Original contribution

Leprosy-specific B-cells within cellular infiltrates in active leprosy lesions

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Summary Leprosy is a spectral disease with polar lepromatous and tuberculoid forms correlating with enhanced humoral and cell-mediated immunity, respectively, against \textit{Mycobacterium leprae} and the borderline forms, borderline lepromatous, midborderline, and borderline tuberculoid showing in-between clinical and immunological characteristics. Histopathologically, the cellular infiltrates of leprosy lesions show predominantly the presence of interacting T-cells and antigen presenting cells like macrophages, whereas the presence of B-cells has only been sporadically reported. The present study demonstrates by immunohistochemical techniques the presence of B-cells, including plasma cells, in active lesions from lepromatous leprosy, skin smear negative borderline lepromatous, and paucibacillary borderline tuberculoid leprosy. Furthermore, the study demonstrates the in situ production of \textit{M leprae}–specific antibodies from BT lesions using an organotypic skin explant culture model. Finally, analysis of the cytokine release profile in supernatants of lesional organotypic skin cultures showed a microenvironment conducive to the differentiation and maturation of B-cells. The results demonstrate the presence of different functionally active B-cell stages within lesions of patients with leprosy, including borderline tuberculoid patients, which could secrete anti-\textit{M leprae}–specific antibodies. However, their role in leprosy pathology remains to be elucidated.

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1. Introduction

Leprosy is a spectral disease with the polar lepromatous (LL) and tuberculoid (TT) forms and the borderline forms including borderline lepromatous (BL), midborderline (BB), and borderline tuberculoid (BT) forms showing clinical and immunological characteristics between the polar forms [1]. Immunity to intracellular infections like leprosy is largely regarded as a T-cell–mediated event. TT characterized by a strong T-cell–mediated immunity resulting in restriction of
Table 1 Characterization of the immune infiltrates

<table>
<thead>
<tr>
<th>Markers</th>
<th>LL/BL</th>
<th>BT</th>
</tr>
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<tbody>
<tr>
<td>B-cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD20</td>
<td>2+</td>
<td>0 to 1+</td>
</tr>
<tr>
<td>CD79</td>
<td>2+</td>
<td>0 to 1+</td>
</tr>
<tr>
<td>CD138</td>
<td>2+</td>
<td>0 to 1+</td>
</tr>
<tr>
<td>T-cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>+</td>
<td>3+</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68</td>
<td>2+ to 3+</td>
<td>+</td>
</tr>
<tr>
<td>M leprae antigens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGL-I</td>
<td>3+</td>
<td>±</td>
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<tr>
<td>LAM</td>
<td>3+</td>
<td>±</td>
</tr>
</tbody>
</table>

a Semiquantitative scores based on distribution of positive cells in the infiltrate: 0 = negative; ± = 1 to 3 cells in the section; 1+ = 3 to 10 cells per granuloma, few granulomas; 2+ = 10 to 50 cells per granuloma; 3+ = 50 to 100 cells per granuloma, many granulomas.

b Membrane and cytoplasmic staining within macrophages.

c Diffused cytoplasmic staining in few epithelioid cells.
d Strong membrane and cytoplasmic staining of macrophages and some extracellular staining.

The orchestrated role of T-cells, macrophages, and antigen presenting cells, such as dendritic cells, are well known to be of primary importance in leprosy pathology [7]. Although presence of plasma cells in LL and BL lesions has been reported [8], the importance of their in situ presence related to the pathology of leprosy lesions has never been elucidated. Interestingly, the presence of plasma cells has also been occasionally reported in histological sections of lepromin reactions and TT/BT lesions [9] despite anti-M leprae antibodies being undetectable in circulation among 50% to 60% TT and BT patients with leprosy [10]. Lai et al [11,12] reported previously the detection of M leprae–speciﬁc antibodies in supernatants of minced leional biopsies from patients with leprosy incubated in medium for 48 hours. However, the experimental setup did not clarify whether active release of antibodies into the supernatant was actually involved. A later study demonstrated secretion of anti-M leprae antibodies from full-thickness skin cultures of lesions from BL/LL patients [13]. Although these studies indicated the presence of M leprae–speciﬁc antibodies in lesional tissues, no systematic attempt was made to relate the presence of antibodies with histological or immunohistochemical identiﬁcation of B-cells/plasma cells within tissue sections of the lesions. Moreover, secretion of antibodies in paucibacillary (BT/TT) lesions has seldom been studied.

