Immune Response to *Mycobacterium tuberculosis* and Identification of Molecular Markers of Disease

Mercedes Gonzalez-Juarrero¹, Luke C. Kingry^{1,2}, Diane J. Ordway¹, Marcela Henao-Tamayo¹, Marisa Harton¹, Randall J. Basaraba¹, William H. Hanneman³, Ian M. Orme¹, and Richard A. Slayden^{1,2}

²Rocky Mountain Regional Center of Excellence, ¹Department of Microbiology, Immunology and Pathology, and ³Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, Colorado

The complex molecular events that occur within the host during the establishment of a *Mycobacterium tuberculosis* infection are poorly defined, thus preventing identification of predictive markers of disease progression and state. To identify such molecular markers during *M. tuberculosis* infection, global changes in transcriptional response in the host were assessed using mouse whole genome arrays. Bacterial load in the lungs, the lesions associated with infection, and gene expression profiling was performed by comparing normal lung tissue to lungs from mice collected at 20, 40, and 100 days after aerosol infection with the H37Rv strain of *M. tuberculosis*. Quantitative, whole lung gene expression identified signature profiles defining different signaling pathways and immunological responses characteristic of disease progression. This includes genes representing members of the interferon-associated gene families, chemokines and cytokines, MHC, and NOS2, as well as an array of cell surface markers associated with the activation of T cells, macrophages, and dendritic cells that participate in immunity to *M. tuberculosis* infection. More importantly, several gene transcripts encoding proteins that were not previously associated with the host response to *M. tuberculosis* infection, and unique molecular markers associated with disease progression and state, were identified.

Keywords: tuberculosis; transcriptional response; immunity

Tuberculosis is a world health problem, with reports estimating that as much as one third of world's population is infected with the tubercle bacilli, and 2 million people die every year as a result. The prevalence and incidence of tuberculosis worldwide remain high despite the intense efforts by the World Health Organization-sponsored directly observed therapy campaign and the availability of routine diagnostic methods, a vaccine, and effective chemotherapy. Disease management has been hindered by the inability to objectively assess disease state, thus preventing a rational guide for patient management aimed at reducing the rate of relapse and spread. The characterization of the complexities of the immune response at different stages of infection, and identification of informative molecular markers, is one of the most difficult aspects of understanding pathogenesis and disease progression and in developing new strategies and tools to diagnose and treat disease.

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Correspondence and requests for reprints should be addressed to Richard A. Slayden, Ph.D., Rocky Mountain Regional Center of Excellence and Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523-1682. E-mail: richard.slayden@colostate.edu

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CLINICAL RELEVANCE

This work characterizes the global host response at different stages of disease and can be used as a foundation for further development of molecular markers that best correlate with disease state or responses to vaccines and chemotherapy.

Pulmonary exposure to *Mycobacterium tuberculosis* elicits both host innate and adaptive immune responses, yet the bacteria are still capable of establishing chronic infections. Much of the information about the immune response to infection and host susceptibility has been compiled from various techniques, including passive cell transfer (1–3), the use of mice with targeted gene disruptions (4, 5), as well as PCR, enzyme-linked immunosorbent assay, and flow cytometric methods (6, 7). Several studies have reported the transcriptional responses to *M. tuberculosis* infection, but none have analyzed global transcriptional changes in the host genes at different stages of chronic pulmonary infection with *M. tuberculosis* (8–14). This has resulted in limited knowledge of the dynamic transcriptional changes that occur during infection and disease progression. These data are needed to better understand the differences in host response at various stages of disease and to correlate these transcriptional changes with lesion morphology and disease progression. Thus, more comprehensive and global studies focusing on the host response to infection with *M. tuberculosis* have the potential to identify previously unrecognized immune mechanisms that better correlate with disease progression and signature profiles that are predictive of protection.

In the present work, transcriptional profiling of uninfected mouse lungs and lungs harvested during development of disease (Day 20 through Day 100 of the infection) allowed for the correlation of the host immune response with the bacterial load and resulting pathology. The results of this study provide a global view of the dynamic changes in the host response throughout the progression of disease and identified gene transcripts expressing molecules that were poorly associated with the host response to *M. tuberculosis* infection. In addition to defining the trends in the immune response during pulmonary infection, molecular markers of disease progression were identified. Together, this work characterizes the host response at different stages of disease and can be used as a foundation for further characterization of molecular mechanisms controlling disease progression as well as further development of molecular markers that best correlate with disease state or responses to vaccines and chemotherapy.

MATERIALS AND METHODS

Low Dose Aerosol Infection

Six- to eight-week-old specific pathogen-free female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were infected with *M. tuberculosis* H37Rv by low-dose aerosol exposure using a Glass-Col (Terre

Haute, IN) aerosol generator calibrated to deliver 50 to 100 viable bacteria into the lungs. Bacterial load in the lungs of representative mice at each time point were determined by plating serial dilutions of organ homogenates on Middlebrook 7H11 medium and enumeration of colony-forming units after incubation at 37°C for 3 weeks.

Histologic Analysis

Lungs from mice ($n = 5$) in the same groups were harvested for histologic analysis on Days 0, 20, 40, and 100 of the infection. The accessory lung lobe from each mouse was fixed with 10% formalin in phosphate-buffered saline (PBS). Sections from these tissues were stained using hematoxylin and eosin. All sections were scored by a board certified veterinary pathologist, blinded to treatment groups. Lesion scores were based on percent lung involvement as well as specific morphologic features like lesion necrosis and proportion of various cell types that make up the granulomatous inflammatory responses. All pictures were taken with a DP70 Olympus camera (Olympus, Center Valley, PA).

Transcriptional Analysis

Global expression analysis was performed using Affymetrix mouse genome 430 2.0 array. For analysis, uninfected mice and mice at 20, 40, and 100 days of the infection ($n = 15$ per group) were killed, and the lungs were excised and subjected to homogenization in Trizol. Nucleic acids were partitioned from other cellular products by addition of chloroform (1:2, vol/vol) and centrifugation at $13,000 \times g$ for 20 minutes at 4°C. The resulting aqueous layer was removed and total RNA was precipitated with isopropanol (1:1, vol/vol). DNase treatment was used to remove DNA contamination, and total RNA was purified using an RNeasy miniprep kit (Qiagen, Valencia, CA). RNA from five mice per biological group was pooled for labeling, resulting in replicates representing uninfected mice and mice at 20, 40, and 100 days of infection. Global expression analysis was performed using Affymetrix mouse genome 4302.0 gene chips (Affymetrix, Santa Clara, CA). RNA labeling and hybridization was per standard protocols provided by Affymetrix.

Data reduction and analysis of uninfected mice compared with mice at 20, 40, and 100 days of the infection was performed using Genesifter software (geospiza, Seattle, WA) (15), and Benjamini and Hochberg was used for adjusting the P value from a comparison test based on the number of tests performed. A principal component analysis (PCA) comparing uninfected mice and mice at 20, 40, and 100 d of the infection was performed to determine the similarity of the gene response to infection at each time point. PCA is a statistical method of analysis for determining the key variables in a multidimensional data set that explain the differences in the observations, and can be used to simplify the analysis and visualization of multidimensional data sets (16, 17). Hierarchical clustering and self-organizing mapping (SOM) was used to identify patterns and partitioning to separate data into discrete groups. Quantitative real-time PCR analysis was performed in triplicate from three biologically independent samples of total RNA from the lungs of uninfected mice and from mice 20, 40, and 100 days after challenge. The fold increase in signal over the 18S housekeeping gene was determined using the $\Delta\Delta\text{Ct}$ calculation.

