NATURAL KILLER CELL TRANSCRIPTOME IN PATIENTS TREATED WITH MULTI-DRUG-THERAPY AND HOST GENETIC FACTORS INFLUENCING LEPROSY OUTCOME

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List of Publications

This thesis is based on the following publications and unpublished manuscripts.

(** corresponding author, # shared first author*)

I. **Marcinek P**, Jha AN, Shinde V, Sundaramoorthy A, Rajkumar R, Suryadevara NC, Neela SK, van Tong H, Balachander V, Valluri VL, Thangaraj K, Velavan TP* (**2013**). LRRK2 and RIPK2 variants in the NOD 2-mediated signaling pathway are associated with susceptibility to Mycobacterium leprae in Indian populations. PLoS One. 2013 Aug 28;8(8):e73103.

II. Shinde V[#], **Marcinek P**[#], Rani DS, Sunder SR, Arun S, Jain S, Nath I, Thangaraj K, Velavan TP, Valluri VL* (**2013**). Genetic evidence of TAP1 gene variant as a susceptibility factor in Indian leprosy patients. Hum Immunol. 2013 Jun;74(6):803-7.

III. **Marcinek P**, de Carvalho EG, Velavan TP*. Interaction of *Mycobacterium leprae* with human monocytes increases L-Selectin expression and influences phagocytosis. (*Unpublished manuscript*)

IV. **Marcinek P**, Engleitner T, Valluri VL, Velavan TP*. Transcriptome profile of natural killer cells in patients treated with multi-drug-therapy. (*Unpublished manuscript*)

Summary

Leprosy is a bacterial disease of the skin and the nerves, caused by *Mycobacterium leprae*. Ever since the introduction of a multi-drug therapy in the 1970's, worldwide incidence has been decreasing and the disease has been eradicated in many countries. However, in recent years occurrence of new cases remains stable at around 200,000 per year. Most new cases are reported in developing countries, and India accounts for more than half of new cases registered every year. In my thesis, I investigated how innate immunity plays an important role in regulating the disease outcome during multi-drug-therapy. This is addressed by examining the transcriptome profile of specific cells of the innate immunity, namely the Natural Killer (NK) cells. Secondly, I investigated innate immunity-associated genes of the human host that are believed to play a significant role in regulating the disease outcome. The thesis is structured into four different chapters.

In **chapter I** of my thesis, published as an original study, I evaluated four specific functional variants in three different genes that are part of the NOD-2 signaling pathway: *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G), and *RIPK2* (rs40457A/G and rs42490G/A). I utilized 211 clinically classified Indian leprosy patients with 230 ethnically matched controls. The genetic variants were chosen for their role in innate immunity and because they had been found to be associated to leprosy in an earlier study carried out in a Han Chinese population. The *LRRK2* locus proved to be associated with leprosy outcome. The *LRRK2* rs1873613A minor allele and respective rs1873613AA genotype were significantly associated with an increased risk whereas the *LRRK2* rs1873613G major allele and rs1873613GG genotypes conferred protection. Also, the reconstructed GA haplotype from the *RIPK2* rs40457A/G and rs42490G/A variants was observed to contribute towards increased risk.

In **chapter II**, also published as an original study, I investigated loci in the heterodimeric transporter associated with antigen processing (TAP) gene, which is known to play a vital role in immune surveillance. Functional variants both in *TAP1* (rs1057141 Iso333Val and rs1135216 Asp637Gly) and *TAP2* (rs2228396 Ala565Thr and rs241447 Ala665Thr) were genotyped using a study group of clinically classified Indian leprosy patients (n = 222) and ethnically matched controls (n = 223). The minor allele of *TAP1*

(637G) contributed to an increased risk of leprosy. In the dominant model, two of the researched genotypes of *TAP1*, rs1135216AG and rs1135216GG, contributed to an increased risk of leprosy as well.

In the unpublished **chapter III** of my thesis, I investigated the interactions of *M. leprae* antigen with monocytes and monocyte-derived macrophages by fluorescence-activated cell sorting. *M. leprae* whole cell antigen up-regulated L-Selectin expression in monocytes by 20%. This is a strong indicator of increased migration of phagocytic cells into the tissue upon identification of a threat by *Mycobacterium leprae*. Additionally, the CD163 expression is increased by 30% in macrophages. As CD163 is an indicator of M2-macrophage activity this underlines the role of possible immuno-evasion by the pathogen through alternative activation of macrophages.

In **chapter IV**, also unpublished, peripheral blood mononuclear cells were extracted from fresh blood of 9 Indian leprosy patients (5 paucibacillary and 4 multibacillary) and three healthy, ethnically matched controls. The cells were then incubated and afterwards mRNA was isolated from them. The Natural Killer-cell transcriptome profile was investigated using microarray technology. *LCN2* and *APOBEC3* transcription was predominantly up-regulated in paucibacillary cases, while multibacillary cases were mostly unchanged and controls were down-regulated. A comparison of paucibacillary and multibacillary patients with controls as a baseline revealed that multibacillary patients generally showed a higher transcription of genes associated to cell-cell-communication, a phenomenon not repeated in paucibacillary patients, which actually presented a down-regulation in comparison with healthy controls. Further, multibacillary patients also showed an up-regulation in gene-products involved in immunity. However, some notable exceptions emerged, such as CD70 and TNFRSF9.

In conclusion, utilizing a combination of molecular genetics, cell-culture experiments, and microarray technology, a number of promising findings can be presented here which could serve in deepening our understanding of host-bacterial interactions in leprosy.

Zusammenfassung

Lepra ist eine bakterielle Erkrankung der Haut und Nerven, die von *Mycobacterium leprae* hervorgerufen wird. Seit Einführung einer Antibiotikatherapie in den siebziger Jahren ist die Anzahl an weltweit Infizierten stark gesunken und die Krankheit ist in vielen Ländern ausgerottet. In letzter Zeit verbleibt die Zahl an registrierten Neuerkrankungen jedoch konstant bei ca. 200.000 Personen pro Jahr. Von den Ländern, die noch immer unter der Lepra leiden, verzeichnet Indien mehr als die Hälfte der jährlichen Neuerkrankungen. Die Erforschung der Besonderheiten im Genom der indischen Bevölkerung, die dieses ermöglichen, sowie die generelle Untersuchung der komplizierten Interaktionen zwischen Erreger und Wirtsimmunität sind die Ziele der hier präsentierten Ergebnisse.

In den Studien für **Kapitel I** verglichen wir 211 klinisch klassifizierte indische Leprapatienten mit 230 ethnisch entsprechenden Kontrollen. Die vier untersuchten genetischen Varianten waren: *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G), und *RIPK2* (rs40457A/G und rs42490G/A). Ihnen wurde bereits eine Assoziation mit Lepra in einer früheren Studie mit Han Chinesen nachgewiesen. Das rezessive Allel *LRRK2* rs1873613A und der entsprechende Genotyp rs1873613AA waren signifikant mit einer erhöhten Infektionsgefahr assoziiert. Andererseits vermittelten das dominante Allel *LRRK2* rs1873613G und der entsprechende Genotyp rs1873613GG in unserer Patientenkohorte einen Schutz gegen Lepra im Allgemeinen und gegen die pauzibazilläre Ausprägung im Besonderen. Zudem beobachteten wir, dass der GA Haplotyp der *RIPK2* rs40457A/G und rs42490G/A Varianten mit einem erhöhten Risiko der Erkrankung einherging, während der AA Haplotyp einen Schutz vermittelte.

Im **Kapitel II** wurden die möglichen Assoziationen von Polymorphismen in den Genen *TAP1* und *TAP2* zur Lepra in einer Kohorte von 222 klinisch klassifizierten indischen Leprapatienten untersucht und mit den Genotypen in 223 ethnisch entsprechen Kontrollen verglichen. Die Genotypisierung fand durch Sequenzierung in vier Loci statt: *TAP1* (rs1057141 Iso333Val und rs1135216 Asp637Gly) und *TAP2* (rs2228396 Ala565Thr und rs241447 Ala665Thr). Das rezessive Allel *TAP1* 637G trug zu einer erhöhten Anfälligkeit für Lepra bei. Im dominanten Model trug der Genotyp *TAP1*

rs1135216AG + GG zusätzlich zu einer Erhöhung der Erkrankungswahrscheinlichkeit bei.

Im **Kapitel III** wurden periphere mononukleäre Blutzellen verwendet, um die Interaktion von *M. leprae*-Antigen mit Monozyten und mit aus Monozyten gewonnenen Makrophagen via „fluorescence-associated cell sorting“ zu untersuchen. Wir fanden heraus, dass das verwendete Antigen die L-Selectin Expression in Monozyten um 20% erhöhte. Hier zeigt sich ein möglicher Hinweis darauf, dass die Migration von phagozytischen Zellen ins Gewebe von der Bedrohung durch *Mycobacterium leprae* gefördert wird. Zudem war die CD163 Expression in aktivierten Makrophagen um 30% höher. CD163 ist ein Indikator für M2-Makrophagenaktivität, daher ergeben sich aus dieser Tatsache weiterführende Fragestellungen über die Rolle von *M. leprae* in der alternativen Aktivierung von Makrophagen. Da alle Experimente mit einem Antigen ausgeführt wurden, das aus fragmentierten Bakterienzellen besteht, kann davon ausgegangen werden, dass die beobachteten Effekte passiver Natur sind.

Für **Kapitel IV** wurden periphere mononukleäre Blutzellen von 9 indischen Leprapatienten (5 pauzibazillär und 4 multibazillär) und 3 ethnisch entsprechenden Kontrollen extrahiert. Die gewonnenen Zellen wurden inkubiert und anschließend deren mRNA isoliert. Hierüber wurde die genetische Aktivität durch Verwendung von „microarray“ Technologie bestimmt. Ein individueller Vergleich von Patienten und Kontrollen zeigte auf, dass *LCN2* und *APOBEC3* hauptsächlich in pauzibazillären Fällen hochreguliert wurden. Ein Vergleich der Patientengruppen enthüllte zudem, dass multibazilläre Patienten im Allgemeinen eine höhere Transkription von Genen zeigten, die mit interzellulärer Kommunikation assoziiert sind, wohingegen pauzibazilläre Patienten hier herunter regulierten. Außerdem präsentierten multibazilläre Patienten erhöhte Transkription von Genen, die in die Immunität involviert sind. Hier waren jedoch ein paar bemerkenswerte Ausnahmen festzustellen, wie zum Beispiel in den Fällen von CD70 und TNFRSF9.

Abschließend lässt sich feststellen, dass diese Dissertation innovative Wege präsentiert, mit denen Lepra trotz der mit der Krankheit assoziierten Einschränkungen untersucht werden kann. Unter Verwendung einer Kombination von Methoden der molekularen Genetik, Zellkulturexperimenten, und „microarray“ Technologie war es uns möglich diverse interessante Entdeckungen zu machen, auf denen zukünftige Experimente fußen können.

General Introduction and Background

1. Leprosy

Leprosy, also known as Hansen's disease, is a chronic infectious disease of the skin and nerves caused by the intracellular pathogenic bacterium *Mycobacterium leprae*.

1.1 History

The disease was first described by the Norwegian physician Gerhard Armauer Hansen in 1873, thus making it the first disease directly contributed to a bacterial causative agent. However, it is very clear that leprosy has been a long-lasting companion for humankind, with the oldest traces in medical texts reaching back all the way to 600 BC [1], though molecular analysis suggests that *M. leprae* has been a human pathogen long before the advent of medical documentation [2]. Until the 1970s, when a working antibiotic therapy was introduced, the disease was a long-standing companion of human societies specifically in the tropical and sub-tropical regions. Yet, despite decreased prevalence in the last four decades, the number of new case detection rates remains moderate to high in 130 countries worldwide [3] (Fig. 1).

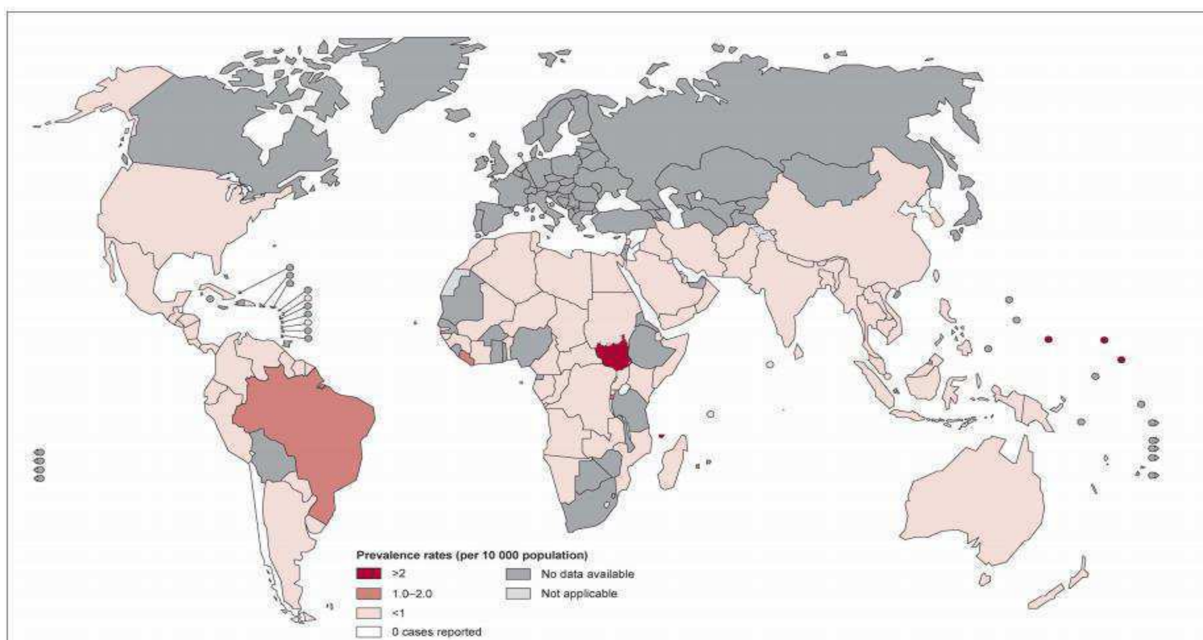


Figure 1: Leprosy prevalence rates as reported to WHO as of January 2012. Source: http://www.who.int/lep/situation/Leprosy_PR_2011.pdf

1.2 Distribution

Amongst the countries still suffering from leprosy, India contributes to half of the new cases detected worldwide, with 120,000-130,000 new cases every year. India surpasses the second-ranking Brazil by four-fold (approximately 30,000 new cases per year) and third-ranking Indonesia by six-fold (approximately 20,000 new cases per year) [4].

The incidence in India has remained constant over the last five years despite an effective multi drug therapy regimen, consisting of a combination of rifampicin, clofazimin, and dapsone, being available and distributed [5]. It can be assumed that this fact is partially due to a multitude of specific host-pathogen interactions, as it has been shown that host immune responses [2, 6] and host genetic factors influence the clinical spectrum of leprosy [7-10]. In addition, a massive gene-loss event contributed to a greatly diminished genetic diversity of *M. leprae* [2, 11]. Taken together, the documented influence of host immune response on disease presentation and the low species diversity of the pathogen hint that host genetics may play a large role in disease outcome and persistence. This could also be a possible explanation for documented relapse during and/or shortly after treatment [12-14]. These listed facts allow for the conclusion that the spectral nature of the disease is largely influenced by host genetics, possibly elucidating the extraordinary role the disease still holds on the Indian sub-continent.

1.3 Disease presentation

What makes it possible to describe the disease as “genetic” is its wide range of manifestations, presumably based on the genetic makeup of the host’s immune system. The disease can range from the relatively mild form of tuberculoid leprosy to the sometimes crippling lepromatous presentation [15]. The lepromatous side of the spectrum is classified into borderline lepromatous (BL) and lepromatous (LL) forms. Following WHO standards, this form is also classified as multibacillary (MB), based on the observed bacillary load in slit skin smears. The tuberculoid form, on the other hand, is separated into tuberculoid (TT) and borderline tuberculoid (BT) forms and is classified as paucibacillary (PB). This classification does not only represent the symptoms during a leprosy, but also directly correlates to the patient’s ability to mount

a successful immune answer, or their failure to do so. In multibacillary, or lepromatous, forms, the absence of Th1 responses leads to an increased bacterial load with a strong humoral immune response [7]. On the other hand, the paucibacillary, or tuberculoid, forms reveal an increased Th1 response with limited bacterial load.

1.4 Mycobacterium leprae

Mycobacterium leprae belongs to the Mycobacteriaceae family, in which we also find *Mycobacterium tuberculosis*. The descriptive unifying element for this family is the incorporation of mycolic acids into the hydrophobic and waxy cell membrane. The lack of an outer membrane classifies Mycobacteriaceae as a Gram-positive bacterium. However, their cell-membrane cannot retain the crystal violet stain commonly used to identify Gram-positive bacteria. The Gram-stain is therefore replaced by the so-called Ziehl-Neelsen-stain, which uses carbol fuchsin for the identification of acid-fast bacilli.

