INFECTION AND IMMUNITY, Oct. 2006, p. 5561–5573 Vol. 74, No. 10
0019-9567/06/$08.00+0 doi:10.1128/IAI.00408-06

Common and Divergent Immune Response Signaling Pathways Discovered in Peripheral Blood Mononuclear Cell Gene Expression Patterns in Presymptomatic and Clinically Apparent Malaria†

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Received 14 March 2006/Returned for modification 8 May 2006/Accepted 14 July 2006

Using genome-wide expression profiles from persons either experimentally challenged with malaria-infected mosquitoes or naturally infected with *Plasmodium falciparum* malaria, we present details of the transcriptional changes that occur with infection and that either are commonly shared between subjects with presymptomatic and clinically apparent malaria or distinguish these two groups. Toll-like receptor signaling through NF-κB pathways was significantly upregulated in both groups, as were downstream genes that function in phagocytosis and inflammation, including the cytokines tumor necrosis factor alpha, gamma interferon (IFN-γ), and interleukin-1β (IL-1β). The molecular program derived from these signatures illuminates the closely orchestrated interactions that regulate gene expression by transcription factors such as *IRF-1* in the IFN-γ signal transduction pathway. Modulation of transcripts in heat shock and glycolytic enzyme genes paralleled the intensity of infection. Major histocompatibility complex class I molecules and genes involved in class II antigen presentation are significantly induced in 90% of malaria-infected persons regardless of group. Differences between early presymptomatic infection and natural infection involved genes that regulate the induction of apoptosis through mitogen-activated protein (MAP) kinases and signaling pathways through the endogenous pyrogen IL-1β, a major inducer of fever. The induction of apoptosis in peripheral blood mononuclear cells from patients with naturally acquired infection impacted the mitochondrial control of apoptosis and the activation of MAP kinase pathways centered around MAPK14 (p38α and p38β). Our findings confirm and extend findings regarding aspects of the earliest responses to malaria infection at the molecular level, which may be informative in elucidating how innate and adaptive immune responses may be modulated in different stages of infection.

The burden of disease resulting from infection with the human malaria parasite *Plasmodium falciparum* is a major contributing factor leading to increased childhood mortality and is a significant obstacle to sustainable economic growth in many developing countries (3, 26). The complex nature of the host-parasite relationship leading to illness, death, or recovery from the disease involves an intricate interaction of the parasite, the *Anopheles* mosquito, and the human host. The specificity, breadth, and intensity of the immune response to infection are dependent upon pathogen-derived molecular patterns that initiate host immune responses and activate adaptive immune mechanisms to thwart the pathogen's spread and its destructive consequences.

Not surprisingly, the host response to infection by the malaria parasite *P. falciparum* elicits a cascade of proinflammatory cytokines that includes tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and gamma interferon (IFN-γ) in both uncomplicated and severe disease. The proinflammatory cytokines drive the host's response to either control the infection or accelerate the disease process (2, 5, 7, 15, 18). Whether or not the magnitude of this cytokine storm is sufficient to explain why only 1 to 2% of malaria infections in children progress to complicated disease that is characterized by either severe anemia or cerebral malaria is not clear (24).

Peripheral blood mononuclear cells (PBMCs) that traffic between the spleen and lymphoid compartments and the peripheral circulation are the first responders to an infectious attack and are potent biological sensors of infection. PBMCs detect molecular patterns on the surfaces of pathogens, transmitting signals into the cell to activate proinflammatory and anti-inflammatory cytokine and chemokine gene expression (14, 20, 22, 34). In extreme circumstances, an excessive unregulated inflammatory response exacerbates the disease process. Although significant strides have been made in understanding the cell-mediated immune mechanisms and cytokine responses in naturally infected patients with acute *P. falciparum* malaria, little is known about the transcriptional events that drive the host response in early preclinical infection and how the kinetics of genome-wide gene expression differ between presymptomatic early infection and uncomplicated febrile malaria.

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† Supplemental material for this article may be found at http://iai.asm.org/.

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To uncover the features of transcriptional events associated with malaria infection in presymptomatic and symptomatic disease, we examined the gene expression profiles of peripheral blood mononuclear cells from two groups. The first group consisted of 22 malaria-naïve volunteers from the United States experimentally infected with a single clone of *P. falciparum* from whom PBMCs were collected immediately prior to infection and again on the day of diagnosis, when the parasite density was extremely low. The second group consisted of 15 adults naturally infected with *P. falciparum* in Cameroon, West Africa, and from whom PBMCs were collected at the time of clinical illness (fever) concurrent with a positive malaria blood smear. A set of 22 samples from uninfected U.S. volunteers was used as the baseline control. We present the patterns of gene expression that are common and unique to early, presymptomatic, experimentally acquired malaria infection and acute, uncomplicated, natural infection. Distinctions in gene ontology (GO) and gene interaction networks between the two groups are examined to reveal intricacies of the immune response and regulation in these two stages of infection.

MATERIALS AND METHODS

Study volunteers and clinical specimens. (i) Group 1: U.S. volunteers. Twenty-two malaria-naïve volunteers (group 1) were recruited through the Walter Reed Army Institute of Research (WRAIR) Clinical Trials Center under protocols approved by the WRAIR Human Use Review Committee and the U.S. Army Human Subjects Research Review Board. Written informed consent was obtained from all volunteers prior to enrollment into the study, and all subjects were fully informed of the risks associated with malaria challenge. Group 1 consisted of 11 enrolled subjects each in two identical studies separated by a 1-year interval for a total of 22 subjects. Volunteers were excluded from participation if they had any significant pulmonary, cardiovascular, hepatic, or renal functional abnormality; had previously undergone splenectomy; had a positive beta human chorionic gonadotropin test; or were immunodeficient. The demographics of the study population included 13 males and 9 females aged 20 to 45 years (9 Caucasians and 13 African-Americans). The malaria challenge model has been used safely at the Walter Reed Army Institute of Research for the last 15 years and has enrolled >500 subjects to participate in malaria drug and vaccine studies. Each malaria challenge study always includes a subset of subjects who volunteer to undergo malaria challenge knowing in advance that they will become infected. The clinical investigators have designed the conduct of such studies to minimize the adverse impact of infection with a potentially lethal pathogen.

Cloned, chloroquine-sensitive *P. falciparum* (strain 3D7) parasites were expanded from a master seed lot and were used to infect laboratory-reared *Anopheles stephensi* mosquitoes. Infection was initiated with the bite of five infected mosquitoes that ensured 100% infectivity of the subjects and that approximated the kinetics of infection as determined by measurement of the prepatent period. PBMCs were separated from whole blood collected from each subject immediately prior to mosquito challenge (uninfected controls, $n = 22$) and at the time that a single asexual blood-stage parasite was identified on a thick blood smear by microscopic detection. In most instances, the parasite load was approximately 1 parasite per microliter of blood. This is approximately 1,000- to 10,000-fold lower than typically observed in children and adults with uncomplicated malaria. In many instances, the subject remained afebrile at the time a parasite was detected on the blood smear. Of the 22 subjects experimentally challenged with malaria, 6 subjects had a low-grade elevated temperature on the day of diagnosis. Clinical evaluation of each subject occurred daily, and subjects were treated with chloroquine to eradicate the infection when a single parasite was detected on daily blood smears. The typical prepatent period from the time of mosquito malaria challenge to the detection of a singe parasite on a blood smear is 9 to 12 days.

Samples from malaria-naïve uninfected subjects ($n = 22$) residing in the United States served as the baseline control for all subsequent analyses assessing gene expression changes in subjects from group 1 and group 2. These baseline uninfected samples included male and female subjects of Caucasian and African-American racial backgrounds.

(ii) Group 2: Cameroonian volunteers. Group 2 consisted of 15 adults, aged 19 to 49 years, from Cameroon, West Africa, presenting with fever and signs of clinical malaria and evaluated for the presence of *P. falciparum* parasites by a positive malaria blood film. Parasite density was not determined. The study was approved by both the Institutional Review Board at the Johns Hopkins Bloomberg School of Public Health and a local Cameroonian institutional review board formed by the Ministry of Health. The consent form was translated into French, and a local physician informed subjects of the purpose of the study. The protocol did not permit the collection of samples from malaria-naïve uninfected subjects living in Cameroon. After obtaining written informed consent, approximately 20 ml whole blood was obtained immediately prior to treatment and processed to obtain PBMCs. PBMCs were separated by Ficoll gradient and cryopreserved. Cameroonian volunteers were treated for malaria infection with the drug chloroguine (colecxin).

