INTRODUCTION

Over the past century, an increase in incidence and prevalence in many autoimmune diseases, such as multiple sclerosis (MS),\(^1\) rheumatoid arthritis,\(^2\) type 1 diabetes\(^3\) and systemic lupus erythematosus,\(^4\) has been documented, and now these diseases impose a very significant public health burden.\(^5\) The etiology of autoimmune disease is highly complex and multifactorial, owing both to increased genetic heterogeneity in human populations and diverse environmental influences. The contribution of the genetic component has been increasingly better defined, with early studies identifying the profound influence of the major histocompatibility complex haplotypes, and linkage studies and the more recent genome-wide association studies (GWAS) identifying hundreds of additional disease-modifying loci.\(^6,7\) In concert with progress in human genetics, appropriate animal models are critical to a mechanistic understanding of complex autoimmune phenotypes. The reverse genetics revolution in the mouse has provided numerous critical insights into gene function, mostly through the use of knockout approaches. However, such ‘wreck-and-check’ approaches yield only limited information applicable to the understanding of the impact of natural genetic variation at the population level. In this regard, classical quantitative trait locus (QTL) mapping studies in inbred mice are more useful, but these have been hampered by the limited genetic diversity of commonly used laboratory mouse strains.\(^8\) To overcome this limitation, the so-called wild-derived inbred mouse strains have been established, such as PWD/PhJ (PWD), belonging to the Mus musculus musculus subspecies. These mice are genetically highly divergent from the standard laboratory strains, thereby more accurately modeling the greater evolutionarily selected genetic diversity seen in human populations.\(^9,10\) Additionally, consomic strains of C57BL/6J (B6) mice carrying chromosomes from PWD (B6.Chr\(^{PWD}\)) have been established,\(^11\) and have been useful in mapping QTL controlling various complex phenotypes.\(^12,13\) We have recently used this approach to begin to map QTL controlling susceptibility to experimental autoimmune encephalomyelitis (EAE), the principal autoimmune animal model of MS.\(^14\)

The consomic model carries predominantly the B6 genome, however, and thus it is also limited by the loss of many genome-wide epistatic interactions and trans-expression quantitative trait loci. Here, using the parental B6 and PWD strains of mice, we assessed the impact of natural genetic variation distinguishing these two strains on basal gene expression in five major immune cell types, as well as the outcomes in an autoimmune disease model and the associated immune responses. We found striking differences in basal immune cell gene expression that were genetically regulated and cell type-specific, and a smaller subset of genes whose expression was regulated in a sex-specific manner. Bioinformatic analyses identified several critical differentially regulated cellular pathways and processes, and predicted a dampened basal immune response in PWD compared with B6. Accordingly, we found that PWD mice were highly resistant to EAE induction, and exhibited altered encephalitogenic immune responses.

\(^1\)Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA, USA; \(^2\)Department of Medicine, University of Vermont, Burlington, VT, USA; \(^3\)The Jackson Laboratory, Bar Harbor, ME, USA; \(^4\)Department of Microbiology and Molecular Genetics, University of Vermont, Burlington, VT, USA; \(^5\)Center for Nuclear Receptors and Cell Signaling, Department of Biology and Biochemistry, University of Houston, Houston, TX, USA and \(^6\)Department of Pathology, University of Vermont, Burlington, VT, USA. Correspondence: Dr DN Krementsov, Department of Medicine, University of Vermont, D305 Given, 89 Beaumont Avenue, Burlington, VT 05405, USA. E-mail: dikrement@uvm.edu

Received 28 June 2016; revised 15 August 2016; accepted 18 August 2016; published online 22 September 2016
RESULTS

Striking differences in immune cell gene expression exist between B6 and PWD mice.

To understand how natural genetic variation and sex impact gene expression in the immune system, we performed extensive transcriptomic profiling of immune cells from male and female B6 and PWD mice. The following five major immune cell types were isolated from the spleen and lymph nodes of naïve mice. B cells were isolated by positive selection for the surface marker CD19. The remaining cell types were isolated by fluorescence-activated cell sorting as follows: CD8 T cells (CD45+NK1.1−CD19 TCRβ−CD8+), CD4 T cells (CD45+NK1.1+CD19 TCRβ+CD4+CD25−), CD25+ T-regulatory cells (Treg; CD45+NK1.1+CD19 TCRβ+CD4+CD25+); and myeloid antigen-presenting cells (APCs; CD45+NK1.1−CD19 TCRβ−CD11b+CD11c+). RNA was isolated, and genome-wide gene expression was assessed using the Illumina BeadArray platform. Principal component analysis was used to reduce the global expression differences between samples into a limited number of vectors, capturing a total of 64% of the variation in gene expression along three principal components (PCs) (Supplementary Figure 1). Cell type-specific differences were captured primarily by PC no. 2 and to a lesser extent PC no. 3, which gave a clear separation between APC, B cells and the three different T-cell subsets, which clustered closer to each other, as expected. Strain-specific differences were captured primarily by PC no. 3, showing distinct separation between B6 and PWD samples. Sex-specific differences were much more subtle, and were captured partly by PC no. 1, although in most cases male and female samples clustered close together by strain and cell type. CD4 T-cell and Treg samples showed the most sample-to-sample variability, which was also partly captured by PC no. 1.

In the first set of analyses, expression data from males and females were pooled to assess the effect of strain. We found striking differences in gene expression between B6 and PWD. Using a conservative filter of false discovery rate (FDR) < 0.05 and a fold change (FC) > 2, hundreds to thousands of genes were differentially expressed (DE), ranging from −267- to 170-fold difference in expression, with 865 DE genes in CD4 T cells, 1044 in CD8 T cells, 1357 in APCs, 1097 in B cells and 557 in Tregs, representing a total of 2764 unique DE genes in at least one cell type (Figure 1 and Supplementary File 1). A pooled analysis of the five cell types revealed a core subset of 822 DE genes between B6 and PWD across all five cell types. Consistent with this, a survey of the top 20 upregulated (defined as having higher expression in PWD compared with B6) or top 20 downregulated (lower expression in PWD compared with B6) DE genes illustrates that some expression differences occurred uniformly across most cell types, for example, Itgb2 or Actb, whereas others were cell type-specific (Figure 1b). Examples of cell type-specific DE genes include CD4-specific Pdlim4; CD8-specific Klrk1; CD4- and CD8-specific Cd163 and Cdln10; Treg-specific Lcn10; APC-specific Fcer1g, Cd59a and Chi3l3; and B-cell-specific Blk and several Igk genes. Additionally, we noted the lower expression of several genes encoded in the mitochondrial genome in PWD compared with B6. Lastly, many hypothetical and/or uncharacterized genes were abundantly represented among the DE genes.

