

The Cutaneous Response in Humans to *Treponema pallidum* Lipoprotein Analogues Involves Cellular Elements of Both Innate and Adaptive Immunity¹

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To extend prior studies implicating treponemal lipoproteins as major proinflammatory agonists of syphilitic infection, we examined the responses induced by intradermal injection of human subjects with synthetic lipoprotein analogues (lipopeptides) corresponding to the N termini of the 17- and 47-kDa lipoproteins of *Treponema pallidum*. Responses were assessed visually and by flow cytometric analysis of dermal leukocyte populations within fluids aspirated from suction blisters raised over the injection sites. Lipopeptides elicited dose-dependent increases in erythema/induration and cellular infiltrates. Compared with peripheral blood, blister fluids were highly enriched for monocytes/macrophages, cutaneous lymphocyte Ag-positive memory T cells, and dendritic cells. PB and blister fluids contained highly similar ratios of CD123⁺/CD11c⁺ (DC1) and CD123⁺/CD11c⁺ (DC2) dendritic cells. Staining for maturation/differentiation markers (CD83, CD1a) and costimulatory molecules (CD80/CD86) revealed that blister fluid DC1, but not DC2, cells were more developmentally advanced than their peripheral blood counterparts. Of particular relevance to the ability of syphilitic lesions to facilitate the transmission of M-tropic strains of HIV-1 was a marked enhancement of CCR5 positivity among mononuclear cells in the blister fluids. Treponemal lipopeptides have the capacity to induce an inflammatory milieu reminiscent of that found in early syphilis lesions. In contrast with *in vitro* studies, which have focused upon the ability of these agonists to stimulate isolated innate immune effector cells, in this study we show that in a complex tissue environment these molecules have the capacity to recruit cellular elements representing the adaptive as well as the innate arm of the cellular immune response. *The Journal of Immunology*, 2001, 166: 4131–4140.

Syphilis, a sexually transmitted disease caused by the spirochetal bacterium *Treponema pallidum*, begins as an ulcer (chancre) at the site of inoculation and, when untreated, progresses through secondary (disseminated), latent (asymptomatic), and tertiary (recrudescent) stages (1). The cell-mediated inflammatory processes triggered by spirochetes within infected tissues have two distinct, yet interrelated, consequences. On the one hand, they cause the tissue damage that ultimately gives rise to clinical manifestations, while on the other, they also are responsible for the clearance of treponemes, a prerequisite for lesion resolution (2, 3). Cellular infiltrates composed of T lymphocytes, macrophages, and plasma cells are the *sine qua non* of syphilitic lesions (1–7). Immunocytochemical and RT-PCR analyses of early syphilitic skin lesions have revealed that these infiltrating cells, as well as keratinocytes and proximal vascular

endothelium, are activated and that the T cells are elaborating cytokines consistent with a Th1 response (4, 8). Because macrophages are both professional phagocytes and a rich source of proinflammatory mediators, their presence in large numbers is thought to be central to both lesion formation and resolution (2, 4, 6).

Host defenses against microbial pathogens involve a complex interplay of innate and adaptive immunity (9). Innate immunity involves preprogrammed responses to diverse microbial constituents, whereas the slower adaptive immune response results in the generation of specific Abs and Ag-sensitized T cells. Although *T. pallidum* lacks LPS, the proinflammatory molecule found in the outer membranes of Gram-negative bacteria, it does contain abundant lipoproteins (10–13). Evidence from both *in vitro* and *in vivo* studies have demonstrated that these lipid-modified proteins are potent activators of effector cells associated with innate immunity, principally monocytes/macrophages and endothelial cells (14–20). Because *T. pallidum* cannot be cultivated *in vitro*, investigation of the proinflammatory properties of treponemal lipoproteins has been hampered by difficulties in isolating sufficient quantities of these molecules. This obstacle has been circumvented by the use of synthetic lipohexapeptides that correspond to the N termini of the full-length proteins. A number of studies have shown that these lipoprotein surrogates possess proinflammatory properties qualitatively similar to those of their native counterparts (17–21). Most recently, we and others have demonstrated that cellular activation by native lipoproteins and synthetic analogues proceeds via the Toll-like receptor 2 (TLR2)³-dependent signaling pathway as opposed to LPS-mediated signaling, which uses TLR4 (22–27).

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³ Abbreviations used in this paper: TLR, Toll-like receptor; CLA, cutaneous lymphocyte Ag; CXCR, CXC chemokine receptor; DC, dendritic cell; FA, fluorescence assay; PAMP, pathogen-associated molecular pattern; PB, peripheral blood.

The rabbit has been the traditional animal of choice for studying the evolution of histopathological changes and the development of cellular and humoral immune responses to *T. pallidum* during infection (2, 3). In a prior study (28), we extended this model to demonstrate that intradermal injection of treponemal lipopeptides elicits cellular infiltrates resembling those observed in acquired syphilis in humans. However, a detailed analysis of this response was precluded by the paucity of reagents directed against rabbit immune cells and immunomodulators. For this reason, we turned to an in vivo human skin model to characterize further the biological properties of *T. pallidum* lipoproteins and to define their role in disease pathogenesis. In this model (29), mild suction is used to elicit blister formation at the dermo-epidermal junction following intradermal injection with treponemal lipopeptides. In contrast with the paucity of cells in fluid from blisters raised over saline-injected skin or unmanipulated normal skin, fluid within blisters elicited over sites of inflammation contains numerous extravasated immune cells that can be analyzed by flow cytometry (30–33); comparison with peripheral blood (PB) enables one to identify selectively recruited leukocyte subsets and to assess the influence of the inflammatory microenvironment on their state of activation/differentiation (31–33). There is now extensive evidence that the composition of cells in blister fluid accurately reflects the cellular infiltrates within the underlying dermis (30–33).

We postulated that studying these agonists in skin, a major target organ of syphilitic infection (1), would yield insights into their biological activities that could not be obtained from in vitro investigations using highly purified leukocyte subtypes or leukocytic cell lines. Our findings demonstrate that treponemal lipopeptides have the capacity to induce in human skin an inflammatory milieu reminiscent of that found in early syphilis lesions. Of particular relevance to the ability of syphilitic lesions to facilitate the transmission of M-tropic strains of HIV-1 (34, 35) was a marked enhancement of CCR5 positivity among mononuclear cell populations, particularly macrophages, in the blister fluids. In contrast with in vitro studies that have focused upon the ability of these agonists to stimulate isolated innate immune effector cells, in this study we show that in a complex tissue environment these molecules have the capacity to recruit cellular elements representing the adaptive as well as the innate arm of the cellular immune response.

