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The Frequency of *Chlamydia trachomatis* Major Outer Membrane Protein-Specific CD8+ T Lymphocytes in Active Trachoma Is Associated with Current Ocular Infection

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Chlamydia*-specific cytotoxic T lymphocytes are able to control model infections but may be implicated in disease pathogenesis. HLA-A2 peptide tetramers to *Chlamydia trachomatis* major outer membrane protein 258-266 (MOMP258-266) and MOMP260-268 were used to characterize HLA class I-restricted CD8+ T cells in Gambian children aged 4 to 15 years with clinical signs of active trachoma and/or infection with *C. trachomatis*. The frequencies of circulating HLA-A2 tetramer binding cells (TBC) were determined in whole blood samples by flow cytometric analysis. Initial screening of subjects with an anti-HLA-A2 antibody confirmed the presence of either HLA-A2 or HLA-A28. These were subsequently further divided by molecular subtyping. The *C. trachomatis*-specific HLA-A2 peptide tetramers were able to bind T cells with receptors from subjects which were restricted by either the HLA-A2 or the HLA-A28 restriction element. In this population, the median value of *C. trachomatis*-specific CD8+ T cells was 0.02%, with frequencies of up to 3.71% of CD8+ T cells reactive with a single tetramer in a minority of subjects. TBC were detected more often in subjects who were infected at the ocular surface, and their presence was associated with infection episodes of longer duration. Detection of *C. trachomatis*-specific TBC was not associated with the presence of disease or with the estimated load of ocular *C. trachomatis* infection at the time of sample collection. High frequencies of *C. trachomatis*-specific cells did not predict subsequent appearance or resolution of the clinical disease signs of active trachoma.

Chlamydia trachomatis is the intracellular bacterium responsible for the conjunctival disease trachoma. Trachoma has two distinct phases with various degrees of severity. Active trachoma, in which *C. trachomatis* is most frequently isolated, usually occurs in young children. Under the World Health Organization grading scheme, this is classified as trachomatous inflammation, follicular (TF), which may appear with or without intense inflammation—trachomatous inflammation, intense (TI) (34). Active disease episodes are more common and last longer in children than in adults from the same community living where the disease is endemic, suggesting that acquired resistance or immunity may develop with age (3). Scarring trachoma, in which *C. trachomatis* can rarely be isolated, occurs most frequently in adults. Development of scar tissue is thought to be related to the adaptive immune response responsible both for the clearance of *C. trachomatis* and in some cases the induction of tissue pathology (22).

HLA class I-restricted CD8+ effector cells (cytotoxic T lymphocytes [CTL]), usually with CD4+ T-cell help, maintain immunity to viral infections (16, 28, 32, 36). Because *C. trachomatis* is an obligate intracellular pathogen, mechanisms similar to those that control viral replication and infection may be

effective in chlamydial infection. For many years, it was considered that chlamydial antigens were not expressed on the surface of infected human cells, since after its entry into host cells *C. trachomatis* is enclosed by a membrane-bound vacuole that resists lysosome fusion. Evidence gathered from several groups since the mid 1990s indicates that CTL can be generated in response to infection with *C. trachomatis*, suggesting that antigens are made available for presentation, though the importance of these CTL in natural disease and infection remains unclear (4, 13, 21, 30). In tissue sections from human conjunctiva (27) and in primate animal models, CD8+ cells clearly accumulate at the site of infection (35). Furthermore, in this primate model, Van Voorhis et al. (35) demonstrated elevated levels of perforin mRNA in cells isolated from the site of infection. In human studies, Burton et al. (5) have also shown that subjects with ocular infection or clinical signs of disease had elevated levels of perforin mRNA in cells collected from the conjunctiva.

Work in vitro and in murine models has demonstrated that *Chlamydia*-specific CTL have the ability to lyse infected targets and to produce cytokines (gamma interferon [IFN-γ]) in response to antigenic stimulation (13, 21, 30). CTL have been used to identify antigens, such as major histocompatibility complex (MHC) class I-accessible protein 1, which were not detected by other techniques (11). In humans, *C. trachomatis*-specific CTL have been identified in subjects exposed to chlamydial infection (12, 13, 17, 19, 20). In these and other studies, CTL generated to

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a number of *C. trachomatis* epitopes that were restricted by common and disease-associated HLA types were used to identify peptide-specific responses (12, 15, 24). Human CTL epitopes have also been mapped in several major *C. trachomatis* and *C. pneumoniae* proteins and their HLA restriction described; for example, multiple overlapping epitope-dense regions have been mapped in *C. trachomatis* major outer membrane protein (MOMP) (18).

The in vitro techniques required to generate and test for CTL are often labor intensive and require significant numbers of cells. HLA-peptide tetramers or pentamers offer an alternative technique to measure the frequency of cells bearing epitope-specific T-cell receptors (TCR) that have the ability to bind to antigen. This direct analysis can describe the frequency of naturally circulating cells without any in vitro restimulation or manipulation.

