CD4+ Lymphocytes and Gamma Interferon Predominate in Local Immune Responses in Early Experimental Syphilis

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The clearance of Treponema pallidum subsp. pallidum from early syphilis lesions involves infiltration of a large number of mononuclear cells and is characteristic of a cell-mediated immune response. In the present study, we sought to determine the relative abundance of different T-lymphocyte populations and Th1/Th2-associated cytokines present in testicular lesions following experimental infection with the Chicago strain of T. pallidum. Using flow cytometry, we examined the proportion of CD4+ and CD8+ T cells present throughout the progression and resolution of primary syphilis in the rabbit model. We related these findings to the results of real-time reverse transcription-PCR quantification of treponemal and cytokine mRNA levels. Treponemal mRNA levels reached peak values on day 18 postinfection, coincident with an initial peak in the level of T cells, which were primarily CD4+ T cells. T-cell levels increased again during resolution of orchitis, and there was an increased proportion of CD8+ T cells. The maximum gamma interferon (IFN-γ) and interleukin-10 (IL-10) mRNA levels were observed on days 11 and 18, respectively, while only negligible amounts of IL-4 and IL-2 were detected throughout the infection. In addition to showing the temporal relationship between treponemal burden and T-cell responses during lesion progression, our results also demonstrate that the composition of the T-cell population changes during lesion resolution. The presence of the mRNA for IFN-γ, but not IL-4, is consistent with cytokine expression in human syphilis and provides further support for the hypothesis that there is a Th1 predominance during the early immune response to T. pallidum.

Natural infection with Treponema pallidum subsp. pallidum, the causative agent of syphilis, results in the formation of a primary lesion, or chancre, at the site of infection. A vigorous immune response develops early during infection and results in local clearance of the majority of treponemes and resolution of the primary lesions. The cell phenotypes and cytokines present in early syphilitic lesions during human infection and experimental infection in the rabbit models suggest that the early immune response to T. pallidum is characteristic of a delayed-type hypersensitivity or Th1 response. A large number of mononuclear cells, consisting mostly of T cells and macrophages, infiltrate early lesions prior to bacterial clearance and lesion healing (15, 23, 24, 28). CD4+ helper T cells are believed to mediate bacterial clearance primarily through the production of cytokines, such as gamma interferon (IFN-γ), which activate macrophages (14, 30). Macrophages then engulf and kill opsonized treponemes (2, 17). There is also evidence that activated cytolytic (CD8+) T lymphocytes participate in the local immune response within lesions, although their role in clearance remains unclear (18, 31). An important role for antibody in bacterial clearance has also been demonstrated (3, 4, 16); however, reports vary on the relative abundance of B cells within the lesions (15, 18, 32, 34).

A study examining the cytokine mRNAs present in human primary and secondary syphilitic lesions showed that Th1-associated cytokines (IFN-γ, interleukin-2 [IL-2], and IL-12) were present, while Th2-associated cytokines (specifically IL-4) were consistently absent (30). Further evidence of Th1 predominance during early infection was obtained by Arroll et al., who demonstrated that in vitro restimulation with whole T. pallidum or recombinant treponemal antigens induced the production of predominantly Th1-associated cytokine mRNAs (IFN-γ and IL-2) in spleen cells from T. pallidum-infected rabbits (1). Furthermore, lipoproteins of T. pallidum, such as TpN17 and TpN47, are potent inducers of proinflammatory cytokines, such as IL-12, which may also promote the initiation of a Th1 response (5, 21).

Previous studies have provided a detailed characterization of the pathogenicity of early lesions and have delineated important aspects of the immune response to T. pallidum. However, our understanding is far from complete due to inherent difficulties in conducting immunologic studies of syphilis in either humans or experimental animal models. Human studies are often limited due to the availability of appropriate clinical samples and do not permit examination of lesion progression and healing following administration of a defined inoculum. Experimental studies in the rabbit model, which is the best-characterized and most extensively used small-animal model for syphilis, have long been hindered by the lack of the reagents and assays for immunologic studies available for other animal models, like the mouse model (29). In the present study, we characterized the temporal patterns of infiltrating T-lymphocyte subsets during experimental testicular infection with the Chicago strain of T. pallidum and related our findings to both cytokine responses and the bacterial burden. This study included the first analysis of T-cell subsets that infiltrate syphilis lesions in the rabbit and the first quantitative measure of the cytokine message in rabbit testes in vivo.
MATERIALS AND METHODS

