Lung response to *Bordetella pertussis* infection in mice identified by gene-expression profiling

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Abstract Host genetics determines the course of *Bordetella pertussis* infection in mice. Previously, we found four loci, Tlr4 and three novel loci, designated *Bps 1–3*, that are involved in the control of *B. pertussis* infection. The purpose of the present study was to identify candidate genes that could explain genetic differences in the course of *B. pertussis* infection, assuming that such genes are differentially regulated upon infection. We, therefore, studied the course of mRNA expression in the lungs after *B. pertussis* infection. Of the 22,000 genes investigated, 1,841 were significantly differentially expressed with 1,182 genes upregulated and 659 genes downregulated. Upregulated genes were involved in immune-related processes, such as the acute-phase response, antigen presentation, cytokine production, inflammation, and apoptosis, while downregulated genes were mainly involved in nonimmune processes, such as development and muscle contraction.

Pathway analysis revealed the involvement of granulocyte function, toll-like receptor signaling pathway, and apoptosis. Nine of the differentially expressed genes were located in *Bps-1*, 13 were located in *Bps-2*, and 62 were located in *Bps-3*. We conclude that *B. pertussis* infection induces a wide and complex response, which appears to be partly specific for *B. pertussis* and partly nonspecific. We envisage that these data will be helpful in identifying polymorphic genes that affect the susceptibility and course of *B. pertussis* infection in humans.

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Introduction

The airway pathogen *Bordetella pertussis* can cause the disease known as whooping cough. Despite high vaccination coverage, pertussis is still an endemic disease with peaks in incidence every 2–3 years during the last decade (Crowcroft and Pebody 2006; de Greeff et al. 2003, 2005). Susceptibility to and severity of *B. pertussis* infection in infants and children vary widely (Kimman 2001). The spectrum of clinical symptoms ranges from subclinical infection to mild disease, severe whooping cough, and death. In previous studies, we examined genetic susceptibility of mice to *B. pertussis* and identified loci in the mouse genome that are involved in restriction of colonization and/or clearance of *B. pertussis*. We and others have provided indications for the role of several polymorphic host genes in the course of *B. pertussis* infection, including the interferon gamma receptor (Mahon et al. 1997), toll-like receptor 4 (Tlr4; Higgins et al. 2003; Mann et al. 2004, 2005), and three putative disease loci, *B. pertussis* suscep-

tibility *locus 1*, *2*, and *3* (*Bps-1*, *2*, and *3*; Banus et al. 2005). *Tlr4* is a major host factor involved in the course of *B. pertussis* infection. C3H/HeJ mice, which carry a nonfunctional *Tlr4* gene, exhibit an aberrant course of infection. Functional *Tlr4* is essential for an efficient IL1-β, TNF-α, and IFN-γ response, efficient clearance of bacteria from the lung, and therewith reduced lung pathology (Banus et al. 2006). The *Bps-1* locus is located on chromosome 12, spanning a region of 185 genes and has a dominant positive effect on the clearance of *B. pertussis* from the lung. The function of most genes in this locus is unknown. Two other loci, *Bps-2* and *Bps-3*, showed genetic interaction and are located on chromosomes 5 and 11, respectively (Banus et al. 2005).

The highest linkage between the number of colony-forming units (CFU) and the *Bps-1* locus that we observed showed a logarithm of the odds (LOD) score of 4.6. This LOD score and the corresponding P value ($P=0.000025$) are statistically significant also after correction for multiple comparisons ($P=0.0019$). We interpreted these data as suggestive for linkage warranting further investigation (Banus et al. 2005). In the present study, we aimed to enhance the insight in the host response and pathogenesis of *B. pertussis* infection by identifying genes that are differentially expressed upon infection. In particular, we were interested in identifying candidate genes that could explain genetic differences in the course of *B. pertussis* infection, assuming that such genes are differentially regulated upon infection. For this purpose, we infected mice and analyzed gene-expression profiles in the lungs on days 1, 3, and 5 after inoculation using microarrays. We were especially interested if genes, located in the *Bps-1*, *2*, and *3* loci, were differentially expressed after *B. pertussis* infection or that the susceptibility of these loci was regulated alternately.

Materials and methods

Experimental design

Female C3H/DISnA mice (eight mice per group, total of 48) were intranasally inoculated with 2×10^7 CFU of the streptomycin-resistant Tohama strain of *B. pertussis* (B213) in 40 μl of Verwey medium (The Netherlands Vaccine institute, Bilthoven, The Netherlands) or as a control with Verwey Medium only. One, 3, and 5 days after inoculation, mice were euthanized. To remove blood cells from the lungs, mice were perfused with phosphate-buffered saline, and the lungs and trachea were collected (King et al. 2001; Willems et al. 1998). For the extraction of RNA, the right lung was collected in the RNA stabilization reagent (RNAlater, Qiagen, Venlo, The Netherlands). The left lung

was fixed intratracheally using 4% formalin for histological examination.

