Genetic Susceptibility to the Delayed Sequelae of Neonatal Respiratory Syncytial Virus Infection Is MHC Dependent

John S. Tregoning, Yuko Yamaguchi, Belinda Wang, Dagmar Mihm, James A. Harker, Ellen S. C. Bushell, Ming Zheng, Guochun Liao, Gary Peltz and Peter J. M. Openshaw

*J Immunol* published online 4 October 2010
http://www.jimmunol.org/content/early/2010/10/04/jimmunol.1001594

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Genetic Susceptibility to the Delayed Sequelae of Neonatal Respiratory Syncytial Virus Infection Is MHC Dependent

John S. Tregoning,*† Yuko Yamaguchi,* Belinda Wang,* Dagmar Mihm,* James A. Harker,*1 Ellen S. C. Bushell,* Ming Zheng,‡ Guochun Liao,§ Gary Peltz,§ and Peter J. M. Openshaw*

Respiratory syncytial virus (RSV) is a major cause of respiratory morbidity, resulting in hospitalization for bronchiolitis in some infected infants that is associated with wheeze in later life. Genetic factors are known to affect the severity of the sequelae after RSV infection, but the complexity of the temporal and genetic effects makes it difficult to analyze this response in studies in man. Therefore, we developed a murine genetic model to analyze the sequelae occurring after RSV infection in early life. Haplotype-based genetic analysis of interstrain differences in severity identified the MHC as an important genetic determinant. This was confirmed by analysis of responses in congenic mice with different MHC haplotypes. We also found that susceptible strains had high CD8 levels during secondary infection. Analysis of first filial generation, second filial generation, and back-cross progeny produced by intercrossing resistant (H-2b, C3H/HeN) and sensitive (H-2d, BALB/c) strains indicated that susceptibility to sequelae after RSV infection was dominantly inherited but also segregated in a non-MHC–dependent manner. Thus, MHC haplotype and its effect on CD8 cell response is an important determinant of the outcome of neonatal RSV infection. The Journal of Immunology, 2010, 185: 000–000.

---

The Journal of Immunology

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00

Received for publication May 20, 2010. Accepted for publication August 25, 2010.

This work was supported by Program Grant 071381/Z/03/Z from the Wellcome Trust, UK and by the MRC & Asthma UK Centre in Allergic Mechanisms of Asthma. G.P. was partially supported by a grant (1R01 GM068885-01A1) from the National Institute of General Medical Sciences.

Address correspondence and reprint requests to Peter J. M. Openshaw, Department of Respiratory Medicine, Centre for Respiratory Infection, National Heart and Lung Institute, Imperial College London, St. Mary’s Campus, London W2 1PG, U.K. E-mail address: p.openshaw@imperial.ac.uk

Abbreviations used in this paper: F1, first filial generation; F2, second filial generation; FFU, focus forming unit; RSV, respiratory syncytial virus; SNP, single-nucleotide polymorphism.

---

Materials and Methods

Mice

Time-mated pregnant mice were obtained at <14 d gestation (Harlan, Hillcrest, U.K.), and the pups were weaned at 3 wk of age. The following strains were used in this study: BALB/cOlaHsd, BALB.B, BALB.K,
B10.A, B10.BR, B10.D2, A/JOlHsd, 129S2/SvHsd, DBA/2JrcHsd, C57BL/6JrcHsd, NZW/OlaHsd, NZB/OlaHsd, AKR/OlaHsd, C3H/HeJ, and MRL/MpOlaHsd. For the cross study, two lots of two BALB/c female mice were paired with one male C3H/HeN. This first filial generation (F1) produced 30 pups; 4 females and 2 males were retained from the litters of these crosses and used to set up the second filial generation (F2). The back-cross (BC1) was set up using males from the F1 BALB/cxC3H/HeN with female C3H/HeN.

For cell depletion, neonatal C57BL/6 mice were treated with 50 μl 1 mg/ml Ab i.p. on day −1, day +2, and day +5 postinfection, starting on day 6 of life. CD4 cells were depleted using clones YTA 191 and YTA 3; CD8 cells were depleted with clone YTS 156; control treatment was an irrelevant matched IgG2b isotype monoclonal (all Abs were a kind gift of S. Cobbold, Oxford University). For TNF depletion, neonatal C3H/HeN mice were treated with 50 μl 1 mg/ml monoclonal rat anti-TNF i.p. (IgG1; clone XT22) on days 0, 2, 4, and 6 postinfection. All work was approved and licensed by the U.K. Home Office.