M. leprae is a slow growing pathogen. Researching the bacteria is made particularly hard due to the fact that they cannot be cultured and the available animal models are restricted to the nine-banded armadillo [16] and the footpads of BALBc mice [17]. Within the human body, *M.* leprae is an intracellular pathogen that resides inside macrophages and Schwann-cells of the nervous system. Both cell-types are directly correlated to disease presentation, with infected macrophages being found in great numbers in the characteristic lesions, while the reduced activity of infected Schwann-cells is directly correlated to decreased sensitivity in limbs of leprosy patients. The reduced activity of infected cells depends in great parts on the introduction of large quantities of low-density lipids (LDLs), the resulting droplets giving the afflicted macrophages and Schwann-cells a “foamy” appearance in microscopic observation. It has been speculated that this phenotype is induced by the pathogen, partially in order to reduce phagocytic potential in infected macrophages, but also for energy production through the glyoxylate-cycle [18]. Interestingly, this “foamy” phenotype can be found in other diseases as well, namely tuberculosis [19], Whipple’s disease [20] and even atherosclerosis [21]. It is therefore possible to speculate on a common genetic predilection in certain individuals that is differentially exploited in various diseases, supporting the cause for genetic analysis in the patients.

This hypothesis is supported by the previously mentioned fact that the *M. leprae* genome is massively impeded in its function, with approximately 40% of the genes inactivated to become pseudogenes [11]. It can be assumed that the host cell overtakes vital duties for the bacteria. This is underlined by the findings that the bacteria massively reprogram infected Schwann-cells into a stem cell-like phenotype [22]. Adding the knowledge about gene-decay in *M. leprae* to the fact that the bacterium only has a genome of 3.31 megabase pairs (Mb), it does not come as a surprise that clonal differences between bacterial isolates from various countries (namely India, Thailand, Brazil, and the USA) amount to a mere 200 base pairs (bp), or just 0.005% of the whole genetic information of the organism [2]. Based on this information about low clonal frequency and the low mutation rate in pseudogenes, it was calculated that the evolutionary shift from a free-living form to an intracellular parasite took place approximately 20 million years ago [23].

Tracing the clonal patterns of the bacteria found in various localities around the globe, it has been deduced that the most likely origin of the disease was located in Eastern Africa, from which it spread towards Europe and Asia, only reaching Western Africa and the Americas through European colonization [2, 24].

The high dependency on the human host explains why *M. leprae* has not yet been grown in culture. Even growing the bacteria in animal models is mostly restricted to the footpads of BALBc-mice and the nine-banded armadillo. The latter is also the only known case of a naturally occurring leprosy in an organism other than humans. However, multilocus typing of the *M. leprae* strains of the armadillo suggests a human origin of not more than a few hundred years ago [24], with the most probable source being early European settlers. Thus, and in this particular setting, leprosy may possibly be of zoonotic origin [25].

1.4.1 Mycobacterium lepromatosis

M. lepromatosis is a recently discovered relative of *M. leprae* and can be predominantly found in Central America [26, 27], though a single case has previously been identified in Singapore [28] and Canada [29], respectively. A study compared six corresponding genes in *M. lepromatosis* and *M. leprae* and found a heterology of 7.4%, strongly indicating a new species [30].

A difference between the two species can also be seen in disease representation. While classical leprosy exhibits the previously mentioned symptoms, *M. lepromatosis* induces disseminated leprosy, a severe disease presentation quite unique for Mexico. It has been present as "Lucio's phenomenon" in the area for at least the last two centuries [26, 31].

2. Innate immunity

The term "innate immunity" covers a wide array of immunological measures that, opposed to acquired immunity, work fast and without priming. The defense against intracellular threats, such as *Mycobacterium leprae*, falls within this topic.

2.1 Genetics in leprosy

Considering its importance in fighting off intracellular threats, it becomes clear that the innate immune system represents the first line of defense against *M. leprae.* Of particular importance in bacterial threats to the immune system are the pattern recognition receptors (PRRs), which recognize microbial motifs and mount an immune answer. Amongst these PRRs, the Toll-like receptors (TLRs) play a prominent role in recognising microbial cell surfaces outside of immune cells, while nucleotide oligomerization domain (NOD)-like receptors are localized in the cytosol and are invaluable for recognising intracellular pathogens [32].

A recent genome-wide study in Chinese leprosy patients has provided vital insights on the role of particularly *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G) and *RIPK2* (rs40457A/G and rs42490G/A) variants in regulating the leprosy infection [10]. In addition, the expression of these genes has been shown to be up-regulated in the tissue of patients with leprosy when compared to healthy individuals [33]. The Nucleotide-binding oligomerization domain 2 (NOD2) is an intracellular microbial sensor for muramyl dipeptide, a component of bacterial peptidoglycan [34]. Dysregulation in NOD2 signalling is associated with pathogenesis in many inflammatory disorders [35] and is also associated with triggering of IL-32-dependent dendritic cell programing in leprosy [36]. It can therefore be summarized that *NOD2* plays an important role in leprosy [37, 38]. Additionally interesting in the context of Zhang et al.'s study [10] is the discovery that both NOD1 and NOD2 also activate the gene encoding receptor-interacting serine-threonine kinase 2 (RIPK2) [39].

RIPK2 belongs to the group of kinases and is essential for signaling through the Toll-like receptors, key regulators of the innate immune system [39, 40]. In addition, NF-κB activity is enhanced by interaction of RIPK2 with NOD2 [41], making it an important player in cellular immune response.

The Leucine-rich repeat serine/threonine-protein kinase 2 (*LRRK2*) belongs to the *PARK* family of genes. While LRRK2 is predominantly known for its interaction with parkin (PARK2) in Parkinson's disease, both LRRK2 and PARK2 have been previously implicated of playing a role in leprosy [42-44]. In fact, the connection between Parkinson's and leprosy goes so far that rifampicin, a staple drug in the multi-drug therapy (MDT) of leprosy, has been found to also be of use in treating Parkinson's in an animal model [45]. LRRK2 also has been proven to be an important player in other inflammatory diseases [46] and in reaction to microbial threat [47, 48].

We investigated the possible association of gene variants in *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G) and *RIPK2* (rs40457A/G and rs42490G/A) with leprosy outcome in a cohort of clinically classified patients from India.

As pointed out above, the decision whether to mount a cellular Th1 or a humoral Th2 defense is a vital part in the fight of the immune system against *M. leprae*. Therefore it is important to observe the points in the immune answer where the switch between Th1- and Th2-response occurs. This statement is not only true in the specific case of leprosy, as observations here can, due to the high impact of host genetics on immunity in this disease, also be extrapolated to other diseases [49, 50]. The delivery of major histocompatibility complex (MHC) class I molecules from cytosol to the endoplasmic reticulum is the first step to mounting a Th2 response through antigen presentation on the cell surface. It is facilitated by the transporter associated with antigen processing (TAP), a heterodimer composed of two subunits TAP1 and TAP2. Polymorphisms in the *TAP* genes are already known to be associated with other inflammatory diseases, including autoimmune diseases such as rheumatoid arthritis, and in tuberculosis [51-55].

The role of the *TAP* genes deserves a special focus in regards to the part they play in tuberculosis, as both the causative agent of this disease as well as leprosy belong to the same genus of *Mycobacterium*. Therefore we investigated *TAP* polymorphisms that were shown to be associated to tuberculosis: *TAP1* rs1057141 (Iso333Val) and

rs1135216 (Asp637Gly), as well as *TAP2* rs2228396 (Ala565Thr) and rs241447 (Ala665Thr) [53, 54].

2.2 The shifting nature of macrophages

In recent years the existence of a macrophage polymorphism that expresses a non-classical phenotype has been discovered [56, 57]. Finally, it was found that macrophages can "polarize" into differently acting phenotypes upon activation through cytokine or Toll-like-receptor (TLR) stimulation [58]. Furthermore, these polarized macrophages can change their phenotype in response to growth factors (e.g. colony stimulating factor 1 (CSF-1) and granulocyte/macrophage colony stimulating factor (GM-CSF)) and other activating impulses, such as microbial threat, phagocytosis, or corticoids [59, 60].

Polarized macrophages are generally categorized into the classically activated M1, and the alternatively activated M2 phenotype [61, 62], replicating the Th1/Th2 dichotomy. However, this separation is not as clear here as it is in T-helper cells.

In general, the M1 phenotype can be differentiated by flow-cytometry through its strong surface expression of CD86 and CD80, which is quantitatively lower in the M2-population [63] (Fig. 2). Further, the M1-phenotype strongly expresses IL-12 and IL-23, while IL-10 production remains meagre. M1-macrophages produce effector molecules (reactive oxygen species and nitrogen intermediates) and inflammatory cytokines (IL-1β, TNF, IL-6) and participate in Th1 responses [64, 65]. In this manner they help mediate resistances against intracellular pathogens.

M2-macrophages present high levels of scavenger, mannose, and galactose-type receptors, particularly CD163 (scavenger receptor) and CD206 (mannose receptor) [66]. Unlike M1-macrophages, the M2 variant acts less directly within the immune system and overtakes a role more akin to a regulating and clean-up force. The cells take part in parasite clearance [67], dampening of inflammation and the promotion of tissue remodelling thereafter [68, 69], as well as general immunoregulation.

2.3 M2-Macrophages in tuberculosis

As mentioned already, the scavenger receptor CD163 is a commonly accepted marker for general M2-macrophage-activity, particularly in tissue-repair after inflammatory challenge. CD163 is characteristically up-regulated by the anti-inflammatory cytokine

IL10 and, consequently, down-regulated by the pro-inflammatory TNF-α and IFN-γ [70].

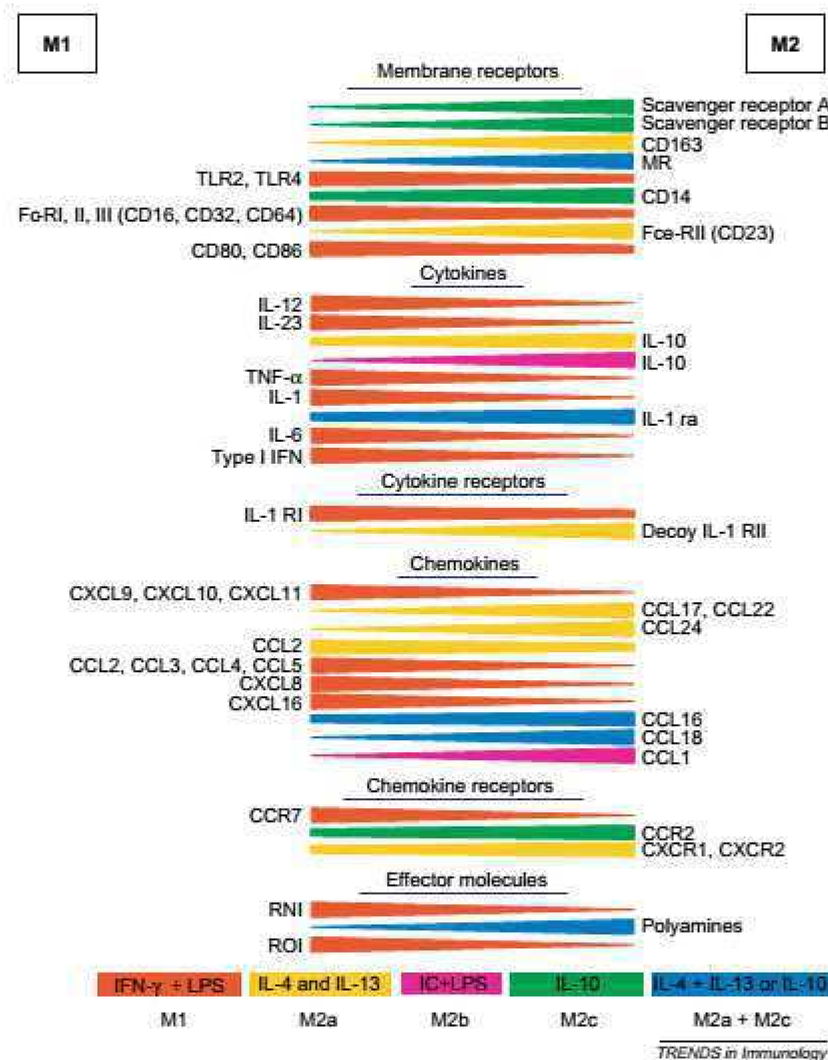


Figure 2: The different parameters used to identify M1 macrophages and M2 macrophage-sub-types as proposed by Mantovani et al. *[71].*

It can be assumed, therefore, that the CD163 profile, in accordance with M2 activity, would generally be low during an infection and increase notably after, in order to participate in immuno-reconstitution measures. This is supported by findings concerning *M. tuberculosis*, where, in a macaque-model of tuberculosis, anti-inflammatory macrophages carrying CD163 were only found in the periphery of granulomas, adjacent to uninvolved tissue, and never associated to a strong bacterial presence [72]. Further, *in vitro* studies of artificially with *M. tuberculosis* infected monocytes showed a notable reduction of CD163 on the cell surface only in

tuberculosis patients, while healthy controls appeared unaffected [73]. The expression of CD163 did not increase either for patients or healthy controls.

In yet another study it was observed that immune-histochemical stains of tuberculous granuloma samples contained only a small percentage of cells that expressed CD163 at all (21%) [74]. Interestingly, upon examination of samples from *Schistosoma mansoni*-induced granulomas, CD163 positive cells made up 73% per-cent of the samples. *S. mansoni* is known for its ability to modulate the immune answer, so this may have played a role here.

As *M. leprae* is an organism that, to date, cannot be grown *in vitro*, a certain degree of conjecture in regards to its effects on the human immune system is necessary. The closely related *M. tuberculosis* serves as a good entry point for understanding *M. leprae*-activity within the human body, as not only do the two organisms share a common ancestor and still have approximately 1400 genes in common [23], they also behave similarly *in situ* [75-77]. Therefore, M2-activity during tuberculosis could present a good indicator for the same activity during leprosy, hinting at CD163 as a probable indicator for measuring M2-activity in human leprosy.

For the reasons mentioned above we investigated macrophage-polarization in leprosy based on CD163 as a reliable marker.

2.4 L-Selectin

L-Selectin, or CD62L, is a part of the extravasation process of leukocytes, including monocytes, into the tissue [78, 79]. It acts as an anchor on the cells' surface, tying them to the epithelium and allowing for the characteristic rolling movement that precedes the migration into the tissue.

The lack of L-Selectin has been found to play a role in the formation of inflammation-causing cardiovascular disease [80, 81]. This is of interest in mycobacterial infection, as a common macrophage-phenotype, which imports massive amounts of low-density lipids, can be found in both *M. leprae* and *M. tuberculosis* infections as well as in arteriosclerosis [18, 82]. Also, the diseases caused by those pathogens both involve inflammatory processes comparable to those found in arteriosclerosis [83-85]. L-Selectin also plays an important role in the clearance of inflammation. This is proven

by the discovery that anti-CD62L antibodies inhibit the recruitment of monocytes and neutrophils to inflammatory sites [78].

Considering its important role in inflammatory processes in humans and particularly in the recruitment of monocytes into the tissue, we decided to observe the role of L-Selectin in the particular case of leprosy.

3. Natural killer cells

Natural killer cells (NK-cells) are lymphoid cells of the innate immunity. They participate in the clearance of tumor cells [86] as well as the clearance of cells affected by intracellular parasites like viruses [87] and bacteria such as *M. tuberculosis* [88]. They are also a major source of the type 1 cytokine IFN-γ, as well as TNF-α, GM-CSF, and other cytokines and chemokines associated with type 1 immunity and inflammation [89, 90].

3.1 NK-cells and macrophages

As both NK-cells and macrophages play important roles in innate immunity, their interaction bears importance in this context. Indeed, it has been found recently that NK-cell-derived interferon γ influences the above mentioned macrophage sub-populations differently [91]. This influence also seems to vary in between the sexes [92]. However, it seems that the interaction between the two cell types is not entirely mutually beneficial, as a recent study revealed killing of macrophage sub-types M0 and M2, though not M1, through lysis by activated NK-cells [93]. The same study found that M1 macrophages showed increased resistance to lysis through their elevated HLA I surface expression.

3.2 NK-cells and mycobacterial diseases

As mycobacteria are intracellular pathogens, NK-cells play an important role in the body's defense against them. They have been proven to heavily channel and edit dendritic cell (DC) activity in *Mycobacterium tuberculosis*-infected tissue, particularly granuloma [94-96]. NK-cells can induce DC-maturation and are often found to kill immature DCs in peripheral tissue.

The above-mentioned interaction of NK-cells with phagocytic cells can also be found in the immune system's fight against tuberculosis. In particular, NK-cell-activated

monocytes that have phagocytised *M. tuberculosis* show a mycobactericidal activity that is 84% greater than in control monocytes [97].

A great part of the NK-cells' antimicrobial activity is conveyed through their ability to produce IFN-γ in great quantities. This was shown in an experiment with mice, where NK-cell-knockout or anti-IFN-γ individuals showed great susceptibility particularly to *M. tuberculosis* [98]. The role of IFN-γ especially in tuberculosis is underscored by its greatly diminished production in acute pulmonary tuberculosis and the rapid return to normal levels after successful treatment [99].