The number of viable *B. pertussis* bacteria was determined in the trachea to confirm a proper infection (Verwey et al. 1949). Therefore, approximately 1 cm of the trachea was collected in 500 μl of Verwey medium. Bacterial suspensions were diluted in Verwey medium, and the number of CFU was determined by plating on Bordet Gengou agar supplemented with 15% sheep blood and 30 μg/ml streptomycin (Tritium Microbiology, Veldhoven, The Netherlands). Plates were incubated for 4 days at 35°C before counting the number of CFUs using a ProtoCOL Colony counter (Synbiosis, Cambridge, UK).

Animals

Female C3H/DISnA mice were kindly supplied by Dr. Peter Demant (Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY). Mice were bred to the appropriate numbers at our animal testing facility in Bilthoven, The Netherlands. Mice were acclimatized for at least 1 week before the start of the experiments. Mice received standard laboratory food (SRM-A, Hope Farms, Woerden, The Netherlands) and tap water ad libitum. All animal experiments were approved by the Institute's Animal Ethics Committee and were performed according to the National Institutes of Health (1996) guidelines.

Clinical and pathological examinations

Mice were weighed before inoculation and subsequently every day after inoculation to determine the relative change in weight. Lung weights were determined postmortem as a parameter for lung inflammation. Formalin-fixed lungs were embedded in paraplast (Monoject, St Louis, MO). Sections (5 μm) were stained with hematoxylin eosin. Lung lesions were examined for infiltration of inflammatory cells in the peribronchiolar space (peribronchiolitis), infiltration of inflammatory cells in the alveoli (alveolitis), infiltration of inflammatory cells in the perivascular space (perivasculitis), hypertrophy of mucus-producing glands, free protein (exudate), and eosinophilia. Lung lesions were scored semiquantitatively as absent, minimal, slight, moderate, marked, or strong as previously described (Barends et al. 2004).

Transcription profiling

Lungs were incubated in RNAlater (Qiagen) at 4°C for 1 day, after which, the tissues were transferred to a fresh tube and stored at −80°C. RNA was extracted by using the midi-RNA isolation kit (Qiagen). RNA concentrations were measured using a NanoDrop Spectrophotometer (Nano-

Drop Technologies, Wilmington, DE). RNA quality was determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and was expressed as the RNA Integrity Number (RIN) as defined by the manufacturer. Only samples with a RIN score of at least eight (on a 1–10 scale) were used for expression profiling.

Microarray slides were spotted at the Microarray Department of the University of Amsterdam. The slides contain 21,997 65-mer oligo nucleotides from the Sigma-Compugen Mouse oligonucleotide library, 192 additional 65-mer oligo nucleotides of genes, of which 185 were located in the *Bps-1* region spotted in duplicate (Isogen Life science, IJsselstein, The Netherlands), as well as appropriate controls and blank spots.

Based on RNA quality and yield, RNA isolated from six mice per group was selected for amplification and labeling. RNA amplification and labeling were carried out using the Amino Allyl MessageAmp II aRNA kit (Ambion, Austin, TX) using 1 μg of total RNA as starting material. RNA samples from individual mice were labeled with Cy3. A common reference containing a RNA pool of all samples isolated was labeled with Cy5. After hybridization and washing, the arrays were scanned using a ScanArray 4000XL microarray scanner (Perkin-Elmer, Wellesley, MA).

Median Cy3 and Cy5 spot signals were determined using ArrayVision (Imaging Research, St. Catharines, ON, Canada). Quality control was performed by means of visual inspection of the scanned images, raw data scatter, and MA plots (M is a mnemonic for the log-ratios of expression, A is a mnemonic for the log-intensity of each spot; Smyth and Speed 2003), as well as a normal probability plot to assess signal distribution (Pennings and Heisterkamp 2004). If more than 10% of the spots were flagged as missing data, slides were excluded from data analysis. At least five slides per group were used for further analysis.

Raw microarray signal data were normalized in the R statistical software environment (R Development Core Team 2005) using a four-step approach of (1) natural log transformation, (2) quantile normalization of all scans, (3) correcting for differences in the reference signal, and (4) averaging replicate spot data.