Kim et al. have described several HLA-peptide tetramers specific for *C. trachomatis* MOMP which were able to bind to $CD8^+$ T cells from blood and in vitro cultures of peripheral blood mononuclear cells (PBMC) of patients with documented chlamydial sexually transmitted infection (19). Additionally, *Chlamydia*-specific HLA-B27 tetramers for a number of antigens have been used to identify specific $CD8^+$ T cells from the synovial fluid mononuclear cells of subjects with a *C. trachomatis*-induced reactive arthritis, albeit at low frequency (2, 20). For *C. pneumoniae*, HLA-A*0201-restricted tetramers specific for T-cell epitopes in heat shock proteins 60 and 70 have described the presence of low-frequency populations in cultured PBMC of some subjects with coronary heart disease (7). One common feature appears to be that without in vitro restimulation, *Chlamydia*-specific tetramer-binding cell (TBC) frequencies are very low.

In order to study a large number of subjects, we selected two tetramers covering overlapping epitopes of MOMP that fall in an epitope-dense region and are restricted by a common HLA type, HLA-A*0201, to screen subjects from a region where trachoma is endemic.

The prevalence of HLA-A2 in the Gambian population is ~25%; however, a further consideration is the fact that a number of HLA-A types can present identical peptide epitopes. For this reason, HLA-A2 is considered a supertype, consisting of HLA-A*0201, -A*0202, -A*0203, -A*0206, and -A*6802. HLA-A*6802 is itself part of a serological determinant, HLA-A28. HLA-A28 has three molecular subtypes, HLA-A*6801, -A*6802, and -A*6901. The prevalence of HLA-A28 in the Gambian population is ~20%. HLA-A*6802 constitutes >95% of the HLA-A28 in Gambian populations (9). Computational models of predictive epitope binding have been validated by binding and presentation of HLA-A2 nonamer peptides by HLA-A*6802 (10, 23, 29). The same techniques can be used to look at cross-reactivity of TCR-pMHC complexes. Thus, if the reactive surfaces of HLA-A2 and -A*6802 are sufficiently similar, any TCR reactive to one should be reactive to the other, such that TCR restricted by HLA-A*6802 may bind HLA-A2–pMHC. The lack of structural data for TCR, however, is a major constraint in the prediction of the reactivity of TCR-pMHC complexes. We show that HLA-A2–peptide tetramers can cross-react with $CD8^+$ TCR from HLA-A*6802 subjects but not with $CD8^+$ TCR from HLA-A2- or -A*6802-negative subjects. We then analyzed whether either the presence or the frequency of TBC is associated with clinical signs of active trachoma, infection, or the conjunctival *C. trachomatis* load in HLA-A2 subjects.

MATERIALS AND METHODS

Subjects. A subgroup of 159 subjects from a larger-cohort study consented to venipuncture (1 to 5 ml). The entire cohort consisted of 346 children, aged 4 to 15 years, from nine villages in The Gambia. Ocular examinations and samples were collected every 2 weeks over a period of 28 weeks. At each visit, a clinical examination of the conjunctiva was made and a digital photograph of the everted eyelid was taken. Trachoma was graded according to the World Health Organization simplified grading scheme by an experienced trachoma examiner. Swabs were used to collect cellular material from the conjunctiva of the right eye, a dry swab for DNA and a swab for RNA collected in RNAlater buffer [Ambion (Europe) Ltd., Cambridge, United Kingdom]. Children with TI were offered treatment immediately with 20-mg/kg oral azithromycin. Children with TF and their household members were offered treatment according to the national program policy at the end of the study period. The joint Gambian Government-Medical Research Council Ethics Committee and the Ethics Committee of the London School of Hygiene & Tropical Medicine approved the design and procedures of this study.

***C. trachomatis* ocular load.** The conjunctival *C. trachomatis* load was determined as described elsewhere (5). Briefly, conjunctival swabs from the right eye were immediately collected into the RNA stabilization buffer RNAlater [Ambion (Europe) Ltd., Cambridge, United Kingdom]. RNA was then extracted according to the manufacturer's instructions with the RNeasy 96 Qvac system (QIAGEN Ltd., Crawley, United Kingdom). Additional precautions were taken to remove copurified DNA by treatment with RNase-free DNase I and further incubations and washes with the buffers RW1 and RPE (QIAGEN). Before the final elution, the 96-well Qvac plates were dried by centrifugation for 15 min at $200 \times g$ and then incubation at 70°C for 15 min. RNA was then eluted with 2×65 μl of RNase-free H_2O for 5 min at room temperature. Samples were stored at −70°C until assayed by quantitative real-time reverse transcription-PCR with QuantiTech SYBR Green reverse transcription-PCR kits (QIAGEN Ltd., Crawley, United Kingdom). The extent of productive chlamydial infection was determined with primers specific for *C. trachomatis* 16S rRNA (25).