Materials and Methods

Human subjects

Eligible participants were healthy volunteers between the ages of 18 and 60 years without clinical or serological evidence of syphilis. Individuals were considered ineligible if they were taking anti-inflammatory medications or had a history of chronic dermatoses. All participants underwent a physical examination before enrollment. Protocols used in this study were approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center and the University of Connecticut Health Center. Informed written consent was obtained from all persons who participated. A total of 45 subjects were enrolled for the various studies described in Results. The participants ranged from 20 to 54 years of age and included 22 males and 23 females, consisting of 41 Caucasians (including 3 Hispanics), 2 Blacks, and 2 Asians.

Synthetic treponemal lipohexapeptides and hexapeptides

Lipohexapeptides (lipopeptides) corresponding to the N termini of the *T. pallidum* 17- and 47-kDa lipoproteins (designated 17-L and 47-L, respectively) were synthesized and extensively characterized as described previously (21). The corresponding nonlipidated hexapeptides (17 and 47, respectively) also were synthesized as controls using standard 9-fluorenylmethoxycarbonyl chemistry on an Applied Biosystems (Foster City, CA) 430A peptide synthesizer. Both hexapeptides and lipopeptides contained undetectable levels of endotoxin (≤ 1 pg LPS/ μ g protein) as measured by the QCL-1000 quantitative, chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD). For intradermal injection, lyophilized

hexapeptides and lipopeptides were suspended by vortexing in sterile H₂O for drug diluent use (Abbott Laboratories, North Chicago, IL).

Intradermal injection of synthetic hexapeptides/lipopeptides and elicitation of epidermal blisters

To establish the dosage of lipopeptides for flow cytometric studies, five volunteers were injected intradermally at four separate sites on the volar surface of the forearm with 25, 50, and 100 μ g of 17-L or 47-L and 100 μ g of the corresponding hexapeptide. Subsequently, volunteers were injected intradermally at separate sites with 100 μ g of each lipopeptide. Twenty-four hours later, the sites were swabbed with alcohol and an acrylic suction cup was applied, as shown in Fig. 1A. A thin coating of high vacuum silicone lubricant (Dow Corning, Midland, MI) on the underside of the suction blister cup ensured an airtight seal with the surface of the skin. Vacuum suction (200 mm Hg) and gentle warming with a 125 W infrared lamp were used for 1.5–2 h to raise epidermal blisters (Fig. 1A, inset). Fluid was syringe aspirated from the blisters the following day (48 h postinjection).

Immunologic reagents

Monoclonal and isotype-matched control Abs conjugated to FITC, PE, PerCP, and allophycocyanin were obtained from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA), with the exception of FITC- γ δ -TCR (Endogen, Woburn, MA).

Cell staining and flow cytometry

Whole blood was either treated with erythrocyte lysis buffer (150 mM NH₄Cl, 1 mM KHCO₃, and 1 mM EDTA) to produce erythrocyte-depleted leukocytes or centrifuged in Vacutainer Cell Preparation Tubes (Becton Dickinson, Franklin Lakes, NJ) to isolate PBMCs. Blister fluids were transferred to 3 ml of fluorescence assay (FA) buffer (Difco Laboratories, Detroit, MI) containing 2 mM EDTA (pH 8) and washed once before resuspension in 0.5 ml of the same buffer. Following enumeration using a hemacytometer, aliquots of $\geq 5 \times 10^4$ freshly isolated erythrocyte-depleted leukocytes, PBMCs, or blister fluid cells were placed into 12 $\times$ 75 polystyrene tubes (Becton Dickinson Labware, Lincoln Park, NJ) and washed once with FA buffer containing 0.1% BSA and 13 mM NaN₃ (FA/BSA/NaN₃), followed by resuspension in 50 μ l of the same buffer. Cells then were blocked with 10 μ g of purified human IgG (Sigma, St. Louis, MO) for 15 min on ice, followed by incubation with fluorochrome-conjugated mAbs for another 30 min protected from light. Additionally, aliquots of erythrocyte-depleted leukocytes were incubated with individual fluorochrome-conjugated Abs or isotype-matched control Abs to compensate for fluorescence emission overlap and nonspecific fluorescence, respectively. The intrinsic and nonspecific fluorescence of negative cell populations was adjusted to fall within the first decade, thus delineating positive cell populations as those whose mean fluorescence intensity falls within the second to fourth decade. Staining of blister fluid cells with isotype-matched control Abs did not produce any greater nonspecific fluorescence than that observed with erythrocyte-depleted leukocytes. After staining, cells were washed once with FA/BSA/NaN₃ and fixed by resuspension in 1 ml of FA buffer containing 1% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) and 13 mM NaN₃. Fluorescence data were acquired on a FACSCalibur dual laser flow cytometer (BDIS) using a threshold of 52 and an appropriate scatter gate to exclude dead cells, cellular debris, and residual erythrocytes. List mode multiparameter files (consisting of forward scatter, orthogonal scatter, and three or four fluorescence parameters) were analyzed using PAINT-A-GATE^{PRO} (version 2.0) software (BDIS). Using this program, events were sequentially “painted” to identify up to seven discrete cell populations based upon phenotypic signatures and their relationship to each other. These populations then were quantified as percentages of the total events (or a gated subset thereof), and their mean channel fluorescence intensities were calculated.

Statistics

An ANOVA followed by a multiple comparisons test was used to determine whether significant differences existed between data sets. Significance was accepted when $p < 0.05$.