Significance of differences in gene expression between the experimental groups was calculated in R with analysis of variance. The false discovery rate (FDR) was calculated according to Benjamini and Hochberg (1995). The FDR is the expected proportion of false positives among the genes considered significantly differentially expressed (Benjamini et al. 2001; Benjamini and Hochberg 1995). Gene-expression data were visualized by hierarchical clustering (using Euclidian distance and Ward linkage) using GeneMaths (Applied Maths, St-Martens-Latem, Belgium). For this purpose, gene-expression values were corrected for the overall average to allow a neutral comparison between all *B. pertussis-*

and mock-inoculated groups. Gene categories were defined by Gene Ontology (GO, http://www.geneontology.org). Classification and enrichment according to GO categories were determined using DAVID/EASE (Dennis et al. 2003; Hosack et al. 2003), using EASE scores as *P* values for enrichment. Additional pathway analysis was performed using Meta-Core™ (GeneGo, http://www.genego.com).

Results

Bacteriologic and pathologic findings

All *B. pertussis*-inoculated mice had between 10^2 and 10^5 CFUs in the trachea during the first 5 days after inoculation thereby confirming a proper infection. A statistically significant decrease in the number of bacteria in the trachea was observed over time during the first 5 days postinoculation ($P=0.022$, linear regression, SPSS, data not shown).

Infected mice lost up to 4% of their body weight during the first 5 days postinoculation, while all mock-inoculated mice gained weight up to 4% during these 5 days. This difference in relative gain of weight between mock- and *B. pertussis*-inoculated mice is significant from day 1 to 5 postinoculation (Supplementary data Figure 2).

We determined lung weights in relation to body weight as a marker for inflammation. All mock-inoculated mice had relative lung weights of approximately 1% during the first 5 days after inoculation, while *B. pertussis*-infected mice had relative lung weights of up to 3% of their total body weight. This difference in relative lung weights between mock- and *B. pertussis*-inoculated mice is significant from day 1 to 5 postinoculation (Supplementary data Figure 3).

A summary of histopathological findings is presented in Table 1. Intranasal inoculation of *B. pertussis* causes an acute inflammatory response that is characterized by influx of polymorphonuclear leukocytes (PMNs) and macrophages starting in perivascular and peribronchiolar areas on day 1 and extending to alveolar walls and lumina on days 3 and 5.

Expression profiling

Gene-expression profiles were determined in at least five animals per group. The three mock-inoculated groups, euthanized at days 1, 3, and 5, were statistically indistinguishable (i.e., there were no genes with FDR<0.6). Of the approximately 22,000 genes tested, 1,841 genes were significantly differentially expressed in *B. pertussis*-inoculated lungs (FDR<0.05, corresponding to *P*<0.0042). Expression levels of all 1,841 genes as well as comparisons to other studies are presented in the supplementary data Table 5.

Table 1 Histological evaluation of lung changes after *B. pertussis* inoculation

Parameter and grade		No. of animals[a]					
		Mock			*B. pertussis*		
		Day 1	Day 3	Day 5	Day 1	Day 3	Day 5
Peribronchiolitis[b]	Minimal	2		3	4	2	2
	Slight				1	2	5
	Moderate					3	
	Total[c]	(25%)	(0%)	(38%)	(63%)	(88%)	(88%)
Perivasculitis	Minimal	2	2	2	2	1	
	Slight				4	3	3
	Moderate					3	5
	Marked					1	
	Total	(25%)	(25%)	(25%)	(75%)	(100%)	(100%)
Hypertrophy	Minimal	1		1	1	2	
	Slight						1
	Moderate					3	1
	Marked					2	4
	Strong						1
	Total	(13%)	(0%)	(13%)	(13%)	(88%)	(88%)
Alveolitis	Minimal					2	1
	Slight				4		2
	Moderate					3	1
	Marked					1	3
	Strong					3	1
	Total	(0%)	(0%)	(0%)	(75%)	(100%)	(88%)
Exudate	Minimal					2	2
	Slight					2	2
	Moderate					1	1
	Marked					1	
	Strong					1	
	Total	(0%)	(0%)	(0%)	(0%)	(100%)	(63%)

[a] Eight mice per group were inoculated with medium (mock) or *B. pertussis*.

[b] Hematoxylin/eosin-stained slides were examined for peribronchiolitis, alveolitis, perivasculitis, hypertrophy of mucus-producing glands, and free protein (exudate). Lung lesions were scored semiquantitatively as absent (not included in table), minimal, slight, moderate, marked, or strong.

[c] Total percentage of animals with at least minimal scoring in the specified category

Hierarchical clustering showed time-dependent changes in gene expression with 1,182 upregulated genes and 659 downregulated genes. Of these genes, 439 were more than twofold upregulated, and 134 were more than twofold repressed. All genes followed a similar trend in time. One day postinoculation differential gene expression was already observed, but the effect was larger on days 3 and 5 postinoculation (Fig. 1). There was no identifiable subset of genes that displayed a different kinetic trend in gene expression.

