Periportal Fibrosis in Human *Schistosoma mansoni* Infection Is Associated with Low IL-10, Low IFN-γ, High TNF-α, or Low RANTES, Depending on Age and Gender


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**Periportal Fibrosis in Human *Schistosoma mansoni* Infection Is Associated with Low IL-10, Low IFN-γ, High TNF-α, or Low RANTES, Depending on Age and Gender**


*Schistosoma mansoni* infection is highly endemic in parts of Uganda, and periportal fibrosis is common in communities along the shore of Lake Albert. In this study, we have identified cellular immune responses associated with fibrosis. A cohort of 199 individuals aged 6–50, resident in the village for at least 10 years or since birth, were examined for evidence of periportal fibrosis by ultrasound using the Niamey protocol. Whole-blood samples were assayed for levels of nine cellular immune molecules (IL-3, IL-4, IL-5, IL-10, IL-13, TNF-α, IFN-γ, IL-1β, and RANTES) in the absence of in vitro Ag stimulation, and after stimulation with egg and worm Ags. A lack of Ag specificity allowed the number of variables in the analysis to be reduced by factor analysis. The resulting factor scores were then entered into a risk analysis using a classification tree algorithm. Children, adult males, and adult females had different factors associated with fibrosis. Most cases of fibrosis in children (eight of nine) were associated with low RANTES factor scores (58th percentile). These results demonstrate that periportal fibrosis is associated with cytokine production profiles that vary with both age and gender. *The Journal of Immunology*, 2004, 172: 1295–1303.

Periportal fibrosis of the liver is a serious consequence of *Schistosoma mansoni* infection that involves remodeling of the extracellular matrix and excessive deposition of collagen, primarily by hepatic stellate cells, along the branches of the portal tract. Disease only occurs in a proportion of infected individuals, with demographic risk factors including age, duration of exposure, and gender (1, 2). The clinical picture is well established (3), and some of the generic cellular and molecular mechanisms of fibrosis have been recently described (4). Experimental murine studies indicate that development of schistosome-associated fibrosis is influenced by several counterregulatory Th1- and Th2-type cytokines, including IL-4, IL-13, TNF-α, and IFN-γ (5). A pivotal role of IL-10 in down-regulating both Th1 and Th2 responses to schistosome Ags, and hence limiting the extent of pathology, has also been demonstrated (6). Importantly, most experiments on animal models have focused on periovular rather than periportal fibrosis, and the relevance of these observations may depend on whether or not the distended lesions typical of fibrosis in humans have the same etiology and molecular mechanisms as localized granulomatous responses.

Although invasive experimental procedures are generally undesirable and often unethical in humans, it is still possible to demonstrate associations between immune responses and disease status that may yield valuable insights into disease mechanisms. The antifibrogenetic cytokine IFN-γ in particular has been associated with protection against severe portal fibrosis. A genetic study mapped a high risk of severe hepatic fibrosis to a locus on chromosome 6 close to the IFN-γR gene (7), and this was followed by a demonstration that cases of severe portal fibrosis were associated with low levels of IFN-γ and high levels of TNF-α (8). Clues to the involvement of other cytokines in the etiology of fibrosis in humans have come from studies of chronic injury in other organs. For example, studies of human pulmonary fibrosis indicate that fibroblasts from diseased tissue are less able to induce IL-10 production than those from normal tissue (9). Other forms of pathology associated with schistosome infection in humans have also identified cytokine responses as important factors, including IL-10 and TNF-α in bladder pathology associated with *Schistosoma haematobium* infections (10), and IL-5, associated with a lack of hepatosplenomegaly attributable to *S. mansoni* infection (11).

In this study, the aim was to identify demographic and cytokine responses associated with ultrasound-detected fibrosis in a population heavily exposed to *S. mansoni* infection (2, 12, 13). Nine cellular immune responses to three crude *S. mansoni* Ag preparations, as well as constitutive responses assayed in the absence of in vitro Ag stimulation, were measured. The potential obstacle of
complex associations being masked in the analysis was overcome by using recursive partitioning techniques (14). Our observations demonstrate that children, adult males, and adult females not only vary in their overall risk of fibrosis, but that, within these strata, there are different levels of risk that are associated with specific cytokine/chemokine response profiles.

Materials and Methods

Study area and population

Booma is a village situated on the eastern shore of Lake Albert, in the district of Masindi. S. mansoni infection is endemic, and is transmitted by Biomphalaria sudanica, and Biomphalaria stanleyi. The village’s proximity to the lake means that the population is in contact with infested lake water on a regular basis. Fishing is the main occupation, and the lake is also used for recreational purposes and bathing. Lake water is drawn for drinking and washing. There are rudimentary health facilities, and treatment for S. mansoni infection is rare.

