Genetic Susceptibility to the Delayed Sequelae of neonatal RSV Infection is MHC-Dependent


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Running Title: MHC determines post RSV sequelae
Abstract

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 make 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 inter-strain differences in severity identified the major histocompatibility complex (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 F1, F2 and backcross progeny produced by intercrossing resistant (H-2\(^k\), C3H/HeN) and sensitive (H-2\(^b\), 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.
Introduction

Respiratory syncytial virus (RSV) is the most significant cause of infant hospitalization due to bronchiolitis (1). There is a strong association between an episode of RSV-induced hospitalization in childhood and the development of wheeze in later life (2). Whilst the majority of children will be infected with RSV during infancy, only a small proportion (2%) will require hospitalization (3). Several candidate gene-based studies have demonstrated that the inter-individual differences in outcome after RSV infection are associated with the genetic background of the infected child (4,5). Alleles that affect the quality of the immune response (e.g. the IL-4 -589T allele – which increases transcription factor binding (6), the IL-8 -251A allele – which increases IL-8 expression (7) and the IL-13 -1112T allele – which transcription factor binding affinity (8)) or the anti-viral response (e.g. the TLR4 Asp299Gly polymorphism – which reduces TLR4 translocation (9), the CD14 -155C allele – which increases levels of soluble CD14 (10) and the IL-6 -174C allele – which decreases IL-6 expression (9)) have been implicated in susceptibility. However, the use of an un-biased, genome-wide association study could uncover other, cryptic, genetic factors.

If we could identify genetic factors affecting respiratory disease severity due to RSV infection, this could lead to improved methods for treatment or prevention of respiratory disease. The genetic interactions between host, virus and environment that contribute to post-bronchiolitic wheeze are likely to be complex (11), which makes it very difficult to uncover the genetic architecture through analysis of human cohorts. We previously developed a murine model to study the delayed consequences of neonatal RSV infection (12). Using this model, we and others have demonstrated that
CD8 cells (13), IL-13 (14), IgE (15) and IFN-γ (16,17) are all important determinants of disease severity after RSV infection.

Murine genetic models of human disease have been repeatedly used to unravel the genetic architecture of complex human diseases (18,19). In fact analysis of mouse genetic models has generated novel approaches for treatment of narcotic drug addiction (20) and for prevention of acetaminophen-induced liver toxicity (21). Differences in response to RSV have been observed among inbred mouse strains, including differences in viral load (22-24) after infection, and in eosinophilic responses after vaccination with the RSV glycoprotein (25,26). Therefore, we examined the effect of neonatal RSV infection on the outcome after re-infection in 10 inbred strains of mice. Haplotype-based genetic analysis indicated that these inter-strain differences in disease severity after re-infection correlated with MHC haplotype, and this was experimentally confirmed using congenic mice. However, subsequent genetic analysis revealed that other genetic loci also modulate the impact of the MHC. Thus, we were able to identify genetic factors of importance in a complex multifactorial disease.
Materials and Methods

Mice. Time mated pregnant mice were obtained at <14 days gestation (Harlan, UK), and the pups were weaned at 3 weeks old. The following strains were used in this study BALB/cOlaHsd, BALB.B, BALB.K, B10.A, B10.BR, B10.D2, A/JOlaHsd, 129S2/SvHsd, DBA/2JRccHsd, C57BL/6JRccHsd, NZW/OlaHsd, NZB/OlaHsd, AKR/OlaHsd, C3H/HeJ, MRL/MpOlaHsd. For the cross study, two lots of 2 BALB/c female mice were paired with 1 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 50μl of 1mg/ml antibody intraperitoneally on d-1, d+2 and d+5 post infection, 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 antibodies were a kind gift of S. Cobbold, Oxford University). For TNF depletion, neonatal C3H/HeN mice were treated with 50μl 1 mg/ml monoclonal rat anti-TNF i.p. (IgG1; clone XT22) on day 0, 2, 4 and 6 post infection. All work was approved and licensed by the UK Home Office.

Viral Infection. RSV A2 strain was grown in HEp-2 cells and viral titer in Plaque Forming Units (PFU) determined by plaque assay. Mice were infected intranasally with an equivalent dose based on their body mass (4x10^4 PFU/g) RSV A2 at 4 days (neonatal ~ 10^5) or 4-6 weeks of age (immature adults ~ 5 x 10^5) under
isoﬂurane anesthesia. Secondary RSV challenge was given intranasally at 8wk, with 5x10^5 PFU in 100µl. Following infection, sickness was monitored by measuring weight daily; we have previously demonstrated that weight loss is valid disease biomarker that correlated 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 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.** After infection, animals were culled using intraperitoneal pentobarbitone. Cells were harvested as described previously (13). To assess airway eosinophilia, bronchoalveolar lavage fluid (100 µl) was centrifuged onto glass slides and stained with haematoxylin and eosin (H&E).
For analysis by flow cytometry cells were initially blocked with CD16/32. For surface staining antibodies against the surface markers CD4, CD8, the MHCI haplotype specific antibodies H-2k\(^k\), H-2k\(^d\), H-2d\(^k\), H-2d\(^d\) and the MHCII haplotype specific antibodies I-A\(^k\), I-A\(^d\) (BD, Bevil’s Hill, UK) were added in 1:100 dilution. For intracellular staining, cells were stimulated for 4h at 37°C in the presence of 10 μg/ml Brefeldin A, 100μg/ml PMA and 10μg/ml ionomycin. Cells were permeabilised with 0.5% saponin and stained with directly conjugated anti-TNF or anti-IFNγ. Samples were run on an LSR (BD) and analyzed using CellQuest (BD).