To assign functions to differentially expressed genes, we classified them according to GO categories. In addition, we determined enrichment for GO categories to identify pathophysiological processes involved in response to *B. pertussis*. The most important GO categories, i.e., with the highest percentage differentially expressed genes, are summarized in Table 2. Most upregulated genes are involved in immune response-related processes, such as the acute phase response, antigen presentation, cytokine activity, inflammation, and apoptosis; whereas downregulated genes are involved in nonimmune processes, such as development, muscle contraction, cytoskeleton, and general processes like metabolism.

To reveal intergene relationships, we performed Meta-Core™ analysis on the 1,841 regulated genes in addition to the GO-based analysis presented above. The most significant pathways are presented in Table 3. This analysis revealed the significance of several pathways, such as regulation of granulocyte function, the toll-like receptor (TLR) signaling pathway, the classic complement pathway, and apoptosis.
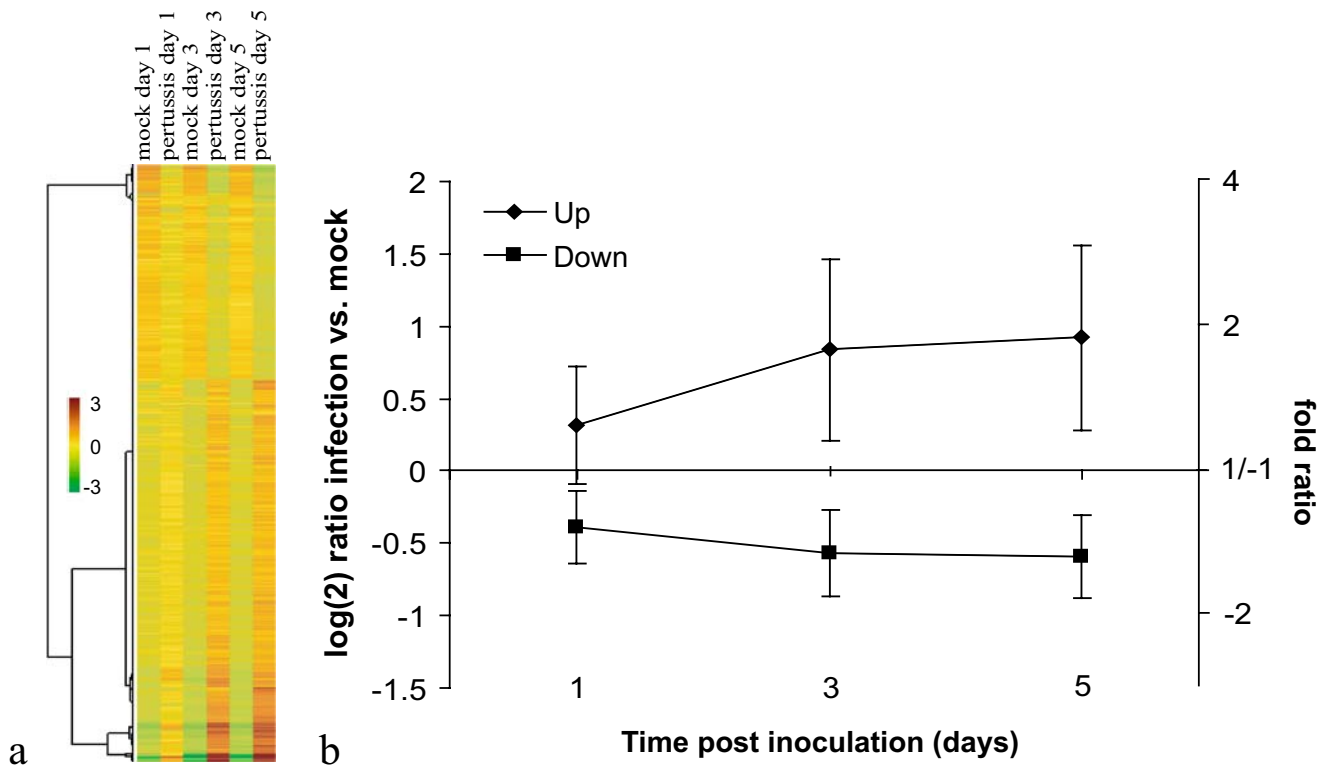
Fig. 1 Presentation of microarray data from mock- and *B. pertussis*-inoculated mice. Analysis was performed on 1,841 genes whose expression was found to be significant at an FDR<0.05. **a** Hierarchical clustering shows the segmentation of the genes clustered by gene expression. Relatively increased gene expression is presented in *red*, while relatively decreased gene expression is presented in *green*. The color scale shows log(2) ratios of gene expression levels compared to the global average. **b** Course of *B. pertussis*-regulated gene expression; *diamonds* represent the average of 1,182 upregulated genes, and *squares* represent the average of 659 downregulated genes. *Error bars* show the standard deviation (SD) of the average gene expression

