Predominance of Interferon-Related Responses in the Brain during Murine Malaria, as Identified by Microarray Analysis

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Cerebral malaria (CM) can be a fatal manifestation of Plasmodium falciparum infection. We examined global gene expression patterns during fatal murine CM (FMCM) and noncerebral malaria (NCM) by microarray analysis. There was differential expression of a number of genes, including some not yet characterized in the pathogenesis of FMCM. Some gene induction was observed during Plasmodium berghei infection regardless of the development of CM, and there was a predominance of genes linked to interferon responses, even in NCM. However, upon real-time PCR validation and quantitation, these genes were much more highly expressed in FMCM than in NCM. The observed changes included genes belonging to pathways such as interferon signaling, major histocompatibility complex processing and presentation, apoptosis, and immunomodulatory and antimicrobial processes. We further characterized differentially expressed genes by examining the cellular source of their expression as well as their temporal expression patterns during the course of malaria infection. These data identify a number of novel genes that represent interesting candidates for further investigation in FMCM.

Malaria is a devastating disease affecting developing countries in the tropical and subtropical regions of the world. Cerebral malaria (CM), a severe manifestation of Plasmodium falciparum infection, can lead to neurological complications and death (64). Murine models of malaria are important tools for studying the pathogenesis of malaria, with numerous clinical and histopathological similarities between human and murine malaria having been described (19). Comparisons between fatal murine CM (FMCM) and noncerebral malaria (NCM) provide valuable insights into the pathogenesis of CM.

A number of significant changes in the murine brain that characterize CM have been described, including breakdown of the blood-brain barrier (62), redistribution and activation of glia (31, 32, 34), apoptosis of endothelial cells and astrocytes (46, 47), metabolic changes (44, 48), and distinct patterns of chemokine gene expression (38).

A recent approach to identifying novel changes within the brain during CM is to profile gene expression changes using microarray technology (9, 30). Microarray studies can generate a plethora of data that pinpoint involvement of unknown or poorly characterized genes, stimulating investigation of their biological functions. Some studies have used microarrays to examine global gene expression patterns during murine malaria, defining gene expression profiles that are associated with susceptibility or resistance to murine CM (26, 27), identifying genes in the brain that discriminate between different outcomes of Plasmodium infection (5), or describing trends in gene expression within the brain (55). Studies on the spleen have been performed to find genes that differentiate between cerebral and noncerebral (55) and between lethal and nonlethal (52) forms of murine malaria. Microarrays also have been used to examine Plasmodium infection in humans (14, 41) and monkeys (68). In these studies, the majority of genes identified have well defined roles in such functions as immunity, erythropoiesis, and metabolism.

In the current study we used microarrays to examine global gene expression patterns in the brains of Plasmodium berghei-infected mice. In contrast to previous studies, which examined the response of genetically divergent murine hosts to the parasite that causes FMCM, P. berghei ANKA (PbA), we investigated the response of genetically identical murine hosts to different, but related, parasite strains, PbA and P. berghei K173 (PbK), the latter being one that results in NCM. Furthermore, we used two different microarray technologies, oligonucleotide and Affymetrix arrays, and compared the outcomes. Using either technique, we found similar patterns, though different degrees, of gene expression in groups irrespective of progression to CM. These included genes belonging to interferon (IFN) signaling pathways and major histocompatibility complex (MHC) processing and presentation, as well as those associated with apoptotic, immunomodulatory, and antimicrobial processes. However, we found that very strong induction of these genes was associated with fatal outcome due to FMCM, as confirmed by real-time PCR. These results contribute to our understanding of the processes occurring in the brain during CM, and the roles of the genes identified deserve further attention in FMCM and other neurological diseases.

MATERIALS AND METHODS

Murine models of malaria. Female CBA/T6 mice (6 to 8 weeks old) were housed in the Blackburn Animal House, University of Sydney, and given food and water ad libitum. Intraperitoneal inoculation of CBA/T6 mice with $1 \times 10^6$
PhB-parasitized red blood cells (PRBCs) (courtesy of G. Grau, University of Sydney, Australia) is a well-described model of FMC, in which mice display neurological symptoms by day 6 to 7 postinoculation (p.i.). On the other hand, mice inoculated with PhK PRBCs (courtesy of I. Clark, Australian National University, Australia) do not show these neurological signs but instead succumb to acute anemia and hyperparasitemia at 2 to 3 weeks p.i. (40). Control mice were uninfected. All procedures involving the use of animals were approved by, and carried out according to the regulations of, the Animal Ethics Committee, University of Sydney.

**Tissue extraction and histopathology.** Mice were anesthetized by isoflurane inhalation and euthanized at each stated time point. Mice were perfused with 10 ml cold sterile phosphate-buffered saline to clear the vessels of blood, and organs were removed. Formalin-fixed, paraffin-embedded sections (7 μm) were used to examine histopathological features, as described previously (47).

**RNA extraction.** Brain tissue from animals was collected in 1 ml TRIzol reagent (Invitrogen, Victoria, Australia) and immediately homogenized using Zirconia beads (Biospec Products, OK) and a Fast Prep homogenizer (O-Bio- gene, CA). Total RNA was isolated according to the manufacturer’s instructions (Invitrogen). The integrity of the RNA samples was tested using Bioanalyzer Nano chips (Agilent Technologies) according to the manufacturer’s instructions. Samples were DNase treated using a DNA-free kit (Ambion, TX). The same RNA samples were hybridized to the oligonucleotide and Affymetrix arrays as were used in the real-time PCR analyses.

**Experimental design of microarray studies.** All experiments were designed to be compliant with Minimum Information About a Microarray Experiment (MIAME) standards. For oligonucleotide array experiments, control samples were pooled and used as a common reference for the test samples. Experimental groups of five mice were either inoculated with PhB or sacrificed for sample preparation at day 6 p.i. [PhB(6)] or inoculated with PhK and sacrificed for sample preparation at day 6 p.i. [PhK(6)] or day 14 p.i. [PhK(14)]. Five individual test samples were used per group and directly compared to the pooled (n = 5) control reference sample. For Affymetrix array experiments, biological replicates (n = 5) from all groups [control, PhB(6), PhK(6), and PhK(14)] were pooled and hybridized to an individual slide. These were compared with the pooled control samples for downstream computational analysis.