Viral infection

RSV A2 strain was grown in HEp-2 cells and viral titer measured in focus forming units (FFU) by 96-well focus forming assay. Mice were infected with RSV A2 intranasally with an equivalent dose based on their body mass (4 × 10^4 FFU/g) at 4 d (neonatal ∼10^5) or 4–6 wk of age (immature adults ∼5 × 10^5) under isoflurane anesthesia. Secondary RSV challenge was given intranasally at 8 wk, with 5 × 10^5 FFU in 100 μl. After infection, sickness was monitored by measuring weight daily; we have previously demonstrated that weight loss is a valid disease biomarker that correlates with pulmonary disease (27). Susceptibility was based on a mean weight loss of ≥3.5% at day 5.

Computational genetic mapping

The computational genetic analysis of the inbred strain data was performed as previously described (18, 28). In brief, a haplotype block map of the mouse genome was constructed, and single-nucleotide polymorphisms...
(SNPs) were organized into haplotype blocks that typically consisted of 2–4 haplotypes (29, 30). The haplotype-based computational analysis identified haplotype blocks in which the haplotypic strain grouping within the block correlates with the distribution of phenotypic data among the inbred strains analyzed. The program calculates a \( p \) value that assesses the likelihood that genetic variation within each block could underlie the observed distribution of phenotypes among the inbred strains (18, 30). The haplotype blocks are ranked based on the calculated \( p \) value. The genomic regions within haplotype blocks that strongly correlated with the phenotypic data are then analyzed. The Roche SNP database (http://mousesnp.roche.com) contains 250,837 SNPs generated from 21 inbred mouse strains covering 3346 genes.

**Cell preparation and flow cytometry**

Postinfection, animals were culled using i.p. pentobarbitone. Cells were harvested as described previously (13). To assess airway eosinophilia, bronchoalveolar lavage fluid (100 \( \mu l \)) was centrifuged onto glass slides and stained with H&E. For analysis by flow cytometry, cells were initially blocked with CD16/32. For surface staining, Abs against the surface markers CD4, CD8, the MHC class I haplotype specific Abs H-2Kk, H-2Kd, H-2Dk, H-2Dd, and the MHC class II haplotype specific Abs I-Ak, I-A^d (BD, Bevil’s Hill, U.K.) were added in 1:100 dilution. For intracellular staining, cells were stimulated for 4 h at 37˚C in the presence of 10 \( \mu g/ml \) brefeldin A, 100 \( \mu g/ml \) PMA, and 10 \( \mu g/ml \) ionomycin. Cells were permeabilized with 0.5% saponin and stained with directly conjugated anti-TNF or anti–IFN-\( \gamma \). Samples were run on a Becton Dickinson Life Sciences Research 2 Flow Cytometer and analyzed using CellQuest (BD).

**Statistical analysis**

The results are expressed as mean ± SEM. Because of the group sizes, non-parametric statistical tests were used. For comparisons of more than two groups, a Kruskal–Wallis test was used followed by a Dunn’s post test to compare significance between groups. When two groups were compared, a Mann–Whitney \( U \) test was used. All data were analyzed using Prism software (GraphPad Software, La Jolla, CA).

**FIGURE 2.** SNP haplotype mapping of susceptibility to weight loss after neonatal RSV infection. Strains were ordered by percentage weight loss at day 5 postinfection (A). A representative set of haplotype blocks having the highest correlation with this data set are shown (B). The haplotype for each strain is represented by a colored block. Where the same haplotype occurs, the same color is used and is presented in the same order as the phenotypic data in the top panel. The calculated \( p \) value measures the probability that strain groupings within an individual block would have the same degree of association with the phenotypic data by random chance. For each block, the chromosomal location, number of SNPs within a block, its gene symbol, and an indicator of gene expression in lung are shown.
Results

MHC is a determinant of severity of sequelae to neonatal RSV infection

Ten inbred mouse strains were used to examine the interstrain differences in response to neonatal RSV infection. Mice from each strain were first infected intranasally with RSV A2 at 4 d (neonatal) or 4–6 wk (adult) of age. These mice were reinfeated 8 wk later, and weight loss was measured as a marker of disease severity. There were substantial differences in disease severity among inbred strains (Fig. 1). Neonatally infected BALB/c and DBA/2 mice exhibited substantial weight loss between days 2 and 5 after secondary infection (Fig. 1A, 1B); of note, adult DBA/2 mice were not protected against rechallenge. Neonatal primed NZB (Fig. 1C), C57BL/6 (Fig. 1D), and NZW (Fig. 1E) mice lost a small amount of weight after secondary RSV infection. 129/sv, MRL, AKR, C3H/HeJ, and A/J mice did not lose any weight after