Table 2 Classification of genes induced upon *B. pertussis* infection[a]

	Up		Down[b]		Total on array	P value[c]
Acute-phase response[d]	12	(55%)	0	(0%)	22	2.4×10^{-7} (up)
Antigen presentation	21	(66%)	0	(0%)	32	7.7×10^{-15} (up)
Apoptosis	47	(15%)	6	(2%)	324	8.0×10^{-5} (up)
Cell cycle	43	(8%)	22	(4%)	510	4.3×10^{-1} (up)
Chemokine activity	21	(58%)	0	(0%)	36	1.2×10^{-13} (up)
Complement activation	8	(26%)	1	(3%)	31	9.7×10^{-3} (up)
Cytokine activity	48	(25%)	6	(3%)	192	7.8×10^{-13} (up)
Cytoskeleton	31	(6%)	40	(8%)	531	2.0×10^{-3} (down)
Development	96	(7%)	94	(7%)	1,433	9.8×10^{-1} (down)
Immune response	162	(40%)	3	(1%)	408	6.3×10^{-75} (up)
Inflammatory response	46	(38%)	2	(2%)	121	1.1×10^{-19} (up)
Metabolism	353	(7%)	220	(5%)	4,771	1.8×10^{-1} (down)
Muscle contraction	0	(0%)	12	(24%)	49	6.7×10^{-6} (down)
Listed in any of the above	576	(9%)	318	(5%)	6,574	
Other	328	(6%)	204	(4%)	5,639	
Unannotated	278	(3%)	137	(1%)	9,744	
Total	1,182	(5%)	659	(3%)	21,957	

[a] Classification according to gene-ontology (GO) categories
[b] Analysis was performed on 1,841 regulated genes.
[c] Ease score for GO category enrichment in the specified set of genes
[d] Some genes are classified into multiple categories.

Table 3 Pathway analysis of genes induced upon *B. pertussis* infection

Name of Pathway involved[a]	Main process	*P* value	No. of genes in pathway		
			Expressed		Total
Transcription regulation of granulocyte development	Immune response	0.000001925	17	(38%)	45
TLR ligands and common TLR signalling pathway	Immune response	0.00001814	16	(34%)	47
ECM remodeling	Metabolism	0.00003906	18	(30%)	60
Role of IAP proteins in apoptosis	Apoptosis	0.00005458	13	(36%)	36
HETE and HPETE diosynthesis and metabolism	Metabolism	0.0003249	13	(31%)	42
TPO signaling via JAK-STAT pathway	Cell cycle	0.0003893	9	(39%)	23
Leukotriene 4 biosynthesis and metabolism	Metabolism	0.0004655	16	(27%)	60
RhoA regulation pathway (extension, GEFs/GAPs)	Immune response	0.002412	10	(29%)	34
MIF in innate immunity response	Immune response	0.002435	14	(25%)	57
Retinol metabolism	Metabolism	0.002842	12	(26%)	46
Classic complement pathway	Complement activation	0.002842	12	(26%)	46
Putative erythropoietin signaling pathway (part 1)	Immune response	0.002905	13	(25%)	52
MAPK cascade	Cell cycle	0.004975	12	(24%)	49
Ceramide-dependent NO antiapoptotic action	Apoptosis	0.006738	13	(23%)	57
IL9 signaling pathway	Cytokine activity	0.006809	9	(27%)	33
Ephrins signaling	Other	0.007847	13	(22%)	58
Polyamine metabolism	Metabolism	0.008731	7	(30%)	23
Integrin outside-in signaling	Cell cycle	0.009444	16	(20%)	79
Chemokines and adhesion	Chemokine activity	0.009657	34	(16%)	209

Analysis was performed on 1,841 genes whose expression was found to be significant at an FDR<0.05. The list is presented by descending *P* values. *P* values represent the probability of particular mapping arising by chance given the numbers of genes in all Metacore pathways, genes involved in the specified pathway, and genes regulated upon *B. pertussis* inoculation.

