

Infiltrating leukocytes surround early Buruli ulcer lesions, but are unable to reach the mycolactone producing mycobacteria

Marie-Thérèse Ruf^{a,b}, Christina Steffen^c, Miriam Bolz^{a,b,†}, Peter Schmid^{a,b}, and Gerd Pluschke^{a,b}

^aSwiss Tropical and Public Health Institute, Basel, Switzerland; ^bUniversity of Basel, Basel, Switzerland; ^cDepartment of Surgery, Cairns Hospital, Cairns, QLD, Australia

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Buruli ulcer (BU) is a chronic necrotizing skin disease caused by *Mycobacterium ulcerans*, which has been reported worldwide from more than 30 mainly tropical and subtropical countries,^{1,2} with the highest burden in West-Africa.³ In Australia, the majority of BU cases are found in 2 endemic areas, Far North Queensland,^{4,5} and temperate coastal Victoria.^{6–11} The macrolide toxin mycolactone is an essential virulence factor of *M. ulcerans*.¹² It causes apoptosis in mammalian cells¹³ and down-regulates local and systemic immune responses by interfering with the activation of immune cells.^{14–21} BU presents with a wide clinical spectrum that includes pre-ulcerative forms (nodules, plaques, edema) and ulcerative lesions.^{2,22,23} The dermis and epidermis overlying the initially closed *M. ulcerans* infection foci eventually degenerate, when mycolactone mediated destruction of the subcutaneous tissue spreads. This leads to the formation of ulcers with undermined edges and a necrotic slough in the base. So far the pathogenesis of BU has primarily been studied by histopathological analyses of advanced lesions. These have revealed contiguous coagulation necrosis of the deep dermis and subcutaneous fat tissue, with destruction of blood vessels and interstitial edema. Presence of clusters of extracellular acid fast bacilli (AFB) and relative lack of infiltrating leukocytes are regarded as further characteristic histopathological hallmarks of BU lesions.^{1,24,25} African BU patients often seek health care late, which may have several reasons, including cultural beliefs, poor access to the formal health system and late recognition of the often painless BU lesions.²⁶ In contrast, most Australian patients seek treatment early with lesions of the World Health Organization (WHO) defined category 1.^{4,11,27} The current

WHO treatment recommendation is a combination chemotherapy with rifampicin and streptomycin for 8 weeks for all forms of the disease, combined with wound care and prevention of disability.²⁸ However, Australian guidelines to treat *M. ulcerans* infections recommend a different regimen that combines rifampicin with oral clarithromycin or an oral fluoroquinolone as first line therapy.^{27,29–31} Furthermore, surgery is accepted as treatment of BU when antibiotics are declined, not tolerated or the patient's preference is excision and direct wound closure of small lesions.^{4,29} For the present study we therefore could get access to early lesions of BU patients from Northern Queensland, which were excised without prior antibiotic treatment. Here we investigated whether the well described lack of infiltrates is a characteristic of *M. ulcerans* infection from the beginning of the infection.

Our study cohort consisted of 12 patients (Table 1) with IS2404 quantitative real-time PCR confirmed early BU lesions. Patients were recruited between 2000 and 2015 in Far North Queensland; their lesions had persisted for only 3 to 12 weeks (mean 7.5 weeks) before excision. In all cases the surgical procedure consisted of a narrow elliptical excision which encompassed the ulcer plus the indurated surrounding skin and subcutaneous tissue. The excised specimens were bisected through the ulcer; one half was used for PCR, smear microscopy and culture, the other half for histopathology. Excisions were closed primarily with non-absorbable sutures which were removed after 7 to 10 d. The patients were reviewed at 4 weeks, 3 months and 12 months. The median age of the patients was 44.5 y (range 5 to 82 years), which is in line with the observation, that the median age of patient cohorts from Queensland and Victoria (45 and 61 years,

CONTACT Prof. Gerd Pluschke  gerd.pluschke@unibas.ch  Swiss Tropical Institute, Socinstr. 57, 4002 Basel, Switzerland.

[†]Current affiliation: New York University School of Medicine, Department of Medicine, Division of Infectious Diseases and Immunology, New York, NY, USA.

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Table 1. Study cohort.