**Oligonucleotide arrays: cDNA labeling, hybridization, scanning, and normalization.** For oligonucleotide arrays, indirect labeling was used to incorporate cyanine (Cy3 and Cy5) dyes into cDNA samples. Labeled probes were then applied to 22.5K Compugen mouse oligonucleotide arrays (~22,500 genes) (Ramaciotti Centre, University of New South Wales). Slides were washed and then scanned, and spot intensities were determined using an Axon scanner (GenePix 3.0; Molecular Devices, CA). Basic normalization procedures for oligonucleotide arrays were carried out in GenePix Pro software, using background subtraction to remove data generated from noise and removal of poor-quality spots due to hybridization artifacts or morphology. Data were then imported into Limma (R, Bioconductor project; http://www.biocoductor.org/) (12) for loess normalization. For full experimental details, including raw data sets, refer to the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/index.cgi), accession number GSE9088.

Prior to normalization, the majority of the arrays revealed systemic biases (i.e., more spots with negative M values), as well as low-intensity spots (i.e., spots with low A values), which can affect ratios. Some arrays also showed fanning effects (see Fig. S1a in the supplemental material), while box plots were used to view the spread of M values (see Fig. S1c in the supplemental material). Many arrays showed variations across the M values, particularly across each print-tip group. These systematic biases suggested that local and not global methods of normalization are more appropriate in this situation. From the raw data, local and not global methods of normalization were applied to 22.5K Compugen mouse oligonucleotide arrays (~22,500 genes) (Ramaciotti Centre, University of New South Wales) before being scanned using the GeneArray Scanner (Affymetrix). Affymetrix arrays were initially assessed and single-array analyses carried out in the GeneChip Operating Software program according to the Affymetrix GeneChip Expression Analysis Data Analysis Fundamentals Manual (Affymetrix) on data generated from the image analyses. For full experimental details, including raw data sets, refer to the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/index.cgi), accession number GSE9088.

**Microarray data analysis.** Following basic normalization procedures carried out using Limma and GeneChip Operating Software, oligonucleotide arrays were imported into Genespring (Agilent Technologies) for further analyses. Within the Genespring program, genes are assigned individual P values, based on a one-sample Student t test and assuming a baseline of 0 on a log scale. For oligonucleotide arrays, genes were selected as differentially expressed if they had a normalized value greater than 2 (upregulated) or less than 0.5 (downregulated) on a log scale, with at least three slides in each group satisfying these criteria. Genes were then ranked according to their P values to generate lists of upregulated and downregulated genes. However, one-way analyses of variance were found to be too stringent when applied to these lists, as no genes were found to be differentially expressed, and thus were not used. For Affymetrix arrays, following normalization using robust multiarray analysis, arrays from P. berghei-infected mice were compared to the uninfected control array. Genes were selected as differentially expressed if they had a normalized value greater than 2 (upregulated) or less than 0.5 (downregulated) on a log scale. Genes were then ranked according to their fold change values to generate lists of upregulated and downregulated genes.

**Functional annotation of genes.** Functional annotation of genes was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp) (6). Genes were annotated based on the three structured vocabularies (ontologies), describing gene products in terms of their “biological processes,” “molecular functions,” and “cellular compartments,” which are considered independent of each other. DAVID has the advantage of clustering genes with similar gene ontology (GO) terms together, while removing the low level of redundancy that exists in the ontologies and making interpretation easier.

**Real-time PCR.** Real-time PCR was performed as described previously (2), except that PCRs were performed with the Corbett Rotor-Gene (Corbett Research, New South Wales, Australia) and using Platinum Syber green qPCR SuperMix UD (Invitrogen). mRNA levels were normalized to a housekeeping gene, that for hypoxanthine guanine phosphoribosyltransferase (HPRT), and expressed relative to the mean for uninfected control samples using the delta threshold cycle method. The specificity of primers was checked using melting curve analyses, and the primer sets used (Table 1) had similar amplification efficiencies.

**Laser capture microdissection.** Laser capture microdissection was performed as previously described (38). Briefly, cerebral microvessels were stained with a fluorescent lectin [fluorescein Griffonia (Bandetanua) Simpleficolia lectin; Vector, CA]. Laser capture was performed on a PixCell II microscope (Arcturus, TX)
using high-sensitivity caps (CapSure HSLCM; Arcturus). Vessels and parenchymal cells were captured onto separate caps, and the RNA was isolated using columns as described in the RNeasy MicroKit (Qiagen, Victoria, Australia). RNA was amplified according to a previously described protocol for degraded RNA (66), and real-time PCR was carried out as described above.

Statistical analyses. Statistical analyses for gene expression data were performed using the Kruskal-Wallis test. Where statistical differences were observed, probabilities were then calculated using the Mann-Whitney test on selected comparisons. These analyses were carried out using GraphPad Prism version 4.00 for Windows (GraphPad Software, CA).

Microarray accession number. The Gene Expression Omnibus accession number for the microarray data reported in this paper, which also includes details of the MIAME-compliant experiments, is GSE9808.

RESULTS

Many genes are differentially expressed in *P. berghei* infection. Many genes were differentially expressed in the brains of mice inoculated with *P. berghei* (Fig. 1), irrespective of whether the animal developed CM. Genes were either upregulated (normalized value greater than 2, red) or downregulated (normalized value less than 0.5, green) according to criteria outlined in Materials and Methods. Similar sets of genes were found to be upregulated on the oligonucleotide (see Table S1 in the supplemental material) and Affymetrix (see Table S3 in the supplemental material) arrays. There were differences in the lists of downregulated genes on oligonucleotide (see Table S2 in the supplemental material) and Affymetrix (see Table S4 in the supplemental material) arrays, partially due to the green dye bias observed in the oligonucleotide arrays. This commonly occurs due to the higher instability of the green dye, resulting in less efficient incorporation of this dye into cDNA.

