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Host Genetics of *Bordetella pertussis* Infection in Mice: Significance of Toll-Like Receptor 4 in Genetic Susceptibility and Pathobiology

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ABSTRACT

The susceptibility to and the severity of *Bordetella pertussis* infections in infants and children varies widely, suggesting that genetic differences between individuals influence the course of infection. We have previously identified three novel loci that influence the severity of whooping cough by using recombinant congenic strains of mice: *Bordetella pertussis* susceptibility loci 1, 2, and 3 (*Bps1*, *-2*, and *-3*). Because these loci could not account for all genetic differences between mice, we extended our search for additional susceptibility loci. We therefore screened 11 inbred strains of mice for susceptibility to a pertussis infection after intranasal infection. Susceptibility was defined by the number of bacteria in the lungs, being indicative of the effect between the clearance and replication of bacteria. The most resistant (A/J) and the most susceptible (C3H/HeJ) strains were selected for further genetic and phenotypic characterization. The link between bacterial clearance and chromosomal location was investigated with 300 F_2 mice, generated by crossing A/J and C3H/HeJ mice. We found a link between the delayed clearance of bacteria from the lung and a large part of chromosome 4 in F_2 mice with a maximum log of the odds score of 33.6 at 65.4 Mb, which is the location of *Tlr4*. C3H/HeJ mice carry a functional mutation in the intracellular domain of *Tlr4*. This locus accounted for all detectable genetic differences between these strains. Compared to A/J mice, C3H/HeJ mice showed a delayed clearance of bacteria from the lung, a higher relative lung weight, and increased body weight loss. Splenocytes from infected C3H/HeJ mice produced almost no interleukin-1ß (IL-1ß) and tumor necrosis factor alpha (TNF-α) upon ex vivo restimulation with *B. pertussis* compared to A/J mice and also showed a delayed gamma interferon (IFN-γ) production. TNF-α expression in the lungs 3 days after infection was increased fivefold compared to uninfected controls in A/J mice and was not affected in C3H/HeJ mice. In conclusion, *Tlr4* is a major host factor explaining the differences in the course of infection between these inbred strains of mice. Functional *Tlr4* is essential for an efficient IL-1-ß, TNF-α, and IFN-γ response; efficient clearance of bacteria from the lung; and reduced lung pathology.

INTRODUCTION

The clinical course of *Bordetella pertussis* infection varies widely. Knowledge about host genetic and immunological factors that influence susceptibility and severity of infection may lead to the identification of new approaches for prevention or treatment of this disease (13). Knowledge of human genetic factors that influence *B. pertussis* infection is still very limited. A number of studies have provided clues for the role of host genes in the susceptibility of mice to *B. pertussis* infection (4, 11, 19, 23). We have recently shown that host genes of mice affect the clearance of bacteria from the lung and that *B. pertussis* infection is under multigenic control. This study resulted in the identification of three novel loci (*Bps1 to -3*) that influence the clearance of *B. pertussis* from the lung. These loci could explain up to 10% of the variation in the lung colonization observed in F_2 mice. Therefore, additional loci are likely to influence the course of *B. pertussis* infection (2). In the present study, we used inbred strains of mice to identify additional genetic factors.

Other researchers have identified Toll-like receptor 4 (*Tlr4*), in addition to *Bps1* to *-3*, to be a major factor that influences the course of *Bordetella bronchiseptica* and *B. pertussis* infection in mice (11, 20, 21). In these studies C3H/HeJ mice were used. These mice carry a functional mutation in the gene coding for Tlr4 rendering them unresponsive to lipopolysaccharide (LPS) (6, 26).

The aims of the present study were (i) to examine whether inbred strains of mice show genetic differences in susceptibility to *B. pertussis*, (ii) to determine whether we could identify genetic loci responsible for such differences, and (iii) to examine the pathobiological mechanisms of the identified susceptibility loci. To do this, we compared the present approach using inbred strains of mice to our previous approach using recombinant congenic strains of mice. We examined lung colonization, lung pathology, and the immune responses of inbred strains after infection to correlate phenotypic characteristics of infection with the genetic background of the mice.

MATERIALS AND METHODS

Experimental design. We examined the course of *B. pertussis* infection in 11 inbred strains of mice. Approximately eight animals of each strain were initially used to determine the number of bacteria in the lung 1 week postinfection. A total of 300 F_2 hybrid mice were obtained by crossing the strain with the highest number of bacteria in the lung (C3H/HeJ) with the strain with the lowest number of bacteria in the lung (A/J) and subsequently intercrossing their F_1 progeny. The 300 F_2 hybrid mice were examined as described below. Due to logistical limitations, we infected 50 mice per day and combined the results. To assure the reproducibility of the infection model, BALB/c mice were included on each day of infection and infected in the same way. The original inbred strains of mice were examined on three different days. The F_2 hybrid mice were examined on six different days.

To confirm that differences in lung pathology and clinical response were caused by Tlr4, *Tlr4* defective (*Tlr4*$^{Lps\text{-}d}$) C3H/HeJ mice, *Tlr4* wild-type (*Tlr4*$^{Lps\text{-}n}$) A/J, and C3H/HeOuJ mice were infected with *B. pertussis* as described below. To control groups (mock) a similar volume of Verwey medium was given.

The number of bacteria in the lungs of the BALB/c control mice, 1 week postinfection, was similar regardless of the day the experiment was performed. Because there was no significant difference between any of the control groups ($P > 0.05$), we combined the results of all experiments (data not shown).

Animals. Female mice were used for the infection experiments. A/J, C57BL/6J, SPRET/Ei, Cast/Ei, DBA/2J, B10.D2.H2/oSnJ, AKR/J, BALB/cJ, 129X1/SvJ, C3H/HeOuJ, and C3H/HeJ strains of mice were supplied by the Jackson Laboratory (Bar Harbor, Maine). BALB/cOlaHsd (referred to as BALB/c) mice were supplied by Harlan (Harlan Europe, Horst, The Netherlands). F_2 hybrid mice were generated by crossing A/J mice with C3H/HeJ mice and subsequently intercrossing their F_1 progeny. Mice were acclimatized for at least 1 week before the start of the experiments. Mice received standard laboratory chow (SRM-A; Hope Farms, Woerden, The Netherlands) and tap water ad libitum. All animal experiments were approved by the Institute's Animal Ethics Committee.

Bacteria. *B. pertussis* strain B213, a Tohama derivative (*ptxA1 prn1*), and *B. pertussis* strain B2566, a clinical isolate from 1997 (*ptxA2 prn2*) (14, 25) were cultured by plating on Bordet-Gengou agar supplemented with 15% sheep blood and 30 µg of streptomycin/ml. Plates were incubated for 4 days at 35°C. The number of CFU was determined with a ProtoCOL SR Colony Counter (Synbiosis, Cambridge, United Kingdom). All dilutions of bacteria were made in Verwey medium (NVI, Bilthoven, The Netherlands).