Results

T. pallidum lipopeptides elicit a cutaneous inflammatory response

At the outset, we characterized the gross inflammatory response to the lipopeptides and determined the dosages for subsequent flow

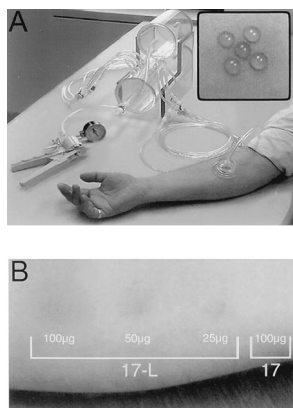


FIGURE 1. A, Suction blister apparatus and epidermal blisters (inset) elicited after 2 h of vacuum at 200 mm Hg. Blister formation occurs at the epidermal-dermal interface. B, Cutaneous inflammatory responses elicited 48 h after intradermal inoculation with the designated doses of lipidated and nonlipidated hexapeptides corresponding to the N terminus of the 17-kDa *T. pallidum* lipoprotein (17-L and 17, respectively).

cytometric studies. Five volunteers were injected on the volar surface of the forearm with graded doses (25, 50, and 100 μ g) of 17-L or 47-L and with 100 μ g of the corresponding hexapeptide. In all five subjects, dose-dependent erythema and induration were observed within 24 h following intradermal injection with either lipopeptide; this response was maximal at $\sim$ 48 h (Fig. 1B) and resolved by 96 h (data not shown). None of the subjects experienced significant discomfort at any of the injection sites. In accordance with prior studies showing that lipid modification is essential for the proinflammatory activity of both full-length lipoproteins and synthetic analogues (16–18, 21, 28), no responses were observed at sites injected with hexapeptides (Fig. 1B). Fluids obtained immediately after the blisters were raised were essentially devoid of cells. However, 24 h later the time point corresponding to the peak gross inflammatory response, dose-dependent increases in white blood cells, and only rare erythrocytes were observed at sites receiving lipopeptides (Table I). Consistent with the lack of gross inflammatory response, sites injected with 100 μ g of either hexapeptide contained $\sim$ 100-fold fewer cells than those receiving

Table I. Total cell counts ($\times 10^5$) in blister fluids following injection with treponemal lipohexapeptides or control hexapeptides^a

Sample	25 μ g	50 μ g	100 μ g
17-L	0.73 $\pm$ 0.17	1.29 $\pm$ 0.80	3.64 $\pm$ 0.68
17	NA	NA	0.035 $\pm$ 0.01
47-L	0.54 $\pm$ 0.37	1.26 $\pm$ 0.80	3.11 $\pm$ 0.63
47	NA	NA	0.024 $\pm$ 0.01

^a Each value represents the mean $\pm$ SE from three or more subjects.

an equivalent amount of either lipopeptide (Table I). These results show that the vast majority of cells in the blister fluids were elicited by the lipopeptides. Based upon these findings, in all subsequent experiments volunteers received 100 μ g doses of 17-L and 47-L. Blisters were raised over sites 24 h after injection, and the fluids were aspirated the following day. Parallel injections with hexapeptides were discontinued because the low numbers of cells elicited precluded reproducible flow cytometric analysis.

Characterization of the major leukocyte subsets in blister fluids

Flow cytometric studies were performed to identify the major leukocyte populations in the blister fluids. Representative results for a single individual are shown in Fig. 2, and a summary of the results is presented in Table II. The absence of significant differences between the lipopeptides is in agreement with prior in vitro studies showing comparable potencies and biological activities for these agonists (17–21). Infiltrates elicited by the lipopeptides consisted predominantly of neutrophils and monocytes/macrophages, but also contained substantial numbers of lymphocytes. Compared with PB, blister fluids were markedly enriched ($\sim$ 8-fold) for monocytes/macrophages, relatively deficient in T lymphocytes, and virtually devoid of B lymphocytes, while the percentages of granulocytes in the two compartments were similar. Monocytic cells in blister fluids were larger and more granular by forward and side scatter characteristics than their circulating counterparts and expressed $\sim$ 3-fold more CD14 (Fig. 2) and HLA-DR (data not shown) on their surfaces, findings that indicated that they had differentiated into activated macrophages within the cutaneous microenvironment. That monocytic cells increased their surface expression of CD14 within inflamed skin also was noteworthy

FIGURE 2. Recruitment of major leukocyte populations into blister fluid. Cells in PB and in blister fluids (17-L and 47-L) were stained with FITC α CD14, PE α CD20, PerCP α CD45, and APC α CD38. A combination of forward (FSC) and side (SSC) scatter characteristics and Ab reactivities was used to define granulocyte (red), monocyte/macrophage (green), T lymphocyte (yellow), and B lymphocyte (blue) populations. All displayed events (10,000) are restricted by FCS threshold and a scatter gate to exclude dead cells, cellular debris, and residual erythrocytes. The identification of cell populations was performed sequentially because Paint-A-Gate^{PRO} has the capability to memorize the analyzed events. The results shown are representative of eight different subjects.

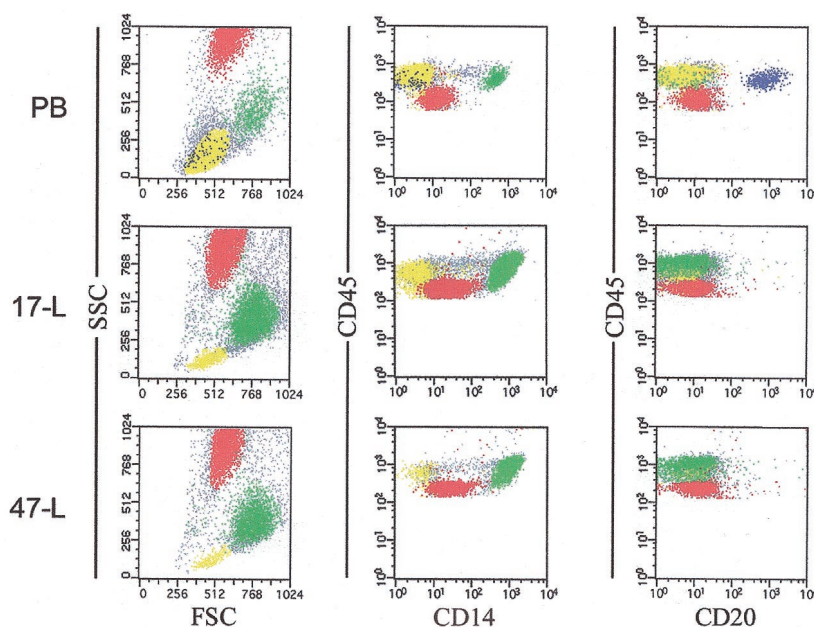


Table II. Immunophenotypic characterization of leukocyte populations in PB and blister fluids^a