Patient number	Age (years)	Gender	Body location of the lesion	Existence of lesion (weeks)
1	7	F	Left leg	<12
2	80	M	Left arm	8
3	82	F	Left thigh	12
4	8	F	Right leg	8
5	5	M	Left arm	4
6	62	M	Right calf	8
7	49	M	Left leg	8
8	9	M	Left leg	3
9	71	F	Left upper arm	4
10	39	F	Left knee	12
11	70	M	Left leg	3
12	40	M	Left leg	5

respectively^{11,27}) is much higher than that of patients from West Africa.³²⁻³⁴ Both in Africa and Australia, BU affects primarily the limbs, in particular the lower legs,^{11,32} and also in our cohort 75% (8/12) of the lesions were located on the lower and the rest (25%; 4/12) on the upper extremities. While 3 of the analyzed lesions showed only a first very small opening, where the epidermal layer had focally broken in (Fig. 1 A, D), ulceration had progressed slightly more in the others (Fig. 1 G, J, M). As exemplified in more detail for patient 5 (Fig. 2), in all lesions the characteristic signs of BU were found, including epidermal hyperplasia (Fig. 2 N), large necrotic areas (Fig. 2 Q) containing fat cell ghosts (Fig. 2 P) and extracellular clumps of AFB (Fig. 2 D, E). A strong TUNEL staining clearly marked the entire necrotic area of all lesions (Fig. 3 A, B1 (red star), B3). While the TUNEL assay has been primarily developed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis, the diffuse staining of the necrotic core of the BU lesions is most likely caused by residual DNA fragments still present in the destroyed tissue.

In all lesions, a belt of infiltrating leukocytes was found, which separated the highly confined necrotic core from the intact tissue which surrounded the lesions (Fig. 1B, E, H, K, N; Fig. 2A). In the infiltration belt, TUNEL staining identified individual apoptotic cells dispersed in the infiltrate (Fig. 3 B1 (green star), B2). In contrast, inside the necrotic area staining was diffuse and not cell-associated (Fig. 3 B1 (red star), B3). The analysis of serial sections from the lesions revealed that CD20 positive B-cells were present in small and large clusters throughout the infiltrated area (Fig. 4 A1, B1). CD3 positive T-cells, neutrophils and CD68 positive macrophages appeared in a more dispersed manner all over the infiltration belts (Fig. 4 A2-A4, B2-B4). The distribution of different cell types was not homogeneous within the belt: while CD3 T-cells (Fig. 4 B2) were primarily located close to the healthy tissue, neutrophils (Fig. 4 B3) were mainly present close to the necrotic core.³⁵ Macrophages

(Fig. 4 B4) were distributed all over the infiltrated area with preponderance to the inner layers toward the lesion core. Besides intact neutrophils in the infiltration belt, neutrophilic debris was observed inside the lesion core, which appears to be the aftermath of a massive early neutrophil infiltration, which is also observed in an experimental *M. ulcerans* pig infection model.^{36,37} Furthermore, in areas with secondary infection (Fig. 2 B, C), accumulations of neutrophils were found (Fig. 4 A3).

Early immune responses may in many cases help to contain *M. ulcerans* infections and prevent clinical BU disease, as indicated by the development of a serological response against *M. ulcerans* in many healthy individuals living in African BU endemic areas.³⁸⁻⁴⁰ We assume that for the development of a chronic infection after inoculation with *M. ulcerans* it may be critical that a large enough bacterial cluster can develop that generates a protective cloud of mycolactone. After intracellular multiplication and killing of the host cell, globi-like bacterial accumulations may represent a starting point for the development of large extracellular clusters of *M. ulcerans*.⁴¹⁻⁴³ In early but nonetheless established lesions, as analyzed here, the infiltrating immune cells can no longer reach the extracellular mycobacteria in the necrotic center, despite massive extravasation of leukocytes. On the other hand the layer of infiltrating cells around the lesion core may – together with other factors, such as the formation of an abundant extracellular matrix⁴⁴ – hinder spread of the mycobacteria into the lymph and bloodstream. This may contribute to the fact that most BU patients present with a single lesion.²