Figure 1. Genetic control of gene expression in immune cells. (a) Top significantly DE probes between B6 and PWD (for each strain biological replicates, male, n = 3; female n = 3) immune cells exhibiting |FC| > 4 at an FDR < 0.05 in any of the five cell types are shown. FC indicates expression in PWD relative to B6. Mean indicates the FC average across all five cell types, which was used to sort the order of the probes. The |FC| > 4 cutoff was chosen to highlight the significantly DE genes with the largest effect size, and to facilitate visualization of the data. (b) Top 40 DE genes in PWD vs B6 cells, identified by top 20 maximum FC (upregulated) and top 20 minimum negative FC (downregulated) in any cell type. (c and d) Distribution and overlap of DE genes between selected cell types is shown. The number of genes indicates DE genes passing the filter of |FC| > 2 and FDR < 0.05, darker shading indicates a higher number of genes.
(e.g., Figure 1b), suggesting that expression and likely function of these genes is genetically determined. Overall, these results demonstrate that the natural genetic variation distinguishing B6 and PWD mice results in a remarkable level of genetic control over the immune cell transcriptomes.

The PWD immune cell transcriptome predicts a lower basal activation state, lower autoimmune susceptibility and promiscuous cell type-specific gene expression

To predict the overall consequences of the altered transcriptome in PWD immune cells, we undertook a bioinformatic analysis of the DE transcripts across each of the five cell types. Pathway analysis revealed that DE transcripts were enriched in immune-related pathways, as expected, such as dendritic cell maturation, lymphotxin and JAK/STAT signaling, and so on. Strikingly, using expression directionality (i.e. whether the transcripts were up- or downregulated in PWD relative to B6) to predict the directionality of change in impacted pathways, we found almost uniform predicted dampening of the activity of enriched canonical pathways in PWD cells, with the notable exceptions of p53, protein kinase A and death receptor signaling, which were predicted to have increased activity (Figure 2a). Similar results were obtained using upstream regulator analysis, which identified upstream regulation by central immune mediators such as interferon-γ (IFNγ), interleukin-2 (IL-2), nuclear factor-κB and IL-1β, most of which exhibited lower activity in PWD cells (Figure 2b). The minority of upstream regulators that were predicted to have enhanced activity in PWD cells, for example, KLF3, FOXP3 and IL-10RA, tended to be immune regulatory or neutral. Altogether, these results predict a lower basal activation state in PWD immune cells.

To test whether the observed genetic regulation of immune cell transcriptomes had any implications for human autoimmunity, we tested whether GWAS candidate genes for MS susceptibility (MS-GWAS) were enriched within the DE gene sets for each cell type. Significant enrichment of MS-GWAS genes was found only for transcripts that were downregulated in PWD cells relative to B6, but not for those that were upregulated (Figure 2c), suggesting that PWD cells express lower levels of autoimmune susceptibility genes. Interestingly, for the downregulated genes, the level of MS-GWAS enrichment varied across cell type, with APCs, CD8 T cells and Tregs showing the highest level of enrichment. To verify the specificity of this observation, we assessed the enrichment of a GWAS candidate gene set from a related immune-mediated disease, inflammatory bowel disease, which exhibits a significant amount of genetic overlap with MS, and a second set of candidate genes for a non-immune-mediated neurological disease, autism spectrum disorder (ASD). We observed some cell type-specific significant enrichment of the inflammatory bowel disease gene set, but unlike the case for the MS-GWAS gene set, the enrichment was less pronounced and exhibited no directionality (similar enrichment of genes up- or downregulated in PWD) (Supplementary Figures 2A and B). With regard to the ASD candidate gene set, no significant enrichment was observed, supporting the specificity of our findings with MS and inflammatory bowel disease candidate genes (Supplementary Figure 2C). Additionally, we compared level of enrichment of the DE genes in PWD cells within the set of transcripts that were reported to be upregulated in CD4 T cells isolated from early onset MS patients (clinically isolated syndrome; MS-CIS) relative to healthy controls. As was the case for the MS-GWAS gene set, genes upregulated in PWD compared with B6 showed no significant MS-CIS enrichment, whereas the downregulated genes exhibited robust enrichment (Figure 2d). Here, the most significantly enriched subset was CD4 T cells, which was expected. As a specificity control, we also compared the enrichment of genes differentially expressed in ASD brains relative to healthy control brains. With the exception of the transcript set upregulated in PWD APCs, no significant enrichment was observed (Supplementary Figures 2D and E). Taken together, these results predict a central nervous system (CNS) autoimmunity-resistant phenotype for PWD immune cells, which is driven by differential expression of MS-GWAS and MS-CIS signature genes across different cell types.

To test how the altered gene expression pattern in PWD cells affects cell type-specific genes, we performed a gene set enrichment analysis using the ImmGen database, comparing the expression of DE genes in our data set across multiple immune cell type-specific data sets. We found that DE genes that were upregulated in PWD CD4 T cells tended to have lower expression in T cells, and higher expression in non-T cells, for example, myeloid lineage and stromal cells, whereas the downregulated genes tended to have a more T-cell-like expression signature (Figure 2e, top). The same was true for APCs, where upregulated transcripts in PWD tended to be expressed by non-myeloid/innate immune cells (e.g., T cells), and downregulated transcripts had a myeloid/innate immune-like signature (Figure 2e, bottom).