Major Cell Populations	Surface Ags	n	PB	17-L	47-L
Granulocytes	CD45 ⁺ 14 ^{lowb}	8	42.07 ± 4.76	34.39 ± 4.99	39.22 ± 5.44
Monocytes/macrophages	CD45 ⁺ 14 ^{hb}	8	4.29 ± 0.29	26.50 ± 5.59**	24.04 ± 3.80*
Activated macrophages	CD14 ⁺ HLA-DR ^{hc}	4	75.28 ± 5.22	92.56 ± 4.54*	93.47 ± 2.55*
T lymphocytes	CD45 ⁺ 3 ^{hb}	8	32.03 ± 4.74	16.68 ± 3.82**	13.78 ± 3.10**
Activated T lymphocytes	CD3 ⁺ CLA ⁺ HLA-DR ^{hc}	4	2.63 ± 0.11	20.75 ± 1.49*	23.48 ± 0.76*
B lymphocytes	CD45 ⁺ 20 ^{hb}	8	3.15 ± 0.72	0***	0***
T lymphocyte subsets					
Virgin	CD3 ⁺ 45RO ⁻ 95 ^{-d}	4	45.03 ± 4.11	0.19 ± 0.19***	0.18 ± 0.18***
Memory/effector-I	CD3 ⁺ 45RO ^{-low} 95 ^{hd}	4	27.36 ± 4.37	27.10 ± 6.41	31.27 ± 7.61
Memory/effector-II	CD3 ⁺ 45RO ^{high} 95 ^{hd}	4	27.53 ± 0.83	71.71 ± 5.54***	68.55 ± 7.65***
Skin-homing	CD3 ⁺ CLA ^{he}	4	16.80 ± 2.72	83.98 ± 3.88***	76.16 ± 6.45***
Helper	CD3 ⁺ 4 ^{hf}	8	67.18 ± 5.20	72.62 ± 5.30	76.64 ± 3.43
Cytotoxic	CD3 ⁺ 8 ^{hf}	8	23.44 ± 2.47	19.75 ± 4.09	17.12 ± 2.41
γδ	CD3 ⁺ γδ TCR ^{hf}	8	7.45 ± 3.36	4.03 ± 1.66	2.85 ± 0.77

^a Data on the major leukocyte populations in PB and blister fluids were acquired by collecting ≥50,000 events using a threshold of 52 and an appropriate scatter gate to exclude dead cells, cellular debris, and residual erythrocytes. Major leukocyte populations were identified using a combination of forward- and side-scatter characteristics and the Ab panels indicated above. Different leukocyte types are presented as a percentage of total cells, whereas activated macrophages are presented as a percentage of monocytes/macrophages and T lymphocyte subsets are presented as a percentage of CD3⁺ cells. Each value represents the mean ± SE from four or more subjects. Asterisks indicate values that are significantly greater than or less than the corresponding values in PB (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

^b FITC-αCD14, PE-αCD20, PerCP-αCD45, allophycocyanin-αCD38

^c FITC-αCD14, PE-αCD3, PerCP-αHLA-DR

^d FITC-αCD45RO, PerCP-αCD3, allophycocyanin-αCD95

^e FITC-αCLA, PE-αCD3, PerCP-αHLA-DR, allophycocyanin-αCD95

^f FITC-αγδ-TCR, PE-αCD8, PerCP-αCD3, allophycocyanin-αCD4

because it contrasted with prior in vitro studies in which incubation of PB monocytes with treponemal lipoproteins/lipopeptides had the opposite effect on CD14 expression (20).

Despite the reduced proportion of T cells in blister fluids, we considered it possible that particular subsets had been recruited selectively into the inflammatory site. Further immunophenotypic analysis of blister fluid T lymphocytes confirmed this supposition (Table II). Only a minuscule percentage of T cells (<1%) in the blister fluids were of the virgin (i.e., CD45RO⁻/CD95⁻) phenotype. In contrast, roughly 70% of the T cells were CD45RO^{high} memory/effector cells, while the remainder displayed the

CD45RO^{low}/CD95⁺ memory/effector phenotype that is associated with repeated antigenic stimulation (36). Approximately 80% of the blister fluid T cells stained positively for the skin-homing receptor cutaneous lymphocyte Ag (CLA) (37), a 4-fold increase above the percentage of CLA⁺ cells found in PB ($p < 0.001$). The proportions of blister fluid T lymphocytes that were positive for CD4, CD8, or the γδ-TCR did not differ significantly from those in PB, indicating that a bias for recruitment of these subsets did not exist. Approximately 10-fold more of the CLA⁺ blister fluid T cells expressed HLA-DR than did their CLA⁺ counterparts in PB, raising the possibility that a subpopulation of T cells was activated

FIGURE 3. The proinflammatory microenvironment established by treponemal lipopeptides is enriched for DCs, including a subset that expresses the maturation/differentiation markers, CD83 and CD1a. To identify DCs, cells in PB and in blister fluids (17-L and 47-L) were stained with a lineage cocktail consisting of FITC-conjugated αCD3, αCD14, αCD16, αCD19, αCD20, and αCD56 and PerCP αHLA-DR. DCs (shown in yellow in the left-most panels) are lineage⁻ and HLA-DR⁺. Staining with CD83 (blue) and CD1a (violet) also was performed to assess the maturational states of the DCs in PB and blister fluids. The arrows in the CD1a panels indicate the small subpopulation of CD1a^{high} cells, which are believed to be locally recruited resident Langerhans cells. The results shown are representative of eight different subjects.

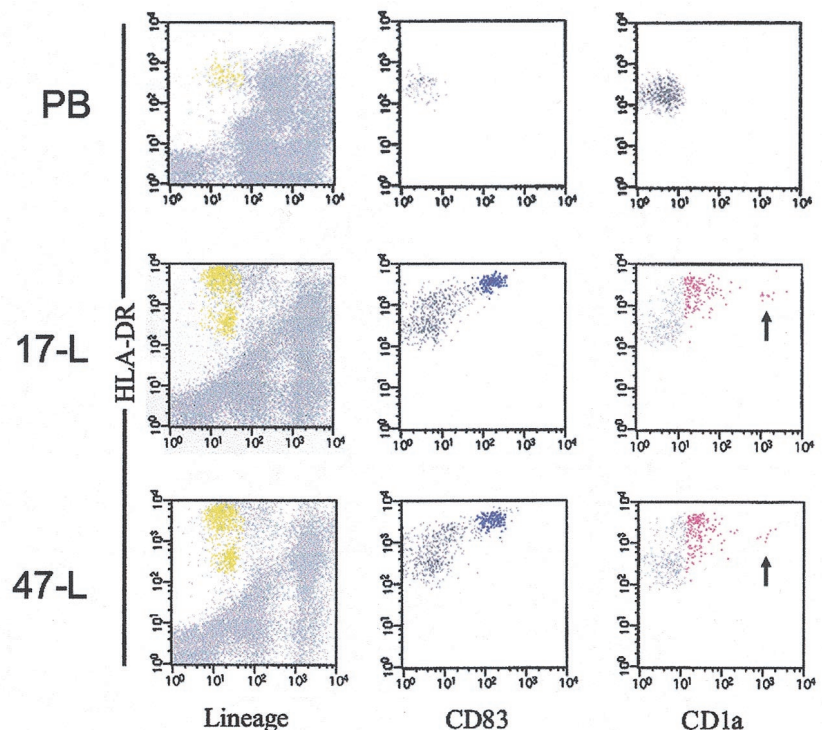


Table III. Expression of maturation markers and costimulatory molecules on DCs in PB and blister fluids^a