The activity of NK-cells in leprosy is less well researched than in tuberculosis. The main focus of studies over the recent years has been on the NK-produced cytokines and their activities. Thus it was found, for example, that interleukins (IL) 12 and 18 induce host-cell death in a murine leprosy model [100]. Further studies revealed that IL13 has a modulatory effect on the activity initiated by IL18, possibly by affecting IFN-γ production by NK-cells [101].

Perhaps unsurprisingly, the human leukocyte antigen (HLA) surface marker plays a dominant role in the interaction of NK-cells with cells hosting *M. leprae* [102, 103], informing NK cells of the host cell status and initiating intervention. Even more so, certain HLA genotypes elicit a protective effect against leprosy [104, 105]. Also of note: The killer immunoglobulin-like receptor (KIR) family is engaged in the recognition of host cells as well and it has been proven that certain genotypes are more beneficial for avoiding persisting infection [106].

Based on results stemming from the investigation of tuberculosis as well as the known role of NK-cells in innate immunity, particularly against leprosy, we decided to investigate the transcriptome-level activity of NK cells in leprosy patients utilizing microarray technology.

Objectives

Innate immunity is of the utmost importance in defending the human body against leprosy. The studies presented in this thesis aim at describing the host-pathogen relationship in the context of innate immunity from a molecular/cell biology point of view in the hope of expanding the scope of future research into treatment of the disease. Our research is focused on the Indian population, as the Indian sub-continent contributes more than half of all newly diagnosed leprosy cases worldwide each year.

Specific Objectives

1. The significance of single nucleotide polymorphisms in the *NOD2*, *LRRK2*, and *RIPK2* genes, which have been proven to be associated with leprosy in past studies, was investigated using a case control study design in an Indian population.
2. The significance of leprosy-associated single nucleotide polymorphisms in the immune surveillance-relevant genes *TAP1* and *TAP2* was investigated in a case control study design.
3. The influence of *Mycobacterium leprae* antigens on monocyte recruitment and macrophage activation was investigated.
4. Natural Killer cells are extremely important in defending against intra-cellular pathogens. The NK cell transcriptome profile in leprosy patients was therefore compared to healthy, ethnically matched controls.

Results

Chapter I

***LRRK2* and *RIPK2* Variants in the *NOD2*-Mediated Signaling Pathway Are Associated with Susceptibility to Mycobacterium leprae in Indian Populations (published)**

The observed genotype and allele frequencies of all studied SNPs in clinically classified patient groups and controls were in Hardy-Weinberg equilibrium ($P > 0.05$) except for the *NOD2* SNP rs9302752 ($P < 0.05$). Therefore the *NOD2* SNP rs9302752 was excluded from further association analysis.

We observed that the minor allele *LRRK2* rs1873613A and the homozygous genotype rs1873613AA were more frequent in leprosy patients than in controls, with odds ratios that imply an increased risk for the development of leprosy (OR = 1.7, 95% CI = 1.25–2.2, $P = 0.0003$ and OR = 2.04, 95% CI = 1.2–3.6, $P = 0.007$, respectively). The major allele *LRRK2* rs1873613G and the homozygous genotype rs1873613GG were observed less frequently in patients compared to controls implicating a decreased risk of leprosy (OR = 0.61, 95% CI = 0.45–0.8, $P = 0.0003$ and OR = 0.56, 95% CI = 0.37–0.83, $P = 0.0028$, respectively). When only clinically classified paucibacillary (PB) patients were compared to controls, a similar trend was observed with a stronger significance (for the minor allele rs1873613A: OR = 2.77, 95% CI = 1.9–4.1, $P < 0.0001$; for the homozygous genotype rs1873613AA: OR = 4.1, 95% CI = 2.1–7.9, $P < 0.0001$). There was no significant association between the multibacillary (MB) presentation of the disease and this genotype.

In the observed leprosy patients both the studied *RIPK2* variants (rs40457A/G and rs42490G/A) were found to be in a high degree of linkage disequilibrium (LD, Leprosy patients: D'= 0.64, LOD = 12.72, $r^2 = 0.2$; MB patients: D' = 0.53, LOD = 5.84, $r^2 = 0.56$; PB patients: D'= 0.84, LOD = 7.58, $r^2 = 0.34$). In controls we only found a low degree (D'= 0.38, LOD = 3.2, r2 = 0.06). Four haplotypes and their frequencies were observed: AG, AA, GA and GG. The *RIPK2* haplotype GA was observed more frequently in leprosy patients compared to healthy controls, inferring an increased risk of leprosy (OR = 1.46, 95% CI = 1.02–2.1, $P = 0.036$), whereas *RIPK2* haplotype AA was observed less frequently in leprosy patients compared to controls, inferring a protection

against leprosy (OR = 0.69, 95% CI = 0.49–0.97, *P* = 0.028). In addition, *RIPK2* haplotype GA was observed more frequently in PB patients in comparison to controls, inferring an increased risk of this particular presentation of the disease (OR = 1.8, 95% CI = 1.1–2.8, *P* = 0.018). Furthermore, there was no significant difference of *RIPK2* haplotype frequencies in comparison between MB patients and controls. This is notable in the context of Zhang *et al.*'s genome-wide association study [10], which found the SNP rs42490 in the *RIPK2* gene to have a stronger association towards the MB presentation of leprosy.

Chapter II

Genetic evidence of *TAP1* gene variant as a susceptibility factor in Indian leprosy patients (published)

Two of the observed loci (one in *TAP1* rs1057141 and one in *TAP2* rs241447) were not in Hardy–Weinberg equilibrium. These were excluded from further analysis and haplotype analysis was not attempted.

The *TAP1* rs1135216 (Asp637Gly) in exon 10 was observed to be a susceptibility factor. The presence of the minor allele of this locus (rs1135216G) was observed to be significantly higher in patients than in healthy control individuals (OR: 1.68, 95% CI 1.2–2.36, *P*= 0.0057). The susceptibility was comparable in both the paucibacillary (PB) and the multibacillary (MB) patient cohorts.

Similar observations were made in the dominant genetic model (AA) for this variant when comparing patients with controls or clinically classified patient groups with controls: Patients vs. Controls (OR: 2.01, 95% CI 1.34–3.03, *P*= 0.0012); MB vs. Controls (OR: 1.92, 95% CI 1.21–3.03, *P*= 0.011); PB vs. Controls (OR: 2.20, 95% CI 1.25–3.90, *P*= 0.015). The recessive model (GG) did not produce any statistically significant associations.

For the *TAP2* SNP rs2228396 (Asp637Gly) in exon 10 we did not observe any significant contributions (after *p*-value corrections) either between patients and controls or between clinically classified patient groups compared to controls.

Chapter III

Interaction of Mycobacterium leprae with human monocytes increases L-Selectin expression and influences phagocytosis (not published)

After six days of incubation, $CD14^+$ cells (at this point macrophages) showed an increase in internal complexity and size in FACS. Only approximately 4.5% of them were still $CD62L^+$ when compared to $CD14^+$ cells on day zero (at this time point they were still monocytes). When monocytes were only incubated for 3 days and then stimulated with *Mycobacterium leprae* whole cell sonicate (WCS) overnight, CD62L-expression across individuals increased by 30%, indicating that transformation into macrophages had not fully occurred yet.

After incubation with WCS for 18 hours, matured macrophages showed a significantly different phenotype when compared to non-activated macrophages. Both size and internal complexity, represented by the FSC and SSC values respectively, changed upon stimulation. Upon activation with WCS, macrophages showed an up-regulation of CD163 that was more than 20% higher than that of non-stimulated controls.

Chapter IV

Transcriptome profile of natural killer cells in patients treated with multi-drug-therapy (not published)

When comparing expression profiles of the patients (NK 2, 4-6, 8, and 11-12) with each other and with the healthy controls (NKK) we found three groups emerging: NK2 and NK11 are both member of the paucibacillary (PB) group, while NK4 and NK6 both are multibacillary (MB) patients. NKK1 and NKK3 both belong to the control group. It is notable that we found a number of outliers that could not be included in any of the mentioned groups based on their expression, even including one of the controls. All of the outliers were excluded from further analysis.

Comparing patients and controls on an individual level, we found a number of genes up-regulated in infected individuals when compared to healthy controls (NKK samples). Of particular prominence were the genes for lipocalin 2 (LCN2) and apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3), which both were more differentially expressed in multibacillary patients than in paucibacillary ones.

We also found a number of Y- and X-chromosomal genes differentially expressed, such as protein kinase, Y-linked (PRKY) and kelch-like 4 (KLHL4). They were adequately expressed in different genders, with PRKY, for example, being only up-regulated in males. This serves as an additional quality control for the microarray data.

When the multibacillary and the paucibacillary patients were compared with the values from the control group as a baseline, we found two distinct clusters where PB and MB patients differed greatly and genetic activity was diametrically opposed, i.e. up-regulated in the PB group and down-regulated in the MB group and vice versa.

The first cluster is dominated by gene-products that play a role in immunity (CD70, phospholipase A2, group IVC (cytosolic, calcium-independent) [PLA2G4C], and tumor necrosis factor receptor superfamily, member 9 [TNFRSF9]). These genes were more strongly expressed in PB patients.

The second, larger cluster on the other hand includes many molecules involved in general cell-cell-communication, like signal-regulatory protein gamma (SIRPG), or pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) (PPBP). The genes in this cluster were more strongly expressed in MB patients.

General discussion

The Indian population is highly represented in the global annual new case registration rate of leprosy. Investigating the variations in immunity-associated genes in Indian patient populations should help to find specific susceptibility loci that regulate disease outcome in general and in the Indian setting in particular.

Research of leprosy is further complicated by the inability to grow *M. leprae* in culture. Even animal models fail for the most part, as outlined in the introduction. Taken together, these facts underline the need for experiments built around cell cultures and isolated patient samples.

The studies described in this work are an attempt to perform a two-pronged analysis of the immune system of the leprosy patient, utilizing molecular as well as cell culture methods, thus circumventing the particular problems mentioned above.

1. Immunogenetics

Individual and ethnical immunogenetics play a greater role in leprosy than in most other diseases, as the causative agent, *Mycobacterium leprae*, presents such little genetic drift. Any variations in disease progression observed can therefore be traced back directly to the host's genetic make-up.

1.1 Chapter I

The results outlined in chapter I are useful for pointing out the influence of population differences in regards to leprosy.

We investigated genetic variants in *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G) and *RIPK2* (rs40457A/G and rs42490G/A). These positions were derived from a whole genome association study of leprosy patients in a Han-Chinese population by Zhang *et al.* [10]. The three genes *NOD2*, *LRRK2*, and *RIPK2* are interlocked in a tightly-knit gene-interaction network (see Fig. 3).

While our study population doesn't compare to Zhang *et al.*'s study in size (211 patients in our study versus 706), we found our locus frequencies to be in accordance with both the Chinese study and the Gujarati Indian population found within the HapMap project (http://hapmap.ncbi.nlm.nih.gov/). It can be assumed that observed differences are based on differences in the populations rather than on differences in sample sizes.

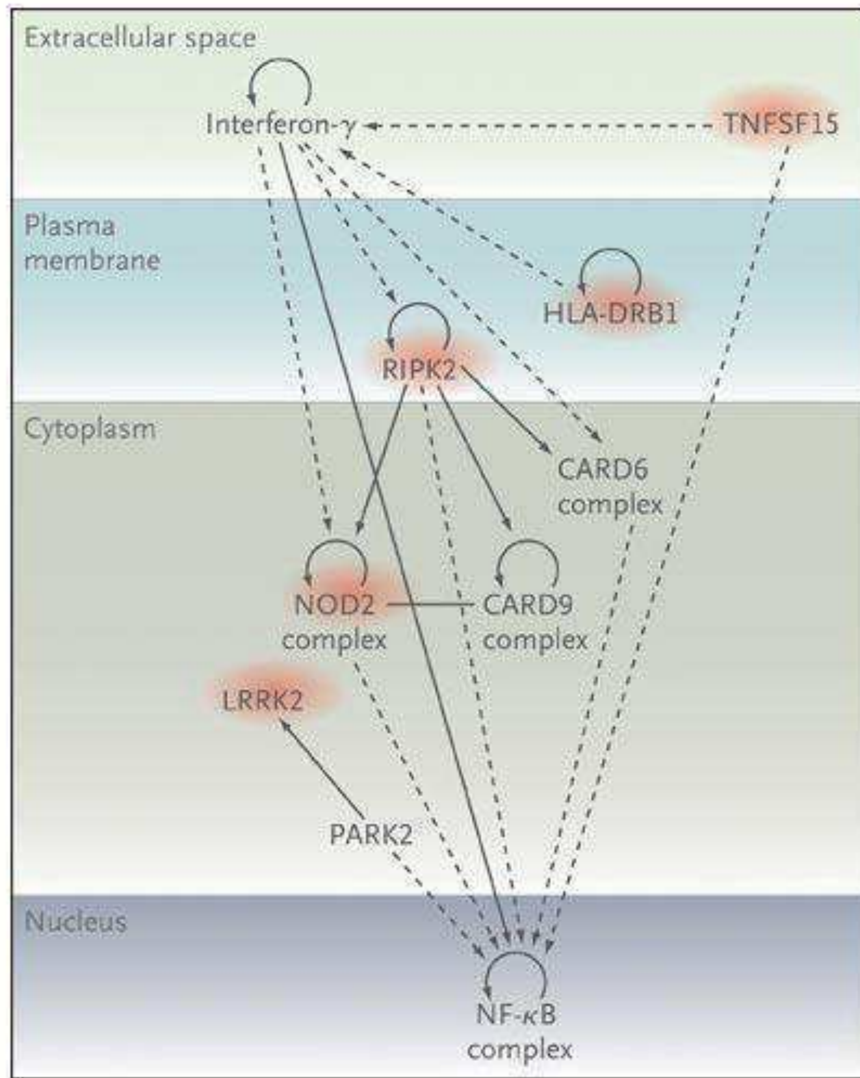


Figure 3: Gene-interaction network including NOD2, LRRK2, *and* RIPK2 *by Zhang* et al. *[10]. Highlighted genes have been proven to be associated to susceptibility towards leprosy.*

The Nucleotide-binding oligomerization domain-containing protein 2 (***NOD2***) plays an extremely important role in the immune-defense, particularly against the threat of intracellular pathogens [107]. Through recognition of peptidoglycan it initiates detection of intracellular bacteria and commences autophagy, pro-inflammatory pathways, and other means of host-defense. Finding genetic variations of this particular gene would open up intriguing lines of questioning its efficiency in the fight against intracellular pathogens such as *M. leprae*.

The researched *NOD2* locus, rs9302752A/G, was not within Hardy-Weinberg-Equilibrium, despite the minor allele frequency in our study being found to be comparable to the one identified by Zhang *et al.* (0.28 and 0.22 respectively). The fact

that we did not find an independent population for this locus is probably based on longstanding Indian marital traditions vis-à-vis the caste system [108]. We attempted to counter this known effect of population stratification by recruiting a great variety of patients and ethnically matched controls within the metropolis region of Hyderabad, Telangana, hoping that an urban mixture of study participants would result in a non-stratified study group. We achieved this goal with the remaining three loci, but we had to exclude locus rs9302752A/G from further analysis.

The major allele of the single nucleotide polymorphism rs1873613A/G of the gene ***LRRK2*** was found with a similar frequency in our study group as reported in Gujarati Indians in Houston, Texas (GIH) in the HapMap project (0.68 and 0.61 respectively). *LRRK2* and its gene-product Leucine-rich repeat kinase 2 are predominantly known for their involvement in the occurrence of Parkinson's disease (PD) [109]. PD is a degenerative affliction of the central nervous system, caused primarily by the as-of-yet poorly understood death of cells in the *substantia nigra* area of the mid-brain. Recent studies have discovered involvement of the gene in inflammatory priming in monocytes [110] and in autophagy [111], both of which help to establish the degenerative phenotype of PD. *LRRK2* has also been implicated in Crohn's disease (CD) [112], an inflammatory bowel disease. Known polymorphisms promoting development of CD have also been proven to play a role in leprosy patients in Vietnam [113].

The influence of *LRRK2* in pathologies that involve inflammation and autophagy make it a prime candidate for research in leprosy, as both processes feature prominently in leprotic morbidity and in the *M. leprae* infectious process respectively [114-116]. We found in our study disease-association of the major allele G, as did Zhang *et al.* Within our population, we also found the very same polymorphism to be particularly associated to the paucibacillary spectrum of the disease, a result that wasn't replicated in the Han Chinese population researched by Zhang *et al*. Here, on the other hand, the polymorphism rs1491938C/T was found to be associated to the multibacillary spectrum of the disease exclusively.

