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Coevolution of Markers of Innate and Adaptive Immunity in Skin and Peripheral Blood of Patients with Erythema Migrans\textsuperscript{1,2}

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We used multiparameter flow cytometry to characterize leukocyte immunophenotypes and cytokines in skin and peripheral blood of patients with erythema migrans (EM). Dermal leukocytes and cytokines were assessed in fluids aspirated from epidermal suction blisters raised over EM lesions and skin of uninfected controls. Compared with corresponding peripheral blood, EM infiltrates were enriched for T cells, monocytes/macrophages, and dendritic cells (DCs), contained lower proportions of neutrophils, and were virtually devoid of B cells. Enhanced expression of CD14 and HLA-DR by lesional neutrophils and macrophages indicated that these innate effector cells were highly activated. Staining for CD45RO and CD27 revealed that lesional T lymphocytes were predominantly Ag-experienced cells; furthermore, a subset of circulating T cells also appeared to be neoallergized. Lesional DC subsets, CD11c\textsuperscript{+} (monocytoid) and CD11c\textsuperscript{−} (plasmacytoid), expressed activation/maturation surface markers. Patients with multiple EM lesions had greater symptom scores and higher serum levels of IFN-\(\alpha\), TNF-\(\alpha\), and IL-2 than patients with solitary EM. IL-6 and IFN-\(\gamma\) were the predominant cytokines in EM lesions; however, greater levels of both mediators were detected in blister fluids from patients with isolated EM. Circulating monocytes displayed significant increases in surface expression of Toll-like receptor (TLR)1 and TLR2, while CD11c\textsuperscript{−} DCs showed increased expression of TLR2 and TLR4; lesional macrophages and CD11c\textsuperscript{−} and CD11c\textsuperscript{−} DCs exhibited increases in expression of all three TLRs. These results demonstrate that Borrelia burgdorferi triggers innate and adaptive responses during early Lyme disease and emphasize the interdependence of these two arms of the immune response in the efforts of the host to contain spirochetal infection. The Journal of Immunology, 2003, 171: 2660–2670.

Lyme disease (LD),\textsuperscript{5} the most prevalent arthropod-borne infectious disease caused by the tick-borne spirochete Borrelia burgdorferi (1). The number and geographic distribution of LD cases continues to increase in the United States due to the expanding geographic range of its primary vector, the hard tick Ixodes scapularis, and the explosive growth in the white-tailed deer population (2). Optimism that these trends might be reversed by vaccination of at-risk individuals has diminished with the commercial failure of the recombinant outer surface protein A vaccine (3).

LD is usually heralded by erythema migrans (EM), an expanding annular rash, which develops in at least 80% of patients following the inoculation of spirochetes into the skin during tick feeding (1, 4). The prognosis is excellent with appropriate therapy (1, 5). If untreated, dissemination of spirochetes from the site of inoculation may result in a wide range of clinical manifestations, typically involving the skin, joints, peripheral and central nervous systems, and heart (1). The most common histological pattern of EM is a superficial and deep dermal infiltrate reported to consist mostly of lymphocytes, but also containing neutrophils, macrophages, and plasma cells, often in a perivascular distribution (6). Although these histological changes undoubtedly reflect the local inflammatory response to the spirochete, relatively little is known about the leukocyte immunophenotypes comprising these infiltrates. Moreover, given the similar histopathological abnormalities in spirochete-infected skin and extratumoral tissues (6), it is plausible that a better understanding of the immunopathological processes triggered by B. burgdorferi at the site of inoculation will provide insights into disease pathogenesis in other affected organ systems.

Traditionally, investigators have used immunocytochemical analysis of biopsy specimens to characterize cellular immune responses within tissues. Because of the small number of Abs that can be applied to individual tissue sections at any one time and the
difficulties in accurately quantifying Ab staining, such techniques provide only a limited amount of information about the leukocyte subpopulations within an inflammatory infiltrate. To circumvent these limitations, we have used the epidermal suction blister technique by which mild suction is used to raise blisters over a naturally or artificially inflamed site; fluid within the blisters contains extravasated immune cells that can be analyzed using multiparameter flow cytometry (7, 8). Previous studies using this method have established that the composition of the cells in the blister fluid (BF) accurately reflects the cellular infiltrates in the underlying dermis (9–11). A particular strength of the technique is that it enables one to conduct qualitative and quantitative comparisons between peripheral blood (PB) leukocytes and leukocyte subsets selectively recruited to skin. Using this method, we previously reported that intradermal injection of synthetic analogs of Treponema pallidum and B. burgdorferi lipoproteins (i.e., lipopeptides), proinflammatory agonists that activate innate immune effector cells via the pattern recognition receptors (PRRs) CD14 and Toll-like receptors (TLRs) 1 and 2 (12, 13–19), elicit infiltrates comprising cellular elements derived from the adaptive as well as the innate arms of the cellular immune system (20) (J. C. Salazar, C. D. Pope, M. W. Moore, and J. D. Radolf, unpublished observations). The results obtained using this model system strongly bolstered the contention that lipoproteins are major proinflammatory agonists during human spirochetal infection (21, 22).

In this report, we extended the use of the epidermal suction blister technique to supplement the paucity of information regarding the cellular and cytokine responses elicited by LD spirochetes following tick inoculation into human skin. Taken as a whole, these results demonstrate that B. burgdorferi has an impressive capacity to mobilize innate and adaptive responses, both systemically as well as locally, during the primary stage of LD. They also emphasize the interdependence of these two arms of the immune response in the generation of clinical manifestations and the efforts of the host to contain spirochetal infection.