Infection experiments. The number of viable *B. pertussis* bacteria was determined in the lungs 1 week after infection (14, 35). Briefly, mice were intranasally infected with 2×10^7 CFU of *B. pertussis* after being anesthetized with isoflurane. Seven days after infection, mice were sacrificed, and the lungs were collected. A ligature was made around the right bronchus, after which the right lobes were removed to count the bacteria (34). The remaining left lung lobe was fixed intratracheally using 4% formalin for histological examination. The right lung lobes were homogenized in Verwey medium and diluted 10 and 1,000 times. The number of CFU in these dilutions was determined by plating on Bordet-Gengou agar supplemented with 15% sheep blood and 30 µg of streptomycin/ml. Plates were incubated for 4 days at 35°C before the numbers of bacteria were counted by using a ProtoCOL Colony Counter (Synbiosis).

Macroscopic and histopathological examination. Mice were weighed 1 week before infection and 3, 7, 14, and 21 days after infection to determine the relative gain or loss of weight. Lung weights were determined after sacrifice of the mice as a parameter for lung inflammation. Lung weights are represented relative to the body weights.
Formalin-fixed lungs were embedded in paraplast (Monoject). Transverse sections of 5 µm were stained with hematoxylin-eosin. In a blinded fashion, an independent observer examined the slides for peribronchiolitis (i.e., infiltration of inflammatory cells in the peribronchiolar space), alveolitis (i.e., infiltration of inflammatory cells in the alveoli), perivasculitis (i.e., infiltration of inflammatory cells in the perivascular space), hypertrophy of mucus-producing glands, free protein, and eosinophilia. Lung lesions were scored semiquantitatively as absent (score 0), minimal (score 1), slight (score 2), moderate (score 3), marked (score 4), or severe (score 5), as previously described (3).

Genotyping. Genomic DNA was isolated from mice tails by using the DNeasy Tissue kit (QIAGEN). The sequences of all primers were obtained from the mouse genome database of the Massachusetts Institute of Technology (24). DNA was amplified in a 10-µl volume using 5 µl of Hotstar 2x Mastermix (QIAGEN), 1.0 µM concentrations of each primer, and ca. 10 ng of tail-DNA. 6-Carboxyfluorescein (FAM)-labeled microsatellite primer-sets were used (Isogen Life Science, Maarssen, The Netherlands). Amplification was performed on a GeneAmp PCR System 9700 (Applied Biosystems) as follows: 15 min at 95°C to denature the DNA and activate the Hotstar Taq; 30 cycles of 45 s at 94°C, 45 s at 57°C, and 1 min at 70°C; and finally 10 min at 72°C. Fragment sizes were determined on a

3700 Capillary DNA Sequencer/Genotyper system (Applied Biosystems) using Genotyper software (Applied Biosystems). The single nucleotide polymorphism (SNP) markers were analyzed by restriction fragment length polymorphism assay on a 2.5% agarose gel (Table 1). PCR was performed as described above, and restriction conditions were used according to the manufacturer's instructions (New England Biolabs).

TABLE 1. SNP assays

Reference SNP identification no.	Chromosome	Position (Mb)	Sequence		SNP allele		Restriction enzyme
			Forward primer	Reverse primer	C3H	A/J	
Rs4224427	4	32	CAAAGAGGCTGAAGCACTTG	GTCTAGGACCTTCCCACAA[a]	A	G	SspI
Rs3023006(*Tlr4*)	4	65.4	GCTTTCACCTCTGCCTTCAC	ATAACCTTCCGGCTCTTGTG	A	C	Hsp92II
Rs3022979	4	75.5	ATAATGGGGCTAACGCAATG	GAAGAGGGCATCAGTGTTCC	A	C	BsrI

[a] A mismatch primer pair was used to create a restriction site for SspI

Cytokine responses. To examine cytokine production by spleen cells after infection, single-cell suspensions were prepared from the spleen by pressing the tissue through a cell strainer (Falcon). The cells were washed once in RPMI 1640 (Gibco-BRL/Life Technologies). The splenocytes (1.5×10^5 cells per well) were cultured (37°C, 5% CO_2) in 96-well tissue culture plates (Nunc, Denmark) in the presence of concanavalin A (ConA; 5 µg/ml; Sigma Chemical Co.) or *B. pertussis* (10^5 heat-inactivated bacteria per well). Bacteria were heat inactivated at 56°C for 30 min. The culture medium was composed of RPMI 1640 (Gibco-BRL/Life Technologies), supplemented with 2 mM glutamine, penicillin, streptomycin, HEPES buffer, ß-mercaptoethanol (50 mM), and 10% fetal calf serum. After 72 h, the culture supernatant was collected and stored at –80°C until analyzed for cytokine production (5, 33).

Interleukin-1ß (IL-1ß), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) concentrations were determined by enzyme-linked immunosorbent assay (ELISA; Biosource International) using the concentrations recommended by the manufacturer. Briefly, 96-well plates (Nunc-Immuno Plate) were coated with 1.25 µg of anti-mouse IL-1ß, IFN-γ, or TNF-α/ml in coating buffer (0.05 M carbonate buffer [pH 9.6]; Sigma). After overnight incubation at 4°C, the plates were incubated in blocking buffer (1% bovine serum albumin in Tris-buffered saline; Sigma) for 2 h at room temperature and washed (0.05% Tween 20; Merck, The Netherlands). Recombinant mouse cytokines (Biosource) were used as a standard. Standards, as well as serial dilutions of splenocyte culture supernatants, were added to the plate. Plates were incubated at 37°C for 2 h and washed. Biotinylated anti-mouse IL-1-ß, IFN-γ, or TNF-α (0.125 µg/ml) was added, followed by incubation for 1 h at room temperature. The plates were washed, and horseradish peroxidase-labeled streptavidin (10,000-fold dilution, Strepta-E+; Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) was added, followed by incubation for 1 h at room temperature. After a washing step, tetramethylbenzidine solution (0.1 mg/ml; Sigma-Aldrich) plus 0.006% H_2O_2 in 0.1 M sodium acetate (pH 5.5) was added. The color reaction was stopped by adding sulfuric acid (10%; Merck). The plates were measured at 450 nm on a Fluostar Platereader (Fluostar Galaxy; BMG Labtech, Germany) (32).

TLR4 and TNF-α mRNA expression. To examine mRNA expression, lungs from infected animals were incubated overnight in RNA-later (QIAGEN) at 4°C and stored at –80°C. Lungs were homogenized in RLT-buffer (QIAGEN) with a rotor homogenizator, and RNA was extracted with an RNeasy kit (QIAGEN) as described by the manufacturer. cDNA was generated by using the High Capacity cDNA archive kit containing random hexamer primers (Applied Biosystems). mRNA expression was measured by using TaqMan gene expression assays (Applied Biosystems) on a 7500 Fast Real-Time PCR System. We used assays on demand for *Tlr4* (Mm00445274_m1) and TNF-α (Mm00443258_m1). For the reference gene, RNA polymerase IIa (Polr2a), the assay was designed using the primer express program (Applied Biosystems) resulting in the probe CATCCGCTTCAATTCAT, the forward PCR primer GCAGTTCGGAGTCCTGAGT, and the reverse PCR primer CCCTCTGTTGTTTCTGGGTATTTGA. TaqMan probes carried a 5' FAM reporter label and a 3' Non Fluoro Quencher group. *Taq* polymerase was activated by heating for 20 s at 95°C. Amplification was for 3 s at 95°C and 30 s at 60°C for 40 cycles in a TaqMan Fast Universal PCR Master mix (Applied Biosystems) containing 5 µl of cDNA template, 1 µl of TaqMan Gene Expression Assay mix (20x mix containing primers and probes) in a total volume of 20 µl. The fluorescence intensity of the reporter label was normalized to the rhodamine derivative ROX as a passive reference label present in the buffer solution (9, 30). The relative concentration of the *Tlr4* and TNF-α mRNA was determined by the

comparative threshold cycle method (ddCt) (1, 9, 30). Each sample was run in triplicate.