Against this background, the present study explored, by immunohistochemical analysis, the in situ presence of B-cell subsets within active lesonal skin of patients with leprosy on multidrug treatment (MDT), particularly in those of a BT or bacteriologically negative BL classification. The functional entity of the B-cell infiltrates within the lesions was determined by studying M leprae–speciﬁc antibody release and the local cytokine milieu within lesional biopsies using the human organotypic skin culture model established in our laboratory [14].

2. Materials and methods

2.1. Patients

Lesional skin biopsies were obtained from a total of 15 patients with leprosy (1 LL, 4 BL, and 10 BT patients) with active lesions attending the outpatient department of the National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra, India. The clinical diagnosis of the patients was conﬁrmed histopathologically according to the Ridley-Jopling classiﬁcation [1]. Patients were treated with World Health Organization-MDT regimens for a minimum of 6 and 12 months, respectively, for the paucibacillary (BT) and multibacillary (BL/LL) patients [15]. All borderline (BT/BL) patients had active lesions at the time of the biopsy, as deﬁned syndromatically by the appearance of swollen lesions with well-demarcated margins, histopathologically showing large numbers of inﬁltrating cells, despite being bacteriologically negative. Only the LL patient was bacteriologically positive with bacteriological index of 3+ (indicating 1-10 acid fast bacilli in 1 oil immersion ﬁeld). Three of the BT patients had reversal reaction at the time of biopsy. All the patients, except one untreated BT, were under MDT treatment, having received between 2 and 6 months of therapy at the time of the biopsy. Skin biopsies from 10 normal individuals, neither having leprosy nor any other skin lesions, being subjected to cholecystectomy, served as healthy controls (HC). Ethical clearance for the present study was obtained following the guidelines of the Indian Council for Medical Research, and informed consent of the patients was obtained before inclusion in the study.

2.2. Antigens

M leprae isolated from armadillo liver were kindly supplied by Prof P.J. Brennan, College of Veterinary Medicine and Biomedical Sciences, Department of Micro-
Fig. 1 Representative immunohistochemical stainings of skin sections from a LL (left panel) and a BT patient (right panel) for B-cell subsets: CD20 (A and E), CD79 (B and F), CD138 (C and G), isotype controls (D and H) (magnification, ×10; areas enclosed by boxes enlarged [×40] in insets).
2.3. Processing of skin biopsies

Skin biopsies (1 × 1 cm) were obtained from the periphery of lesions and divided into 2 equal halves. One half was fixed in 10% formaldehyde and processed further for histopathological and immunohistochemical analysis. The other half was used for organotypic culture as described previously [14].

2.4. Immunohistological techniques

Formalin-fixed, paraffin embedded tissues (6 μm) were stained with the hematoxylin-eosin technique for a light microscopic examination of the tissue morphology. The immune infiltrate was characterized by immunohistochemical staining as described earlier [17]. Briefly, the sections were preincubated with 3% H$_2$O$_2$ in 0.01% sodium azide to inhibit endogenous peroxidase activity. The sections were pretreated, when required, with enzymes or heat treatment to expose the antigenic epitopes followed by blocking with normal goat serum. Primary mouse monoclonal antibodies to different leukocyte surface antigens or to mycobacterial antigens, cross-reactive lipoarabinomannan (LAM), and $M$ leprae–specific PGL-I were applied to the specimen followed by incubation with biotinylated rabbit antimouse immunoglobulin (Ig) and subsequently horse radish peroxidase (HRP)-labeled streptavidin. The color reaction was developed using 3 amino-9 ethyl carbazole as a substrate. The sections were counterstained with hematoxylin. The percentage of the various cellular populations in the lesions was determined by light microscopy in 3 randomly selected fields of sequential sections. The antibodies used for the stainings were CD3 (Immunologic, the Netherlands); CD4 (Lab Vision Corp, UK); CD8, CD20, CD79, CD68 (Dako, Denmark); CD138 (IQ products, the Netherlands); LAM (clone F30-5), PGL-I (clone DZ-1) (Dr A. Kolk, the Netherlands); cutaneous lymphocyte antigen (CLA; clone HECA-452, Dr A. Duijnvenstijn, the Netherlands). Antigen retrieval for CD3, CD8, CD79, and CD138 antigens was carried out by microwaving the sections in 0.01 mol/L sodium citrate buffer, pH 6.0, for 10 minutes at 99°C; whereas for CD68 staining, the slides were treated with 0.25% pepsin in 0.01 mol/L HCl for 10 minutes at 37°C.