RESULTS

Progress of Disease and Development of Lung Pathology

C57BL/6 mice were infected with a low dose aerosol of *M. tuberculosis* H37Rv to determine the host response at different time intervals after infection. The bacterial burden, pathology, and host transcriptional response was determined at 20, 40, and 100 days of the infection. Consistent with previous observations, after aerosol exposure the bacteria in the lungs grew in an exponential manner for 20 days, after which time the number of cultivable bacteria remained constant, giving rise to a characteristic chronic infection (Figure 1A) (20). Examination of the histopathology revealed that lung lesions were mild at Day 20 and mostly restricted to peribronchial and perivascular parenchyma (Figure 1B). As the infection progressed, lesions developed into organized structures containing large aggregates of lymphocytes and epithelioid macrophages, with increasing numbers of highly vacuolated cells (referred to as foamy cells) (Figure

1C). By Day 100 of the infection, lesions were extensive and consisted of coalescing foci of mixed inflammation containing predominately lymphocytes, macrophages, and numerous foamy cells (Figure 1D). During the acute (Day 20), subacute (Day 40), and chronic stages of infection (Days 40 and 100), histologic findings illustrate the dynamic nature of the immune and inflammatory responses as the disease progresses. To further confirm that the infection in this study was consistent with previous reports, we verified that IFN- γ and TNF- α expression increased over the course of infection (Figure 2). This information allows us to make a connection between the stage of infection, development of lesions, and activation of the host adaptive immune response.

Global Changes in the Transcriptional Response during the Chronic State of Infection

The global transcriptional response in the lungs of mice to *M. tuberculosis* infection was assessed through whole mouse genome DNA microarray analysis. Compared with uninfected C57BL/6 mice (Day 0), a total of 3,308 open reading frames (ORFs), displayed a 1.5-fold or greater change in expression (P value < 0.05) in the lungs from infected C57BL/6 mice over a 100-day infection (see Table E1 in the online supplement for a complete list of data). This represents altered expression of approximately 9% of the annotated transcripts in the mouse genome. To determine the similarity of the gene response to infection at each time point, we used the principal components analysis (PCA) to cluster the transcriptional response of uninfected mice and of mice at Day 20, Day 40, and Day 100 after exposure and visualized the analysis with a scatter plot (Figure 3A). This multivariate technique reduces the complexity of the transcriptional response data and preserves closeness between biological data sets, so that time points residing in close proximity in many dimensions are configured close to each other in the scatter plot. Accordingly, data analysis indicated that the overall host transcriptional response in the lungs during *M. tuberculosis* infection was significantly different between uninfected mice and mice after 20 days, 40 days, or 100 days of infection, with the later time points being highly concordant. The global ontology profile of the differentially expressed genes revealed that there is a dynamic change in genes involved in cellular metabolism and physiology, and genes involved in regulation and response to stimulation being the next dominant response (Figure 3B). Ontology analysis of the transcriptional response of immune-specific genes substantiate this global analysis because genes associated with stimulus and physiologic processes are the most altered in expression, followed by cellular metabolism, regulation, and development (Figure 3C). Together, global analysis demonstrates that there is a large transcriptional response and that the response is progressive from Day 20, to Days 40 and 100, and in particular a massive induction of genes involved in host defense, including both cell-mediated and humoral responses.

Trends in the Host Immune Response to Infection with *M. tuberculosis* over 100 Days

Host-pathogen interaction. *M. tuberculosis* infection in the lungs elicited components of the innate immune response involved in bacterial recognition. The Toll-like Receptor *tlr2* and CD14 were induced at 20 days after infection and remained elevated throughout the infection, whereas *tlr1*, *tlr13*, *tlr4*, and *tlr12* were only induced at 40 and 100 days after infection (Table 1, section I). These data are in agreement with previous reports indicating the importance of the TLR2 (21, 22) in recognition of *M. tuberculosis*. Although TLR4 have also been reported in this process (9, 23), our study suggests that these receptors, as well as *tlr12* and *tlr13*, only become expressed late in infection. The

Figure 1. Development of pulmonary granulomatous lesions in mice exposed to *Mycobacterium tuberculosis*. Growth curve of *M. tuberculosis* in C57BL/6 mice after low dose aerosol exposure. (A) Bacterial load in the lungs was monitored at Day 1, Day 20, Day 40, and Day 100 of infection by plating and enumeration of colony-forming units (CFU). Sections of formalin-fixed and paraffin-embedded lung tissue of C57BL/6 mice exposed to *M. tuberculosis* on Days 20, 40, and 100 after *M. tuberculosis* challenge were visualized with hematoxylin and eosin (H&E) staining. (B) Mild interstitial pneumonia and moderately sized lesions observed at Day 20 of the infection with *M. tuberculosis*. (C) Large aggregates of lymphocytes were seen within the epithelioid macro-

phage and increasing numbers of foamy highly vacuolated cells at Day 40. (D) Development of organized inflammatory multifocal granulomas containing lymphocytes and macrophages and large numbers of foamy cells by Day 100 of infection. Pictures were taken with an IX70 Olympus microscope with an attached ZP70 digital camera. Total magnification: A–C, $\times 4$; insets, $\times 2$.

differential up-regulation of Toll-like receptors over time supports the notion of a change in bacterial recognition pathways and subsequent activation of immune responses between the early and chronic stage of infection. Complement receptors (CR3 or CD11b/CD18 and CR4 or CD11c/CD18), and various Fc receptor transcript elements expressing for FcγRIIIA (CD16), FcγRI, FcγRII (CD64), FcγRIIB (CD32), and FcγRIII (CD16), were also induced throughout the infection. Furthermore, several studies have reported an important role of C-type I lectins such as *Ly75* (DEC-205;CD205), *Mrc1* (mannose receptors;CD206), and *Cd209* (DC-SIGN;CD209) in the recognition of *M. tuberculosis* (24, 25). However, our data indicated that while C-type I lectins displayed

only modest induction, the very poorly studied C-type II lectins transcripts expressing for Mincle, DECTIN-2, MDL-1, and DECTIN-1 were highly up-regulated. (Table 1, section I). This observation is consistent with a recent report that described Dectin-1 as promoter of mycobacterial-induced IL-12p40 production by dendritic cells (26). Furthermore, we believe this information could be used as a foundation for further characterization of molecular mechanisms involved in bacterial recognition.

T cell response. At 20 days after infection, the T cell response was already polarized toward a TH1 response, which is thought to be predominantly targeted toward elimination of the bacteria.

Figure 2. Real-time PCR of IFN-γ and TNF-α at different times of infection. (A) IFN-γ expression at Day 0 (D0), Day 20 (D20), Day 40 (D40), and Day 100 (D100). (B) TNF-α expression at Day 0 (D0), Day 20 (D20), Day 40 (D40), and Day 100 (D100). The primer and probe sequences for murine IFN-γ and TNF-α were previously published (18, 19). Data are presented using the mean values ($n = 5$) using replicated samples and duplicate or triplicate assays. A parametric method, the Student *t* test, was used to assess statistical significance between groups of data.

Figure 3. Analysis of gene expression ontology of global response and physiology of immune responses. (A) Principal component analysis and scatter plot of the transcriptional response of uninfected mice (D0) and of mice at Day 20 (D20), Day 40 (D40), and Day 100 (D100) of infection with *M. tuberculosis*. (B) Global ontology profile and (C) ontology of the immune associated genes. Data displayed are (A) based on 1,310 genes and (B) based on 183 immune discriminant genes (1.5-fold or greater alteration; P values < 0.01).