The variation between the Han Chinese and the Indian population in regards to the observed *LRRK2* polymorphism rs1873613A/G is not surprising, considering the already mentioned great dependency of the pathogen on the genetic makeup of a population. Therefore it is possible to have, within considerable close proximity in Central to Southern Asia, three different ethnicities which present three different effects

of the polymorphism on disease progression: While in a Vietnamese population rs1873613A/G does not play a statistically significant role [113], in our Indian population the major allele is directly associated to the disease, even to a particular representation (paucibacillary). And in a Han Chinese population we find a general association, but it is an entirely different polymorphism in the gene, rs1491938C/T, which influences disease representation (towards multibacillary) [10].

Our results regarding *LRRK2*, when put in a context of similar studies covering different ethnicities, demonstrate how ethnic variability can influence leprosy outcome.

RIPK2 was investigated at the positions rs40457A/G and rs42490G/A. This gene encodes receptor-interacting serine/threonine-protein kinase 2. The enzyme is of great importance in innate inflammatory pathways [117-119]. It has also been proven to be involved in various inflammatory morbidities, such as inflammatory bowel disease [120], Crohn's disease [113, 118], sarcoidosis [121], or inflammatory arthritis [122]. The initial discovery by Zhang *et al.* of *RIPK2* playing a role in leprosy in a Han-Chinese population was re-established by the same group through independent experiments involving a gene-expression study [33] as well as a gene-polymorphism association study involving only the rs40457A/G locus [123].

In this context of established results regarding leprosy and the overall involvement of *RIPK2* in various inflammatory disorders we decided to include the two loci identified in Zhang *et al.*'s whole genome association study in our experiments. However, within our Indian population we did not find any association of the single polymorphisms towards either presentation of the disease. We assume that this is partially due to sample size, as even in the study by Zhang *et al.*, including 706 patients and 1225 controls, the discovered *p*-values for the loci were, considering the sample sizes, moderately significant, with rs42490G/A at *p*-value = $1.23*10^{-3}$ and rs40457A/G at *p*-value = $1.43*10^{-2}$. Still, we are certain that part of the lack of association is due to the already discussed great variation of ethnic genetic differences.

This line of thought is corroborated by findings in a Vietnamese population, where in 474 patients only the rs42490G/A locus, and not the rs40457A/G counterpart, was found to be significantly associated to leprosy, with a *p*-value of just 0.043 [113]. Our own results within an Indian population show an association to leprosy only when observing the haplotypes of the *RIPK2* loci. The combination of major allele A in

rs40457 and minor allele A in rs42490 exhibited a moderate association with a *p*-value of 0.028. The odds ratio found was 0.69, indicating an association of this haplotype towards protection against the disease. When examining the combination of minor allele G in rs40457 and minor allele A in rs42490 we achieved a moderate association of 0.036. However, in this case the odds ratio was found to be 1.46, conveying a tendency towards disease progression.

Taken together the results of the haplotype analysis of the *RIPK2* gene imply that major allele A in rs40457 conveys a protective effect against leprosy in the Indian population, while minor allele G in this locus supports disease progression. Minor allele A in rs42490 exaggerates the respective effect to a degree that makes the association visible even in a smaller population such as ours. This is in agreement with Zhang *et al.*'s findings both in the whole genome association study and in the context of a *RIPK2-NOD2* interaction study [123] where rs40457 was found to be associated with disease protection. In the Vietnamese study rs42490G/A alone was associated to disease progression, with an odds ratio of 1.2 [113]. Additionally, it appears that the combination of rs40457G and rs42490A particularly supports disease progression towards the paucibacillary spectrum, as we found a significant *p*-value of 0.018 and an odds ratio of 1.8 when examining the haplotypes in the context of the paucibacillary sub-population within our study.

In conclusion it can be stated that the results of chapter I particularly point out the great value of re-examining results of immunogenetics studies of leprosy in various ethnic contexts, as even geographically close populations can show varying and sometimes even opposing results. It is of utmost importance to be mindful of the differences in genetic make-up of the various ethnic populations under threat by leprosy today when attempting to design new treatments attempting new drug regimes.

1.2 Chapter II

Chapter II serves to highlight the similarities of the role of immunogenetics in the context of leprosy and tuberculosis.

The transporter associated with antigen processing (***TAP***) is specialized in transporting cytosolic peptides to HLA class I molecules in the endoplasmic reticulum (ER) [124] (See Fig. 4). It consists of two subunits, TAP1 and TAP2, which belong to the ATP-binding cassette family of transporter molecules. The transporter's elevated

importance in the context of immunity can easily be proven by the fact that deletion or mutation of either one or both subunits severely reduces translocation of peptides to the ER, resulting in the so-called “TAP-deficiency syndrome” (TDS) [125]. Resulting clinical manifestations are, amongst others, recurrent bacterial infections of the upper respiratory tract within the first six years of life, progressing into infections of the lower respiratory tract by the second decade of life. Antiviral immunity does not seem to be affected. The most striking symptom of TDS in the context of leprosy is the occurrence of necrotizing granulomatous skin lesions, typically located on the extremities or in the mid-face. The lesions heal very slowly and often leave hyperpigmented scars behind. In their behavior and appearance they seem very close to the lesions found on many leprosy patients.

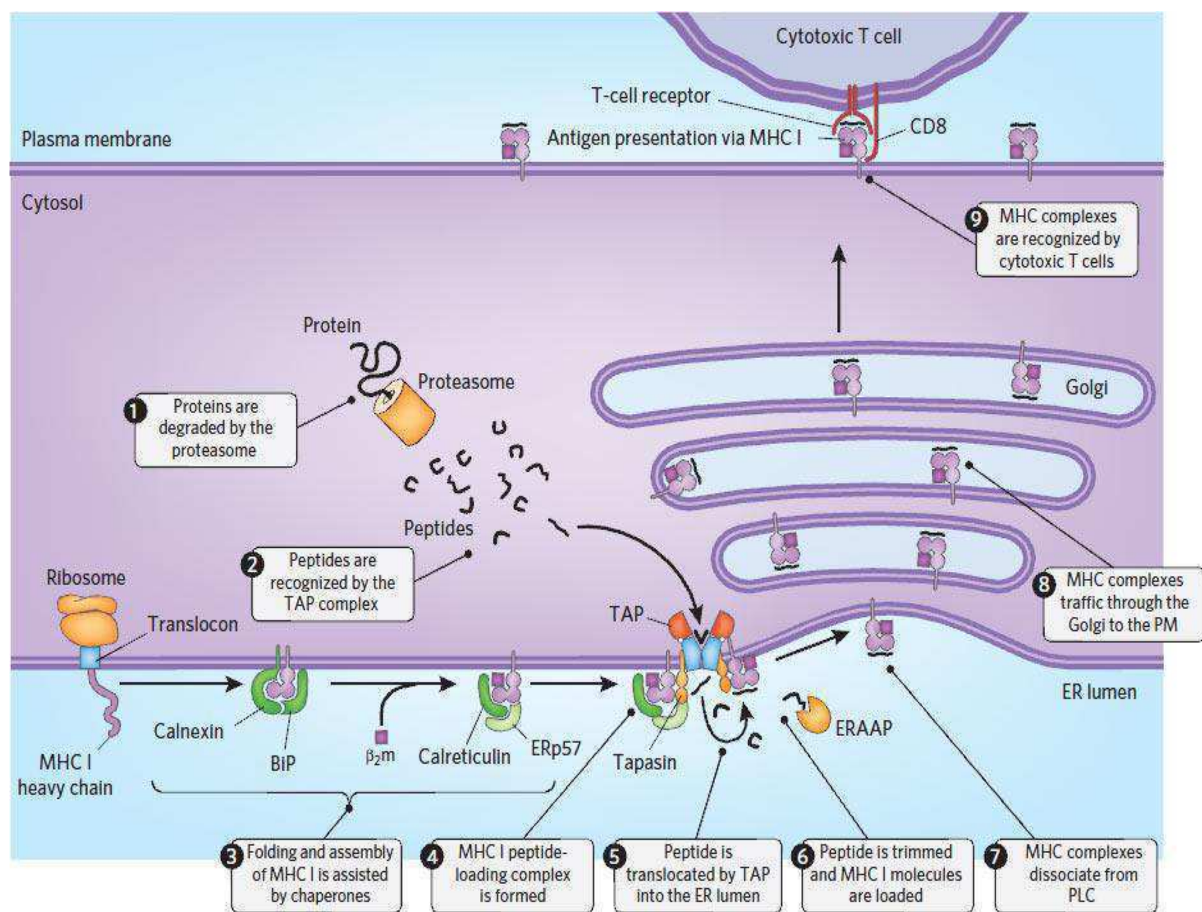


Figure 4: The central role of the TAP-complex in the processing of antigen-peptides by Parcej et al. *[126].*

TAP has also long been known to be involved in various diseases, such as ankylosing spondylitis [51], a chronical inflammatory disease of the joints, vitiligo [127], HIV-infection [128], or, in particular, tuberculosis [53, 129]. Based on Sunder *et al.*’s discovery of the involvement of *TAP1*-polymorphisms rs1057141A/G and

rs1135216A/G, as well as *TAP2*-polymorphisms rs2228396G/A and rs241447A/G, in tuberculosis-HIV-co-infection [53], we decided to investigate the influence of these polymorphisms in the context of leprosy. Furthermore, the population in their study was recruited from the same area as ours, i.e. the greater metropolitan area of Hyderabad, India. Our approach is additionally supported by findings that connect some of the very same polymorphisms described above to susceptibility towards both pulmonary tuberculosis and tuberculoid leprosy in a population in New Delhi, India [55].

The minor allele combination GG in *TAP1* rs1057141A/G was found to be significantly associated with HIV-TB-co-infection when compared with controls (*p*-value = 0.01) and HIV-only individuals (*p*-value = 0.03) by Sunder *et al*. [53]. Also, the major allele combination AA was represented at a significantly lower concentration in the co-infection group than in HIV-only individuals (*p*-value = 0.05), indicating protective activity at least against tuberculosis. In general, they found allele G to be positively associated to HIV-TB-co-infection, while allele A appeared to be negatively associated, conveying a certain degree of protection. We, on the other hand, could not find this SNP to be within Hardy-Weinberg-equilibrium and thus excluded it from further analysis. We assume that this fact is based on the size of the examined population, 222 patients in our study versus 345 for Sunder *et al*. This assumption is supported by the low significance of the observations in the latter study, with *p*-values ranging from 0.01 at best to 0.05. This should improve with sample size.

For the *TAP1* polymorphism rs1135216A/G both Sunder *et al*. as well as our study found significances. In our study, we found the minor allele G to be highly positively associated to the disease in general. Examining the MB and PB sub-groups separately revealed less significant *p*-values for both. While association to the MB group was just barely significant with a *p*-value of 0.054, the association towards the PB group was moderately high, with a *p*-value of 0.013. Observing the results utilizing a dominant model (AA versus AG + GG) resulted in mostly the same, though general association towards the disease appeared stronger while association towards the MB group now was of a higher significance than that of the PB group. All of the above reveals that, while G is the minor allele at position rs1135216 in the Indian population, its effect on leprosy manifestation and disease outcome dominates that of the major allele A when present.

The findings of Sunder *et al*. support ours in some regards. They found the heterozygous genotype AG to be significant in the HIV-TB-co-infected individuals, particularly when compared to HIV-positive ones. This significant association can be traced back, as in our case, to the minor allele G, where they found a moderate association with a *p*-value of 0.03 when comparing HIV-TB-co-infected individuals with HIV-only controls. Of course, the genotype associations of a study involving HIV-TB-co-infected individuals cannot be entirely equal to our findings in the context of leprosy. This would explain why Sunder *et al.* found a negative association of the AA genotype and HIV-TB-co-infection at this position, while we couldn't identify a significant contribution of the major allele A at all. Still, identifying the same association for the G allele at this position for a study involving the same population, from the greater metropolitan area of Hyderabad, India, underscores the close relationship of tuberculosis and leprosy in regards to immunogenetics.

Rajalingam *et al*. in their study involving patients with tuberculoid leprosy and pulmonary tuberculosis did not find any significant associations for the *TAP1* genotypes researched within our study [55]. Considering that their study populations consisted of 50 individuals with tuberculoid leprosy and 57 with pulmonary tuberculosis, this demonstrates the need for sufficient numbers of samples to identify the effect of the researched *TAP1* genotypes.

Despite the relatively small number of individuals enrolled into the study, Rajalingam *et al*. found significant associations of the researched *TAP2* genotypes to both tuberculoid leprosy and pulmonary tuberculosis. These results couldn't be repeated within either Sunder *et al*.'s or our study. For both studies, *TAP2* rs2228396G/A revealed no significant associations.

We could not find Hardy-Weinberg-equilibrium for the polymorphism rs241447A/G within our study population and thus did not calculate associations. Sunder *et al*. also did not establish a Hardy-Weinberg-equilibrium, yet still calculated associations. They found the AA genotype to be moderately associated with HIV-TB-co-infection. At the same time, the AG genotype was found to be associated with protection. Rajalingam *et al.* found the minor allele G to be generally associated with protection against pulmonary tuberculosis, mirroring the results of Sunder *et al*., while in the case of tuberculoid leprosy the minor allele conveyed disease susceptibility in a highly

significant manner. However, Rajalingam *et al.* did not test for Hardy-Weinberg-equilibrium either.

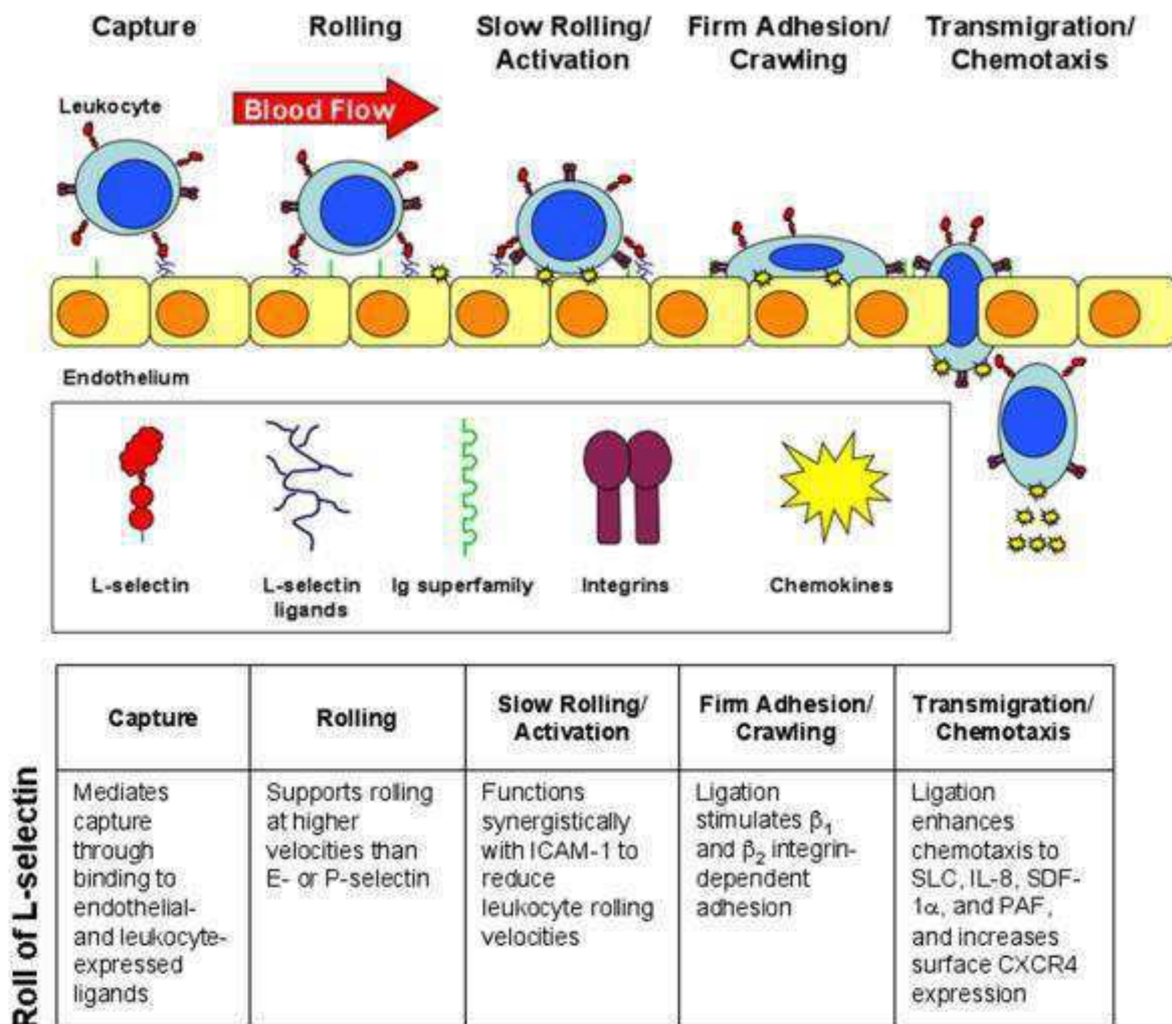
The studies by Sunder *et al.* and Rajalingam *et al.* in the context of our results lead to a variety of conclusions: While the results in Rajalingam *et al*. in regards to the *TAP2* polymorphisms are interesting and seemingly build a bridge between the immunogenetics of leprosy and tuberculosis, the small sample size and the neglect of establishing Hardy-Weinberg-equilibrium hampers the conclusions that can be drawn. On the other hand, Sunder *et al*.'s results in the context of HIV-TB-co-infection mirror very well our own results in the context of leprosy. As the study populations in both publications can be considered as generally the same, also studying the same polymorphisms, this leads to intriguing insights into the similarities of the immunogenetics of both leprosy and tuberculosis. Future research will have to take into account the possibility that discoveries regarding one of these two diseases may be able to translate into the research of the other, and vice versa.