Materials and Methods

Human subjects

A network of experienced primary care and emergency medicine physicians recruited patients from Connecticut towns known to be highly endemic for LD during the months of May through October, 2000–2002. EM was defined as an expanding annular lesion, at least 5 cm in diameter, often with partial central clearing (1). The decision to initiate antimicrobial therapy at the time of presentation was left to the discretion of the referring physician. Patients were referred to the University of Connecticut Health Center General Clinical Research Center (Farmington, CT) where a study physician (J. C. Salazar, J. G. Pope, H. M. Feder, or J. D. Radolf) corroborated the diagnosis. After written informed consent was obtained, patients filled out a questionnaire and a complete physical examination was performed. The questionnaire was designed to capture information relating to 16 symptoms well-recognized to be associated with acute LD (23). This information was used to devise a symptom score for each patient that was subsequently correlated to other study parameters. Blood was then drawn for complete blood count with differential as well as serological and PCR diagnostic studies. Epidermal blisters were raised as described below. Patients were re-evaluated during a convalescent visit scheduled within 30–60 days of presentation. Healthy volunteers without a history of LD, or serological evidence for LD, were recruited by the General Clinical Research Center to provide PB for flow cytometry and to raise epidermal blisters on healthy skin. The University of Connecticut Health Center Institutional Review Board approved the protocols used in this study.

Elicitation of epidermal suction blisters

Epidermal blisters were raised as previously described (20) by applying mild suction (200 mm of Hg) through acrylic cups applied to the skin surface and gentle warming with a 125 W infrared lamp for 2 h. One or more suction cups were applied to the periphery of the primary EM lesion. A thin coating of high vacuum silicone lubricant (Dow Corning, Midland, MI) was applied to the underside of the suction cup to ensure an airtight seal with the surface of the skin. In two patients with disseminated EM lesions, it was not possible to identify the primary lesion or the primary lesion was poorly situated for eliciting blisters. In these instances, blisters were raised over one or more secondary lesions. Fluid was aspirated from the blisters 24 h later at which time 10 ml of blood also was drawn.

Serological assays

Acute and convalescent sera were studied for LD, human anaplasmosis, and babesiosis. ELISA and immunoblot assays for LD were performed by the University of Connecticut Health Center clinical laboratory using standard methodologies and interpreted based on criteria established by the Centers for Disease Control and Prevention (24, 25). A serologic diagnosis of LD was established if diagnostic levels of IgM or IgG Abs were measured by an ELISA of acute and/or convalescent sera and confirmed by immunoblot analysis. Babesial Ab was assayed serologically by using a previously described indirect immunofluorescence assay. Titers of 1:64 or greater were considered reactive (26). Serum Abs directed against Anaplasma phagocytophila were detected by ELISA using recombinant HGE-44 Ag as previously described (27).

Isolation of B. burgdorferi

Plasma from 18–30 ml of blood was inoculated into one to three 50-ml screw-cap plastic tubes that contained 40–45 ml of BSK-H complete medium (Sigma-Aldrich, St. Louis, MO) containing phosphomycin (20 μg/ml), amphotericin B (2.5 μg/ml), and rifampicin (50 μg/ml). Portions of BF s were inoculated directly into the same medium. Cultures were incubated at 33°C for up to 12 wk and examined weekly by dark field microscopy.

PCR detection of pathogens

DNAs from blood and BF s of LD patients and, in one instance, from a tick removed from a bite site were purified using the IsoQuick nucleic acid extraction kit (Orca Research, Bothell, WA). The PCR assays targeted a 294-bp portion of the B. burgdorferi ospA gene, a 238-bp portion of the Babesia microti small subunit ribosomal RNA gene and a 247-bp portion of the A. phagocytophila small subunit ribosomal RNA gene (28–30). Cycling parameters for detection of B. burgdorferi and B. microti consisted of one 5-min cycle at 94°C, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Cycling parameters for detection of A. phagocytophila consisted of one 5-min cycle at 94°C, followed by 45 cycles of denaturation at 94°C for 45 s, annealing at 65.5°C for 45 s, and extension at 72°C for 45 s. Amplicons were detected using the GEN-ETI-K DNA enzyme immunoassay according to the instructions of the manufacturer (DiaSorin, Stillwater, OK). In preliminary standardization experiments, each assay detected between 1 and 10 copies of its respective target (data not shown).

Antibody conjugates

The following Abs were purchased from BD Immunocytometry Systems (San Jose, CA): CD3-PerCP, CD1-allophycocyanin, CD4-PerCP, CD4-allophycocyanin, CD8-PE, CD8-PerCP, CD11c-allophycocyanin, CD14-FITC, CD20-PE, CD27-PE, CD38-allophycocyanin, CD45-PerCP, CD69-FITC, CD80-PE, HLA DR-PerCP, and lineage mixture-FITC, CD83-PE, CXCR3-PE, and CCX3-FITC were obtained from BD Pharmingen (San Francisco, CA). Caltag Laboratories (Burlingame, CA) was the source for CD1a-PE. CD45RO-FITC was purchased from DAKO (Carpinteria, CA). FITC-yö TCR was obtained from Endogen (Woburn, MA). Isotype-matched Ab conjugates were obtained from BD Immunocytometry Systems. Table I presents a summary of the Ab panels used to identify the various cell populations examined in this study. Each panel was used on a minimum of four patients or control specimens.

Cell staining and flow cytometry

Erythrocyte-depleted leukocytes from PB and BF were prepared for FACS analysis as previously described (20). Cells were blocked with 10 μg of purified human IgG followed by incubation with the fluorochrome-conjugated Abs listed in Table I. Aliquots of erythrocyte-depleted leukocytes were also incubated with a single fluorochrome-conjugated Ab or isotype-matched control Abs to compensate for fluorescence emission overlap and nonspecific fluorescence, respectively. FACS analysis was performed on a FACS Calibur dual laser flow cytometer (BD Immunocytometry Systems) using a threshold of 52 and an appropriate scatter gate to exclude dead cells, cellular debris, and residual erythrocytes. A minimum 60,000 events were collected from BF s for each staining panel. List mode multiparameter files (consisting of forward and orthogonal scatter and three or four fluorescence parameters) were analyzed using PAINT-A-GATE® (version
3.0) software (BD Immunocytometry Systems). Events can be sequentially “painted” with this program to identify up to seven discrete cell populations. Subpopulations identified in this manner then were quantified as percentages of the total events (or a gated subset thereof) and their mean fluorescent intensities (MFIs) were calculated.