A global quantitative expression analysis supported these observations, revealing significant differences in lineage-specific gene expression between genes that were upregulated in PWD relative to B6 vs those genes that were downregulated, typically in opposite directions across different cell lineages, for example, higher expression of genes upregulated in PWD CD4 cells by innate immune cell lineages, and higher expression of downregulated in PWD CD4 cells by α-β T-cell lineage genes (Figure 2f). This pattern also held true for other cell types, where downregulated transcripts in PWD cells typically belonged to the corresponding cell type, whereas the upregulated transcripts tended to be expressed by other cell types (data not shown). Additionally, this is supported by some significant enrichment of transcripts upregulated (but not downregulated) in PWD cells within the ASD brain transcript data set (Supplementary Figure 2E). Altogether, these results demonstrate that PWD immune cells exhibit more promiscuous cell type-specific gene expression profiles, upregulating genes that are typically expressed by other cell types at the expense of cell type-specific genes.

Analysis of sex-specific gene expression and sex-by-strain interactions reveals minimal impact of sex on gene expression

The incidence and prevalence of many autoimmune diseases, such as MS, rheumatoid arthritis and systemic lupus erythematosus, exhibit a profound sexual dimorphism, with females being affected 3–10 times more often than males. The reasons for this are unclear, but it is thought that sex hormones and sex chromosomes influence gene expression in immune cells, which gives rise to sexual dimorphism in autoimmunity. To test this idea, and to see how it interacts with genetic control of gene expression, we compared the transcriptomes of immune cells isolated from male and female B6 and PWD mice. We first sought to identify genes that exhibited differential sex-specific expression as a function of strain (sex-by-strain interaction), that is, those genes that exhibited a significantly different male/female (M:F) expression ratio in B6 vs PWD (see Materials and methods section). Surprisingly, even using a relatively relaxed filter of FDR < 0.05, and |FC| > 1.5 (here the FC is in M:F ratio between PWD vs B6), this analysis identified only two unique genes across all five cell types, Xist and Kdm5d (Figure 3), encoded on the X and Y chromosomes, respectively, and well known to exhibit sexually dimorphic expression (SDE). Although these two genes exhibited SDE in both strains (see below), Xist exhibited ~2.3-fold higher M:F ratio in PWD compared with B6, whereas Kdm5d exhibited ~2-fold lower M:F ratio (Figures 3b and c). This pattern held true for these two genes across all five cell types, but did not reach the
Next, we sought to identify transcripts whose expression was sexually dimorphic, independent of genetic background. As genetic background exerted little interaction with SDE (see above), B6 and PWD data were pooled for this analysis. Using a filter of \( |FC| > 1.5 \) and an FDR \( \leq 0.05 \), this analysis identified four genes exhibiting SDE: \( Y \)-encoded \textit{Kdm5d}, \textit{Ifnar}, \textit{Ddx3y} and \textit{Xist} (Table 1), all well known to be expressed in a sexually dimorphic manner. The SDE of these genes was similar across different cell types. The heat map indicates the significance of differences between genes upregulated in PWD (lower panels) and downregulated genes (upper panels) (as a measure of enrichment) across different cell lineages was performed as described in the Materials and methods section. Significance of differences between genes upregulated in PWD relative to B6 versus downregulated is indicated as follows: *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \). ILC, innate-like lymphoid cells; GN, granulocytes; Stem, stem cells; Str, stroma; TC, T cells.

**Figure 2.** Predicted activation state of PWD immune cells. (a) Canonical pathway analysis of differential gene expression between PWD and B6 immune cells. The top significantly enriched canonical pathways (\( P < 0.01 \)) are shown. The heat map indicates the Z-score, indicative of predicted direction of change (orange, upregulated; blue, downregulated). (b) Upstream analysis of differential gene expression between PWD and B6 immune cells. Top predicted activators (genes and proteins only) (\( P < 0.01 \)) are shown. Enrichment analysis of MS-GWAS (c) or MS-CIS (d) genes within the DE transcripts between B6 and PWD was performed as described in the Materials and methods section. Enrichment \( P \)-values (displayed as negative log\( (P) \)) for each cell type are shown. (e) Gene set enrichment analysis of DE genes in CD4 T cells and APCs was performed as described in the Materials and methods section. Top panels show genes upregulated in PWD, and the bottom panels show downregulated genes. (f) Quantitation of cell lineage-specific gene expression (as a measure of enrichment) across different cell lineages was performed as described in the Materials and methods section. Significance of differences between genes upregulated in PWD relative to B6 versus those downregulated is indicated as follows: *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \). ILC, innate-like lymphoid cells; GN, granulocytes; Stem, stem cells; Str, stroma; TC, T cells.
hemoglobin genes Hba-a1 and Hbb-b1. Further lowering the stringency of the filter to \(|FC| > 1.5\) and nominal \(P < 0.01\) identified 16 additional genes: two on the X chromosome (Utx and Alas2), and the rest on autosomes (Supplementary Table 2). Interestingly, most of these genes exhibited SDE only in one cell type, and most tended to have higher expression in males.

PWD mice display resistance to EAE and altered associated immune responses

Our gene expression results above suggested a dampened basal immune activation state in PWD mice compared with B6, as well as lower expression of MS susceptibility genes. This led us to hypothesize that this would result in decreased susceptibility to EAE, the principal autoimmune model of MS. To test this hypothesis, B6 and PWD mice were immunized with mouse spinal cord homogenate in complete Freund’s adjuvant, together with pertussis toxin as an ancillary adjuvant. The primary EAE readout, cumulative disease score, differed significantly by strain independent of sex (two-way analysis of variance, sex, \(F = 1.2,\)

\(P = 0.47\); strain, \(F = 21.3, P = 0.004\); sex × strain interaction, \(F = 0.29, P = 0.72\)); therefore, EAE data for males and females were pooled by strain. Compared with B6, PWD mice were highly resistant to EAE, as illustrated by reduced disease incidence, cumulative disease score and other EAE quantitative trait variables (Figures 4a–f).