The main cytokine of this pathway, IFN- γ , was induced throughout the infection along with 19 known IFN-regulated genes. In addition, the induction of IFN- γ -associated GTPases (*Ifi47*, *Ifit1*, *Ifi35*, *Ifi44*, *Ifit2*, *Ifit3*, *Ifit4*, *igtp*) and two members of the IFN-signaling pathway (*Stat1* and *Irf7*) was observed. Importantly, *ifi27*, *ifi44*, *ifit1*, *ifit3*, *ift3*, and *irf7* were induced at 20 days after infection, but were down-regulated as the infection progressed (Table 1, section II). Altogether, these data indicated that despite an increased expression of the IFN- γ , there was not a corresponding increase in the activation of the IFN- γ pathway throughout the 100 days of infection. This information suggests that the IFN- γ pathway reaches a (maximal) saturation level of activation during later chronic infection which is not enhanced by continued stimulation.

The soluble mediator TNF- α with strong inflammatory and apoptotic capacity synergizes with IFN- γ during the TH1 response (27). While TNF- α transcriptional activity as determined by microarray analysis was modest, other TNF- α -associated genes were induced during infection. Specifically, TNF family-like genes *Tnfaip2*, *Tnfaip3*, *Tnfrsf*, *Tnfrsf1b*, *Tnfrsf9*, *Tnfrsf12*, *Tnfrsf1*, *Traf1*, *Traf3*, *Traf3ip3*, and *Trafid1* were induced, substantiating the contribution of TNF- α in the inflammatory process in response to *M. tuberculosis* infection (Table 1, section II).

Antimycobacterial activity and arrest of bacterial growth. The cytokines IFN- γ stimulated the production of effector molecules such as inducible nitric oxide synthase (iNOS) and the phagocyte oxidase (phox) which are the major source of antimicrobial reactive nitrogen and oxygen intermediaries, respectively, known to kill intracellular *M. tuberculosis* (28–31). Specifically, *nos2* (iNOS) was induced throughout infection while *ncf1* (p47 Phox), *ncf2* (p60 Phox), *ncf4* (p40 Phox) induction being limited to day 20 and day 40 of infection (Table 1-III). Similarly, there were substantial changes of several transcripts encoding chelators of proteins also known to influence bacterial growth. Thus, the transcriptional response of type II arginase, (*arg*), lactotransferrin and indoleaminepyrrole 2,3 dioxygenase (IDO) which are known to deplete the environment of arginine, iron and tryptophan respectively were also upregulated (32–34). The hypoxia-responsive factor, HIF1a was upregulated. While HIF-1a is induced under hypoxic conditions, there are oxygen-independent mechanisms that can also induce HIF-1a expression. This is consistent with the fact that *M. tuberculosis* lesions in mice fail to develop hypoxia as do other species (35). However, along with it, the induction of *Lip1* (lysosomal acid lipase 1), *Laptm5*

(lysosomal-associated protein transmembrane), the *Cd68* (macrosial lysosomyl glycoprotein), the *Cd53* (membrane late endosomes) and the Rab proteins whose expression are known to favor a niche for bacteria survival were also observed (36, 37) (Table 1- III). Together, these data indicate that as the infection progresses, the host-bacterial interaction is a dynamic process resulting in a limitation of available nutrients and development of an adequate niche capable of promoting bacterial survival.

Cellular activation mechanisms and differentiation of immune cell populations. Activation markers associated with antigen-presenting cells and with T cells were also induced through the course of the infection. Leukocyte specific antigens CD2, CD45 and CD52, and T cell-specific markers CD3 γ , CD3 δ , CD4, CD8 β , CD8 α , and CD44, IL7r, or CD5 associated with activation of memory T cells were induced by Day 20 and continued to be transcriptionally active throughout the infection. Importantly, other genes encoding proteins with either unknown or poorly described roles in tuberculosis immunity displayed altered expression as well. Specifically, the signaling lymphocyte activating molecule (*Slam*)-related receptors (SRR) *Slamf6*, *Slamf7*, and *Slamf8* (CD150) and CD244 (2B4) molecules and its ligand *Cd48* molecules were induced during infection. Similar trends were observed for *Cd274* (also known as B7-H1 and PD-L1), a co signaling molecule involved in regulating T cell immunity *in vivo* (Table 1, section IV).

The main killing mechanism of CD8 T cells is through secretion of cytotoxic granules (38). An interesting observation was that among the nine granzymes included in this study, the gene encoding Granzyme K (*Gzmk*) and the gene encoding the perforin gene *Prfl* were highly up-regulated in response to infection (Table 1, section IV). While previous work in the murine model of tuberculosis reported a nonessential role of perforin and granzyme cytotoxic granules during the course of the infection (38), GzmK was not included in these studies. Interestingly, recent reports described that circulating levels of GzmK are significantly elevated in virus-infected patients and that it triggers rapid cell death independently of caspase activation similar to GzmA (39, 40).

Genes encoding the markers CD40, CD83, CD86, and class II MHC antigens associated with activation of lung-resident antigen cell presentation were also up-regulated. Although previous reports describe decreased production of MHC class II antigens during an *M. tuberculosis* infection, this disagreement is explained by the fact that down-regulation of MHC antigen