IFN-stimulated genes (ISGs) associated with signaling and immune functions were predominant on lists of upregulated genes, including IFN regulatory factor 1 (IRF1), Ia-associated invariant chain (Ii), β2-microglobulin (β2-m), granzymes A and B, and IFN-γ-induced GTPase (IGTP), as well as poorly an-
notated ISGs such as IFN-induced protein with tetratricopeptide repeats 2 (IFIT2) and IFN-induced transmembrane protein 3 (IFITM3), IFN-induced gene 30 (I30), I365, and I644 (see Tables S1 and S3 in the supplemental material). Genes associated with cellular compartments as well as unannotated genes were found on lists of downregulated genes (see Tables S2 and S4 in the supplemental material).

**Genes are differentially expressed between the PbA(6), PbK(6), and PbK(14) groups.** On closer inspection, many genes were common to each group [i.e., PbA(6), PbK(6), or PbK(14)], indicating that gene expression patterns may overlap. In order to identify genes specific to each group, different lists were directly compared using Venn diagrams in the GeneSpring program (Fig. 2; see Tables S5 and S6 in the supplemental material).

Altogether, there were 205 upregulated genes on the oligonucleotide arrays due to *P. berghei* infection. Of these, 88 genes were specifically upregulated 6 days after PbA infection (group A), including genes associated with signaling (e.g., GATA binding protein 2, signal transducers and activators of transcription 2, and transcription factor Id1B), immune functions (e.g., cathepsin S, glial fibrillary acidic protein, ISG15, low-molecular-weight polypeptide 7, and 2'-5' oligoadenylate synthetase-like 2 [OAS-2]), and metabolism (e.g., fructose bisphosphatase and lactate dehydrogenase). Seventeen genes were specifically upregulated 6 days after PbK infection (group B), and 50 genes were specifically upregulated 14 days after PbK infection (group C), including genes associated with immune functions (e.g., CCL5 and vascular cell adhesion molecule 1) and hemopoiesis (e.g., β-globin and hemoglobin α). Four genes were upregulated in both PbA(6) and PbK(6) (group D), 16 genes were upregulated in both PbA(6) and PbK(14) (group E), 6 genes were upregulated in both PbK(6) and PbK(14) (group F), and 24 genes were commonly upregulated in all three groups (group G). In these intersecting groups (i.e., D, E, F, and G), genes were commonly associated with immune functions (e.g., β2-m, CCL12, CXCL9, CXCL11, cytotoxic-T-lymphocyte-associated protein 2α [CTLA-2α], granzymes A and B, guanylate nucleotide binding 1, histocompatibility 2, Ii, IFIT1, IFIT3, IGTP, IRF1, and metallothionein 1) (Fig. 2; see Table S5 in the supplemental material).

Similarly, many of these genes were found on lists of upregulated genes on the Affymetrix arrays following *P. berghei* infection (see Table S6 in the supplemental material).

**Functional clustering reveals distinct groups of differentially expressed genes.** Upregulated genes were clustered using the DAVID program, based on level 5 GO terms. For oligonucleotide arrays, some clusters were nonspecifically associated with *P. berghei* infection, including clusters of genes for biological processes related to immune functions (e.g., antigen processing and response to pathogen) as well as molecular functions associated with signaling (e.g., nucleotide binding). There were a few clusters of genes that were specific to PbA(6), including those for biological processes (e.g., catabolism, negative regulation of biological and cellular processes, and signal transduction) and cellular compartments (e.g., nuclear lumen and non-membrane-bound organelle). While no clusters were specific to PbK(6), a cluster for a cellular compartment (e.g., hemoglobin complex) was found to be specific to PbK(14) (see Table S7 in the supplemental material).

Clustering of Affymetrix arrays revealed similar groups of genes, particularly genes for biological processes related to immune functions (e.g., antigen processing and response to pathogen) and molecular functions associated with signaling (e.g., nucleotide binding and receptor binding). Again there were a few clusters of genes that were specific to PbA(6), including those for biological processes (e.g., cytokine biosynthesis, regulation of enzyme activity, and endocytosis) and molecular functions (e.g., ligase activity) (see Table S8 in the supplemental material).

Clustering groups were diverse in downregulated genes, and unannotated genes were prevalent (see Tables S7 and S8 in the supplemental material). There was no discernible pattern and little overlap in the annotations of downregulated genes during...
**TABLE 2.** Selected lists of functionally annotated genes that are upregulated during PbA infection