HLA-A2/28 staining. Fluorescein isothiocyanate-labeled anti-HLA-A2/28 (VH Bio Ltd., Newcastle, United Kingdom) was used to rapidly identify subjects from the study population with the appropriate HLA type. Briefly, 5 μl of fluorescein isothiocyanate-conjugated anti-HLA-A2/28 was added to 50 μl of whole blood and the mixture was incubated at 4°C for 15 min. Red blood cells were then lysed by addition of 1.5 ml of 1× fluorescence-activated cell sorter (FACS) lysing solution by vortex mixing and 10 min of incubation at room temperature. Cells were harvested by centrifugation and resuspended in 100 μl of FACS wash before immediate acquisition of fluorescence in the mononuclear cell gate (50,000 events). Results were expressed as histogram plots of event counts versus fluorescence intensity. Cells from previously confirmed HLA-A2/28-positive and -negative individuals served as controls.

HLA-A2 and -A28 (HLA-A*6801, -A*6802, and -A*6901)-specific typing by PCR. HLA class I typing was carried out by PCR with the sequence-specific primers shown in Table 1. All samples were checked for inhibition of PCR and DNA integrity by amplification of the β2-microglobulin gene. Template DNA was prepared by extraction and purification of DNA from conjunctival swabs as described elsewhere (6). Two to 10 ng of genomic DNA in 2 μl of H_2O was added to 23 μl of a standard hot-start PCR mixture (QIAGEN Ltd., Crawley, United Kingdom) containing primers and cycled under the conditions described in Table 1, footnote *a*.

Tetramer staining and FACS analysis. Previously identified CTL epitopes from the MOMP CS4 epitope cluster were selected; HLA-A2 MOMP258 ($_{258}$R LNMFTPYI$_{266}$)- and HLA-A2 MOMP260 ($_{260}$NMFTPYIGV$_{268}$)-restricted tetramers were synthesized by Proimmune (Oxford, United Kingdom). Both of these peptide sequences contain the preferred residues for HLA-A*0201; these are L or M at P2 and I or V at P9. One microliter of phycoerythrin-conjugated tetramer was added to 150 μl of heparinized whole blood, and the mixture was incubated at 37°C for 15 min. Fluorochrome-conjugated anti-CD8 monoclonal antibody PerCP (BD Sciences, Oxford, United Kingdom) was then added, and the mixture was incubated for a further 15 min at room temperature. Red blood cells were lysed by the addition of 3 ml of 1× FACS lysing solution (BD Sciences, Oxford, United Kingdom). Samples were vortexed and allowed to stand for 15 min at room temperature in the dark. Cells were then collected by centrifugation at $800 \times g$ for 5 min. The supernatant was discarded and the pellet resuspended with 3 ml of FACS wash solution. Cells were then collected by centrifugation as before. The pellet was finally resuspended in 200 μl of FACS fix (phosphate-

TABLE 1. Primer sequences, binding positions, product sizes, and thermal cycling conditions used in HLA PCR typing[a]

Primer	Sequence	Target (relative exon binding position)	PCR parameters		Product size (bp)
			Annealing temp (°C)	No. of cycles	
HLA#A2F	GTGGATAGAGCAGGAGGGT	HLA-A exon 2 (149–167)	70	7	491
HLA#A2R	CCAAGAGCGCAGGTCCTCT	HLA-A exon 3 (110–127)	65	30	
			55	3	
AL#6	CGGAATGTGAAGGCCCAG	HLA-A exon 2 (192–209)	66	5	447
AL#H	CAAGAGCGCAGGTCCTCT	HLA-A exon 3 (110–127)	56	30	
			50	5	
β2M#5	AGATTCAGGTTTACTCACG	β2-M exon 2 (132–150)	66	5	264
β2M#6	TAACTATCTTGGGCTGTGA	β2-M exon 3 (377–395)	50	35	

[a] All PCRs included 15 min of heat activation of DNA polymerase enzyme at 95°C. Each denaturation and extension phase was 15 s at 94°C and 30 s at 72°C. The annealing temperatures and the cycle numbers are indicated. All exon locations and product sizes are based on sequences with the following accession numbers: HLA-A*0201, AJ575565; HLA-A*68, AJ633570; β2-M, M17987.

buffered saline containing 2% formaldehyde). Fluorescence data were acquired immediately or within 12 h on a FACScalibur flow cytometer (BD Sciences, Oxford, United Kingdom). For each analysis, 250,000 events gated on lymphocyte size and granularity were acquired. The percentages of tetramer^{+} cells in the CD8^{+} positive gate were then expressed. All FACS data were analyzed with FCSExpress v2.0 software. HLA-A2-negative subjects were tested in the same manner to control for nonspecific tetramer binding. Each tetramer staining experiment included this negative control, which was later used to define the range of non-tetramer-specific binding. This percentage was not subtracted from the values obtained in HLA-A2 or -A28 subjects. The data are presented as unadjusted percentages. Laboratory staff were blind to clinical disease status and infection status.

Statistical applications. The data from the longitudinal study were managed by Microsoft Access. For statistical analysis, data were extracted, manipulated, and analyzed in Stata v8.0. The tetramer frequencies were highly skewed, even after log transformation; therefore, results were tested for significance with nonparametric Kruskal-Wallis and Mann-Whitney tests. In the subgroup of subjects selected for tetramer studies, the potential number of data collection points was 1,288. We made 1,132/1,288 clinical observations and 1,084/1,288 PCR tests for ocular *C. trachomatis*. For the construction of disease duration data, concordant observations of disease/infection status at 2-weekly follow-up were taken to imply that the state was unchanged between the observations. Where a single observation is missing between concordant observations, continuity was assumed and where a missing observation intervened between changes of state, the state was assumed to have changed midway between the observations.