Fieldwork

Selection of individuals took place according to the criteria that residency in the village was at least 10 years, or since birth if younger. Pregnant women were excluded from the study. A cohort of 206 individuals aged 7–50 years, balanced for age and sex, were enrolled in surveys that recorded parasitological and ultrasound parameters. The extent of liver fibrosis was assessed by experienced ultrasonographers using a portable ultrasound machine (SSD 500) with a 3.5 MHz convex probe; Aloka, Tokyo, Japan). Image patterns scores were assigned to each cohort member using the modified Niaeye protocol (15). This protocol grades patients on an ordered categorical scale from A through F, depending on the extent of fibrotic tissue around the portal branch and throughout the parenchyma. Grades A and B are considered to be normal livers, whereas grades C, D, E, and F represent increasingly severe manifestations of the disease. Liver damage due to cirrhosis, which has a different appearance under ultrasound examination, was recorded alongside any lesions recorded on the Niaeye scale. Each member of the cohort harvested three stool samples in the week before venous blood samples were taken. These samples were processed by the modified Kato-Katz procedure (16), with two microscope readings per stool sample. An average egg count was then estimated for each individual.

Parasite Ag preparation

Parasite Ags were prepared from a Puerto Rican isolate of S. mansoni maintained in outbred mice and albino Biomphalaria glabrata. Adult worms were isolated by portal vein perfusion (17), and eggs were obtained from liver tissue. The soluble fraction of homogenized adult worms (SWA)* was prepared as described previously (18). The outer tegumental layer of adult worms (TEG) was collected from the supernatant after incubating freshly isolated adult worms in PBS at room temperature (RT) for 1 h (19). Soluble egg Ag (SEA) was prepared as described previously (18).

In vitro whole-blood cultures

Venous blood from each cohort member was collected into heparinized tubes (20). Whole blood, diluted 1/4 with RPMI 1640 (Sigma-Aldrich, Poole, U.K.) supplemented with 2 mM l-glutamine (Sigma-Aldrich), 50 μM penicillin, and 50 μg/ml streptomycin (Sigma-Aldrich), was used to set up 1-ml cultures in 48-well tissue culture plates (Falcon; BD Biosciences, Oxford, U.K.). Duplicate cultures of diluted blood were stimulated with SWA (10 μg/ml), TEG (10 μg/ml), or SEA (10 μg/ml), or left without Ag stimulation, for each cohort member. Plates were incubated at 37°C in 5% CO₂, for 48 or 96 h. After culturing, 800 μl of each supernatant was transferred into 1.1-ml microtubes (Bioscope, York, U.K.), which were kept at −20°C before being transported frozen to U.K., where they were stored at −80°C before being assayed.

Cytokine/chemokine assays

Capture ELISA was used to assay cytokine/chemokine in supernatants from whole-blood 48-h (IL-4, TNF-α) or 96-h (IL-3, IL-5, IL-10, IL-13, IFN-γ, RANTES, IL-1β) cultures. Commercially available paired mAb, the second Ab biotinylated, were used to detect IL-3 (clone number BV8D-3G11, at a final concentration of 1 μg/ml; BVD3-1F9, 1 μg/ml), IL-4 (8D-4, 1 μg/ml; MP4-2SD2, 1 μg/ml), IL-5 (TRFK5, 1 μg/ml; JES1-5A10, 0.5 μg/ml), IL-10 (JES-10D7, 1 μg/ml; JES-10G8, 1 μg/ml); IL-13 (JES10-5A2, 0.5 μg/ml; B69-2, 0.25 μg/ml), IFN-γ (N1B42 1 μg/ml; 45B3, 0.5 μg/ml), and TNF-α (MAb1, 2 μg/ml; MAb11, 1 μg/ml), all purchased from BD PharMingen (Oxford, U.K.). For measurement of IL-1β, IL-6, Rantes, Accl 2805, Antibody epitope U.K., was used as first Ab and biotinylated polyclonal goat anti-human IL-1β (200 ng/ml) as second Ab (R&D Systems). For measurement of RANTES, polyclonal rabbit anti-human RANTES Ab (2 μg/ml; BD PharMingen) and biotinylated polyclonal rabbit anti-human RANTES Ab (2 μg/ml; BD PharMingen) were used. The first, capture, Ab was coated onto Immuno 2H ELISA plates (Dynex; Thermo Life Sciences, Basingstoke, U.K.) in carbonate/bicarbonate buffer (pH 9.6), 50 μl/well, and incubated overnight at 4°C. Plates were washed three times in washing buffer (PBS plus 0.03% Tween 20) and blocked with 100 μg/ml 1% Marke skimmed milk powder (Premier Foods, St. Albans, Hertfordshire, U.K.) diluted in incubation buffer (PBS plus 0.05% Tween 20) (Sigma-Aldrich) and incubated at RT for 1 h. Duplicate assays on 50-μl aliquots of culture supernatant were quantified for each cytokine/chemokine by reference to recombinant human standards (BD PharMingen) added to each plate. Plates were incubated overnight at 4°C, washed four times, and biotinylated second Ab (diluted in incubation buffer plus 0.1% Marke) was added. After incubation at RT for 2 h, plates were washed four times, and streptavidin-labeled poly-HRP (Mast Group, Bootle, U.K.), diluted 1/4000 in incubation buffer plus 0.1% Marke, was added and the plates were incubated at RT for 1 h. After further washing, 100 μl/well o-phenylenediamine dihydrochloride substrate solution (Sigma-Aldrich) was added. Color development was stopped by addition of 25 μl/well 2 M sulfuric acid. Absorbance was read at 490 nm on a BioTek EL 312e plate reader (BioTek Instruments, Winooski, VT). Calculation of cytokine and chemokine concentrations was made by interpolation from standard curves (20).