| GO term and gene | GO term and gene |
|------------------|------------------|
| Transcription    | Proteosome (prosome, macropain) subunit, beta type 8 (large multifunctional peptidase) 7 |
| Activating transcription factor 3 | Proteosome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase) 2 |
| B-cell translocation gene 2, antiproliferative | TAP-binding protein |
| Caudal type homebox 1 | Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP) |
| CCAAT/enhancer binding protein, beta | Transporter 2, ATP-binding cassette, subfamily B (MDR/TAP) |
| CCAAT/enhancer binding protein, delta | Immune innate |
| Chromatin homolog 1 (Drosophila HP1 beta) | Complement component 1, Q subcomponent, alpha polypeptide |
| CLPB casenoetic peptide b homolog (Esherichia coli) | Complement component 1, Q subcomponent, beta polypeptide |
| Early growth response 4 | Complement component 1, Q subcomponent, C chain |
| Entenogen-related receptor gamma | Complement component 1, R subcomponent |
| ETS variant gene 4 (EEl1a enhancer binding protein, El1d) | Complement component 2 (within H-2S) |
| FBJ osteosarcoma oncogene | Complement component 3 |
| FBZ osteosarcoma oncogene B | Complement component 4B (Childo blood group) |
| GATA binding protein 2 | Complement factor B |
| General transcription factor II 1 repeat domain-containing 1 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 |
| Hairy and enhancer of split 1 (Drosophila) | FC receptor, IgG, high affinity I, gamma polypeptide |
| Inhibitor of DNA binding 1 | FC receptor, IgG, high affinity I |
| IFN-activated gene 204 | FC receptor, IgG, low affinity III |
| IFN-dependent positive-acting transcription factor 3 gamma | IFN induced with helicase C domain 1 |
| IRF1 | Serine (or cysteine) peptidase inhibitor, clade G, member 1 |
| IRF7 | Toll-like receptor 3 |
| IRF8 | Chemotaxis |
| Lipopolysaccharide-induced TN factor | Chemokine (C-C motif) ligand 2 |
| Methyl-CPP binding domain protein 2 | Chemokine (C-C motif) ligand 3 |
| Minichromosome maintenance deficient 5, cell division cycle 46 | Chemokine (C-C motif) ligand 5 |
| Neuronal D4 domain family member | Chemokine (C-C motif) ligand 6 |
| Nuclear X box binding factor 1 | Chemokine (C-C motif) ligand 7 |
| Procollagen (type III) N-endopeptidase | Chemokine (C-C motif) ligand 8 |
| Retinoblastoma-like 1 (P107) | Chemokine (C-C motif) ligand 9 |
| Signal transducer and activator of transcription 1 | Chemokine (C-C motif) ligand 10 |
| Signal transducer and activator of transcription 2 | Chemokine (C-X-C motif) ligand 11 |
| Signal transducer and activator of transcription 3 | Chemokine (C-X-C motif) ligand 12 |
| SEN3-associated polypeptide | Chemokine (C-C motif) ligand 13 |
| Timeless homolog (Drosophila) | Chemokine (C-C motif) ligand 14 |
| Transcription factor 20 | Chemokine (C-X-C motif) ligand 15 |
| Tripartite motif protein 30 | Chemokine (C-X-C motif) ligand 16 |
| V-MAF musculoaponeurotic fibrosarcoma oncogene family, protein F | Chemokine (C-X-C motif) ligand 17 |
| Zinc finger protein, subfamily la, 1 (Ikaros) | Chemokine (C-X-C motif) ligand 18 |
| Apoptosis | Chemokine (C-X-C motif) ligand 19 |
| ATM up-regulated 1 | Chemokine (C-X-C motif) ligand 20 |
| B-cell leukocyte/lymphoma 2 related-protein A1A | Chemokine (C-X-C motif) ligand 21 |
| B-cell translocation gene 2, antiproliferative | Chemokine (C-X-C motif) ligand 22 |
| Caspase 4, apoptosis-related cysteine peptidase | Chemokine (C-X-C motif) ligand 23 |
| Caspase 5 | Chemokine (C-X-C motif) ligand 24 |
| Caspase 8 | Chemokine (C-X-C motif) ligand 25 |
| Caspase 12 | Chemokine (C-X-C motif) ligand 26 |
| CD74 antigen (invariant polypeptide of MHC, class II antigen associated) | Chemokine (C-X-C motif) ligand 27 |
| Granzyme A | Chemokine (C-X-C motif) ligand 28 |
| Granzyme B | Chemokine (C-X-C motif) ligand 29 |
| Growth arrest and DNA damage-inducible 45 beta | Chemokine (C-X-C motif) ligand 30 |
| Growth arrest and DNA damage-inducible 45 gamma | Chemokine (C-X-C motif) ligand 31 |
| IFN-activated gene 204 | Chemokine (C-X-C motif) ligand 32 |
| IFN-activated gene 205 | Chemokine (C-X-C motif) ligand 33 |
| IFN-activated gene 206 | Chemokine (C-X-C motif) ligand 34 |
| Lipopolysaccharide-induced TN factor | Chemokine (C-X-C motif) ligand 35 |
| Peptidoglycan recognition protein 1 | Chemokine (C-X-C motif) ligand 36 |
| Phosphatidylinositol receptor | Chemokine (C-X-C motif) ligand 37 |
| PYD and CARD domain containing | Chemokine (C-X-C motif) ligand 38 |
| Serine (or cysteine) peptidase inhibitor, clade A, member 3G | Chemokine (C-X-C motif) ligand 39 |
| Serum/glucocorticoid-regulated kinase | Chemokine (C-X-C motif) ligand 40 |
| Tumor necrosis factor receptor superfamily, member 1A | Chemokine (C-X-C motif) ligand 41 |
| Antigen presentation | Chemokine (C-X-C motif) ligand 42 |
| β2-m | Chemokine (C-X-C motif) ligand 43 |
| Cathepsin S | Chemokine (C-X-C motif) ligand 44 |
| CD74 antigen (invariant polypeptide of MHC, class II antigen associated) | Chemokine (C-X-C motif) ligand 45 |
| Heat shock protein 1B | Chemokine (C-X-C motif) ligand 46 |
| Histocompatibility 2, class II, antigen A, alpha | Chemokine (C-X-C motif) ligand 47 |
| Histocompatibility 2, class II, antigen A, beta | Chemokine (C-X-C motif) ligand 48 |
| Histocompatibility 2, class II, antigen B | Chemokine (C-X-C motif) ligand 49 |
| Histocompatibility 2, class II, locus DMA | Chemokine (C-X-C motif) ligand 50 |
| Histocompatibility 2, class II, locus MBI | Chemokine (C-X-C motif) ligand 51 |
| Histocompatibility 2, class II, locus MB2 | Chemokine (C-X-C motif) ligand 52 |
| Histocompatibility 2, B region locus 1 | Chemokine (C-X-C motif) ligand 53 |
| Histocompatibility 2, D region locus 4 | Chemokine (C-X-C motif) ligand 54 |
| Histocompatibility 2, K1, K region | Chemokine (C-X-C motif) ligand 55 |
| Histocompatibility 2, Q region locus 2 | Chemokine (C-X-C motif) ligand 56 |
| Histocompatibility 2, Q region locus 5 | Chemokine (C-X-C motif) ligand 57 |
| Histocompatibility 2, Q region locus 7 | Chemokine (C-X-C motif) ligand 58 |
| Histocompatibility 2, Q region locus 10 | Chemokine (C-X-C motif) ligand 59 |
| Histocompatibility 2, T region locus 9 | Chemokine (C-X-C motif) ligand 60 |
| Histocompatibility 2, T region locus 10 | Chemokine (C-X-C motif) ligand 61 |
| IFN-γ-induced protein 30 | Chemokine (C-X-C motif) ligand 62 |
| MHC A1/C1/H-2K-F1 class I antigen | Chemokine (C-X-C motif) ligand 63 |
| Proteasome (prosome, macropain) 28 subunit, alpha | Chemokine (C-X-C motif) ligand 64 |
| Proteasome (prosome, macropain) 28 subunit, beta | Chemokine (C-X-C motif) ligand 65 |
P. berghei infection, due in part to the green bias on oligonucleotide arrays. Moreover, since many of the genes found in these lists were not annotated, these were not explored further here.