2. Cell culture experiments

As *Mycobacterium leprae* cannot be grown in culture and the best available animal model, the nine-banded armadillo, necessitates extensive animal facilities, cell culture experiments are the tool of choice for elucidating direct impact of the bacteria on the immune system. We believe an activation via *M. leprae* whole cell sonicate (WCS) achieves a reaction of immune cells that is quite close to the one *in situ*. Our particular goals in this study were to identify the activation potential of monocytes and macrophages *in vitro* in the context of leprosy and to estimate the role of macrophage sub-populations in this setting.

2.1 Chapter III

CD62L, or L-Selectin, plays a major role in the leukocyte extravasation [79]. Of particular interest is its involvement in the migration of monocytes into inflamed tissue (Fig. 5). Here they mature into macrophages [130, 131], losing L-Selectin [132].



	Capture	Rolling	Slow Rolling/ Activation	Firm Adhesion/ Crawling	Transmigration/ Chemotaxis
Roll of L-selectin	Mediates capture through binding to endothelial- and leukocyte-expressed ligands	Supports rolling at higher velocities than E- or P-selectin	Functions synergistically with ICAM-1 to reduce leukocyte rolling velocities	Ligation stimulates β_1 and β_2 integrin-dependent adhesion	Ligation enhances chemotaxis to SLC, IL-8, SDF-1α, and PAF, and increases surface CXCR4 expression

Figure 5: The functional roles of L-Selectin in leukocyte recruitment and extravasation by Grailer et al. *[133].*

Due to its properties we chose L-Selectin as a marker of both maturation of monocytes into macrophages as well as monocyte activation. After three days of incubation, our cells showed a marked increase of L-Selectin expression by 30% on their surface when stimulated with WCS. On the one hand, this demonstrates that after three days, monocytes in culture do not turn into macrophages yet, as they still present a great quantity of L-Selectin. On the other hand, it can be assumed that the increase in L-Selectin expression means that *Mycobacterium leprae* WCS has monocyte-migration inducing properties. The increase in L-Selectin could be a direct reaction to the presence of antigen and a first step to initiate tissue-migration. This theory is supported by findings in mice that showed monocytes with high L-Selectin-expression as the first responders to acute inflammation [134]. Further, studies in elderly patients with chronic inflammation found a trade-off between reduced L-Selectin-expression, and thus reduced migration of monocytes, and an increased susceptibility to acute infection [135, 136].

In summary, this underlines the importance of L-Selectin in estimating the activation status of monocytes. It is important to note that our findings could also hint at an up-regulation of monocyte migration as a survival strategy of *M. leprae*, as this would serve to increase the number of macrophages at the infection site. This would leave the bacteria with a greater population of host-cells to infect.

After incubation for six days, a further examination for CD62L expression on the cell surface revealed a strong decrease. For the reasons stated above we considered this as a sign that monocyte maturation into macrophages had taken place. The newly matured monocyte-derived macrophages (MDMs) were incubated with *M. leprae* whole cell sonicate for eighteen hours. This resulted in changes of the internal plasticity as well as the surface expression of CD163. Changes of the internal plasticity of cells can be measured in fluorescence-associated cell sorting by observing the so-called side scatter (SSC). This measures the refraction of light on its way through the cell. The greater the amount of internal structures, the greater is the refraction, and therefore the measured SSC. In our case we found an increase of SSC in MDMs after treatment with whole cell sonicate when compared to untreated controls. The sonicate consists of broken up whole bacterial cells in a solution. It can be assumed that the observed increase in SSC is due to an increase in phagosomes as a result of phagocytosis of the bacterial debris by MDMs. CD163 is a well-known scavenger receptor that in recent years has been identified as an indicator for alternatively activated, i.e. M2, macrophages [71]. It has been documented as being strongly expressed in tissues of lepromatous leprosy patients [137, 138]. A recent study established an in vitro model for granuloma formation in leprosy, similar to the one utilized in our manuscript [139]. An up-regulation of CD163 after activation with live *M. leprae* bacilli occurred here as well.

Moura *et al.* hypothesized that CD163 may function as an alternative route of entry for *M. leprae* and that the observed up-regulation under influence of the bacteria is a mean of supporting the infection via facilitating the entry into host cells [140]. They found significantly higher CD163-mRNA levels in lesions of lepromatous patients than in those of borderline tuberculoid ones. Further, 41.7% of macrophages isolated from lepromatous lesions were shown to be CD163-positive. This is more than our 21.1% increased CD163-expression in stimulated MDMs, but this may be due to the

differences of comparing isolated macrophages from patients and MDMs that were stimulated *in vitro*.

The hypothesis that *M. leprae* utilizes CD163 to its own advantage is lent credence through comparison to the closely related *M. tuberculosis*. Here we find the expected decrease or stagnation in expression during active threat [70, 73]. These two species generally cause similar immune answers and morbidities, yet they show completely opposite effects on the polarization of macrophages. We conclude that it is possible that *Mycobacterium leprae* enhances Macrophage polarization towards M2 populations in order to facilitate invasion via CD163. *Mycobacterium tuberculosis*, where this mode of invasion hasn't been encountered yet, would have no need to influence immunity this way.

Alternatively, as the literature clearly reveals a bias towards M2 polarization on the lepromatous side of the spectrum, it can be assumed that enhancing alternative activity in macrophages over classical, anti-pathogen activity provides an added benefit for the pathogen, as it hinders an effective immune answer. M2 macrophages, more associated to clean up after an infection is over, would be less effective at fighting a leprosy than their classically activated counterparts.

The integral role of M2 polarization in sustaining an ongoing leprosy of the lepromatous type is made even clearer as a successful treatment causes a shift from the lepromatous towards the tuberculoid end of the disease spectrum which is accompanied by a shift from predominantly M2 macrophages towards a predominantly M1 population [137].

Finally, we can state that the influence of the bacteria on macrophage polarization is passive rather than active. This is shown by our own results, where the effect was caused by sonicated bacteria, as well as the literature, where often whole, but dead, bacteria are being used for *in vitro* experiments [140, 141]. It would be logical to assume that surface factors on the bacterial cells are influencing the shift in macrophage populations, as the effect can be seen with whole dead bacteria.

In conclusion we can state that, even though our study population is too small to make statistically significant statements, it serves as an interesting proof of principle about the influence of *Mycobacterium leprae* on L-Selectin and macrophage polarization. Further studies will have to be conducted to support our theses.

3. Microarray of Natural Killer cells

Cell culture experiments utilizing activating antigens are one way to research the interaction of *Mycobacterium leprae* with the human immune system. However, they are still only an approximation of the real events *in situ* and therefore have to be corroborated by additional methods that enable us to see what occurs from different perspectives. One method through which this might be achieved is the transcriptome analysis of immune cells in leprosy patients.

We chose Natural Killer (NK) cells as our target as they occupy a very central role in cellular immunity, killing infected cells and interacting with macrophages in various ways (Fig. 6).

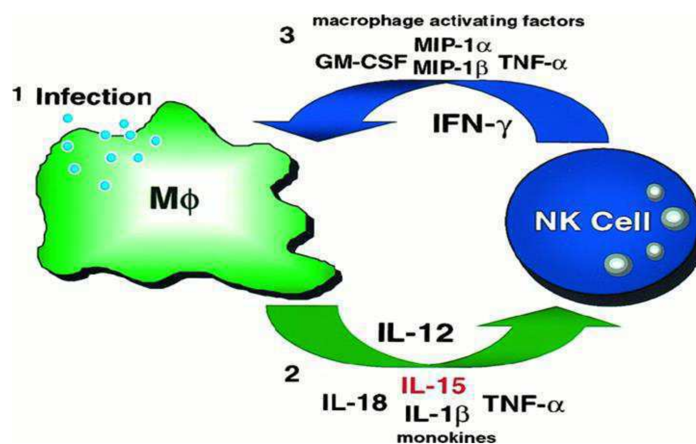


Figure 6: The interaction of macrophages and Natural Killer cells by Fehniger et al. *[142].*

3.1 Chapter IV

This chapter describes a study involving 9 patient samples and 3 healthy controls from the greater Hyderabad city area in India. As the numbers of individuals are comparatively low, any results achieved should be regarded as exploratory groundwork to build on for planning and executing future studies. Figure 7 shows the relationships in expression patterns in between samples in a dendrogram.

Due to the small sample size we decided to discard outliers completely and only concentrate analysis on the groups NK2 + Nk11, NK4 + NK6, and the control group NKK1 + NKK3. The group involving NK12 and NKK2 was not included as it includes a patient and a control sample. This may be because samples were taken after completing a WHO-approved course of antibiotics, so immunity of individual NK12 could have returned to pre-infection levels at this point.

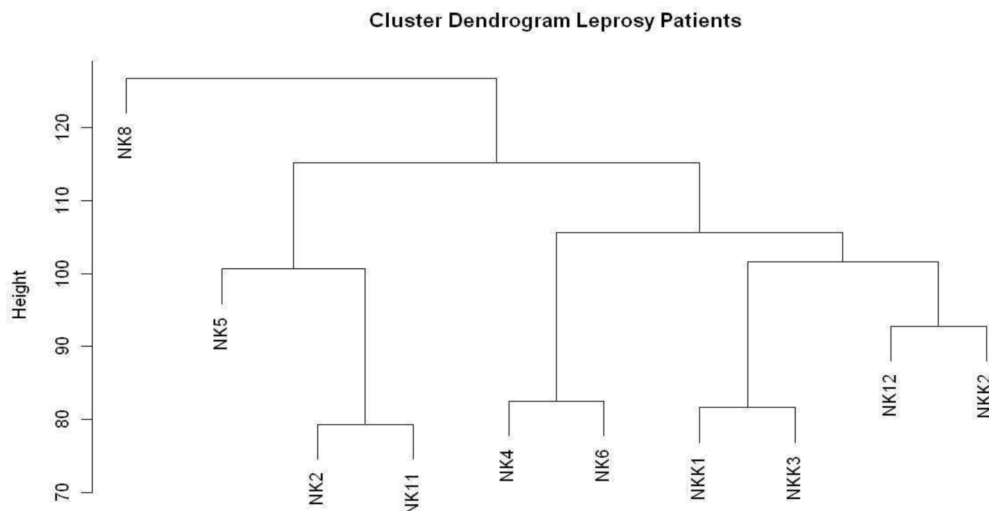


Figure 7: Cluster dendrogram of leprosy patients and controls according to microarray data.

NK2 and NK11 represent the paucibacillary patients, while NK4 and NK6 are both multibacillary. For the remainder of this text they will be referred to as the “paucibacillary” and the “multibacillary” group respectively.

Comparing gene-expression of patients versus controls reveals a number of genes that are differentially expressed, the most prominent one being ***SPP1***. This gene codes for secreted phosphoprotein 1, also known as osteopontin. The protein can act as a cytokine that up-regulates production of interferon gamma and interleukin 12, both great influences in type 1 immunity [143]. Up-regulation in the context of leprosy is therefore not surprising.

We also found that ***LCN2*** was differentially expressed. The gene product, lipocalin 2, has been implicated in anti-bacterial activity, acting as a siderophore [144, 145]. The expression is more pronounced in the paucibacillary than in the multibacillary group, implying a possible role in fighting off *M. leprae* by association to the patient group that generally manages the pathogen better.

It is remarkable that ***APOBEC3***-expression is almost the same as that of *LCN2*. The gene-product, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3, has five sub-groups in humans (Ab, B, C, F, G). We couldn’t identify the sub-group. All APOBEC3 sub-groups are generally associated to retroviral defense [146, 147]. The

fact that we find very similar expression in both the anti-bacterial *LCN2* and *APOBEC3* could hint at an as of now undescribed immunological activity by the latter.

We also compared the paucibacillary with the multibacillary patient groups, utilizing expression in the control group as a baseline. As the number of individuals was small, we opted to only include genes in the analysis that had a fold-change of greater than $\log_3$ when compared to the expression in the control group.

The gene which shows the greatest differences in fold-changes in the two groups is ***CD70***, being strongly down-regulated in the multibacillary and slightly up-regulated in the paucibacillary group. The product of this gene belongs to the tumor necrosis factor (TNF) ligand family and is a ligand for CD27 on T-cells, where the CD70/CD27 interaction is critical for the cell's expansion and survival [148]. CD70 also enhances the direct cytotoxic activity of natural killer and T-cells [149, 150]. The negative fold-change of -2.8 in multibacillary cases, which fail to mount an effective immune answer against the pathogen in leprosy, is of interest in this context.

SIRPG (signal-regulatory protein gamma), with a slight up-regulation (0.9) in the multibacillary group and a strongly negative fold-change of -2.2 in the paucibacillary group, shows a mirror picture of *CD70*. The gene product of *SIRPG* is indicated in regulatory processes, especially with CD47 [151]. CD47 is a central player in, amongst other things, apoptosis and migration.

Also strongly indicated in regulatory processes and up-regulated by a fold-change of 2.1 in the paucibacillary cohort of our study is ***RMRP*** (RNA component of mitochondrial RNA processing endoribonuclease), which has been proven to play a role in cell growth and division [152].

PPBP (pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) is up-regulated 0.6-fold in the multibacillary patients, compared with a down-regulation of -1.7 in the paucibacillary individuals. Derivatives of PBP (platelet basic protein), such as PPBP, have demonstrated an anti-microbial activity in monocytes [153]. PPBP also works as a chemo-attractant for neutrophils [154]. Considering its beneficial role in immunity, the fact that PPBP is down-regulated in paucibacillary individuals, which are considered as having a working immune answer against *M. leprae*, has to be investigated further.

Another gene whose transcription is down-regulated in the paucibacillary group is ***EMR4***. The gene product is a member of the EGF-TM7 receptor gene family, though sequencing results indicate that it may be soluble instead of membrane-bound in humans [155]. It could be a possible mean of G-protein-mediated cellular signaling, a central part of signal-transmission in humans.

In the same context as above, the down-regulation of the ***FPR1*** gene product in the paucibacillary group becomes noteworthy. The negative fold-changes of EMR4 and FPR1 (-1.9 versus -1.8) are very similar, and FPR1 is a member of the G-protein coupled receptor family 1. FPR1 plays a role in natural killer cell-mediated cytolytic activity [156] and in anti-inflammatory activity [157].

KIR2DS3 is a member of the killer cell immunoglobulin-like receptor family and transfers activating signals into the Natural Killer cell. In our study it shows a positive fold-change of roughly 1.9 in the multibacillary group. The gene also plays a role in other pro-inflammatory diseases, such as hepatitis C [158]. Given this information, it appears that transfer of activating signals into natural killer cells works in multibacillary patients and therefore doesn't represent a fault line that is exploited by the bacteria.

Another gene of interest due to its importance in immunity is tumor necrosis factor receptor superfamily, member 9 (***TNFRSF9***). In our study, transcription was up-regulated in paucibacillary patients and the gene product is known for part-taking in the recognition of antigen presenting cells by T- and B-lymphocytes [159]. It has also been implicated in increasing dengue virus-mediated apoptosis in vitro [160].

Conclusions

The preceding chapters represent alternative ways to investigating the interplay of *Mycobacterium leprae* and human immunity in the context of leprosy, circumventing the lack of an *in vitro* model for the pathogen. They show that the bacteria both directly and indirectly influence the host's immune answer and that particularly the Indian population presents very distinct immunogenetics that influence the progress of the disease and make it more receptive.

The genetic association towards leprosy and its progression in the Indian population will have to be intensively studied so that we may understand why the disease proves

so particularly resilient here. Our results hint at a particular role of innate immunity in this context, giving a direction to future research endeavors.

In vitro activation of immune cells via antigens and the careful investigation of patient samples allows us a window into host-pathogen interactions and will help us identifying novel ways to fighting off the disease. Here, again, innate immunity appears as a mayor player beyond its standard role as a defense mechanism, possibly taking over the role as a facilitator of leprosy.

The results presented within this document are only step stones towards identifying how leprosy still persists to such a great degree on the Indian sub-continent, and we will have to further our research based on the results achieved towards more effective personal therapy of the individual and widespread eradication of leprosy.

Personal Contributions

My contributions to the listed papers include:

Chapter I

- Initial ideas and polymorphism selection.
- DNA isolation for the majority of the samples.
- Screening of genetic variants for all single nucleotide polymorphisms.
- Data exploration and statistical analysis in cooperation with Velavan TP.
- Writing the manuscript in cooperation with Velavan TP.