Cytokine measurements

The Cytokine Bead Array (BD Biosciences, San Diego, CA) kit was used for simultaneous measurement of IFN-γ, TNF-α, IL-10, IL-6 or IL-5, IL-4, and IL-2 in sera and BFs (31). Fifty microliters of each sample (serum or BF) were added to an equal volume of the cytokine bead mixture and detection reagent followed by a 3-h incubation at room temperature in the dark. Ten additional tubes, each containing equal volumes of beads, detection reagent, and graded amounts of the six cytokines, were prepared in parallel to generate a standard curve for each cytokine. Unstained, FITC-, or PE-labeled cytometer setup beads were prepared toward the end of the sample incubation period. At the end of the incubation period, beads were washed with the wash buffer provided in the kit, centrifuged at 200 × g for 5 min, and the supernatants were carefully aspirated. The pellets were suspended in 300 μl of the wash buffer provided in the kit and assayed immediately on the FACSCalibur; cytokine concentrations were determined into 1) the entire cohort (n = 30 patients were enrolled before the administration of antibiotics; blood cultures for B. burgdorferi were positive in 2. BF were isolated from the 7 untreated patients also were cultured in BSK-H medium, and B. burgdorferi was isolated from 2. One patient had a titer of 1:512 for IgG Abs against A. phagocytophilum but was PCR-negative in blood, suggesting a previous infection. Three patients were coincided with B. microti based upon PCR and serological studies. PB smears at presentation were available for 2 and both were negative, consistent with low-grade piroplasmal infection. Similar to other case series (1, 4, 32), localized and constitutional symptoms were a common accompaniment of acute LD regardless of whether the EM was solitary or disseminated. Patients with disseminated disease, however, had significantly higher symptom scores than did those with solitary EM (p = 0.04).

Selective recruitment of leukocyte populations into EM lesions

Yields from EM BFs averaged 1 × 10^7 cells (range: 6 × 10^5–6 × 10^7 cells). In contrast, BFs from normal skin contained, on average, 100-fold fewer cells, confirming that the vast majority of cells in the EM BFs were elicited by the inflammatory response to the spirochete. Fig. 1 presents a summary of the principal leukocyte populations in PB from 6 normal volunteers and PB and BFs from 6 patients gave a history of a tick bite where the EM appeared, and one removed a tick that was PCR-positive for B. burgdorferi DNA from the site where EM subsequently developed. Borrelial DNA was detected in 1 of the 29 blood specimens analyzed by PCR and in 3 of the 26 BFs. The mean duration of antibiotic therapy at the time of enrollment was 1 day (range 0–4 days). Seven of the 30 patients were enrolled before the administration of antibiotics; blood cultures for B. burgdorferi were positive in 2. BF were isolated from the 7 untreated patients also were cultured in BSK-H medium, and B. burgdorferi was isolated from 2. One patient had a titer of 1:512 for IgG Abs against A. phagocytophilum but was PCR-negative in blood, suggesting a previous infection. Three patients were coincided with B. microti based upon PCR and serological studies. PB smears at presentation were available for 2 and both were negative, consistent with low-grade piroplasmal infection. Similar to other case series (1, 4, 32), localized and constitutional symptoms were a common accompaniment of acute LD regardless of whether the EM was solitary or disseminated. Patients with disseminated disease, however, had significantly higher symptom scores than did those with solitary EM (p = 0.04).

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### Table II. Clinical and epidemiologic features (n = 30)

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Number (Percentage/Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>22 (73%)</td>
</tr>
<tr>
<td>Mean age</td>
<td>47.1 (range 26–71 years)</td>
</tr>
<tr>
<td>EM (average diameter)</td>
<td>14.6 (range 5–30 cm)</td>
</tr>
<tr>
<td>Multiple EM</td>
<td>7 (23%)</td>
</tr>
<tr>
<td>Duration of EM at enrollment (days)</td>
<td>6 (range 1–17)</td>
</tr>
<tr>
<td>Days of therapy prior to enrollment</td>
<td>1 (range 0–4)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>23 (77%)</td>
</tr>
<tr>
<td>Fever</td>
<td>21 (70%)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>21 (70%)</td>
</tr>
<tr>
<td>Headache</td>
<td>21 (70%)</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>17 (57%)</td>
</tr>
<tr>
<td>Chills</td>
<td>14 (47%)</td>
</tr>
<tr>
<td>Neck pain</td>
<td>8 (27%)</td>
</tr>
<tr>
<td>Sore throat</td>
<td>6 (20%)</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>6 (20%)</td>
</tr>
<tr>
<td>Nausea</td>
<td>5 (17%)</td>
</tr>
<tr>
<td>Lymphadenopathy (local)</td>
<td>3 (10%)</td>
</tr>
<tr>
<td>Serologic evidence of Lyme disease</td>
<td>26 (87%)</td>
</tr>
<tr>
<td>Babesia coinfection</td>
<td>3 (10%)</td>
</tr>
<tr>
<td>Ehrlichia coinfection</td>
<td>1 (3%)</td>
</tr>
</tbody>
</table>
the 30 LD patients. There was excellent agreement between PB white cell differentials determined by flow cytometry and from Wright-stained smears; with either method, no significant differences were noted between normal individuals and EM patients. In contrast, differences were readily evident between circulating and lesion-derived leukocyte populations in LD patients. In comparison to PB, lesional infiltrates were 1) enriched for T cells, monocytes/macrophages, and DCs, 2) contained a lower percentage of neutrophils, and 3) were virtually devoid of B cells. There were no significant differences in the cellular composition of BFs from primary lesions elicited in patients with solitary EM vs multiple EM or between patients not previously treated with antibiotics and those who had received antibiotics at enrollment (data not shown). Although the cell yields from blisters raised on healthy volunteers were too low for accurate flow cytometry, manual differentials performed on Wright-stained smears from two people revealed that the overwhelming majority (98 and 93%) of cells were mononuclear. Additional evidence that the EM FACS data reflected a distinctive inflammatory process was obtained by analyzing fluids from two patients with cutaneous lesions initially misdiagnosed as EM. One individual with a history of psoriasis had an erythematous plaque with an infiltrate comprising mostly T cells (34%) and monocytes/macrophages (55%). Fluid from the second patient, who developed cellulitis following an insect bite, contained neutrophils (29%), eosinophils (21%), T cells (42%), and monocytes/macrophages (8%).