RESULTS

Study population demographics, clinical disease, prevalence of infection, and frequency of HLA-A2/28. There were 114 females (33%) and 232 males (67%) in the cohort; the median age of study participants was 8 years (range, 4 to 15 years). Seventy-four children (21%) had clinical signs of trachoma (TF and/or TI) at their first examination, and 18 children (5.2%) had evidence of trachomatous scarring. Eighty-two subjects had incident active trachoma at follow-up. The frequency of HLA-A2/28 by serology in those subjects from whom blood samples were obtained was 75/159 (47.1%) by FACS analysis. Molecular subtyping of HLA-A2 and -A28 (HLA-A*6801, -A*6802, and -A*6901) identified 45/159 (28.3%) and 39/159 (24.5%), respectively. Eight samples that were HLA-A2/28 positive by FACS could not be confirmed by PCR. Sixteen samples that were negative by FACS were either HLA-A2 positive (n = 10) or HLA-A28 positive (n = 6) by PCR. Eighty-six subjects were included in the tetramer analysis. None of these subjects had TI at the time the blood sample was

collected. Of these, 42 were HLA-A2 positive, 24 were HLA-A28 positive, and 20 were HLA-A2/28 negative controls. In the subgroup analyzed for tetramer staining with a PCR-confirmed HLA type (n = 86), there were 35 females and 51 males, with a median age of 9 (range, 4 to 15) years at the time of venipuncture. Seventeen children had signs of active trachoma, and three had evidence of trachomatous scarring. Infection (*C. trachomatis* 16S rRNA) was present in 27.9% (24/86) of the subjects in which tetramer staining was undertaken.

A minority of subjects from communities where trachoma is endemic have circulating *C. trachomatis* MOMP-specific CD8^{+} cells. Sixty-six HLA-A2/28-positive subjects and 20 HLA-A2/28-negative samples were tested for reactivity with the HLA-A2 *Chlamydia*-specific tetramers. HLA-A2/28-negative subjects were used to establish the range of nonspecific background obtained by
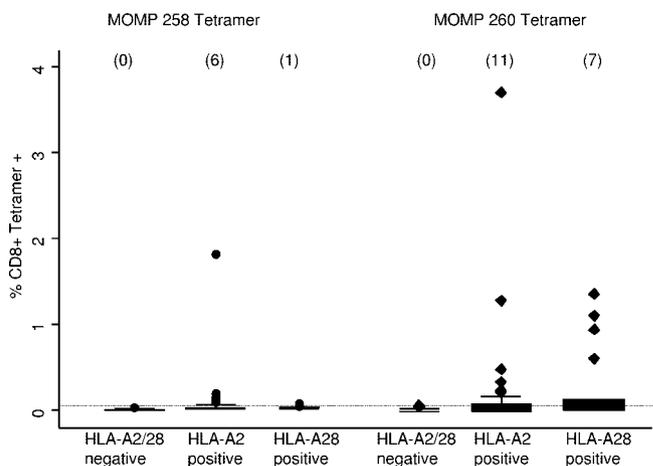

