fig. 2A. This observation suggests that chronic exposure to low doses of LPS may prime the response to subsequent TLR2 stimulation.

The priming effect of LPS on subsequent responses to TLR2 ligands, which has also been noted by others [17], raises the possibility that the heightened TLR2-dependent TNFα response of wild-type macrophages in comparison to TLR4 mutant macrophages may be related to chronic exposure to low doses of environmental LPS. If that were the case, it would be expected that this priming effect would diminish once the exposure to LPS was ter-

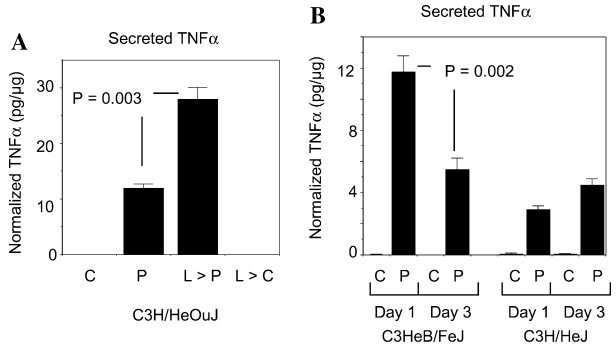

Fig. 2. (A) Effect of LPS pre-treatment on the PGN-induced TNFα response in C3H/HeOuJ macrophages. Peritoneal macrophages from C3H/HeOuJ mice were left without pre-treatment or treatment (C), or were pre-treated overnight with 10 ng/ml LPS. The following morning the cells were washed, cultured in fresh medium, and then left untreated (L > C), or were treated 1 h later with 25 μg/ml PGN for 2 h (L > P). Another set of cells was treated for 2 h with 25 μg/ml PGN without any pre-treatment (P). Cell supernatants were collected and analyzed by ELISA for TNFα. Means and standard deviations of the supernatant TNFα from triplicate wells, normalized to cellular protein, are shown. The results shown are representative of three separate experiments. (B) Effect of time on the PGN-induced TNFα response of C3HeB/FeJ and C3H/HeJ macrophages. Peritoneal macrophages were collected from either C3HeB/FeJ or C3H/HeJ mice and either left untreated as controls (C), treated with 25 μg/ml PGN overnight (P), either on the day of collection (day 1), or after 48 h (day 3). Supernatants were collected and analyzed by ELISA for TNFα. Means and standard deviations of the supernatant TNFα from triplicate wells, normalized to cellular protein, are shown. The results shown are representative of two separate experiments.

minated. Accordingly, we examined the PGN-induced TNFα response in wild-type and C3H/HeJ macrophages immediately after explantation (day 1) and 48 h later (day 3), reasoning that LPS in the environment of the animal would still exert its effects at the first time point, but not at the later one. The results shown in Fig. 2B are consistent with this idea, since it can be seen that the amount of TNFα elicited from the wild-type C3HeB/FeJ macrophages is significantly higher on day 1 than on day 3. The day 1 and day 3 amounts did not differ significantly in the C3H/HeJ macrophages.

These results suggest that wild-type macrophages are exposed to subliminal environmental stimuli that chronically activate TLR4 signals. The exact source of such stimulation is not clear, but one possibility is the large number of bacteria that live as commensals in the mammalian gut [18]. Endotoxin levels in the cell culture medium and serum are too low to be the cause of such stimulation, but we cannot rule out that LPS contamination of the thioglycolate used to elicit the macrophages might contribute to the priming effect. Regardless of the source of the stimulation, our observations suggest that chronic exposure to TLR4-dependent activating signals enhances the TNFα response to TLR2 ligands in wild-type macrophages. The lack of such signals in TLR4 mutant animals impairs the production of TNFα in response to TLR2 activation.