We next tested whether the relevant encephalitogenic T-cell responses were affected in PWD mice. EAE and MS are thought to be initiated and driven by CNS autoantigen-reactive CD4 T cells of the Th1 or Th17 phenotype, identified by their signature cytokines, IFN\(\gamma\) and IL-17, respectively.\(^{26}\) Granulocyte–macrophage colony-stimulating factor (GM-CSF) is another cytokine that can be produced by either Th1 or Th17 cells, and its expression correlates with their encephalitogenic potential. In contrast, FoxP3+ Treg cells are immune regulatory in EAE. Therefore, we examined the expression of these three signature cytokines and the frequency of FoxP3+ Treg cells. B6 and PWD mice were immunized with mouse spinal cord homogenate as above, and T-cell responses in the spleen and draining lymph nodes were assessed by flow cytometry and intracellular staining. We found that in the spleen, compared with B6, PWD mice had more GM-CSF+ CD4 T cells, and comparable numbers of IFN\(\gamma\) and IL-17 producers (Figure 5a). Interestingly, PWD CD8 T cells in the spleen produced significantly lower amounts of IFN\(\gamma\), but higher amounts of IL-17 (Figure 5b). In contrast, in the draining lymph nodes, PWD CD4 and CD8 T cells produced much lower amounts of all three cytokines compared with B6 (Figures 5c and d). Treg frequency largely followed the magnitude of the effector T-cell responses, with PWD mice having more FoxP3+ Treg in the spleen, but fewer in the draining lymph nodes (Figure 5e). These findings suggest that PWD mice are capable of mounting a potent T-cell response, but it is weaker in the lymph nodes compared with spleen, where B6 mice exhibit a much more robust T-cell response. The reduced effector T-cell responses in PWD do not appear to be due to an enhanced Treg expansion, as the FoxP3+ Treg frequency is proportional to the effector T-cell responses in both strains.

Genetic control of immune cell transcriptomes

F Bearoff et al

Figure 3. Genes exhibiting significant sex-by-strain interaction across five cell types. (a) Genes exhibiting sex-by-strain interactions were identified as outlined in the Materials and methods section. Genes passing the filter of \(|FC| > 1.5\) and \(FDR < 0.05\) are shown. FC represents the change in male/female ratio between PWD and B6, calculated as \((FC_{PWD\text{Male}} - FC_{PWD\text{Female}}) - (FC_{B6\text{Male}} - FC_{B6\text{Female}})\), see the Materials and methods section. Thus, a positive value is indicative of more male biased expression of a gene in PWD compared with B6. (b and c) Relative expression values of the indicated genes exhibiting significant sex-by-strain interaction in CD8 cells. Relative expression values represent log 2-scaled normalized raw expression values. Error bars indicate standard error of the mean.

Table 1. Genes exhibiting significant SDE

| Gene  | Chr | All CD4 | CD8 | Treg | APC | BC |
|-------|-----|---------|-----|------|-----|----|
| Elf2s3y | Y  | 16  | 14  | 14  | 16  | 21 |
| Kdm5d  | X  | 1.7  | 1.8 | 1.5 | 2.2 |
| Xist   | X  | -4.8  | -6.5 | -6.5 | -7  |
| Ddx3y  | Y  | 3.1 | 2.9 | 1.9 | 2.0 |
| Hbb-b1 | 7  | 1.9 | 2.0 | 1.9 | 2.0 |
| Hba-a1 | 11 | 2.0 | 2.0 | 2.0 | 2.0 |

Abbreviations: APC, antigen-presenting cell; BC, B cells; Chr, chromosome; FC, fold change; FDR, false discovery rate; SDE, sexually dimorphic expression; Treg, T-regulatory cell. FC (MF) is shown for those genes reaching the cutoff filter \(|FC| > 1.5, FDR < 0.05\) for a given cell type. All’ indicates a combined analysis of all five cell types. Direction and strength of FC is also indicated: bold represents higher expression in males; italics represent higher expression in females). An absence of an FC value indicates a failure of a gene to reach the cutoff for a given filter.

Figure 4. PWD mice are resistant to EAE. EAE was induced and evaluated in B6 (female, \(n = 6\); male \(n = 10\)) and PWD (female, \(n = 5\); male \(n = 15\)) mice as described in the Materials and methods section. The following EAE quantitative traits were calculated: (a) incidence, (b) cumulative disease score (CDS), (c) severity index, (d) peak score, (e) days affected and (f) day of onset. Significance of differences in (a) was determined by Fisher’s exact test. Significance of differences in (b–f) was determined by two-tailed Student’s \(t\)-test. Significance of differences between B6 and PWD is indicated using asterisks as follows: \(*P < 0.05, **P < 0.01\) and ***\(P < 0.001\). Error bars indicate standard error of the mean. The data are pooled from two independent experiments, both of which yielded similar results.

---
**DISCUSSION**

Although standard inbred laboratory strains of mice exhibit a low level of genetic diversity, the wild-derived PWD strain is highly divergent compared with the standard B6 strain, similar perhaps to the genetic differences between ethnically distinct human populations such as, for example, Europeans, Africans or Asians. Importantly, our mouse model eliminates environmental factors that have profound influences on gene expression in human populations, and allows for the study of genetic control only. Our results demonstrate that the level of this genetic control over gene expression in immune cells is profound, with thousands of genes differentially expressed between B6 and PWD strains at baseline, some cell-specific and others conserved across different cell types. Of note, some of the genes highly upregulated in PWD cells compared with B6 included anti-viral or interferon-induced genes, such as Mx1 and Mx2, several genes encoding IFITM proteins (interferon-induced transmembrane) and IFIT (interferon-induced proteins with tetratricopeptide repeats) proteins (with the notable exception of Ifi27, which was highly downregulated in PWD), which may reflect a loss of evolutionary pressure exerted by viral infection in the laboratory B6 strain (Figure 1b and Supplementary File. 1). Moreover, laboratory strains of mice, unlike wild-derived mice, carry a non-functional and often poorly expressed alleles of these two genes in B6 compared with PWD cells in our data set.

**Figure 5.** PWD mice display skewed peripheral immune responses. B6 (n = 6) and PWD (n = 9) mice were immunized as in Figure 3. At day 10 postimmunization, cells were isolated from the spleen (a, b and e) and draining lymph nodes (c–e), restimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (except in e), stained for surface markers, followed by fixation and intracellular staining for the indicated cytokines (a–d) or FoxP3 (e), and flow cytometric analysis. Percentages of cytokine-positive cells among the live CD19- TCRβ+CD4+ (a, c and e), or CD19-TCRβ+CD8+ (b and d) populations are shown. Significance of differences was determined by two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test. The data represent one independent experiment.