DCs	n	PB	17-L	47-L
Total DCs	8	0.41 ± 0.07	2.71 ± 0.62**	4.66 ± 1.14**
DC1	8	64.10 ± 4.50	56.40 ± 6.70	64.60 ± 5.90
DC1/CD83 ⁺	8	0	31.27 ± 11.57**	27.40 ± 7.05**
DC1/CD1a ⁺	8	0	29.64 ± 4.95**	31.10 ± 5.05**
DC1/CD80	5	0	64.84 ± 7.75**	66.89 ± 12.44**
DC1/CD86 ^{low}	5	96.14 ± 0.60	47.23 ± 7.26*	48.30 ± 15.59*
DC1/CD86 ^{int}	5	0	44.42 ± 8.37*	46.86 ± 17.84*
DC2	8	35.90 ± 4.50	43.60 ± 6.70	35.40 ± 5.90
DC2/CD80	5	0.73 ± 0.47	0	0
DC2/CD86 ^{low}	5	35.34 ± 11.08	37.65 ± 23.06	57.50 ± 28.85
DC2/CD86 ^{high}	5	45.50 ± 11.27	0**	0**

^a The lineage mixture used, in conjunction with PerCP-αHLA-DR, to identify DCs consisted of FITC-conjugated Abs directed against CD3, CD14, CD16, CD19, CD20, and CD56. DCs are presented as a percentage of the total cells from PB and blister fluids. Those populations labeled DC1 and DC2 are presented as a percentage of the total DCs and refer to subsets distinguished by their reciprocal expression of CD123 and CD11c, respectively. DC1 and DC2 subsets were characterized further on the basis of staining for CD83, CD1a, CD80, and CD86. Each value represents the mean ± SE from five subjects. Asterisks indicate values that are significantly greater than or less than the corresponding values in PB (*, $p < 0.01$; **, $p < 0.001$).

within the proinflammatory environment created by the lipopeptides. CC chemokines, particularly RANTES, which are produced in abundance by lipoprotein/lipopeptide-activated macrophages (20), could have been responsible for this effect (38).

Lipopeptides promote recruitment of dendritic cells (DCs) and maturation of the myeloid- but not lymphoid-derived DC subset

DCs, professional APCs whose primary function is to capture and process Ags for presentation to T and B cells (39, 40), can be recruited to peripheral sites by a broad range of inflammatory stimuli (41). Because of their unique function at the interface between the innate and adaptive immune responses, we next asked whether lipopeptides could recruit these potent inducer cells into skin. DCs were identified in PB and blister fluids by their lack of staining with FITC-conjugated lineage markers (αCD3, αCD14, αCD16, αCD19, αCD20, and αCD56) and their positive staining for HLA-DR. Although numerically minor components in both compartments, DCs were between 5- and 10-fold more abundant in blister fluids than in PB, differences that were highly significant ($p < 0.001$) (Fig. 3 and Table III). Staining for the maturation/differentiation markers CD83 (42) and CD1a (43) was next performed to assess the developmental state of DCs in the two compartments. Neither Ag was detected on circulating DCs, whereas both surface molecules were expressed by a HLA-DR^{high} subpopulation of cells in the blister fluids (Fig. 3 and Table III).

Reciprocal expression of the surface Ags CD123 and CD11c has been used to distinguish two different DC lineages in PB (43–48). CD123⁺/CD11c⁺ (DC1) cells express myeloid markers (e.g., CD13 and CD33), respond well to inflammatory stimuli, and give rise to Langerhans cell precursors (43, 47, 49). CD123⁺/CD11c[−] (DC2) cells, in contrast, display lymphoid features, are less responsive to inflammatory stimuli, and have been localized to lymphoid tissues (45, 47, 49, 50). Based upon staining with these two Ags, PB and blister fluids contained similar proportions (~2:1) of DC1 and DC2 subsets (Fig. 4A and Table III), a ratio comparable with that reported by others (43, 44, 47). Little is known about the trafficking patterns of DC2 cells, although there is evidence that they migrate from the circulation into lymphoid tissues via high endothelial venules (45, 49, 50). To our knowledge, this is the first report in which DC2 cells were identified in extralymphoid inflammatory sites. With regard to DC1 cells, several differences were noted between this subpopulation in PB and blister fluids. Compared with their circulating counterparts, DC1 cells in the blister fluids 1) were larger and more granular (data not shown), 2)

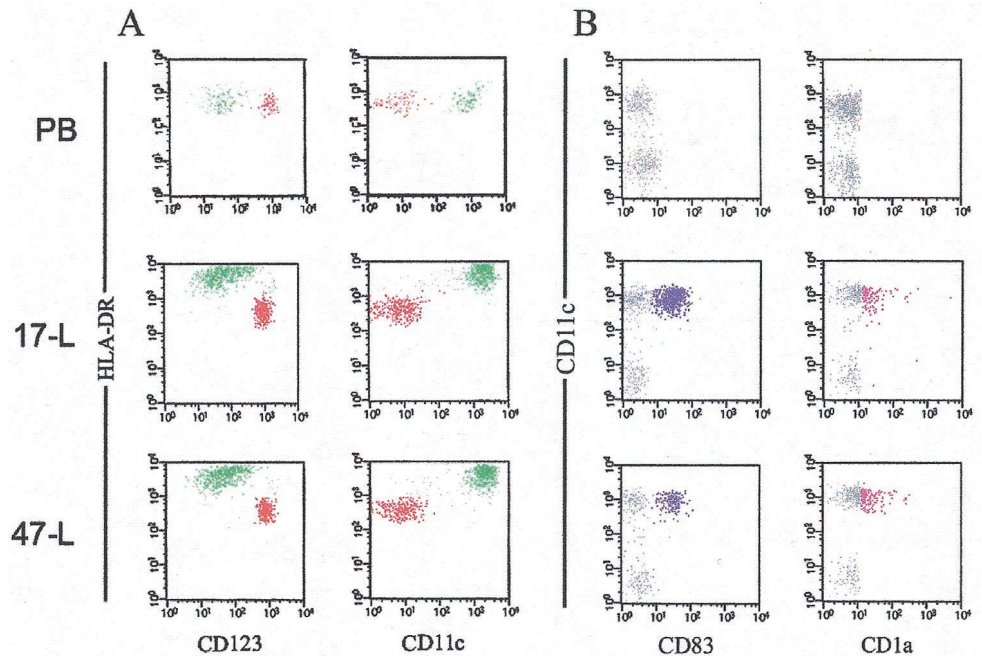
expressed ~2-fold greater levels of CD11c, and 3) expressed ~7-fold greater levels of HLA-DR (Fig. 4A). The observation that expression of CD83 and CD1a was confined largely to a HLA-DR^{high} subpopulation suggested that these Ags were associated with the DC1 subset in the blister fluids (Fig. 3). This was confirmed directly by staining blister fluid DCs for CD11c, CD83, and CD1a (Fig. 4B and Table III).

