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Human Beta-Defensin 3 Is Up-Regulated in Cutaneous Leprosy Type 1 Reactions

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Abstract

**Background:** Leprosy, a chronic granulomatous disease affecting the skin and nerves, is caused by *Mycobacterium leprae* (*M. lepra*). The type of leprosy developed depends upon the host immune response. Type 1 reactions (T1Rs), that complicate borderline and lepromatous leprosy, are due to an increase in cell-mediated immunity and manifest as nerve damage and skin inflammation. Owing to the increase in inflammation in the skin of patients with T1Rs, we sought to investigate the activation of the innate immune system during reactionary events. Specifically, we investigated the expression levels of human beta-defensins (hBDs) 2 and 3 in the skin of patients with T1Rs, in keratinocytes, and in macrophages stimulated with *M. leprae* and corticosteroids.

**Results:** Skin biopsies from twenty-three patients with Type 1 reactions were found to have higher transcript levels of hBD3 as compared to fifteen leprosy patients without Type 1 reactions, as measured by qPCR. Moreover, we observed that keratinocytes but not macrophages up-regulated hBD2 and hBD3 in response to *M. leprae* stimulation *in vitro*. Corticosteroid treatment of patients with T1Rs caused a suppression of hBD2 and hBD3 in skin biopsies, as measured by qPCR. *In vitro*, corticosteroids suppressed *M. leprae*-dependent induction of hBD2 and hBD3 in keratinocytes.

**Conclusions:** This study demonstrates that hBD3 is induced in leprosy Type 1 Reactions and suppressed by corticosteroids. Furthermore, our findings demonstrate that keratinocytes are responsive to *M. leprae* and lend support for additional studies on keratinocyte innate immunity in leprosy and T1Rs.

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Introduction

Leprosy, caused by the pathogen *Mycobacterium leprae* (*M. leprae*), is a chronic granulomatous infection that causes skin lesions and potentially disabling neuropathy. Whilst the widespread use of multi-drug therapy (MDT) has reduced the disease burden globally, 213,036 new cases were reported in 2009 and leprosy continues to be an important health concern especially in areas such as India, Brazil, and Africa [1].

The clinical manifestations and classification of leprosy correlate with the type of immune response generated by the host. Tuberculoid leprosy (TT), on one pole of the spectrum, results from patients having an intense cell-mediated immune response and is characterized by an abundance of Th1 cytokines. On the opposite end of the spectrum are the patients with lepromatous leprosy (LL), characterized by a predominant humoral response and lack of *M. leprae*-specific cell-mediated immunity [2]. Patients with the borderline forms of leprosy fall between the two poles (TT and LL), are immunologically unstable, and are more prone to complicating reactions.

Type 1 reactions (T1Rs) correlated with an increase in Th1-mediated immunity while Type 2 reactions (also known as erythema nodosum leprosum, ENL) are associated with Th2 humoral complications. Reactions can occur in up to 30–50% of leprosy patients as single or recurrent episodes anytime before, during or years after effective multi-drug therapy (MDT). Due to the lipid content of the cell wall, *M. leprae* infection can leave behind persistent antigens within affected skin and nerve tissues rendering patients vulnerable to unpredictable leprosy reactions years after technical cure. Leprosy reactions, therefore, are a major factor of permanent neuropathy and disability development for patients. In response to the upgrades in cell-mediated immunity, T1Rs are often treated using oral corticosteroids, usually prednisolone. One of the effects of corticosteroids is the suppression of skin inflammation (edema and erythema) associated with cutaneous T1Rs.