Statistical analysis. The differences in numbers of CFU in the lungs between the different inbred strains of mice were examined by analysis of variance (ANOVA; SPSS) and tested with the Student-Newman-Keuls test for multiple comparisons or the Bonferroni post hoc test. The Bonferroni post hoc test is known to be the most conservative test for ANOVA. The test is similar to for instance the Fisher least significant difference test, but the observed significance levels are adjusted for the number of comparisons made. If a difference is significant according to the Bonferroni test, it is for all other tests. The Student-Newman-Keuls test was used to test the difference between multiple groups. To stabilize variances and to obtain approximately normal distributions, the CFU were square root (sqrt) or natural definition (ln) transformed. In F_2 mice, the link between the CFU in the lung and the genetic markers and the effect on the total phenotypic variation were calculated by ANOVA, with genotype as a fixed factor and CFU as a dependent variable. To correct for the influence of the experiment, the experiment was included as a random factor. All single markers and all pairs of nonlinked markers were tested for linkage with another marker or interaction between markers. Interaction, or epistasis, is defined as the combined effect of two or more genes on a phenotype that could not have been predicted as the sum of their separate effects (8). Linkage is presented as the *P* value and the log of the odds (LOD) score. The latter was calculated as −log of the significance (*P* value).
All markers and interactions were tested at the level of 0.05 ($P < 0.05$). *P* values were corrected for multiple comparisons using the formula (15, 16):

$$P_{\text{corrected}} = [C + 2\rho G T^2] P$$

where $P_{\text{corrected}}$ is the desired corrected *P* value, *C* is the number of chromosomes segregating in the cross (20 for mice), ρ is the crossover rate (1.5 for a F_2 hybrid generation), *G* is the genome length of the segregating part of the donor genome in Morgans (the mouse genome is 16 M), T^2 is the threshold (the F-value from ANOVA for the observed *P* value is used as T^2), and *P* is the observed uncorrected *P* value.
The estimated effect of a linked locus on the total of the observed phenotypic variation is presented as R^2 (ANOVA; SPSS). To test the reproducibility of the infection protocol, we performed a *t* test between the CFU of control mice infected in different experiments.
The difference in the survival of animals was calculated by Kaplan-Meier analysis (Survival; SPSS). We used time after infection when death occurred as time, death as status, and group as factor.

RESULTS

Colonization. To examine whether inbred strains of mice differ in susceptibility to *B. pertussis*, we examined 11 different inbred strains of mice. The number of viable *B. pertussis* bacteria in the lung 1 week after infection was used as phenotype. The number of CFU varied from 2.4×10^5 to 1.2×10^7 CFU per lung (Fig. 1), therewith confirming the genetic basis for modulation of the course of *B. pertussis* infection. Significant differences in lung colonization were observed between some of these strains of mice. From these strains we selected A/J as the most resistant strain and C3H/HeJ as the most susceptible strain to generate an F_2 intercross generation.

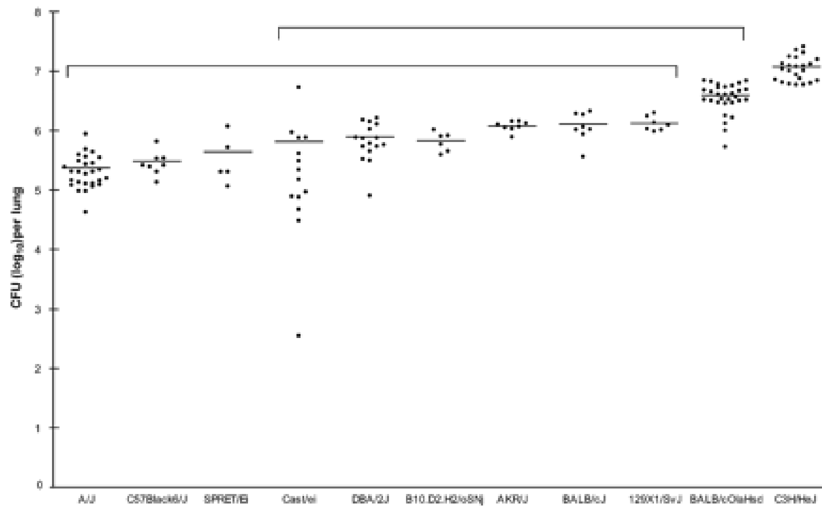

FIG. 1. Colonization of inbred strains of mice by B. pertussis. Inbred strains of mice were infected intranasally with B. pertussis strain B213, and 7 days later lungs were removed, and the number of viable B. pertussis B213 was determined. Each dot represents the number of bacteria in the lung of an individual mouse. Horizontal lines indicate the group average. Upper horizontal lines connect groups of mice who are mutually not significantly different according to the Student Neuman-Keuls test. From these strains, A/J and C3H/HeJ mice were selected for generating F_2 hybrid generations.

Linkage analysis. To search for genetic loci that could explain the differences between C3H/HeJ and A/J mice, 300 (A/J x C3H/HeJ) F_2 hybrid mice were infected and examined as described above. As expected, the F_2 mice showed a greater variation in the numbers of bacteria compared to the parental strains (Fig. 2). The number of CFU per lung ranged from 10^2 (detection limit) to almost 10^8. The average number of CFU was 4.5 x 10^6. All mice were individually genotyped, and we compared the genotypes with phenotypes to identify possible susceptibility loci by linkage analysis.

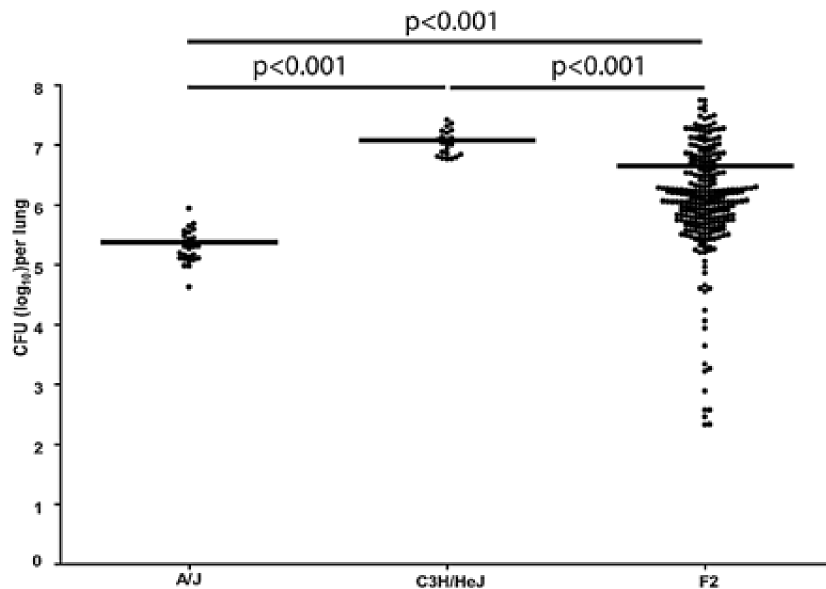

FIG. 2. Colonization of B213 in A/J x C3H/HeJ F_2 mice. The F_2 generation,

obtained by crossing C3H/HeJ mice with A/J mice and subsequently intercrossing their F_1 progeny, was infected intranasally with B. pertussis. Seven days after infection, lungs were removed, and the number of viable B. pertussis was determined. Each dot represents the number of bacteria in the lung per individual mouse. Horizontal lines indicate the group average. Horizontal connecting lines represent the level of significance of difference between groups. The experiment was performed twice, and a representative result was shown.