Analysis

Cases were identified as individuals with an image pattern score of grade C, D, E, or F. Exclusions consisted of those individuals who had grade C fibrosis and cirrhosis, because the etiological agent of the fibrosis could not be definitively identified as S. mansoni infection.

A total of nine cytokines and chemokines were assayed for in vitro constitutive production, and for responses to stimulation with three crude Ag extracts of S. mansoni. This yielded a total of 36 responses per individual, too large a number of variables to give any power in a logistic regression analysis of raw data that would include detection of complex interactions. Therefore, we first reduced the number of variables by factor analysis in SPSS. Extraction of factors was by principal components, and factor-specific loadings for each assay were estimated. These loadings indicated the degree of correlation between each factor and the raw data of each assay. Finally, standardized factor-score variables were derived from the factor loadings and raw baseline values for the factor, and replaced the raw data. Each factor-score variable was interpreted as a summary of those variables with high factor loadings associated with the factor from which the scores were derived. They were maintained for further analysis only if the eigenvalue of the factor (an estimate of the variance in the raw data explained by the factor) was >1, and there was a significant linear correlation between the factor scores and each of the variables associated with that factor.

Age, sex, egg count, and factor scores for each of the factors were entered into a classification tree analysis that involved application of recursive partitioning algorithms in S-Plus (version 6; Insightful, Seattle, WA) using the Rpart library. The aim of this analysis was to identify factors associated with either a high or low risk of periporal fibrosis. A detailed explanation of the methods used in the classification trees in the context of health sciences is given elsewhere (14). Briefly, the algorithm is designed to identify clusters of records that are almost or entirely homogeneous in terms of containing either cases or noncases. This is achieved by recursively partitioning the data until some stopping criteria are reached. The partitions are created by identifying breakpoints in explanatory variables that, when used as a selection criteria, separate the remaining records into two groups, one of which contains most or all of the cases. The variable is selected on the basis of producing the best improvement to the misclassification rate, which is defined as the percentage of records in the subgroups formed by the split that do not follow the classification of the majority of records in that group.

Because recursive-partitioning analysis requires several ad hoc parameter values to define the size and shape of the trees, a sensitivity analysis

* Abbreviations used in this paper: SWA, soluble fraction of homogenized adult worms; TEG, outer tegumental layer of adult worms; SEA, soluble egg Ag; RT, room temperature; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP.
was conducted by varying key parameters (data not shown), the most sensitive of which was the minimum node size. Varying this parameter between 10 and 20 changed the number of nodes per tree as well as the size of each node. Larger values for the minimum node size produced trees with a lower number of nodes. The rule that applied to records with missing values for a particular factor-score variable was exclusion from the analysis at the point where the factor-score variable was selected as the partitioning variable.

**Post hoc analysis**

For each tree that was produced, the significance of each node was formally tested by conducting a $\chi^2$ analysis comparing the prevalence of cases between the groups above and below the breakpoint.

**Results**

**Parasitology**

The prevalence of infection was almost 100% in each age group, and did not differ significantly by either age or sex. Intensity of infection peaked in young adults. Further details of the cohort with respect to parasitological observations are given elsewhere (2).

**Factor analysis**

The aim of this analysis was to reduce the number of variables used in later analysis by producing new variables that summarized several correlated assays. Table I summarizes the results. In the following description, cytokine and chemokine levels in the absence of in vitro stimulation are denoted as -MED. Responses after stimulation with SEA, SWA, or TEG are denoted as -SEA, -SWA, and -TEG, respectively.