ECM Extracellular matrix, *GAP* GTPase-accelerating protein, *GEF* guanine nucleotide exchange factor, *HPETE* hydroperoxyeicosatetraenoic acid, *HETE* hydroxyeicosatetraenoic acid, *IAP* inhibitor of apoptosis, *IL9* interleukin 9, *JAK* Janus tyrosine kinase, *MIF* macrophage migration inhibitory factor, *MAPK* mitogen-activated protein kinase, *NO* nitric oxide, *RhoA* ras homolog gene family member A, *STAT* signal transducers and activators of transcription

[a] Classification according to main gene-ontology (GO) categories

Genes located in *Bps-1* that were differentially regulated upon *B. pertussis* inoculation and their function according to the GO database are presented in Table 4. *Bps-1* contains 185 genes, of which nine were differentially expressed. The strongest upregulated genes in this locus were trypto-phanyl-tRNA synthetase (*Wars*, fourfold) and tumor necrosis factor alpha-induced protein 2 (*Tnfaip2*, 5.4-fold). Zinc finger FYVE domain containing 21 (*Zfyve21*, 1.5-fold) and kinesin family member 26A (*Kif26a*, 1.7-fold) were the strongest downregulated genes.

Genes located in *Bps-2* and *-3* that were differentially regulated upon *B. pertussis* inoculation are presented in Supplementary data Table 6. *Bps-2* is located on chromosome 5 and contains 262 genes, of which 13 were differentially expressed. Interleukin-6 (IL-6) was the strongest upregulated gene (4.7-fold) in this locus, and general transcription factor IIIC, polypeptide 2, beta was the strongest downregulated gene (twofold) in this locus. *Bps-3* is located on chromosome 11 and contains 922 genes, of which 62 were differentially expressed. Chemokine (C–C motif) ligand 8 was the strongest upregulated gene (15.3-fold) in this locus, and RIKEN cDNA 9530033F24 gene was the strongest downregulated gene (2.3-fold) in this locus.

Discussion

We have recently shown that genetic differences of the host play an important role in the restriction of colonization and/or clearance of *B. pertussis* from the lungs of mice (Banus et al. 2005, 2006). In the present study, we wanted to extend the understanding of the host response to *B. pertussis* and identify genes and pathways that play a role in the pathophysiological response to *B. pertussis* infection. In particular, we were interested in identifying candidate genes that could explain genetic differences in the course of *B. pertussis* infection, assuming that such genes are differentially regulated upon infection. For these purposes, we studied the time course of mRNA-expression in the lungs of mice after a *B. pertussis* infection by microarray analysis. We were especially interested if genes, located in the *Bps-1*, *2*, and *3* loci, were differentially expressed after *B. pertussis* infection or that the susceptibility conferred by these loci was regulated alternately.

Of the approximately 22,000 genes investigated, we found 1,841 (8.4%) genes to be significantly differentially expressed. Differential gene expression was already ob-

Table 4 Genes located in *Bps-1* that are differentially regulated upon *B. pertussis* inoculation

GenBank accession	Gene symbol	Location[a]	Fold ratio change			P value	Description	Pathway[b]
			Day 1	Day 3	Day 5			
NM_011705	Vrk1	106	1.29	1.52	1.41	0.00003	Vaccinia-related kinase 1	Protein kinase activity
NM_011710	Wars	109	1.95	3.58	4.01	0.00000	Tryptophanyl-tRNA synthetase	Aminoacyl-tRNA ligase activity
NM_010480	Hspca	111	-1.14	1.40	1.27	0.00064	Heat shock protein 1, alpha	Protein folding
NM_009396	Tnfaip2	112	1.43	4.48	5.40	0.00000	Tumor necrosis factor, alpha-induced protein 2	Angiogenesis
XM_138272	Gm266	112	-1.08	-1.25	-1.35	0.00110	Gene model 266, (NCBI)	G-protein coupled receptor activity
NM_026752	Zfyve21	112	-1.33	-1.41	-1.50	0.00001	Zinc finger, FYVE domain containing 21	Zinc ion binding
XM_138275	Kif26a	113	-1.32	-1.68	-1.73	0.00030	Kinesin family member 26A	Microtubule motor activity
NM_178911	Pld4	113	1.21	1.82	2.52	0.00000	Phospholipase D family, member 4	Catalytic activity
XM_619046	LOC434166	113	1.13	1.42	1.42	0.00141	Similar to hypothetical protein MGC37588	Unknown

[a] *Bps-1* is located on chromosome 12; location is presented as distance from centromere (Mb).
[b] Classification according to gene-ontology (GO) categories

served on day 1 postinoculation, but the maximum effect was observed on day 3 postinoculation. This effect remained constant till at least day 5 postinoculation. The observed course in gene expression after *B. pertussis* inoculation was similar to that induced by another gram-negative lung pathogen, *Francisella tularensis* (Andersson et al. 2006).In a study of expression profiles in the lungs of *F. tularensis*-infected C57BL/6 mice, 424 (2.3%) genes out of the 18,500 genes investigated were upregulated. Genes involved in immune responses were activated strongly on day 4 postinoculation but hardly or not at all on days 1 and 2 (Andersson et al. 2006).