FIGURE 3. Importance of CD8 on disease during secondary infection. Neonatal mice were infected with $4 \times 10^3$ FFU RSV per gram body weight intranasally. Eight weeks later, mice were reinfected with $5 \times 10^5$ RSV. Percentage of lung lymphocytes that were CD8 (white bars) or CD4 positive (black bars) (A), number of airway eosinophils (B), and number of lung cells (C) on day 7 postinfection. C57BL/6 mice were infected with RSV as neonates and challenged 8 wk later with RSV. During primary neonatal RSV infection, mice were treated i.p. with T cell-depleting Abs on days −1, +2, and +5 postinfection. Weight change after secondary RSV challenge (D). Cell number (E), CD4 (F), and CD8 (G) T cells in lungs 7 d after secondary RSV challenge. $n \geq 4$ mice per group ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.
secondary infection (Fig. 1F–J). These interstrain differences demonstrate that disease sequelae after primary neonatal RSV infection is genetically controlled.

The degree of weight lost on day 5 after secondary RSV infection was used as a measure of disease severity, because it was maximal at this time point among the most severely affected strains (Fig. 2A). Haplotype-based computational genetic analysis of this disease severity measure was used to identify genes with a correlated pattern of genetic variation (Fig. 2B). As described (31), the pattern of genetic variation within a number of genomic regions will correlate by chance with the phenotypic data, which can make it difficult to identify correctly the genomic region with the causative genetic difference from among those that are randomly correlated. However, 9 of the 18-haplotype blocks that were most highly correlated \((p < 0.0033, \text{ genetic effect size } >0.8)\) with severity were all within a small (2.4 megabase) region \((33.550–35.922)\) on chromosome 17 that was within the MHC.

We previously demonstrated that CD8 (but not CD4) depletion during the primary neonatal RSV infection prevents the development of disease during adult challenge (13). Because MHC affects the T cell immune responses, we examined T cell recruitment into the lungs of different strains after secondary adult RSV infection of neonatally sensitized mice. We found that genetically susceptible strains had more CD8 cells in their lungs (mean \(30.5 \pm 2\%\)) than that in resistant mice (mean \(19.1 \pm 0.8\%\)) (Fig. 3A; \(p < 0.001\)), whereas there was no significant difference in eosinophil recruitment (Fig. 3B) or total lung cell number (Fig. 3C). To test further the pathogenic role of CD8 cells, we depleted T cell subsets during primary neonatal infection in C57BL/6 (H-2b) mice. Whereas CD4 depletion had no effect, CD8 depletion inhibited weight loss (Fig. 3D), reduced airway cellularity (Fig. 3E), increased the percentage of CD4 T cells (Fig. 3F) and decreased the percentage of CD8 T cells (Fig. 3G) in the lungs during secondary challenge. Therefore, CD8 T cell recruitment into the lung after secondary infection is associated with severe disease. These data indicate that the MHC association with disease may be through an effect of MHC class I alleles on the CD8 T cell response.

Consistent with the computational finding, we observed a strong association between MHC haplotype and disease severity. Strains with H-2d (BALB/c, DBA/2, NZB), H-2b (C57BL/6), or H-2z (NZW) haplotypes were susceptible, whereas those with H-2129 (129/sv), H-2k (C3H, AKR, MRL), or H-2a (A/J) haplotypes were resistant. To test this experimentally, we used congenic mice with different MHC loci on the BALB/c genetic background. BALB.K (H-2k) mice were resistant, whereas BALB.B (H-2b) and BALB/c (H-2d) mice lost significantly more weight on days 3–6 after re-infection (Fig. 4A; \(p < 0.05\)). The susceptible strains had a greater CD8 (Fig. 4B) and eosinophilic (Fig. 4C) cell infiltrate than the resistant BALB.K strain. The MHC association was also observed in B10 congenic strains, but the magnitude of the effect was diminished compared with the BALB background congenics. B10.A (H-2a) mice lost no weight, B10.BR (H-2k) lost 2.8% body weight, and B10.D2 (H-2d) lost 3.8% on day 5 after secondary infection (Fig. 4D). The B10.D2 strain had significantly more CD8 T cells (Fig. 4E; \(p < 0.05\)) and eosinophils (Fig. 4F; \(p < 0.05\)) than the other two strains.