Chapter II

- DNA isolation for the majority of the samples.
- Screening of genetic variants for all single nucleotide polymorphisms.
- Data exploration and statistical analysis.
- Writing the manuscript.

Chapter III

- Ideas and study design.
- Sample collection.
- Cell culture experiments and analysis of results.
- Writing the manuscript.

Chapter IV

- Sample collection.
- Pre-processing for microarray.
- Data exploration in cooperation with Engleitner, T.
- Writing the manuscript.

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Manuscripts

I. LRRK2 and RIPK2 variants in the NOD 2-mediated signaling pathway are associated with susceptibility to Mycobacterium leprae in Indian populations. PLoS One. 2013 Aug 28;8(8):e73103.

II. Genetic evidence of TAP1 gene variant as a susceptibility factor in Indian leprosy patients. Hum Immunol. 2013 Jun;74(6):803-7.

III. Interaction of Mycobacterium leprae with human monocytes increases L-Selectin expression and influences phagocytosis. (*Unpublished manuscript*)

IV. Transcriptome profile of natural killer cells in patients treated with multi-drug-therapy. (*Unpublished manuscript*)

LRRK2 and *RIPK2* Variants in the *NOD* 2-Mediated Signaling Pathway Are Associated with Susceptibility to *Mycobacterium leprae* in Indian Populations

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Abstract

In recent years, genome wide association studies have discovered a large number of gene loci that play a functional role in innate and adaptive immune pathways associated with leprosy susceptibility. The immunological control of intracellular bacteria *M. leprae* is modulated by NOD2-mediated signaling of Th1 responses. In this study, we investigated 211 clinically classified leprosy patients and 230 ethnically matched controls in Indian population by genotyping four variants in *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G), *RIPK2* (rs40457A/G and rs42490G/A). The *LRRK2* locus is associated with leprosy outcome. The *LRRK2 rs1873613A* minor allele and respective *rs1873613AA* genotypes were significantly associated with an increased risk whereas the *LRRK2 rs1873613G major* allele and *rs1873613GG* genotypes confer protection in paucibacillary and leprosy patients. The reconstructed *GA* haplotypes from *RIPK2* rs40457A/G and rs42490G/A variants was observed to contribute towards increased risk whereas haplotypes *AA* was observed to confer protective role. Our results indicate that a possible shared mechanisms underlying the development of these two clinical forms of the disease as hypothesized. Our findings confirm and validates the role of gene variants involved in *NOD2*-mediated signalling pathways that play a role in immunological control of intracellular bacteria *M. leprae.*

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Introduction

Leprosy is a chronic infectious disease of the skin and nerves, caused by the bacterium *Mycobacterium leprae*. Despite decreased prevalence in last two decades, the number of new case detection rates remains high as far as in 130 countries, with India contributing to half of the new cases detected worldwide [1]. Host immune responses [2,3] and genetic factors had been shown to influence the clinical spectrum of leprosy [4–7]. Most notable is inter individual variability in disease development, with a wide range of manifestations ranging from lepromatous to tuberculoid leprosy [8]. The lepromatous leprosy is distinguished as borderline lepromatous (BL) and lepromatous (LL) forms and is classified as multibacillary (MB) based on the bacillary load, whereas the tuberculoid leprosy is distinguished as tuberculoid (TT) and borderline tuberculoid (BT) and are classified as paucibacillary (PB) by WHO standards. A difference in immune responses between multibacillary and paucibacillary forms are predicted. In multibacillary or lepromatous forms, the absence of Th1 responses increases the bacilli load with strong humoral immunity [4]. On the other hand, the paucibacillary or the tuberculoid forms reveal an increased Th1 response with limited bacterial load.

The intracellular *M. leprae* has an extended incubation period for up to 30 years. The pathogen driven selection can potentially alter the primed sequence and can direct to substantial changes in gene expression [9]. The pathogen recognition receptors such as *TLRs* (Toll-like receptors) ably recognize the microbes at cell surfaces, whereas PRRs such as nucleotide oligomerization domain (*NOD*) like receptors that are localized in the cytosol can sense and recognize the intracellular pathogens [10]. A recent genome wide study in Chinese leprosy patients has provided vital insights on the role of *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G) and *RIPK2* (rs40457A/G and rs42490G/A) variants in regulating the leprosy infection [7]. In addition, the expression of these genes has been shown to up regulate in leprosy in comparison to normal tissues [11]. The Nucleotide-binding oligomerization domain 2 (*NOD2*) located on the long arm of chromosome 16

(16q21) is an intracellular microbial sensor for muramyl dipeptide, a component of bacterial peptidoglycan [12]. Dysregulation in *NOD2* signalling is associated with pathogenesis of many inflammatory disorders [13] and is also associated with triggering of *IL-32* dependent dendritic cell programming in leprosy [14]. The cytosolic pattern recognition receptor *NOD1* and *NOD2* also activates the *RIPK2* gene [15]. The gene encoding receptor- interacting serine-threonine kinase 2 (*RIPK2*) located on the long arm of chromosome 8 (8q21) is essential for signaling through the Toll-like receptors [15,16]. In addition, the *RIPK2* interaction with NOD2 enhances NF-κB activity making it an important player in cellular immune response [17]. The Leucine-rich repeat serine/threonine-protein kinase 2 (*LRRK2*) variants located on the long arm of chromosome 12 (12q12) are well documented as a common cause for parkinson disease [18]. Also *LRRK2* gene variants were also documented for their role in inflammatory diseases [19] and to microbial infections [20,21]. During onset of early leprosy infection, *M. leprae* antigens are presented to $CD4^{+}$T cells, which activate the Th1 responses resulting in interferon gamma production leading to macrophages maturation and subsequent killing. *NOD2* and *RIPK2* regulate the interferon- gamma production [7].

Initiation of NOD2 signalling is mediated by *RIPK2* by an ubiqutination process and involvement of TAK1 and nuclear factor-κB essential modulator to the NOD2-RIPK2 complex leads to the movement of NF-κB to the nucleus and subsequent activation of NF-κB target genes [22]. NOD2-mediated signaling pathway plays an essential role in the immunological control of intracellular bacteria. We investigated the possible association of gene variants *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G) and *RIPK2* (rs40457A/G and rs42490G/A) that are vital for NOD2 signalling and subsequent activation of the NF-kB complex in a cohort of clinically classified leprosy patients.

Materials and Methods

Ethical Statement

Informed written consent was received from all leprosy patients. The study was approved by the research advisory committee and institutional ethical committee of LEPRA- Blue peter public health research centre, Hyderabad, India. Informed written consent was also obtained from all the normal individuals and the institutional ethical committee of CCMB has approved this study.

Sampling

All the leprosy patients studied (n = 211) were outpatients and were recruited at the LEPRA- Blue Peter Public Health and Research Centre (BPHRC) in Hyderabad, India [23]. Patients were clinically evaluated and graded by the physicians either as a paucibacillary (PB, n = 74) or multibacillary (MB, n = 137) group, based on WHO standards [24]. Based on the number of lesions and presence of acid-fast bacilli (AFB) in skin slit smears taken from at least five different places of the body (both earlobes, both halves of the forehead, at least one from one of the lesions) leprosy patients were classified. More than five lesions as well as presence of acid fast bacilli in any of the smears precludes a multibacillary diagnosis, while a number of less than five lesions and, more importantly, a lack of acid fast bacilli in any of the smears is considered as the paucibacillary presentation. In addition to the patients, blood samples from individual controls (n = 230) were collected from adult males and females (18–35 years) form the same ethnicity.

Genotyping

DNA was isolated from blood utilizing the DNeasy Blood and Tissue kit (Qiagen, Germany) following the protocol of the manufacturer. The primer sequences utilized for genotyping with primer specific annealing temperatures is summarized in Table 1. In brief: PCR was carried out in a 20 µl reaction volume with 5 ng of genomic DNA, 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM of MgCl2; Qiagen), 0.125 mM of dNTPs, 0.5 mM of each primer and 1 U Taq DNA polymerase (Qiagen, Hilden, Germany) on a PTC-200 Thermal cycler (MJ Research, USA). Thermal cycling parameters for amplification were: initial denaturation at 94°C for 5 min, followed by 35 cycles of respectively 15 sec at 94°C for denaturation, 60 sec at primer specific annealing temperature, and 60 sec at 72°C extension. This was followed by a final extension of 10 min at 72°C. PCR products were cleaned up using Exo-SAP-IT (USB, Affymetrix, USA) and 1 µl of the purified product were directly used as templates for sequencing, using the BigDye terminator v. 2.0 cycle sequencing kit (Applied Biosystems, USA) on an ABI 3130 XL and ABI 3730×L DNA sequencer, according to the manufacturer's instructions. Polymorphisms were identified by assembling the sequences with respective reference sequences obtained from SNPper database (http://snpper.chip.org) using Codon code Aligner 4.0 software (http://www.codoncode.com/) and were reconfirmed visually from their respective electropherograms.

Statistical Analysis

Data was analyzed using STATA and the level of significance was set to a p-value of <0.05. The distribution of genotypes between control and leprosy patients, as well as between controls and clinically classified patients were analyzed by two tailed fisher exact tests. Genotype or haplotype frequencies were analyzed by simple gene counting and expectation-maximum (EM) algorithm and the significance of deviations from Hardy-Weinberg equilibrium was tested using the random-permutation procedure as implemented in the Arlequin v. 3.5.1.2 software. (http://lgb.unige.ch/arlequin). The comparison of Linkage disequilibrium (LD) for *RIPK2* variants for each patient group as well as for controls were computed using the Haploview v4.2 software that utilizes a default algorithm and this algorithm ignores markers with minor allele frequencies (MAF) <0.05.

Results

The observed distribution of genotypes and alleles in both clinically classified patients and controls were summarized in Table 2. The observed genotype and allele frequencies of all studied SNPs in clinically classified patient groups and controls were in Hardy-Weinberg equilibrium ($P>0.05$) except for the *NOD2* SNP rs9302752 ($P<0.05$). Therefore the *NOD2* SNP rs9302752 was excluded for further association analysis.

In *LRRK2* gene locus, we observed that the minor allele *LRRK2 rs1873613A* and homozygous genotype *rs1873613AA* were more frequent in leprosy patients than in controls conferring an increased risk of leprosy (OR = 1.7, 95% CI = 1.25–2.2, P = 0.0003 and OR = 2.04, 95% CI = 1.2–3.6, P = 0.007, respectively). Whereas major allele *LRRK2 rs1873613G* and homozygous genotype *rs1873613GG* were observed less frequently in patients compared to controls conferring a decreased risk of leprosy (OR = 0.61, 95% CI = 0.45–0.8, P = 0.0003 and OR = 0.56, 95% CI = 0.37–0.83, P = 0.0028, respectively). When clinically classified paucibacillary (PB) patients were compared to controls, a similar trend was observed with a stronger significance (for the minor allele *rs1873613A*: OR = 2.77, 95% CI = 1.9–4.1,

Table 1. Investigated SNP variants in leprosy patients and controls.

SNP ID	Locus	Gene	SNP	Primer Pairs (5′–3′)	Tm [°C]
rs9302752	16q21	*NOD2*	A/G	F: GCCTTTGTTTTCGCAGTTCCTTCAG	55
				R: CCTCGGTGACCACTTCTCTGCATTC	
rs1873613	12q21	*LRRK2*	A/G	F: CACCCAAGACACACAAGGAAAAAGCATATA	55
				R: GCCTTCTTACGTTTTACCTCCCCCTCTT	
rs40457	8q21	*RIPK2*	A/G	F: GATTTTCCCCCAGAAGAAGG	50
				R: GCAGGAAAATGAATCCATGA	
rs42490	8q21	*RIPK2*	G/A	F: ACCCACTTCCTCCCTACCACAATCTG	55
				R: GCGGAATAGCTGGATCTCTCACACA	

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$P<0.0001$; for the homozygous genotype *rs1873613AA*: OR = 4.1, 95% CI = 2.1–7.9, P = $P<0.0001$). These results may confer that the *LRRK2 rs1873613A/G* contributed to the progression of paucibacillary leprosy. However, there was no significant difference of allele and genotype frequencies in comparison to multibacillary (MB) patients with controls (Table 2). In addition, we did not observe any significant difference of heterozygous genotype frequency in all comparisons.

Two loci of *RIPK2* gene (rs40457A/G and rs42490G/A) were investigated in this study. However, no significant difference of allele and genotype frequency of neither SNP rs40457A/G or rs42490G/A was observed in all the comparisons. In leprosy patients, both the studied *RIPK2* variants were observed to be in high LD [Leprosy patients (D′ = 0.64, LOD = 12.72, r^2 = 0.2), MB patients (D' = 0.53, LOD = 5.84, r^2 = 0.56), PB patients (D′ = 0.84, LOD = 7.58, r^2 = 0.34)], whereas in controls it was observed in a low degree of linkage disequilibrium (D′ = 0.38, LOD = 3.2, r^2 = 0.06)]. We reconstructed haplotype based on these two studied *RIPK2* SNPs (rs40457A/G and rs42490G/A). Four haplotypes and their frequencies were observed including *AG*, *AA*, *GA* and *GG*. The *RIPK2* haplotype *GA* was observed more frequently in leprosy patients compared to controls inferring an increased risk of leprosy (OR = 1.46, 95% CI = 1.02–2.1, P = 0.036), whereas *RIPK2* haplotype *AA* was observed less frequently in leprosy patients compared to controls inferring a protection against leprosy (OR = 0.69, 95% CI = 0.49–0.97, P = 0.028). In addition, *RIPK2* haplotype *GA* was observed more frequently in paucibacillary (PB) patients in comparison to controls inferring an increased risk of leprosy (OR = 1.8, 95% CI = 1.1–2.8, P = 0.018). Furthermore, there was no significant difference of *RIPK2* haplotype frequencies in comparison between multibacillary (MB) patients with controls (Table3).

Discussion

All the four studied innate immune gene variants were hypothesized to play a significant role in controlling the interferon-gamma production and considered as vital modulators for NF-κB [7]. We investigated the role of gene variants in *NOD2* (rs9302752A/G), *LRRK2* (rs1873613A/G) and *RIPK2* (rs40457A/G and rs42490G/A) based on a recently published genome-wide association study (GWAS) that utilized Han Chinese population [7]. Although the studied sample size was lower, the frequencies of all four studied variants were in accordance with Han Chinese population and also in a Gujarati Indian population as reported in HapMap database. The minor allele of *NOD2* rs9302752A/G variant was observed at a similar frequency (0.28) in the studied Indian population to reported frequencies in Han Chinese population (0.22). However, the *NOD2* rs9302752A/G variant was not in Hardy-Weinberg equilibrium in Indian clinically classified leprosy patients and marginally significant in control group. The major allele of *LRRK2* rs1873613A/G variant was also observed at a similar frequency (0.68) compared to reported frequencies in Gujarati Indians in Houston, Texas (GIH) (0.61) as reported in the NCBI Hapmap database. The minor allele of studied *RIPK2* variant rs40457A/G was observed at a similar frequency (0.21) when compared to reported frequencies in Han Chinese (0.25) and in Gujarati Indians in Houston, Texas (GIH) (0.30), whereas the minor allele of *RIPK2* rs42490A/G was observed at a similar frequency (0.41) when compared to reported frequencies in Han Chinese (0.45) and in Gujarati Indians in Houston, Texas (GIH) (0.40).

NOD2 is an intracellular microbial sensor of the innate immune system that can act as a potent activator and regulator of inflammation in mycobacterial infections [10,25]. Mutations in the gene encoding *NOD2* in humans have been associated with Crohn's disease (CD) [26], Blau syndrome (BS) [27], and early onset sarcoidosis (EOS) [28]. In addition, the studied *NOD2* variant (rs9302752A/G) was associated with susceptibility to tuberculosis and leprosy in Chinese and Vietnamese population [7,29,30]. NOD2 signaling pathways are activated by a CARD effector domain that causes inflammation by the activation of NF-kB and MAP kinase pathways [10]. Studies have documented the fact that stimulation of NOD proteins are associated with enhanced pro inflammatory cytokine production to *M. leprae* infections [31] and this is well achieved by the recognition of conserved microbial domains by PRRs. Therefore, any alteration in the gene function of PRR domain may possibly reflect towards leprosy susceptibility. Also it was shown that phenotypes of mouse deficient with *NOD2* and *RIPK2* revealed a failure to produce inflammatory cytokines to initiate Th1 responses [32]. In contrast to Han Chinese population [7] we observed that *NOD2* rs9302752A/G variant was not in Hardy-Weinberg equilibrium in studied Indian population, therefore the contribution of this variant to leprosy susceptibility remains unclear. The possibilities for the studied NOD2 variant not in HWE can be due to the fact that Indian populations have been following strict endogamy marriage practices for last tens of thousands years, hence every population acquired unique set of genetic variations. In this study, the leprosy patients represent different ethnic groups from south India and the number from each group is very less. Nevertheless in this study, we always employed ethnically matched control individuals from the same population to avoid the role of

Table 2. Distribution of investigated *NOD2*, *RIPK2*, and *LRRK2* variants in clinically classified leprosy patients and controls.