**Ag-experienced T cells predominate in EM infiltrates**

We next asked whether particular T lymphocyte subsets were selectively recruited into *B. burgdorferi*-infected skin. Table III presents a summary of these results. The proportions of CD4+ and CD8+ T cells in the PB and skin compartments were not significantly different. Similarly, significant differences in the percentage of CD4+/CD8- T cells in PB and BFs also were not observed. Specific staining for γδ T cells in one patient also failed to reveal

### Table III. Immunophenotypes of T cells in LD blood (PB-LD) and EM BF (EM-BF)*

<table>
<thead>
<tr>
<th>Surface Ags</th>
<th>PB-NL</th>
<th>PB-LD</th>
<th>EM-BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4/CD87</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>CD3+CD4+</td>
<td>57 (48.1–65.9)</td>
<td>65.5 (55.4–75.6)</td>
<td>68.2 (53.0–83.5)</td>
</tr>
<tr>
<td>CD3+CD8+</td>
<td>34.1 (22.7–45.3)</td>
<td>27.4 (19.5–35.3)</td>
<td>20.6 (14.1–27.1)</td>
</tr>
<tr>
<td>CD3+CD4+CD8-</td>
<td>0.5 (0.3–0.8)</td>
<td>0.7 (0.2–0.9)</td>
<td>1.2 (0.6–1.7)</td>
</tr>
<tr>
<td>CD3-CD4-CD8-</td>
<td>8.4 (4.8–11.9)</td>
<td>5.6 (0–11.4)</td>
<td>9.3 (0–21.6)</td>
</tr>
<tr>
<td>CD27+CD45RO-</td>
<td>n = 5</td>
<td>n = 13</td>
<td>n = 5</td>
</tr>
<tr>
<td>CD4+CD27+CD45RO-</td>
<td>28.7 (0.7–56.7)</td>
<td>42.7 (34.2–51.2)</td>
<td>7.0 (2.3–11.7)</td>
</tr>
<tr>
<td>CD4+CD27-CD45RO+</td>
<td>64.2 (39.8–88.6)</td>
<td>47.5 (42.2–52.9)</td>
<td>68.7 (53.3–84.2)</td>
</tr>
<tr>
<td>CD4-CD27-CD45RO-</td>
<td>6.6 (3.1–10.1)</td>
<td>7.2 (4.4–10.0)</td>
<td>21.5 (7.2–35.8)</td>
</tr>
<tr>
<td>CD4-CD27+CD45RO-</td>
<td>0.6 (0.1–1.1)</td>
<td>2.6 (0–5.7)</td>
<td>2.8 (0–5.7)</td>
</tr>
<tr>
<td>CD8+CD27-CD45RO-</td>
<td>49.3 (23.3–75.4)</td>
<td>21.2 (3.2–39.3)</td>
<td>27.1 (9.9–33.6)</td>
</tr>
<tr>
<td>CD8-CD27+CD45RO+</td>
<td>33.4 (12.9–53.9)</td>
<td>26.8 (19.6–34.1)</td>
<td>50.1 (36.9–63.2)</td>
</tr>
<tr>
<td>CD8-CD27-CD45RO-</td>
<td>3.3 (1.2–5.5)</td>
<td>2.1 (1.4–2.9)</td>
<td>11.3 (4.9–17.6)</td>
</tr>
<tr>
<td>CD8-CD27+CD45RO-</td>
<td>13.7 (2.4–25.0)</td>
<td>10.3 (5.0–20.0)</td>
<td>11.4 (0–24.4)</td>
</tr>
<tr>
<td>CXCR3/CCR5</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>CD3+CXCR3+</td>
<td>4.6 (0.3–8.8)</td>
<td>27.8 (5.7–49.8)</td>
<td>34.1 (2.4–66.1)</td>
</tr>
<tr>
<td>CD3+CCR5+</td>
<td>8.1 (3.2–12.9)</td>
<td>4.5 (0.3–8.8)</td>
<td>8.2 (0–24.8)</td>
</tr>
<tr>
<td>CD3+CXCR3-CCR5-</td>
<td>2.8 (0–5.8)</td>
<td>9.6 (0.3–8.8)</td>
<td>43.6 (10.4–76.8)</td>
</tr>
</tbody>
</table>

*p < 0.05 when comparing LD patients, blood (PB-LD) to EM BF (EM-BF)."
Surface expression of CD45RO and the costimulatory molecule CD27 has been used previously to distinguish naïve from Ag-experienced T lymphocytes (34–36). Simultaneous staining for these two surface markers revealed that cells belonging to the memory (CD27+/CD45RO+) and memory-effector (CD27−/CD45RO−) subsets, were enriched among lesional T cells (Table III, see also Fig. 2). For both CD4+ and CD8+ T cells elicited from infected skin, the enrichment in memory cells achieved statistical significance. Also noteworthy in this regard was the significantly decreased percentage of lesional CD4+ T cells derived from the CD27+/CD45RO− subset that contains naïve lymphocytes in PB. Interestingly, although the relative proportions of circulating T cells delineated by staining for CD45RO and CD27 did not differ between normal volunteers and patients (Table III), the CD27+/CD45RO− and CD27−/CD45RO− subsets from patients expressed appreciably more CD27 on their surfaces (Figs. 2 and 3). As shown in Fig. 3, statistically significant differences were found in MFI values for both the CD4+ (p = 0.001) and CD8+ (p = 0.04) subsets in the PB of patients as opposed to that of controls. Substantial numbers of lesional T cells, particularly CD4+ lymphocytes, also expressed high surface levels of CD27 although the MFIs were not different from their PB counterparts. MFI values for patient PB lymphocytes fell appreciably when re-examined 4- to 6-wk postenrollment (data not shown), indicating that these elevations in surface CD27 expression were infection-related.