The enhanced expression of differentiation/maturation markers by CD11c⁺ DCs in the blister fluids prompted us to assess whether differences also existed between the two subsets with respect to the expression of CD80 (B7-1) and CD86 (B7-2), costimulatory molecules that are essential for the priming of naive T cells (51). The results of these experiments are summarized in Table III, while representative flow cytograms are presented in Fig. 5. In accord with previous studies (52), CD80 was not detected on DCs in PB. However, it was strongly induced on most DC1, but not on DC2 cells, in the blister fluids. The inducibility of CD80 expression on DCs also has been noted previously (52), although, to our knowledge, not specifically related to DC1 and DC2 subpopulations. The expression patterns for CD86 were more complex. Consistent with prior reports stating that CD86 is constitutively expressed by DCs in PB (51), most circulating DCs expressed this molecule; it was noteworthy that the highest levels of CD86 were expressed by the DC2 cells. Most of the DC1 cells from the cutaneous compartment expressed CD86 at levels moderately or markedly greater than their PB counterparts. The CD86^{high} subset of DC2 cells seen in PB was not evident in the blister fluids. In fact, all of the blister fluid DC2 cells expressed relatively low levels of this Ag.

Differential expression of CCR5 and CXCR4 chemokine receptor 4 (CXCR4) by mononuclear cells within inflamed skin

In a recent in vitro study (20), we demonstrated that incubation of PBMCs with *T. pallidum*, treponemal lipoproteins, or synthetic lipopeptides resulted in enhanced expression of CCR5 and a reciprocal decrease in the expression of CXCR4. Based upon these results, we proposed that up-regulation of CCR5, the coreceptor for M-tropic strains of HIV-1 (35), in response to *T. pallidum* and its lipoprotein constituents was a potential biological correlate to the epidemiological observation that M-tropic strains of the virus are typically involved in establishing primary infection by the sexual route (53). To garner additional support for this idea, as well as to shed light on the role(s) these chemokine receptors might play in leukocyte trafficking into inflamed skin, we compared the expression of CCR5 and CXCR4 by mononuclear cells within the PB

FIGURE 4. PB and blister fluids contain two distinct lineages of DCs identified by their reciprocal expression of CD11c (DC1 cells) and CD123 (DC2 cells), only one of which (DC1 cells) expresses the maturational markers CD83 and CD1a. A, DCs, identified as lineage⁺ and HLA-DR⁺ cells as described in Fig. 3, were stained with Abs directed against CD11c and CD123 to distinguish DC1 and DC2 lineages. B, DCs were stained with Abs against CD11c and either CD83 or CD1a. The results shown in A and B are representative of eight different subjects.



and cutaneous compartments. An increase in the percentage of cells expressing CCR5 was observed for all three mononuclear cell populations in blister fluid (Fig. 6A). This effect was most pronounced for monocytic cells, in which marked increases in both the percentages of cells expressing CCR5 and in their mean fluorescence intensity occurred. Although the percentage of T cells expressing CCR5 was significantly increased in blister fluid, the intensity of CCR5 expression was unchanged, suggesting that lipopeptides promoted selective recruitment of CCR5⁺ T cells into the dermis. We confirmed this by finding that CLA⁺ T cells in both compartments expressed identical levels of CCR5 (data not shown). A modest, although statistically significant, increase in the percentage of CCR5⁺ DCs in blister fluid also was observed.

However, more striking was the 3- to 4-fold increase in the levels of this chemokine receptor expressed by blister fluid DCs. In contrast to CCR5, the percentage and mean fluorescence intensity of mononuclear cells within blister fluids expressing CXCR4 were either decreased (monocytes/macrophages) or unchanged (T cells and DCs) (Fig. 6B). Finally, we also determined whether DC1 and DC2 cells differed with respect to the expression of CCR5. Surprisingly, the increase in the proportion of CCR5⁺ cells among blister fluid DCs was confined to the DC2 subset (Fig. 7A), whereas both subsets manifested significantly increased levels of CCR5 expression (Fig. 7B).

Discussion

A principal objective of our study was to assess the relationship between the innate cellular response to *T. pallidum* lipoproteins and the histopathological abnormalities of syphilis. The underlying premise was that the spirochete's abundant lipoproteins induce a proinflammatory backdrop against which specific cellular responses develop to complete the histologic picture characteristic of this sexually transmitted disease (5). In keeping with this idea, we found that there was indeed overlap between the cellular response to the lipopeptides and the elements that comprise the infiltrates of early syphilis lesions. A prominent example was the marked enrichment for macrophages in the blister fluids, a finding that parallels immunohistochemical analyses showing that activated macrophages are the predominant mononuclear cell in secondary syphilis lesions (4). Lymphocytes are a nearly ubiquitous component of early syphilis lesions (1, 5) and were readily identified in blister fluids. Moreover, in accordance with immunohistochemical analyses of syphilis lesions (4, 6, 54), T cells, but not B cells, were elicited by the lipopeptides. Granulocytes were the most abundant cell type in the blister fluids. Although syphilis is not normally considered an acute inflammatory process, neutrophils are frequently present in skin lesions of early syphilis and may predominate in some histological patterns (1, 5). Our data suggest that the acute inflammatory component of syphilis lesions is lipoprotein induced. Given that plasma cells are a histologic hallmark of syphilis infiltrates (1, 5), the absence of B cells from both syphilis lesions (4, 54) and blister fluids was noteworthy and suggests that