FIG. 1. The number of TBC detected is associated with the presence of HLA-A2 or -A28. The number of subjects with a percentage of TBC above the maximum obtained with HLA-A2/28 (shown in parentheses) was as follows for HLA-A2 subjects: MOMP258 level of >0.04, n = 6 (range, 0.05 to 1.71%); MOMP260 level of >0.08, n = 11 (range, 0.09 to 3.8%). For HLA-A28 subjects, it was as follows: MOMP258 level of >0.04, n = 1 (0.06%); MOMP260 level of >0.08, n = 7 (range, 0.09 to 1.35%). Cumulative total TRC, i.e., percent MOMP258 plus MOMP260 of >0.1: HLA-A2 subjects, n = 11 (range, 0.11 to 5.51); HLA-A28 subjects, n = 8 (range, 0.13 to 1.36) (data not shown).
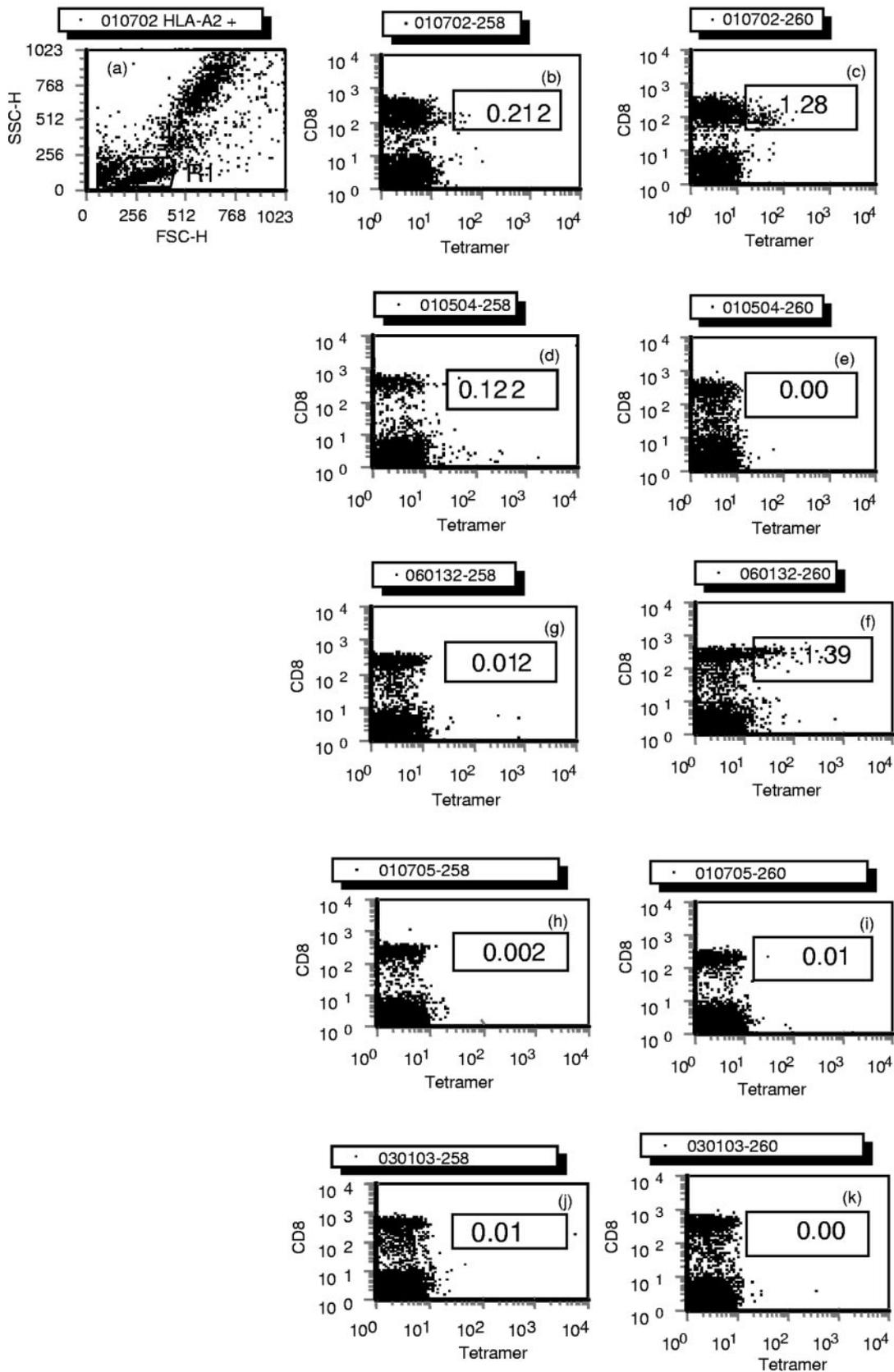
FIG. 2. FACS plots from four different HLA-A2-positive subjects (a to i). Panels j and k are plots from a single HLA-A2/A28-negative subject. The subject number is shown above each plot. Panel a shows the R1 gate for lymphocytes used in all subsequent plots. Panels b and c show the percent positive for either MOMP258 or MOMP260 for the subject shown in panel a. The gate and the percent CD8^{+} tetramer^{+} are indicated in each plot. Panels b and c show results for a subject positive for both MOMP258 and MOMP260. Panels d and e show results for a subject positive for MOMP258 but not MOMP260. Panels f and g show MOMP260-reactive but not MOMP258-reactive cells. Panels h and i show no cells with reactivity with either MOMP258 or MOMP260.
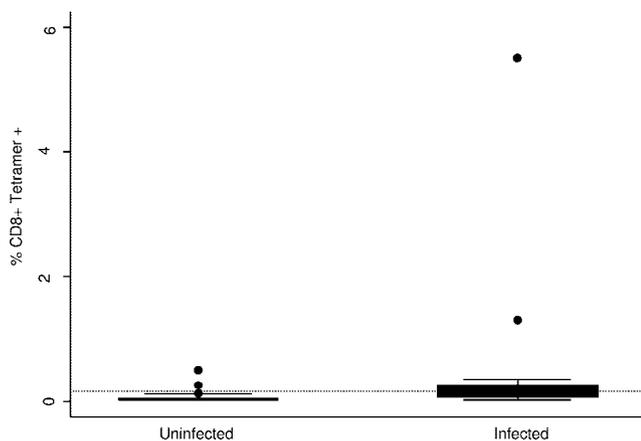
FIG. 3. TBC are associated with the presence of infection. Median values (ranges) are as follows: uninfected subjects, 0.03% (0.02 to 1.31%); infected subjects, 0.15% (0.02 to 5.51%) (Mann-Whitney $P = 0.02$). Of 42 HLA-A2-positive subjects, 10 had ocular infection. Of these, eight had TBC values of $>0.1\%$. Of the 32 subjects without infection, only 2 subjects had TBC values of $>0.1\%$.
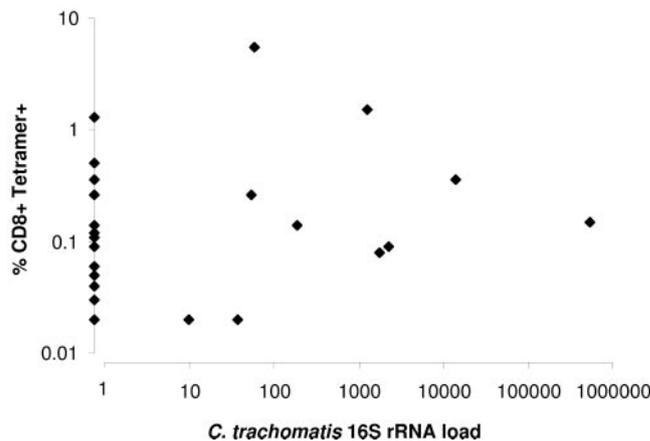

