Genetic control of immune cell types in fungal disease

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Edited by Ralph R. Isberg, Tufts University School of Medicine, Boston, MA, and approved November 2, 2010 (received for review September 1, 2010)

Millions of people harbor latent infections of the fungus *Histoplasma capsulatum*. Such persistent infections represent a state-ment between mechanisms of virulence and the immune re-sponse. The differing responses of inbred mouse strains to the same pathogen reflect variation in the genes that control the out-come of infection. Here we show that a 250-fold difference in *H. capsulatum* susceptibility between inbred mouse strains is attributable to the genotype at the MHC H2 locus. Gene expression analysis of strains varying only at the H2 locus identified genotype-specific and genotype-independent expression signatures, including infection-induced genes such as the fungal pattern recognition receptor Clec7a. Surprisingly, B-cell-specific gene expression was negatively correlated with fungal burden, whereas neutrophil-specific genes were correlated with superior disease outcome. Indeed, disease outcome improved when B cells were eliminated and neutrophils were more active, a previously unknown aspect of the host response. These data refine the understanding of genetic influences on histoplasmosis, reveal how shifts in the composition of immune cell populations compel different disease outcomes, and uncover how innate immunity modulation alters histoplasmosis.

Results

Gene Expression Analysis of Congenic Mice. We used microarray expression analyses to identify signatures that correlated informative genotypes with fungal burden. Differential expression profiles of only the parental strains would generate artifacts like allele-specific hybridization where SNPs occurred in the probe sequences. Instead, a circuit analysis that compared parental with H2-swapped congenic mice allowed reference of expression

*Author contributions: J.A.M. designed research; J.A.M. and M.F.F. performed research; M.F.F. contributed new reagents/analytic tools; J.A.M. and J.R. analyzed data; and J.A.M. and J.R. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1012925108/DICSupplemental.
levels against identical sequences. Whole spleen RNA from groups of four uninfected controls or infected animals was harvested at 5 d postinfection. Fungal burden in the lung was determined to ensure infection, although strain-specific trends were already apparent (Fig. S1).

Three interesting groups of differentially expressed genes were identified using statistical analysis of the microarray data (Fig. S2): genes induced by infection in all strains (Dataset S1A), genes differentially expressed in uninfected animals because of the H2 genotype (Dataset S1B), and genes with altered expression during infection in a strain-specific manner (Dataset S1C). We compared all uninfected samples with all infected samples to identify genotype-independent, infection-altered genes. This comparison revealed 196 genes that were up-regulated during infection, many with known immunological roles, including the chemokine receptor Ccr2, which was recently shown to influence murine histoplasmosis (11). Many genes included in this subset are IFN-γ-induced, including Stat1 and genes encoding six guanylate-binding proteins (12).

Five C-type lectins also demonstrated increased expression during infection, including Clec7a (Dectin-1) and Clec4e, pattern-recognition receptors critical to combating other fungal pathogens (13–15). Indeed, a splice variant of Clec7a was identified as a major determinant of Coccidioides immitis infection outcome in mice (15). Conflicting data regarding CLEC7a recognition of H. capsulatum suggests a more complex story than for other pathogens (16, 17). Intriguingly, the C-type lectins cluster together on chromosome 6, a region identified in our trait-mapping experiment. Although less influential than H2, chromosome 6 loci from the A/J strain have been shown to decrease the fungal burden relative to B6 controls (3).

A second, prominent expression pattern was characterized by lower expression in the A strain only. This group includes many B-cell–expressed genes (Dataset S1B and Fig. S3A). A transposon insertion in the Tnfrsf13c gene on chromosome 15 in the A background that reduces B-cell populations accounts for this expression cluster (18). We confirmed a decrease in B-cell number in the A strain using flow cytometry (Fig. S3B). Surprisingly, the A.B strain had normal B-cell gene expression despite an A heritage (Fig. S3A). Genotyping the A.B strain revealed an intact Tnfrsf13c gene (Fig. S3D). The lack of transposon insertion in the A.B strain likely derives from the mixed A and A/Lilly heritage of this recombinant: Not all A strains carry the Tnfrsf13c mutation.