The double stainings were carried out to study the localization of T-cells in relation to the B-cells and to observe the extent of skin homing receptor CLA expression on B-cells and T-cells. The staining methods were similar to that used by Bos et al [18]. The immunostained sections were evaluated by 3 independent observers, including 1 experienced pathologist, and scored as indicated in Table 1.

2.5. Organotypic culture of the skin

The protocol used was in accordance to that used by Pistoor et al [14]. Briefly, 5-mm full-thickness skin biopsies were placed on cellulose nitrate membrane filters (Millipore, Billerica, MA; 1.2-μm pore size) on a sterile stainless steel grid in a 25-mm disposable Petri dish. Five hundred
microliters of serum-free RPMI 1640 medium (GIBCO, Invitrogen, Carlsbad, CA, USA) was added underneath the grid, thus maintaining the tissue in an air/liquid interface during the culture period. The tissue was incubated at 37°C in a 5% carbon dioxide atmosphere. The culture fluid was collected every 24 hours from beneath the biopsy and replenished with fresh medium. Collected culture fluids were immediately frozen at −20°C until further analysis.

2.6. Measurement of anti-M leprae IgG antibodies by ELISA

The anti-M leprae IgG antibodies released into the culture fluid from the skin biopsies were measured by ELISA as described earlier with some modifications [13]. Briefly, flat-bottomed ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 μL of MLSA at a concentration of 10 μg/mL in 0.05 mol/L carbonate buffer, pH 9.6, and incubated at 37°C for 4 hours, then overnight at 4°C. Plates were preblocked with 1% bovine serum albumin (Sigma Chemical Co, St. Louis, MO, USA) followed by addition of 50 μL of 1:10 diluted culture fluid in duplicate for 2 hours at 37°C. The plates were probed with 50 μL of HRP-conjugated rabbit antihuman IgG (1:1000, Dako, Carpinteria, CA, USA) for 90 minutes at 37°C. The color reaction was developed with o-phenylene diamine dihydrochloride substrate solution (Sigma Chemical Co), stopped with 7% H2SO4, and the optical density was measured at 492 nm using a Titrertek Multiskan Plus ELISA reader (Flow Laboratories, High Wycombe, UK).

2.7. Measurement of cytokines

Levels of a panel of cytokines, that is, interleukin 4 (IL-4), IL-6, IL-10, interferon γ (IFN-γ), and tumor necrosis factor (TNF-α) were measured in the sequential culture fluid of the organotypic cultures by specific sandwich ELISA systems described previously [19]. Briefly, flat-bottomed ELISA microtiter plates (Costar, Cambridge, MA, USA) were coated overnight with the specific mouse antihuman cytokine monoclonal capture antibody in 0.1 mol/L carbonate buffer (pH 9.6; U-Cytech, Utrecht, Netherlands). The plates were washed, preblocked with 3% bovine serum albumin, and incubated with the organotypic culture fluids or standard antigens in duplicate. The plates were washed and incubated with biotinylated detection antibody followed by HRP-labeled streptavidin (CLB, Amsterdam, The Netherlands). Tetramethyl benzidine (Sigma Chemical Co) was used as the color substrate, and the reaction was stopped with an equal volume of 1 mol/L H2SO4. The absorbance was read in a Bio-Rad microplate reader (Richmond, CA, USA) at 450 nm with a reference wavelength of 655 nm.