FIG. 4. Percent TBC (total TBC) and extent of *C. trachomatis* shedding or load of infection at the conjunctiva. For presentation on a log scale, zero values were treated by the addition of a constant (*C. trachomatis* 16S rRNA load + 1) to each value in the data set examined (42 HLA-A2-positive subjects with or without infection).

flow cytometry with these tetramers. Figure 1 shows the distribution of tetramer reactivity and the percentage of TBC to each individual tetramer, irrespective of clinical status or infection, in HLA-A2 and HLA-A28 subjects compared to non-HLA-A2/28 subjects. This clearly indicates that tetramer-specific cells are present in the PBMC of HLA-A2 subjects in a population resident where trachoma is endemic. The range of tetramer reactivity in non-HLA-A2/28 subjects was 0 to 0.04% (MOMP258) and 0 to 0.08% (MOMP260). Only responses above these values were considered indicative of tetramer-reactive cells in HLA-A2 subjects. For further analysis, the values obtained with both tetramers were summed (MOMP258 plus MOMP260) to yield a total TBC percentage. As a cutoff, the highest combined level obtained in a single individual was used (0.1%).

Of 42 HLA-A2 subjects, 10 subjects had total TBC frequencies above 0.1%. The remaining 32 subjects had levels of $\leq 0.1\%$ and so were considered negative. Of 24 HLA-A28 subjects, 8 were positive by these criteria. Since HLA-A28 subjects may represent a subset of subjects whose T cells may cross-react with tetramers containing HLA-A2, these subjects were eliminated from subsequent analysis but were not considered suitable as HLA-A2 negative controls. Figure 2 shows the typical staining obtained by flow cytometry in whole blood from four separate HLA-A2 individuals and a single non-HLA-A2/28 subject. This demonstrates the complete range of binding patterns. Typical FACS plots of TBC to each tetramer are shown in Fig. 2a to k, with frequencies of TBC for individual tetramers as follows: MOMP258, 0.002 to 0.212%; MOMP260, 0 to 1.39%.

TBC are detected more often and at higher frequencies in the presence of *C. trachomatis* infection. Tetramer frequencies and their relationship with clinical signs of active trachoma and the presence of *C. trachomatis* infection at the time the blood sample was taken were examined. Detection of TBC in HLA-A2 subjects was associated with the presence of infection, and higher TBC frequencies (percent) were found in the

presence of clinical signs (Fig. 3). However, TBC frequency and the load of ocular infection (16S rRNA quantity) could not be objectively summarized with a simple summary statistic. Correlation is the most appropriate analysis tool; however, a significant number of observations are below the sensitivity of each test (<10 copies of *C. trachomatis* 16S rRNA and $<0.1\%$ TBC). This leads to clustering of the data, which generates bias in a simple correlation. Given the small sample size, this is not open to more complex modeling to account for clustering. Data are therefore presented in a simple scatterplot (Fig. 4). Detection of TBC and the frequency of total TBC were associated with infection but not associated with clinical disease signs. Thus, clinical signs without coincident infection (*C. trachomatis* 16S rRNA) or clinical disease signs with infection
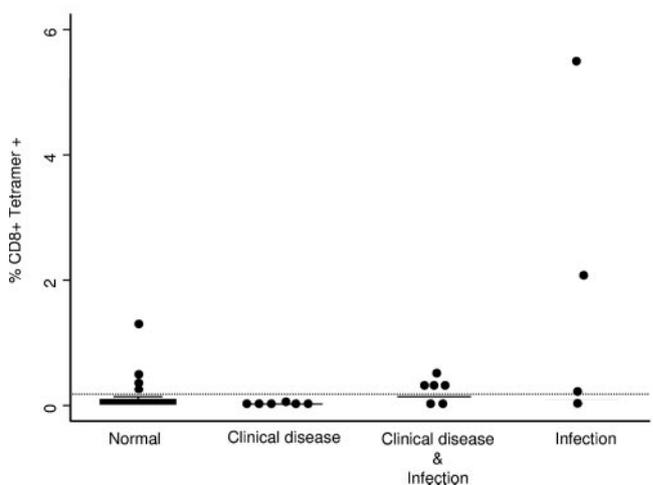

FIG. 5. Percent TBC is not associated with clinical observation of disease ($P = 0.52$) but is associated with infection ($P = 0.05$). The numbers of subjects in whom the percent TBC was >0.1 were as follows: normal, 4/26; clinical disease signs only, 0/6; clinical disease signs and infection, 4/6; infection only, 2/4. The numbers of subjects in the affected groups were limited, and thus the statistical inference is underpowered.