Loci	Patients n = 211(%)	MB (LL+LB) n = 137(%)	LL n = 62 (%)	LB n = 75 (%)	PB (BT+TT) n = 74 (%)	BT n = 70 (%)	TT n = 4 (%)	Controls n = 230 (%)	Patients vs. Controls		MB vs. Controls		PB vs. Controls	
									OR (95% CI)	*P value*	OR (95% CI)	*P value*	OR (95%C I)	*P value*
NOD2_rs9302752														
AA	120(56.9)	84 (61.3)	52(83.9)	32 (42.7)	36 (48.6)	34 (48.6)	2 (50)	114(49.6)		NA		NA		NA
AG	58 (27.5)	32 (23.4)	9 (14.5)	23 (30.7)	26 (35.1)	25 (35.7)	1 (25)	104(45.2)		NA		NA		NA
GG	33 (15.6)	21 (15.3)	1 (1.6)	20 (26.6)	12 (16.2)	11 (15.7)	1 (25)	12 (5.2)		NA		NA		NA
A	298(70.6)	200(73)	113 (91)	87 (58)	98 (66.2)	93 (66.4)	5 (62.5)	332(72.2)						
G	124(29.4)	74 (27)	11 (9)	63 (42)	50 (33.8)	47 (35.6)	3 (37.5)	128(27.8)		NA		NA		NA
LRRK2_rs1873613														
GG	73 (34.6)	56 (40.9)	27(43.6)	29 (38.7)	17 (23)	16 (22.8)	1 (25)	112(48.7)	0.56 (0.37–0.83)	0.0028		NS	0.31 (0.16–0.6)	0.0001
AG	93 (44.1)	62 (45.2)	25(40.3)	37 (49.3)	31 (41.9)	30 (42.9)	1 (25)	91 (39.6)		NS		NS		NS
AA	45 (21.3)	19 (13.9)	10(16.1)	9 (12)	26 (35.1)	24 (34.3)	2 (50)	27 (11.7)	2.04 (1.2–3.6)	0.007		NS	4.1 (2.1–7.9)	0.000014
G	239(56.6)	174 (63.5)	79(63.7)	95 (63.3)	65 (43.9)	62 (44.3)	3 (37.5)	315(68.5)	0.61 (0.45–0.8)	0.0003		NS	0.36 (0.240.54)	0.00000017
A	183(43.4)	100 (36.5)	45(36.3)	55 (36.7)	83 (56.1)	78 (55.7)	5 (62.5)	145(31.5)	1.7 (1.25–2.2)	0.0003		NS	2.77 (1.9–4.1)	0.00000017
RIPK2_rs40457														
AA	117(54.5)	77 (56.2)	32(51.6)	45 (60)	40 (54)	37 (52.9)	3 (75)	144(62.6)		NS		NS		NS
AG	79 (37.4)	50 (36.5)	25(40.3)	25 (33.3)	29 (39.2)	28 (40)	1 (25)	74 (32.2)		NS		NS		NS
GG	15 (7.1)	10 (7.3)	5 (8.1)	5 (6.7)	5 (6.8)	5 (7.1)	0	12 (5.2)		NS		NS		NS
A	313(74.2)	204 (74.5)	89(71.8)	115(76.7)	109 (73.6)	102(72.9)	7 (87.5)	362(78.7)						
G	109(23.8)	70 (25.5)	35(28.2)	35 (23.3)	39 (26.4)	38 (27.1)	1 (12.5)	98 (21.3)		NS		NS		NS
RIPK2_rs42490														
GG	82 (38.9)	55 (40.1)	23(37.1)	32 (42.7)	27 (36.5)	26 (37.1)	1 (25)	85 (37)		NS		NS		NS
GA	90 (42.7)	59 (43.1)	23(37.1)	36 (48)	31 (41.9)	28 (40)	3 (75)	104(45.2)		NS		NS		NS
AA	39 (18.4)	23 (16.8)	16(25.8)	7 (9.3)	16 (21.6)	16 (22.9)	0	41 (17.8)		NS		NS		NS
G	254(60.2)	169 (61.7)	69(55.6)	100(66.7)	85 (49.2)	80 (57.1)	5 (62.5)	274(59.6)						
A	168(39.8)	105 (38.3)	55(44.4)	50 (33.3)	63 (50.8)	60 (42.9)	3 (37.5)	186(40.4)		NS		NS		NS

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Table 3. Distribution of investigated RIPK2 haplotypes in clinically classified leprosy patients and controls.

RIPK2 haplotype (rs40457/rs42490)	Patients n = 422 (%)	MB (LL+LB) n = 274(%)	LL n = 124(%)	LB n = 150(%)	PB (BT+TT) n = 148(%)	BT n = 140(%)	TT n = 8(%)	Controls n = 460 (%)	Patients vs. Controls		MB vs. Controls		PB vs. Controls	
									OR (95% CI)	*P value*	OR (95% CI)	*P value*	OR (95% CI)	*P value*
AG	234 (55.5)	152 (55.5)	66 (53.2)	86 (57.3)	82 (55.4)	77 (55.0)	5 (62.5)	247 (53.7)		NS		NS		NS
AA	79 (18.7)	52 (19.0)	23 (18.6)	29 (19.3)	27 (18.3)	25 (17.9)	2 (25.0)	115 (25.0)	0.69 (0.49–0.97)	0.028		NS		NS
GA	89 (21.1)	53 (19.3)	32 (25.8)	21 (14.0)	36 (24.3)	35 (25.0)	1 (12.5)	71 (15.4)	1.46 (1.02–2.1)	0.036		NS	1.8 (1.1–2.8)	0.018
GG	20 (4.7)	17 (6.2)	3 (2.4)	14 (9.4)	3 (2.0)	3 (2.1)	0	27 (5.9)		NS		NS		NS

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population stratification in allelic difference between cases and controls.

For the investigated *LRRK2* rs1873613A/G variant the contribution was observed of this particular variant in the studied Indian population. The minor allele *A* and homozygous genotype *AA* contributed towards an increased risk of leprosy whereas the major allele *G* and homozygous genotype *GG* predisposed as protective factors for leprosy. Our results also showed that *LRRK2* rs1873613A/G variant significantly contributed to the development of paucibacillary leprosy but not multibacillary leprosy. Earlier studies have demonstrated that a trend towards an association between the variant and susceptibility to leprosy [7]. The association study of *LRRK2* rs1873613A/G variant with leprosy was also conducted in a Vietnamese population, however the association was not statistically significant [30]. *LRRK2* is associated with various diseases, including Parkinson's disease, cancer, and leprosy [33] and also known to be associated with susceptibility to the chronic autoimmune Crohn's disease, which is an inflammatory disorder [34]. Higher expression of LRRK2 is observed in macrophages and monocytes revealing its significance in the innate immune system [35] and in leprosy per se. Most of the replication studies do not document similar effects of a particular gene variant contribution when investigated in different ethnicities [36–38]. In the reported GWAS study from Han Chinese population, the *LRRK2 rs1873613A* allele was observed as a major allele whereas the *rs1873613G* variant is a minor allele and confers decreased risk towards leprosy in Chinese patients. [7]. Additionally similar allele frequencies were reported in the Vietnamese population [30]. However in the studied Indian population, the *LRRK2 rs1873613G* allele was observed as a major allele whereas the *rs1873613A* variant increased the risk towards leprosy in the investigated Indian cohort. There seems a switch of allele frequencies between populations and the clinical significance of the studied variant likely depends on the context of the studied ethnicities. Additionally India is inhabited by the very first out-of-Africa modern human about 65,000 years ago. Since then they remain unmixed, therefore the genome of Indian populations are unique and the allele frequency differ significantly when compare to the rest of the world [39]. *LRRK2* gene has been shown to play an important role in different diseases including Parkinson, Crohn's and inflammatory diseases [18,19,21,33]. Interestingly, LRRK2 has been also demonstrated to be an IFN-gamma target gene, involve in different immune response signaling such as NF-kB pathways, and contribute to the antibacterial activity of the macrophages, in which LRRK2 plays a role in the killing of intracellular bacteria such as *S. typhimurium* [20]. In addition, the expression of *LRRK2* gene was significantly higher in leprosy compared to normal tissues [11]. This suggested that LRRK2 might contribute crucially to the immune response against intracellular bacteria *M. leprae*.

The *RIPK2* rs40457A/G and rs42490G/A variants did not confer any significance results at allele and genotype levels. A larger sample size will be required in order to detect any significance for the both studied *RIPK2* variant at allele level. We observed the reconstructed haplotypes *AA* and *GA* based on two variants rs40457A/G and rs42490G/A to be significantly associated with leprosy susceptibility. In addition, the haplotype *GA* also conferred to an increased risk of paucibacillary leprosy development. Recently, a replicate study conducted in a Vietnamese population showed that the variant *RIPK2* rs42490G/A was significantly associated with leprosy whereas the significance was not revealed for the variant *RIPK2* rs40457A/G [30].Studies have established the fact that RIPK2 interaction with NOD2 enhances NF-κB activity making it an important

player in cellular immune response [17]. A recent study has also documented on the interaction between the *NOD2* and *RIPK2* loci (NOD2-RIPK2 complex) in activating the NF-κB pathway as a part of the host defence response to leprosy infection [40]. Therefore, our results indicate that a possible shared mechanisms as a basis for the development of these two clinical forms of the disease as hypothesized earlier [7].

In conclusion, our study validated the association of gene variants involved in intracellular sensing that are believed to play a role in immunologic control of intracellular bacteria *M. leprae* in Indian leprosy patients. Overall the study increases our understanding on complex molecular and cellular mechanisms that are regulated by the intracellular pathogen *M. leprae* during its clinical course.

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Author Contributions

Conceived and designed the experiments: KT TPV VLV. Performed the experiments: PM ANJ VS AS NCS SKN HVT RR. Analyzed the data: HVT TPV. Contributed reagents/materials/analysis tools: VB KT TPV. Wrote the paper: PM TPV HVT.

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Genetic evidence of *TAP1* gene variant as a susceptibility factor in Indian leprosy patients

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The heterodimeric transporter associated with antigen processing (*TAP*) gene loci is known to play a vital role in immune surveillance. We investigated a possible association of gene polymorphisms both in *TAP1* and *TAP2* in a cohort of clinically classified leprosy patients ($n = 222$) and in ethnically matched controls ($n = 223$). The *TAP1* and *TAP2* genes were genotyped for four single nucleotide polymorphisms *TAP1* (rs1057141 Iso333Val and rs1135216 Asp637Gly) and *TAP2* (rs2228396 Ala565Thr and rs241447 Ala665Thr) by direct sequencing and ARMS-PCR. The minor allele of *TAP1 637G* contributes to an increased risk to leprosy compared to controls (OR: 1.68, 95% CI 1.2–2.36, $P = 0.0057$). An increased risk for the variant minor allele of the *TAP1 637G* to multibacillary (BL + LL) or paucibacillary (BT + TT) infections was also observed [multibacillary vs. controls (OR: 1.56, 95% CI 1.07–2.28, $P = 0.054$); paucibacillary vs. controls (OR: 1.92, 95% CI 1.21–3.01, $P = 0.013$)]. In the dominant model, the genotypes of the *TAP1* rs1135216AG + GG additionally contributed to an increased risk. Overall our findings demonstrate that the *TAP1* gene variant (rs1135216 Asp637Gly) influences the susceptibility to clinically classified leprosy patients in Indian population.
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1. Introduction

Leprosy is caused by an obligate intracellular pathogen *Mycobacterium leprae.* The clinical presentation is governed by various host attributes, which presents leprosy over a five point Ridley–Jopling spectrum with stable lepromatous (LL) and tuberculoid (TT) at the polar ends and unstable Borderline tuberculoid (BT), Borderline (BB) and Borderline lepromatous (BL) between the polar ends. By standards of WHO classification, all BL and LL individuals represented as multibacillary (MB) patients, whereas the BT and TT individuals represented the paucibacillary (PB) patients. Susceptibility to leprosy upon exposure to the pathogen is far limited to 1% [1] and host genetic factors had been shown to influence the infection of *M. leprae* and its clinical outcome [2,3].

The pathogen driven selection on immune genes can potentially alter the primed sequence in the promoter regions and can direct to substantial changes in gene expression [4]. Family studies, segregation analyses, and twin studies have provided evidence that, host genetic factors influence the susceptibility to leprosy [5–7]. Recent studies have demonstrated that gene variants in the chromosome 6 and 10 are linked to susceptibility to leprosy [8–10,1]. In addition, candidate gene analysis and genome wide studies have implicated various variants in the *HLA* locus and SNPs (single nucleotide polymorphisms) in genes regulating the cytokines influence susceptibility or resistance towards the infection [8,11,12]. In particular, a variant in the promoter region of the TNF-α gene has revealed contradictory results within populations [6,13–16]. A recent study from India reported a strong association of SNP variants located in *BAT1*, *LTA*, *TNF* genes and *BTNL2-DRA* interval within *HLA* (6p21.3) with leprosy susceptibility in two independent sets of North Indian population which was replicated in a geographically distinct East Indian population [17]. The first genome wide linkage scan from India mapped leprosy susceptibility to chromosome 10p13 followed by further studies indicating the association of this locus with tuberculoid leprosy [9,10,1].

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The *MRC1* gene encoding the human mannose receptor (MR) is located in the 10p13 region and non-synonymous SNP in exon 7 of the *MRC1* have been suggested as leprosy a susceptibility factor [18]. The SNPs in the immunomodulatory gene Interleukin-10 (*IL10-819C/T*) promoter has been suggested to be associated with leprosy susceptibility in Brazil and India [15,19,20]. In addition, chromosome 20p12, 6q25 have been reported to be associated with susceptibility to leprosy [20,21]. Investigation of such SNPs in the immune genes will possibly reflect the level of susceptibility to any infectious disease. One such gene of interest is Transporter associated with antigen presentation (*TAP*) gene locus that map to the long arm of chromosome 6 (6p21.3).

The delivery of major histocompatibility complex (MHC) class I molecules from cytosol to the endoplasmic reticulum is facilitated by transporter associated with antigen processing (TAP), a heterodimer composed of two subunits *TAP1* and *TAP2*. Earlier studies suggested that polymorphisms in the immune genes such as human leukocyte antigen (*HLA*), mannose binding lectin (*MBL2*), lymphotoxin-α (*LT-α*), ficolin-2 (*FCN2*) and Interferon-gamma receptor-1 (*IFNγR1*) are significantly associated to leprosy outcome [2,22–25]. In addition, polymorphisms in the *TAP* genes are known to be associated with other diseases including autoimmune diseases such as ankylosying spondilytis, rheumatoid arthritis and in tuberculosis [26–29]. Earlier studies on *TAP* gene polymorphisms have been associated to tuberculosis [29,30,27]. Since the causative organism of both leprosy and tuberculosis represent the same genus *Mycobacterium*, we aim to investigate the association of *TAP1* (rs1057141 Iso333Val and rs1135216 Asp637Gly) and *TAP2* (rs2228396 Ala565Thr and rs241447 Ala665Thr) gene polymorphisms to the infection outcome in Indian leprosy cohort with clinically classified patient groups.

2. Materials and methods

2.1. Study subjects

Leprosy patients ($n = 222$) reporting to LEPRA projects, BPHRC-Blue Peter Public Health Research Centre were enrolled for the study. Individuals were characterized either as Borderline (BL) [$n = 80$, Male/Female ratio = 61/19, Mean age = 36.9 ± 14.1], Lepromatous (LL) [$n = 66$, Male/Female ratio = 51/15, Mean age = 58.1 ± 14.5], Borderline Tuberculoid (BT) [$n = 73$, Male/Female ratio = 32/41, Mean age = 33.3 ± 12], and Tuberuculoid (TT) [$n = 3$, Male/Female ratio = 1/2, Mean age = 44 ± 6.5], based on their clinical manifestations as graded by the physicians by slit skin smear examination, histopathology and routine diagnostic procedures. All recruited individuals are further grouped on WHO classification either as paucibacillary or multibacillary patients. All BL and LL individuals represented the mutibacillary group [$n = 146$, Male/Female ratio = 112/34, Mean age = 40.4 ± 16.1], whereas BT and TT individuals represented the paucibacillary group [$n = 76$, Male/Female ratio = 33/43, Mean age = 45 ± 18.8]. A total of 223 ethnically matched control individuals from the same socio-ethnic and geographical region have been utilized from DNA data bank of the Centre for Cellular and Molecular Biology [Male/Female ratio = 148/75, age: 30–55]. None of them had family history of leprosy or tuberculosis or other related disorders. Informed written consent was obtained from all study subjects. The study was approved by the Institutional Ethical Committee (IEC) of LEPRA-BPHRC.