A total of 10 factors were produced with eigenvalues $>1$, but 3 were excluded due to nonsignificant correlations between factor scores and assays most strongly associated with those factors. This resulted in the exclusion of factors associated with certain assays (IL-5-MED, IL-5-SEA, TNF-\(\alpha\)-MED, IL-1\(\beta\)-MED, and IL-1\(\beta\)-SWA) from any further analysis. The remaining 7 factors are described in Table I. Each cytokine or chemokine response was highly correlated with 1 factor only, but each factor had high loadings associated with several responses.

Strong correlations between the constitutive and Ag-specific responses for specific cytokines resulted in some factors with heavy loadings for the entire range of responses associated with a particular cytokine or chemokine. Thus, factor 3 was strongly correlated with constitutive IL-10 production and all IL-10 responses, and factor 7 was correlated with constitutive RANTES production and all RANTES responses (Fig. 1). Some factors were correlated with a limited subset of constitutive levels or responses associated with specific cytokines; thus, factor 4 was strongly correlated with IL-13-MED and IL-13-SEA only, and factor 6 was strongly correlated with TNF-\(\alpha\)-SEA, TNF-\(\alpha\)-TEG, and TNF-\(\alpha\)-SWA (Fig. 1). Other factors were correlated with several cytokines or chemokine assays; thus, factor 1 was correlated with IL-3-MED, IL-3-SEA, IL-3-TEG, IL-4-SEA, IL-4-TEG, IL-1\(\beta\)-MED, and IL-1\(\beta\)-TEG; factor 2 was moderately correlated with IL-3-SWA and IL-4-SWA, and strongly correlated with all IFN-\(\gamma\) responses (Fig. 1). Factor 5 was correlated with IL-5-TEG, IL-5-SWA, IL-13-TEG, and IL-13-SWA. The loadings within each factor were used to create new factor-score variables as outlined in Materials and Methods. This step produced seven factor-score variables that effectively summarized the dataset.

Although most assays were strongly correlated with only one factor, some assays were only partially correlated with several factors. This particularly affected IL-3 and IL-4 responses. Thus, IL-3-SWA was partially correlated not only with the second factor, but had a loading of 0.44 on the fourth factor, 0.38 on the first factor, and 0.30 on the third factor. IL-4-SWA was not only partially correlated with factor 2, but was also partially correlated with the fourth factor (loading, 0.40), and the first factor (loading, 0.3). IFN-\(\gamma\) assays were partially correlated with factor 3, but to a lower extent than factor 2 (loading range, 0.32–0.46). Given these observations, inferences about IFN-\(\gamma\) were limited to observations concerning the second factor, and no inferences were made about IL-3 and IL-4 responses to worm Ag from observations related to this factor.

**Logistic regression**

A preliminary regression analysis was conducted separately on three demographic strata: children, adult females, and adult males. In each stratum, fibrosis was entered as the dependent variable, and the factor-score variables detailed above were entered as explanatory variables. No factor-score variable was observed to have a significant association with fibrosis in any of these strata when entered as a continuous variable.

**Classification trees**

The recursive partitioning procedure identified three demographic groups with different overall risks of fibrosis, irrespective of the level of stratification. These three groups were children, adult females, and adult males. Fig. 2 depicts the rise in prevalence of fibrosis with age, and shows the clear difference in prevalence by

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**Table I. Summary of factor analysis including factor loadings that indicate the degree of correlation between assays and the factors**

<table>
<thead>
<tr>
<th>Cytokine assay (loading)</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
<th>Factor 4</th>
<th>Factor 5</th>
<th>Factor 6</th>
<th>Factor 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>MED (0.72)</td>
<td>IL-10 MED (0.93)</td>
<td>IL-13 MED (0.86)</td>
<td>IL-5 TEG (0.80)</td>
<td>TNF-(\alpha) SEA (0.83)</td>
<td>RANTES MED (0.77)</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>MED (0.74)</td>
<td>SWA (0.78)</td>
<td>SE (0.81)</td>
<td>TEG (0.87)</td>
<td>SWA (0.86)</td>
<td>TEG (0.87)</td>
<td>SEA (0.68)</td>
</tr>
<tr>
<td>IL-13</td>
<td>MED (0.80)</td>
<td>TEG (0.81)</td>
<td>SE (0.78)</td>
<td>TEG (0.87)</td>
<td>TEG (0.83)</td>
<td>SWA (0.78)</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>MED (0.72)</td>
<td>IL-10 MED (0.93)</td>
<td>IL-13 MED (0.86)</td>
<td>IL-5 TEG (0.80)</td>
<td>TNF-(\alpha) SEA (0.83)</td>
<td>RANTES MED (0.77)</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>MED (0.74)</td>
<td>SWA (0.78)</td>
<td>SE (0.81)</td>
<td>TEG (0.87)</td>
<td>SWA (0.86)</td>
<td>TEG (0.87)</td>
<td>SEA (0.68)</td>
</tr>
<tr>
<td>IL-13</td>
<td>MED (0.80)</td>
<td>TEG (0.81)</td>
<td>SE (0.78)</td>
<td>TEG (0.87)</td>
<td>TEG (0.83)</td>
<td>SWA (0.78)</td>
<td></td>
</tr>
</tbody>
</table>