TABLE 2. Longitudinal data showing the relationship(s) of disease signs and infection in HLA-A2 subjects with detectable TBC compared to those without detectable TBC

Parameter and TBC level	n	Median	Range	P value
Duration of infection (days)				
>0.1	10	33.5	0–146	0.0048
≤0.1	32	14	0–91	
Duration of disease signs (days)				
>0.1	10	13	0–190	0.72
≤0.1	32	0	0–203	
No. of infection episodes				
>0.1	10	2	0–3	0.013
≤0.1	32	1	0–5	
No. of disease episodes				
>0.1	10	2	0–6	0.96
≤0.1	32	1.5	0–5	

were also independent of TBC frequency (Fig. 5). In this analysis, only infection at the time of blood sampling is considered. The control group was infection free at the time of sampling. Because these communities live where trachoma is endemic, all individuals within this age range will experience infection and are likely to be antigen experienced. This is shown by equal levels of whole-blood IFN-γ recall responses to chlamydial elementary bodies (data not shown) between infected and uninfected subjects.

TBC are more often detected during longer infection episodes. From the longitudinal data, it is possible to search for associations between TBC frequency and the number of disease episodes, duration of disease episodes, number of infection episodes, and duration of infection and to examine these together (Table 2). Clinical disease sign durations were estimated in the same manner for each subject in which tetramers were tested. Subjects in whom TBC were detected had longer infection episodes: the median duration (range) of current infection in TBC-positive subjects was 33.5 (0 to 146) days, compared to 14 (0 to 96) days in the TBC-negative group (P = 0.0048). A larger number of infection episodes was also associated with detection of TBC (P = 0.013). The range of disease sign episodes was zero to six, but the frequency of TBC was not associated with the number of disease episodes during follow-up. The duration of disease episodes was also independent of TBC frequency.

DISCUSSION

We have demonstrated that TBC are present in the peripheral blood of patients exposed to ocular *C. trachomatis* and that the presence of TBC is dependent on the presence of HLA-A2/28. This was important in the absence of a T-cell line reactive to these epitopes which could be used to confirm tetramer specificity when each staining reaction was conducted. The prevalence of HLA-A2/28 in this population was 48% by FACS analysis, which is similar to that found in previous studies (1, 9). We used molecular typing to confirm the presence of HLA-A2 and -A28 after tetramer staining. Thus, by eliminating cross-reactive HLA-A28 subjects from the analysis and studying a number of non-HLA-A2/28 subjects with

and without infection or clinical disease signs, it was possible to establish a range of reactivity and define a stringent positive binding result.

In two separate studies, Kim et al. (17, 19) described *C. trachomatis* MOMP-specific T cells with CTL activity and addressed the question of whether such cells are representative of in vivo responses to infection. Cells reactive to several MOMP epitopes were characterized with HLA-A2–peptide tetramers in patients with documented genital *C. trachomatis* infection. Frequencies in a range of 0.01 to 0.2% were identified, and it was possible to culture and isolate TBC with CTL activity. Two MOMP epitopes were predominantly studied, MOMP258-266 and MOMP260-268, but it was noted that the frequencies obtained were significantly lower than those observed with some viral infections. With these same epitopes in HLA-A2–peptide tetramers, we obtained frequencies in a range 0.01 to 3.7% (for 42 HLA-A2 subjects, mean and median values of 0.18% and 0.02%, respectively, were obtained for a single tetramer [MOMP260-268]) in peripheral blood of subjects in communities where trachoma is endemic. This indicated that subjects in communities where trachoma is endemic recognized these epitopes; however, it is still not established if these are immunodominant epitopes. In other human studies in which *Chlamydia*-specific tetramers have been used, even cells at the site of inflammatory disease (isolated from the synovial fluid of reactive-arthritis patients) were present at very low frequencies (0.02 to 0.09%). In both cases, only after isolation and expansion in vitro were appreciable levels of TBC obtained (2, 19).

Nevertheless, our data indicate that in subjects exposed to the ocular serovars, TBC to these conserved MOMP epitopes are generated. Among the possible explanations for the low frequency of TBC in patients with genital or synovial chlamydial infection are the low number of subjects tested and the inability to characterize these subjects in terms of the amount of *C. trachomatis* exposure, antigen load, and temporal observations of disease and infection. A longitudinal cohort study in communities where trachoma is endemic has allowed us the opportunity to observe disease and infection in a temporal manner and measure microbiological correlates such as antigen load. In this case, we can address the questions posed by Kim et al. (19) about the use of HLA-peptide tetramers to identify, quantify, and study immunopathogenic consequences of infection.