**B. burgdorferi infection of skin promotes recruitment and activation of two distinct DC subsets**

DCs, professional APCs whose primary function is to capture, process, and present Ags to naïve T cells within secondary lymphoid tissues (37), can be recruited to peripheral sites by a broad range of inflammatory stimuli (38). As noted earlier (Fig. 1), DCs were enriched ~5-fold in EM infiltrates. Staining with a panel of markers (Table I) was performed to further characterize the impact of *B. burgdorferi* infection on this small, but critically important, leukocyte population. In recent years, surface expression of CD11c has been used to delineate DC subsets derived from monocytic and presumptive lymphoid precursors (39–41). There were no significant differences in the percentages of CD11c+ (monocytoid) and CD11c− (plasmacytoid) DCs in PB (47.2 vs 39.1%) and BF (51.9 vs 40.3%) of LD patients, indicating that neither DC subset was selectively recruited to the site of infection. There were, in contrast, marked differences in the activation/maturation states of DCs in the two compartments. Though most evident for the CD11c+ subset with respect to both percentage (Fig. 4) and MFI values (data not shown), a significant increase also was noted in the percentage of CD83+ CD11c+ DCs in lesional infiltrates. Expression of CD1a denotes monocytoid DCs with the capacity to differentiate into Langerhans cells (40); the lack of CD1a staining by CD11c− DCs from infected skin was consistent with their presumed lymphoid origin. Also noteworthy was a lesional subpopulation of CD11c+/CD1ahigh DCs that most likely consists of resident epidermal Langerhans cells recruited into the dermis (42) (Fig. 5). Person-to-person variability notwithstanding, there were no significant differences between DCs in the PB of LD patients and healthy volunteers for any of the surface markers examined (Figs. 4 and 5).

**Serum and skin cytokine responses differ between patients with solitary vs disseminated EM**

Serum and BF cytokine levels were determined for 21 patients: IFN-α was included in the cytokine panel because plasmacytoid

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**FIGURE 2.** Characterization of T lymphocytes based upon surface expression of CD45RO and CD27. Cells in PB of a normal volunteer (PB-NL) or in PB and EM of a patient (PB-LD and EM, respectively) were stained with Ab conjugates specific for CD45RO, CD27, CD4, and CD3 (see Table I). CD8+ T lymphocytes were identified as CD3+/CD4− cells.

**FIGURE 3.** Levels of CD27 expression on (A) CD4+ or (B) CD8+ T lymphocytes from PB of healthy controls (PB-NL) and LD patients (PB-LD) and BF derived from EM lesions (EM) as measured by MFI. Cells were stained with Ab conjugates specific for CD45RO, CD27, CD4, and CD3 (see Table I). CD8+ T lymphocytes were identified as CD3+/CD4− cells. *p values < 0.05. Values of p denote comparison between PB-NL and PB-LD MFI.
DCs have been shown to be capable of secreting large amounts of type I IFNs (39, 43). The resulting data are summarized in Table IV. Overall, serum cytokine levels were low. In contrast, BFIs contained markedly elevated levels of IFN-γ and IL-6 and modest elevations in IL-10 and TNF-α. To confirm that the cytokine elevations in the lesional fluids were due to spirochete-induced inflammation rather than the blistering process, we assessed fluids from blisters raised over the volar aspect of the forearms of five healthy volunteers. Only elevations in IL-6 were detected and at levels (mean 178.9 ± 82 pg/ml) significantly lower (p = 0.007) than those in EM BFs. Striking relationships emerged when serum cytokine and BF levels were compared between solitary EM patients. Subjects with multiple EM had significantly higher serum levels of IFN-α, TNF-α, and IL-2. With the exception of IFN-α, cytokine levels in BFs from solitary EM patients were significantly higher than the corresponding sera. Remarkably, there were no significant differences between serum and BF cytokine levels in the patients with disseminated disease. Except for IFN-α, cytokine levels in lesional fluids from patients with solitary EM were significantly greater than levels in BFs from patients with disseminated disease. We further analyzed the cytokine data in an effort to obtain evidence for a causal relationship(s) between inflammatory mediators and symptoms. For the patient cohort, a significant correlation was found between symptom score and serum levels of IFN-γ (r = 0.4, 95% confidence interval (CI): 0.04–1.0; p < 0.05, single tail). The correlation between symptom score and serum levels of IL-6 was close to significance (r = 0.3, 95% CI: 0.03–1.0; p = 0.06, single tail). No correlations were found between symptom scores and BF levels for any of the cytokines measured.

Expression of the chemokine receptors CCR5 and/or CXCR3 by T lymphocytes correlates with their capacity to elaborate Th1 cytokines (44–46). As a corollary to the cytokine analyses demonstrating a strongly polarized Th1 response within most EM lesions, we also examined PB and skin T cells for expression of these two chemokine receptors. Greater than 80% of lesional T cells expressed one or both of these receptors as compared with ~25% of circulating T lymphocytes (Table III).

Enhanced surface expression of TLRs by phagocytic cells in PB and skin of EM patients

*B. burgdorferi* expresses an abundance of lipoproteins (47, 48), and it is now well-established that these lipid-modified proteins are potent proinflammatory agonists (21, 22). In light of in vitro studies demonstrating that these spirochetal constituents activate innate immune cells via their interactions with TLR1 and TLR2 (14–19), we sought to determine the effect of *B. burgdorferi* infection on the in vivo surface expression of these PRRs. Because of its importance as a prototypical PRR, and to assess changes in TLRs other than those that mediate spirochete- and lipoprotein-induced cellular activation, we also stained cells for TLR4, the LPS receptor (49). The results of these experiments are summarized in Table V, while representative flow cytometry for DCs is shown in Fig. 6. Compared with their counterparts in control PB, circulating monocytes from patients displayed significant increases in surface expression of TLR1 and TLR2, while circulating CD11c+ DCs showed increases in surface expression of TLR2 and TLR4. A downward trend for surface TLR expression by both immune cell types also was apparent during convalescence (data not shown), supporting the notion that these increases were infection-related. Even more dramatic increases were observed for EM-derived cells. For lesional macrophages and CD11c+ DCs, surface expression of

**FIGURE 4.** Expression of activation/differentiation markers by dendritic cells from PB of healthy controls (PB-NL) and LD patients (PB-LD) and BF derived from EM lesions (EM). DCs were identified as lineage−, HLA-DR+ cells and then further gated with respect to the expression of CD11c to identify monocytoid (CD11c+CD11c−) and plasmacytoid (CD11c−) subsets. Values of p denote comparison between PB-LD and EM populations.