Affymetrix GeneChip preparation. Preparation of cellular RNA for GeneChip analysis, cDNA preparation, in vitro transcription, staining, and scanning of Affymetrix U133A GeneChips (Affymetrix, Santa Clara, CA) were carried out essentially as described previously (45). The Affymetrix U133A GeneChip contains 22,283 probe sets including 18,400 transcripts and 14,500 well-characterized human genes. Details of the preparation and characterization of RNA and the specifics of sample preparation and GeneChip development for Affymetrix GeneChip analysis are given in the MIAME spreadsheet included in Table S1 in the supplemental material.

Data normalization. Gene expression profiles from both groups of subjects were harvested from Affymetrix U133A GeneChips. The scanned images were analyzed using Affymetrix MAS 5.0 to generate CEL files (fluorescence intensity files), which were normalized at the probe level using the robust multichip average method (19), with the average fluorescence intensity of each probe expressed as $\log_2$. The data sets from all groups (22 data sets from experimentally infected U.S. volunteers, 22 data sets from healthy U.S. volunteers, and 15 data sets from naturally infected Cameroonian volunteers) were normalized together in order to permit direct comparisons of gene expression patterns in the two groups relative to the same baseline.

Identification of differential gene expression patterns. Differentially expressed genes in the experimentally infected (group 1) and naturally infected (group 2) groups were identified by comparison to the same baseline of malaria-naïve U.S. volunteers. The two groups were evaluated independently using the two-class paired feature for group 1 and the two-class unpaired feature for group 2 in the statistical program SAM (Significance Analysis of Microarrays), version 2.10 (44), with the false-discovery rate (FDR) set at 1%.

Derivation of gene ontology and functional associations. The functions and biological classifications of differentially regulated gene sets were further analyzed by the Web-based online tool Onto Express using the "corrected" *P* value feature to control for multiple-comparison testing (http://vortex.cs.wayne.edu:8080/index.jsp) (21). This application sorts gene lists into functional profiles using broad gene ontology categories by associated biological processes, cellular component, and molecular function. The application calculates statistical significance values for each functional category to reveal gene groups that are significantly enriched in a given data set. Pathway Architect (Stratagene, La Jolla, CA) software was used for biological pathway analysis (30). It allows the visualization of pathways, gene regulation networks, and gene-gene interaction maps.

Transcription factor centric clustering. Interferon regulatory factor 1 (*IRF-1*) transcription factor binding sites in sets of differentially expressed genes in PBMC transcripts from malaria-infected and uninfected subjects were computationally identified using a method developed previously by Zhu and colleagues (49) and analyzed using the Web-based program CLOVER (*cis* element overrepresentation) (http://zlab.bu.edu/clover/as) described previously by Frith et al. (11). CLOVER (11, 12) identifies sites within the DNA query sequences for known *IRF-1* binding sites that are statistically overrepresented as determined by JASPAR. JASPAR is an open-access database for eukaryotic transcription factor binding profiles (36). TRANSFAC 7.0, a public database of eukaryotic transcription factors, their genomic binding sites, and DNA-binding profiles, was used to confirm known IRF-1 binding sites (47).

This approach is based upon the hypothesis that the higher the correlation in gene expression by GeneChip microarrays between *IRF-1* and a set of genes with a similar correlation in gene expression, the greater the likelihood of identifying an *IRF-1* sequence motif or transcription factor binding pattern that is statistically overrepresented.

In our study, the following steps (shown in Fig. S1 in the supplemental material) were used to identify putative target genes regulated by the transcription factor *IRF-1* exclusively from the microarray data from group 1 subjects: (i) the correlation coefficient between the expression pattern of *IRF-1* with each of the other 22,283 probe sets from 44 Affymetrix U133A GeneChips (22 uninfected

and 22 infected samples from subjects with experimental malaria) was calculated, and the genes were ranked from the highest to the lowest correlation (a cutoff score of 0.7 was used to yield a set of 326 genes with correlation coefficients of >0.7); (ii) the GenBank accession number was used to extract 1,000 bp of the upstream promoter region of each gene; (iii) CLOVER was used to search for *cis*-regulatory elements within the promoters of sequential 25-gene sets (the promoters within the sets of genes suspected to be coexpressed with *IRF-1* were compared to a set of chromosome 20 sequences as background controls); (iv) clusters with overrepresented ($P < 0.001$) IRF-1 binding sites were further analyzed by using sets of 10 genes overlapping by 1 to identify all the potential genes regulated by *IRF-1* with a high correlation in gene expression as determined on the GeneChip microarrays; and (v) the putative binding sites within the promoter regions of the 12 genes identified with this approach (mean correlation coefficient of 0.96 for correlation to *IRF-1*) were aligned using a sequence logo generator (http://weblogo.berkeley.edu/).

Flow cytometry. Peripheral blood mononuclear cell analysis was performed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) using a panel of mouse anti-human monoclonal antibodies as described by the manufacturer.

Chemokine detection in plasma. Measurements of the levels of CXCL10, CXCL9, and CCL2 chemokines in paired plasma samples collected prior to and after malaria challenge were performed using commercial kits (R&D Systems, Minneapolis, MN).

Microarray accession numbers. The Affymetrix data sets can be accessed at http://www.ncbi.nlm.nih.gov/geo/ under accession numbers GSE5418.

RESULTS

Patterns of gene expression were measured in subjects with presymptomatic, experimentally induced, early blood-stage malaria (group 1) and in adults with naturally acquired symptomatic malaria residing in Cameroon, West Africa (group 2). A baseline set of data derived from 22 malaria-naïve uninfected U.S. volunteers was used for comparisons of gene expression in samples from groups 1 and 2. We considered this baseline sample set appropriate for these comparisons in the absence of data from samples from malaria-naïve Cameroonian subjects, which could not be collected under the approved clinical protocol. The correlation of the mean gene expression level of baseline samples to the mean gene expression level of samples from 22 subjects in group 1 and 15 subjects in group 2 exceeded 97%, indicating the comparability of gene expression between data sets collected and processed at the Walter Reed Army Institute of Research and at the Johns Hopkins School of Public Health for both U.S. and Cameroonian volunteers.

Differentially expressed genes from groups 1 and 2 relative to the baseline sample data set were identified in separate analyses using SAM with a 1% FDR. Differentially expressed genes identified by SAM were grouped according to whether they were induced or repressed relative to the baseline. The differentially expressed genes were sorted into lists of genes that were induced/repressed in group 1 but not in group 2 and vice versa or that were differentially induced or repressed in both groups. The results of this analysis are shown in Table 1.

TABLE 1. Numbers of differentially expressed genes[a]

Data set	No. of genes	
	Induced	Repressed
Group 1 only	408	1,125
Group 2 only	3,895	7,763
Group 1 and 2 (shared)	1541	938

[a] Differentially expressed genes from groups 1 (experimentally infected volunteers) and 2 (naturally infected volunteers) relative to the baseline sample data set were identified in separate analyses using SAM with a 1% FDR.

Innate immunity. As shown in Fig. 1, transcripts of the Toll-like pattern recognition receptors (PRR) Toll-like receptor 1 (TLR1), TLR2, TLR4, and TLR8 were significantly induced in both experimentally and naturally acquired malaria infections. In contrast, TLR3 and TLR5 were significantly repressed (Fig. 1A) in both groups. TLR9, a known non-DNA ligand for *P. falciparum*-purified hemozoin (6), is not represented on the U133A GeneChip. Other cell surface PRRs such as CD14 and CD36 and intracellular PRRs are likewise differentially induced in both groups (Fig. 1B and C). These observations are consistent with antigen-presenting cells in the peripheral circulation being the first to recognize PRRs in parasites (Fig. 1B and F). The activation or repression of TLR gene expression greatly affects the downstream expression of proinflammatory cytokines (46). The activation of TLRs by parasites or components thereof, such as glycosylphosphatidylinositol (GPI) (23) and hemozoin (6), transmit signals intracellularly through a common molecular pathway anchored by the adapter protein MyD88, which is upregulated in both groups (Fig. 1D). MyD88 associates with the intracellular tail of TLRs and functions to activate the transcription factor NF-κB, which in turn propagates signals to the nucleus to regulate the expression of proinflammatory cytokines (Fig. 1D and E) (13). Five categories of Fc receptors (*CD16*, *CD32*, *CD64*, *FCAR*, and FcεRI) are transcriptionally activated in both presymptomatic, experimentally acquired infection and acute, uncomplicated, naturally acquired infection (Fig. 1F). Associated with the activation of Fc receptor gene expression is the concomitant upregulation of membrane-bound and cytosolic genes critical to the functioning of the NADPH oxidase system, which generates reactive oxygen intermediates to which parasites are exquisitely sensitive (28, 35) (Fig. 1G).