Similarly, although the A/J and A strains are comparable, A/J mice have an intact Tnfrsf13c gene. To evaluate concordance between our earlier mapping experiments and the microarray experiments reported herein, we performed validation experiments using A/J and B6 mice. Quantitative RT-PCR validation of the infection-induced gene Ly6i varied as expected (Fig. S4A). In contrast, H2-Ob and H2-C, two genes identified in the B-cell expression cluster, did not vary between A/J and B6 strains (S3C), confirming an A strain–specific B-cell defect. Mutation of Tnfrsf13c could not explain the lower fungal burden, because A/J and A mice behaved similarly despite differing Tnfrsf13c alleles. The significant B-cell depletion indicated that these cells were not instrumental in reducing fungal burden, however.

A third cluster of genes shared high constitutive expression in uninfected resistant A mice and high induction during infection in all strains (Fig. 1B, Fig. S2, and Dataset S1A–C). This group of genes, referred to here as the neutrophil gene expression set, encodes many neutrophil granule proteins and the canonical neutrophil marker Ly6g (Dataset S1B). Neutrophil granule proteins have potent antimicrobial activity, including against H. capsulatum (19), and knockouts of several genes encoding granule contents worsen infections with diverse pathogens (20). Given that all mouse strains tested showed increased expression of this gene set during infection, the high initial expression state in A strain mice was uniquely correlated with improved outcome. Quantitative RT-PCR confirmed differential expression of Elane, Ctsg, and Mpo (Fig. S4B) using the A/J and B6 backgrounds, confirming a neutrophil expression signature independent of the Tnfrsf13c mutation. In addition, the more sensitive qRT-PCR assay showed greater fold changes in the mRNA levels of these genes than were evident in the microarray analysis. We extended the differences in transcript levels to differences in protein levels using an ELISA for MPO (Fig. 1C and D). MPO levels were sig-
significantly higher in the lungs ($P < 0.05, t$ test) and spleen ($P < 0.01, t$ test) of A/J mice. These data imply a correlation among neutrophil differences, reduced histoplasmosis outcome, and $H2$ genotype.

**Enumeration of Cell Types in Different Mouse Strains.** Our data are consistent with two models: (i) individual A neutrophils transcribe granule genes at higher levels, and/or (ii) there are more neutrophils in A mice. A higher neutrophil level in A/J mice compared with B6 mice was previously suggested in uninfected lung by histology (21). We used flow cytometry to quantify the number of neutrophils present in the spleens of uninfected A/J and B6 mice (Fig. 2A, Fig. S5 A–H, and Table 1). Spleens from A/J mice had significantly more neutrophils, with no significant differences in the size (forward scatter) or granularity (side scatter) of cells. The fluorescence intensity of Ly6G-positive cells was also greater in A/J cells, indicating increased levels of neutrophil-specific proteins per cell (Fig. 2B and Table 1). The trends in both neutrophil number and Ly6G level extended to the lung and bone marrow (Fig. 2 B and C, Fig. S5 I–X, and Table 1). Intriguingly, the lymphocyte antigen complex containing the Ly6g gene was significantly linked to fungal burden (3).

Neutrophil population differences were apparent with monoclonal antibodies recognizing two different Ly6G epitopes or the myeloid marker CD11b (Table 1); thus, strain-specific antigen reactivity could not explain the data. Both Ly6G antibodies showed increased fluorescence in A-derived mice. In flow cytometry comparisons using the A strain, changes due to the Tnfrsf13c-/-mutation could not be distinguished from $H2$-related differences. Nonetheless, analysis of the congenic strains by flow cytometry revealed that the numbers of neutrophil cells closely paralleled those of neutrophil-specific transcripts, with the highest levels of both in the A strain (Table 1 and Fig. S6).