Annotations and classes of differentially expressed genes.

To gain a clearer overview of genes differentially expressed during P. berghei infection, gene lists from oligonucleotide and Affymetrix arrays were combined. Importantly, both oligonucleotide and Affymetrix array determinations were performed using the same RNA samples obtained from the same biological experiment. Genes were annotated using level 3 GO terms in DAVID and the percentage of functional classes determined, together with their statistical significance (see Table S9 in the supplemental material).

There was a large overlap in the annotations for genes that were upregulated during P. berghei infection, many of which were overrepresented. These were associated with GO terms from biological processes such as “immune response” [PbA(6), 110 genes, P = 1.98 × 10^{-49}; PbK(6), 56 genes, P = 1.19 × 10^{-38}; and PbK(14), 74 genes, P = 1.56 × 10^{-39}], molecular functions such as “cytokine activity” [PbA(6), 16 genes, P = 2.63 × 10^{-4}; PbK(6), 5 genes, P = 6.84 × 10^{-2}; and PbK(14), 10 genes, P = 4.13 × 10^{-3}], and cellular compartments such as “immunological synapse” [PbA(6), 13 genes, P = 3.45 × 10^{-8}; PbK(6), 13 genes, P = 1.80 × 10^{-14}; and PbK(14), 14 genes, P = 3.68 × 10^{-12}] (see Table S9 in the supplemental material).

Overall, there were more genes upregulated by PbA infection than by PbK infection. Genes belonging to selected annotation categories are shown in Table 2.

When the expression patterns in the oligonucleotide and Affymetrix arrays were compared, a high degree of concordance between the two platforms was observed, although a higher number of upregulated genes were observed with the Affymetrix arrays (Table 3).

PCR validation of differentially expressed genes on the arrays. Real-time PCR was carried out to validate the results obtained from the oligonucleotide and Affymetrix arrays. Initially, validation by real-time PCR was used to confirm the oligonucleotide array data and to determine at which point genes were no longer differentially expressed, by randomly selecting genes at set intervals within the ranked gene lists. This improved the selection criteria for differentially expressed genes and increased confidence in the selected lists of differentially expressed genes (data not shown).

The majority of genes, including Il, β2-m, OAS-1b, ISG15, and IGTP, were found to be expressed predominantly by vessel-associated cells compared with parenchymal cells, which expressed mRNAs for these genes at much lower levels. However, CTLA-2α and Fas were very highly expressed by vessel-associated cells but were not detectable in parenchymal cells (Table 4).

Temporal expression of differentially expressed genes. The expression patterns of a number of genes validated by real-time PCR also were examined during the progression of PbA infection. OAS-1b (Fig. 4A) and ISG15 (Fig. 4B) mRNAs were both found to increase dramatically at day 4 p.i. (22.1 ± 5.5-fold compared with uninfected [mean ± SEM] for OAS-1b mRNA (Fig. 3C) was greatly upregulated in PbA(6) (133.5 ± 7.8-fold) compared with PbK(6) (18.4 ± 2.2-fold) and PbK(14) (23.6 ± 2.6-fold).

There also were some genes that were predominantly upregulated at the end stage of the disease [PbA(6) and PbK(14)]. These included Il mRNA (Fig. 3D), which was strongly induced in PbA(6) (54.1 ± 3.9-fold) and PbK(14) (58.4 ± 16.2-fold) compared with PbK(6) (19.8 ± 3.2-fold). CTSW mRNA (Fig. 3E) was upregulated in the PbA(6) (31.5 ± 9.1-fold) and PbK(14) (29.2 ± 11.4-fold) compared with PbK(6) (11.5 ± 0.7-fold). CTLA-2α mRNA (Fig. 3F) had a similar expression pattern, being upregulated in PbA(6) (28.0 ± 4.8-fold) and PbK(14) (44.2 ± 17.8-fold) compared with PbK(6) (4.0 ± 0.2-fold).

Overall, the real-time PCR data confirmed the upregulation of genes found on both the oligonucleotide and Affymetrix arrays and showed concordance between these two different platforms. However, the real-time PCR results also revealed that differential expression was not always easily identified by arrays, particularly by oligonucleotide arrays, which are less sensitive than Affymetrix arrays. By comparing the results from the arrays with the real-time PCR results, it is evident that, during hybridization of the samples, a threshold is reached on the oligonucleotide arrays that does not allow highly upregulated or downregulated genes to be distinguished from genes that are also differentially expressed at lower levels. Thus, a number of genes found to be upregulated by P. berghei infection on the arrays also were found to be differentially expressed between PbA(6), PbK(6), and PbK(14) upon validation with real-time PCR (Fig. 3).

There also appeared to be a significant problem associated with the detection of genes that are of low abundance. For example, tumor necrosis factor is known to be highly upregulated in the central nervous system during PbA infection (35) but was not identified as an upregulated gene on the arrays, despite being represented. With real-time PCR, tumor necrosis factor mRNA (data not shown) was found to be strongly upregulated in PbA(6) compared with PbK(6) and PbK(14). These findings suggest that a number of differentially expressed genes are not detected above background levels even though they may play critical roles during PbA infection.