The mode of transmission of *M. ulcerans* is currently not clear.⁴⁵ Both insect bites and direct inoculation of bacteria into the skin from an environmental reservoir after skin trauma have been implicated. After inoculation, an early intra-macrophage growth phase may play a role in disease development.^{15,46} In the early lesions analyzed here, AFB were exclusively found in the necrotic core of the lesions (red stars in Fig. 1B, E, H, K, N) and appeared mainly as extracellular cluster (Fig. 1I,

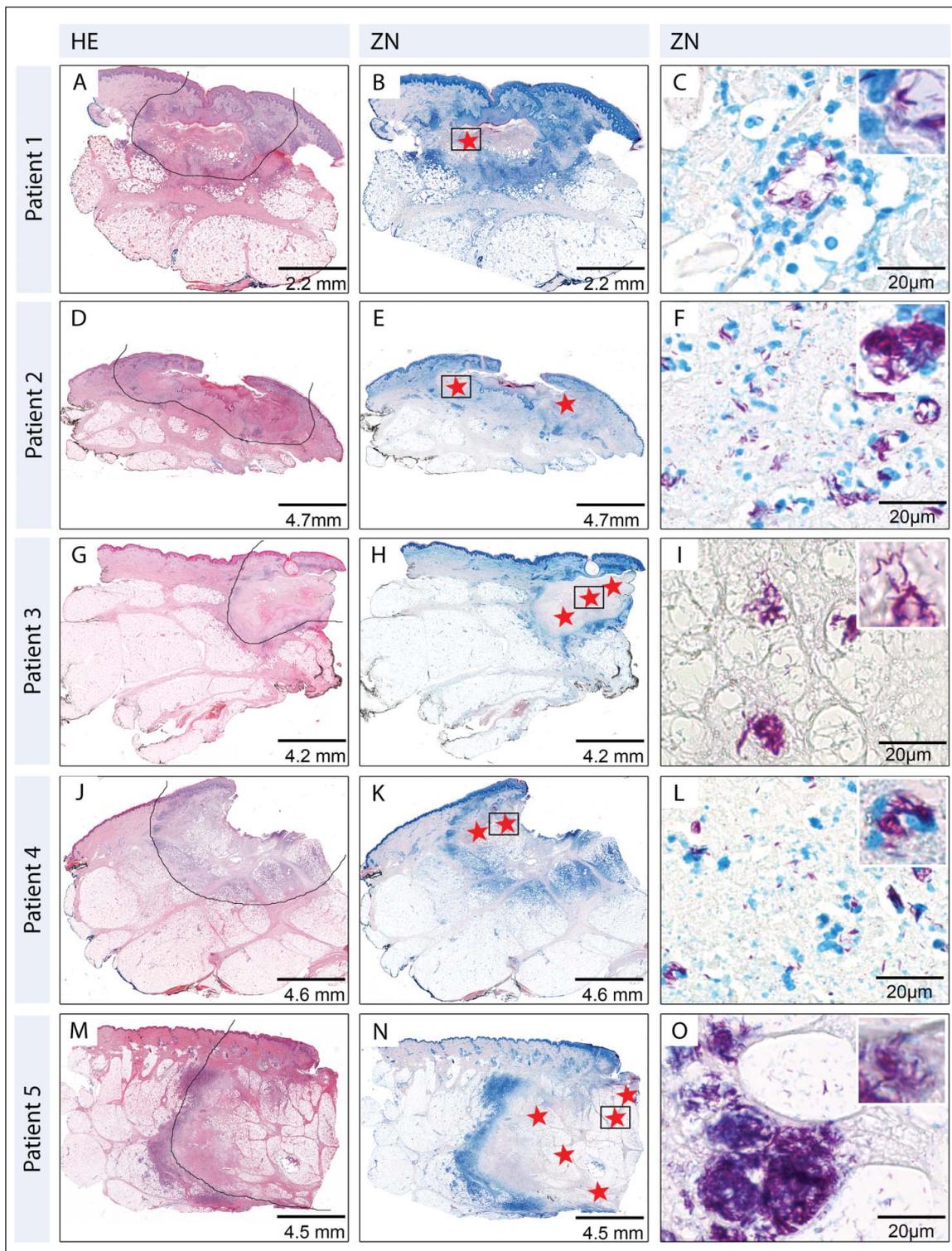


Figure 1. Histopathological overview over 5 of the early ulcerative lesions. Sections were either stained with Haematoxylin-Eosin (HE; A, D, G, J, M) or Ziehl-Neelsen/Methyleneblue (ZN-M; B, C, E, F, H, I, K, L, N, O) according to the WHO standard protocol.¹ Pictures were either taken with a Leica DFC 420C camera or with an Aperio ScanScope XT. The border of the tissue area affected by the disease is indicated with a black line in the HE staining (A, D, G, J, M). For patients one and 2 cross sections through the entire lesions are shown (A, D), lesions of patients 3 to 5 (G, J, M) were too large to be entirely processed and therefore only half of the lesion is shown with the necrotic lesion core in the upper right corner. ZN staining (B, E, H, K, N) revealed that AFB were exclusively found in the necrotic areas (red stars). Predominantly clusters of extracellular bacteria were present (C, I, O), however close to the infiltration belt extra- as well as intracellular bacteria (F, L) were observed. Similar findings were made for all 12 lesions analyzed.

O). However, when present close to the infiltration belt, some mycobacteria appeared to be intracellularly located (Fig. 1C, F, L). Like in more advanced lesions,⁴⁷ AFB were typically found in the subcutaneous tissue and only rarely in the dermis. Preferential location of *M. ulcerans*

in the deeper subcutis may either reflect the inoculation route or a tropism related to nutritional needs only provided by the destroyed fat tissue.⁴⁷

Taken together histopathological data from our Australian patients clearly show that infection by *M.*