IFN-γ signaling pathway activation in early infection. As shown in Fig. 2A, IFN-γ gene expression and members of the IFN-γ signaling pathway, which include IFN-γ receptors, JAK2 kinases, and the transcription factors *STAT1* and *IRF-1*, are significantly induced in presymptomatic subjects very early in infection, when the parasite burden is at the limit of detection. IFN-γ is a major proinflammatory cytokine that has direct antiparasitic activity and indirect immunoregulatory effects on many genes in experimental and human malaria infection (18, 25).

We adapted a strategy developed previously by Zhu and colleagues (49) to computationally identify a set of genes whose transcription during infection correlated strongly to that of *IRF-1* in order to assess whether the promoter regions of such genes contained similar DNA-binding sites for *IRF*. This approach identified 12 putative target genes whose expression correlated highly ($R = 0.96$) with that of *IRF-1* and whose consensus binding sites matched the known *IRF-1* binding sites from both the TRANSFAC (47) and JASPAR (36) databases (Fig. 2B and C). A heat map showing the relative expression for the 12 predicted genes and for *IRF-1* showed a high level of correlation in both uninfected and infected subjects (Fig. 2D).

IFN-γ induces the expression of several chemokines that play pivotal roles in the host's response to infection. We examined the transcriptional activation of three such chemo-

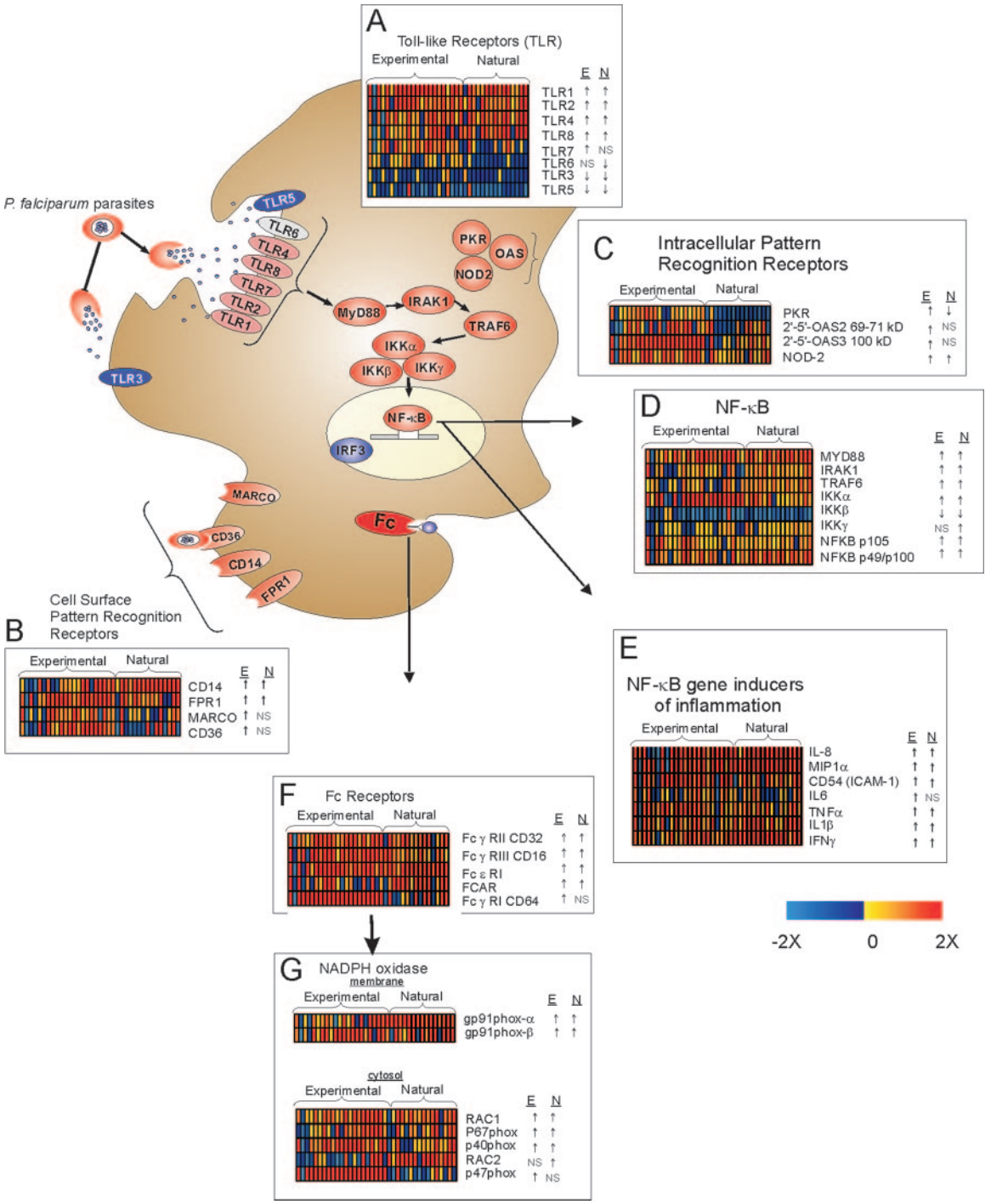

FIG. 1. Induction of innate immune responses in subjects experimentally (E) or naturally (N) infected with *P. falciparum* superimposed onto a conceptual model sketch of an antigen-presenting cell. Differentially expressed genes are represented by shaded boxes indicating the change in expression (*n*-fold) (upregulated, red; downregulated, blue) relative to the mean expression levels from uninfected subjects. Boxes of gene sets show the major pattern recognition receptors (A to C) that recognize and initiate the signaling pathways through NF-κB (D) leading to proinflammatory cytokine (E) production. Activation of the Fc receptor (F), which recognizes antibody-coated parasites, is induced early in infection and triggers reactive oxygen intermediates (ROIs) formed by the induction of the NADPH oxidase system (G). NS, not significant; PKR, double-stranded RNA-dependent protein kinase; MIP1α, macrophage inflammatory protein 1α.

kines, CCL2, CXCL9, CXCL10, in presymptomatic infection and correlated gene expression to protein expression. As shown in Fig. 3B, there is a direct correlation between the levels of transcriptional activation in these three chemokine genes and the amount of chemokine detected in the plasma. Importantly, these patterns distinguish the malaria-infected and uninfected samples.

Adaptive immunity. We investigated the scope of major histocompatibility complex (MHC) gene activation in malaria infection by analyzing the expression of genes that process and