TABLE 1. DIFFERENTIALLY EXPRESSED HOST GENES DISCUSSED IN TEXT

Annotation	Name	Unique ID	Days of the Infection		
			20	40	100
I. Host-Pathogen Interaction					
Toll-like receptor 1	<i>Tlr1</i>	AF316985	1.66	2.78	3.3
Toll-like receptor 2	<i>Tlr2</i>	NM_011905	3.6	3.47	5.14
Toll-like receptor 4	<i>Tlr4</i>	AF185285	-1.9	1.93	2.88
Toll-like receptor 12	<i>Tlr12</i>	BB745017	-1.01	1.89	3.1
Toll-like receptor 13	<i>Tlr13</i>	BI655907	1.25	2.31	2.22
CD11a/Integrin alpha L	<i>Itgal</i>	NM_008401	2.84	3.86	5.36
CD11b/ Integrin alpha M	<i>Itgam</i>	NM_021334	2.04	2.94	4.22
CD11c/ Integrin alpha X	<i>Itgax</i>	NM_009841	1.58	3.18	4.86
CD14	<i>Cd14</i>	NM_008404	2.26	2.45	3.8
CD18/Integrin β 2	<i>Itgb2</i>	NM_007642	2.66	4.06	5.51
Fc γ RIIIA (CD16)	<i>Fcgr3a</i>	BC027310	8.87	7.91	17.17
Fc ϵ R γ	<i>Fcer1g</i>	NM_010185	3.42	3.55	6.81
Fc γ RI (CD64)	<i>Fcgr1</i>	AF143181	3.55	3.42	6.14
Fc γ RIIB (CD32)	<i>Fcgr2b</i>	M14216	2.58	5.3	5.9
Fc γ RIII (CD16)	<i>Fcgr3</i>	NM_010188	3.17	4.29	7.93
C-type lectin/ DEC-205	<i>Ly75</i>	NM_013825	1.19	1.15	1.96
C-type lectin /DC-SIGN/CD209	<i>Cd209a</i>	AF374470	-1.85	-1.39	-1.97
CD206 Mannose receptor, C type 1	<i>Mrc1</i>	BB528408	-1.64	-1.09	-1.14
Macrophage activation 2 like	<i>Mpa2l</i>	BG092512	11.85	5.39	11.49
C-type lectin domain family 4, member a3	<i>Clec4a3</i>	AK014135	2.47	3.61	4.95
C-type lectin domain family 4, member e/MINCLE	<i>Clec4e</i>	NM_019948	15.54	38.68	52.78
C-type lectin domain family 4, member n/DECTIN-2	<i>Clec4n</i>	NM_010819	5.15	11.04	15.58
C-type lectin domain family 5, member a/MDL-1	<i>Clec5a</i>	NM_021364	2.36	2.94	2.73
C-type lectin domain family 7, member a/DECTIN-1	<i>Clec7a</i>	NM_020008	2.69	3.42	5.11
II. Immune Response					
Interferon gamma	<i>Ifng</i>	K00083	6.13	6.62	13.98
Interferon activated gene 205	<i>Ifi205</i>	AI481797	4.01	3.15	4.55
Interferon, alpha-inducible protein 27	-	NM_019440	4.82	4.58	6.05
Interferon gamma inducible protein 30	<i>Ifi27</i>	AY090098	3.35	1.04	1.57
Interferon-induced protein 35	<i>Ifi30</i>	NM_023065	2.26	2.18	3.35
Interferon-induced protein 44	<i>Ifi35</i>	AW986054	2.28	1.6	2.39
Interferon gamma inducible protein 47	<i>Ifi44</i>	BB329808	7.47	2.18	4.5
Interferon-induced protein with tetratricopeptide repeats 1	<i>Ifi47</i>	NM_008330	5.14	3.95	6.13
Interferon-induced protein with tetratricopeptide repeats 2	<i>Ifit1</i>	NM_008331	5.03	2.58	3.68
Interferon-induced protein with tetratricopeptide repeats 3	<i>Ifit2</i>	NM_008332	3.97	2.16	3.02
Interferon induced transmembrane protein 3	<i>Ifit3</i>	NM_010501	5.33	2.37	3.65
Interferon-induced protein 44	<i>Ifitm3</i>	BC010291	2.42	1.48	2.26
Interferon gamma induced GTPase	<i>Igtp</i>	NM_018738	6.69	4.75	7.98
Interferon inducible GTPase 1	<i>Iigp1</i>	BM239828	12.51	7.7	14.34
Interferon regulatory factor 1	<i>Irf1</i>	NM_008390	2.64	2.4	3.17
Interferon regulatory factor 5	<i>Irf5</i>	NM_012057	2.47	2.38	3.17
Interferon regulatory factor 7	<i>Irf7</i>	NM_016850	6.43	3.18	3.85
Interferon regulatory factor 8	<i>Irf8</i>	BG069095	2.45	3.48	4.69
Signal transducer and activator of transcription 1	<i>Stat1</i>	AW214029	6.77	6.25	10.63
Signal transducer and activator of transcription 2	<i>Stat2</i>	AF088862	4.03	2.81	3.4
TNF- α -induced protein 2	<i>Tnfaip2</i>	NM_009396	2.24	5.53	7.28
TNFreceptor superfamily, member 1b	<i>Tnfrsf1b</i>	M60469	2.75	2.83	4.16
Fas ligand (TNF superfamily, member 6)	<i>Fasl</i>	NM_010177	1.71	3.06	2.9
Lymphotoxin B	<i>Ltb</i>	NM_008518	2.47	3.77	6.57
Tnf receptor-associated factor 1	<i>Traf1</i>	BG064103	2.4	1.63	2.73
Tnf receptor-associated factor 3	<i>Traf3</i>	U21050	1.68	2.35	3.1
III. Host Antimycobacterial Activity and Bacterial Cell Growth Arrest					
Nitric oxide synthase 2, inducible, macrophage	<i>Nos2</i>	AF065921	1.9	3.81	5.67
Neutrophil cytosolic factor 1/p47phox	<i>Ncf1</i>	AI844633	4.39	6.12	2.36
Neutrophil cytosolic factor 2/p67 phox	<i>Ncf2</i>	NM_010877	1.73	2.83	1.44
Neutrophil cytosolic factor 4/p40phox	<i>Ncf4</i>	NM_008677	3.3	4.85	2.08
Arginase 1, liver	<i>Arg1</i>	NM_007482	1.5	-1.11	-1.01
Arginase type II	<i>Arg2</i>	NM_009705	2.15	3.13	3.08
Lactotransferrin	<i>Ltf</i>	NM_008522	2.67	1.02	1.31
Indoleamine-pyrrole 2,3 dioxygenase/IDO	<i>Indo</i>	NM_008324	7.26	12.44	11.26
Lysosomal acid lipase 1	<i>Lip1</i>	AI596237	2.71	4.11	1.13
Lysosomal-associated protein transmembrane 5	<i>Laptm5</i>	AF364050	3.37	4.87	3.25
CD68/macrosial lysosomal glycoprotein	<i>Cd68</i>	AK014135	3.62	6.26	11.48
CD53 antigen/membrane late endosomas	<i>Cd53</i>	BM239715	2.13	1.98	3.11
RAB GTPase activating protein 1-like	<i>Rab10</i>	BF465974	1.45	1.49	2.09
RAB guanine nucleotide exchange factor 1	<i>Rab8a</i>	BC019990	-1.03	1.95	2.35
RAB geranylgeranyl transferase, b subunit	<i>Rab20</i>	BG066967	1.81	1.82	3.06

(Continued)

TABLE 1. (CONTINUED)