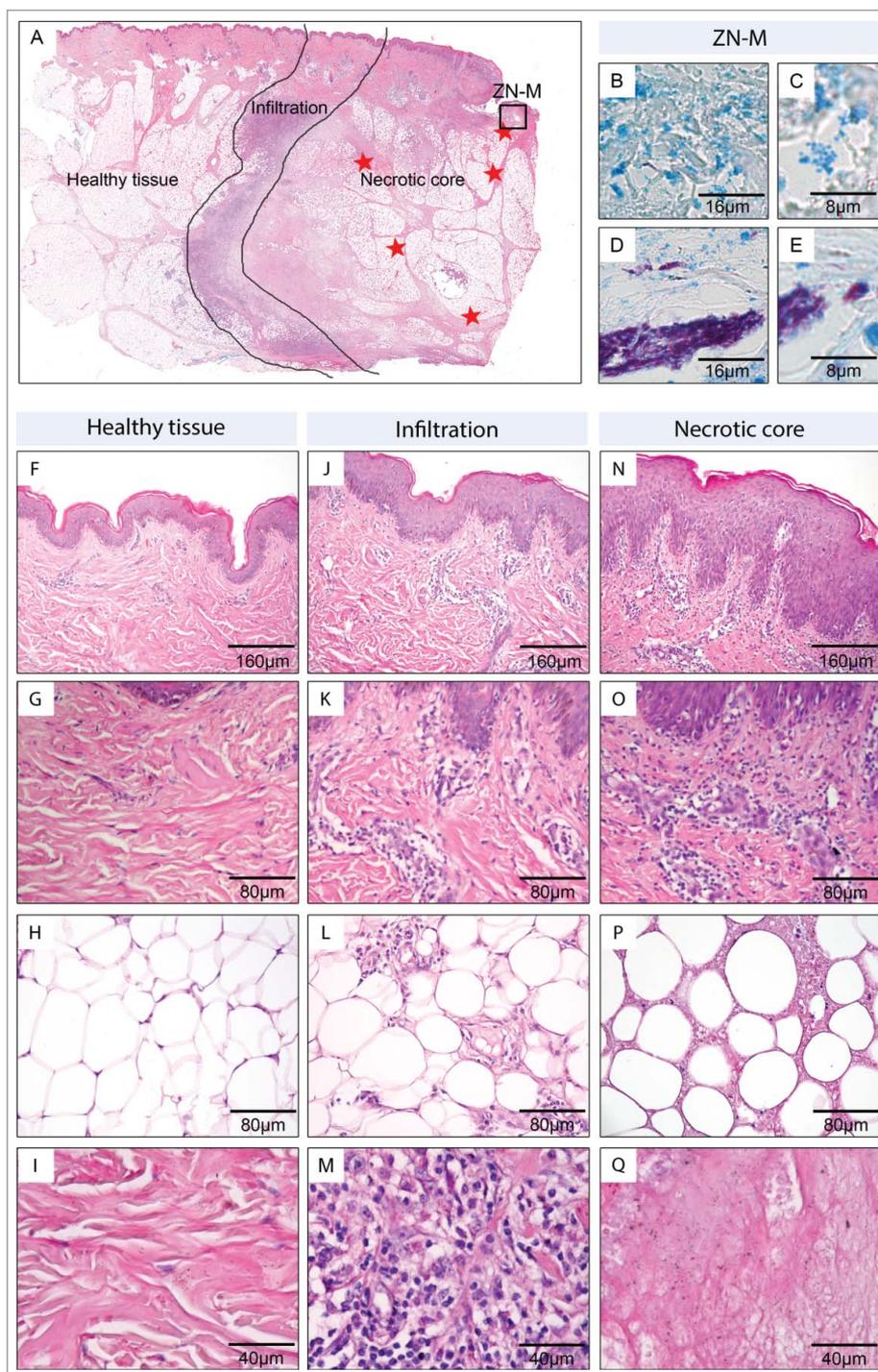


Figure 2. (For figure legend, see page 1922.)