We started testing markers in the *Bps1* region (2) and a mutation in the *Tlr4* gene that was described in C3H/HeJ mice (11, 21). We found no linkage with markers from the *Bps1* region (data not shown), but strong linkage was found with the *Tlr4* locus with a LOD score of 33.6 (P = 2.324 x 10^{-34}, $P_{corrected}$ = 1.188 x 10^{-30}). Additional markers surrounding this gene were tested, and almost all markers located on chromosome 4 showed a linkage with reduced clearance of bacteria from the lungs, with a maximum LOD score of 33.6 at 50.3 cM, which is the location of *Tlr4* (Fig. 3). Forty-five percent (R^2 = 0.451) of the variation in the number of bacteria in the experiments could be ascribed to *Tlr4*. Due to logistical limitations, the F_2 hybrid mice were examined in six experiments. To assure the reproducibility of the infection model each experimental group contained BALB/c mice that were infected in the same way. There was no significant difference in CFU in BALB/c mice between different experiments (P = 0.365). However, the residual mean square error of the results in the BALB/c mice (σ^2 = 1.35 x 10^6) is larger than the residual mean square error of the *Tlr4* genotype (σ^2 = 1.07 x 10^6), which means that the residual variation is due to environmental variation. Thus, virtually all of the genetically detectable variation in lung clearance in these experiments could be ascribed to the *Tlr4* gene. Although we set out to test approximately five markers per chromosome, we decided that further testing was no longer informative.

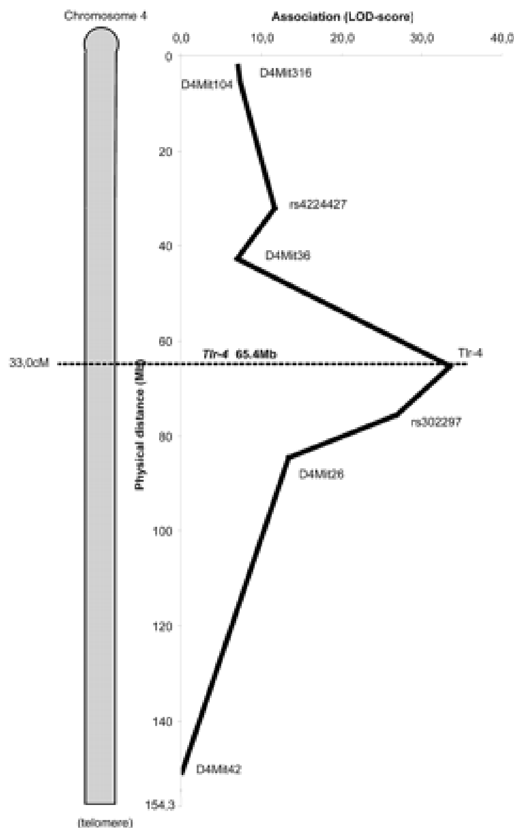

FIG. 3. Linkage (LOD score) between the degree of lung colonization by B. pertussis (phenotype) and chromosomal loci (genotype). The association was calculated by ANOVA with genotypes as a fixed factor and the square-root CFU as a dependent variable. The LOD score is plotted as –log P against the physical distance of chromosome 4. The region has a maximum LOD score of 33.6 (P = 2.324 x 10^{-34}, R^2 = 0.451) for the p712h mutant in Toll-like receptor 4 (Tlr4).

Influence of bacterial genetic differences. To examine whether genetic differences in *B. pertussis* also affect the course of infection in these mice, we infected A/J and C3H/HeJ mice with *B. pertussis* strain B213 and a recent clinical isolate (B2566). The two strains differ with respect to pertussis toxin and pertactin. The number of bacteria in the lungs of mice infected with *B. pertussis* strain B2566 was similar to the number of bacteria in the lungs of mice infected with *B. pertussis* strain B213. A/J mice showed an average of 2 x 10^5 bacteria per lung, whereas C3H/HeJ mice showed an average of 2 x 10^7 CFU per lung (Fig. 4). So, the observed difference between these inbred strains of mice does not appear to be *B. pertussis* strain specific.

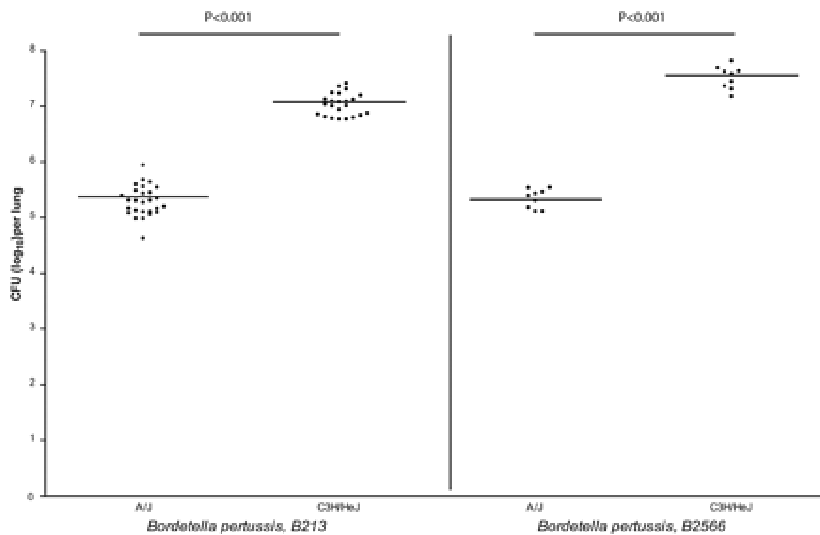

FIG. 4. Colonization in two inbred strains of mice by distinct B. pertussis strains. Seven days after inoculation with B. pertussis B213 (Tohama) and B2566 (a recent clinical isolate), the lungs were removed, and the number of viable B. pertussis organisms was determined. Each dot represents the number of bacteria in the lung of an individual mouse. Horizontal lines indicate the group average. Horizontal connecting lines represent the level of significance of difference between groups. Mice inoculated with B. pertussis B213 are displayed in the left panel; mice inoculated with B. pertussis B2566 are displayed in the right panel.