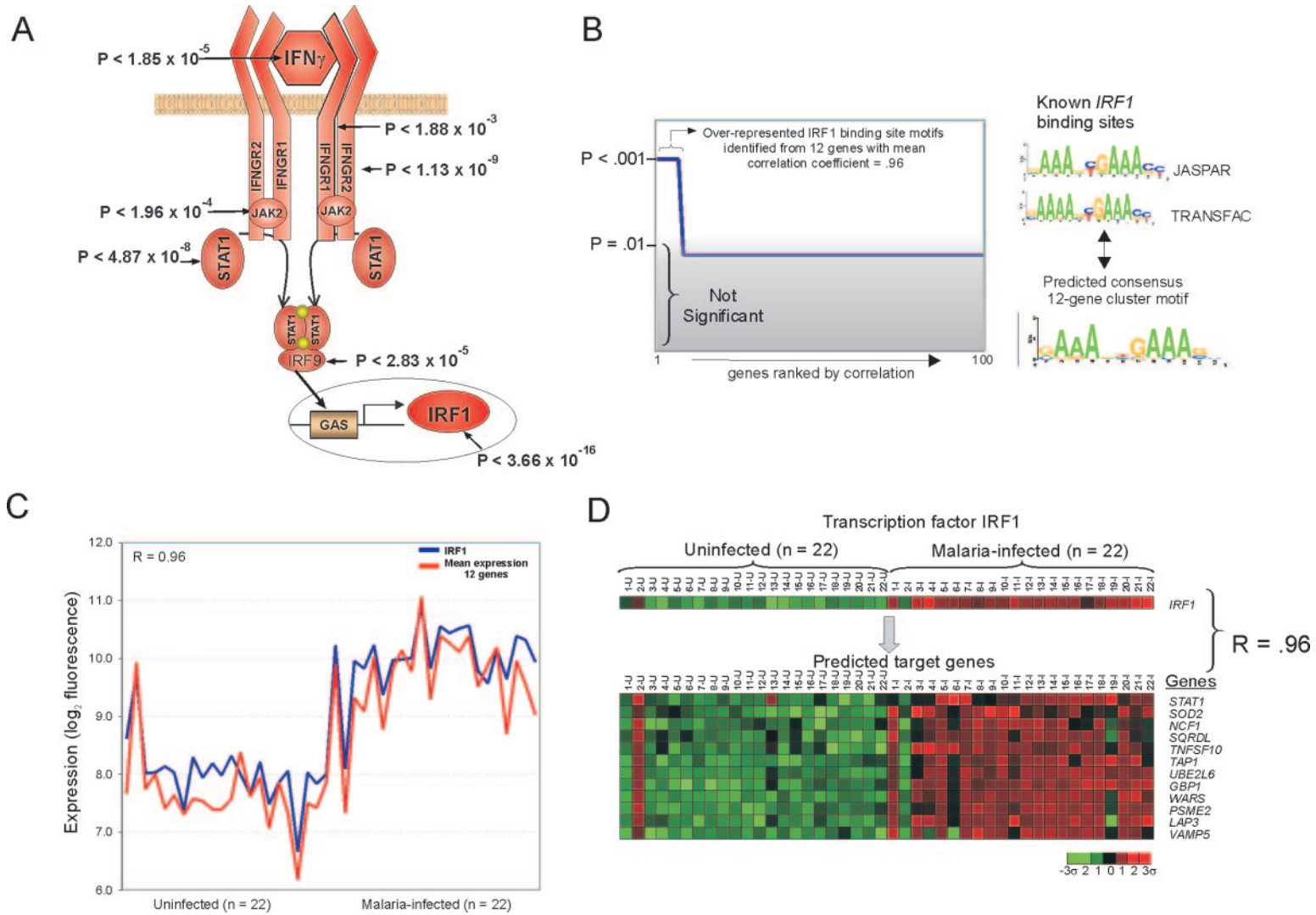

FIG. 2. IFN-γ signaling pathway and gene regulation by *IRF-1*. (A) IFN-γ pathway genes induced (red) in 44 PBMC samples from 22 paired infected and uninfected subjects. (B) Identification of statistically significant ($P < 0.001$) sets of genes (12 genes identified) whose promoter region binding sites are homologous to the known *IRF-1* DNA-binding site using CLOVER with the default cutoff significance set at a *P* value of 0.01. Promoter regions were aligned, and the consensus IRF-1 binding site was determined and depicted using a sequence logo generator. (C) Correlation plot of the normalized expression value of *IRF-1* from 22 uninfected and 22 malaria-infected samples with the mean gene expression level of 12 genes. (D) Heat map showing individual normalized gene expression of *IRF-1* with each of the genes identified by clover as having an overrepresented *IRF-1* binding site in the gene promoter.

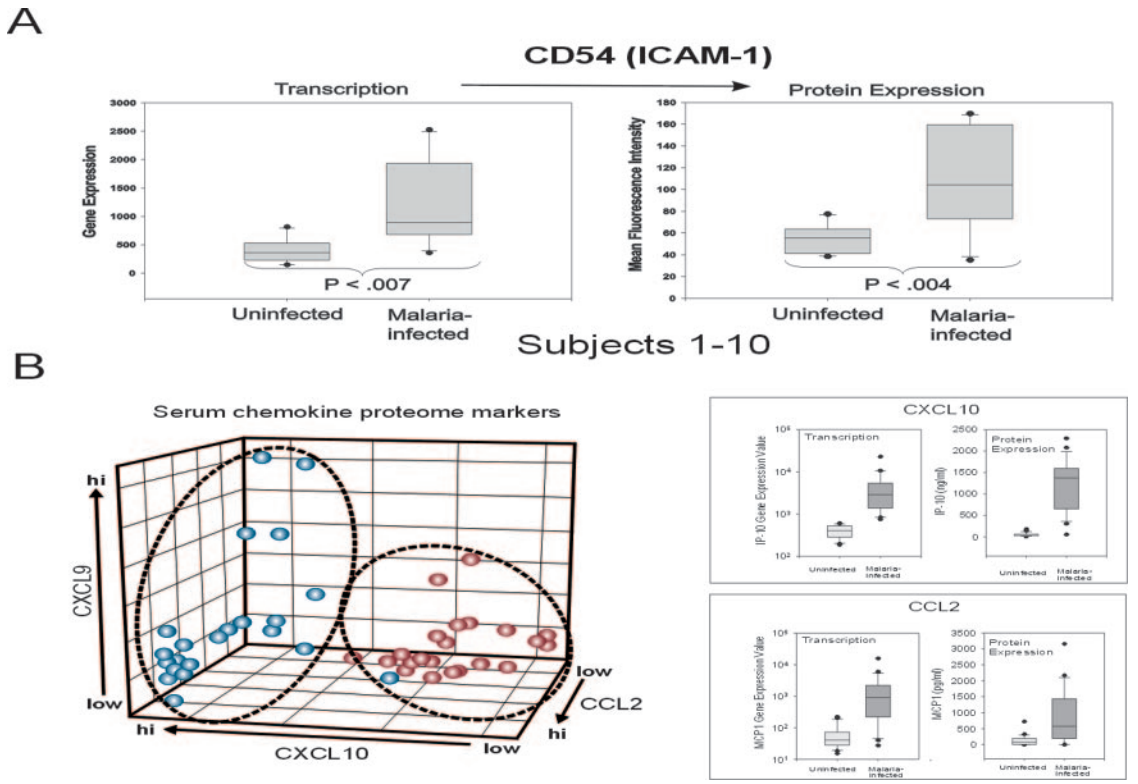

FIG. 3. Concordance between transcription of genes and translation of proteins in early malaria infection. (A) Induction of intracellular adhesion molecule 1 (ICAM-1) transcription and expression on the surface of PBMCs as detected by flow cytometry before and after malaria challenge. (B). CXCL10, CXCL9, and CCL2 chemokine expression in plasma samples from 22 subjects with significantly induced gene expression (left panel) and comparison to gene expression (right panel). MCP1, monocyte chemoattractant protein 1.

present antigens through either the class I (Fig. 4A and C) or class II (Fig. 4B and D) pathway. The 26S proteasome is composed of subunits comprising both a regulatory particle and the 20S catalytic core particle. As shown in Fig. 4A, 13 of the 14 protein subunits comprising the 20S catalytic component and genes of the immunoproteasome are significantly induced in >90% of the volunteers regardless of group. The loading of small peptide fragments onto MHC class I molecules, all of which are highly induced, requires efficient transport by chaperone molecules such as TAP1, TAP2, calreticulin, and tapasin. As shown in Fig. 4C, these genes are coordinately upregulated. Likewise, class II antigen-processing genes including cathepsins (Fig. 4B) and genes involved in class II presentation such as *HLA-DR*, *HLA-DQ*, *HLA-DM*, and *HLA-DP* are highly expressed in samples from infected individuals relative to samples from uninfected individuals (Fig. 4D).

Functional associations of differentially expressed genes. As shown in Table 2, processes associated with the immune and inflammatory responses were among the most prominently overrepresented GO terms. To derive a working model of the system biologies of the immune response to human malaria infection shared between presymptomatic and symptomatic infection, we selected specific subsets of genes belonging to the statistically significant GO categories of immune response, inflammatory response, and positive regulation through the NF-κB signaling pathway. The extent to which these genes bind to or are regulated by other genes within the data set was determined using the Pathway Architect software package, which creates biological interaction networks using a natural language processing algorithm to extract knowledge from known molecular interactions (30). Of the 126 genes that were analyzed, 100 were retained within the network layout, which is shown in Fig. 5.