Animals. The animals used in this study were outbred adult male New Zealand White rabbits obtained from R&R Rabbity (Stannwood, WA). Only animals that were seronegative in tests for syphilis, thus excluding infection with Treponema pallidum (rabbit syphilis), were used. Rabbits were housed separately at 15 to 18°C and provided with antibiotic-free food and water. All studies were approved by the University of Washington Animal Care Committee and conducted in accordance with institutional guidelines.

Antibodies. For flow cytometry experiments, T-cell populations were stained with a rat anti-rabbit Pan-T fluorescein isothiocyanate (FITC) conjugate (Ken5 clone; Antigenix, Huntington Station, NY), which has previously been shown to be highly specific for rabbit T cells (10, 11), either alone or in conjunction with anti-rabbit CD4-phycocerythrin (PE) conjugate (Antigenix) or anti-CD8-PE conjugate (Antigenix). Optimal antibody dilutions were determined by titration using spleenocytes from uninfected rabbits prior to the use of antibodies in syphilis experiments. Isotype-matched control antibodies were purchased from the same manufacturer and used at the same dilution as the specific antibodies.

Experimental infection with T. pallidum. A total of 16 rabbits were infected intratestically with 7 × 107 treponemes of the Chicago strain of T. pallidum as described elsewhere (7, 15). Groups of four uninfected animals or animals infected for 11, 18, 25, and 39 days were euthanized, and the testes of each animal were aseptically removed as previously described (15). The first time point for the infected groups was determined by the gross appearance of the testes indicating orchitis and was followed by two weekly harvests of tissues and final harvest at lesion resolution, when the testes returned to the normal size.

Cellular extraction and staining for flow cytometry. Testes from each animal were collected individually in sterile 100-mm tissue culture dishes (BD Falcon, Bedford, MA) and placed on ice throughout processing. Testes were cut in half by mechanical disruption, which first involved fine mincing and agitation of the tissue. Next, the tissue homogenate was passed through a disposable 70-μm nylon cell strainer (BD Falcon), and cells were further released into a disposable 50-ml tube (BD Falcon) by firmly pressing the tissue against the nylon mesh of the strainer using the plunger of a disposable 10-ml syringe (BD Falcon). Cells were washed using 20 ml of FACS staining buffer and pelleted by centrifugation at 200 × g for 10 min, and the red blood cells were lysed by incubation in 0.85% NH4Cl at 28°C for 7 to 10 min. After three more washes with cold FACS staining buffer, cells were passed through a fresh 70-μm cell strainer (BD Falcon) to remove clumps of dead cells. Viable cells were enumerated by light microscopy using a hemocytometer and trypan blue exclusion (Sigma, St. Louis, MO).

RNA extraction and cDNA synthesis. A total of 16 rabbits were infected intratestically with 7 × 107 treponemes of the Chicago strain of T. pallidum as described elsewhere (7, 15). Groups of four uninfected animals or animals infected for 11, 18, 25, and 39 days were euthanized, and the testes of each animal were aseptically removed as previously described (15). The first time point for the infected groups was determined by the gross appearance of the testes indicating orchitis and was followed by two weekly harvests of tissues and final harvest at lesion resolution, when the testes returned to the normal size.

RESULTS

T. pallidum burden in testicular lesion and clinical course of early experimental syphilis with the Chicago strain. The gross changes in the testicular tissue observed during experimental infection with the Chicago strain of T. pallidum closely resembled the changes described in previous studies performed with the Nichols laboratory strain, except that lesion progression was slightly delayed (15). Enlargement and firmness of the testes indicative of orchitis became apparent at day 11 and reached maximal levels at day 18. We chose to examine cellular infiltration and cytokine expression at times corresponding to early (day 11) and maximal (day 18) orchitis, as well as during (day 25) and following (day 39) healing of the lesion; this range encompasses the period of local bacterial clearance. Copies of TpN47 mRNA, a highly expressed treponemal gene used in this study as a marker for the relative burden of viable treponemes in the testes, were easily detectable at the first assay time after infection (day 11) and reached peak levels at day 18 (Fig. 1A). At day 18, the gross inflammation was greatest, with focal hemorrhage and a serous exudate throughout the testes. Although signs of inflammation were still apparent at day 25, very little treponemal mRNA was detected at this time and healing was becoming apparent, with areas of the testes exhibiting fibrosis or a gross appearance similar to that of normal tissue. The testes returned to an overall firmness and size similar to the overall firmness and size of uninfected testes by day 39 postinfection. No treponemal mRNA was detectable at this time, and the gross appearance resembled that of normal, healthy testis tissue.