* Each assay appears once, underneath the heading for the factor with which the assay was most strongly correlated.
sex, particularly in older age groups. Egg count was not a significant factor in this analysis. Factor-score variables that did not contribute significantly to variation in disease prevalence were also excluded from the final classification trees.

Varying the minimum node size between 10 and 20 produced only two different trees. Fig. 3 is an illustration of these trees with the breakpoints given in terms of the factor scores. In the following description, the breakpoints have been transformed into percentiles to facilitate comparisons between studies. Additionally, to facilitate inference in terms of cytokine production, factor-score values are henceforth given in conjunction with those assays that had the highest loadings.

Fig. 3A depicts a tree with very few nodes due to a high value assigned to the minimum node size parameter \( n = 20 \). In this analysis, age and sex were identified as the most important risk factors. Within the child stratum, the majority of cases of fibrosis \( (8 \text{ of } 40 \text{ vs } 1 \text{ of } 45; \chi^2 = 5.3, p = 0.021) \) had low scores \((<47\text{th percentile})\) on the third factor (IL-10). Within the adult female stratum, a significantly higher risk of fibrosis \((8 \text{ of } 14 \text{ vs } 6 \text{ of } 34; \chi^2 = 5.7, p = 0.017) \) was associated with high scores \((>62\text{nd percentile})\) on the sixth factor (TNF-\( \alpha \)). In adult males, a relatively low score \((<60\text{th percentile})\) on the seventh factor (RANTES) was associated with a significantly increased risk of fibrosis \((22 \text{ of } 30 \text{ vs } 9 \text{ of } 22; \chi^2 = 4.3, p = 0.038) \).

Decreasing the value for minimum node size increased the number of nodes in the analysis, and the resulting classification tree is depicted in Fig. 3B. Concomitant with increased number of nodes was identification of subgroups within each demographic stratum that had particularly high or low risks of fibrosis for that stratum. Thus, among children, a group of 10 individuals was identified, of whom 6 had fibrosis. The cytokine profile associated with this group of 10 individuals was a combination of low third-factor scores \((<47\text{th percentile})\), corresponding to low IL-10 production, combined with moderate scores \((34\text{th to } 57\text{th percentile})\) on the fifth factor, corresponding to moderate IL-5 and IL-13 production in response to stimulation with worm Ag.

In the adult female stratum, there were two subgroups with very low probability of fibrosis. One subgroup with no cases \((0 \text{ of } 10; 0.00) \) had the highest scores \((>83\text{rd percentile})\) on the second factor, corresponding to relatively high IFN-\( \gamma \) production. The other subgroup contained one case only \((1 \text{ of } 13; 0.08) \) and consisted of those individuals with scores \(<32\text{nd percentile}\) on the same factor. An intermediate-probability group \((6 \text{ of } 19; 0.32) \) consisted of women with a combination of intermediate scores on the second factor \((32\text{nd to } 83\text{rd percentile})\), and low-to-moderate scores \((1\text{st to } 60\text{th percentile})\) on the sixth factor, corresponding to intermediate IFN-\( \gamma \) production and low TNF-\( \alpha \) production on stimulation with Ag. The highest probability of fibrosis among females \((7 \text{ of } 10; 0.7) \) was associated with a combination of intermediate \((32\text{nd to } 83\text{rd percentile})\) second-factor scores, combined with high \((>60\text{th percentile})\) sixth-factor scores, corresponding to moderate IFN-\( \gamma \) production and high TNF-\( \alpha \) production on stimulation with Ag. The distribution of factor scores for these subgroups is shown in Fig. 4.