The difference in the TNFα response to PGN between C3H/HeJ and wild-type macrophages is at a step subsequent to generation of TNFα mRNA

The results presented so far indicate an impairment in the production of secreted TNFα in response to PGN in the TLR4 mutant macrophages. To elucidate the step of biosynthesis at which the impairment occurs, we examined the levels of intracellular TNFα protein and mRNA in the two sets of macrophages. Fig. 3A shows a comparison of the amounts of TNFα protein present in

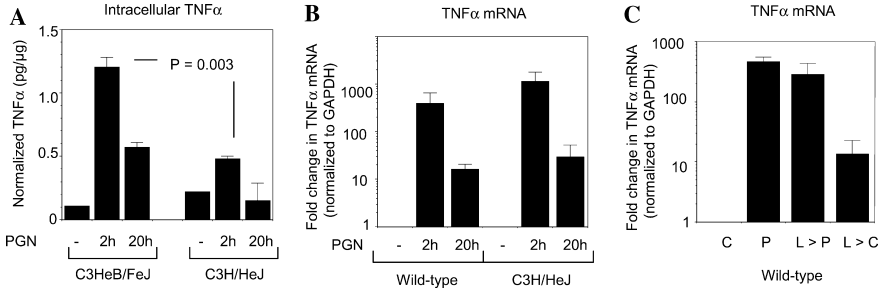

Fig. 3. (A) Comparison of PGN-induced TNFα in cell lysates of C3HeB/FeJ and C3H/HeJ macrophages. Peritoneal macrophages from either C3HeB/FeJ or C3H/HeJ mice were treated with 25 μg/ml PGN for either 2 or 20 h. After collecting the supernatants, the cells were washed and lysed. The TNFα content of the cleared lysates was determined by ELISA. Means and standard deviations of the lysate TNFα from triplicate wells, normalized to lysate protein, are shown. The results shown are representative of two separate experiments. (B) Comparison of PGN-induced TNFα mRNA levels in wild-type (C3H/HeOuJ or C3HeB/FeJ) and C3H/HeJ macrophages. Peritoneal macrophages from either wild-type or C3H/HeJ mice were treated for 2 or 20 h with 25 μg/ml PGN. Total RNA was prepared and used for quantitative RT-PCR with TNFα or GAPDH-specific primers. The fold-change in TNFα mRNA (normalized to GAPDH mRNA) is indicated. The means and standard deviations of four separate experiments (two using C3H/HeOuJ as the wild-type and two with C3HeB/FeJ as the wild-type) are shown. Note that the scale is logarithmic. (C) Effect of LPS pre-treatment on PGN-induced TNFα mRNA levels in wild-type (C3H/HeOuJ or C3HeB/FeJ) macrophages. Peritoneal macrophages from wild-type (C3H/HeOuJ or C3HeB/FeJ) mice were left without pre-treatment or treatment (C), or were pre-treated overnight with 10 ng/ml LPS. The following morning the cells were washed, cultured in fresh medium and then left untreated (L > C), or treated 1 h later with 25 μg/ml PGN for 2 h (L > P). Another set of cells was treated for 2 h with 25 μg/ml PGN without any pre-treatment (P). Total cellular RNA was prepared 2 h later and used for quantitative RT-PCR with TNFα or GAPDH-specific primers. The fold-change in TNFα mRNA (normalized to GAPDH mRNA) is indicated. The means and standard deviations of three experiments (two with C3H/HeOuJ mice and one with C3HeB/FeJ) are shown. Note that the scale is logarithmic.

the cell lysates of C3HeB/FeJ and C3H/HeJ macrophages at 2 and 20 h of PGN treatment. As was seen for the comparison of secreted TNFα (Fig. 1A), the level in the C3H/HeJ cells is significantly lower than in the C3HeB/FeJ macrophages. This result suggests that the difference between C3H/HeJ and C3HeB/FeJ is unlikely to be at the level of TNFα secretion. (Note that in Fig. 1A, progressive accumulation of secreted TNFα in the supernatant makes the difference between C3H/HeJ and C3HeB/FeJ greatest at 20 h, whereas in Fig. 3A, the difference in intracellular TNFα is greatest at 2 h, presumably reflecting the high rate of synthesis at this time in the wild-type cells.)