Previous studies have suggested a prominent role for toll-like receptor (TLR) 2 in susceptibility to infection by *M. leprae* and *Mycobacterium tuberculosis* (Mt). TLR2 is a receptor that recognizes
Human Beta-Defensins in Leprosy Type 1 Reactions

Author Summary

Leprosy, caused by Mycobacterium leprae (M. leprae), is a chronic infection leading to potentially debilitating nerve damage. Although the infection is curable with multi-drug therapy, many patients continue to suffer from episodes of inflammation, called Type 1 reactions. These reactions may lead to nerve damage and deformation. Patients with Type 1 reactions are treated with corticosteroids to reduce the inflammation. Elucidating the immune factors contributing to leprosy Type 1 reactions will further clarify why many leprosy patients develop nerve and skin inflammation. We conducted a study to understand whether the antimicrobial peptides, human beta-defensins (hBD) 2 and 3, are increased in the skin of leprosy patients with Type 1 reactions. We also investigated keratinocyte expression of hBD2 and hBD3 in response to M. leprae. In this study, we found that hBD2 was significantly increased in the skin of leprosy patients with Type 1 reactions. Moreover, we demonstrated that corticosteroid treatment reduced the expression of hBD3 in patients with Type 1 reactions. We also found that keratinocytes incubated with M. leprae upregulated hBD2 and hBD3 in vitro; corticosteroids were found to suppress this response. These studies suggest a role for the innate immune system in leprosy Type 1 reactions.

Materials and Methods

Ethics statement

This study was approved by the Nepal Health Research Council and the Ethics Committee of the London School of Hygiene and Tropical Medicine (Number 4022). All subjects provided informed written consent.

Skin biopsies

Full-thickness skin biopsies were taken from leprosy patients with and without Type 1 Reactions at Anandaban Hospital in Kathmandu, Nepal [26]. The case definitions and Ridley-Joplin classifications for the patients with leprosy and Type 1 reactions in this study have been previously published [26]. In a double blind parallel-group randomized controlled trial (RCT), patients with Type 1 reactions or nerve function impairment were randomized to receive high dose intravenous methylprednisolone (MP) followed by oral prednisolone or intravenous normal saline followed by oral prednisolone. Control subjects were untreated newly diagnosed leprosy patients who presented to Anandaban Hospital during the period of recruitment and follow-up of the methylprednisolone study. Biopsies from control subjects and T1Rs subjects were stored in RNA Later (Sigma) until processing. This study utilizes biopsies taken at day 0 (before corticosteroid treatment) and day 113 (at the end of corticosteroid treatment). We analyzed skin biopsies from 28 patients with T1Rs and 15 patients without T1Rs that served as controls. Of these 28 patients with T1Rs, 23 patients received corticosteroid treatment (oral prednisolone ± intravenous methylprednisolone). Skin biopsies of
these 23 patients (with T1Rs and receiving corticosteroid treatment) were analyzed on day 0 and day 113, before and after corticosteroid treatment, respectively. Cellular infiltration, the relative abundance of infiltrated cells in the dermis (whose nuclei stain with hematoxylin), was performed as previously described [27].

Cell culture
A human keratinocyte cell line, HaCaT cells, kindly supplied by Dr. Edel O’Toole (St. Barts and The London), were maintained in DMEM with 10% (v/v) heat-inactivated fetal calf serum (FCS, Fisher scientific), 100 µg/ml (100 IU) penicillin/streptomycin, and 2 mM L-glutamine on T75 ml flasks at 37°C at 5% CO₂. At 80% confluency, cells were lifted using Accutase (Sigma) and resedded at a 1:10 ratio. For stimulation assays, HaCaT cells were seeded into 12-well plates and at 50% confluency, were stimulated for 24 hours with whole cell sonicated M. leprae (supplied by Colorado State University) at 1 µg/ml, 10 µg/ml, 100 µg/ml or prednisolone (Sigma) at 1 nM, 10 nM, or 100 nM or 1 µM Pam3CSK4 (Toll-like receptor 2/1 ligand). Assays were performed 3 times in triplicate.