Annotation	Name	Unique ID	Days of the Infection		
			20	40	100
RAB guanine nucleotide exchange factor 1	<i>Rab24</i>	NM_009000	1.17	1.48	2.3
Rab geranylgeranyl transferase, a subunit	<i>Rab32</i>	NM_026405	1.46	2.7	2.76
IV. Immune Cell Populations and Cellular Activation					
CD2	<i>Cd2</i>	NM_013486	2.13	2.06	2.58
CD45	<i>Ptpcr</i>	BM239436	2.26	2.25	4.39
CD52 antigen	<i>Cd52</i>	NM_013706	3.68	3.86	8.1
CD3 γ antigen,	<i>Cd3g</i>	BB398671	4.02	5.63	7.13
CD3 δ antigen	<i>Cd3d</i>	M58149	3.88	3.56	5.31
CD4	<i>Cd4</i>	NM_013487	2.12	3.21	4.16
CD8 β ₁	<i>Cd8b1</i>	NM_013488	2.19	2.36	3.14
CD8 α	<i>Cd8a</i>	BB154331	1.66	2.73	2.28
CD5 antigen	<i>cd5</i>	NM_007650	3.23	4.06	6.6
CD44	<i>Cd44</i>	U12434	1.88	1.93	4.01
CD127/Interleukin 7 receptor)	<i>Il7r</i>	AI573431	2.01	3.01	5.1
CD150 (SLAM family member 8)	<i>Slamf8</i>	U34882	7.72	16.54	28.39
CD319 (SLAM family member 7)	<i>Slamf7</i>	X67128	2.71	2.64	4.95
CD150 (SLAM family member 6)	<i>Slamf6</i>	X66083	2.65	2.77	4.14
CD177	<i>Cd177</i>	BC024587	4.18	3.57	4.66
CD244 (2B4-SRR)	<i>Cd244</i>	AK016183	2.56	1.83	2.5
CD247	<i>Cd247</i>	AF248636	2.32	1.55	2.55
CD274 antigen PD-L1 (B7-H1 or CD274),	<i>Cd274</i>	BC027283	7.77	7.12	10.63
CD300A antigen	<i>Cd300a</i>	BE634960	2.26	2.86	3.7
CD300 antigen like family member F	<i>Cd300lf</i>	AK017904	2.51	1.97	4.62
Granzyme A	<i>Gzma</i>	NM_010370	3.24	1.74	2.05
Granzyme B	<i>Gzmb</i>	NM_013542	3.13	2.64	2.8
Granzyme K	<i>Gzmk</i>	AB032200	6.98	8.91	13.5
Lymphocyte antigen 6 complex, locus I	<i>Ly6i</i>	AF232024	15.86	16.43	28.53
Immunoresponsive gene 1	<i>Irg1</i>	L38281	20.78	12.68	25.21
V. Inflammatory Response					
IL-1 β	<i>Il1b</i>	BC011437	3.27	2.18	3.3
IL-2	<i>Il2</i>	AF065914	-1.14	-1.04	-1.09
IL-10	<i>Il10</i>	NM_010548	1.52	1.12	1.23
IL-12 β	<i>Il12b</i>	AF128214	1.24	2.73	2.14
IL-15	<i>Il15</i>	NM_008357	1.08	1.6	2.13
IL-16	<i>Il16</i>	BC026894	2.14	2.35	2.98
IL-21	<i>Il21</i>	NM_021782	2.07	1.36	1.94
IL18bp	<i>Il18bp</i>	AF110803	4.44	7.7	12.3
IL-4i	<i>Il4i1</i>	NM_010215	1.6	2.56	3.81
Interleukin 10 receptor, alpha/CD210	<i>Il10ra</i>	NM_008348	-1.02	2.85	3.29
Interleukin 12 receptor, beta 1/CD212	<i>Il12rb1</i>	NM_008353	1.92	2.09	3.19
Interleukin 12 receptor, beta 2/CD212	<i>Il12rb2</i>	NM_008354	2.16	1.21	-1.04
Interleukin 13 receptor, alpha 1/CD213A	<i>Il13ra1</i>	S80963	1.71	2.38	2.15
Interleukin 17 receptor A/CD217	<i>Il17ra</i>	AK010040	2.44	2.06	3.02
Interleukin 18 receptor accessory protein	<i>Il18rap</i>	AV247387	2.24	2.42	2.31
Interleukin 1 receptor antagonist	<i>Il1rn</i>	M57525	3.82	4.16	8.19
Interleukin 2 receptor, alpha chain/CD25	<i>Il2ra</i>	AF054581	-1.01	1.08	1
Interleukin 2 receptor, beta chain/CD122	<i>Il2rb</i>	M28052	4.44	2.78	4.56
Interleukin 2 receptor, gamma chain/CD132	<i>Il2rg</i>	L20048	2.04	2.77	4.05
Interleukin 3 receptor, alpha chain/CD213	<i>Il3ra</i>	NM_008369	1.43	1.31	2.03
Interleukin 7 receptor/CD127	<i>Il7r</i>	AI573431	2.01	3.01	5.1
Chemokine (C-C motif) ligand 2	<i>Ccl2</i>	AF065933	2.07	1.21	1.64
Chemokine (C-C motif) ligand 5	<i>Ccl5</i>	NM_013653	7.21	16.02	21.76
Chemokine (C-C motif) ligand 7	<i>Ccl7</i>	AF128193	2.94	1.53	2.61
Chemokine (C-C motif) ligand 8	<i>Ccl8</i>	NM_021443	16.46	15.72	32.95
Chemokine (C-C motif) ligand 12	<i>Ccl12</i>	U50712	2.59	2	2.73
Chemokine (C-C motif) ligand 19	<i>Ccr1</i>	AV231648	4.25	3.15	4.01
Chemokine (C-C motif) receptor 2	<i>Ccr2</i>	BB148128	2.47	2.39	3.47
Chemokine (C-C motif) receptor 5	<i>Ccr5</i>	D83648	4.08	5.22	7.91
Chemokine (C-C motif) receptor 7	<i>Cxcl1</i>	NM_008176	2.54	3.6	6.59
Chemokine (C-X-C motif) ligand 5	<i>Cxcl5</i>	NM_009141	3.97	1.91	4.58
Chemokine (C-X-C motif) ligand 9	<i>Cxcl9</i>	NM_008599	54.11	72.14	122.61
Chemokine (C-X-C motif) ligand 10	<i>Cxcl10</i>	NM_021274	17.33	14.98	18.95
Chemokine (C-X-C motif) ligand 13	<i>Cxcl13</i>	AF030636	2.9	3.75	5.97
Chemokine (C-X-C motif) ligand 16	<i>Cxcl16</i>	BC019961	2.93	4.14	7.83
Chemokine (C-X-C motif) receptor 3	<i>Cxcr3</i>	NM_009910	4.56	5.08	7
Chemokine (C-X-C motif) receptor 6	<i>Cxcr6</i>	AF301018	5.38	7.75	10.67
Serum amyloid A 3	<i>Saa3</i>	NM_011315	57.97	47.09	76.47
Caspase 1	<i>Casp1</i>	BC008152	2.47	2.96	4.25

(Continued)

TABLE 1. (CONTINUED)

Annotation	Name	Unique ID	Days of the Infection		
			20	40	100
Caspase 4,	<i>Casp4</i>	NM_007609	3.54	2.89	4.53
Caspase 7	<i>Casp7</i>	NM_007611	1.89	2.09	2.41
VI. Immunosuppression					
TGF- α	<i>Tgfa</i>	M92420	3.56	1.54	-1.51
TGF- β ₁	<i>Tgfb1</i>	NM_011577	2.84	4.54	5.39
TGF- β -induced	<i>Tgfb1</i>	NM_009369	4.03	3.12	3.84
CD274 antigen PD-L1	<i>Cd274</i>	BC027283	7.77	7.12	10.63
CD72 antigen/ antibody switching	<i>Cd72</i>	BC003824	3.59	3.97	6.5
Indoleamine-pyrrole 2,3 dioxygenase/IDO	<i>Indo</i>	NM_008324	7.26	12.44	11.26
BCL2-antagonist/killer 1	<i>Bak1</i>	NM_007523	3.31	2.98	3.89
Bcl2-associated X protein	<i>Bax</i>	BC018228	1.42	1.66	2.21
B-cell leukemia/lymphoma 10	<i>Bcl10</i>	AF100339	2.17	2.02	2.56
B-cell leukemia/lymphoma 2	<i>Bcl2</i>	BM119782	1.62	-1.04	2.05
B-cell leukemia/lymphoma 2 related protein A1a	<i>Bcl2a1a</i>	L16462	3.51	3.97	6.41
B-cell leukemia/lymphoma 3	<i>Bcl3</i>	NM_033601	2.47	2.69	3.69

All open reading frames were analyzed statistically using Genesifter software. All open reading frames listed have *P* values < 0.05.

production during *M. tuberculosis* is a post-translational event (41–43).

Members of the Ly-6 superfamily (Ly-6SF), specifically Ly-6i, were highly up-regulated (Table 1, section IV). Although the role of Ly6i is unknown, it has been proposed as a maturation marker for T and B lymphocytes as well as for subsets of monocytes and granulocytes (44). The Immunoresponsive gene1 (*Irg1*) was highly up-regulated as well. Although its function is also unknown, it has been proposed to act as an adhesion molecule by binding cell surface ligands. Several studies have identified a peculiar regulation of the *Irg1* gene in *M. tuberculosis*-infected macrophages (8) (Table 1, section IV).