Time course of colonization. Because *Tlr4* appeared to be a dominant genetic factor, we subsequently characterized the role of Tlr4 in more detail. We first examined the number of bacteria at different time points after infection in different strains of mice.

The number of bacteria in the lung was determined 3, 7, 14, and 21 days after infection of A/J(*Tlr4*$^{Lps-n}$), C3H/HeOuJ(*Tlr4*$^{Lps-n}$), and C3H/HeJ (*Tlr4*$^{Lps-d}$) mice (Fig. 5). C3H/HeOuJ mice are genetically identical to C3H/HeJ mice except for the mutation in the *Tlr4* gene (31). A/J and C3H/HeOuJ showed the same course of clearance of bacteria from the lung, while C3H/HeJ mice showed a significantly slower clearance as evidenced by a higher number of CFU per lung up to 14 days postinfection. At 21 days after infection, almost all bacteria were cleared from the lung in all groups of mice.

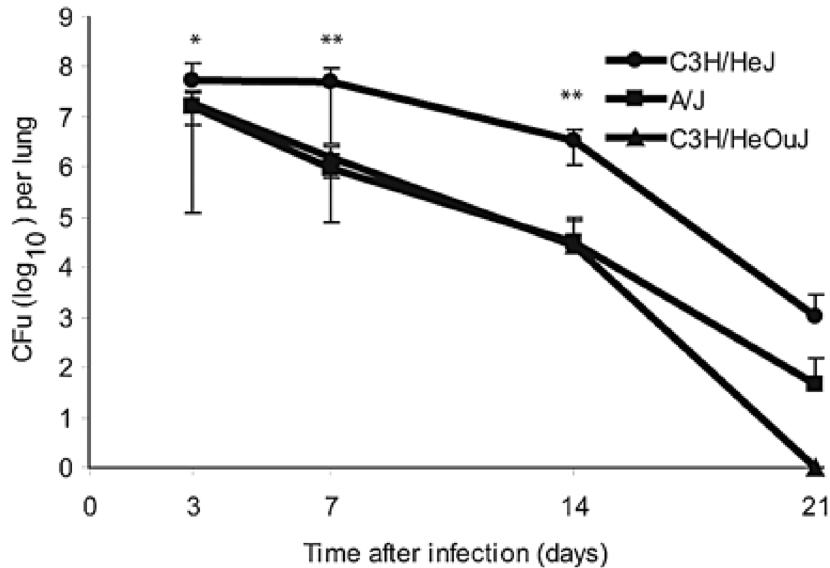

*FIG. 5. Time course of colonization by B. pertussis in different strains of mice. CFU in the lungs of A/J, C3H/HeOuJ (both $Tlr4^{Lps\text{-}n}$), and C3H/HeJ ($Tlr4^{Lps\text{-}d}$) mice. At 3, 7, 14, and 21 days after inoculation with B. pertussis, the lungs were removed, and the numbers of viable B. pertussis organisms were determined. The mean of the number of CFU was plotted with the standard deviation. Asterisks indicate a significant difference in the number of CFU between C3H/HeJ mice and A/J or C3H/HeOuJ mice. *, $P < 0.05$; **, $P < 0.01$. The data are representative of two different experiments.*

Clinical observations. To characterize differences in the clinical course of infection between C3H/HeJ ($Tlr4^{Lps\text{-}d}$) and A/J ($Tlr4^{Lps\text{-}n}$) mice, 24 mice of both strains were inoculated with *B. pertussis* (6.1×10^7 CFU per mice). Unexpectedly, several animals died after infection (Fig. 6). Nine A/J mice died within the first 4 days after infection, while eleven C3H/HeJ mice died after 4 days.

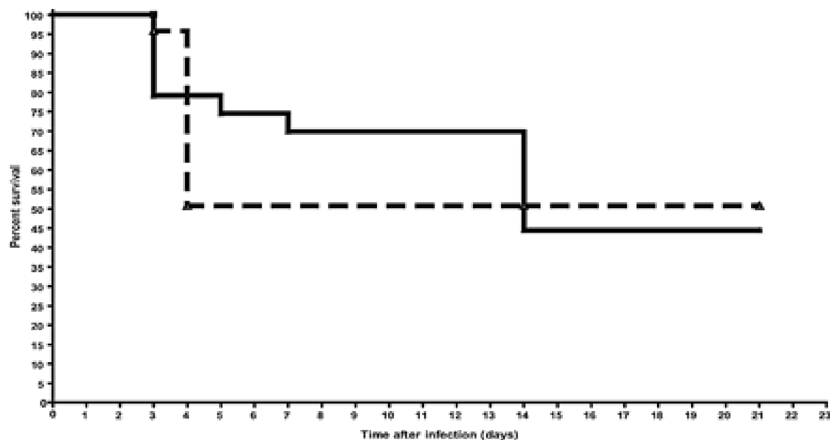

FIG. 6. Survival of mice after infection with B. pertussis. The solid line represents C3H/HeJ mice ($Tlr4^{Lps\text{-}d}$), and the dashed line represents A/J mice ($Tlr4^{Lps\text{-}n}$).

All mice were weighed 1 week before infection and 3, 7, 14, and 21 days after infection. Differences in body weight are presented in Fig. 7. Infected mice lost 5 to 15% of their body weight in the first 7 days after infection. At 14 days after infection, A/J mice had regained most of their initial weight, while C3H/HeJ mice

were still losing weight. At 21 days after infection all mice had regained their weight. Thus, in these two strains of mice, mortality appears to be inversely related to weight loss after infection, i.e., A/J mice died earlier but surviving mice recovered more quickly compared to C3H/HeJ mice.

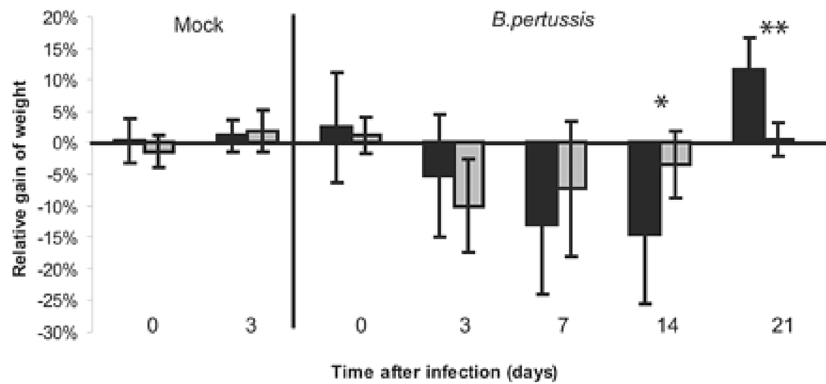

*FIG. 7. Gain of weight after B. pertussis inoculation. Solid boxes represent the mean of C3H/HeJ mice (Tlr4^{Lps-d}); gray boxes represent A/J mice (Tlr4^{Lps-n}). Asterisks represent the significance between the two inbred strains at a specific point of time. *, P < 0.05; **, P < 0.001. The data are representative of two different experiments.*

Lung pathology. The role of *Tlr4* in lung pathology was examined by comparing differences in lung lesions between C3H/HeJ (*Tlr4*$^{Lps-d}$) and A/J (*Tlr4*$^{Lps-n}$) mice. Lungs were weighed as a marker for inflammation. Relative lung weights are presented in Fig. 8. Mock-infected mice had an average relative lung weight of 1%, while *B. pertussis*-infected mice had higher relative lung weights. C3H/HeJ mice had significantly higher relative lung weights after infection compared to A/J mice.