To obtain primary human macrophages, peripheral blood mononuclear cells (PBMCs) were first isolated from 50 ml whole blood using a Ficoll gradient. PBMCs were cultured in RPMI with 10% FCS on a T75 flask for 24 hours at 37°C to allow monocyte adherence. Nonadherent cells were gently removed with warm media, adherent monocytes were washed with PBS and cultured for 48 hours. Cells were lifted with Accutase, washed, and re-cultured in 12 well plates for 48 hours at 10⁶ cells/ml. Stimulation assays were performed as above. Assays were performed 2 times in triplicate.

RNA isolation
After HaCaT and macrophage stimulation, RNA was isolated using TRIzol Reagent (Invitrogen). According to manufacturer’s instructions, 0.5 µg of total RNA was used for cDNA synthesis by the iSCRIPT cDNA synthesis kit (Bio-Rad). For the skin biopsies, RNA was isolated by first disrupting the skin using disposable pestles and lysis buffer from the RNeasy Fibrous Tissue Mini Kit. The skin was subjected to a Qiashredder (Qiagen) and RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen) per manufacturer’s instructions. RNA concentration was ascertained using Nanodrop (ND-1000). RNA was converted to cDNA using the iScript kit (Invitrogen) per manufacturer’s instructions.

Real-time quantitative RT-PCR
Real-time quantitative RT-PCR was performed on an ABI 7900HT Fast Real-Time PCR (ABI) using Power SybrGreen (ABI) and primers at a final concentration of 0.2 µM. The primer sequences are as follows: 36B4 F: 5’-TCGAAACCTTGCCTGATGAC, 36B4 R: 5’-CCACGGCTGCTGAAATGCT, DEFB4 (hBD2) F: 5’-GGTGTGGTTTGTGGTAGGC, DEFB4 (hBD2) R: 5’-AGGGAAAAGACTGGATGACA, DEFB103 (hBD3) F: 5’-GGCTGCTTTCAAAAGGAGGA, DEFB103 (hBD3) R: 5’-TCTTTCCGGACAGTATTTG, TLR2 F: 5’-CATTCCCTCAGGGCCTACAG, TLR2 R: 5’-TTGTTGGA-CAGGTCAAGGCTT, TLR1 F: 5’-GAGGGAATGCTGCGTTCGAC, TLR1 R: 5’-CCTGGTACCCCTATTAGGT. The ΔΔCt method was used for quantification of gene expression. The target genes were normalized to the endogenous reference gene that encodes an acidic ribosomal phosphoprotein P0 (RPLP0) also known as 36B4. Target genes are reported as the fold difference relative to the reference gene. All of the stimulation experiments were performed in triplicate and repeated at least two times. All of the experiments using skin biopsies were analyzed by real-time PCR in triplicate.

Immunoﬂorescence
HaCaT cells were seeded into chamber slides and stimulated at 50% confluence with 1 µM Pam3CSK4, 100 µg/ml M. leprae whole cell sonicate, or 100 µg/ml prednisolone as indicated. After 24 hours, the supernatant was removed and cells were fixed with acetone for 15 minutes at room temperature. The slides were subsequently washed 3 times with 1× phosphate-buffered saline (PBS), blocked for 1 hour at room temperature with 3% bovine serum albumin in 1× PBS, and stained with primary antibodies rabbit anti-hBD3 (Abcam) or rabbit IgG in 3% bovine serum albumin/1× PBS overnight at 4°C. After washing the cells 3 times with PBS, the cells were stained with secondary antibodies goat anti-rabbit IgG (Sigma), anti-rabbit IgG in 3% bovine serum albumin in 1× PBS for 1 hour at room temperature. Slides were washed three times in 1× PBS and were mounted in ProLong Anti-Fade reagent (Molecular Probes).

Statistical methods
All statistical analyses were performed using GraphPad Prism 4.0. An unpaired t-test was used when appropriate. Values of p<0.05 were considered significant.

