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Role of B Cells in Vaccine-Induced Immunity against Coccidioidomycosis

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We investigated secondary immunity against coccidioidomycosis by using gene expression microarrays. Surprisingly, a high percentage of B-cell-related genes were associated with protective immunity. A functional confirmation of the importance of B cells against coccidioidomycosis was achieved by demonstrating that vaccination was not fully protective in B-cell-deficient MuMT mice.

Coccidioidomycosis is a pulmonary fungal disease caused by dimorphic fungi in the genus *Coccidioides* (8, 13). The cumulative interpretation of studies performed in humans and mice indicates that cell-mediated immune responses are essential in primary host defenses against *Coccidioides* and that recovery is associated with antigen-specific delayed-type hypersensitivity and the production of T helper 1 (Th1)-associated cytokines, such as gamma interferon (IFN-γ) and interleukin 2 (1, 2, 7–10, 19, 20). Conversely, disease progression is associated with low delayed-type hypersensitivity levels to *Coccidioides* antigens but high levels of anti-*Coccidioides* antibodies.

The optimum method to induce protective immunity in mice is vaccination with formalin-killed spherules (FKS) (6, 11, 12, 18). We showed that vaccination of susceptible BALB/c mice with FKS results in both reduction in fungal tissue burden and increased survival (10, 15). In secondary responses, IFN-γ levels and antibody levels increased, indicating that protective mechanisms could utilize both T cells and B cells. This study further dissected these secondary immune mechanisms using Affymetrix gene expression microarray analysis.

Female BALB/c mice were immunized with either FKS (by using spherules from *Coccidioides posadasii* strain Silveira; the FKS-immune group) or saline (the sham-immune group) and intranasally challenged with 26 arthroconidia (10). In two independent experiments, time-matched lungs were removed at day 12 postinfection and processed for RNA isolation by using TRIZOL (Invitrogen) and RNeasy kits (QIAGEN, Valencia, CA). Nonimmunized, noninfected BALB/c mice (control) lung tissue was also processed. Biotin-labeled amplified antisense RNA samples were generated and hybridized to individual Affymetrix murine MG-U74Av2 GeneChip microarrays (Affymetrix, Santa Clara, CA). Expression analysis was performed using GeneSpring 5.1 (Agilent, Redwood City, CA) and statistical analyses, including Pearson correlated measurements of gene expression similarity, were performed with P values of <0.05 considered significant. Following normalization, initial data filtering, performed to identify probe sets with signal intensities of >25 that were present in at least two of the six conditions, generated a list of 6,836 genes. We then performed filtering (n-fold change) using all possible condition-to-condition comparisons and identified 1,096 genes that were changed at least twofold in expression between any two conditions. Hierarchical cluster analysis separated these genes into two categories: those that exhibited increased gene expression (716 genes) and those that exhibited decreased gene expression (380 genes) relative to control samples (Fig. 1A).

Subcluster analysis was performed using VxInsight (Sandia National Labs, Albuquerque, NM) (5). This analysis creates a two-dimensional plot of genes with similar expression patterns between the conditions on the x-y plane. A third dimension represents an accumulation of genes revealing a topographical terrain of gene cluster "mountains." The 716 up-regulated genes segregated into nine peaks (Fig. 1B), and the 380 down-regulated genes segregated into three peaks (Fig. 1C). Individual lists representing the gene populations in each of the nine peaks were imported into GeneSpring for expression pattern visualization. Peaks 1, 2, 3, and 4 (528 genes) exhibited increased gene expression in sham-immune mice relative to FKS-immune mice, and thus these genes could be associated with a failing host response following intranasal challenge. In contrast, the mean expression patterns of the genes in peaks 5, 6, 7, 8, and 9 (188 genes) exhibited increased expression in the FKS-immune mice and thus could be associated with protected host responses. Analyses of the lists revealed a marked increase in genes controlling immunoglobulin production or B-cell markers with 95 of the 188 genes (49.47%) in peaks 5 to 9, compared to 23 of the 528 genes (4.35%) in peaks 1 to 4 ($P < 0.001$ by chi-square analysis). While IFN-γ gene expression did not segregate in these analyses, there was an increase in interferon-regulated genes relative to control mice (Table 1). In addition, interleukin-4 and other Th2-associated gene inductions were seen in each of the up-regulated peaks. Thus, while there was no discernible Th1-Th2 cytokine bias in the FKS-protected response, both cytokine pathways were increased throughout the peaks.

We sought to confirm that B-cell-associated genes were

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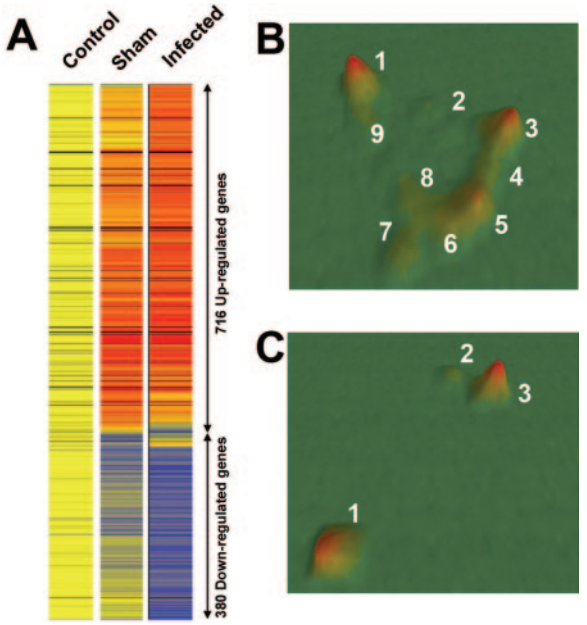

FIG. 1. (A) Initial hierarchical clustering of the 1,096 genes identified as >2-fold changed in expression in infected samples relative to mock-infected and control samples segregated this population into two groups: 716 up-regulated genes (red) and 380 down-regulated genes (blue). Up-regulated (B) and down-regulated (C) groups were further analyzed using VxInsight software to generate topographical terrain maps corresponding to subclusters of genes exhibiting similar expression.