As expected, most upregulated genes are involved in immune and inflammation-related processes, such as antigen presentation, complement, cytokine, and chemokine activity. Other processes involved include the acute phase response and metabolism. Most downregulated genes are involved in nonimmune processes, such as metabolism, development, cell cycle, or muscle contraction. We speculate that by downregulating these genes, the energy balance is shifted in favor of the immune response. Evidently, cellular influx in the lungs is likely to influence the gene-expression profile.

Our data provide more detailed information on the central role of PMNs in the pathogenesis of *B. pertussis* infection. Pathogenesis of *B. pertussis* infection is characterized by colonization and proliferation of the ciliated mucosal cells resulting in damage of the respiratory epithelium and an acute increase in the levels of inflammatory cytokines resulting in cellular infiltrate in the alveolar spaces (Mattoo and Cherry 2005; Mills 2001; Preston 2005). Circulating PMNs are rapidly recruited to the lungs to bind and ingest *B. pertussis*, subsequently killing the bacteria by a combination of reactive oxygen and granule components. Finally, the PMNs undergo apoptosis (Kobayashi et al. 2005a). TLR ligands such as lipopolysaccharides (LPS) are critical components for the recruitment and priming of PMNs. Serum antibody-mediated clearance of *B. pertussis* also requires a TLR-induced early recruitment of PMNs. However, pertussis toxin limits this rapid serum antibody-mediated clearance by inhibiting PMN recruitment (Kirimanjeswara et al. 2005). As determined by pathway analysis in this study, the most significant pathways involved in the response to *B. pertussis* infection are regulation of PMN function, TLR signaling pathway, extracellular matrix remodeling, and apoptosis. It has been described that apoptosis of PMNs, in which TLR2 plays an important role (Jablonska et al. 2006), is accelerated after phagocytosis of bacteria (Kobayashi et al. 2005a, b). Therefore, the involvement of three out of four pathways may, at least partly, be explained by the influx of PMNs. Our data thus further illustrate the central role of PMNs in the course of *B.*

pertussis infection and the involved mechanisms in PMN function.

We found the response to *B. pertussis* to be wide and complex. Some remarkable findings were the involvement of mucin genes, intracellular pathogen resistance gene, and the excessive upregulation of serum amyloid A (SAA) genes. We found the mucin (*Muc*) genes *Muc4* and *Muc5b* to be significantly upregulated. Expression of mucin in the respiratory tract is one important innate host defense mechanism that respiratory pathogens must overcome (Belcher et al. 2000). *Muc4*, which encodes a respiratory tract mucin glycoprotein, was expressed 3.3-fold at day 5 upon *B. pertussis* inoculation. *Muc5b*, which encodes mucin 5, subtype B protein, in tracheobronchial epithelial cells, was expressed 1.8-fold 3 days upon inoculation. Belcher et al. (2000) described that *B. pertussis* induces mucin gene transcription by BEAS-2B cells. Evidently, gene expression studies do not allow to define the functional role of regulated genes. Further studies should define whether the mucin response is favorable to the host, the pathogen, or both.

In our study, we found a threefold upregulation of the intracellular pathogen resistance gene 1 (*Ipr1*). *Ipr1* mediates innate immunity to *mycobacterium tuberculosis* (Pan et al. 2005). Pan et al. (2005) speculated that the *Ipr1* gene product might function in integrating signals generated by intracellular pathogens with mechanisms controlling innate immunity, cell death, and pathogenesis. Our data therefore suggest that *Ipr1* may be involved in intracellular presence of *B. pertussis*. Interestingly, *B. pertussis* was shown to survive intracellularly in macrophages, PMNs and bronchoalveolar lavage (BAL) cells of mice (Hellwig et al. 1999; Mills 2001).

Among the upregulated genes upon *B. pertussis* inoculation, SAA genes were upregulated to a remarkably high extent. SAA is an acute phase protein whose expression is markedly upregulated during inflammation and infection (Uhlar and Whitehead 1999). Among other functions, this protein plays an important role in high density lipoprotein cholesterol metabolism by promoting cellular cholesterol efflux through a number of different efflux pathways (van der Westhuyzen et al. 2005). In our study, SAA1, 2, and 3 were induced 14-, 54-, and 44-fold, respectively, after *B. pertussis* infection, again underlining the important role for the acute phase response after *B. pertussis* infection. Although strongly upregulated, it is clear that the acute-phase response is not specific for *B. pertussis* infection.