In Fig. 5, genes linked to each other by binding or regulatory interactions are depicted as interconnecting lines between nodes. The central nodes of the biological network included the inflammatory cytokines TNF-α, IFN-γ, and IL-1β, which orchestrate the immune response signature program in both presymptomatic early malaria and clinically apparent natural malaria. These gene interactions were not unexpected, as their importance in malaria pathophysiology has previously been reported; nevertheless, the application of this approach provided a framework to examine gene interaction networks from lists of differentially expressed genes that differ between the two groups.

We next applied the same network analysis to construct a biological interaction pathway from a set of genes differentially induced in clinically apparent natural infection (group 2) but whose transcripts were found to be largely unchanged in presymptomatic malaria. As shown in Fig. 6, a complex set of interacting genes whose central nodes included genes involved in immune regulation (IL-10), apoptosis, and mitogen-activated protein (MAP) kinase signal transduction pathways was derived. The upregulation of the transcript that encodes the

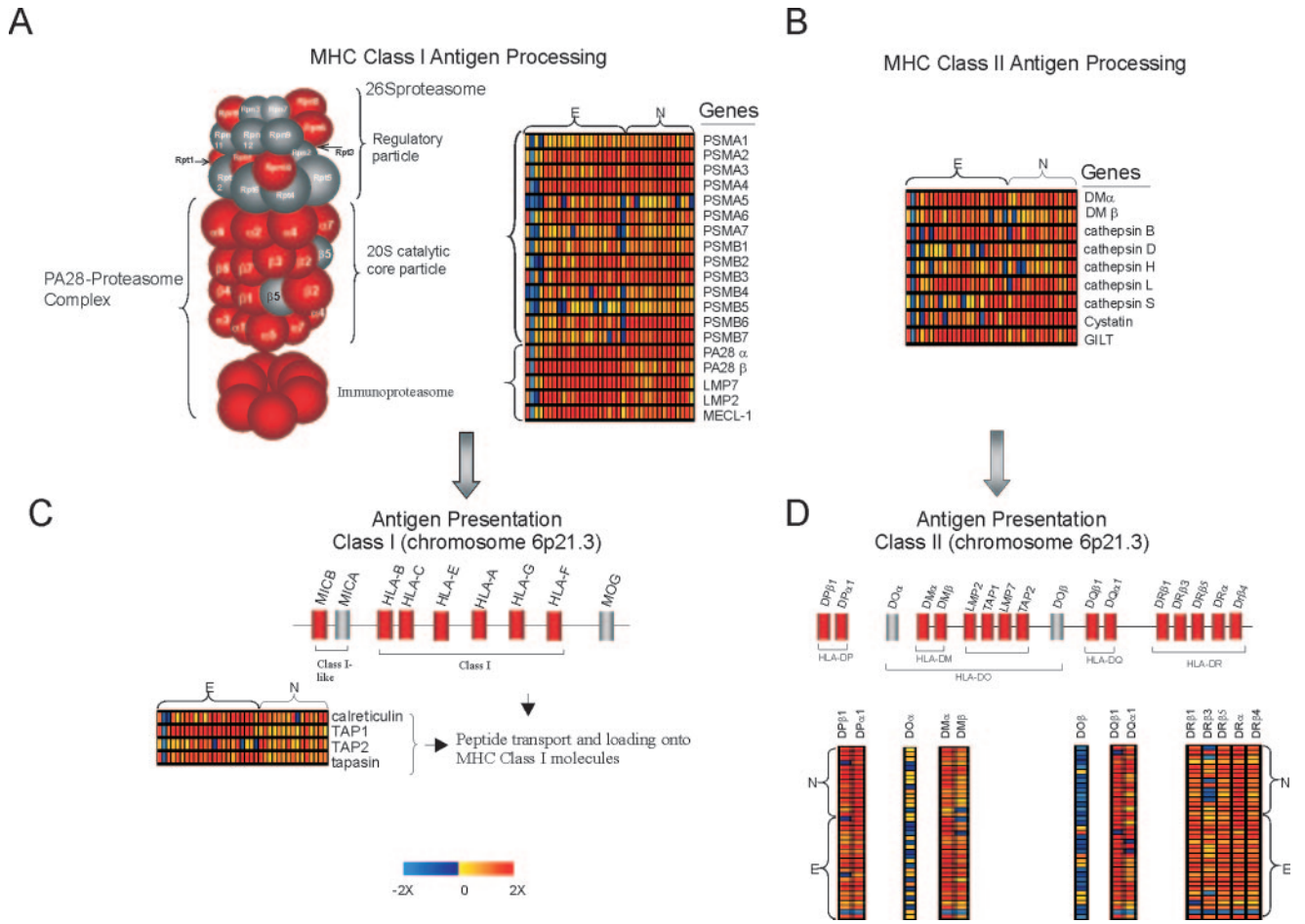

FIG. 4. Concordant gene expression and transcriptional activation of genes involved in antigen processing (A and B) and presentation to MHC class I (C) and class II (D) molecules in early experimental (E) and naturally acquired (N) malaria infection. Subunits of proteasome with increased (red), and decreased (blue) gene transcription levels relative to those of uninfected subjects are depicted in the figure and represent the consensus pattern of transcription. MHC class I and class II genes during malaria infection are color coded according to the change in gene expression and are positioned relative to each other on chromosome 6. Heat maps from individual subjects depict changes in gene expression relative to uninfected subjects.

cytokine IL-10 plays a central regulatory role that influences a wide variety of other genes specific to patients with acute, uncomplicated, naturally acquired infection (17).

The observations summarized in Fig. 7 for genes involved in the death receptor pathway regulated by *TNFSF6* (FasL) and genes known to be activated by MAP kinases including *MAPK14* (*p38*α and *p38*β) indicate that these genes are up-regulated in clinically apparent malaria but not in presymptomatic infection. These genes converge on the same functional process of cellular apoptosis whereby mitochondrial genes belonging to the Bcl-2 family of proteins act to regulate apoptosis by the release of cytochrome *c* along with changes in mitochondrial permeability leading to the activation of caspase 3. Furthermore, the up-regulation of *p38* (MAP) kinases leads to the activation of a set of transcription factors, also shown in Fig. 7, that regulate cytokine production in PBMCs and sustain the proinflammatory cytokine stimulus in the infected host. The hypothesis that mitochondrial control of apoptosis is an important process in clinically apparent, naturally acquired malaria was supported by an independent analysis of overrepresented gene ontology terms shown in Table 2. The upregulation of genes involved in apoptosis may reflect the normal feedback loops in regulatory T cells that undergo programmed cell death after exposure to antigen, which occurs temporally after blood-stage malaria infection.

Fever, heat shock, and glycolysis. Fever is frequently the host's initial response to infection, which is triggered by parasites interacting with host surface receptors (TLRs) that activate a signaling cascade resulting in the elaboration of the inflammatory cytokine most responsible for fever, namely, IL-1β (8, 9). As shown in Fig. 8A, the inflammatory cytokine IL-1β and CASP1, the interleukin 1-converting enzyme responsible for cleaving the IL-1β precursor into its mature form, were significantly induced in both early, presymptomatic, experimentally acquired infection and clinically apparent, naturally acquired infection independent of the presence of fever. However, the major type I IL-1 receptor was significantly upregulated in naturally infected febrile persons, while no statistical change in gene expression was observed in the afebrile subjects from group 1.

TABLE 2. Significantly overexpressed GO terms derived from differentially expressed genes upregulated in presymptomatic experimental and naturally acquired malaria[a]

Data set, GO term, and description	No. of genes	*P* value
Group 1 only		
GO:0006955, immune response	22	3.03E−05
GO:0006954, inflammatory response	17	4.84E−07
GO:0016567, protein ubiquitination	12	1.54E−02
GO:0007267, cell-cell signaling	12	3.92E−02
GO:0000074, regulation of cell cycle	10	4.83E−02
GO:0042981, regulation of apoptosis	7	3.81E−03
Group 2 only		
GO:0006355, regulation of transcription	324	5.49E−03
GO:0005739, mitochondrion	202	6.13E−09
GO:0006412, protein biosynthesis	104	2.56E−09
GO:0006915, apoptosis	79	5.03E−03
GO:0000398, nuclear mRNA splicing	73	3.57E−07
GO:0006886, intracellular protein transport	71	1.60E−04
Groups 1 and 2 (shared)		
GO:0006955, immune response	108	<1E−10
GO:0006118, electron transport	52	2.21E−04
GO:0006915, apoptosis	48	4.19E−04
GO:0006512, ubiquitin cycle	44	1.62E−04
GO:0006886, intracellular protein transport	43	5.29E−05
GO:0006457, protein folding	41	6.41E−05

[a] The table shows six representative statistically significant (corrected *P* value) GO terms derived from Onto Express for each analysis. A full list of GO terms are available in Table S2 in the supplemental material.