We next examined the immune response in the relevant target organ for EAE, the CNS. Mice were immunized with mouse spinal cord homogenate as above, and immune cells were isolated from the CNS on D30 post-EAE induction, and analyzed by flow cytometry. Compared with B6, PWD mice had a profound reduction in the infiltration of immune cells into the CNS, as demonstrated by reduced numbers of CD45+ and TCRβ+ cells (Figures 6a and b). In addition to the reduced CNS T-cell numbers in PWD mice, a lower proportion of CD4 T cells in the CNS produced IFNγ and IL-17 (Figure 6c). Collectively, these results suggest that the EAE resistance of PWD mice is associated with altered and/or reduced encephalitogenic T-cell responses in peripheral lymphoid organs, and the inability of these T cells to enter the CNS efficiently.

**Figure 6.** PWD mice display reduced immune responses in the CNS during EAE. B6 (n = 4) and PWD (n = 6) mice were immunized as in Figure 3. At day 30 postimmunization, mononuclear cells were isolated from the CNS by Percoll gradient, and counted and enumerated by flow cytometry. Numbers of cells positive for the indicated markers were calculated by multiplying the total number of isolated mononuclear cells by the percentage of CD45+ cells (a) or by the percentage of CD45+ TCRβ+ cells (b). In (c), mononuclear cells were restimulated with PMA/ionomycin and analyzed by intracellular staining and flow cytometry, as in Figure 4. Percentages of CD45+ TCRβ+CD4+ cells positive for the indicated cytokines are shown. Significance of differences was determined using the Student’s t-test. The data represent one independent experiment.
Several DE genes are encoded by the mitochondrial genome, with lower expression in PWD cells. This may reflect different metabolic states or requirements in the two different strains, suggesting a lower mitochondria-dependent metabolic demand in the naive state of PWD immune cells, which may be important in the conservation of the available resources that may be limited due to scarce food sources in the wild. Notably, the dynamics of metabolic state(s) in immune cells has recently emerged as a critical regulator of immune cell effector function. The DE of mitochondrial genes is also consistent with the idea that the non-recombining nature of the mitochondrial genome is likely to result in highly divergent genome sequence and expression profiles between these two distantly related strains.

The ImmmGen Consortium recently published a large microarray-based study profiling gene expression in two immune cell types, CD4 T cells and granulocytes, across a panel of 39 inbred strains of (male) mice, including several wild-derived strains. The results of this analysis are in line with ours, demonstrating a high level of genetic control over gene expression, and widespread variation in expression across different strains, with the largest differences seen between wild-derived and conventional laboratory strains. Interestingly, many of the most profound DE genes in wild-derived mice in the ImmmGen study match ours, for example, ifitm1, ifitm2, Rf27, Cd163, Klrd1, Anxa3, Cd59a and Chi3l3 (Figure 1b), as well as Tlr1 and Th7 (Supplementary File 1).

Our published work using B6.ChrPwD concisomic strains to map EAE QTL revealed striking sex differences in the genetic control of EAE. Based on these differences, we expected to find large sex differences in immune cell transcriptomes. However, in stark contrast to the marked effect of genetic background on gene expression, we found very few genes exhibiting significant SDE or sex-by-strain interactions, with the majority of these localized on the sex chromosomes. Interestingly, Kdm5d, an ancestral single-copy gene that resides on the short arm of mouse chromosome Y, was the only gene found to exhibit significant strain-by-sex interaction. This highlights Kdm5d as a potential candidate gene responsible for the EAE phenotype in B6. ChrYPwD concisomic mice, which display augmented EAE susceptibility. Collectively, these results are consistent with previous reports of SDE across non-immune tissues, where relatively subtle differences in SDE of autosomal genes have been detected. Taken together, our results suggest that genetic background has a profound influence on gene expression, but its influence on SDE is relatively subtle. This is also in line with the findings from human GWAS, for example, in MS, where no sex-specific autosomal candidate genes have been reported to date, although notably, loci on sex chromosomes have not been included for technical reasons. Given the marked phenotypic differences between the sexes in immunity, these findings are surprising, yet they suggest that sex may exert a relatively minor influence on transcript expression levels in the naive state of immune cells. It is possible that more profound differences in gene expression are observed after immune activation. It is also possible that there is a higher level of sex-specific control at post-transcriptional levels, which is supported by the robust SDE of two Y-linked translation regulator genes, Eif2s3y and Ddx3y, observed in our study (Table 1) and many others. Both of these genes, similar to the other handful of Y-linked ancestral single-copy ubiquitously expressed genes (2–4 other genes in the mouse, including Kdm5d), have been proposed to function as dosage-sensitive regulators of gene expression, translation and protein stability, and as such likely have essential roles in male viability, development and sexual dimorphism in health and disease far beyond male gamete function and sexual differentiation. Moreover, the notion that bigger sex differences can be seen at the protein level is also supported by a recent systems proteomics approach, which revealed that sex is a major factor in determining protein levels of autosomal genes.

The recent explosion in GWAS has identified hundreds of genetic variants associated with complex polygenic diseases, including autoimmune diseases. Integrating these data with expression quantitative trait loci studies (see below) has suggested that many GWAS candidates modify autoimmune susceptibility by driving differential expression in autoimmune disease. Using MS as a prototypical autoimmune disease, we show enrichment of MS-GWAS genes in our DE gene sets, with striking directionality: only those genes that exhibit lower expression in PWD are significantly enriched with MS-GWAS genes. This suggests that the B6-PWD genetic model appropriately models natural genetic variation that is relevant to human autoimmune disease. It also predicts reduced susceptibility of PWD mice to CNS autoimmunity, a hypothesis that is supported by our functional data using the EAE model. It is important to note that most of the candidate MS-GWAS genes are typically identified by imputation analysis using the nearest single-nucleotide polymorphism marker with a significant effect; therefore, it is likely that not all current candidates represent the true MS genes, and improved fine-mapping and candidate gene identification continues to be a work in progress. Nonetheless, the results from our model support the functional importance of at least a majority of the current GWAS candidates included in our analysis. Future studies can include such analyses of our data sets using emerging results from follow-up GWAS and fine-mapping studies, which should prove informative.