In a previous study, we observed a 1.5-fold upregulation of the membrane-receptor *Tlr4* and a fivefold upregulation of the downstream cytokine gene *Tnf-α* 3 days post-*B. pertussis* inoculation by means of quantitative real-time polymerase chain reaction (PCR). Thus, a slight upregulation (1.5-fold) of this receptor induces a more pronounced (fivefold) induction of this downstream proinflammatory cytokine (Banus et al. 2006). In the present study, we obtained similar results by using microarray analysis. *Tlr4* expression was increased 1.6-fold, while the expression of *Tnfaip* was increased 4.5-fold 3 days postinoculation. The results of the present study hereby confirm our previous observations. In a recent study, a high correlation between gene-expression values determined by quantitative real-time PCR and microarray was observed (Canales et al. 2006). Given these results and our observations, we conclude that the results, at least for the two given genes, are similar for both techniques.

It is very likely that the expression profile to *B. pertussis* is partly unique to *B. pertussis* and partly nonspecific. To examine the specificity of *B. pertussis*-regulated gene expression, we compared our data to other studies. In a recent review by Jenner and Young (2005), a comparison was made between published transcriptional profiling data from 32 studies that involved 77 different host–pathogen interactions. A set of 417 genes that comprise a common host transcriptional response was defined. We found that 122 genes affected by *B. pertussis* infection were listed in this common host–pathogen response, further illustrating that many genes regulated by *B. pertussis* are indeed not specific for *B. pertussis* infection but are involved in a common response to pathogens. One explanation for the broad response in expression profiles is the complexity of the bacterium. It is, for instance, known that LPS is a very potent immune stimulus, which induces a strong inflammatory response (Kawai et al. 2001; Qin et al. 2005; Wells et al. 2003). Transcriptional profiling of LPS-induced acute lung injury in mice resulted in 71 immune-related upregulated genes (Jeyaseelan et al. 2004). Of these 71 genes, 43 were also regulated upon *B. pertussis* inoculation in this study.

Previously, we have demonstrated that the *Bps-1* locus has a dominant positive effect on the clearance of *B. pertussis* from the lung. Of the 185 genes in this locus, nine were differentially expressed. Some of these genes could explain the linkage of this locus with susceptibility to *B. pertussis* infection. Tumor necrosis factor, alpha-induced protein 2 (*Tnfaip2*), has been described as a tumor necrosis factor-alpha-inducible primary response gene in endothelial cells (Sarma et al. 1992). Aminoacyl-tRNA synthetases catalyze the aminoacylation of tRNA with their cognate amino acid. Tryptophanyl-tRNA synthetase (*Wars*) catalyzes the aminoacylation of tRNA(trp) with tryptophan and is induced by interferon (Ibba and Soll 2000).The role of the nine genes in the *B. pertussis* infection process is unknown and will be investigated in future experiments. Thus, by using a microarray approach, we selected nine possible candidate genes from the 185 present in *Bps-1*. However, by this approach, we focused on transcriptionally regulated genes, and the possibility remains that suscepti-

bility to *B. pertussis* is regulated in a different way, such as phosphorylation or protein binding.

In comparison to the *Bps-1* region, the *Bps-2* and *-3* regions contain considerably more genes, namely, 262 and 922 genes, respectively, of which 13 and 62 were differentially expressed post-*B. pertussis* inoculation. Among these differentially expressed genes are several interesting candidate genes that may explain the linkage of these loci with susceptibility to *B. pertussis* infection. IL-6 was the most strongly induced gene in the *Bps-2* region (4.7-fold). IL-6 is a proinflammatory cytokine that can be secreted by macrophages after Tlr4 activation (Akira and Takeda 2004). Among the genes differentially expressed in the *Bps-3* region was a large group of chemokines, of which Chemokine (C–C motif) ligand 8 was the strongest upregulated gene (15.3-fold). Upon *B. pertussis* infection, this large group of chemokines is induced to promote immune cells to migrate to the site of infection. The role of the other *B. pertussis*-induced genes in these loci is still unknown and will be investigated in future experiments.

In conclusion, we found 1,841 genes in the lungs of mice that are differentially expressed after *B. pertussis* inoculation. Most upregulated genes are involved in immune and inflammation-related processes or in nonspecific processes. Most downregulated genes are involved in nonimmune processes. A substantial number of genes and pathways provide more detailed information on the central role of PMNs in the pathogenesis of *B. pertussis*. In particular, the transcriptional profiles indicate the significance of genes involved in TLR activation, the recruitment and activation of PMNs, and apoptosis.

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