T-cell infiltration in testicular lesion during early experimental syphilis. Most information regarding the T cells within early lesions during experimental syphilis has resulted from
immunohistological evaluations, where quantification of lymphocyte populations is difficult. Here we employed flow cytometry with antibodies specific to rabbit T-cell subpopulations both to characterize the temporal pattern of the T-cell responses in the lesion and to determine the relative proportions of CD4\(^+\)/H11001\(\) and CD8\(^+\)/H11001\(\) T cells present during lesion progression and healing. In uninfected testes (day 0) less than 0.2% of cells stained positive as T lymphocytes. Elevated levels of T cells were evident at day 11, which corresponded to the time during infection when clinical orchitis was first apparent (Fig. 1B).

The proportion of T cells continued to increase and reached peak levels at day 18, when the treponemal burden in the tissue was also greatest (Fig. 1A), and the proportion remained greater than the background proportion through day 39 (Fig. 1B).

CD4\(^+\) T cells comprised the largest proportion of T cells infiltrating the testes during the early stages of infection, particularly when viable treponemes were present (Fig. 2), accounting for 8.7% of the total cells at the peak. The proportion of CD8\(^+\) T cells steadily increased throughout early infection and lesion resolution (Fig. 2A), reaching 3.6% of the total population at day 39. At days 25 and 39, CD8\(^+\) T cells also comprised a larger proportion of the T-cell population, as indicated by the increased ratio of CD8\(^+\) T cells to CD4\(^+\) T cells (Fig. 2B).

**Cytokine expression in the lesion.** A previous study examining the cytokine milieu present in early human lesions showed that mRNAs for Th1 cytokines were present, while cytokines indicative of a Th2 response, namely, IL-4, were absent (30). Because the biopsy samples represented only a snapshot of each infection, nothing is known about cytokine expression during progression and resolution of early syphilis. Furthermore, there is a lack of information concerning the temporal patterns of the production of different Th1 and Th2 cytokines in relation to lymphocyte infiltration and bacterial burden. To examine the cytokines produced locally during experimental infection, we utilized a new method for the relative quantification of rabbit cytokine mRNA by real-time RT-PCR (9a). We observed early and strong induction of
IFN-γ in early lesions, with high mRNA levels temporally overlapping the maximum treponemal burden at days 11 and 18, followed by sharp decline at day 25, when organisms had been cleared (compare Fig. 1A and 3A). The pattern of IFN-γ induction resembled that observed for both CD4+/H9253 and IL-4 levels (A) or IL-10 levels (B) were determined using the real-time RT-PCR relative quantitation method. Data were normalized using the eukaryotic HPRT housekeeping gene. The values are the means ± standard errors from triplicate reactions for two separate tissue samples from four separate animals per time point.

FIG. 3. Cytokine levels during early experimental syphilis. Day 0 represents uninfected rabbits. Total RNA was extracted from the tissues of four animals per time point, and IFN-γ, IL-2, and IL-4 levels (A) or IL-10 levels (B) were determined using the real-time RT-PCR method. Data were normalized using the eukaryotic HPRT housekeeping gene. The values are the means ± standard errors from triplicate reactions for two separate tissue samples from four separate animals per time point.