Additional genetic studies were then performed to analyze further the genetics of this response. Female BALB/c mice (sus-
ceptible, H-2d) were crossed with male C3H/HeN mice (resistant, H-2k) to produce F1 mice that were infected as neonates or adults and then reinfeected as adults. C3H/HeN mice were used because they do not have the missense (ProHis712) mutation within the third exon of the Tlr4 gene, which causes defective TLR4 signaling in C3H/HeJ mice (32). All neonatally infected F1 mice lost a significant amount of weight after reinfection (Fig. 5A), and this was substantially more than in adult-infected mice ($p < 0.01$). No significant differences in CD8 T cell responses were observed in the lung on day 7 postinfection (Fig. 5B).

F2 progeny were also produced and infected as neonates and reinfeated as adults (Fig. 5C). Using the same criteria as in our initial studies, susceptibility was defined as a weight loss $\geq 3.5\%$ on day 5; 17% (12 of 70) of the F2 mice were resistant, and 83% (58 of 70) F2 mice were susceptible. The MHC alleles in the F2 mice could not explain the pattern of susceptibility/resistance in these progeny (Fig. 5E), nor was there an association with coat color or sex (data not shown). However, the MHC alleles in F2 mice did affect CD8 cell infiltration into the lung; there were significantly more CD8 cells in mice with one or more H-2d alleles (H-2k/d or H-2d) than in H-2k/k mice (Fig. 5D; $p < 0.01$).

The F1 mice were back-crossed with C3H/HeN mice (Fig. 5F); and 72% (21 of 29) of the neonatally infected BC1 mice were resistant, whereas 28% (8 of 29) were susceptible to reinfection. However, the peak weight loss in BC1 mice was smaller than in the F1 or F2 mice. Similar to the results in the F2 mice, there was no correlation between MHC haplotype and susceptibility in the BC1 mice (data not shown). When the BC1 mice were grouped by MHC haplotype, there were significantly more CD8 cells in the H-2k/d mice than in the H-2k/k mice (Fig. 5G; $p < 0.01$). The analyses of intercross progeny confirm that the MHC haplotype plays a role in susceptibility to the delayed effects of RSV infection but also indicates that other genetic regions contribute.

Because the MHC haplotype was not the sole determinant, we investigated whether other aspects of the immune response could affect disease severity after reinfection. Because the TNF gene is

![Figure 5](http://www.jimmunol.org/)

**Figure 5.** The effect of MHC haplotype on susceptibility to neonatal RSV priming. Mice were intranasally infected as neonates or as immature adults with $4 \times 10^5$ FFU RSV per gram body weight. Eight weeks later, mice were reinfeected with $5 \times 10^5$ FFU RSV. Weight change after adult rechallenge of neonatally or adult primed F1 (A), neonatally primed F2 (C), and neonatally primed back-cross (F1×C3H; F) generations. Lung CD8 T cells on day 7 postinfection in F1 (B), F2 (D), and back-cross (F1×C3H; G). F2 and back-cross are grouped by MHC haplotype. Day 5 weight loss of each individual mouse, indicating the MHC haplotype in the F2 challenge experiment (E). Bars/points represent $n = 4$ mice per group $\pm$ SEM. $sp < 0.05$; $***sp < 0.01$; $***sp < 0.001$. 
colocated within the MHC cluster, we investigated whether there were differences in the production of proinflammatory cytokines or infiltrating cells between resistant (C3H/HeN) and susceptible (BALB/c) strains after primary RSV infection in neonatal mice. On day 7 postinfection, there was no difference in the proportions of CD4 and CD8 T cells (Fig. 6A) between strains, but there were more IFN-γ-producing CD8 T cells (Fig. 6B) and significantly more TNF-producing CD4 T cells ($p < 0.05$; Fig. 6C) in the lungs of C3H/HeN mice. We hypothesized that the increased TNF seen was protective against the delayed effects. To test this, TNF was depleted during primary neonatal infection of C3H/HeN mice by Ab and the mice compared with control treated mice. Mice were reinjected as adults; there was no significant difference in weight loss after secondary infection between TNF-depleted and control mice (Fig. 6D).