2.8. Statistical analysis

The Student t test was used to assess the statistical significance of differences in antibody and cytokine levels over the culture period.

3. Results

3.1. Characterization of lesional cells

A comparison of BL/LL and BT lesions with respect to distribution of cell types and antigens is shown in Table 1. Distribution of the infiltrating cells and M leprae antigens PGL-I and LAM in the lesions are described below.

3.1.1. Macrophages (CD68)

CD68 staining was intense as both membrane and cytoplasmic staining and widely distributed over the granuloma and in foamy macrophages in BL/LL patients. In BT
granulomas, CD68 staining was diffused within epithelioid cells in the center of the granulomas (Table 1). Double staining of CD68+ cells for \textit{M. leprae} antigens LAM and PGL-I showed the antigens in association with the CD68+ cells, some of which were near the T-cells and B-cells (data not shown).

### 3.1.2. T-cells (CD3, CD4, and CD8)

Single stainings for CD3 showed intense positive staining in BT patients, with 70% to 80% of the infiltrating cells showing this marker, whereas the staining was moderate in the BL/LL patients. Double stainings for CD3/CD8 showed moderate numbers of double-positive cells in BL/LL patients and in BT patients (data not shown). However, a ratio of CD4 to CD8 cell numbers showed bias toward CD8 in BL/LL patients (0.6:1-0.3:1), whereas CD4 cells predominated in BT patients (1.25:1-2:1) as reported earlier [20].

### 3.1.3. B-cells (CD20, CD79, and CD138)

Single stainings of skin sections showed consistent presence of varied numbers of CD20-positive cells in BL/LL patients, whereas in BT patients, the presence was sporadic (Fig. 1A and E). Similarly, moderate positivity for CD79 and CD138 was seen in BL/LL patients, whereas it was only sporadically positive or negative in BT patients (Fig. 1B, F and C, G). Presence of B-cells was seen both as single cells and as small clusters of cells both in and outside T-cell areas in BL/LL patients, and B-cells were found in contact with T-cells in BT patients (data not shown). Double staining for the skin homing CLA with B-cells (CD20/CD79/CD138) showed CLA-positive cells lying in close juxtaposition with B-cells in BT patients (Fig. 2A and C, inset). However, few B-cells were also positive for CLA (Fig. 2B and C, inset).

### 3.1.4. Mycobacterial antigens

Strong positive staining for mycobacterial LAM and \textit{M. leprae}–specific PGL-I was seen in BL/LL patients, whereas it was sporadically positive to negative in BT patients (Table 1). This indicated the presence of \textit{M. leprae} antigens in active lesions despite absence of acid fast bacilli.

### 3.2. Antibody secretion of skin lesions is an active phenomenon

One biopsy from a BL was bisected with one of the halves being autoclaved. Both pieces were used for organotypic culture as described in Materials and methods. Although no antibody secretion was observed from the autoclaved biopsy, antibody secretion from the untreated biopsy followed a time-bound response (Fig. 3A), indicating that the antibody detected in the culture supernatant was a result of active secretion and not a result of serum contamination of the biopsy.

### 3.3. Kinetics of antibody production in organotypic culture supernatants

Anti-\textit{M. leprae} antibodies (IgG) against MLSA were measured in organotypic skin culture supernatants from...
10 HC and 8 patients with BT leprosy. Although the antibody levels remained low throughout the culture period in the HC, the antibody level in the BT patients peaked at 24 hours, and by 96 hours, there was a considerable fall in the yield of the antibody (Fig. 3B). The mean antibody levels were significantly more in BT lesions compared with biopsies of HC at 24, 48, 72, and 96 hour time points ($P < .01$).