Results
Human beta-defensin 3 is up-regulated in cutaneous leprosy type 1 reactions
To determine the levels of hBD2 and hBD3 expression in Type 1 reactions, the relative stable transcript levels were assessed in skin biopsies of control leprosy patients and leprosy patients with Type 1 reactions. We found a trend towards increased hBD2 expression in the skin of patients with Type 1 reactions by 16.6 fold-change, though it was not statistically significant (p = 0.087) (Figure 1A). Moreover, we found that hBD3 was significantly up-regulated in the skin of patients with Type 1 reactions by 5.96 fold-change (p<0.05) (Figure 1B).

Since T1Rs are clinically characterized by edema and erythema, we investigated whether the increased hBD expression was due to an increase in cellular infiltration. We found that the cellularity score of the controls and T1Rs are similar (Figure 1C). These data demonstrate that the increase in hBD2 and hBD3 expression is due to activation of the innate immune response rather than due to an increase in the number of hBD-expressing cells in T1Rs.

Mycobacterium leprae induces human beta-defensins in keratinocytes but not macrophages in vitro
Since the skin is comprised of multiple cell types that play a role in cutaneous innate immunity, we sought to determine in which cell type the hBDs are being up-regulated. Keratinocytes, the primary cell in the epidermis, were first investigated for their ability to up-regulate the hBDs in response to M. leprae. We found that HaCaT cells, immortalized keratinocytes, increased their expression of hBD2 and hBD3 in a dose-dependent manner in response to M. leprae whole cell sonicate (Figure 2A and 2B). To determine if macrophages induce a similar antimicrobial program HaCaT cells, primary macrophages were stimulated with M. leprae whole cell sonicate at the indicated concentrations for 24 hours. We found that HaCaT cells but not macrophages up-regulate hBD2 and hBD3 in response to M. leprae (Figure 2C and 2D). We also attempted to stain skin biopsies from patients with...
Type 1 reactions and from control leprosy patients with two different anti-hBD3 antibodies. Unfortunately, both antibodies exhibited non-specific staining and could not be used to assess the temporal or relative quantity of hBD3 in the biopsies (data not shown).

Since hBD production likely results from TLR2/1 activation by M. leprae, we investigated whether TLR2 and TLR1 are induced in M. leprae or TLR2/1 agonist (Pam3CSK4)-treated keratinocytes. We found that M. leprae, as well as synthetic triacylated-lipopetide Pam3CSK4, induce TLR2 but not TLR1 stable transcript levels (Figure 3A and 3B). Taken together, these data demonstrate that M. leprae activates keratinocytes through up-regulating the human beta-defensins and TLR2 in vitro.

Corticosteroids suppress human beta-defensin expression in the skin of patients with Type 1 reactions

To determine if oral corticosteroid treatment suppresses the innate immune response in leprosy patients with Type 1 reactions, the expression of hBD2 and hBD3 was evaluated from the skin of patients at day 0 and at day 113 of corticosteroid treatment. For hBD2, we observe a mean relative transcript suppression of 78.5% with 13/23 (56.5%) patients showing a decrease in hBD2 expression (Figure 4A). For hBD3, we observe a mean relative transcript suppression of 61.3% with 16/23 (69.6%) patients showing a decrease in hBD3 expression (Figure 4B). In addition, 14 of the 23 patients with Type 1 reactions received an initial dose of intravenous methylprednisolone before a course of oral prednisolone. We found that methylprednisolone caused no additional suppressive effect on
hBD2 and hBD3 and in fact, the two patient groups were nearly identical in regards to their cutaneous suppression of hBD2 and hBD3 (data not shown). Thus, all patients receiving corticosteroids (prednisolone and methylprednisolone) were grouped together for collective analyses (Figures 4A and 4B). These data illustrate that oral corticosteroids suppress hBD2 and hBD3 in the skin of patients with Type 1 reactions.

**Discussion**

In this study, we have demonstrated that during Type 1 reactions, hBD3 is significantly up-regulated in the skin. This up-regulation appears to be due to an increase in gene expression rather than an increase in cellular infiltration (Figure 1). In addition, we found that keratinocytes but not macrophages up-regulate hBD2 and hBD3 in response to *M. leprae* in vitro. This result was unanticipated since macrophages are known to play a major role in leprosy pathogenesis. Moreover, we find it interesting that keratinocytes were robustly responsive to *M. leprae*.