**Alteration of B-Cell and Neutrophil Levels.** B cells are critical to limiting secondary histoplasmosis (22), but our data suggest that they have little influence in primary infections. The B6.129S2-Igh6$^{a/}\text{ama}^g$ (Igh6$^{-/-}$; previously $\mu$MT) mouse strain lacks conventional B cells, due to disruption of the IgM gene; however, it manifests an increase in neutrophil migration during infection and is more resistant to *Leishmania* infection than wild type controls (23). Indeed, mutations of CD19 or Btk similarly lower B-cell levels and increase neutrophil migration, suggesting that B cells oppose neutrophil migration (24). To test both B cell and neutrophil roles in histoplasmosis, we infected Igh6$^{-/-}$ mice. B-cell deficiency improved fungal resistance, showing a 3-fold lower fungal burden at 5d postinfection and a 10-fold lower fungal burden at 10d postinfection in Igh6$^{-/-}$ mice compared with controls (Fig. 3A: $P < 0.005$, ANOVA). Thus, in these mice, B cells impaired the immune response to *H. capsulatum*.

Flow cytometry confirmed a drastic reduction of B cells, from 33% of nucleated spleen cells in control mice to 0.2% in Igh6$^{-/-}$ mice (Fig. S3E; $P < 0.0005$, $t$ test). The proportion of neutrophils in uninfected Igh6$^{-/-}$ mice also was significantly increased relative to controls (Table 1, Fig. 3B, and Fig. S6), with the caveat that removing B cells from the spleen altered the ratios of all remaining cells. Importantly, two unrelated mechanisms exerted control over neutrophils: B-cell level and an A/J intrinsic mechanism. In either case, when neutrophil numbers were elevated, the fungal burden was decreased. The A strain combined both mechanisms and consequently had a higher neutrophil level than the A/J strain (Table 1 and Fig. S3F). However, the two strains had a similar fungal burden (Fig. 1A), possibly reflecting either an epistatic relationship or a threshold for the genetic control of fungal burden at this infective dose. The concordance between neutrophils and fungal burden tempted us to assign the A/J intrinsic mechanism to the $H2$ locus. Although the high fungal burden in the A.B strain is clearly $H2$-related, the lower neutrophil level relative to A mice could be due to an intact Tnfrsf13c gene. Overall, we confirmed the dispensability of B cells in the primary response to histoplasmosis and instead uncovered an antagonistic role with respect to immune protection.

**Discussion**

The identification of multiple genes from quantitative trait locus mapping, each with additive phenotypes, is common for disease-susceptibility phenotypes and often thwarts the transition from mapping to understanding mechanisms. Instead of focusing on individual genes, we combined genetically determined disease resistance with genomic methodology to identify immunological components correlated with disease. By applying rigorous statistics in place of hierarchical clustering, we found the gene expression correlates of disease outcome, with no allelic variation to confound the results. This robust method revealed the strain-specific expression signatures of two different immune cell types: B cells and neutrophils. The signatures were evident even when using the mRNA from the mixed-cell-type population of whole spleen. These signatures led to two hypotheses regarding histoplasmosis: (i) Depleting B cells should have little influence on primary histoplasmosis, and (ii) increasing neutrophil levels should decrease the fungal burden. In fact, B cells appeared to hinder the primary immune response to *H. capsulatum*, and their depletion resulted in elevated neutrophil levels. Previous elegant

![Fig. 2.](image-url)
work showed that neutrophils have potent antifungal activities (19); our genetic experiments support a role in lowering fungal burden. As additional gene expression information is cataloged, similar parsing of expression signatures will become a powerful tool for diagnosing cellular phenotypes.

We have shown that the H2 locus powers differences in fungal burden, and that the consomic strains offer a logical path for determining the causal genes. Although confounding a causative relationship between H2 alleles and neutrophil levels, the Tnfrsf13c mutation led us to test the contribution of B cells to histoplasmosis. With additional recombinant mice lacking the Tnfrsf13c insertion, the locus modifying the H2 affect on fungal burden can be determined, the correlation between H2 genotype and neutrophil level tested, and a causal relationship between neutrophils and fungal burden established. In general, the mechanisms for genetic control of immune cell levels can be determined similarly, with important medical implications. For example, the recent identification of a Duffy cytokine receptor allele as the cause of lower circulating neutrophil levels in people of African descent (25) raises the distinct possibility of a parallel mechanism for genetic control of immune cell levels that can be determined similarly, with important medical implications. For example, the recent identification of a Duffy cytokine receptor allele as the cause of lower circulating neutrophil levels in people of African descent (25) raises the distinct possibility of a parallel mechanism for genetic control of immune cell levels that can be determined similarly, with important medical implications.