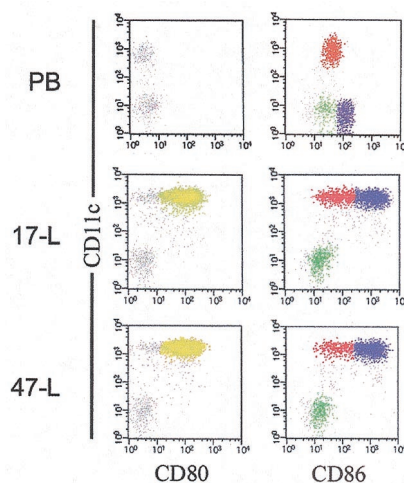
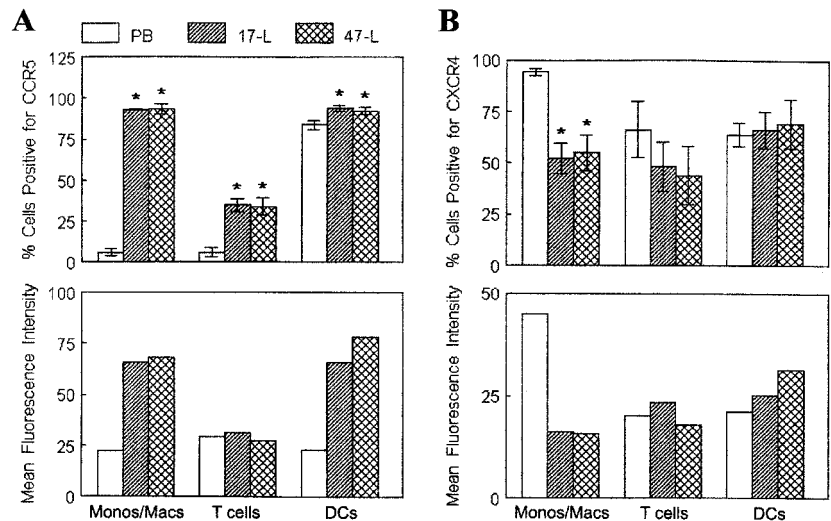


FIGURE 5. The cutaneous proinflammatory response to treponemal lipopeptides results in the modulation of CD80 and CD86 costimulatory molecules on blister fluid DCs. DC1 and DC2 cells, identified as described in Fig. 3, were stained with PE α CD80 or PE α CD86. DC1 cells expressing CD80 are shown in yellow (left panels). Four distinguishable CD86⁺ subpopulations are shown in red (DC1/CD86^{low}), blue (DC1/CD86^{high}), green (DC2/CD86^{low}), and violet (DC2/CD86^{int}). The results shown are representative of five different subjects.

FIGURE 6. Enhanced expression of CCR5 on mononuclear cells in blister fluids. Mononuclear cells were stained with PE α CCR5 (A) and APC α CXCR4 (B). *Upper panels*, Show the percentages of monocytes/macrophages, T cells, and DCs expressing CCR5 and CXCR4 (mean $\pm$ SE from six subjects), while the *lower panels* show representative mean fluorescence intensities. Asterisks indicate values that are significantly greater than or less than the corresponding values in PB (*, $p < 0.01$, except for DCs in A, in which $p < 0.05$).



plasma cells traffic into skin after B cells mature within secondary lymphoid tissues. This finding was consistent with the fact that B lymphocytes are not found in normal skin and are rarely observed in cutaneous inflammatory processes (55).

Activation of innate immunity by microbial constituents, now frequently termed pathogen-associated molecular patterns or PAMPs (56), is considered a prerequisite for the development of the slower adaptive responses that are often essential for pathogen elimination (9, 56, 57). Indeed, in the rabbit model of experimental

syphilis, clearance of treponemes correlates with the local influx of T cells and the appearance of opsonic Ab (2, 3). Therefore, a second major objective of our study was to examine the capacity of lipoprotein-mediated responses to bridge innate and adaptive immunity in cutaneous lesions of early syphilis. Along these lines, we found that two synthetic lipoprotein analogues were highly effective at recruiting cellular elements, DCs and memory/effector T lymphocytes, required for primary and/or secondary immune responses. Trafficking of DCs, as well as T cells, into skin is mediated by CLA and other isoforms of P-selectin glycoprotein ligand 1, which are expressed constitutively in PB (37, 58). Therefore, it is highly likely that the enrichment for CLA⁺ T cells and DCs in the blister fluids reflected the expression of receptors for these molecules (i.e., E- and P-selectin) by activated vascular endothelium. It also is plausible that the innate response to treponemal lipoproteins helps to set the stage for the Th1 bias observed in primary and secondary syphilis lesions (8, 59). DC1 cells, the subpopulation that expressed maturation markers and both costimulatory molecules within blister fluid, promote Th1 differentiation (48, 60) and would be expected to do so during infection when presenting processed treponemal Ags to naive T cells within draining lymph nodes. In support of this contention, spirochetal lipopeptides and the 19-kDa lipoprotein from *Mycobacterium tuberculosis* stimulate monocyte-derived DCs to release IL-12, which directs the selective development of a Th1 response (61). Furthermore, the expression of CCR5 on the surface of activated DCs has been shown to provide a signal for this microbial-induced production of IL-12 (62). Finally, both CLA⁺ and CCR5⁺ T lymphocytes, which were highly enriched in the blister fluids, also are associated with Th1-type reactions (63–68).

Although increased numbers of DCs have been reported in different tissue models of inflammation (41, 58, 69), the relative importance of recruitment from blood vs resident tissue sources has not been well established. For this reason, the source of DCs in the blister fluids is an important question. We believe that the blister fluid DCs were derived from both the PB and skin compartments. The fact that PB and blister fluids consistently contained highly similar ratios of DC1 and DC2 cells suggests that the overwhelming majority of DCs extravasated from PB. At the same time, a small percentage of blister fluid DCs expressed very high levels of CD1a (designated by arrows in Fig. 3) and are likely to represent locally recruited, resident Langerhans cells (43, 70). Inasmuch as

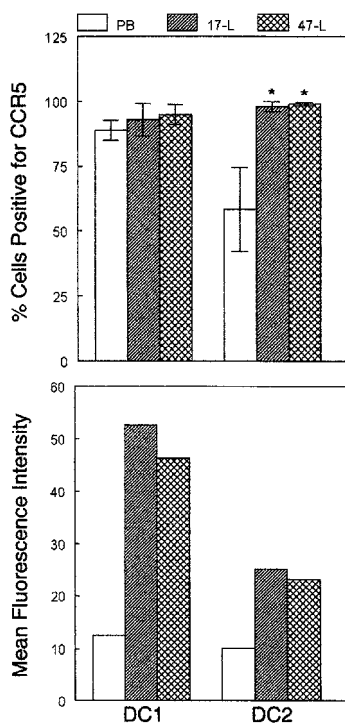


FIGURE 7. The lipopeptide-induced proinflammatory response results in enhanced expression of CCR5 on both DC1 and DC2 cells. The *upper panel* shows the percentage of DC1 and DC2 cells expressing CCR5 (mean $\pm$ SE from six subjects), while the *lower panel* shows representative mean fluorescence intensities. Asterisks indicate values that are significantly greater than the corresponding value in PB (*, $p < 0.01$).