Several recent seminal studies in humans have examined the effect of natural genetic variability on gene expression in adaptive and innate immune cells in large cohorts of genetically diverse individuals. Thousands of cell type- and non-cell type-specific expression quantitative trait loci were identified, some of which were restricted to specific ethnic groups, and others that were shared across ethnic groups. Although it is difficult to compare our results with these studies directly, it is clear that natural genetic variation exerts a strong influence on gene expression in immune cells in both humans and mice. In the PWD:B6 mouse comparison, this influence is very robust, as we are able to eliminate variability introduced by environmental influences and heterogeneous genetic backgrounds in the human studies. This also highlights the utility of the mouse model in studying gene-by-environment interactions in a setting where the genetic and environmental factors can be tightly controlled and manipulated, to support or refute cause–effect relationships, which are more challenging to assess in human studies. Such future studies will complement human studies, providing a better mechanistic understanding of the genetic basis of complex diseases and their environmental modulators.

**MATERIALS AND METHODS**

RNA isolation and microarray analysis of RNA expression

For the microarray analysis on basal expression differences in immune cell subsets, three biological replicates for each strain and sex combination were created by pooling cells from three different individual naive 8–10-week-old mice into each biological replicate. Cells were isolated from Liberace/DNase I-digested spleens and combined with total cells from lymph nodes (axillary, brachial and inguinal) for each mouse. B cells were isolated using the EasySep B-cell-positive selection kit and EasySep magnet (STEMCELL Technologies, Vancouver, BC, Canada). The remaining live cells from the flow through were purified by fluorescently activated cell sorting using fluorophore-conjugated antibodies against cell surface markers as follows: CD4 T cells (CD45 NK1.1CD19 TCRβ CD4 CD25), CD8 T cells (CD45 NK1.1CD19 TCRβ CD8), Treg cells (CD45 NK1.1CD19 TCRβ CD4 CD25) and APCs (CD45 NK1.1CD19 TCRβ CD11b CD11c). Antibodies were purchased from BioLegend (San Diego, CA, USA); catalog numbers were as follows: CD45 NK1.1, CD19, CD4, CD25, CD8, CD11b, CD11c, CD45RA 03121, 103112, 108707, 115534, 100531, 102016, 101206, 117319, 110922, respectively. High-quality RNA was isolated using the Qiagen RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and the transcriptomes were analyzed using Illumina BeadArray Technology (Illumina, San Diego, CA, USA), using 45 281 unique probes.
Illuminon BeadArray

Hundred nanograms of RNA was amplified and converted to cRNA using Illumina TotalPrep 96 DNA Amplification Kit (Ambion, Carlsbad, CA, USA). Five hundred nanograms of cRNA was used for hybridization onto the Illumina Whole-Genome Gene Expression Array Hybromat microarray (Illumina MouseWG-6 v2.0 R2 Expression BeadChip; Illumina). A total of 45,281 probes from the microarray were included for analysis. All microarray data were uploaded to Gene Expression Omnibus, under accession number GSE85418.

Statistical analyses of microarray data

Probe-level intensities were calculated using the lumi and limma packages in R specifically for Illumina arrays (http://www.basic.northwestern.edu/publications/lumi/lumi.pdf), including background correction and quantile normalization for each probe set and sample. Summarized intensity data were imported into Partek Genomics Suite, version 6.6 (Copyright 2009; Partek Inc., St. Louis, MO, USA) for multivariate and univariate analyses. Principal component component analysis, using the covariance matrix, was performed to (1) look for outliers samples that would potentially introduce latent variation into the analysis of differential expression across sample groups, and (2) assess sample-based differential expression within and between sample groups. One outlier sample (female B6 Treg) was identified by principal component analysis and excluded from all analyses. Univariate linear modeling of sample groups was performed using analysis of variance as implemented in Partek Genomics Suite. The magnitude of the response (FC calculated using the least-squares means) and the P-value associated with each probe set and binary comparison are calculated, as well as a ‘step-up’, adjusted P-value for the purpose of controlling the FDR.48 In all analyses, the FDR was at least five times larger than the nominal/uncorrected P-value. For downstream analysis, for example, identification of the number of DE genes, pathway analysis and so on, multiple probes probing for the same gene were averaged.

For identification of sex-by-strain interactions, to identify genes where male/female expression ratio was significantly different between B6 and PWD cells, the following comparison was made for each cell type, to calculate FC, P-values and FDR: (PWD Male and PWD Female) minus (B6 Male and B6 Female), which is algebraically equivalent to (PWD Male and B6 Female) minus (B6 Male and PWD Female). FC here represents the FC in male/female ratio between PWD and B6.

Bioinformatic analyses

Pathway analysis was performed using Ingenuity Pathway Analysis (IPA) software. The expression data set for all five cell types was uploaded into IPA and filtered by FC > 2 and FDR < 0.05, then subsequently analyzed using the Core Analysis function in IPA, followed by the Comparison Analysis function to compare across the five cell types, as follows. The Canonical Pathway function was used to identify the top canonical pathways (P < 0.01, Z-score > 0.5) affected by the DE genes between B6 and PWD. The Upstream Analysis function was similarly used to identify top upstream predicted regulators (P < 0.01, Z-score > 2). Top 20 pathways (ranked by Z-score) were shown.

Enrichment of DE genes in the MS-GWAS candidate gene list was performed in IPA software as follows. The current published best list of MS-GWAS candidate genes14 was imported into IPA. The Core Analysis function was used to determine the significance of enrichment of DE genes (up- or downregulated separately) within the MS-GWAS list. The same procedure was carried out on the following data sets: (1) a list of transcripts reported to be upregulated in CD4 T cells from MS-CIS subjects vs controls,20 (2) a GWAS candidate gene set for inflammatory bowel disease,15 (3) a set of candidate genes for ASD,16 (4) a set of genes differentially expressed in ASD brains relative to healthy control brain.17,21

Cell type-specific gene set enrichment analysis was performed using the ImmGen Geneset MyGeneSet function (http://rstats.immgen.org/MyGeneSet). The top 200 (ranked by FC; FDR < 0.05) upregulated genes in PWD (relative to B6) were used in the W Plot function, and then the same procedure was repeated for the top 200 downregulated genes. To generate quantitative comparisons of enrichment, the expression of the top 200 up- and downregulated genes for each of the five cell subsets was analyzed across the ImmGen cell type-specific data set (version 1). A global average was obtained by averaging the expression of all 200 genes for a given ImmGen subtype, and then by determining the average of all of these cell subtypes within a specific lineage/category, for example, monocytes. This average expression thus serves as a quantitative measure of enrichment of gene expression within a particular ImmGen population, and this measure was compared between 200 top genes upregulated in PWD relative to B6 vs 200 top genes downregulated in PWD, for each of the five cell types analyzed in our study. Significance of differences was determined in the GraphPad Prism (GraphPad Software, La Jolla, CA, USA), using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 5%.