Inflammatory response: soluble factors and cellular infiltration. While interleukins were induced, interleukin receptors were altered to a larger degree in general. Specifically, interleukins *1 β* , *IL-12b*, *IL-15*, *IL-16*, and *IL-21* were induced during the course of infection (Table 1, section V). Interestingly, *IL-18bp* and the *IL41I* involved with the regulation of interleukin expression and functions were highly up-regulated at all time points. IL-1 is a major mediator of inflammation and, in general, initiates and/or amplifies a wide variety of effects associated with innate immunity and host responses to microbial invasion and tissue injury. In addition, TNF and IL-6 and the interleukin receptors *IL12rb2*, *IL17ra*, *IL18rap*, *IL1rap2*, *IL1rn*, *IL2rb*, *IL2rg*, and *IL7r* were induced early in infection, while *IL10ra*, *IL13ra1*, and *IL3ra* induction was limited to later stages of infection (Table 1, section V).

The extent of the inflammatory process is support by induction of chemokines. Among the four chemokine families studied (the C-, XCL, C-x-C, and the C-C), some members of the C-x-C and C-C families were highly up-regulated (Table 1, section V). These included the chemokines *Cxcl9*, *Cxcl4*, *Cxcl10*, *Cxcl13*, and *Cxcl16*, and receptors for this family, the *Cxcr3* and *Cxcr6* (Table 1, section V). In particular, the chemokine CXCL9, which is known to be induced by IFN- γ , and which recruits activated TH1 CD4 cells as well as monocytes, was significantly induced during infection (45–49). This is consistent with the observed increased serum levels of this chemokine in patients with pulmonary tuberculosis (50). A secondary role of chemokines is the promotion of angiogenesis. Other molecules, including CXCL10, CXCL13, CXCR3, CCL5, CCR1, and CCR5, have all been identified as acting as T cell recruitment molecules (51–56). A further molecule identified here, CXCL16, is induced by TNF- α and plays a pleiotropic role both by acting as a recruiting molecule and by influencing (via CXCR6) local blood vessel

integrity (57, 58). This probably represents a mechanism whereby the host attempts to maintain the local vasculature despite the consolidating effects of the developing granuloma. Of the C-C motif (CCL) family of chemokines, *Ccl8* (MCP-2) had the highest expression, followed by *Ccl5* (RANTES). Other chemokines from the same group, *Ccl12*, *Ccl19* (MIP-3), and *Ccl4*, also had increased expression. Interestingly, among the family of receptors used by these chemokine families, only the *CCR5* was greatly up-regulated (and, to a lesser extent, the *Ccr2* and *Ccr7* receptors).

Saa3, which belongs to the SAA family of proteins and encodes the serum amyloid protein A3 (SAA3), an acute-phase protein, displayed increased expression. The role of serum amyloid is to facilitate phagocytosis of dying cells, thus ensuring their swift disposal. This acute phase protein is primarily regulated by IL-1 and TNF, and serves an important tissue-specific function in the lung during both bacterial infection and tissue remodeling (59). Other genes involved in inflammation (as well as in apoptosis) are the caspases family; however, among the 14 caspases analyzed in this study, only caspases 1 and 4 had increased expression, whereas caspases 6, 9, and 14 displayed reduced expression (Table 1, section V).

Immunosuppression. One of the most significantly induced genes was serpin 3 g, a member of the mouse serpins family (Table 1, section VI). Serpins are serine proteinase inhibitors that are irreversible suicide inhibitors of protease enzymes regulating processes of coagulation, fibrinolysis, complement activation, angiogenesis, apoptosis, inflammation, and neoplasia (60). An important cytokine family to be included under this title is the transforming growth factor family. Within this family, only *Tgfb1* and *Tgfb1* (but not *Tgfb2* *Tgfb3*) were progressively induced during the infection, whereas TGF- α (*Tgfa*), a molecule with potent cell proliferative capacity, was up-regulated at 20 days and reduced thereafter. Another gene transcript encoding IDO was highly up-regulated. IDO has recently been described in the mechanism of deactivation and conversion of dendritic cells into regulatory and immunosuppressive dendritic type of cells (33). The immunoglobulin-like receptors CD72 and Fc γ RIIB that counter-balances chemokine signaling (61, 62); that negatively regulate B cell receptor signaling (50, 63, 64); and CD274, the ligand for CD273, a member of the B7 family and regarded as an “exhaustion molecule,” were also up-regulated during infection. CD273 was originally described in viral infections (65, 66), but we have recently shown CD273 expression on CD8 cells that accumulate in the lungs during chronic tuberculosis infection

TABLE 2. QUANTITATIVE REAL-TIME PCR ANALYSIS OF SELECT IMMUNOLOGICALLY SIGNIFICANT GENES AT DAY 20, DAY 40, AND DAY 100 OF INFECTION WITH *Mycobacterium tuberculosis*

Biological Name	Gene	Day 20		Day 40		Day 100	
		MA	qPCR	MA	qPCR	MA	qPCR
Interleukin 1b	<i>IL-1b</i>	3.27	1.84 ± 0.24	2.18	2.98 ± 0.38	3.3	4.04 ± 0.12
Interleukin 2	<i>IL-2</i>	-1.14	-0.79 ± 0.59	-1.04	-0.73 ± 1.21	-1.09	-1.30 ± 0.29
Interleukin 4	<i>IL-4</i>	-1.17	-0.80 ± 1.31	1.12	-1.66 ± 1.51	1.1	-0.21 ± 0.73
Interleukin 10	<i>IL-10</i>	1.52	-0.93 ± 0.63	1.12	-0.91 ± 0.33	1.23	-0.54 ± 0.51
Interleukin 13	<i>IL-13</i>	1.13	-0.95 ± 0.82	1.09	-1.19 ± 0.52	-1.4	-1.26 ± 0.64
Interleukin 15	<i>IL-15</i>	1.08	-1.60 ± 0.49	1.6	-1.10 ± 0.59	2.13	0.66 ± 0.40
Monocyte Chemotactic Protein 1	<i>Ccl2</i>	2.07	0.84 ± 0.25	1.21	-0.30 ± 0.64	1.64	-0.48 ± 0.50
Rantes	<i>Ccl5</i>	7.21	0.22 ± 0.70	16.02	2.11 ± 0.25	21.76	2.39 ± 0.26
Interferon activated gene 10	<i>Cxcl10</i>	7.92	4.97 ± 0.13	7.71	6.58 ± 0.28	8.05	6.39 ± 0.36
Interferon gamma	<i>Ifng</i>	6.13	-1.60 ± 0.41	6.62	-0.45 ± 0.52	13.98	0.90 ± 0.17
Nitric oxide synthase 2, inducible, macrophage	<i>Nos2</i>	1.9	5.74 ± 0.11	3.81	9.94 ± 0.17	5.67	9.17 ± 0.29
Transforming growth factor, beta 1	<i>Tgfb1</i>	2.84	0.26 ± 0.37	4.54	1.42 ± 0.51	5.39	2.47 ± 0.16
Tumor necrosis factor	<i>Tnf</i>	-1.18	3.43 ± 0.63	1.14	5.98 ± 0.42	-1.05	5.72 ± 0.41

Definition of abbreviations: MA, microarray; qPCR, quantitative real-time PCR.

Quantitative real-time PCR analysis was performed in triplicate from three biologically independent samples of total RNA from the lungs of uninfected mice and from mice at 20, 40, and 100 d after challenge. Values represent fold changes from uninfected controls corrected to 18s rRNA.