We then proceeded to compare the levels of TNFα mRNA in C3H/HeJ and wild-type macrophages. As shown in Fig. 3B, quantitative real-time PCR analysis indicated that the levels of TNFα mRNA at both 2 and 20 h of PGN stimulation were similar in both types of cells. These observations suggest that the impairment in PGN-induced TNFα biosynthesis in TLR4 mutant macrophages involves mechanisms that operate subsequent to the generation of transcript. TLR4-activated signals also appear to act on these mechanisms. As shown in Fig. 3C, LPS pre-treatment of wild-type macrophages had no effect on TNFα mRNA levels induced by PGN, whereas, as was seen in Fig. 2A, it had a clear enhancing effect on secreted TNFα.

Conclusion

The data presented here indicate that macrophages from two different TLR4 mutant mouse strains, C3H/HeJ and C.C3-Tlr4$^{Lps\text{-}d}$, have impaired TNFα biosynthesis in response to treatment with PGN. The impairment is at a step subsequent to the generation of transcript and appears to result from a lack of chronic TLR4-dependent activating signals delivered by environmental stimuli. The exact post-transcriptional mechanism that is regulated by the chronic TLR4 signals remains to be elucidated. In experiments not shown, we examined the proportion of cytoplasmic to total TNFα mRNA, and TNFα mRNA stability, and found no differences between C3H/HeJ and C3H/HeOuJ or C3HeB/FeJ macrophages, ruling out a requirement for TLR4 in nucleo-cytoplasmic transport or turn-over of the transcript. Although TNFα biosynthesis is known to be regulated at multiple levels [19–21], our findings are consistent with TLR4 signals being involved in regulating some aspect of translation. The complexity of TNFα translation, a process that involves the binding of multiple positively and negatively acting proteins to the AU-rich elements in the 3′ untranslated region of the transcript [21], is in keeping with the idea of TLR4-dependent regulatory influences acting at this step. Thus, we propose a mechanism in which environmental stimuli acting through TLR4 provide low-level, persistent signals that are required to enhance TNFα translation. These TLR4 signals, which are activated only weakly by TLR2, are presumed to regulate the expression and/or function of one of the numerous factors that influence translation by interacting with the TNFα 3′ untranslated region [21]. The impairment of PGN-induced TNFα biosynthesis in the C3H/HeJ and C.C3-Tlr4$^{Lps\text{-}d}$ macrophages can then be explained as a consequence of the lack of TLR4-dependent stimulation.

Although the difference in PGN-induced TNFα production between wild-type and TLR4 mutant macrophages is only 2–3-fold, such differences have been associated with marked changes in susceptibility to infection in vivo, consistent with the importance of this cytokine in restricting the early growth and spread of microorganisms [22–24]. In the context of this information, our data suggest that hyporesponsiveness to TLR2 stimulation may contribute to the unusually low TNFα production of C3H/HeJ macrophages to *Salmonella* infection, and the susceptibility of this strain to salmonellosis [12,25–27].

Our findings also provide an explanation for some unexpected observations made by others in the C3H/HeJ strain. Several groups have reported increased susceptibility of this strain to in vivo infection with mycobacteria and *Candida*, in association with decreased macrophage inflammatory cytokine production in vitro [22,23,28,29]. Since the mutation in the C3H/HeJ strain is in TLR4, these observations were explained by postulating the existence of LPS-like molecules in mycobacteria and *Candida*. While this possibility has not been excluded, our results offer an alternative explanation, i.e., that the lack of TLR4-dependent signals derived from environmental sources impairs responses to TLR2 ligands, a number of which are expressed by both mycobacteria and fungi. We suggest that one source of this chronic TLR4 activation is the commensal microflora of the intestinal tract, an idea that is consistent with recent observations on the role of these organisms in modulating immune responses in the gut [30,31].

TLR4 mutant mice, particularly the C3H/HeJ strain, have become widely used in studies of innate and adaptive immunity. The results presented here indicate that an abnormal response to activation of TLR2 may occur secondary to the lack of TLR4 signals, making it important to use caution when interpreting results obtained with these animals.

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