Cellular expression of differentially expressed genes. The cellular sources of a number of these genes were then determined using laser capture microdissection. Vessels were separated from parenchymal cells of the brain (neurons and glia) in PbA-infected mice and real-time PCR performed to determine relative gene expression.

The majority of genes, including Il, β2-m, OAS-1b, ISG15, and IGTP, were found to be expressed predominantly by vessel-associated cells compared with parenchymal cells, which expressed mRNAs for these genes at much lower levels. However, CTLA-2α and Fas were very highly expressed by vessel-associated cells but were not detectable in parenchymal cells (Table 4).
TABLE 3. Comparisons between selected gene expression data from oligonucleotide and Affymetrix arrays

| Category and GenBank accession no. | Ifi no. | Name   | Synonym(s) | Expression on: | Oligonucleotide array* | Affymetrix array* |
|-----------------------------------|---------|--------|------------|----------------|------------------------|------------------|
|                                   |         |        |            |                | PbA(6) PbK(6) PbK(14)  | PbA(6) PbK(6) PbK(14) |
| IFN signaling                     |         |        |            |                |                        |                  |
| NM_008337                         | IFN-γ   |        |            |                |                        |                  |
| NM_009283                         | STAT1   |        |            |                | +                      |                  |
| NM_019963                         | STAT2   |        |            |                |                        |                  |
| NM_213659                         | STAT3   |        |            |                | +                      |                  |
| NM_008390                         | IRF1    |        |            |                | ++                     | ++               |
| NM_016850                         | IRF7    |        |            |                |                        |                  |
| NM_008220                         | IRF8    |        | ICSBP      |                | ++                     | ++               |
| NM_008394                         | IRF9    | p48, ISGF3-γ |            |                | ++                     | ++               |
| NM_007707                         | SOCS3   |        |            |                |                        |                  |
| MHC class II                      |         |        |            |                |                        |                  |
| NM_010378                         | H2-Aa   |        |            |                | ++                     | ++               |
| NM_010381                         | H2-Ea (Aa/Ea) | |             |                |                        |                  |
| NM_010387                         | H2-DMb1/DMb2 | |            |                | +                      |                  |
| X00496                            | li      |        |            |                | +                      | ++               |
| NM_009982                         | CTSC    |        |            |                |                        |                  |
| NM_021261                         | CTSS    |        |            |                | +                      | +                |
| MHC class I                       |         |        |            |                |                        |                  |
| NM_001001892                      | H2-K1/K2|        |            |                |                        |                  |
| NM_010380                         | H2-D1/Q1|        |            |                | ++                     | ++               |
| NM_008200                         | H2-D3/D4|        |            |                |                        |                  |
| NM_010393                         | H2-Q5   |        |            |                | +                      | +                |
| X16214                            | H2-T8/T14|       |            |                |                        |                  |
| NM_010398                         | H2-T23/T24|       |            |                | ++                     | ++               |
| NM_009735                         | β2m     |        |            |                |                        |                  |
| NM_010724                         | PSMB8   |        | LMP-7      |                | ++                     | ++               |
| NM_013585                         | PSMB9   |        | LMP-2      |                | +                      | ++               |
| NM_013640                         | PSMB10  |        | MECL-1     |                |                        |                  |
| NM_011189                         | PSME1   |        | PA28α      |                | +                      |                  |
| NM_011190                         | PSME2   |        | PA28β      |                |                        |                  |
| NM_134013                         | PSME4   |        | TEO        |                |                        |                  |
| NM_013683                         | TAP1    |        |            |                | ++                     | +                |
| NM_011530                         | TAP2    |        |            |                | +                      |                  |
| Apoptosis                         |         |        |            |                |                        |                  |
| NM_009807                         | CASP1   |        |            |                |                        |                  |
| NM_007611                         | CASP7   |        |            |                |                        |                  |
| NM_009812                         | CASP8   |        |            |                |                        |                  |
| Y13089                            | CASP11  |        |            |                |                        |                  |
| NM_009808                         | CASP12  |        |            |                |                        |                  |
| NM_010370                         | GZMA    |        |            |                | +                      | ++               |
| NM_013542                         | GZMB    |        |            |                | +                      | ++               |
| NM_007601                         | CAPN3   |        |            |                | +                      | ++               |
| NM_016816                         | OAS-1   |        |            |                |                        |                  |
| NM_145211                         | OAS-1a  |        |            |                |                        |                  |
| NM_011854                         | OAS-L2  |        |            |                |                        |                  |
| Immunomodulation                  |         |        |            |                |                        |                  |
| NM_010851                         | MyD88   |        |            |                | +                      |                  |
| NM_126166                         | TLR3    |        |            |                | +                      |                  |
| NM_008324                         | IDO     |        |            |                |                        |                  |
| NM_007796                         | CTLA-2α |        |            |                |                        |                  |
| Apoptosis/antiproliferation       |         |        |            |                |                        |                  |
| NM_011940                         | Ifi202b | Ifi202a |            |                |                        |                  |
| NM_008328                         | Ifi203  |        |            |                |                        |                  |
| NM_008329                         | Ifi204  | Ifi16   |            |                | +                      |                  |
| NM_172648                         | Ifi205  | D3      |            |                |                        |                  |
| Immunomodulation                  |         |        |            |                |                        |                  |
| NM_015783                         | ISG15   |         | G1p2       |                | +                      | ++               |

Continued on following page
and 43.9-±6.7-fold compared with uninfected [mean ± SEM] for ISG15) before reaching maximal expression at day 6 p.i. (42.6-±8.7-fold for OAS-1b and 76.1-±8.6-fold, ISG15).

On the other hand, IGTP (Fig. 4C) mRNA expression showed a more gradual increase from day 4 p.i. (34.0-±2.1-fold), increasing at day 5 p.i. (87.8-±28.2-fold) before reaching a maximal expression level at day 6 p.i. (261.3-±85.3-fold).