For these *in vitro* experiments, nonviable, sonicated *M. leprae* was employed. While the possibility cannot be ruled out that live infection may generate immune variation, leprosy reactions appear to occur or persist regardless of *M. leprae* viability [28]. In addition, most patients receiving corticosteroids for leprosy reactions are concurrently receiving MDT or have completed MDT, rendering *M. leprae* essentially nonviable within the study time period [29]. Yet, in the future, viable *M. leprae* could be useful to assess the host response during an active infection.

Few previous studies have suggested that the epidermis plays a role in leprosy or reactions, in spite of the cutaneous manifestation. This may be attributed to the fact that keratinocytes are often thought of as part of the barrier rather than as an immune organ. In fact, keratinocytes play a significant role in immune homeostasis and innate immunity. Our data demonstrating that HaCaT’s up-regulated TLR2 in response to *M. leprae* (C) suggests that the cells are preparing to respond to pathogens. The downstream effect of increased TLR2 expression would likely lead to an enhanced ability to sense lipo-polypeptides and subsequent up-regulation of antimicrobial peptides.

There have been a number of important studies on the cytokine milieu in leprosy [2,30]. It has also been demonstrated that proinflammatory cytokines induce the expression of antimicrobial peptides in gingival keratinocytes [31]. It is possible that the cytokine environment is causing an induction of hBDs, rather than direct mycobacterial interaction with keratinocytes. Yet, mycobacterial lipids are known to directly induce TLR-mediated antimicrobial responses. For leprosy, electron microscopy studies demonstrating *M. leprae* inhabitance in keratinocytes further suggest a direct interaction. Finally, recent studies on cutaneous microbiota demonstrate that an intricate relationship exists between bacterium and keratinocytes [32,33,34]. In the future, it would be interesting to elucidate the specific cellular and molecular interactions between keratinocytes and *M. leprae*.

The effects of steroids on the expression of hBDs were not surprising, yet we included these studies to correlate the hBD expression with T1Rs. Corticosteroids that bind the glucocorticoid receptor have been shown to suppress the expression of proinflammatory cytokines through NFkB [35]. Toll-like receptor 2 initially signals through MyD88 and IRAK, subsequently leading to NFkB activation and production of hBDs [36]. Thus, TLR2-mediated suppression of hBDs by steroids is expected but also confirms that steroids directly affect the dermis and epidermis. It would be interesting to determine the status of keratinocytes in the skin of patients with T1Rs in the presence and the absence of steroids. These studies would further clarify the relative importance of keratinocytes in T1Rs.

Based on our data, we hypothesize that the epidermis plays a more substantial role than previously thought. Many studies have demonstrated that keratinocytes can kill bacteria, viruses, fungi, and parasites through the production of antimicrobial peptides. We hypothesize that the up-regulation of hBDs in keratinocytes leads to reduction of *M. leprae* during Type 1 reactions for several
reasons. First, since the hBDs are directly antimicrobial, they may directly kill the *M. leprae* living in the keratinocytes or may be secreted and kill the bacteria in the upper papillary dermis near the basement membrane. Unfortunately, as we are currently unable to grow *M. leprae in vitro*, we are consequently not able to conclusively test the antimicrobial role of the hBDs without a mouse footpad or an armadillo model of infection. Another means by which the hBDs may lead to the reduction of *M. leprae*, which occurs during Type 1 reactions, is due to their pro-inflammatory potential. If the basal keratinocytes release the hBDs into the dermis, they may activate dermal macrophages, subsequently leading to cytokine production and an antimicrobial response. An increase in Th1 cytokines will activate macrophages, leading to a further reduction of *M. leprae*. It would be interesting to determine whether defenses promote the differentiation of macrophages in Type 1 reactions.

### References