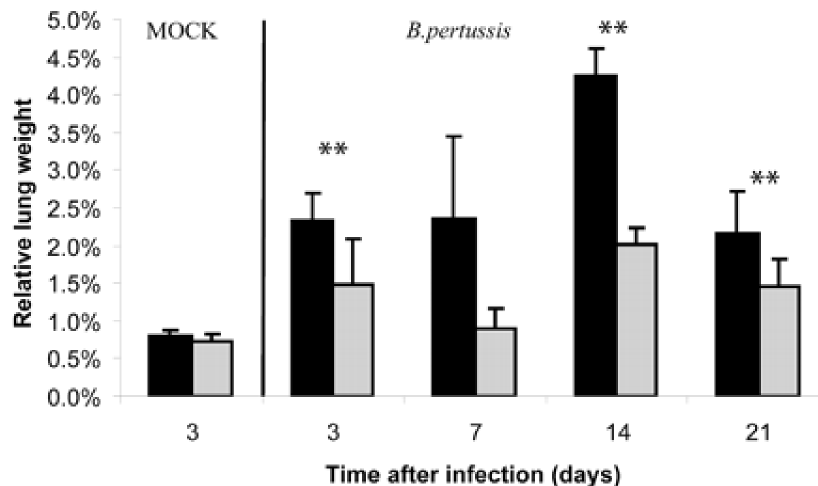

*FIG. 8. Relative lung weights of mice after infection with B. pertussis. Solid boxes represent the mean of C3H/HeJ mice (Tlr4^{Lps-d}); gray boxes represent A/J mice (Tlr4^{Lps-n}). Asterisks represent the significance between the two inbred strains at the specific point of time: *, P < 0.05; and **, P < 0.001. The data are representative of two different experiments.*

Lung lesions are summarized in Table 2. Examples of typical lesions are presented in Fig. 9. The inflammatory response to *B. pertussis* in the two strains of mice examined were similar during the first week after infection. After 3 and 7 days, the peribronchiolar and perivascular inflammation remained slight to moderate.

However, 2 and 3 weeks after infection, perivasculitis was stronger in the A/J strain than in the C3H/HeJ strain. Also, a remarkable difference in the quantity of free protein in the alveoli, which is indicative for inflammatory exudate, was observed. C3H/HeJ mice had a marked inflammatory exudate at 3 days postinfection, which was even more pronounced at days 14 and 21. This inflammatory exudate is very likely responsible for the increased lung weight. In contrast, the A/J mice had no or only a slight inflammatory exudate. No strain differences were seen in peribronchiolitis, alveolitis, the extent of the hypertrophy of the bronchiolar epithelium, and eosinophilia.

TABLE 2. Summary of histological lung changes in two strains of mice infected with *B. pertussis*

Parameter or rank	No. of animals[a]									
	C3H/HeJ					A/J				
	Mock	3	7	14	21	Mock	3	7	14	21
No. of intercurrent deaths			2	2	3			5	2	1
No. examined	6	6	4	4	3	6	6	1	4	5
No. with peribronchiolitis[b]										
Minimal										
Slight		6	3	1			2	1		
Moderate			1	3	3		3		2	5
Marked									2	
No. with perivascular infiltrate										
Minimal	3									
Slight		5					1			
Moderate		1	4	3	3		3	1		1
Marked				1			1		2	3
Strong									2	1
No. with hypertrophy of the bronchiolar epithelium										
Minimal										
Slight										
Moderate					1		2			1
Marked		6	1	2	2		3	1	1	4
Strong			3	2					3	
No. with alveolitis										
Minimal					1					2

Slight				1	2			2
Moderate	6	2	2	1	2	1		1
Marked		2	1		1		4	
Strong			1					
No. with protein in the alveoli								
Minimal					5			
Slight			1					
Moderate	2	1	1					
Marked	2					1		
Strong	2	3	2					

[a] Data are presented grouped by mouse strain and days postinfection. Mock, mock infected.

[b] Hematoxylin-eosin-stained slides were examined for peribronchiolitis, alveolitis, perivasculitis, hypertrophy of mucus-producing glands, free protein, and eosinophili. Lung lesions were scored semiquantitatively as follows: 0, absent; 1, minimal; 2, slight; 3, moderate; 4, marked; or 5, severe.

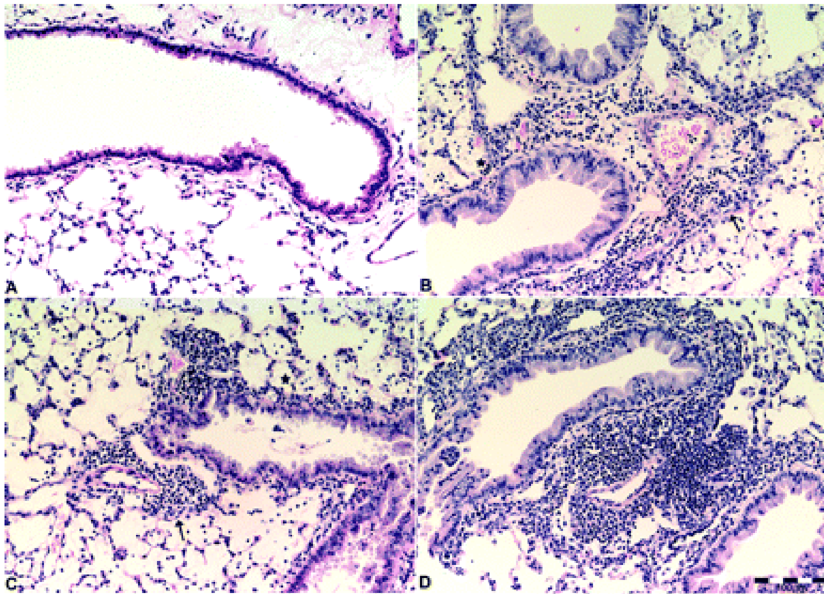

FIG. 9. Examples of time dependency of lung pathology after infection. Hematoxylin-eosin-stained lung sections are shown. Bar, 100 µm. (A) Control A/J mouse bronchiole with a thin epithelial layer surrounded by empty alveoli. (B) At 3 days postinfection of a C3H/HeJ mouse, slight peribronchiolar (star, score 2) and perivascular infiltrates (arrow) are obvious, together with a moderate hypertrophy of the bronchiolar epithelium (center right, score 3). The alveoli show a moderate infiltrate and free protein in the alveolar space. (C) At 7 days postinfection a C3H/HeJ mouse shows slight peribronchiolitis (star) and moderate perivasculitis (arrow). The hypertrophy of the bronchiolar epithelium is very

strong (score 5). In all alveoli free cells as well as protein are present. (D) At 2 weeks after infection an A/J mouse has a moderate peribronchiolitis (score 3) and a marked perivasculitis (center, score 4). A strong hypertrophy of the bronchiolar epithelium is shown with inflammatory cells inside. Some alveolar macrophages are observed (top, right).