In the adult male stratum, the lowest probability of fibrosis \((5 \text{ of } 17; 0.29) \) was associated with moderate scores \((55\text{th to } 82\text{nd percentile})\) on the sixth factor, corresponding to moderate production of TNF-\( \alpha \) on stimulation with Ag. A group with a slightly higher probability of fibrosis \((4 \text{ of } 10; 0.4) \) had a combination of low scores on the sixth factor \((<55\text{th percentile})\), and relatively high scores \((>58\text{th percentile})\) on the seventh factor, corresponding to low TNF-\( \alpha \) production on stimulation with Ag and relatively high RANTES production. Individuals within the 55th to 82nd percentile range of the sixth-factor (TNF-\( \alpha \)), but \(<58\text{th percentile}\) on the seventh-factor (RANTES) scores had the second highest probability of fibrosis \((12 \text{ of } 14; 0.86) \). The highest probability of fibrosis \((10 \text{ of } 11; 0.91) \) was observed among a group of males with high scores \((>82\text{nd percentile})\) on the sixth factor, corresponding to

![FIGURE 1. Scatter plots illustrating the relationship between three factor-score variables and the cytokine assays most strongly correlated with the factors from which the scores were derived.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)}
high TNF-α production on stimulation with Ag. Fig. 5 depicts the distribution of factor scores for these subgroups.

Discussion
In this study, we focused on associations between ultrasound-detectable periportal fibrosis attributable to S. mansoni infection, and seven cytokines and two chemokines in terms of both their constitutive levels and their responses induced by crude schistosome Ag extracts. The results from the present analysis offer new insights into the complexity of the disease process in humans.

A major challenge presented to researchers of human immunopathology lies in uncovering the complexity of immunoregulatory networks that are associated with specific phenotypes, without being able to perform any experimentation of the type conducted in animal models. The appropriate approach in human studies is to investigate interactions, statistically, between immune responses in relation to disease status, but the power to detect any interactions is limited primarily by sample size. As the number of variables in an analysis increases, so does the required sample size to maintain analytical power. We overcame this problem by first reducing the number of variables through factor analysis. Strong correlations among cytokine or chemokine responses allowed us to reduce 40 measurements to 7 uncorrelated factors. From these factors, we created factor-score variables that represented assays strongly associated with the factors. This analysis also confirmed that only certain responses were Ag specific (20). Constitutive RANTES levels and RANTES responses to different antigenic stimuli were highly correlated, and hence one factor-score variable represented all RANTES assays. A separate factor-score variable represented all IL-10 assays for the same reason, although responses to Ag were higher than constitutive levels (20). IFN-γ responses were also correlated across assays with different antigenic stimuli, and hence were represented by one factor-score variable. However, due to a partial correlation between the IFN-γ responses and IL-3 and IL-4 responses to worm Ags, one factor-score variable also represented these two latter responses to a lesser degree. Previous analysis also revealed that anti-SEA IL-5 and IL-13 responses were considerably weaker than the anti-SWA or anti-TEG responses (20), indicating that worm Ags are the major stimulatory factors in the in vitro production of these cytokines. This observation was reflected in the factor analysis, whereby the constitutive and anti-SEA responses were separated from the responses to adult worm Ags. In vitro TNF-α levels in the absence of Ag stimulation were higher than responses to Ag stimulation, and were poorly correlated with these Ag-specific responses (20). Hence, in the factor analysis, one factor-score variable represented the Ag-stimulated responses, and another represented the constitutive levels. However, because the overall variation explained by this latter factor was very low, it was excluded from the analysis. From here onwards, reference to TNF-α is in the context of cytokine production after stimulation with Ag.

The application of a recursive partitioning algorithm that included factor-score variables as well as demographic variables allowed a detailed examination of the pathways leading to particularly low or high risks of periportal fibrosis in the cohort. We have previously observed by a different analysis that age and sex are critical demographic risk factors for the disease (2), and it was therefore not surprising to observe that the classification tree procedure also identified these factors as important. A considerably more striking observation was that the risk of fibrosis was associated with different cytokine profiles depending on age and gender. The use of classification trees was the enabling feature of the analysis that allowed these observations to be made, because the preliminary logistic regression failed to identify any associations. One reason for the striking difference in the output of the two analyses is due to the fact that logistic regression is a parametric analysis that assumes a sigmoidal relationship between the dependent and independent variables, whereas the classification tree carries no such assumption. Furthermore, the classification tree approach does not attempt a global fit of one model to the data, and has more power to detect complex interactions or the clustering of cases within a narrow range of explanatory data. Classification trees can also handle records with missing values in one or more variables of interest more capably, by not excluding such records by default.

Children had the overall lowest risk of fibrosis, the most likely explanation being that they had not been exposed long enough for the cumulative effects of injurious lesions to manifest an excessive collagen secretion in the peripheral parenchyma of the liver or around the portal tract. However, all cases of fibrosis in children had in common a low production of IL-10 either with or without Ag stimulation. In the more stratified analysis, low IL-10 combined with a moderately high IL-5 and IL-13 response to stimulation with whole-worm or tegument Ags was the most important risk factor. IL-10 is a down-regulatory molecule that can inhibit production of both IL-5 and IL-13, and has been specifically associated with an increased risk of hepatic fibrosis in mice deficient in IL-10 (6). IL-13 has been demonstrated in mouse models to play a fundamental role in pathogenesis of fibrotic lesions associated with experimental murine schistosomiasis (21), possibly mediated by activation of the TGF-β pathway (22).