**Discussion**

These studies demonstrate that host genetics has a significant effect on susceptibility to the delayed sequelae after neonatal RSV infection. Specifically, we show an association between MHC haplotype and disease severity after neonatal RSV infection and provide data indicating that the MHC effect is likely to be mediated by its action on CD8 T cells. We and others have previously shown that the CD8 response is critical for driving the pathological immune response to RSV (13, 33) and that CD8-driven immunopathology is associated with a response to a single immunodominant epitope in an RSV protein (M2-1 82–90) (34–37). Our genetic and experimental data indicate that the MHC and CD8 T cells play a significant role in determining disease severity after secondary RSV infection in adults.

Previous studies exploring the delayed effects of neonatal RSV infection have used predominately BALB/c mice, but our current studies demonstrate that other strains of mice are also susceptible to the same effect to various degrees. This is important because the use of BALB/c mice suggested that a Th2 imbalance might be of importance in determining susceptibility, but C57BL/6 have a Th1 skewed response to infection and are also susceptible. Alongside the lack of relation between eosinophils and disease observed in the current study and our recent study that showed that IL-4 does not enhance disease (16), our current view is that delayed sequelae of neonatal infections are not caused by Th2 bias in early life. This contrasts with that observed in vaccine-induced immunopathology, which is also pathogenically distinct from enhanced RSV disease after priming with the RSV G protein expressed by recombinant vaccinia viruses (38). The model we now describe here may be a better choice for screening live vaccine candidates for immunopathology after early life exposure.

As observed from the genetic cross studies, the inheritance of susceptibility is complex and not solely determined by MHC. One possibility is that certain MHC genes play a stronger role than others. For example, the resistant A/J and B10.A mice are I-Kk, I-Ak, I-Ek, but I-Dk. There were also differences within the same MHC haplotype—DBA/2 adult mice were not protected against RSV rechallenge unlike BALB/c mice. A second possibility is that susceptibility/resistance is determined by the TNF gene, which lies within the MHC cluster. We (39) and others (40) have demonstrated that TNF is a key mediator in disease after RSV infection. But TNF was not identified as a positive association in the haplotype map. Furthermore, differences in TNF expression have been observed between congenic mice on both BALB and B10 backgrounds (41, 42), but these do not correlate with susceptibility; H-2k mice produce more TNF than H-2b mice but were resistant to rechallenge disease, and B10 mice produce more TNF than BALB mice but had reduced disease. The alternative possibility is that TNF protects against disease sequelae; T cells from C3H/HeN mice produced more TNF after neonatal infection, but depleting TNF during neonatal infection did not induce disease susceptibility (Fig. 6D).

Human CD8 epitopes have been identified in adults (43) and children (44) including epitopes in the M2 protein (45). It is therefore possible that individuals carrying HLA types that recognize the immunopathogenic CD8 epitopes in RSV are more prone to severe RSV or postbronchiolitic wheeze. Associations between MHC haplotype and disease have been observed in a number of different infections (46). Although a previous study did not observe any link between HLA type and RSV disease severity (47), we hypothesize that this might be due to the complexity of the genetic control observed here; the timing of infection and at least one other (non-MHC) factor affects severity. Nevertheless, the current study demonstrates the value of mouse genetic studies for examining complex biomedical responses; multiple contributing factors and candidate genes and pathways can be identified. This study clearly demonstrates that sequelae after neonatal RSV infection has a genetic component and that the MHC haplotype is an important, but not the only, contributor to this response. Understanding this may allow us to develop vaccination strat-

**FIGURE 6.** Role of proinflammatory cytokines in susceptibility to delayed effects of neonatal RSV priming. Neonatal C3H and BALB/c mice were infected with $4 \times 10^3$ FFU RSV per gram body weight. Percentage CD4 and CD8 cells (A) IFN-γ+ CD8+ T cells (B), and TNF+ CD4 T cells (C) in lungs on day 7 postinfection. Neonatal C3H mice were treated with anti-TNF (○) or control (●) during primary RSV infection. Eight weeks later, mice were challenged with RSV and weight challenge postinfection measured (D). Bars represent the mean of more than four mice per group ± SEM. ***$p < 0.001$.**
egies that target the “good” epitopes without inducing immune responses to the immunopathogenic ones.

Acknowledgments

We thank Ita Askonas and Tracy Huss for advice and encouragement and Steve Cobbold (Oxford University) for depleting Abs.

Disclosures

The authors have no financial conflicts of interest.

References