(unpublished data). The tetraspanin CD151 is a cell-surface molecule known interfere with cell adhesion via interaction with the laminin-binding integrin $\alpha 3 \beta 1$. Other transcripts within the Bcl-2 family and close homologs were also changed during the infection. It is known that activation of transcription factors such as Bcl-xL promote cell survival, while other relatives such as Bax antagonize this function (67). We identified up-regulation of both proapoptotic (Bax, Bak) as well as antiapoptotic (Bcl-2, Bcl-XL) transcription factors, specifically Bcl2-A1, which is known to prevent apoptosis (Table 1, section VI).

To confirm the transcriptional response of immunologically significant genes identified in the global analysis, the transcriptional response of the cytokines *IL-1b*, *IL-2*, *IL-4*, *IL-10*, *IL-13*, *IL-15*, *Tnf*, *ifng*, and *Tgfb1* and the chemokines *ccl2* (MCP-1), *ccl5* (RANTES), and *cxcl10*, and *nos2* where accessed in uninfected and at Days 20, 40, and 100 after challenge by quantitative real-time PCR (Table 2). Analysis revealed that the microarray data and the real-time PCR was 82% concordant. Although the values obtained by microarray analysis for IL-10, IL-13, and IFN- γ were different from those determined quantitative real-time PCR, the overall trends over the course of infection were similar. This information allows us to make a connection between the stage of infection, development of lesions, and activation of the host adaptive immune response.

Transcriptional Differences between Day 20, Day 40, and Day 100

Inspection of the transcriptional response of genes encoding immune function revealed some interesting trends at early compared with later states of disease. *Anxa11* (Annexin 11), *Hrh1* (Histamine receptor H1), *Ppap2b* (Phosphatidic acid phosphatase type 2B), *Cd2ap* (CD2-associated protein), *Itgb1* (Integrin β 1), *Fnrh* (fibronectin receptor β), *Tcrb-J* (T cell receptor β , joining region), *Cyp4a10* (Cytochrome P450), and *TGFfa* (Transforming growth factor α , TGF- α) were all induced at Day 20 but repressed at later time points (Table 3). The other trends are those genes that were repressed early in infection but induced by Day 40 and Day 100. In this group are *Gpr35* (G protein-coupled receptor 35), *Tlr4* (Toll-like receptor 4) and *Tlr12* (Toll-like receptor 12), *Ly6 d* (Lymphocyte antigen 6 complex, locus D), *Ly9* (Lymphocyte antigen 9, CD229), *Il10ra* (Interleukin 10 receptor, α), *Hk3* (Hexokinase 3), *Trem2* (Triggering receptor expressed on myeloid cells 2), and many members of the immunoglobulin family (see below). Importantly, later stages of disease was characterized

by B cell and antibody expression. Specifically, the B cell-specific genes *cd5*, *Cd19*, *Cd22*, *Cd79a*, *CD5*, *CD19*, *CD22*, *CD79a*, *CD79b*, and *CD52* were increased at 40 days after infection and remained transcriptionally active to time of killing (Table 3). This observation is consistent with our previous findings indicating that the B lymphocytes in the granulomatous lesions appear in clusters similar to those found in the germinal center and constitute the predominant type of lymphocyte infiltration during pulmonary chronic infection with *M. tuberculosis* (68). The marker CD72 associated with regulatory B cell function, and antibody switching was also up-regulated during the course of the infection (67). In addition, Bcl10 and Bcl3 associated with B cell differentiation and proliferation were also induced late in infection. Importantly, this study revealed that there was a negative regulation or no changes in the expression of immunoglobulin genes at 20 days after infection, but after 40 days, the immunoglobulin heavy and light chain families—namely *igh-6*, *Igj*, *Igh-VJ558*, *Igk-V32*, *IgkV28*, *Igk-V1*, *Igl-V1* specific for heavy chain of IgM, join and kappa chain variable protein, and heavy lambda chain, respectively—were significantly induced. In some instances at 100 days after infection, *Igh-6*, *Igk-V32*, and *Igj* were induced as much as 10 to 30 times. Altogether, when analyzing the B cell response during this infection, we identified a phenotype of genes expressing for IL-21, CD22, CD52, and CD5 and activation of transcription factors from the Bcl family such as Bcl 10 and Bcl 3, which are factors reported for the progression of particular forms of B cell lymphomas (67).

Identification of Molecular Markers of Disease State and Progression

While trends in the immune response were identified for different times of disease, there is a need for the identification of molecular markers of disease state and progression. Knowing molecular markers provides a means to monitor disease progression, particularly during treatment. Accordingly, tandem-SOM analysis was performed to identify molecular markers characteristic of disease state and progression. These features can be used to inform disease state and progression. When the host transcriptional response to infection was analyzed using SOM, the 1,854 genes were grouped into 20 global SOM-groups (gSOM) (Figure 4A). This analysis generally clustered genes induced at Day 40 or Day 100 into groups 0 to 2, genes induced at Day 20 into groups 11 and 12, and genes induced at Days 40 and 100 and at Days 20, 40, and 100 into groups 14 to 19 based on expression trends. However,

TABLE 3. TRANSCRIPTIONAL DIFFERENCES BETWEEN DAY 20, DAY 40, AND DAY 100 OF INFECTION WITH *Mycobacterium tuberculosis*