TABLE 1. Th1- and Th2-associated gene expression occurs in all peaks of the up-regulated genes

Peak	GenBank accession no.	Description
1	M29697	Interleukin-7 receptor mRNA
	M33266	Interferon-inducible protein mRNA
	AA144469	Interferon-inducible protein 1-8U gene (human)
	Y09864	mRNA for alpha/beta interferon receptor, IFNaR2b
	M31419	204-interferon-activatable-protein mRNA
	M31418	202-interferon-activatable-protein mRNA
2	U22339	Interleukin-15 receptor precursor mRNA
3	M27960	Interleukin-4 receptor mRNA
	M21065	Interferon regulatory factor 1 mRNA
	AB019505	Interleukin-18 binding protein gene
	X56602	Interferon-induced 15-kDa protein gene
4	U69599	Gamma interferon receptor second chain (ifngr2) gene
5	U70430	Interleukin-4-induced gene-1 partially spliced (Fig1ps) precursor RNA
	M34815	Mouse monokine induced by gamma interferon (MIG) mRNA
	U43428	Inducible nitric oxide synthase mRNA
6	U06924	Signal transducer and activator of transcription (Stat1) mRNA
	AF065947	RANTES
7	U04379	Protein tyrosine kinase ZAP-70 mRNA
	M12056	Protein tyrosine kinase gene (lck gene)
8	M32489	Interferon consensus sequence binding protein mRNA
	U56819	MCP-1 receptor mRNA
9	M21038	Interferon-induced mutant Mx1 protein pseudo gene mRNA

strongly up-regulated in FKS-immunized BALB/c mice by testing the efficacy of FKS to protect MuMT mice which are deficient in mature B cells but have relatively normal T-cell function (16). As described above, FKS-immune and sham-immune MuMT mice and C57BL/6 (wild-type controls) were intranasally infected with 30 arthroconidia and monitored (Fig. 2). All sham-immune mice succumbed within 20 days. In contrast, immunization of the C57BL/6 mice with FKS induced complete protective immunity as measured by 100 percent survival. FKS-immune MuMT mice exhibited only 50 percent survival over the observation period. This reduction in the level of protection, while significantly higher than that for the non-immunized controls ($P < 0.001$; Mantel-Haenszel survival curve analysis), is significantly less than wild-type C57BL/6 mice with sufficient B cells ($P < 0.007$). The fact that FKS-immunized MuMT mice were significantly less protected than wild-type controls confirms the microarray results and establishes that B cells and/or antibody play a role in acquired protective immunity to *Coccidioides*.

The results of this study appear to contrast with early studies of host defenses against *Coccidioides* which revealed that passive transfer of immune splenocytes conferred protection and that elimination of T cells from the population before transfer abrogated protection (3, 4, 17). While mice receiving whole-spleen-cell populations were completely protected, recipients given T-cell-depleted (B-cell-enriched) spleen cells succumbed by day 34 postinfection. The authors concluded that this was a total abrogation of protection; however, we extracted the data (3) and analyzed them to reveal a significant increase in the number of days the mice survived after passive transfer of immune B cells compared to nonimmune cells ($P = 0.0097$; Mantel-Haenszel analysis). While T cells are of major importance in host resistance to *Coccidioides*, when combined with the reanalysis of the results of Beaman et al. (3), our current results indicate that B cells or antibodies are also significant contributors to vaccine-induced protection against *Coccidioides*.

The ability to measure thousands of individual host responses can provide a biosignature that reflects the health

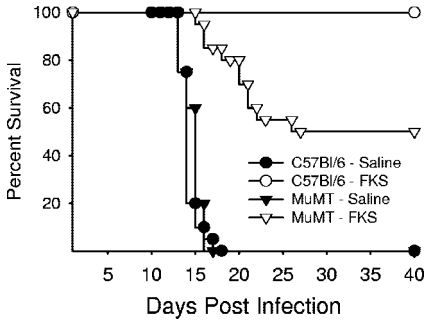

FIG. 2. Capacity for the FKS vaccine to induce immunity in B-cell-deficient MuMT mice. Results are presented as the combination of two independent experiments for a total of 15 mice per group. Triangles represent MuMT mice, and circles represent C57BL/6 (wild-type) mice, with open symbols representing those with FKS-immune treatment and solid symbols those with sham-immune treatment.

status of the host. In this report, we utilized gene expression analysis, but this strategy could be adapted to directly detect changes in the proteome of the host for a more rapid assessment of the biosignature. These types of studies could also be used to establish the biosignature of protective versus nonprotective vaccine regimens. In addition, it may be possible to determine the infecting agent based upon the host biosignature response. For example, studies of gene expression profiles in dendritic cells challenged with viruses, bacteria, or fungi revealed distinct differences in their expression profiles (14). Based upon the biosignature, it may be possible to predict the outcome of infection and categorize those who would survive or those at high risk of mortality. The acquisition of a predictive biosignature would allow targeting therapy towards those who are at greatest risk.

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