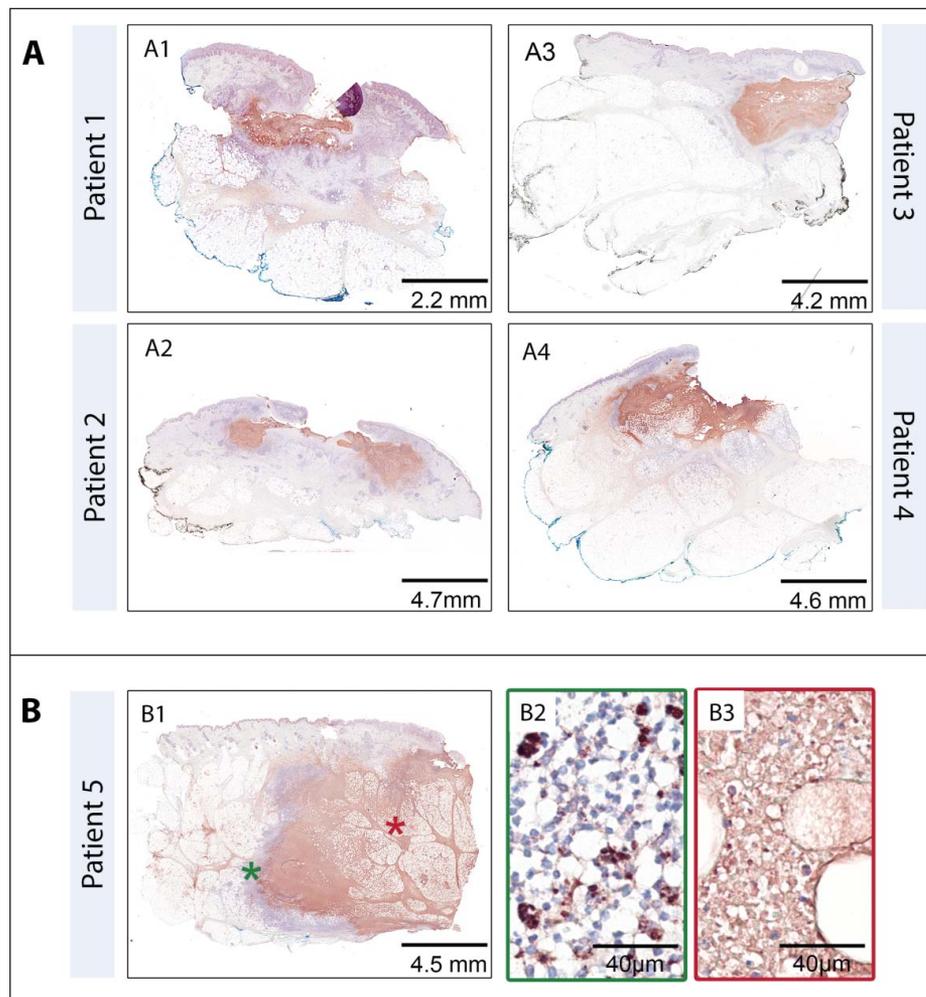


Figure 3. TUNEL staining. The TUNEL protocol allows the staining of low molecular weight DNA fragments typically occurring during apoptosis (brown staining). Sections of patients one to five were stained with the “*In Situ* Cell Death Detection Kit, POD” (Cat. No. 11684817910) from Roche according to the manufacturer’s protocol (protocol used was for tissue sections with high unspecific background). Counter stain was done with Haematoxylin. Pictures were either taken with a Leica DFC 420C camera or with an Aperio ScanScope XT. Overview over tissue sections reveals a diffuse staining of the necrotic lesion core (A1-A4, B1) and no staining of the healthy tissue area. Increased magnification (B2, B3) showed a distinct staining of single cells in the belt of infiltrating cells (B1 green star, B2) representing cells currently undergoing apoptosis and a more dispersed staining inside the necrotic core (B1 red star, B3) representing leftover DNA fragments from the initial infiltration. Similar findings were made for all 12 lesions analyzed.

Figure 2. (see previous page) In depth analysis of the skin tissue in the necrotic core, the infiltrated area and the healthy tissue area (patient 5). Sections were either stained with Haematoxylin-Eosin (HE; A, F-Q) or Ziehl-Neelsen/Methyleneblue (ZN-M; B-E) according to the WHO standard protocol.¹ Pictures were either taken with a Leica DFC 420C camera or with an Aperio ScanScope XT. A: Overview of the histopathological section from patient 5, depicting the 3 regions (healthy tissue, infiltration, necrotic core) which were found in all early ulcerative lesions. Healthy tissue (F-I) presented with a normal epidermal layer (F), no sub-epidermal infiltration (G), healthy fat cells (H) and intact collagen fibers (I). The infiltrated area (J-M), separating the healthy tissue from the necrotic core, reflected a transition state with a slight thickening of the epidermal layer (J), some sub-epidermal infiltration (K), fat cells which started to round up and shrink (L) and the accumulation of large numbers of infiltrating cells (M). The necrotic core (N-O) presented with strongly elongated rete ridges and an epidermal layer which was more than 3 times thicker than the healthy epidermis (N), a strong sub-epidermal infiltration which was largest above the necrotic core (O), fat cell ghosts which displayed signs of cell death (rounding up, shrinkage, absence of nuclei) (P) and necrotic connective tissue without collagen fibers (Q). In the necrotic core focally clustered AFB (D, E, red stars in A) as well as a secondary infection (B, C; at the open ulcer surface (black box A)) could be observed.