### 3.4. Kinetics of cytokine production in organotypic culture supernatants

#### 3.4.1. Proinflammatory cytokines IFN-γ, TNF-α, and IL-6

As depicted in Fig. 4A and B, respectively, peak levels of IFN-γ and TNF-α were detected in culture supernatants of BT lesions at 24 hours gradually declining to negligible levels by 120 hours. On the other hand, biopsies from BL/LL lesions showed significantly lower levels of IFN-γ and TNF-α, which were comparable to levels seen in HC. IL-6 secretion (Fig. 4C) in culture supernatants of BT lesions was maximal at 48 hours and dropped sharply at 72 hours of culture. The IL-6 secretion was lower in BL/LL lesions, although the difference was not statistically significant ($P = .18$). In HC, IL-6 secretion increased over time, peaking at 96 hours before dropping, although it still remained higher than in both groups of patients at the end of 120 hours.

#### 3.4.2. IL-4 and IL-10

IL-4 levels were similar in both groups of patients and were lower than the levels in HC. However, the difference was not statistically significant (Fig. 5A). IL-10 was low in culture supernatants of both BT and BL biopsies, and no significant difference in the levels of secretion was observed between the 2 groups of patients (Fig. 5B). However, significantly higher levels of IL-10 were observed in the supernatants from HC at 48 hours of culture, declining gradually at later time points but remaining higher than in the patient groups at 120 hours.

### 4. Discussion

The present study focused on identifying functionally active B-cells in the cellular infiltrate in leprosy lesions by immunohistochemistry and organotypic tissue culture. Histopathological presence of plasma cells within skin lesions of patients with leprosy [21,22] and within biopsies of lepromin reaction lesions [9] has been sporadically reported in the literature. However, the presence and functional activity of these cells in the lesions has not been critically studied. We here demonstrate, using immunohistochemical analysis, the presence of B-cells indicated by markers CD20, CD79, and CD138, in skin from active lesions of patients with leprosy including skin smear negative BL and paucibacillary BT leprosy patients. Among B-cell markers, the CD20 antigen is a 33- to 35-kDa phosphoprotein expressed on B-lymphocytes from the early pre-B to the late B stage, although its expression ceases when they differentiate into plasma cells [23]. Expression of CD79, the B-cell receptor, precedes Ig heavy-chain gene rearrangement and CD20 expression during B-cell ontogeny and disappears later than CD20 in the late (plasma cell) stage of B-cell differentiation [24]. CD138 is used often as a marker of plasma cells in tissues [25]. The positive scores for CD20, CD79, and CD138 were higher in BL/LL lesions as compared with BT patients. Cells with CD79 and CD138 staining were seen in moderate numbers in BL/LL and BT patients in serial sections, indicating presence of mature B-cells and plasma cells, respectively, within the lesions. Immunostainings of skin sections for immunoglobulins (IgA, IgG, and IgM) were not performed because in our preliminary experiments, these stainings, especially in skin sections, resulted in high background, making it difficult to judge the specificity of the stainings. The presence of relatively more B-cells toward the lepromatous pole in this study is consistent with the higher antibody levels demonstrated in this form of leprosy [26]. Double stainings showed T-cells often lying in close juxtaposition to the
B-cells. Furthermore, immunostained serial sections of the biopsies showed most T-cells to be positive for the skin homing CLA (data not shown), whereas a few of the B-cells were also positive for CLA in double stainings. CLA+ T-cells in skin inflammation as reported in literature [27] are compatible with the present finding. However, CLA-positive T-cells and, in particular, CLA positive, skin homing B-cells in leprosy have never been reported earlier.

Moreover, at a functional level, organotypic skin cultures of active BT lesions were shown to release anti-MLSA IgG antibodies into culture supernatant. In contrast to previous studies [11-13], the demonstration of anti-MLSA antibody secretion in in vitro lesional skin culture in the present study was accompanied by the concomitant immunohistochemical demonstration of mature B-cells within tissue sections from the same lesion. Furthermore, the lesional biopsy was subjected to minimal processing before culture, and the experimental setup was so designed that the tissue was in contact with culture medium only at the air-liquid interface. Thus, the antibodies detected in the culture fluid suggest that this was a result of active and ongoing secretion of antibody by in situ B-cells within lesional tissue. In contrast, in the previous studies [11,12], the biopsies were minced and incubated in the medium for 48 hours before detection of antibodies by crossed immunoelectrophoresis, which did not necessarily reflect an ongoing antibody production. Moreover, no data on the status of different B-cell stages within these lesional biopsies were available.