Associations of frequency of TBC with disease and infection parameters were tested both in a cross-sectional study and longitudinally. At the time of sample collection, the frequency of TBC was dependent on evidence of ocular *C. trachomatis* infection but independent of clinical disease signs. A number of subjects had subclinical infection, i.e., were *C. trachomatis* 16S rRNA positive without clinical signs of trachoma. Similar findings have been found in other studies in which TBC and viral replication have been examined. In these cases, it has been suggested that TBC have little role in the inhibition of local infection but are effective in protection against dissemination (8). Evidence which may support this comes from the observation that strong *C. trachomatis* CTL responses are induced in animal models by *C. trachomatis* serovar L when introduced by a route which leads to disseminated infection (30).

Examination of disease and infection over the study period

revealed an interesting pattern of responses linked to infection. Longer durations of infection were associated with the detection of peripheral blood TBC, suggesting either that TBC result from long-duration infections or that they could be responsible for increasing the duration of infection.

Since the duration and number of disease episodes were independent of TBC frequency, this may indicate that TBC are not directly immunopathogenic. Kim et al. (19) offered the explanation that low levels of TBC in genital *C. trachomatis* infection were due to the fact that the sexually transmitted infection subjects studied were not persistently infected or re-exposed or reinfected. We demonstrate that, in subjects exposed to ocular *C. trachomatis* infection, the levels of TBC for these MOMP epitopes in trachoma are dependent on current infection but not disease signs.

Longer duration of ocular *C. trachomatis* infection is associated with a higher frequency of TBC, perhaps suggesting that the rapid expansions and contractions of this cell population are in response to infection; however, it is likely that a number of infection episodes are required before TBC can be detected. A further possibility is that the TBC quantitative peripheral response is not an important parameter. Recent evidence that CD4$^+$ CD25$^+$ T regulatory cells can prevent CTL homing to sites of infection implies that these cells control the effectiveness of the response (33). Thus, differing levels of peripheral TBC observed in disease and infection may not be representative of TBC that are able to migrate to the site of infection in the conjunctival epithelium. It is also a possibility that a balance between T regulatory cells and T effector cells (CTL,T$_H$1, or T$_H$2) may lead to chronic infection, such that over time the number of TBC expands and they become more easily detectable.

There is no direct demonstration that MOMP can serve as a substrate for the chlamydial type III secretory apparatus and thus gain access to the cytosol, where it may enter antigen presentation pathways. Several studies have shown that chlamydial substrates of type III secretion can be translocated into the cytosol of infected host cells (14) and that these can become targets for CTL. In addition, CD8$^+$ T cells specific for MOMP have been repeatedly demonstrated and these can produce IFN-γ and/or lysis of infected target cells. These CD8$^+$ T cells most likely occur as a result of antigen cross-presentation (31). This is consistent with the finding that the levels of TBC reflect current infection status. Once TBC are stimulated by cross-presentation, in order to be effective they must be able to recognize *C. trachomatis*-infected cells at the conjunctival surface. Infected conjunctival epithelial cells are not known to cross-present antigen, and therefore the MOMP-specific T cells may not recognize these as target cells. With no mechanism to respond to these cells, expand at these sites, or effect clearance of the pathogen, they will not be maintained once the organism load declines. Infected epithelial cells can serve as target cells in vitro. Beatty and Stephens (4) have shown that infected L cells transfected with ICAM-1 can serve as targets for class I-restricted cells. However, in vitro-infected human fibroblasts could not be lysed by *Chlamydia*-specific CD8$^+$ T cells restricted by polymorphic MHC class Ia antigens, irrespective of ICAM-1 status. Only T cells restricted by less polymorphic MHC class Ib molecules could lyse cells. Interestingly, infected fibroblasts were able to stimulate either

CD8$^+$ T-cell population to secrete IFN-γ (13). Complementary and contradictory results were presented by Matyszak and Gaston (26), who also demonstrated the development of two types of CD8$^+$ CTL restricted by either classical MHC class I or nonclassical MHC class I. These clones, however, were equally able to lyse infected target cells (U937). Few studies have used infected epithelial cells as CTL targets; however, Kim et al. (19) successfully used *C. trachomatis* serovar E-infected, HLA-A2-transfected ME180 human cervical epithelial cells as a target cell population for peptide-restimulated and tetramer-enriched effector cells. It follows, then, that host cell targets must play an important and major role in the effectiveness of the response.

In diseases in which persistent or repeated infection appears important in morbidity, these data show the importance of longitudinal follow-up, since we find that TBC are associated with duration of infection but not signs of disease. The frequencies of MOMP-specific, class I-restricted T cells detected in peripheral blood are not the determinants of immune response-mediated resolution. It is possible that the observed HLA-A*6802 association with severe conjunctival scarring in Gambian populations living where trachoma is endemic (9) is due to a class I-restricted T cell, e.g., a TBC which curtails the effective immune response. Since it is the accepted view that the number or intensity of infection episodes drives scarring, our data could support the view that subclinical or inapparent infection is also important in this process.

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