Materials and Methods

Mice. The mice were obtained from The Jackson Laboratory. Their full names and the abbreviations used here were as follows: A/J, C57BL/6J (B6), A/WSnJ (A), C57BL/10SnJ (B10), B10.A-H2H2-T18* lsSnJ (B10.A), A.B, B10.A-H2H2-T18* lsSnJ (B10.A), B10.A-H2H2-T18* lsSnJ (B10.A), B10.A-H2H2-T18* lsSnJ (B10.A), and B6.129S2-Igh6tm1cgn/J (B6). The experiments were performed with 8- to 12-wk-old female mice, age-matched when possible. Animal experiments were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals and were approved by the University of California Berkeley’s Animal Care and Use Committee.

Mapping. Recombination breakpoints in the congenic strains were mapped using the markers defined on the Mouse Genome Informatics Web site (http://www.informatics.jax.org). H2 swaps had exchanges of fewer than 300 genes, as follows: between the markers D17Mit233 and D17Mit198 for A/B, between D17Mit198 and D17Mit100 for B10.A, between D17Mit100 and D17Mit125 for B10.A(R), and between D17Mit29 and D17Mit176 for B10.A(R). The primers Tnfrsf13cLe2 (CCGT-

Table 1. Statistical analysis of neutrophil and Ly6G fluorescence levels

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tissue</th>
<th>Neutrophils, %</th>
<th>P value, t test</th>
<th>Log Ly6G fluorescence</th>
<th>P value, t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>Spleen</td>
<td>1.44 ± 0.22</td>
<td>&lt;0.05</td>
<td>18.3 ± 3.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>B6</td>
<td>Spleen</td>
<td>0.83 ± 0.32</td>
<td>&lt;0.01</td>
<td>18.3 ± 3.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>A/J</td>
<td>Bone marrow</td>
<td>63.1 ± 2.5</td>
<td>&lt;0.001</td>
<td>228 ± 47</td>
<td>NS</td>
</tr>
<tr>
<td>B6</td>
<td>Bone marrow</td>
<td>45.9 ± 2.3</td>
<td>&lt;0.05</td>
<td>165 ± 7</td>
<td>NS</td>
</tr>
<tr>
<td>A</td>
<td>Spleen</td>
<td>5.28 ± 1.7</td>
<td>&lt;0.05</td>
<td>165 ± 8.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>A.B</td>
<td>Spleen</td>
<td>0.45 ± 0.098</td>
<td>NS</td>
<td>131 ± 28</td>
<td>NS</td>
</tr>
<tr>
<td>B10</td>
<td>Spleen</td>
<td>0.60 ± 0.13</td>
<td>NS</td>
<td>47.5 ± 2.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>B.A</td>
<td>Spleen</td>
<td>0.47 ± 0.12</td>
<td>NS</td>
<td>55.7 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td>B6*</td>
<td>Spleen</td>
<td>2.0 ± 0.03</td>
<td>&lt;0.05</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Cells stained double-positive for CD11b and the Ly6G antibody 1A8; fluorescent antibody level of the 1A8 antibody is shown.

NA, not applicable; NS, not significant. Shown are the average numbers of neutrophils or Ly6G fluorescence level for mouse strain pairs ± SD (n = 3 or 4). Cells stained positive with both the 1A8 and R6-8C5 antibodies against Ly6G were considered neutrophils, except where noted. Fluorescence for the R6-8C5 antibody is shown. The significance of t tests for pairs is indicated. Lung samples show very large differences, but high variance.