Because children at highest risk were not those with the lowest or highest IL-13 production, it is also possible that both the level of cytokine production and either expression of its receptor or other molecules in the signaling pathway to collagen gene transcription are important determinants of disease risk. Of particular importance may be the expression of the decoy receptor IL-13Rα2 (23). This high-affinity receptor binds to IL-13 but undergoes internalization without productive signaling, whereas the low-affinity receptor IL-13Rα1 in combination with the IL-4R does lead to signaling. The protective role of the IL-13Rα2 receptor may lie with its high affinity for IL-13 combined with its ability to block the IL-13-mediated transcription of collagen genes. Evidence of this effect has emerged from the mouse model, where blocking of
IL-13 signaling using a soluble inhibitor, soluble IL-13Rα2-Fc, prevents the development of fibrosis (21). Children with relatively high constitutive levels of IL-13 may therefore be protected from excessive collagen production if they also express sufficient IL-13Rα2 to absorb the excess cytokine.

Among adult women, the analysis conducted with a high minimum node size indicated that relatively high levels of TNF-α were associated with a significantly increased risk of fibrosis. After allowing finer partitioning of the data, by decreasing the minimum node size, one subgroup that contained no cases was revealed. These women were characterized as having a particularly high production of IFN-γ. Most cases of fibrosis among women were then observed to have intermediate IFN-γ production, and the association between fibrosis and TNF-α was strongest in those women that had a combination of intermediate IFN-γ and highest TNF-α production. IFN-γ is a type I cytokine involved in the down-regulation of extracellular matrix protein production, and up-regulation of collagenase activity in the liver. Furthermore, treatment with IFN-γ in murine schistosomiasis can inhibit collagen deposition (24). TNF-α is counterregulatory to IFN-γ in that it up-regulates extracellular matrix protein production, and it is also influential in murine granuloma development (25, 26).

Whereas IFN-γ inhibits fibroblast proliferation, TNF-α has the opposite effect. This functional characterization of the two cytokines is consistent with our own and others’ observations of lower IFN-γ and higher TNF-α levels in cases of periportal fibrosis. However, TNF-α is a potent inducer of the synthesis of matrix metalloproteinases (MMP), a family of zinc-dependent endopeptidases involved in the metabolism of collagen found in the extracellular matrix. Whereas this function of TNF-α is likely to be important in the pathogenesis of tissue-destructive diseases such as rheumatoid arthritis (27), it is still unclear how production of TNF-α, MMPs, and their inhibitors (tissue inhibitors of MMPs (TIMPs)) relate to the risk of hepatic fibrosis attributable to S. mansoni infection in humans. Attempts to understand their relationship in the mouse model have suggested that TIMPs do not inhibit fibrogenesis (28), and the intuitive roles for MMPs and TIMP to increase and decrease the risk of hepatic fibrosis, respectively, are therefore questionable. One reason for the polarized effects of high TNF-α levels may be that the function of this cytokine is partly dependent on the local Th1/Th2 balance (29), but its functional role in the development of periportal fibrosis is still unknown.

Our observation that adult women at highest risk of fibrosis had a combination of moderate IFN-γ and high TNF-α production is

![Diagram of classification trees](http://www.jimmunol.org/)

**FIGURE 3.** Classification trees produced as described in Materials and Methods. The vertical length of each branch indicates the relative importance of the split above. At each split involving a cytokine or chemokine factor, the breakpoint score is shown in a box along with the assays most strongly correlated with that factor. The left part of each split leads to the stratum with factor scores lower than the breakpoint, and to the right is the stratum with higher values. The terminal nodes show the proportion of individuals in that node who were cases, and the size of that node is given in brackets. A was produced with a minimum node size of 15, whereas B was produced with a minimum node size of 10.
assessed according to the Cairo classification (32), which involves
and subsequent case definition. Fibrosis in the Sudanese study was
including variation of ultrasound procedures used to identify cases,
Sudan, despite some important differences between the studies,
significantly lower IFN-γ production in sera and
controls by having significant differences in the cytokine assays that
were strongly correlated with that factor. To facilitate interpretation, factor
numbers have been replaced with cytokines or chemokines most strongly
associated with that factor.