**FIGURE 5.** Flow cytometric analysis of activation/differentiation markers on DCs in PB and BF. The control panels (PB-NL) are from a normal donor, whereas the LD panels are a composite of matched PB and EM results for HLA-DR, CD83, and CD80 from one individual and matched PB and EM results for CD1a from another. Note that the percentage of CD80+ plasmacytoid cells in the PB of the LD patient is unusually high. Presumptive Langerhans (CD11c+CD1a+) cells are demarcated by a box.
all three TLRs was significantly elevated compared with PB counterparts. Although TLR surface expression by CD11c^+ DCs in cutaneous infiltrates was increased relative to PB, these differences were not statistically significant. However, it is worth noting that the percentages of TLR1- and TLR2-positive CD11c^+ DCs in infected skin were well above the values for circulating CD11c^+ DCs in normal volunteers. Unlike monocytes and DCs, circulating neutrophils did not display enhanced surface expression of TLRs. However, lesional neutrophils did show increased surface TLR2 expression compared with their counterparts in PB, and all TLRs were increased relative to neutrophils in control PB. Only miniscule percentages of T cells in either PB or lesions expressed detectable levels of TLRs on their surfaces.

### Discussion

Early LD and EM have been characterized extensively from the standpoint of epidemiology, clinical characteristics, microbiology, and serology. Diagnosis, and therapeutic outcomes (1, 4, 25, 50, 51). Rather surprisingly, EM infiltrates have not been subjected to intensive immunocytological scrutiny despite the accessibility of lesions in most patients and the likelihood that the immunopathological processes induced by *B. burgdorferi* within skin can yield valuable insights regarding disease development in other organ systems. In this study, we used a minimally invasive method for extracting dermal immune cells in numbers amenable for flow cytometry to conduct the first comprehensive analysis of leucocyte subsets and cytokines in PB and skin of individuals with early LD associated with EM (20).

It is generally presumed that, in addition to evoking specific B and T cell responses, borrelial lipoproteins exert proinflammatory effects during infection (21, 22). Flow cytometric characterization of dermal leucocyte subsets has enabled us to obtain evidence for the existence of this “lipoprotein effect” in human disease. Intradermal injection of LD-seronegative individuals with spirochetal lipopeptides elicits cellular infiltrates comprising neutrophils, activated macrophages, monocytoid and plasmacytoid DCs, and memory and memory/effector T cells (Ref. 20 and J. D. Salazar, C. D. Pope, M. Moore, and J. D. Radolf, unpublished observations), the same immune cell types that were identified in EM in the present study. LD spirochetes and borrelial lipoproteins induce vascular endothelium to express leucocyte adhesion molecules and secrete chemotactic factors that promote transmural migration of leucocytes (52–55). Most lymphocytes (> 80%) recruited to skin following intradermal injection of lipopeptides express the skin homing receptor cutaneous lymphocyte Ag (20). Moreover, spirochetal lipoproteins/lipoprotein also induce enhanced expression of CCR5 by monocytes/macrophages and DCs as well as secretion of β-chemokines by monocytes harvested from PB (20, 56). Thus, the ability of these pathogen-associated molecular patterns (PAMPs) to engender a local response that promotes the recruitment and retention of diverse leucocyte populations with skin homing capability is a highly plausible explanation for the substantial overlap in the composition of lipoprotein/lipopptide-induced infiltrates and those documented in our EM patients. The most notable difference between the two types of infiltrates, an ~3-fold greater percentage of T cells in EM vs lipopeptide-injected BFs, 50 vs 15% (20), respectively, is consistent with the expectation that EM lesions will accumulate *B. burgdorferi*-specific CD4^+ and CD8^+ T cells (57, 58).

Current paradigms for lymphocyte trafficking maintain that T cells become Ag-sensitized in secondary lymphoid tissues, re-enter the circulation, and home to inflamed peripheral sites (59, 60). The most precise method for quantifying and tracking the migration of *B. burgdorferi*-specific T cells from PB to skin, tetramer staining

### Table IV. Cytokine levels (picograms/milliliter) in serum and EM stratified according to presentation (solitary vs multiple EM)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>All EM (n = 21)</th>
<th>Solitary EM (n = 15)</th>
<th>Multiple EM (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>EM</td>
<td>Serum</td>
</tr>
<tr>
<td>IFN-α</td>
<td>24 (2.1–47.7)</td>
<td>12.2 (0–34.4)</td>
<td>3.9 (0–10.2)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>19.8 (8.5–31.1)</td>
<td>377.8 (417–7138.6)</td>
<td>12 (6.9–17.1)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5.9 (1.6–10.1)</td>
<td>32.5 (9.9–55.1)</td>
<td>1.7 (1.2–2.1)</td>
</tr>
<tr>
<td>IL-2</td>
<td>3.5 (1.1–6.1)</td>
<td>13.2 (5.7–20.7)</td>
<td>0.3 (0–1.0)</td>
</tr>
<tr>
<td>IL-4</td>
<td>5.9 (3.1–8.7)</td>
<td>14.8 (9.6–20.0)</td>
<td>3.8 (3.2–4.3)</td>
</tr>
<tr>
<td>IL-6</td>
<td>19.8 (0–43.8)</td>
<td>3596.9 (1108–6085)</td>
<td>9.3 (6.5–12.1)</td>
</tr>
<tr>
<td>IL-10</td>
<td>5.6 (2.6–8.6)</td>
<td>81.2 (0.8–161.7)</td>
<td>3.3 (2.4–4.1)</td>
</tr>
</tbody>
</table>

* Numbers shown are mean picogram concentrations with 95% CI in parentheses.
* p < 0.05 when comparing EM to control (PB-NL).
* p < 0.05 when comparing PB-LD to EM BF (BF-EM).
CD27high/CD45RO with a model of linear T cell differentiation in which neosensitized viral infection in humans (36, 66) is detectable (34, 64, 65). Extrapolating from ex vivo studies of acute LD in the first weeks) diminishes until this regulatory molecule becomes undetectable. Our staining panels revealed that both CD4+ and CD8+ T cells in patient PB, and, to a lesser extent in EM lesions, express higher levels of CD27 than their counterparts in normal PB. In vitro stimulation of naive T cells via the TCR/CD3 complex results in enhanced expression of CD27 that gradually (i.e., over several weeks) diminishes until this regulatory molecule becomes undetectable (34, 64, 65).