Fig. 3. Igh6−/− mice lacking mature B cells had lower fungal burdens and a higher proportion of neutrophils than isogenic controls. (A) The log of fungal burden measured in the spleen at 5 d and 10 d postinfection for Igh6−/− and control (B6) mice. Individual mice (circle) and the average group (bar) are shown. (B) Histogram of the number of neutrophils per 50,000 nucleated spleen cells. The double-positive population after staining with Ly6G (1A8) and CD11b plotted against 1A8 fluorescence. Data from the representative B6 (blue) and Igh6−/− (yellow) mouse closest to the group average are shown (n = 4).
CCTCTCTAGGGGCGG and Trnrsf13cRE1 (CTGGTTCGCGGGGTTGCGG) were used to detect the transposon in Trnrsf13c.

Infections. Infection was done with intranasal inoculation of 8 x 10^3 yeast cells of H. capsulatum strain G217B. Infection and determination of fungal burden were performed as described previously (3). The entire spleen was homogenized, and serial dilations were plated.

Expression Analysis. All mice within an experiment were infected at the same time in biological quadruplicate. Spleens were removed into RNAlater (Qiagen), and the RNA was purified using Qiazol reagent, Qiashredder columns, and the RNeasy Kit (Qiagen). RNA was quantified using an Agilent 2100 BioAnalyzer before hybridization on Affymetrix Mouse Gene 1.0 ST arrays, both at the Gladstone Institute at University of California San Francisco. Probes were grouped and RMA normalized using Expression Console software (Affymetrix). These data and R code are available as Dataset S2. Expression analysis used the R Bioconductor package Limma (26) to identify genes that met statistical (P < 0.05 after adjustment) and fold-change criteria (at least a 2-fold change) for differential expression using the method of Benjamini and Hochberg (27). Overlaps with other published gene sets used the Molecular Signatures Database (28). The expression pattern of several genes were confirmed by TaqMan gene expression analysis (Applied Biosystems) using independent RNA samples from A/J and B6 mice. Applied Biosystems assays for Actb (Mm00447886_m1), and Mpo (Mm00447886_m1), and Lys (Mm00522346_m1) were used.

MPO ELISA. Whole fresh organs were ground in 1.0 mL (spleen) or 1.5 mL (lungs) of lysis buffer (100 mM NaCl, 10 mM Tris (pH 7.6), 5 mM EDTA, and 10% glycerol) plus Complete Protease Inhibitor (Roche). Homogenate was snap-frozen and then thawed, and debris was removed by centrifugation. Protein level was determined by ELSIA (HyCult) using serial dilutions of whole organs (ρ = 5).

Flow Cytometry. Whole fresh spleens were disrupted using the frosted ends of two microscope slides. Lung tissue was digested with Collagenase D (Roche) before disruption using needles. Bone marrow was isolated from a femur using a mortar and pestle. Homogenates were filtered through a 70-μm nylon strainer, and red blood cells were lysed using ammonium chloride. The purification and staining buffer was HBSS plus 0.5% BSA and 1 mM EDTA. Viable cells were counted using a hemocytometer after trypan blue dye exclusion. A total of 1 million viable cells were stained after blocking with CD16/32 antibody. All antibodies were obtained from eBioscience except the Ly6G monoclonal antibody 1A8, which was from BD Biosciences. 7-AAD was added to exclude dead cells. Enumeration was performed at the University of California Berkeley Flow Cytometry Facility using a Beckman-Coulter FC-500 analyzer to count 30,000 cells (bone marrow) or 50,000 cells (spleen and lung). FlowJo software (Tree Star) was used for analysis.

ACKNOWLEDGMENTS. We thank Leonid Teytelman and Kasper Hansen for their help with R, Hector Nolla and Damian Trujillo for their help with protocols and discussion. We also thank Daniel Portnoy and Russell Vance for their critical reading of the manuscript. This work was supported by National Institutes of Health Grant GM31105 (to J.R.), a Howard Hughes Professorship (to J.R.), and a Damon Runyon Cancer Research Fund fellowship (to J.A.M.).