Since regulatory and counterregulatory genes act to control excessive inflammation resulting in IL-1β production, we next assessed the expression of several genes associated with hereditary autoinflammatory disorders that result in periodic fever syndromes, such as familial Mediterranean fever, in which a mutation in the pyrin gene is associated with excessive IL-1β production (9, 40). Indeed, downregulation of pyrin gene expression was observed in all 15 adults with symptomatic, naturally acquired clinical malaria compared to presymptomatic, experimentally infected subjects with malaria, while the *NALP3* gene that opposes the action of the pyrin gene was significantly induced in both these groups (Fig. 8A).

Heat shock proteins, known as stress proteins, are induced in cells exposed to a wide range of environmental stressors such as infection and temperature. We examined the gene expression profiles for a wide array of heat shock genes in early, presymptomatic, experimentally acquired infection. Gene expression levels for these genes were elevated in early presymptomatic disease and in acute uncomplicated disease (Fig. 8B). Using 10 human orthologous genes that comprise the glycolytic enzyme pathway, we mapped the corresponding gene expression patterns in each of these groups. As shown in Fig. 8C, gene activities for 9 of 10 genes were elevated in early infection and in uncomplicated disease, mirroring the pattern of gene activity observed previously in experimental murine models of both *Plasmodium berghei* (39) and *Plasmodium yoelii* (37) malaria. The concordance in gene expression patterns observed between experimental murine models of malaria and human

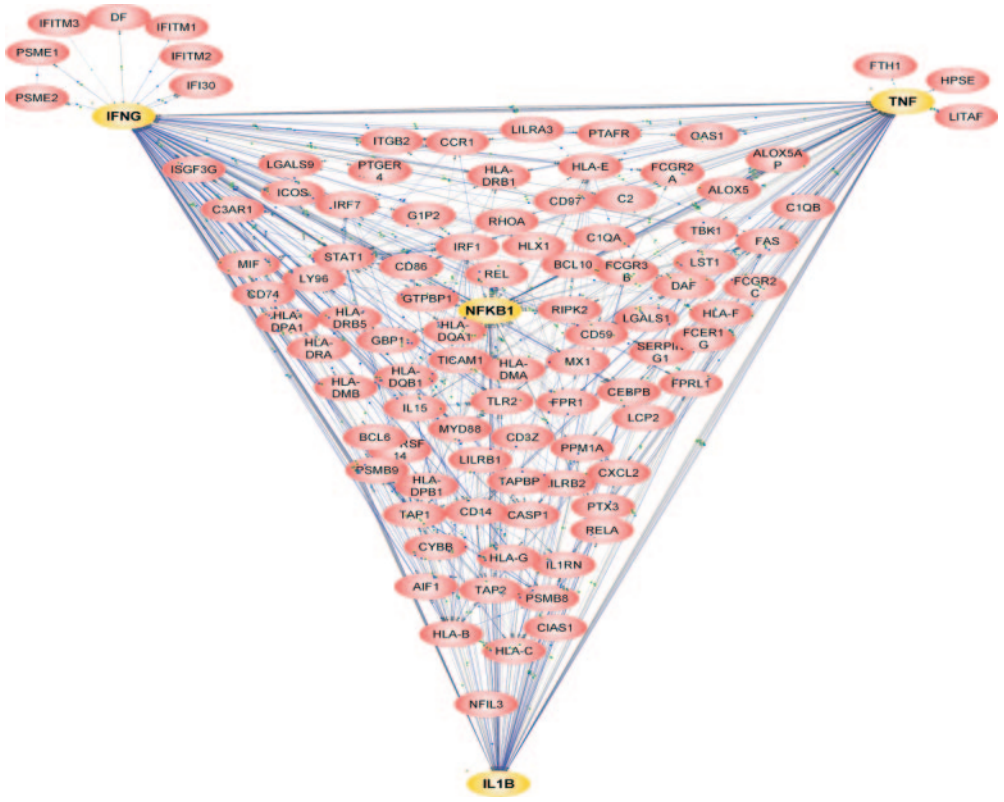

FIG. 5. Central nodes of the biological network generated by Pathway Architect assembly of GO designations for the most prominently overrepresented genes shared in presymptomatic and symptomatic infections including the inflammatory cytokines TNF-α, IFN-γ, and IL-β.

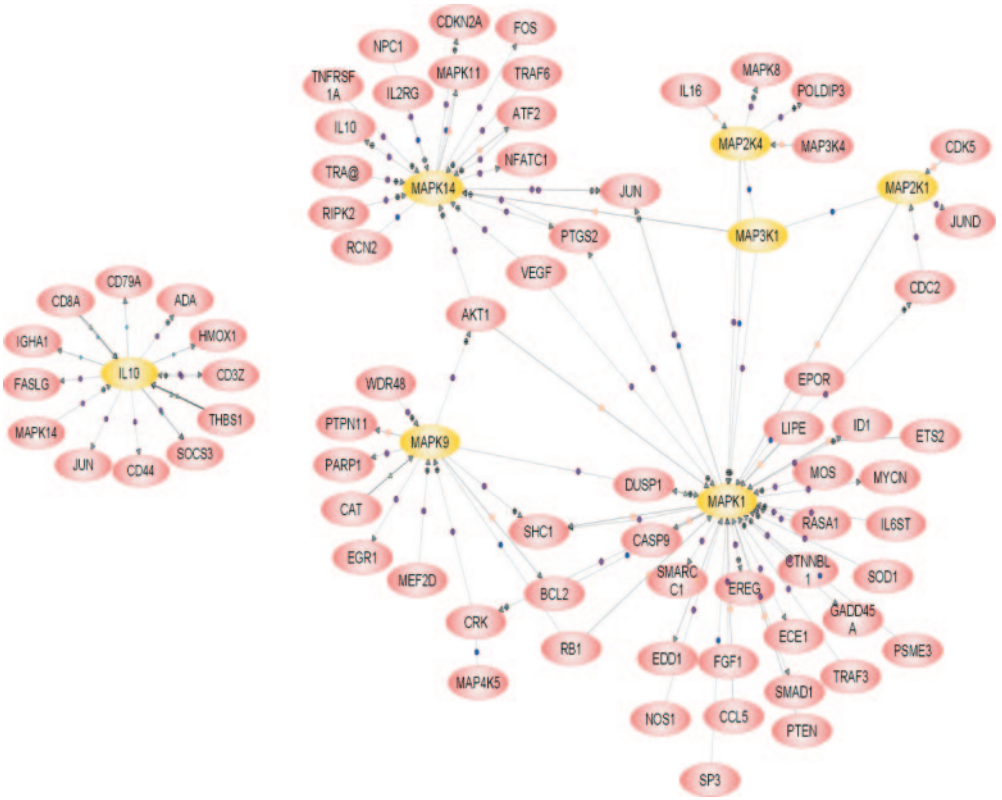

FIG. 6. Biological interaction pathways of genes induced in natural but not in experimental infection representing the immunological regulatory IL-10 and MAP kinase pathways created using Pathway Architect software. VEGF, vascular endothelial growth factor.

malaria for both metabolic and immune response pathways provides a practical and convenient avenue to explore complex gene interaction pathways in animal models followed by clinical investigation of human severe malaria.

DISCUSSION

Gene expression patterns in presymptomatic persons with parasite density levels lower than the clinical threshold (<10 parasites/μl blood) and in persons with naturally acquired, clinically apparent infection were remarkably similar despite differences related to intensity of infection, clinical illness, and length of infection. The results of our study are appropriately considered in the context of both the microarray methodology employed to generate the global expression patterns that we observed and the PBMC compartment that was sampled. It is difficult to predict with certainty what levels of change in transcript result in meaningful changes in the function of a gene product, as this may differ for individual proteins and may depend on complex interactions and metabolic processes. Sampling of mRNA levels at any given time point is a static measure and may not completely capture patterns of expression that might be punctate or cyclical. Expression profiles in this study were drawn from a mixed population of cells and thus cannot be attributed to a specific cell type or lineage. The composition of this compartment reflects the conditions in the periphery and not those in germinal centers, lymph nodes, or remote sites of infection and inflammation.