<FIGURE 6. Expression of TLRs by DCs in PB from a healthy control (PB-NL), PB from a LD patient (PB-LD), and the corresponding EM BF (EM). In each panel, CD11c+/TLR− are shown in red, CD11c+/TLR+ are shown in blue, CD11c−/TLR− cells are shown in green, and CD11c−/TLR+ cells are shown in violet.>

(61, 62), could not be used here because of the diversity of HLA haplotypes in our patient population and the fact that the antigenic specificities of human T cells during early LD are poorly defined (63). As an alternative approach, we used expression of CD45RO and CD27 to define subsets of memory and effector cells (34–36). Our staining panels revealed that both CD4+ and CD8+ T cells in patient PB, and, to a lesser extent in EM lesions, express higher levels of CD27 than their counterparts in normal PB. In vitro stimulation of naive T cells via the TCR/CD3 complex results in enhanced expression of CD27 that gradually (i.e., over several weeks) diminishes until this regulatory molecule becomes undetectable (34, 64, 65). Extrapolating from ex vivo studies of acute viral infection in humans (36, 66–68), our results are consistent with a model of linear T cell differentiation in which neosensitized CD27high/CD45RO+ T lymphocytes differentiate into memory, memory/effector, and effector subsets as they circulate and traffic to sites of infection where they can interact with their cognate borrelial Ags. In animal models of parasitic (69) and bacterial (70–72) infection, as well as tetramer-based studies of acute viral infections in humans (36, 73), have demonstrated that expansion of Ag-specific T cell populations occurs within days following inoculation. This time scale is compatible with the mean duration of infection in our patient population. For the above scenario to occur, spirochetes must track to regional lymph nodes and/or disseminate to more distant secondary lymphoid tissues. Recovery of B. burgdorferi-sensitized T cells from regional lymph nodes within 1 wk of inoculation has been documented in the mouse model (74).

Although spirochetes are difficult to detect in the small volumes of blood typically assessed by PCR, large volume culture methods indicate that hematogenous dissemination of spirochetes is a common event in early LD (1, 75).

EM is frequently accompanied by a multitude of influenza-like symptoms (1, 4) that are generally believed to be cytokine-mediated and indicative of spirochetal dissemination (1). Three findings made here lend support to this notion: 1) patients with clinical evidence for disseminated disease (and presumably higher total spirochetal burdens) were more symptomatic than those with solitary EM; 2) patient symptom scores correlated with serum levels of IL-6 and, particularly, IFN-γ; and 3) serum cytokine levels in patients with disseminated disease were significantly greater than those in patients with solitary EM. In contrast to Mullegger et al. (76), who used in situ hybridization to identify cytokine transcripts in dermal immune cells in paraffin-embedded skin biopsies, we did not observe any correlation between lesional cytokines and symptomatology. However, we did find a striking dichotomy in cytokine levels in lesions from patients with disseminated as opposed to solitary EM. Further study is required to determine whether failure of patients with disseminated disease to mount a robust local cytokine response is due to host genetic factors (21), infection with highly invasive B. burgdorferi subtypes (77), or even the immunosuppressant activities of tick salivary proteins (78, 79).

IFN-γ and IL-6 were the predominant cytokines detected in BFs from solitary EM lesions. Interactions between these two cytokines very likely promote spirochetal clearance while mitigating tissue damage. IL-6 promotes the local recruitment of monocytes (80) as well as their subsequent differentiation into macrophages once within the cutaneous microenvironment (81), while the macrophage-activating effects of IFN-γ will enhance their capacity for clearance of spirochetes (82–84) as well as their responsiveness to lipoproteins and other spirochetal PAMPs (85, 86). Although IL-6 is generally considered to be proinflammatory, this pleiotropic cytokine also can exert profound local and systemic anti-inflammatory effects (87); indeed, Anguita et al. (88) found that IL-6-deficiency worsens Lyme arthritis in the experimental mouse model. The modest amounts of IL-10 found in our patients might potentiate the ability of IL-6 to limit local cytokine production and, hence, tissue damage. The dominance of IFN-γ is in accord with previous analyses of specimens from patients with noncutaneous forms of LD (58, 89–91). Whereas inbred strains of mice will...
manifest either a Th1 or Th2 cytokine pattern depending on genetic background, with the latter profile correlating with milder disease (92–94), LD in humans appears to be invariably Th1-polarized regardless of the organ system involved. In fact, contrary to what has been reported for mice (95, 96), our data raise the possibility that local elaboration of IFN-γ is required for a salutary outcome in humans. Because all of our patients acquired their disease via tick bite, our results also argue against the contention that immunomodulators in tick saliva can reorient the cutaneous response toward the Th2-skewed profile induced by uninfected ticks during feeding (97, 98).