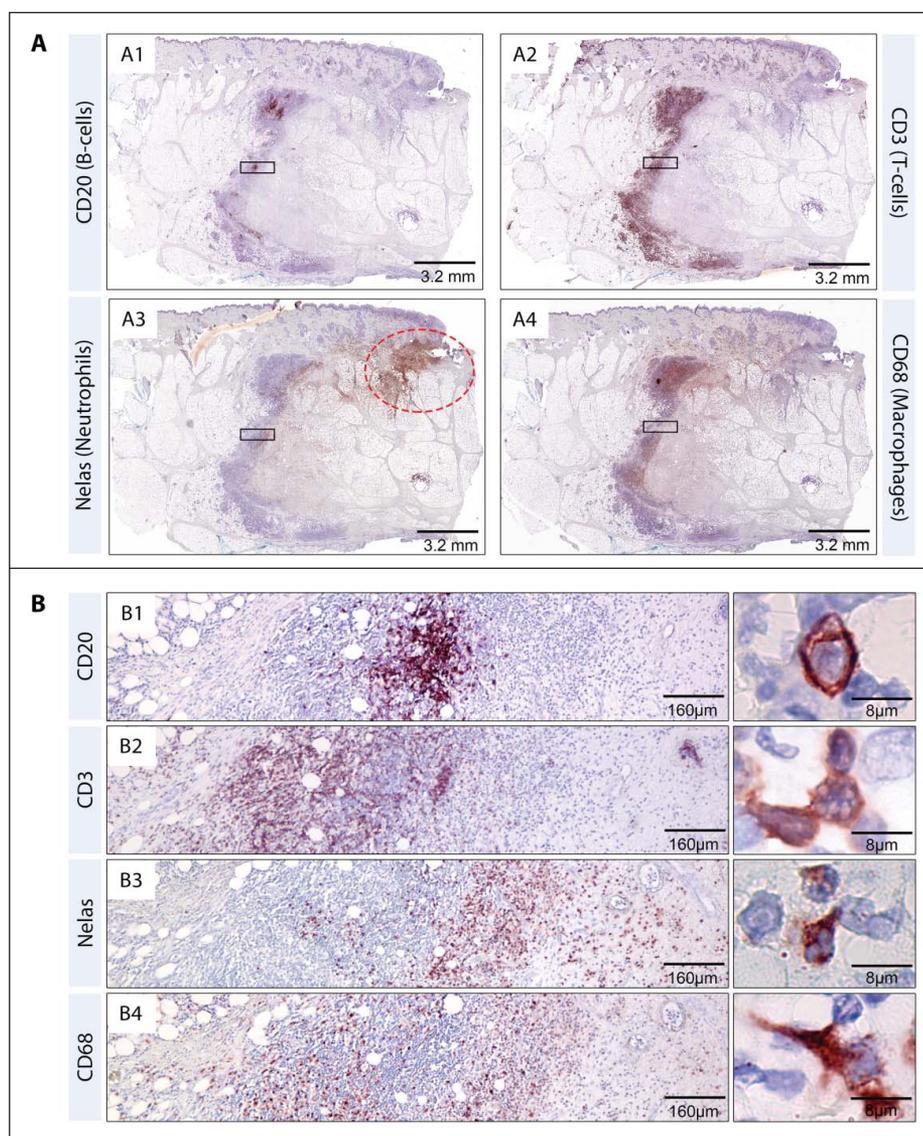


Figure 4. Composition of the immune infiltrate which surrounds the necrotic core. Serial sections from the lesion of patient 5 were stained by immunohistochemistry. The following antibodies were used according to the manufacturer's protocol: Elastase (polymorphonuclear neutrophils, NP57, Dako, M0752), CD3 (T lymphocytes, Dako, A0452), CD68 (macrophages/monocytes, KP1, Dako, M0814), and CD20 (B lymphocytes, 7D1, Novocastra, NCL-CD20-7D1). Pictures were either taken with a Leica DFC 420C camera or with an Aperio ScanScope XT. CD20 positive B-cells (A1, B1) were present in clusters while CD3 positive T-cells (A2, B2), N-elastase positive Neutrophils (A3, B3) and CD68 positive macrophages (A4, B4) were present throughout the infiltration in a layered manner. Accumulations of neutrophils were additionally present close to the secondary infection (A3, red dotted circle).

ulcerans is accompanied by massive leukocyte infiltration. The apparent lack of infiltration described as a hallmark of BU is obviously restricted to the lesion core, where mycolactone causes death of all resident and infiltrating cells by apoptosis. If diagnostic punch biopsies are not taken from the center of the lesion, but more from its periphery, massive infiltration may thus be found. Together with an apparent lack of AFBs, which are not evenly spread and often missed in small tissue biopsies of BU patients⁴⁷ this may mislead histopathological diagnosis. However these results should be reconfirmed by analyzing also

African early BU lesions (plaque, nodules). *M. ulcerans* strains from Australia primarily produce mycolactone C while a mixture of mycolactone C and the more potent mycolactone A/B⁴⁸ is found in lipid extracts from African strains.⁴⁹ Despite these differences, the pathogenesis of BU seems to be comparable in patients from both continents and also in Australian patients large ulcers develop when treatment is delayed. In line with this, the typical histopathological features found in the lesions of our Australian patients were indistinguishable from those observed in samples from African patients.

When early immune responses do not eliminate a *M. ulcerans* inoculum, a chronic infection may develop and the extracellular clusters of the mycobacteria can no longer be reached by the infiltrating cells. However, the formation of the infiltration belt around the necrotic core of the lesion described here, most likely helps to prevent systemic spread of the infection; but it cannot prevent local multiplication of the bacteria followed by progressive ulceration.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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