It is also important to consider that samples from subjects naturally infected with malaria are likely to have been obtained later in the disease stage than those obtained from persons monitored closely for manifestations of infection acquired in a clinical trial. This temporal difference may be reflected in the exacerbation of markers associated with inflammation and cell death in the samples from Cameroon. Caution should be taken to prevent an overinterpretation of the results, as it should also be considered that the general health of the individuals from the Cameroon as well as their genetic makeup are likely to be different from those of American subjects.

Considering the limitations of the technique and the compartment sampled, the observations drawn from the simultaneous assessment of transcripts by microarrays in a body compartment easily obtained are nevertheless reliable and powerful tools to guide the building of conceptual frameworks of what processes are associated with the immune response to malaria infection. Patterns of gene expression that were common to presymptomatic individuals and clinical infection volunteers provide important clues into how the innate and adaptive immune systems respond to parasite antigens that evoke inflammation.

Alterations in gene expression levels assessed at the transcript level cannot directly predict corresponding alterations in the levels of functional proteins. Increases or decreases in mRNA detected on microarrays do not reflect the downstream processes of protein synthesis, modification, folding, stability, and appropriate cellular location that comprise the criteria for

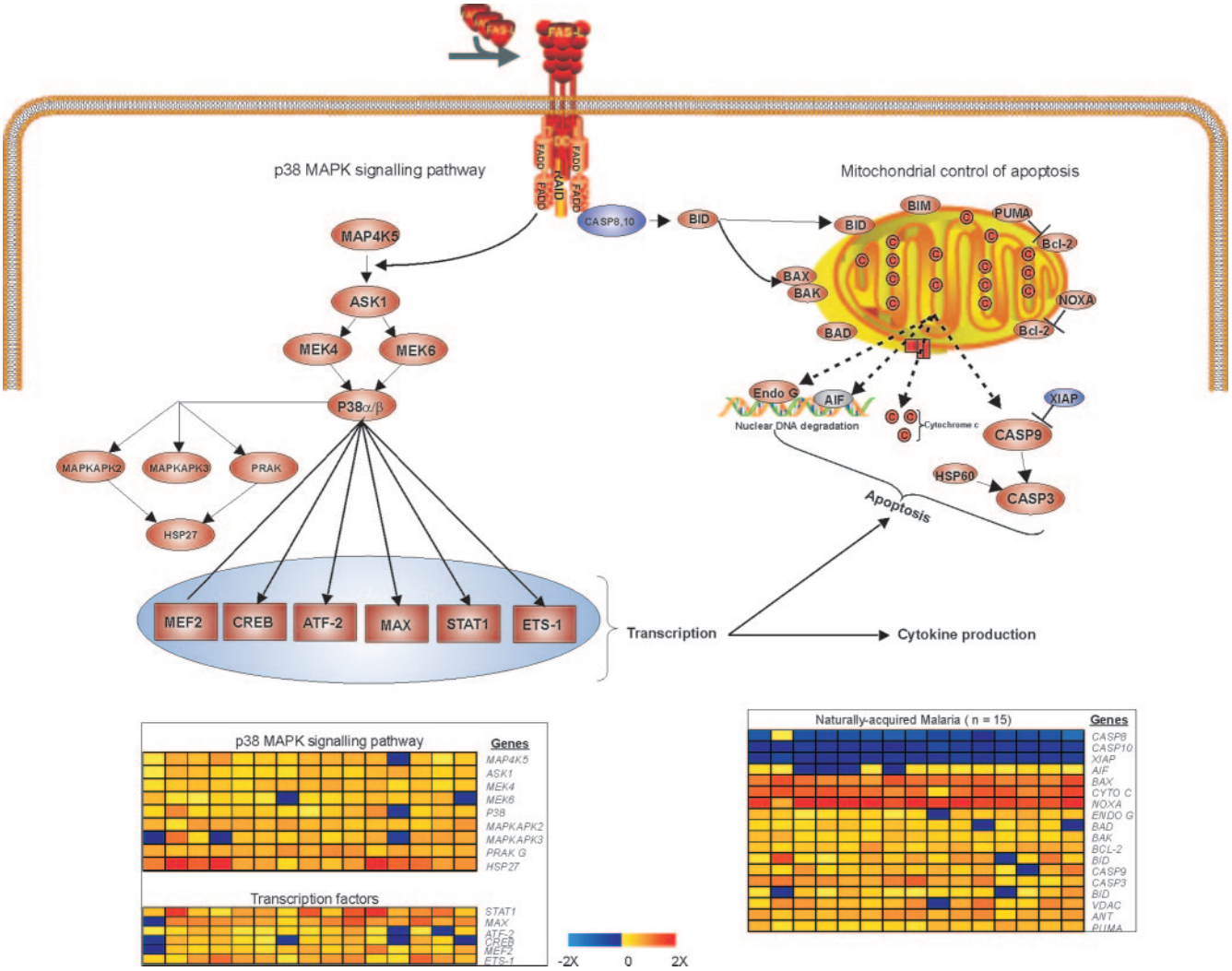

FIG. 7. Schematic showing transcriptional upregulation in genes from the p38 MAP kinase signaling pathway (red shaded circles) initiated by Fas ligand and its receptor, the transcription factors (red shaded boxes) regulated by the kinases, and the genes regulating mitochondrial control of apoptosis from subjects with naturally acquired malaria infection from whom genes were significantly upregulated relative to the mean expression levels from uninfected subjects.

a functional gene product. Despite such limitations, high-density microarrays offer a broad, system-wide window into molecular processes that regulate gene expression, as illustrated in this paper by a set of genes induced by the transcription factor *IRF-1* that were predicted solely from transcriptional profiles.

Patterns of gene expression that are common to both groups provide important clues to how innate and adaptive immune responses evolve by sensing parasite antigens that evoke inflammation and protective immune responses. GPIs from *P. falciparum* are major mediators that contribute to the pathogenesis of malaria by inducing proinflammatory cytokines and mediators such as TNF-α, IL-6, IL-12, and nitric oxide (41, 48). Our observations from PBMCs collected from presymptomatic or symptomatic individuals recapitulate recently reported findings in vitro when macrophages were stimulated by purified GPIs from *P. falciparum* parasites (48). Whether subjects are exposed to parasites in vivo, or whether cells are stimulated in vitro with parasite-derived GPIs, similar intracellular signaling pathways such as p38 kinase and NF-κB are activated, resulting in proinflammatory responses.

It has been previously established that the level of proinflammatory cytokine secretion in malaria infection correlates directly with the severity of disease (1, 2, 18). In particular, TNF-α has numerous biological effects on host cells including the upregulation of adhesion molecules such as intracellular adhesion molecule 1 (CD54) on the surface of postcapillary endothelium (4, 10, 43) to which malaria-infected erythrocytes sequester (31). In addition, CD54 upregulation does not necessarily require TNF-α for CD54 gene expression, since glycosylphosphatidylinositol from malaria parasites is sufficient to induce CD54 expression (38). CD54 on PBMCs is significantly upregulated in early presymptomatic infection as well as in clinical malaria, and the transcriptional activity correlates directly with the protein's translation and cell surface expression as detected by flow cytometry, corroborating the