Animals and induction and evaluation of EAE

C57BL/6J and PWD/J mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) and bred and housed in the vivarium at the University of Vermont (Burlington, VT, USA). The experimental procedures used in this study were approved by the Animal Care and Use Committee of the University of Vermont.

EAE was induced in male and female B6 and PWD mice as follows. Mice were injected subcutaneously with 0.1 ml of emulsion containing 2.5 mg of mouse spinal cord homogenate in PBS and 50% complete Freund’s adjuvant (Sigma-Aldrich, St Louis, MO, USA) on day 0. Complete Freund’s adjuvant was supplemented with 4 mg ml−1 Mycobacterium tuberculosis H37Rv (Difco, BD Biosciences, Franklin Lakes, NJ, USA). On day 0 and day 2, the mice also received an intraperitoneal injection of 200 ng pertussis toxin (List Biological Laboratories, Campbell, CA, USA) as an ancillary adjuvant. Starting on day 10, mice were scored visually as described previously.49 Briefly, the clinical scores were as follows: 0.5—partially curled tail; 1—full loss of tail tone; 2—loss of tail tone and weakened hind limbs; 3—hind limb paralysis; 4—hind limb paralysis and incontinence; 5—quadriplegia or death. EAE scoring was not performed in a blinded manner, as B6 and PWD mice are visually distinct. EAE quantitative traits were calculated as described previously,49 as follows. The incidence of EAE was recorded as positive for any mouse with clinical signs of EAE for 1 or more consecutive days. Cumulative disease score was calculated as the sum of all daily scores over the course of 30 days. Days affected was calculated as the number of days an animal displayed a clinical score > 0, and day of onset was the day a clinical score > 0 was first observed (not calculated for animals without clinical signs). Severity index (assessed in affected animals only) was generated by averaging the clinical scores for each animal over the number of days that it exhibited clinical symptoms. Peak score represents the maximum daily score.

Flow cytometry

For intracellular cytokine staining ex vivo, mice were immunized for EAE induction as above. Spleen and draining (for the immunization site) lymph nodes (axillary, brachial and inguinal) were harvested on day 10 postimmunization, and cells were stimulated with 5 ng ml−1 of phorbol 12-myristate 13-acetate, 250 ng ml−1 of ionomycin (Sigma-Aldrich) and brefeldin A (Golgi Plug reagent; BD Biosciences) for 4 h. Cells were then stained with the UV-Blue Live/Dead Dye (Dread compatible stain, BD, CA, USA) and then surface stained for the following markers: CD4, CD8 and TCRβ. Cells were then fixed with 1% paraformaldehyde (Sigma-Aldrich), permeabilized with buffer containing 0.2% saponin and stained with anti-IL-17A, anti-IFNγ and anti-CD45R0 (BioLegend, San Diego, CA, USA). For surface marker analysis and FoxP3 staining, unstained isolated cells were stained directly ex vivo with the UV-Blue Live/Dead fluorescent stain and then surface labeled for different combinations of following markers: CD25, CD19, CD4, CD8 and TCRβ (BioLegend) and fixed using the FixFixation/permeabilization buffer (eBioscience, San Diego, CA, USA), followed by intracellular staining for FoxP3. Antibodies used for flow cytometry were directly conjugated to fluorophores and obtained commercially (BioLegend; catalog numbers were as follows: CD19, CD4, CD8, FoxP3, CD45R0, 1082016, 101206, 109222, 506904, 5050813, 505404, respectively). Anti-FoxP3 antibody was purchased from eBioscience (catalog number 12-5773-82). Labeled cells were analyzed using an LSR II cytometer (BD Biosciences). Compensation was calculated using appropriate single-color controls. Data were analyzed using the FlowJo software (Tree Star Inc., Ashland, OR, USA).

CNS-infiltrating mononuclear cell isolation

Animals were perfused with PBS and brains and spinal cords were removed. A single-cell suspension was obtained and passed through a 70 μm cell strainer. Mononuclear cells were obtained by Percoll gradient (37%/70%) centrifugation and collected from the interphase. For intracellular cytokine analysis, cells were washed and stimulated with 5 ng ml−1 of phorbol 12-myristate 13-acetate and 250 ng ml−1 of ionomycin in the presence of brefeldin A (Golgi Plug reagent; BD...
Bioscience) for 4 h. Cells were labeled with the UV-Blue Live/Dead fixable stain (Invitrogen) followed by surface staining (CD45, CD11b, CD4, CD8, TCRγδ and TCRβ). Afterwards, cells were fixed, permeabilized and stained for intracellular IL-17 A, IFNγ and GM-CSF as described above. Alternatively, unstained CNS cells were surface labeled for CD45, CD11b, TCRβ, CD4 and CD8 and then fixed and stained for FoxP3, as above.

General statistical analyses

Statistical analyses not pertaining to microarray data were carried out using the GraphPad Prism software, version 6 (GraphPad Software). Details of the analyses are provided in the figure legends. All statistical tests were two sided, and adjustments for multiple comparisons were made as indicated. All center values represent the mean, and error bars represent the standard error of the mean. P-values < 0.05 were considered significant. Sample sizes for animal experiments were chosen based on previous experience with similar analyses. No randomization was used to assign animals to different treatment groups as no differential treatment was performed between the two different strains or the two sexes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Health Grants NS069628 and NS076200, and National Multiple Sclerosis Society (NMSS) Grants RG 51706A1 and Pilot Project Grant PP2123 (to CT); NMSS Grant RG-1501-03107 (to EBP); postdoctoral fellowship FG1911-A-1 from the NMSS and a UVM FISAR award (to DNM).