**Statistical analysis.** The results are expressed as mean ± S.E.M. Due to the group sizes non-parametric statistical tests were used. For comparisons of more than 2 groups a Kruskal-Wallis test was used followed by a Dunn’s post test to compare significance between groups. When 2 groups were compared a Mann Whitney test was used. All data was analyzed using GraphPad Prism software.
Results

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

Ten inbred mouse strains were used to examine the inter-strain differences in response to neonatal RSV infection. Mice from each strain were first infected intranasally with RSV A2 at 4 days (neonatal) or 4-6 weeks (adult) of age. These mice were re-infected 8 weeks 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,b), of note adult DBA/2 mice were not protected against rechallenge. Neonatally 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, A/J mice did not lose any weight after secondary infection (Fig.1f,g,h,i,j). These inter-strain differences demonstrate that disease sequelae following 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, since 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 correctly identify the genomic region with the causative genetic difference from amongst those that are randomly correlated. However, 9 of
the 18-haplotype blocks that were most highly correlated (P-value <0.0033, genetic effect size >0.8) with severity were all within a small (2.4 MB) 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). Since MHC affects the T cell immune responses, we examined T cell recruitment into the lungs of different strains following secondary adult RSV infection of neonatally sensitized mice. We found that genetically susceptible strains had more CD8 cells in their lungs (mean 30.5% ± 2) than resistant mice (mean 19.1% ± 0.8) (Fig.3a, P<0.001), while there was no significant difference in eosinophil recruitment (Fig.3b) or total lung cell number (Fig.3c). To further test the pathogenic role of CD8 cells, we depleted T cell subsets during primary neonatal infection in C57BL/6 (H-2b) mice. While CD4 depletion had no effect, CD8 depletion inhibited weight loss (Fig.3c), reduced airway cellularity (Fig.3d), increased the percentage of CD4 T cells (Fig.3e) and decreased the percentage of CD8 T cells (Fig.3f) 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) were susceptible, while those with H-2^{129} (129/sv), H-2^k (C3H, AKR, MRL) or H-2a (A/J) haplotypes were resistant. To experimentally test this, we used congenic mice with different MHC loci on the BALB/c genetic background. BALB.K (H-2^k) mice were resistant; while BALB.B
(H-2b) and BALB/c (H-2d) mice lost significantly more weight on days 3-6 post 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 to the BALB background congenics. B10.A (H-2a) mice lost no weight, B10.BR (H-2b) lost 2.8% body weight and B10.D2 (H-2d) lost 3.8% on day 5 following 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 further analyze the genetics of this response. Female BALB/c mice (susceptible, 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 re-infected as adults. C3H/HeN mice were used because they do not have the missense (ProHis712) mutation within the 3rd 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 re-infection (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 d7 post infection (Fig.5b).

F2 progeny were also produced, and infected as neonates and re-infected as adults (Fig.5c). Using the same criteria as in our initial studies, susceptibility was defined as a weight loss ≥ 3.5% on day 5, 17% (12/70) of the F2 mice were resistant, and 83% (58/70) 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-2<sup>d</sup> alleles (H-2<sup>k/d</sup> or H-2<sup>d</sup>) than in H-2<sup>k/k</sup> mice (Fig. 5d, p<0.01).

The F1 mice were back-crossed with C3H/HeN mice (Fig. 5f); and 72% (21/29) of the neonatally infected BC1 mice were resistant, while 28% (8/29) were susceptible to re-infection. 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 depicted). When the BC1 mice were grouped by MHC haplotype there were significantly more CD8 cells in the H-2<sup>k/d</sup> mice than the H-2<sup>k/k</sup> (Fig. 5g, P<0.01). The analyses of inter-cross 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.

Since the MHC haplotype was not the sole determinant, we investigated whether other aspects of the immune response could affect disease severity after re-infection. Because the TNF gene is co-located within the MHC cluster, we investigated whether there were differences in the production of pro-inflammatory cytokines or infiltrating cells between resistant (C3H/HeN) and susceptible (BALB/c) strains after primary RSV infection in neonatal mice. On day 7 after infection there was no difference in the proportions of CD4 and CD8 T cells (Fig. 6a) between strains but there were more IFNγ 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 antibody compared to control treated mice. Mice were re-infected as adults, there was...
no significant difference in weight loss following 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 indicates that the MHC and CD8 T cells play a significant role in the determining disease severity after secondary RSV infection in adults.