Annotation	Name	Unique ID	Days of the Infection		
			20	40	100
Annexin A11	<i>Anxa11</i>	AU019881	2.21	-1.73	-3.31
Cytochrome P450, family 4, subfamily a, polypeptide 10	<i>Cyp4a10</i>	BC013476	2.28	-1.02	-1.14
Early B-cell factor 3	<i>Ebf3</i>	NM_010096	2.07	-1.06	-1.18
Fibroblast growth factor 7	<i>Fgf7</i>	AK015893	2.81	-1.19	-1.17
G protein-coupled receptor 22	<i>Gpr22</i>	BB232423	2.26	-1.01	-1.21
Heparan sulfate 6-O-sulfotransferase 2	<i>Hs6st2</i>	AW536432	2.17	1.14	-1.05
Histamine receptor H 1	<i>Hrh1</i>	AF388053	2.03	-1.4	-1.36
Histone cluster 1, H3a	<i>Hist1h3a</i>	NM_013550	2.54	-1.24	-1.12
Histone cluster 1, H4i	—	BC019757	2.01	1.12	-1.11
Integrin alpha V	—	AK011583	2.32	-1.17	-1.14
Interleukin 12 receptor, beta 2	<i>Il12rb2</i>	NM_008354	2.16	1.21	-1.04
Phosphatidic acid phosphatase type 2B	<i>Ppap2b</i>	BB312387	2.07	-1.25	-1.03
Pre B-cell leukemia transcription factor 3	<i>Pbx3</i>	BG066541	2.22	-1.15	-1.04
Procollagen, type V, alpha 3	<i>Col5a3</i>	AB040491	2	1.07	-1.25
Surfactant associated protein C	—	AV169310	2.7	-4.68	-1.44
Transforming growth factor alpha	<i>Tgfa</i>	M92420	3.56	1.54	-1.51
Triggering receptor expressed on myeloid cells 1	<i>Trem1</i>	NM_021406	2.28	-1.07	-1.15
G protein-coupled receptor 35	<i>Gpr35</i>	NM_022320	-1.09	2.28	2.86
Toll-like receptor 4	<i>Tlr4</i>	AF185285	-1.9	1.93	2.88
Lymphocyte antigen 6 complex, locus D	<i>Ly6d</i>	NM_010742	-2.96	1.59	3.09
Toll-like receptor 12	<i>Tlr12</i>	BB745017	-1.01	1.89	3.1
Solute carrier family 7, member 6 opposite strand	<i>Slc7a6os</i>	AK010254	-1.04	1.91	3.15
Hexokinase 3	<i>Hk3</i>	BB334625	-1.02	2.2	3.2
Lymphocyte antigen 9	<i>Ly9</i>	NM_008534	-1.56	2.09	3.26
Interleukin 10 receptor, alpha	<i>Il10ra</i>	NM_008348	-1.02	2.85	3.29
Triggering receptor expressed on myeloid cells 2	<i>Trem2</i>	NM_031254	-1.04	2.24	3.42
Membrane-spanning 4-domains, subfamily A, member 1	<i>Ms4a1</i>	BB236617	-1.35	1.98	4.05
CD86 antigen	<i>Cd86</i>	NM_019388	-1.11	3.04	4.98
CD5 antigen	<i>cd5</i>	NM_007650	3.23	4.06	6.6
CD19 antigen	<i>Cd19</i>	NM_009844	-1.13	1.79	2.38
CD22 antigen	<i>Cd22</i>	AF102134	1.69	2.88	4.71
CD5 antigen-like	<i>Cd5</i>	NM_009690	1.35	2.14	3.35
CD72 antigen/antibody switching	<i>Cd72</i>	BC003824	3.59	3.97	6.5
Cd79a/B cell receptor	<i>Cd79a</i>	NM_007655	1.22	2.31	4.62
Cd79b/B cell receptor	<i>Cd79b</i>	NM_008339	1.05	1.46	2.8
CD52 antigen	<i>Cd52</i>	NM_013706	3.68	3.86	8.1
B-cell leukemia/lymphoma 10	<i>Bcl10</i>	AF100339	2.17	2.02	2.56
B-cell leukemia/lymphoma 3	<i>Bcl3</i>	NM_033601	2.47	2.69	3.69
Immunoglobulin heavy chain 6 (heavy chain of IgM)	<i>Igh-6</i>	BC025447	2.84	16.05	36.19
Immunoglobulin joining chain	<i>Igj</i>	BC006026	-1.2	3.8	10.61
Immunoglobulin kappa chain variable 1-135	<i>Igkv1-135</i>	BF301241	1.06	2.56	4.34
Immunoglobulin kappa chain variable 28 (V28)	<i>Igk-V28</i>	BI107286	1.52	4.67	13.08
Immunoglobulin kappa chain variable 32 (V32)	<i>Igk-V32</i>	U25103	2.34	5.52	10.48
Immunoglobulin lambda chain, variable 1	<i>Igl-V1</i>	AK008145	-1.19	4.3	13.39

All open reading frames were analyzed statistically using Genesifter software. All open reading frames listed have *P* values < 0.05.

to achieve more resolution, further grouping was accomplished by subjecting genes from these groups to another round of SOM analysis (sSOM) that, when inspected, revealed five discriminant groups (Figure 4B). These discriminant groups correspond to Day 20 (discriminant group 1; mean expression = 2.3), Day 40 (discriminant group 2; mean expression = 2.4), Day 100 (discriminant group 3; mean expression = 2.7), Days 40 and 100 (discriminant group 4; mean expression = 2.6 [D40], 2.6 [D100]), and Days 20, 40, and 100 (discriminant group 5; mean expression = 5.4 [D20], 14.1 [D40], 20.8 [D100]). This analysis resulted in the identification of 712 genes that can serve as predictive markers for disease state and can be used to inform disease progression (Table E2).

CONCLUSIONS

One of the most challenging questions in *M. tuberculosis* research is the dynamic interplay between the host and pathogen. Much work has been performed to define the immune response to infection, and while these studies have provided a wealth of

information, it is difficult to truly analyze the host response to infection in an unbiased way. An approach often used to visualize global trends in the response to infection is the use of whole genome microarrays. Accordingly, we used this post-genomic approach to identify global trends of the host response to infection with *M. tuberculosis* and to identify molecular markers of disease progression. The results of this study are consistent with a massive mobilization of IFN- γ -related genes, transcription factors, inflammatory signals dominated by a strong chemokine profile, and activated T cell and macrophage cell responses during the chronic phase of the disease process, and are in keeping with the established demonstration of an ongoing activation of protective immunity associated with strong inflammatory process during the chronic infection (20). The trends in the responses were progressively increased over time and were still in progress during the late chronic stage of infection. However, the transcriptional response indicated that the host response to *M. tuberculosis* infection at 20 days was different than that at 40 and 100 days after infection. Presumably, the early modulated genes are host responses related to *M. tuberculosis*-induced

Figure 4. Identification of molecular markers of disease state and progression. Tandem self-organizing mapping (tandem-SOM) analysis was performed to categorize genes and identify discriminant groups of disease state and progression. (A) gSOM analysis of transcriptional active genes differentially regulated > 1.5 -fold ($P < 0.01$). This analysis distributed genes into 20 groups (0–19). (B) Discriminant groups identified from sSOM analysis. Discriminant groups correspond to Day 20 (discriminant group 1; mean expression = 2.3), Day 40 (discriminant group 2; mean expression = 2.4), Day 100 (discriminant group 3; mean expression = 2.7), Days 40 and 100 (discriminant group 4; mean expression = 2.6 [D40], 2.6 [D100]), and Days 20, 40, and 100 (discriminant group 5; mean expression = 5.4 [D20], 14.1 [D40], 20.8 [D100]).

primary changes rather than a more complex scenario formed by concomitant *M. tuberculosis*-induced inflammation and anti-inflammatory host responses as observed at Days 40 and 100.

Visualization of bacterial growth, pathology, and the PCA analysis revealed that although the bacterial load reaches a plateau around 20 days after exposure, the pathology and host response continues to progress. These data confirm that the progressive inflammatory response in the subacute and chronic stages of infection in mice is independent of the total number of cultureable bacilli. The solid or nonnecrotic lesions that typify experimental *M. tuberculosis* infection in mice reflect the early tuberculosis lesions of humans. However, in the chronic stages of infection, lesions in most susceptible and resistant strains of mice fail to progress to necrosis and cavitation, where bacilli are often extracellular admixed with degenerate cells and necrotic cellular debris. While no one animal model consistently develops the spectrum of lesions seen in the naturally occurring disease in humans, comparative studies including those in mice reveal important clues in the complex pathogenesis of tuberculosis and the host response to infection.

The overall message derived from this study is that limiting bacterial replication occurs at the cost of progressive and poorly regulated cellular influx that compromises lung function and is thus detrimental in the chronic stages of infection. While there are limitations to the mouse model, the overall general trends observed therein are likely to be similar to the response in other hosts, including humans, thus allowing for the characterization of immune response to infection and the identification of molecular markers of disease progression. These markers may prove useful for discerning disease progression and development and characterization of vaccines with increased efficacy against *M. tuberculosis* infection. Indeed, the availability of molecular markers indicative of early, middle, and chronic infection may provide a foundation for tools that can be used to follow disease and response to chemotherapy. Overall, knowledge of the global response to *M. tuberculosis* at different stages of disease provides much-needed knowledge for antigen discovery, and vaccine development, and can be applied to other clinically relevant research questions, including the identification of markers that can be used to monitor the success or failure of therapy.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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