FIGURE 4. Box-and-whisker plots of factor scores for adult females
across four risk strata as identified in the classification tree of Fig. 3A. The
median response is represented by a horizontal black line within a box that
represents the 25th to 75th percentile range. Whiskers represent the range
of data excluding outliers (●). Scores below/above 0 for a particular factor
indicate lower/higher than average responses in the cytokine assays that
were strongly correlated with that factor.

partially comparable with observations made in a case-control
study of both sexes and all ages in Brazil, where TNF-α in sera and
IFN-γ production in PBMC assays were higher and lower, respectively,
in cases of periportal fibrosis when compared with uninfected controls (30, 31). Similar observations were also made in a
case-control study of all ages and both sexes conducted in the
Sudan by Henri et al. (8). There, cases were differentiated from the
controls by having significantly lower IFN-γ production in re-
response to SEA stimulation of PBMCs, and marginally increased production of TNF-α. Our results are comparable with those from the
Sudan, despite some important differences between the studies,
including variation of ultrasound procedures used to identify cases,
and subsequent case definition. Fibrosis in the Sudanese study was
assessed according to the Cairo classification (32), which involves
staging by measuring the wall thickness of the peripheral portal
vein branches. Only individuals recorded as grades II and III fibrosis on the Cairo scale were included in the Sudanese analysis as
cases. The Ugandan cohort was assessed according to the modified
Niamey protocol (15), and individuals were designated as cases according to qualitative rather than quantitative criteria. By in-
cluding individuals with image pattern C and above, we were covering
the spectrum of early-to-late fibrosis. Second, we only observed
the protective association of IFN-γ against fibrosis in adult
women, whereas the other studies did not stratify by age or sex.
Furthermore, our observations were made from in vitro whole-
blood assays rather than PBMCs. Finally, the use of factor scores
rather than raw data from individual assays means that the IFN-γ factor was weighted toward the IFN-γ responses, but was also
partially correlated with other responses including IL-3 and IL-4 responses to adult worms.

An association between TNF-α production and fibrosis was also
observed in males, an observation that underscores the importance of
this cytokine as potential mediator in the development of peri-
portal fibrosis. High TNF-α in adult males was the single most
important risk factor in the analysis with a low minimum node
size, but we also observed that adult males with a combination of
low TNF-α and low RANTES production were at high risk.
RANTES is a chemokine primarily recognized as a promoter of
Th2 allergic responses through attraction of leukocytes to sites of
inflammation, but recent observations lend weight to the idea that
RANTES has pleiotropic functionality involving regulation of
both Th1 and Th2 responses (33). In a study of humans, levels of
plasma-associated RANTES were higher in S. mansoni-infected
individuals (34), possibly due to the Th2-mediated granulomatous
inflammation around eggs. However, a comprehensive analysis of
chemokine expression in the mouse model of S. mansoni infection
observed that RANTES levels were higher in studies where the
Th1 response was experimentally enhanced, for example by ad-
mistration of IL-12 in combination with egg Ag (35). The associa-
tion of a low RANTES response with a high risk of fibrosis in
the present study supports the hypothesis that RANTES has a
down-regulatory effect on Th2 responses.

It is interesting to note that we observed a differential associa-
tion between TNF-α or IFN-γ and fibrosis, whereas in a previous
case-control study in S. mansoni-infected children in Kenya, we
found that relatively high levels of both IFN-γ and TNF-α were
associated with severe hepatosplenomegaly (11). These results are
not inconsistent, because this previous study was based on clinical
examination rather than ultrasonography-detectable periportal
fibrosis. Schistosomiasis-associated hepatosplenomegaly can occur
in the presence of little or no periportal fibrosis (36), particularly
in children, and there is no evidence that the underlying immuno-
logical mechanisms are the same for both conditions. Furthermore,
we do not yet know whether hepatosplenomegaly in childhood is
a risk factor for periportal fibrosis in adults. One possibility is that
exposure to malaria in childhood exacerbates this form of schis-
tosomiasis-associated hepatosplenomegaly (37), and this may
cause a skewing of the immune response toward a Th1 phenotype.

Although we included some cases of periportal schistosomiasis
mixed with cirrhosis in the analysis, it is highly unlikely that the
associations between TNF-α and RANTES and fibrosis are an
artifact of the effects of alcohol-induced liver disease. The scoring
system of the Niamey protocol clearly delineates between cirrhotic
damage to the liver induced by alcohol, and fibrosis around the
portant cytokine produced in the microenvironment of portal fibrosis is hepatocyte growth factor (HGF). We have found that HGF is produced by activated Kupffer cells and sinusoidal endothelial cells and that it promotes the proliferation and migration of hepatic stellate cells, which are thought to be involved in the development of portal fibrosis. These findings suggest that HGF is a key cytokine in the pathogenesis of portal fibrosis.

In conclusion, our observations suggest that the molecular etiology of portal fibrosis associated with schistosomiasis is complex and multifactorial. Further studies are needed to elucidate the role of HGF in the development of portal fibrosis and to identify potential therapeutic targets.