Previous studies exploring the delayed effects of neonatal RSV infection have used predominantly 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 to 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 following 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-K^k, I-A^k, I-E^k but I-D^d. There were also differences within the same MHC haplotype – DBA/2 adult mice were not protected against RSV rechallenge unlike BALB/c. 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 following 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-2^k mice produce more TNF than H-2^b mice but were resistant to re-challenge 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 following 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 post-bronchiolitic wheeze. Associations between MHC haplotype and disease have been observed in a number of different infections (46). Whilst 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 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 strategies that target the ‘good’ epitopes without inducing immune responses to the immunopathogenic ones.
Acknowledgements.

We thank Ita Askonas and Tracy Hussell for advice and encouragement and Steve Cobbold (Univ. of Oxford) for depleting antibodies. The authors have no conflicting financial interests.
Reference List


identifies Bhmt2 as a diet-dependent genetic factor protecting against acetaminophen-induced liver toxicity. *Genome Res.*


Footnotes

1 This work was funded by programme grant number 071381/Z/03/Z from the Wellcome Trust, UK. G.P. was partially supported by a grant (1 R01 GM068885-01A1) from the NIGMS. This work was supported by the MRC & Asthma UK Centre in Allergic Mechanisms of Asthma.

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4 Abbreviations: RSV, Respiratory Syncytial Virus.
**Figure Legends**

**Figure 1. The effect of primary neonatal RSV infection on secondary infection in different strains of mice.** Mice were infected with $4 \times 10^4$ PFU RSV per gram body weight intranasally as neonates (age 4 days; □) or as immature adults (age 4 weeks; ▲). 8 weeks later, mice were reinfected with $5 \times 10^5$ PFU RSV. Weight change after secondary RSV challenge in the following strains: BALB/c (a), DBA/2 (b), NZB (c), C57BL/6 (d), NZW (e), 129/sv (f) MRL (g), AKR (h), C3H/HeJ (i), and A/J (j). Each point represents n>4 mice ± SEM. MHC haplotype of strain is included in each figure.

**Figure 2. Single nucleotide polymorphism (SNP) haplotype mapping of susceptibility to weight loss following neonatal RSV infection.** Strains were ordered by percentage weight loss at day 5 post infection (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.
Figure 3. Importance of CD8 on disease during secondary infection.
Neonatal mice were infected with $4 \times 10^4$ PFU RSV per gram body weight intranasally. 8 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 post infection. C57BL/6 Mice were infected with RSV as neonates and challenged 8 weeks later with RSV. During primary neonatal RSV infection, mice were treated intraperitoneally with T cell depleting antibodies on days -1, +2 and +5 after infection. Weight change after secondary RSV challenge (d). Cell number (e), CD4 (f) and CD8 (g) T cells in lungs 7 days after secondary RSV challenge. $n \geq 4$ mice per group ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4. The effect of MHC haplotype on susceptibility to neonatal RSV priming. BALB and B10 congenic mice were intranasally infected as neonates with $4 \times 10^4$ PFU RSV per gram body weight. 8 weeks later, mice were reinfected with $5 \times 10^5$ PFU RSV. Time course of weight change after secondary RSV challenge (a, d). Percentage of lung lymphocytes that were CD8 (white bars) or CD4 positive (black bars) (b, e), Number of airway eosinophils on day 7 post infection (c, f). $n \geq 4$ mice per group ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 5. The effect of MHC haplotype on susceptibility to neonatal RSV priming. Mice were intranasally infected as neonates or as immature adults with $4 \times 10^4$ PFU RSV per gram body weight. 8 weeks later, mice were reinfected with $5 \times 10^5$ PFU RSV. Weight change following adult rechallenge of neonatally or adult primed F1 (a), neonatally primed F2 (c) and neonatally primed Back Cross (F1xC3H;
f) generations. Lung CD8 T cells on d7 post infection in F1 (b), F2 (d) and Back Cross (F1xC3H; g). F2 and backcross 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 ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Figure 6. Role of pro-inflammatory cytokines in susceptibility to delayed effects of neonatal RSV priming. Neonatal C3H and BALB/c mice were infected with 4x10⁴ PFU RSV per gram body weight. Percentage CD4 and CD8 cells (a) IFNγ+ CD8+ T cells (b) and TNF+ CD4 T cells (c) in lungs on d7 post infection. Neonatal C3H mice were treated with anti-TNF (□) or control (▼) during primary RSV infection, 8 weeks later mice were challenged with RSV and weight challenge post infection measured (d). Bars represent the mean of >4 mice per group +/- SEM; * p<0.05.
Figure 1
Figure 2

(b) Table showing P-values, genetic effect, haplotypes, chromosome (Chr), position, number of SNPs (#SNPs), gene symbol, and lung involvement. The table includes columns for P-value, genetic effect, haplotype, chromosome, position, number of SNPs, gene symbol, and lung involvement. The lung involvement column indicates whether the lung was involved (Yes) or not (No) for each sample.
Figure 3
Figure 4
Figure 5
Figure 6