Furthermore, the analysis of the cytokine profiles within culture fluids of the lesional organotypic cultures showed the in situ existence of a proinflammatory microenvironment, which was conducive to maturation of B-cells. Higher levels of cytokines IFN-γ, TNF-α, and IL-6 were observed in BT patients as compared with BL/LL or HC. IL-6 is cytokine produced by multiple cell types including macrophages, B-cells, and T-cells maintaining the granulomatous response by its proinflammatory role and may further be involved in an autocrine or paracrine manner in differentiation of B-cells to antibody-producing plasma cells [22,28]. In this regard, IL-6 levels in HC also increased at 48 hours and remained higher than in patients at 120 hours. IL-6 detection in the HC, although surprising, is compatible with its proinflammatory function and could probably be related to endemicity of various infectious diseases, including leprosy, in India from where the tissues were obtained. In comparison, no IL-6 could be detected in organotypic cultures of normal skin from healthy individuals from nonendemic areas, like the Netherlands (data not shown). Moreover, the high TNF-α levels in this study and reported previously in plasma cells and macrophages could also help in the maintenance of the granulomatous response [22].

With regard to the role of *M. leprae*–specific antibodies in relation to the pathology of BT lesions, the following speculations can be put forward. The locally secreted antibodies may form immune complexes with *M. leprae* antigens or with cross-reactive host molecules [29,30]. These complexes can then be internalized by antigen presenting cells through specific membrane receptors and be presented to CD4+ or CD8+ T-cells through major histocompatibility complex (MHC) class II or I, respectively [31,32], and maintain the BT granuloma. On the other hand, at the lepromatous pole, where high level of *M. leprae* antigens and specific antibodies are detected, the high concentrations of immune complexes may suppress specific T-cell responses to antigen as suggested by Tyagi et al [33]. An interesting observation reported previously suggested that the ratio of antigen to the antibody in the immune complexes may also influence T-cell activation [34]. Immune complexes close to equivalence or in moderate antibody excess provided optimal T-cell activation. In contrast, complexes in extreme antibody excess, although taken up efficiently by antigen presenting cells (macrophages) fail to activate T-cells, probably because antigen processing is disturbed by such antibody excess [34]. However, it should be noted that the implications for leprosy are, at this juncture, purely speculative.

Previous reports have suggested that B-cells might contribute to the development and maintenance of the granuloma [22] in diseases like cutaneous leishmaniasis [35] and other granulomatous conditions such as nonarthritic psoriasis [36]. In this respect, a study by Bosio et al [37], in a B-cell–deficient mouse model of tuberculosis, showed markedly less severe pulmonary granuloma formation, smaller lesions, little cellular infiltrate, and delayed dissemination of bacteria to spleen and liver despite having a bacterial burden comparable to wild-type mice in the lungs. It was suggested that B-cells played an important role in granuloma formation probably by influencing recruitment of other inflammatory cells such as macrophages and T-cells [37]. However, in relation to leprosy, paradoxically, TT leprosy shows a strong granulomatous response [1] despite having few B-cells within lesional biopsies. However, any of the previously mentioned speculations should be interpreted with caution in relation to leprosy until further functional studies on the role of B-cells are carried out. In this regard, it should be emphasized that in our experience, the isolation of antibody-secreting B-cells from skin granulomas was not possible, in contrast to T-cells. For this reason, functional studies on skin B-cells except by organotypic skin culture as described in the article will be a limiting factor.

In summary, this study demonstrated conclusively the presence of different functionally active B-cell stages within lesions of patients with leprosy, including the BT region of the spectrum, which could secrete anti-*M. leprae*–specific antibodies. However, their role in leprosy pathology remains to be elucidated.

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References