A fundamental property of DCs is their capacity to migrate from PB into inflamed tissues where, along with resident APCs, they serve as sentinels for foreign Ags, including microbial invaders (99, 100). We recently demonstrated that intradermal injection of spirochetal lipopeptides results in the local recruitment of monocyte and plasmacytoid DCs, the two principal circulating DC subsets, in ratios highly similar to those in PB (Ref. 20 and J. C. Salazar, C. D. Pope, M. W. Moore, and J. D. Radolf, unpublished observations). An essentially identical phenomenon was noted here for EM, further implicating borrelial lipopeptides as principal inducers of DC migration from PB to skin. The enrichment of plasmacytoid DCs in both types of cutaneous infiltrates is important, given that noncirculating members of this DC subset have been localized predominantly to inflamed lymph nodes (39, 101). Indeed, to the best of our knowledge, LD is the first bacterial infection in which plasmacytoid DCs have been identified in skin. However, following lipopeptide injection only the CD11c+ DCs expressed activation/maturity markers (20), whereas both the CD11c+ and CD11c− DC subsets in EM infiltrates were more developmentally advanced than their respective PB counterparts. Thus, whole spirochetes either indirectly or directly induce immunophenotypic alterations in plasmacytoid DCs distinct from those induced by lipopeptides/lipoproteins. In contrast to monocyte DCs, plasmacytoid DCs express TLR9 and are activated by CpG oligonucleotides and unmethylated bacterial DNAAs, including B. burgdorferi DNA (Ref. 102 and M. W. Moore and J. D. Radolf, unpublished observations). Thus, it is tempting to speculate that B. burgdorferi DNA is responsible for the activation of plasmacytoid cells in EM lesions. What functional role do plasmacytoid cells play in EM? Earlier studies indicated that plasmacytoid DCs induce a Th2 response, hence their designation DC2s (103). How might these DCs influence the cutaneous response?

By 1993, when many Lyme disease patients were presenting to EM clinics, it was recognized that the majority carried low titer antibodies to B. burgdorferi. By 1995, it became apparent that serologic markers such as IgG and IgM antibody reactivity had limited sensitivity and specificity to determine the presence and stage of Lyme disease. A better and more useful approach to the diagnostic evaluation of Lyme disease patients was developed by the Lyme Disease Clinical Trials Research Group of the National Institute of Allergy and Infectious Diseases (NIAID) in NIH in 1993. This approach was the discovery of Borrelia-specific T-helper type 1 (Th1) cytokines and lymphokines. The discovery that lesional plasmacytoid DCs expressed TLR9 and are activated by CpG oligonucleotides and unmethylated bacterial DNAAs, including B. burgdorferi DNA (Ref. 102 and M. W. Moore and J. D. Radolf, unpublished observations). Indeed, to the best of our knowledge, LD is the first bacterial infection in which plasmacytoid DCs have been identified in skin. However, following lipopeptide injection only the CD11c+ DCs expressed activation/maturity markers (20), whereas both the CD11c+ and CD11c− DC subsets in EM infiltrates were more developmentally advanced than their respective PB counterparts. Thus, whole spirochetes either indirectly or directly induce immunophenotypic alterations in plasmacytoid DCs distinct from those induced by lipopeptides/lipoproteins. In contrast to monocyte DCs, plasmacytoid DCs express TLR9 and are activated by CpG oligonucleotides and unmethylated bacterial DNAAs, including B. burgdorferi DNA (Ref. 102 and M. W. Moore and J. D. Radolf, unpublished observations). Thus, it is tempting to speculate that B. burgdorferi DNA is responsible for the activation of plasmacytoid cells in EM lesions. What functional role do plasmacytoid cells play in EM? Earlier studies indicated that plasmacytoid DCs induce a Th2 response, hence their designation DC2s (103). However, more recent findings have emphasized the capacity of this DC subset to secrete copious amounts of type I IFNs, often in the context of viral infection, as well as its association with Th1-polarized responses (39, 104, 105). The low levels of IFN-α in the EM BF suggest that plasmacytoid DCs may serve some other function within cutaneous sites infected by LD spirochetes (39, 106).

Ten members of the human TLR family have been identified to date (107, 108) and their patterns of expression in various cells and tissues have been intensively investigated under a variety of conditions (102, 109–114). By contrast, comparatively little is known about TLR expression by immune cells in situ during inflammatory and infectious processes. Although the small number of TLR-specific Ab conjugates currently available limits FACS-based analyses, flow cytometry is a convenient and powerful methodology for quantitating surface expression of TLRs. We observed enhanced surface expression of TLR1, TLR2, and TLR4 by neutrophils, macrophages, and DCs in lesional infiltrates. B. burgdorferi lacks LPS (47, 115) and TLR4 does not play a role in cellular activation by this bacterium (14, 15, 17). Therefore, up-regulation of this PRS suggests that the danger signal(s) imparted by lipoproteins, perhaps in concert with other borrelial PAMPs, causes a coordinate up-regulation of PRRs recognizing diverse microbial ligands. Although far less dramatic, enhanced TLR expression by monocytes and CD11c+ DCs in PB was also observed. This finding was surprising given that neither circulating cell type displayed other evidence for activation, and it suggests that, despite small numbers of TLRs on their surfaces (110), monocytes and monocyte DCs are exquisitely sensitive to low level spirochetal and/or minute concentrations of lipoproteins circulating during acute LD (116). Most surprising was the finding that lesional plasmacytoid DCs expressed TLR1, TLR2, and TLR4, a result that is at odds with published in vitro studies which hold that these TLRs are not part of the plasmacytoid DC TLR repertoire (39, 102). Whether these newly expressed TLRs confer altered PAMP responsiveness will require further investigation.

Flow cytometry data acquired from individual LD patients represent isolated snapshots of their responses to B. burgdorferi infection at a single time point during acute illness. However, together they have enabled us to assemble a picture of the remarkable capacity of the bacterium to orchestrate a parallel mobilization and recruitment of innate and adaptive cellular elements. One implication of these findings is that both arms of the cellular immune system act in a coordinated and flexible manner to contain the pathogen following tick inoculation. This may be of particular importance given the poor or delayed specific Ab responses typically observed during acute infection (1). The early arrival of professional phagocytes provides an immediate defense against the bacterium, while infiltrating DCs will take-up spirochetes for processing and presentation to T cells within regional lymph nodes. The presence of mature DCs, as well as activated macrophages, would foster a robust secondary cellular immune response in situ as neosensitized T cells traffic into the EM lesion.

Acknowledgments

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