**DISCUSSION**

**ISGs are induced during *P. berghei* infection.** These results show that ISGs (7, 8) are predominantly induced during *P. berghei* infection, particularly genes associated with IFN signaling pathways and immune responses. mRNAs for many of these genes were highly upregulated in the brain during PbA infection (FMCM), but some also were induced during PbK infection (NCM), largely at the end stage of the disease. The relative induction of many genes was generally found to be greater in PbA (6 days p.i.) than in PbK (6 days p.i.) or PbK (14 days p.i.) upon real-time PCR validation, highlighting the importance of IFN-γ during the pathogenesis of FMCM (1, 13, 51).

Some processes that were evident from gene expression patterns on the arrays included antigen processing and presentation, apoptosis, and antimicrobial pathways. While some of these genes previously have been established to play a role in the pathogenesis of FMCM, for example, intercellular adhesion molecule-1 (28) and granzyme B (46), there also were a number of upregulated genes, previously unreported, that represent interesting candidates for further investigation.

(i) **IFN signaling pathways.** Microarray analysis demonstrated that genes for essential components of the IFN signaling pathway, including members of the signal transducers and activators of transcription family and the IRF family, were induced during *P. berghei* infection. IRFs are secondary transcription factors that are induced early following IFN-γ stimulation and drive the transcription of IFN-γ-responsive genes, including other ISGs (54). IRF1, a transcriptional activator, was upregulated on the arrays, whereas IRF2, a transcriptional repressor, was not. IRF1 is required for the development of a Th1-type immune response, and its absence leads to the induction of a Th2-type response (25, 59). Negative regulators of the IFN signaling pathway, for example, the family of suppressor of cytokine signaling proteins, also were upregulated (see Tables S1 and S3 in the supplemental material).

(ii) **MHC processing and presenting pathways.** Components of the MHC I and MHC II pathways were found by microarray analysis to be expressed during *P. berghei* infection (see Tables S1 and S3 in the supplemental material). This was not surprising given the upregulation of genes associated with IFN signaling pathways, particularly IRF1, and the importance of CD4+ and CD8+ T cells in malaria immunity and immunopathology (16, 46, 50, 58, 67). The expression of genes involved in antigen-processing and -presenting pathways known to be involved in the pathogenesis of murine malaria, for example, MHC peptides and costimulatory molecules (24), lends confi-
dence to the validity of the upregulation of novel genes found by microarray analysis without further confirmation.

(iii) Apoptosis and cell proliferation. A number of genes associated with apoptotic pathways were expressed on the arrays following *P. berghei* infection. These included caspases, granzymes, cathepsins, calpains, protease inhibitors, OASs, and members of the p200 family (see Tables S1 and S3 in the supplemental material). Significantly, these were much more strongly induced in PbA infection than in PbK infection. Apoptosis occurs in the brain during human and murine CM in endothelial cells (45, 46, 65), astrocytes (47), and neurons (23, 53, 65). Both the death receptor-mediated pathway and the

![FIG. 3. mRNA expression of OAS-1b (A), ISG15 (B), IGTP (C), I(4) (D), CTSS (E), and CTLA-2α (F) in whole-brain homogenates of uninfected control mice and PbA(6)-, PbK(6)-, and PbK(14)-infected mice. Expression, measured by real-time PCR as described in Materials and Methods, is relative to the mean expression value in uninfected control samples. Values are means ± SEMs (n = 6). Samples were initially determined to be significantly different using the Kruskal-Wallis test (P < 0.001), and then the Mann-Whitney test was used: ++, P < 0.01 (values compared with uninfected controls); ##, P < 0.01 [values compared with PbA(6)]; **, P < 0.01 [values compared with PbK(6)]; *, P < 0.05 [values compared with PbK(6)].](image)

### TABLE 4. Relative mRNA expression in microdissected cells during FMCM

| mRNA | β2-m | CTLA-2α | Fas | IGTP | I(4) | ISG15 | OAS-1b |
|------|------|---------|-----|------|------|-------|--------|
| Parenchymal | 1.0 | ND* | ND | 1.0 | 1.0 | 1.0 | 1.0 |
| Vessel | 5.3 ± 2.3 | 35.21 ± 10^4 ± 22.6 ± 10^4 | 7.0 ± 10^4 ± 1.9 ± 10^4 | 20.0 ± 10.5 | 17.5 ± 6.5 | 5.1 ± 1.4 | 5.1 ± 2.3 |

*a* Vessels were separated from parenchymal cells (neurons and glia) in PbA-infected mouse brains as described previously (38).

*b* Expression, measured by real-time PCR, is relative to the mean expression value in PbA-infected parenchymal cell samples.

*c* ND, not detected. When PCR products were not detected after 50 cycles of PCR, an arbitrary threshold value of 50 was assigned for the purposes of calculating a relative induction.
stress-induced pathway of apoptosis have been implicated in FMCM (21, 42, 45, 47). Both pathways converge on caspase 3, which also is involved in the pathogenesis of FMCM (23, 36, 45–47).

Other proteases, including granzymes, cathepsins, and calpains, also play a role in apoptosis, primarily by activating caspases. Some of these genes also have been implicated in neuronal death in neurological diseases (11, 17, 60), including CM (33, 56). A number of protease inhibitors were expressed on the arrays, including CTLA-2 (22), and may represent a host mechanism to limit the damage mediated by proteases, particularly since these proteolytic processes can lead to development of intracerebral hemorrhaging (63), which is an invariable finding in human and murine CM.

(iv) Immunomodulatory and antimicrobial genes. Genes involved in immune responses, such as Fc receptors and components of the complement system, were upregulated during P. berghei infection (see Tables S1 and S3 in the supplemental material), reflecting the important role of the immune system in this disease (19). Genes involved in adaptive immunity and other immunomodulatory functions, including cytokines, chemokines, leukocyte surface antigens, and adhesion molecules, also were induced. There were a number of upregulated ISGs with interesting but poorly defined functions, including the family of IFIT genes and the family of IFITM genes. It remains to be seen whether these genes play other roles in host defense, particularly against parasites.