REFERENCES

1. Alonso A, Hernán MA. Temporal trends in the incidence of multiple sclerosis: a systematic review. Neurology 2008; 71: 129–135.
2. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet 2016; e-pub ahead of print 3 May 2016; doi:10.1016/S0140-6736(16)30173-8.
3. Maath DM, West NA, Lawrence JM, Mayer-Davis EJ. Epidemiology of type 1 diabetes. Endocrinol Metab Clin North Am 2010; 39: 481–497.
4. Uramoto KM, Michet Jr CJ, Thumboo J, Sunku J, O’Fallon WM, Gabriel SE. Trends in the incidence and mortality of systemic lupus erythematosus, 1950–1992. Arthritis Rheum 1999; 42: 46–50.
5. American Autoimmune Related Diseases Association. The Cost Burden of Autoimmune Disease: The Latest Front in the War on Healthcare Spending. National Coalition of Autoimmune Patient Groups (NCAPG), 2011. Available at: www.aard.org.
6. Hollenbach JA, Oksenberg JR. The immunogenetics of multiple sclerosis: a comprehensive review. J Autoimmun 2015; 64: 13–25.
7. Hussman JP, Beecham AH, Schmidt M, Martin ER, McCauley JL, Vance JM et al. Genetic control of immune cell transcriptomes and TCR γδ and then

et al. 2010; 32: 71–77.
8. Segal BM. Th17 cells in autoimmune demyelinating disease. Semin Immunopathol 2010; 32: 71–77.
9. FAIR BP, Knight JC. Genetics of gene expression in immunity to infection. Curr Opin Immunol 2014; 30: 63–71.
10. Staeihl P, Grob R, Meier E, Sutcliffe JG, Haller O. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. Mol Cell Biol 1988; 8: 4518–4523.
11. Staeihl P, Sutcliffe JG. Identification of a second interferon-regulated murine Mx gene. Mol Cell Biol 1988; 8: 4524–4528.
12. Haller O, Staeihl P, Kochs G. Interferon-induced Mx proteins in antiviral host defense. Biochimie 2007; 89: 812–818.
13. Hivroz C, Sartakis M. Biophysical aspects of T lymphocyte activation at the immune synapse. Front Immunol 2016; 7: 46.
14. Uhlemann R, Gerz K, Boehmerle W, Schwarz T, Nolte C, Freyer D et al. Actin dynamics shape microglia effector functions. Brain Struct Funct 2016; 221: 2717–2734.
15. Huang W, Ghisletti S, Saijo K, Gandhi M, Aouadi M, Tesz GJ et al. Corazon 2 A mediates actin-dependent de-repression of inflammatory response genes. Nature 2011; 470: 414–418.
16. O’Neill LA, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. J Exp Med 2016; 213: 15–23.
17. Buck MD, O’Sullivan D, Pearce EL. T cell metabolism drives immunity. J Exp Med 2015; 212: 1345–1360.
18. Mostafavi S, Ortiz-Lopez A, Bogue MA, Hattori K, Pop C, Koller D et al. Variation and genetic control of gene expression in primary immunocytes across inbred mouse strains. J Immunol 2014; 193: 4485–4496.
19. Shoh YQ, Alfoldi J, Pyntikova T, Brown LG, Graves T, Minx PJ et al. Sequencing the mouse Y chromosome reveals convergent gene acquisition and amplification on both sex chromosomes. Cell 2014; 159: 800–813.
20. Case LK, Wall EH, Dragon JA, Saligram N, Krementsov DN, Moussavi M et al. The Y chromosome as a regulatory element shaping immune cell transcriptomes and susceptibility to autoimmune disease. Genome Res 2013; 23: 1474–1485.
21. Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L et al. Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res 2006; 16: 995–1004.
22. Sawcer S, Herron E, Riddoll D, Spencer CC, Patonov NA, Moutsianas L et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 2011; 476: 214–219.
23. International Multiple Sclerosis Genetics, Calabrese C, Amor S, Sherlock S, Daly MJ et al. Risk alleles for multiple sclerosis identified by a genomewide study. N Engl J Med 2007; 357: 851–862.
24. Bellott DW, Hughes JF, Skatesley H, Brown LG, Pyntikova T, Cho TJ et al. Multiple Y chromosomes retain widely expressed dosage-sensitive regulators. Nature 2014; 508: 494–499.
25. Chick JM, Munger SC, Simecek P, Huttlin EL, Choi K, Gatti DM et al. The consequences of genetic variation on a proteome-wide scale. Nature 2016; 534: 500–505.
26. Raj T, Rothamel K, Mostafavi S, Ye C, Lee MN, Replodge JM et al. Polarization of the autoimmune and neurodegenerative risk alleles in leucocytes. Science 2014; 344: 519–523.
Genomic modulators of gene expression in human neutrophils. Nat Commun 2015; 6: 7545.

46 Andiappan AK, Melchiotti R, Poh TY, Nah M, Puan KJ, Vigano E et al. Genome-wide analysis of the genetic regulation of gene expression in human neutrophils. Nat Commun 2015; 6: 7971.

47 Dimas AS, Deutsch S, Stranger BE, Montgomery SB, Borel C, Attar-Cohen H et al. Common regulatory variation impacts gene expression in a cell type-dependent manner. Science 2009; 325: 1246–1250.

48 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B (Methodological) 1995; 57: 289–300.

49 Kremensov DN, Case LK, Hickey WF, Teuscher C. Exacerbation of autoimmune neuroinflammation by dietary sodium is genetically controlled and sex specific. FASEB J 2015; 29: 3446–3457.

50 Butterfield RJ, Sudweeks JD, Blankenhorn EP, Korngold R, Marini JC, Todd JA et al. New genetic loci that control susceptibility and symptoms of experimental allergic encephalomyelitis in inbred mice. J Immunol 1998; 161: 1860–1867.

Supplementary Information accompanies this paper on Genes and Immunity website (http://www.nature.com/gene)