Cytokine response. IL-1ß, TNF-α, and IFN-γ production were determined in supernatants of splenocytes isolated at various days after infection from *B. pertussis*-infected A/J and C3H/HeJ mice after 3 days of ex vivo restimulation with heat-inactivated *B. pertussis* or ConA. Because *Tlr4* engagement also modulates the adaptive Th1 immune response (7, 12, 18, 22), IFN-γ was taken as a parameter of the acquired immune response.

As expected, supernatants of cells that were cultured in the absence of antigens did not show cytokine production, whereas supernatants of cells that were cultured in the presence of ConA showed the production of all three cytokines. There was no difference in ConA-induced cytokine production between mouse genotypes or the various days after infection.

The cytokine production of splenocytes restimulated with heat-inactivated *B. pertussis* is shown in Fig. 10. Culture supernatants from splenocytes isolated from A/J mice showed an increase of IL-1ß, TNF-α, and IFN-γ from 3 days till 14 after infection. At day 21 after infection, the cytokine concentrations decreased. In contrast, culture supernatants from splenocytes isolated from C3H/HeJ mice only showed a small cytokine response. No IL-1ß response and only a slight increase in TNF-α and IFN-γ production was seen from 14 days after infection onward.

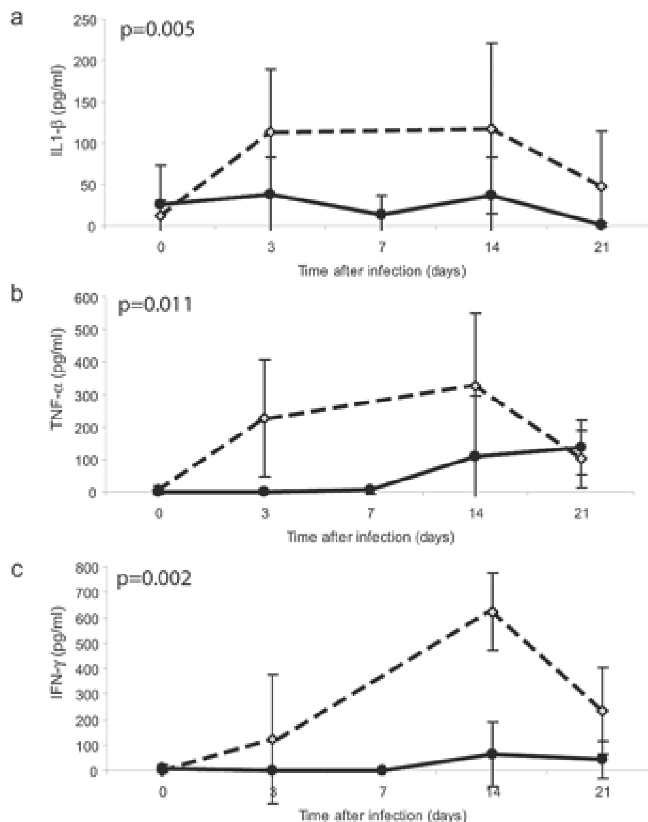

FIG. 10. IL-1ß (a), TNF-α (b), and IFN-γ (c) cytokine production of splenocytes isolated from B. pertussis-infected mice after 3 days of ex vivo restimulation with heat-inactivated B. pertussis. The solid line represents the mean of C3H/HeJ mice ($Tlr4^{Lps-d}$); the dashed line represents A/J mice ($Tlr4^{Lps-n}$). The plotted P value represents the overall difference in cytokine production between the mice.

TLR4 and TNF-α gene expression. The Tlr4-dependent induction of TNF-α has been shown to be critical for the early host response to *B. bronchiseptica* (20, 21). Therefore, we examined the gene expression of *Tlr4* and TNF-α in lung tissue of mice 3 days after infection with *B. pertussis*. The data are presented in Fig. 11 as the fold expression relative to mock-treated A/J mice (shaded gray). All expression data are calculated relative to the housekeeping gene RNA polymerase IIa (Polr2a). C3H/HeJ mice, either mock treated or *B. pertussis* infected, showed no upregulation of expression of *Tlr4*, whereas *B. pertussis*-infected A/J mice showed a 1.5-fold upregulation of this receptor. In addition, *B. pertussis*-infected A/J mice showed a fivefold upregulation of TNF-α, whereas infected C3H/HeJ mice only showed a slight, insignificant upregulation in expression of this gene.

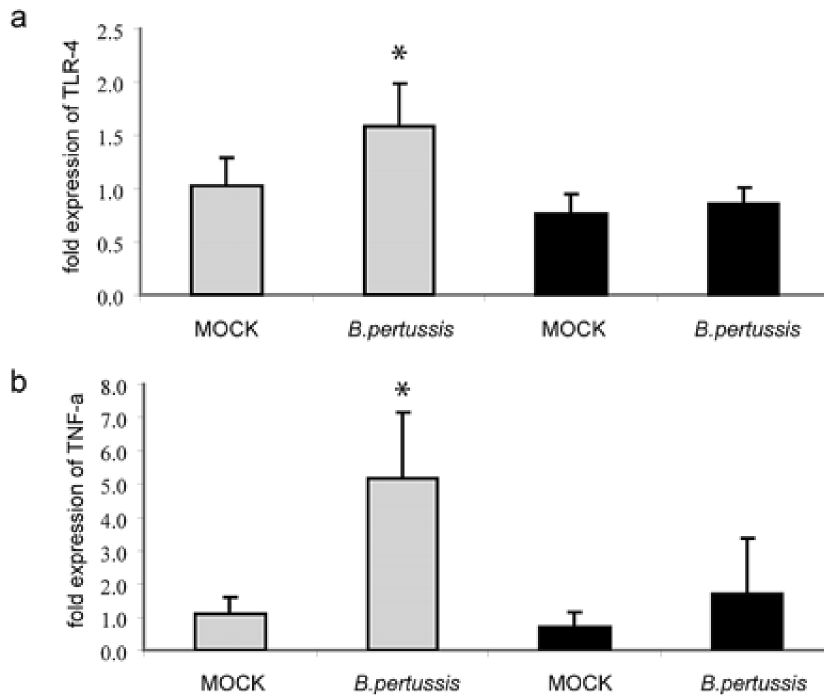

FIG. 11. Gene expression of TLR-4 and TNF-α in infected lung tissue. Gene expression of TLR-4 (a) and TNF-α (b) in the lung tissue of mice 3 days after inoculation with B. pertussis. Gene expression was compared relative to mock-treated A/J mice. Solid boxes represent the mean of five C3H/HeJ mice ($Tlr4^{Lps-d}$); gray boxes represent the mean of five A/J mice ($Tlr4^{Lps-n}$). Asterisks indicate a significant difference ($P < 0.05$) determined according to the Bonferroni test between groups.