A number of interesting immunomodulatory genes expressed on the arrays have not yet been assigned a role in CM. Some of these genes are expressed on the arrays, including CTLA-2 (22), and may represent a host mechanism to limit the damage mediated by proteases, particularly since these proteolytic processes can lead to development of intracerebral hemorrhaging (63), which is an invariable finding in human and murine CM.

For example, ISG15 is released by lymphocytes and monocytes (4, 20) and is inducible by IFN (20) but can itself induce the secretion of IFN-γ from T cells and stimulate the activity of indoleamine 2,3-dioxygenase 1 (49), which has complex roles in malaria infection (18), as well as lymphocyte and NK proliferation, and can induce cytolytic activity (3). Other ISGs identified by arrays belong to the p47 and p65 GTPase (i.e., IGTP) families (29, 61). These GTPases have antimicrobial functions and are preferentially induced by IFN-γ, a cytokine that is essential for the pathogenesis of FMCM through ill-understood mechanisms. While the roles of these genes are not well described, specific gene knockout mice have differences in their susceptibilities to infectious diseases, including those of the central nervous system (29, 61).

Real-time PCR Validation. Selected genes were validated using real-time PCR (e.g., OAS-1b [Fig. 3A], ISG15 [Fig. 3B], IGTP [Fig. 3C], II [Fig. 3D], CTSW [Fig. 3E], and CTLA-2α [Fig. 3F]), confirming the pattern of gene expression seen in the arrays (i.e., upregulated in P. berghei infection). However, real-time PCR also revealed that the expression of some of these mRNAs was greater in PbA(6) and PbK(14) than in PbK(6).

Overall these results suggest that there are similar processes occurring in the brain at the end stages of P. berghei infection, independent of the occurrence of cerebral symptoms. These include those associated with MHC antigen-processing and -presenting pathways, although there was a striking difference in the mRNA upregulation of costimulatory molecules on the arrays. While PbA infection was associated with increased...
mRNAs for a number of costimulatory molecules (e.g., CD40 and CD86; see Table S3 in the supplemental material), this was not seen during PbK infection. In the absence of costimulation, T cells often undergo anergy and apoptosis instead of proliferation and differentiation. Since costimulation is an essential component of the antigen presentation pathway, this suggests that there is an absence of T-cell activation during PbK infection.

The location of gene expression in the brain clearly is important. The expression of Ii and β2-m mRNAs was greater in vessel-associated cells than in parenchymal cells (Table 4). This suggests that endothelial cells are most likely to express malaria antigens associated with either MHC I or MHC II. Indeed, brain endothelial cells are capable of expressing MHC I and MHC II, and their expression levels are associated with the development of FCMC (39). Micoglobins also can express MHC I, but not costimulatory molecules, during FCMC (43). Similarly, mRNAs of genes associated with apoptotic processes, such as Fas, OAS-1b, and CTLA-2a, were localized predominantly to vessel-associated cells compared with parenchymal cells (Table 4), suggesting that important interactions occur at the blood-brain barrier during FCMC. Furthermore, ISG15 and IGTP mRNAs also are predominantly localized to cells associated with cerebral vessels, and given the cytokine-like activities of ISG15 and its potential nonimmune roles in the brain, as well as the antimicrobial actions of IGTP, these are interesting candidates to examine in malaria infection.

These findings emphasize the key role played by cells of the blood-brain barrier, particularly endothelial cells, during FCMC. These cells are located at the key biological interface between the blood compartment, including the circulating intraerythrocytic malaria parasite, and the brain parenchyma. Changes to the blood-brain barrier during malaria during FCMC are well described (18, 19) and are thought to be both a cause and an effect of inflammatory processes. The prominent localization of gene induction to cells of the blood-brain barrier supports the idea that key interactions between the parasite and the host occur at the endothelium during FCMC, and as a consequence, pathological events are concentrated primarily within cerebral microvessels, with lesser and later involvement of cells from the brain parenchyma.

Concluding remarks. Overall, these data are supportive of an inflammatory reaction occurring in the brain during FCMC, which is typified by high circulating levels of inflammatory mediators, an activated endothelium, and adherent leukocytes (19). There was a high degree of concordance between results obtained from the oligonucleotide and Affymetrix arrays, which revealed similar patterns of gene expression. Some gene expression changes typical of inflammatory responses also occurred at the end stage of PbK infection, in the absence of neurological signs. These changes likely result from high circulating levels of PRBCs and cytokines (37) but, overall, were small compared with changes due to PbA infection and presumably are insufficient for immunopathological processes. A number of genes, such as intercellular adhesion molecule-1 and CXCL9, were upregulated at the end stage of PbA and PbK infection and are largely indicative of an activated endothelium. Moreover, indoleamine 2,3-dioxygenase-1 is very strongly induced in endothelial cells during the late stages of PbK (15), perhaps as a tissue-protective response (18), suggesting that important responses also can occur in the brain during NCM.

These results support the view that leukocytes play a crucial role in the pathogenesis of FCMC. Leukocytes do not adhere to the microvasculature during noncerebral models of malaria, including late stages of PbK infection, and during PbA infection in genetically resistant mice, for example, those deficient in IFN-γ or its receptor (1, 51), LT-α (10), and CXCR3 (38), despite continued expression of adhesion molecules by the endothelium. This suggests that leukocytes are unable to adhere to the endothelium during PbK infection, perhaps because of a lack of integrin activation or an absence of costimulation by antigen-presenting cells, thus leading to leukocyte anergy.

Overall, these data highlight the significant gene expression changes in the brain during PbA infection (FCMC). Furthermore, certain critical mediators that were upregulated in PbA were absent during PbK infection. In FCMC, several induced pathways were evident from the gene expression profiles on the arrays involving signaling and transcription, apoptosis, immunomodulation, and antimicrobial activities. Some of these have been identified previously in FCMC using arrays (5, 55), but without comparisons having been made with late stages of NCM. The results from this study contribute to our understanding of the complex pathogenic processes that occur during malaria.

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