monocytes can differentiate into DC1 cells upon extended incubation with monocyte-conditioned medium (71) or various combinations of cytokines (e.g., IL-4, GM-CSF, TNF- α) (49, 72–76), one could argue alternatively that the DC1 subset actually arose from infiltrating monocytes that differentiated in situ. Two lines of evidence indicate that this is unlikely to be the case. One is that the inflammatory milieu within the sites of injection induced monocytes to mature into activated macrophages rather than DCs, as evidenced by their increased expression of CD14, a surface marker lost when monocyte progenitors become DCs (72–76). Second, in accord with our in vivo findings, Randolph and coworkers (77) showed using an elegant in vitro model that monocytes differentiate into macrophages when they traverse endothelial monolayers, while only those cells that subsequently egress in the basal-to-apical direction differentiate into DCs.

In vitro studies of DC maturation and function typically use either blood or bone marrow-derived progenitors that respond as a homogeneous population to inflammatory stimuli, including LPS and other PAMPs (78–80). However, in recent years it has become apparent that circulating DCs are comprised of two distinct lineages denoted by reciprocal expression of CD123 and CD11c, and that this heterogeneity has profound implications for the induction of T cell differentiation during the primary immune response (43–46, 48, 49, 60, 81). Therefore, a striking observation was the remarkable dichotomy in differentiation potential displayed by these two DC lineages within the inflammatory milieu induced by the lipopeptides. At first blush, one might conclude from the absence of maturation/differentiation markers on DC2 cells that they are unresponsive to inflammatory stimuli and, therefore, functionally inactive at the cutaneous site. However, this interpretation is at odds with the findings that DC2 cells within the skin down-regulated CD86 expression and markedly up-regulated expression of CCR5, a principal receptor for inflammatory chemokines (82). Whereas a variety of surface markers and cytokines appear to be useful for assessing activation of DC1 cells (47), evaluation of the responsiveness of DC2 cells to cytokines and PAMPs appears to be critically dependent upon an awareness of the more limited biosynthetic repertoire of these cells under stimulatory conditions. Recently, Cella and coworkers proposed that DC2 cells maintain the efficiency of T cell responses and protect against the cytopathic effects of virus by producing large amounts of IFN- α within inflamed lymph nodes (49). In light of in vitro findings that lipoprotein-mediated responses are proapoptotic (22), it is tempting to speculate that DC2 cells might fulfill a similar protective role during syphilitic infection.

The epidemiologic relationship between genital ulceration due to syphilis and the acquisition of HIV-1 infection was recognized early in the AIDS epidemic (83). Although initially attributed to the disruption of epithelial barriers, it soon was appreciated that genital ulcers comprise a unique inflammatory niche well endowed with HIV-1 target cells (1, 54). Herein we used our human skin model to extend prior in vitro experiments demonstrating that treponemal lipoproteins/lipopeptides induced reciprocal changes in the expression of CCR5 and CXCR4 on PB monocytes (20). We found, in fact, that these proinflammatory agonists have the capacity to establish a microenvironment highly enriched for mononuclear cells, particularly macrophages and DCs, expressing the CCR5 coreceptor for sexually transmitted, M-tropic strains of HIV-1. These observations also have broad implications for our understanding of the ontogeny of the inflammatory response in early syphilis. During infection, diverse immune cells responding to the presence of these chemoattractants would be trapped in the inflammatory focus until the local concentration of lipoproteins is diminished by clearance of organisms, at which point they would

be free to respond to other chemoattractants, directing their return to the circulation or migration to skin-draining lymph nodes. This scenario, interestingly, is at odds with the current paradigm for DC migration stating that CC chemokine receptors, including CCR5, are essential for the homing of DCs to inflamed tissues, but are rapidly down-regulated once within sites of inflammation (82, 84). It should be noted that this paradigm is derived largely from in vitro studies with LPS and proinflammatory cytokines (e.g., IL-1 β and TNF- α) (85, 86), whereas our findings reflect the complex immunomodulatory networks and cell-to-cell interactions occurring in vivo. Moreover, LPS and lipoproteins/lipopeptides engender divergent effects on CCR5/CXCR4 expression (20), with the former down-regulating and the latter up-regulating the expression of CCR5. These converse effects on CCR5 expression reflect the use by these two PAMPs of different TLRs for stimulating innate immune cells (T.J.S. and J.D.R., unpublished observations) (22–26, 87). Because Gram-positive bacterial skin pathogens, such as spirochetes, activate cells via TLR2 (24, 27, 88), our findings also may pertain to other clinically relevant cutaneous infections.

Histochemical studies have established that primary and secondary syphilitic lesions contain highly similar cellular infiltrates consisting predominantly of lymphocytes and macrophages (1, 4–8). Moreover, preliminary evidence from application of the suction blister technique to syphilis patients demonstrates that, similar to lipopeptide injection sites, secondary skin lesions are enriched for macrophages, CLA⁺ T cells, and DCs (data not shown). These findings lead us to propose that the similarities between primary and secondary syphilis lesions reflect, to a large extent, the stereotypical nature of the proinflammatory response to treponemal lipoproteins. If so, it is tempting to speculate that the character of the subsequent adaptive response would be determined by whether the memory/effector T lymphocytes elicited as part of this process possess immunologic memory for *T. pallidum* Ags. Early in the development of a chancre, a primary immune response would ensue because the infiltrating memory/effector T cells are not sensitized to treponemal Ags. However, infiltrating DCs will take up Ag for presentation to naive T cells within regional lymph nodes. Trafficking of these neo-sensitized cells back to the genital ulcer would then accelerate bacterial clearance and subsequent healing of the primary lesion. In secondary syphilis lesions, the presence of mature DCs, as well as other APCs (i.e., activated macrophages), coupled with the influx of *T. pallidum*-sensitized T cells produced during the primary stage of infection, would foster a robust and rapid local secondary immune response, signaling a potential turning point in the host's efforts to contain the pathogen and establish latency.

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