DISCUSSION

By previously identifying three susceptibility loci (*Bps1* to *-3*) in recombinant congenic strains of mice we showed that the clearance and/or replication of *B. pertussis* in mice is under multigenic control. These loci could explain up to 10% of the variation in the lung colonization observed in F_2 mice (2). Because the contribution of these loci to genetic variation was small, we started the present study using inbred strains of mice instead of recombinant congenic strains of mice. By using this approach, we expected to examine a broader range of genetic variation, possibly leading to the identification of additional susceptibility loci. Significant differences in clearance were observed between these strains of mice. From these strains we selected A/J as the most resistant strain and C3H/HeJ as the most susceptible strain to generate an F_2 intercross generation, to

subsequently identify susceptibility loci, and to examine phenotypic characteristics.

We had originally planned to use a low-density genome-wide scan, with SNP markers, but mainly due to the study by Poltorak et al. (26) we started by analyzing this particular mutation in *Tlr4*. It turned out that the association between CFU and this mutation was so prominent that we calculated that possible other genetic effects could not be detected in this F_2 generation. We therefore have not performed a whole genome-wide scan but concentrated on markers on chromosome 4 as displayed in Fig. 2.

We subsequently identified Tlr4 as a major factor that influences the course of *B. pertussis* infection in mice. We calculated by linkage analysis that a large part of chromosome 4 was linked with reduced clearance of bacteria from the lungs, with a maximum LOD score of 33.6 at 65.4Mb, which is the location of *Tlr4*.
Since the number of mice was too large for analysis on a single day, the experiments were executed on multiple days. Because BALB/c control mice were included in each of these days, we were able to estimate the contribution of *Tlr4* to the total variation. We calculated that the residual mean square error of the BALB/c mice is larger than the residual mean square error of the *Tlr4* genotype. This means that the residual variation is smaller than the variation in inbred strains, strongly suggesting that no additional detectable genetic variation was left. We therefore concluded that the residual variation is due to environmental variation and that all of the genetically described variation in these experiments could be ascribed to the *Tlr4* gene. Although other (modifier) genes are expected to influence the course of pertussis, it is not possible to detect the effect of these genes in F_2 hybrids generated from these two strains of mice due to the strong effect of *Tlr4*. We did, however, analyze markers located in *Bps1* but did not find any linkage, probably due to the reason mentioned above. Thus, *Tlr4* dominates the variation in clearance of bacteria from the lungs in the first week after infection in these strains of mice.

To confirm the importance of *Tlr4* function in *B. pertussis* infection, we infected A/J(*Tlr4*$^{Lps-n}$), C3H/HeOuJ(*Tlr4*$^{Lps-n}$), and C3H/HeJ (*Tlr4*$^{Lps-d}$) mice. C3H/HeOuJ mice are genetically identical to C3H/HeJ mice except for the pro712his mutation in the gene encoding for Tlr4 (31). A/J and C3H/HeOuJ mice showed the same course of clearance of bacteria from the lung, while C3H/HeJ mice showed a delayed clearance of bacteria from the lung. Thus, this experiment unequivocally confirmed the significance of Tlr4.

Remarkably, although A/J mice cleared *B. pertussis* more efficiently, we observed no difference in overall mortality after *B. pertussis* infection between A/J and C3H/HeJ mice. However, A/J mice died earlier than C3H/HeJ mice. Thus, mortality appeared associated in time with the inflammatory response. As described below, additional inflammatory and pathobiological parameters confirmed the association between bacterial clearance, inflammation, and clinical effects.
In A/J mice, cytokine production of splenocytes restimulated ex vivo with heat-inactivated *B. pertussis* showed an increase in IL-1ß, TNF-α, and IFN-γ production at 3 days after infection, with a maximum at day 14 after infection. In C3H/HeJ mice, however, there was very little production of IL-1ß, and little production of TNF-α, and no IFN-γ production. Although Higgins et al. (11) suggested a role for Tlr4 in inhibiting Th1 responses by activating IL-10 production, our results indicate that *Tlr4* engagement is essential not only for the innate response but also for stimulation of the ensuing Th1 response, which is in line with other reports (7, 12, 18, 22). Functional Tlr4 may be required for the production of IL-10, which is associated with limiting the inflammatory pathology (11). In the present study we observed enhanced lung pathology in Tlr4-defective mice,

especially enhanced lung weight, which underlines the significance of Tlr4 limiting bacterial growth and probably also inflammation. However, it is likely that in C3H/HeJ mice the pathology resulting from infection differs from that in Tlr4-competent mice as a result of different levels of expression or production of IL-1ß, TNF-α, and IFN-γ. In response to LPS inhalation a TNF-α-dependent neutrophil influx in the BALF is seen in C3H/HeN but not in C3H/HeJ mice (10). In addition, gene profiling of lung tissue showed that 74% of the genes responsive to a *Klebsiella pneumoniae* infection were Tlr4 dependent (28). Interestingly, during lung inflammation the C3H/HeJ mice did not show perivascular accumulation of inflammatory cells, whereas A/J mice did (29). In addition, *Escherichia coli* inhalation resulted in a similar clearance in C3H/HeJ and in C3H/HeSnJ mice but resulted in lower proinflammatory cytokine levels and reduced neutrophils accumulation in C3H/HeJ mice (17).

Three days after infection, *B. pertussis*-infected C3H/HeJ mice showed no expression of *Tlr4* whereas similarly treated A/J mice showed a 1.5-fold upregulation of this receptor. This upregulation may be suggestive of actual receptor engagement. *B. pertussis*-infected A/J mice showed a fivefold upregulation of TNF-α, whereas C3H/HeJ mice only showed a slight, not significant, upregulation of this gene. This suggests that Tlr4-dependent TNF-α expression in the lung is an innate response to *B. pertussis* infection, which is similar to the observations for *B. bronchiseptica* (20, 21). It has been shown that early elicited TNF-α release is critical for host defense against *B. bronchiseptica* and that this process is Tlr4 dependent (20, 21). Although the course of infection with *B. bronchiseptica* is different from infection with *B. pertussis*, our data underline the significance of early TNF-α production in limiting bacterial growth. In C3H/HeJ mice other, possibly compensatory mechanisms might be operational. Functional Tlr4 seems to affect TNF-α levels both in the lung and the spleen.
Also in humans the role of *Tlr4* in infectious diseases has been investigated in genetic association studies (27). Associations were usually investigated for two SNPs, Asp299Gly and Thr399Ile, that result in amino acid changes in the extracellular domain of the receptor. Associations were found in some reports, but not in others, as recently reviewed by Schroder and Schumann (27).
In conclusion, we have shown that C3H/HeJ mice have delayed clearance of bacteria from the lung, increased lung pathology, more weight loss, and less IL-1ß, TNF-α, and IFN-γ production after infection compared to A/J mice. We showed that *Tlr4* is a major genetic factor that is sufficient to explain all detectable genetic differences in bacterial clearance between these mice. Functional Tlr4 is required for an early TNF-α, IL-1ß, and IFN-γ response that may enhance bacterial clearance, and thus, despite the proinflammatory nature of these cytokines, may limit pathology. This early cytokine response was fatal for ca. 50% of the mice, but the surviving mice had efficiently cleared the bacteria from the lungs, and little lung pathology and rather quickly regained their body weight. A lack of this response resulted in delayed mortality, but these mice were less efficient in clearing the bacteria from the lungs, became very ill from lung edema, and only slowly regained their body weights.

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FOOTNOTES

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