IFN-γ in early lesions, with high mRNA levels temporally overlapping the maximum treponemal burden at days 11 and 18, followed by sharp decline at day 25, when organisms had been cleared (compare Fig. 1A and 3A). The pattern of IFN-γ induction resembled that observed for both CD4+ and total T-cell populations (Fig. 1B and 2), with peak levels occurring when viable treponemes were present in the lesion at day 11 and day 18, followed by a sharp decline at day 25 (Fig. 3A). No significant increases in mRNA levels above the background levels were observed at any time during early infection for either IL-4 or IL-2 mRNA (Fig. 3A). Finally, strong induction of the levels of mRNA for IL-10, a cytokine produced by both CD4+ T cells and macrophages, was observed, correlating well with the level of T. pallidum in the lesions (compare Fig. 1A and 3B).

DISCUSSION

The early immune response to T. pallidum during both natural human infection and experimental infection in animal models involves a strong cellular component. T cells sensitized to T. pallidum antigens are first detected in the lymphoid organs of infected animals as early as 3 days following intratesticular infection and infiltrate early lesions shortly before the peak number of treponemes is detected. Peak infiltration of T cells following intratesticular infection with the rabbit-adapted Nichols strain of T. pallidum was reported to occur between 10 and 13 days postinfection (15, 22, 23). In the present study, we examined the local immune events occurring during lesion progression and resolution in rabbits infected with the Chicago strain, which was isolated in 1951 and is less well adapted for growth in rabbits (29). Initial gross changes indicative of orchitis were first observed at day 11 postinfection. However, the maximal treponemal burden and infiltration of T cells in the tissue were not seen until day 18, which is later than the time described for strain Nichols infection. These results suggest that lesion progression is slower following infection with the Chicago strain than following infection with the Nichols strain, as the number of treponemes used for infection in this study was within the dose range reported in the previous study of Lukehart et al. (15). The rates of lesion development and resolution vary widely from patient to patient in humans, implying that different combinations of strain and host yield different courses of disease. Another possibility is that the Nichols strain has become more adapted for rapid growth in the rabbit due to decades of repeated continuous passages at short intervals (8 to 10 days). Thus, peaks in bacterial numbers and the subsequent infiltration of responder T cells may occur sooner after infection with the Nichols strain than after infection with more recent human isolates that have been passed far less frequently, such as the Chicago strain used here.

In human disease, both CD4+ T cells and CD8+ T cells are observed in primary and secondary lesions, although reports indicate that their relative proportions vary (8, 18, 28, 31). Importantly, very little is known about the kinetics of these important T-cell subsets during lesion progression and resolution. Our results show that CD4+ T cells are the predominant T-cell subset infiltrating lesions early during experimental infection, which is consistent with the observation of CD4+ cell predominance in primary human lesions. A more intriguing finding was the change in the composition of the T-cell population during lesion progression. CD8+ T cells first infiltrated the lesion early during infection, when the treponemal burden was greatest on days 11 and day 18. However, they comprised a greater proportion of the total T-cell population during healing and resolution of lesions between day 25 and day 39. One explanation for the increased presence of CD8+ T cells later in infection may be that residual exogenous T. pallidum antigens from nonviable treponemes remaining in the tissue continue to enter alternative endocytic pathways and are presented to CD8+ T cells via the major histocompatibility complex class I pathway. Another possibility is that later during infection a greater number of treponemes reside intracellularly and enter alternative endocytic pathways and are presented to CD8+ T cells via the major histocompatibility complex class I pathway. While T. pallidum is generally considered to be an extracellular pathogen, evidence obtained from microscopic evaluations has suggested that some T. pallidum cells are present inside cells in early lesions (13, 25, 26). Activated CD8+ T cells within a lesion could contribute to the pool of IFN-γ available for macrophage activation or could lyse cells containing intracellular treponemes. The presence of mRNAs for both perforin and granzyme B in early lesions during human infection supports the possibility of a cytolytic function for the CD8+ T cells (31).
The period of peak IFN-γ production occurred from day 11 to day 18 and temporally overlapped both the treponemal burden and the early peak in T cells within the lesions. Furthermore, the early CD4+ T-cell response mirrored the total T-cell response. In addition to the CD4+ and CD8+ cells observed here, other IFN-γ-producing cells, such as NK cells, may be present in the lesion. However, the temporal correlation between the infiltration of large numbers of CD4+ T cells and the peaks of both IFN-γ induction and treponemal burden strongly suggests that these cells are stimulated by *T. pallidum* and produce IFN-γ. Arroll et al. previously demonstrated that T cells sensitized to *T. pallidum* produced IFN-γ upon restimulation (1). In our studies, peak IFN-γ production also directly preceded the clearance of treponemes and the beginning of lesion healing. Although macrophage infiltration was not examined during this study due to a lack of available reagents specific for rabbit macrophages, it has been reported extensively by other workers and was assumed here that macrophages are present in large numbers within the lesion directly prior to clearance (15, 18, 22, 32). Thus, it is likely that bacterial clearance is mediated by the proposed mechanism involving the activation of macrophages by IFN-γ produced by T cells, which leads to the engulfment and killing of opsonized treponemes and lesion resolution (2, 14).