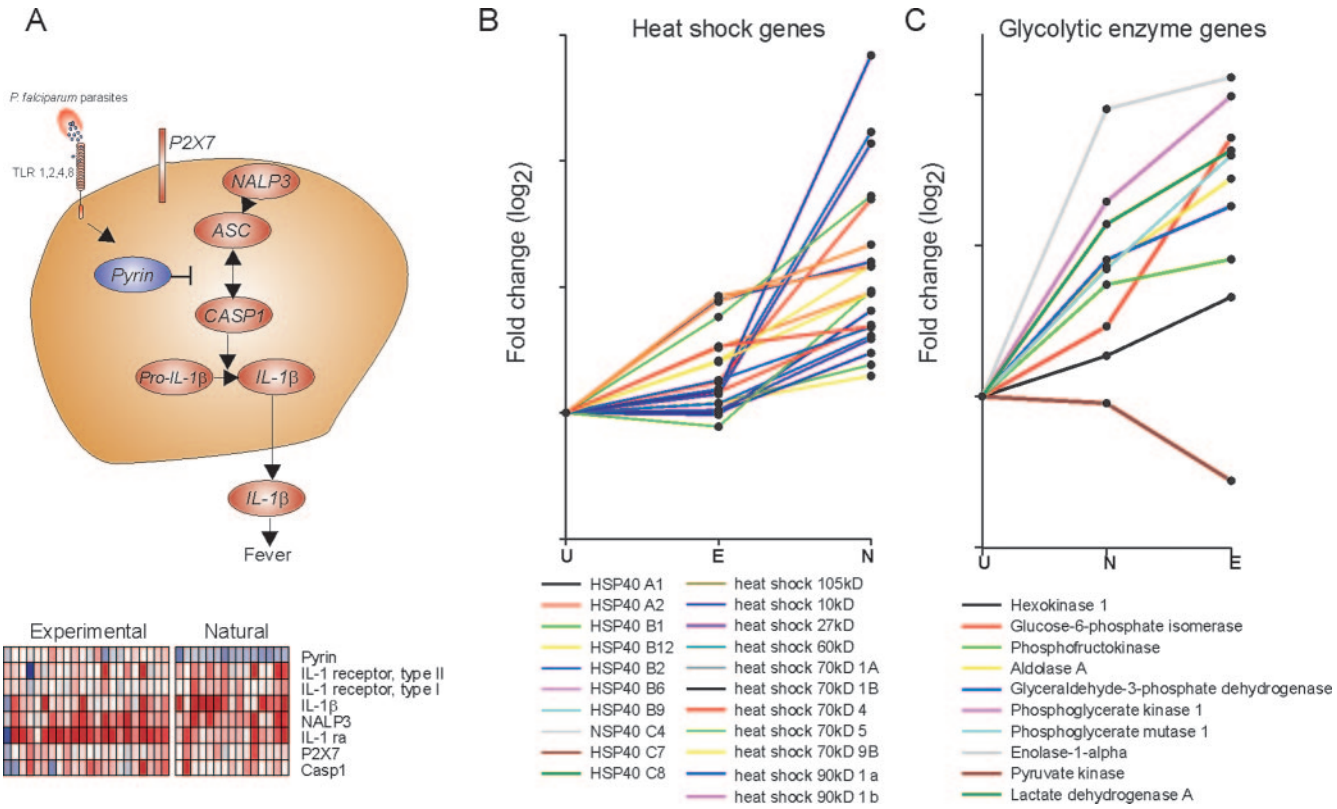

FIG. 8. Differential expression in functional processes related to the regulation of fever (A), heat shock gene expression (B), and lactate production via the glycolytic enzyme pathway (C) in experimental (labeled E) and naturally acquired (labeled N) malaria infection relative to uninfected subjects (labeled U). The shaded circles represent differentially expressed upregulated (red) or downregulated (blue) genes.

concordance between transcriptional profiling and protein expression (Fig. 3A).

The mechanisms by which innate immunity responds to protozoan pathogens and induces protective immune responses are initially dependent upon a the pathogen's interaction with TLRs and other pattern recognition receptors on the cell surface. In the conceptual model of a putative dendritic cell (Fig. 2), TLRs 1, 2, 4, and 8 are significantly upregulated at an early phase of infection, when the parasite density is at the limit of detection and the majority of subjects are afebrile. Several parasite virulence factors that interact with Toll-like receptors, including hemozoin with TLR9 (6) and GPIs with TLR2 and TLR4 (23, 29), that induce signal transduction pathways through the transcription factor NF-κB, which in turn propagates signals to the nucleus to regulate the expression of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 and chemokines such as macrophage inflammatory protein 1α and IL-8 (22, 46), have recently been demonstrated. Significantly, expression levels of these proinflammatory cytokines correlate directly with the severity of disease (1, 18).

However, from results presented in this study, it appears that the mere presence and expression of the cytokine transcripts TNF-α, IL-1β, and IFN-γ alone is not sufficient to explain the pathogenesis of malaria, as these are similarly induced in presymptomatic and symptomatic subjects. Multiple other factors that influence disease severity may include the magnitude (*n*-fold change over baseline) of gene expression, the level of protein expression of inflammatory mediators, and host genetic polymorphisms. Furthermore, immune regulatory genes that converge upon the upregulation of IL-10 in naturally infected, clinically ill patients with uncomplicated disease but that are not induced in early infection suggest a critical role of regulatory cytokines that act to dampen and counter excessive inflammation.

Adaptive immune mechanisms are induced prior to the development of symptomatology and include the initiation of processes related to phagocytosis and antigen processing and presentation. The upregulation of Fc receptor transcription before antibodies appear may function to prepare the adaptive immune system to counter parasite multiplication as specific antibodies are produced. The internalization of parasitized erythrocytes through scavenger receptors such as CD36 and the opsonization of antibody-coated organisms by Fc receptors may cooperate to dampen parasite multiplication (27, 32, 42). During phagocytosis, macrophages produce reactive oxygen intermediates through the activation of a multicomponent NADPH oxidase, a gene family that was strongly induced in both early, presymptomatic, experimentally infected and naturally infected clinically ill persons with uncomplicated disease. Parasite multiplication is initiated by the phagocytosis of free merozoites or malaria-infected erythrocytes by monocytes or tissue macrophages. The multicomponent NADPH oxidase is composed of subunits located in the cytosol and plasma membrane. When phagocytes are stimulated by parasites or

their degradative products, the cytosolic subunits comprising $p67^{phox}$, $p40^{phox}$, and $p47^{phox}$ are translocated to the plasma membrane along with the small GTPases RAC1 and RAC2, forming a new complex with $p22^{phox}$ and the alpha and beta subunits of $gp91^{phox}$ (28, 35). This fully assembled complex uses cytosolic NADPH to reduce oxygen to form superoxide anion, which exerts both antimalarial and cytotoxic effects on the host (33).

In a recently reported study that examined genome-wide expression in children from Kenya with acute uncomplicated malaria or other febrile illness, Griffiths and colleagues assessed expression profiles in whole blood using a cDNA lymphochip microarray (16). Significant differences between that study and ours, which include experimental design (acute pediatric malaria and other febrile illnesses versus experimental and adult uncomplicated malaria), microarray platform (cDNA lymphochip versus genome-wide oligonucleotide microarray), cell population sampling (whole blood versus PBMCs), statistical basis for selection of differentially expressed genes (2.5-fold change difference in 4 of 28 samples versus SAM and *t* tests), and gene clustering methods (hierarchical clustering versus gene ontology functional classification and network construction), make extensive comparisons difficult. The lack of baseline samples from uninfected healthy subjects in the study reported previously by Griffiths et al. and its focus on associations between gene features and clinical parameters within whole-blood samples, which includes a large population of transcripts derived from neutrophils, likely account for some of the differences in expressed genes between the two studies. There was, however, concordance between the two studies when changes in individual genes were more closely examined. For instance, similar changes in genes associated with Toll-like receptors (TLR1 and TLR4), heat shock proteins, transcription factors, MAP kinases (MAPK14), T-cell activation (granzymes), Fc receptors, death domain receptors (TNFRSF6), and markers of inflammation (guanylate-binding protein and IL-1β) were upregulated in both studies.

Human challenge models of infectious disease offer opportunities to identify the earliest transcriptional events in individuals exposed to lethal pathogens to which they are completely naïve at a point in the infectious process that precedes the development of symptomatic disease. Biomarkers detected by this process may help direct therapeutic interventions that target and kill pathogens prior to the onset of inflammatory or pathological processes that lead to illness and even death. Our long-term goal is to understand the immunogenetics of infectious diseases by defining both the usual and the aberrant transcriptional responses in persons exposed to pathogens.

ACKNOWLEDGMENTS

We thank M. Nau and A. Arnold for expert technical assistance. We acknowledge Roger Moyou for access to patients in Cameroon.

This work was supported in part by Cooperative Agreement number W81XWH-04-2-0005 between the U.S. Army Medical Research and Materiel Command and the Henry M. Jackson Foundation for the Advancement of Military Medicine and by the U.S. Army Medical Research and Materiel Command. Financial support was also provided by a pilot grant from the Malaria Research Institute of Johns Hopkins University. N.D.W. is supported by an NIH Director's Pioneer Award and Fogarty International Research Scientist and AIDS Training Research Program awards. Additional support was provided by the U.S. Embassy Camaroon, the JHU Cameroon Program, and the government of Cameroon.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. The authors have no commercial or other association that might pose a conflict of interest.

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Editor: W. A. Petri, Jr.