Our results strengthen the conclusion that there is Th1 predominance in the early cellular immune response to *T. pallidum*, since mRNA for the strong Th1-mediating cytokine IFN-γ was consistently detected and IL-4 mRNA was absent. IL-2 is also produced by Th1 cells, and while IL-2 mRNA was previously detected in early human lesions, the quantity of IL-2 mRNA was not determined in that study (30). In our study, we occasionally observed measurable levels of IL-2 mRNA, but these levels were just at the threshold of sensitivity for real-time RT-PCR. A similar result was obtained for dermal lesions during experimental infection (9a). One possibility is that IL-2 production at the lesion site is transient and our failure to detect significant levels may have been due to IL-2 expression occurring at a different time during lesion progression. The fact that the IL-2 message was detected in all specimens in the previous study examining human lesions argues against this possibility since it is likely that the biopsies were collected at different times over the course of lesion development. Another possibility is that IL-2 is down-regulated or suppressed during lesion development in the rabbit and thus is either absent or expressed at low levels. Fitzgerald and Tomai demonstrated that there was down-regulation of IL-2 synthesis by splenic T cells from *T. pallidum*-infected rabbits following activation in vitro by concanavalin A in the presence of macrophages (9), and they concluded that IL-2 down-regulation was mediated by products secreted by macrophages, such as prostaglandins and cytokines, like transforming growth factor (9, 27). A similar mechanism of IL-2 suppression may also occur at the lesion site.

IL-10, a regulatory cytokine produced primarily by macrophages and T cells, was also consistently detected in human lesions, and synthesis of IL-10 was significantly increased after intradermal infection in the guinea pig model of syphilis (33). In the present study, peak levels of IL-10 were detected at day 18, when the treponemal burden in the tissue was very high and following the period of high IFN-γ levels beginning at day 11.

Previous studies have shown that macrophages infiltrate syphils lesions shortly after T cells infiltrate the lesions (15). Thus, in the context of lesion resolution, IL-10 may be a product of the large numbers of infiltrating macrophages and may help down-regulate the strong IFN-γ-mediated inflammatory response after the bacteria are ingested. IL-10 is also a product of certain subsets of regulatory T cells, such as CD4+ CD25+ regulatory T cells Transforming growth factor β, another immunosuppressive cytokine produced by this regulatory T-cell subset, while not studied here, was suggested by Fitzgerald and Tomai to play a role in the regulation of the inflammatory response during *T. pallidum* infection (9). One may speculate that these regulatory T cells comprise a portion of the CD4+ T cells observed within lesions, particularly after bacterial clearance, and are partially responsible for the down-regulation of the very active inflammatory response during lesion healing. A role for CD4+ CD25+ regulatory T cells in modulating the inflammatory responses during arthritis induced by the related spirochete *Borrelia burgdorferi* has been suggested (19, 20).

In order to develop an effective syphils vaccine, we must expand our understanding of the natural immune response to *T. pallidum*. To this end, the rabbit has been a crucial experimental model for understanding the host response to this infection. In the present study we defined the kinetics of the rabbit cytokine responses during the course of primary infection. The results presented here demonstrate that CD4+ T cells are the primary responders to *T. pallidum* in early lesions and likely mediate clearance through the production of IFN-γ. We also demonstrate that the composition of the T-cell population within the lesion changes over the course of lesion progression, with CD8+ T cells becoming more prominent during resolution and healing. This information, along with information concerning the dynamics of cytokine production during early experimental infection, should aid future efforts to develop vaccines that more effectively target the protective immune response that mediates bacterial clearance.

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