2.2. Genotyping

2.2.1. Direct sequencing

DNA was extracted using Qiagen DNeasy kit (Qiagen, Hilden, Germany) following manufacturer's instructions. For the TAP1 gene locus, the SNP #rs1057141 (A/G) in exon 4 and #rs1135216 (A/G) in exon 10; whereas for TAP2 gene locus the SNP #rs2228396 (G/A) and the SNP #rs241447 in exon 10 and were genotyped by direct sequencing. The primer pairs utilized for TAP1_rs1057141 was 333F: 5′-CCCTGGCATCCTGGCTCATTGTTAG-3′ and 333R: 5′-AGCGGGCCAACTCCATGAACATAC-3′ whereas for TAP1_rs1135216 was 637F: 5′-CCCCTATCCAGCTACAACCGTCAGA-3′ and 637R: 5′-GAAGCAAGATTGGGTGGGATATAGC-3′. For the TAP2 loci, the primer pairs utilized were TAP2_rs2228396 was 565F: 5′- GCCCCTGTCCCTGCTGCAC-3′ and 565R: 5′-GGGCAGAG-GAACAGCAAACATCAAG-3′ whereas for TAP2_rs241447 was 665F: 5′-GCCTTTATCTACTGCCCTTTCCTACCT-3′ and 665R: 5′-GGCCGCA-CAGCTCTAGGGAAACTCA-3′. In brief: 10 ng of genomic DNA was amplified in a 20 μl volume of reaction mixture containing 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM of $MgCl_2$), 0.125 mM of dNTPs, 0.25 mM of each primer and 1 U Taq DNA polymerase (Qiagen, Hilden, Germany) on a PTC-200 Thermal cycler (MJ Research, USA). Thermal cycling parameters for amplification of both *TAP1* and *TAP2* loci were: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C denaturation, 45 s at loci specific annealing temperature (60 °C for TAP1_rs1057141, TAP1_rs1135216, TAP2_rs2228396 and 65 °C for TAP2_rs241447), 1 min 30 s at 72 °C extension, followed by a final extension of 2 min at 72 °C. PCR products were cleaned up using Exo-SAP-IT (USB, Affymetrix, USA) and 1 μl of the purified products were sequenced using the BigDye terminator v. 1.1 cycle sequencing kit (Applied Biosystems, USA) and ABI 3130XL and ABI3730 DNA sequencer, according to the manufacturer's instructions. Polymorphisms were identified by assembling the sequences with respective reference sequences (for TAP1: NM_000593 and for TAP2: NM_018833) using Codon code Aligner 4.0 software (http://www.codoncode.com/) or AutoAssembler (Applied Biosystems, USA) and were reconfirmed visually from their respective electropherograms.

2.3. ARMS PCR (Amplification refractory mutation system)

Initially we employed a tetra primer amplification refractory mutation system – PCR (ARMS-PCR) using previously published primer pairs were performed to genotype four SNP variants in the *TAP1* and *TAP2* gene loci [31] in one hundred controls and one hundred patients. For the *TAP1* gene locus, the SNP #rs1057141 (A/G) in exon 4 and #rs1135216 (A/G) in exon 10; whereas for *TAP2* gene locus the SNP #rs2228396 (G/A) and the SNP #rs241447 in exon 10 were genotyped by ARMS-PCR. Thermal cycling parameters and programming conditions were 95 °C for 1 min followed by 30 cycles of 95 °C for 90 s, appropriate annealing for 90 s (68 °C for *TAP1* and 60 °C for *TAP2*) and 72 °C for 60 s and a final extension at 72 °C for 10 min: Amplicons were separated on a 2% agarose gel by applying a constant voltage of 90 V. To reconfirm for their reproducibility for SNPs, fifty individual samples each from control and patient group were sequenced using the BigDye terminator v. 2.0 cycle sequencing kit (Applied Biosystems, USA) on an ABI 3130 DNA sequencer, according to the manufacturer's instructions.

2.4. Statistical analysis

The sample size was calculated using with OpenEpi program (http://www.openepi.com) with 80% power and 95% confidence interval. Statistical analysis was performed using Intercooled Stata v. 9.1 (STATA Corporation, Texas, USA) to determine the differences in allele frequencies and genotype distributions. The level of significance was set at $P < 0.05$. In addition, different genetic models including dominant (major genotype vs. heterozygous + homozygous minor genotype), recessive (major genotype + heterozygous vs. homozygous minor genotype) and allelic model were

investigated to detect any possible associations of then studied variants between clinically classified patients compared to controls. *P-values* were corrected for multiple comparisons using Bonferroni procedure. Genotype frequencies were determined by simple gene counting and by using the expectation-maximum (EM) algorithm. The significance of deviation from Hardy–Weinberg equilibrium was tested using the random-permutation procedure as implemented in the Arlequin v. 3.5.1.2 software [32].

3. Results

Two of the observed locus (one in *TAP1* rs1057141 and one in *TAP2* rs241447 Ala665Thr) was not in Hardy–Weinberg equilibrium. Hence these two loci (TAP1 rs1057141 and TAP2 rs241447 Ala665Thr) were not considered for further analysis. The distribution of *TAP1* genotypes and allele frequencies in clinically classified patients and in matched controls based on different genetic models is summarized in Table 1. The *TAP1* rs1135216 (Asp637Gly) in exon 10 was observed to be a susceptibility factor. The variant minor allele of the *TAP1 rs1135216G* locus was observed to be higher in patients than in control individuals (OR: 1.68, 95% CI 1.2–2.36, $P = 0.0057$) Table 1. When clinically classified paucibacillary (BL + LL) or multibacillary (BT + TT) patients were compared to controls, the variant minor allele of the *TAP1 rs1135216G* was also observed to increase the risk of infection [MB vs. Controls (OR: 1.56, 95% CI 1.07–2.28, $P = 0.054$); PB vs. Controls (OR: 1.92, 95% CI 1.21–3.01, $P = 0.013$)] Table 1. Similar observations were inferred in dominant genetic model for this variant *TAP1* rs1135216 when compared between patients and controls or between clinically classified patient groups and controls [Patients vs. Controls (OR: 2.01, 95% CI 1.34–3.03, $P = 0.0012$); MB vs. Controls (OR: 1.92, 95% CI 1.21–3.03, $P = 0.011$); PB vs. Controls (OR: 2.20, 95% CI 1.25–3.90, $P = 0.015$)] Table 1. No significant contributions were observed when compared between any of the clinically classified patient groups. For the *TAP2* rs2228396 (Asp637Gly) in exon 10, we did not observe any significant contributions (after *p* value corrections) either between patients and controls or between clinically classified patient groups compared to controls. Since each locus in *TAP1* (rs1057141) and *TAP2* (rs241447) were not in Hardy–Weinberg, hence reconstruction of haplotypes was not executed.

4. Discussion

Our aim of the study was to investigate the association of *TAP* gene polymorphisms with susceptibility to leprosy. The *TAP* gene encoding the TAP protein lies between *HLA-DP* and *HLA-DQ* of chromosome 6 which has been implicated as a leprosy susceptibility locus in genome wide scan studies [8]. The *HLA* class I haplotype *HLA-A*1102-B*4006-Cw*1502* is reported to be significantly associated with leprosy susceptibility in Indian population [11]. Among the class II genes the *HLA-DRB1* locus, more specifically *DRB1*15* and *DRB1*16*, are associated with leprosy susceptibility in India [33,34], Thailand [35] and Brazil [36,7] and *HLA DRB*15* has been reported to be associated with leprosy in Chinese population [8]. The *HLA-DRB1*0405* has been reported to be associated with resistance to multibacillary leprosy in Taiwanese population [37]. In Indonesian population susceptibility to lepromatous leprosy has been reported to be associated with *HLA-DRB1*02*, while resistance to leprosy is associated with *HLA-DRB1*12* [38].

The incompetence of the immune system to clear parasites despite antigen recognition may be attributed to host genetic factors [39]. One such factor can be polymorphisms in the immune recognition domains. The *HLA* class I molecules play a crucial role in adaptive immune system by presenting antigen peptides on the cell surface. The active transportation of antigen peptides to the

Table 1
Distribution of *TAP1* rs1135216 (Asp637Gly) genotypes and allele(s).

rs1135216 TAP1	Patients ($n = 222$)	MB ($n = 146$)	PB ($n = 76$)	BL ($n = 80$)	LL ($n = 66$)	BT ($n = 73$)	TT ($n = 3$)	Controls ($n = 223$)	Patients Vs. controls		MB (BL + LL) vs. controls		PB (BT + TT) vs. controls	
Asp637Gly exon 10	M/F (145/77)	M/F (112/34)	M/F (33/43)	M/F (61/19)	M/F (51/15)	M/F (32/41)	M/F (1/2)	M/F (148/75)	OR (95% CI)	P*	OR (95% CI)	P*	OR (95% CI)	P*
Genotypes														
AA	119 (54%)	80 (55%)	39 (51%)	50 (63%)	37 (56%)	32 (44%)	0	156 (70%)						
AG	92 (41%)	61 (42%)	31 (41%)	26 (33%)	28 (42%)	35 (48%)	3 (100%)	58 (26%)						
GG	11 (5%)	5 (3%)	6 (8%)	4 (5%)	1 (2%)	6 (8%)	0	9 (4%)						
Allele														
A	330 (74%)	221 (76%)	109 (72%)	126 (79%)	102 (77%)	99 (68%)	3 (50%)	370 (83%)	Reference		Reference		Reference	
G	114 (26%)	71 (24%)	43 (28%)	34 (21%)	30 (23%)	47 (32%)	3 (50%)	76 (17%)	1.68 (1.2–2.36)	0.0057	1.56 (1.07–2.28)	0.054	1.92 (1.21–3.01)	0.013
Dominant														
AA	119 (54%)	80 (55%)	39 (51%)	50 (63%)	37 (56%)	32 (445)	0	156 (70%)	Reference		Reference		Reference	
AG+GG	103 (46%)	66 (45%)	37 (49%)	30 (37%)	29 (44%)	41 (56%)	3 (100%)	67 (30%)	2.01(1.34-3.03)	0.0012	1.92 (1.21–3.03)	0.0112	2.20 (1.25–3.90)	0.015
Recessive														
AA+AG	211 (95%)	141 (97%)	70 (92%)	76 (95%)	65 (98%)	67 (92%)	3 (100%)	214 (96%)	Reference		Reference		Reference	
GG	11 (5%)	5 (3%)	6 (8%)	4 (5%)	1 (2%)	6 (8%)	0	9 (4%)	NS		NS		NS	

MB, multibacillary; PB, paucibacillary; BL, borderline; LL, lepromatous, BT, borderline tuberculoid; TT, tuberculoid; M/F, male/female; NS, not significant.
* *P* value corrected for multiple comparisons.

endoplasmic reticulum is facilitated by transporters associated with antigen presentation proteins. Hence the rationale is that any polymorphism in this domain may influence the ability of antigen recognition. A recent study reported that the heterozygous genotype *TAP1* 637AG was observed to be a risk factor to tuberculosis in a Chinese population which is in accordance with our observed results [29]. In a yet other study from our group in the context of *TAP* polymorphisms and HIV positive individuals revealed that genotypes GG (Val/Val) at position *TAP1* 333 and individuals with heterozygous genotypes GA (Gly/Asp) at *TAP1* 637 variants remain at higher risk of developing tuberculosis [27]. Our results indicate that the heterozygous genotype AG (*TAP1 637*) contributed towards increased susceptibility to leprosy in clinically classified groups. The *TAP1637* AG genotype has been reported to be a risk factor in ankylosing spondilytis and cystic echinococcosis [26,40] and the association of *TAP1333* AA genotype with protection in dengue hemorrhagic fever and dengue shock syndrome [41]. However, another study revealed a significant increase of homozygous AA genotype in *TAP1333* and *TAP1637* in Polish and UK patients affected with sarcoidosis and in Korean patients with allergic rhinitis [42,43]. The clinical significance of these alleles may likely vary depending on the disease context and also on the allele distribution in world populations. The observed allele frequencies for both *TAP1* (#rs1057141 and #rs1135216) and *TAP2* gene (#rs2228396 and #rs241447) loci in this study remained consistent with allele frequencies observed in other world populations as inferred from the HapMap database. The AG genotype indicating the presence of both isoleucine and valine at position 333 (codon) of *TAP1* gene might affect peptide transportation by down regulation of antigen presentation and concerned immune responses, that may lead to a diffused immune response against leprae antigen. In this context, the AG genotype at *TAP1 333* appears to be a risk factor for the disease and was substantiated by the fact that subjects with homozygous genotype AA appear to be less susceptible. Our data revealed that for *TAP1 637*, Aspartic acid/Glycine heterozygote (AG genotype) remained a risk factor for the leprosy. Inline with our results, other studies on cystic echinococcosis, pneumonitis also reported AG (*TAP1 637*) genotype to be a risk factor [40,44].

Our results suggest that there is no significant contribution of *TAP2* variants to leprosy. Majority of the patient and control groups expressed homozygous GG genotype coding for Alanine/Alanine at position 565, which was in accordance with other studies on sarcoidosis and ankylosing spondilytis [26,42,43]. Genotype AA coding for Threonine was not detected in either the patient or control groups. These findings were well in accordance with a study on ankylosing spondilytis where a very low frequency of AA genotype was reported in the patient group [26]. The 665AA genotype was observed less in both patients and controls. However, a positive association of 665AA genotype has been reported in inflammatory rheumatoid arthritis in Finnish population [28]. Our results provide genetic evidence that polymorphism in the *TAP1* gene influences the susceptibility to leprosy in Indian population. A plausible explanation on the effect of this variant may be that the microbial peptides may not be recognized or ably bind to MHC class I molecules thereby affecting the antigen transport leading to a detrimental or minimal immune response.

To conclude, the *TAP1* heterozygous genotype AG (#rs1135216 Asp637Gly) and the minor allele variant #rs1135216G contribute towards an increased risk and infection levels. However, these observations need to be confirmed and validated in larger populations. Further studies on functional characterization of SNPs at transcriptional level may shed light on the association of these polymorphisms. Overall, our study demonstrates a significant association of *TAP1* polymorphisms with leprosy outcome and *TAP1* may be a contributing factor in leprosy.

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1 # Interaction of *Mycobacterium leprae* with human monocytes
2 # increases L-Selectin expression and influences phagocytosis

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7 **Short title:** Effects of *M. leprae* on monocytes

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17 **Keywords**: CD163, Leprosy, L-Selectin, M2 macrophages, Monocytes, *Mycobacterium leprae*

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Abstract

Introduction: Leprosy, caused by *Mycobacterium leprae*, is a neglected tropical disease of the nerves and skin and is a major public health problem in tropical and sub-tropical countries. With the pathogen unable to grow *in vitro* and with a lack of a suitable animal model in vitro experiments provides a platform to understand the host-pathogen-interaction at the cellular level.

Methods: Utilizing the peripheral blood mononuclear cells from individuals of different ethnic descent, we investigated the interactions of *M. leprae* antigen on monocytes and monocyte-derived macrophages by fluorescence-activated cell sorting.

Results*: M. leprae* whole cell antigen up-regulated L-Selectin expression in monocytes by 20%. Additionally the CD163 expression is increased by 30% in macrophages.

Discussion: Increased L-Selectin expression in activated monocytes indicates a higher mobility towards affected tissues, and up-regulation of CD163 provides a vital clue on the role of M2 macrophages during the clinical progression.

45 **Introduction**

46 Leprosy is one of the neglected tropical diseases of the skin and the nerves and is caused by
47 *Mycobacteriumleprae*. *M.leprae* depends highly on macrophages and Schwann cells as they are
48 intracellular pathogens (1). Despite concerted efforts to eradicate the disease, leprosy in tropical and
49 sub-tropical countries still remains amongst the most common causes for debilitating disabilities (2).
50 But more than a health problem, leprosy is also a social stigma that decreases quality of life and
51 indeed life expectancy of those affected (3). The disease exerts its influence predominantly in
52 countries with a great disparity in access to medical services, namely Brazil, Indonesia, and India (2).
53 It is also notable that in the aforementioned countries the new case detection rate has remained
54 virtually stagnant since 2006, despite great efforts by World health organization's "final push" program.

55 *Mycobacterium leprae* survives in the human Schwann cells and macrophages by thoroughly
56 changing their phenotype (4-6). This change in phenotype goes so far, that a recent discovery could
57 prove that the bacteria are capable to change the phenotype of mouse Schwann cells back into a
58 stem cell-like one (7). Those susceptible to the bacterium show no uniform reaction, making leprosy a
59 disease with a spectrum of symptoms that is dependent on how a given immune system reacts to the
60 threat (8). For a better understanding towards leprosy pathogenesis, investigation on the interaction of
61 innate immune cells to *M. leprae* during the onset of infection at a cellular level is essential.
62 Macrophages are important in this setting, as they are on the one side a main part of the cellular
63 immunity, which is supposed to kill infected cells. On the other side they are one of the two main cell
64 types in which *Mycobacterium leprae* can survive. Macrophages in leprosy are predominantly found in
65 skin lesions. However, extraction of macrophages from the tissue biopsies remains complicated and
66 cumbersome, a labor intensive exercise. Additionally, infected macrophages are scarce in patients
67 with paucibacillary spectrum. Experimentation with *Mycobacterium leprae* is further aggravated by the
68 small number of functioning animal models. The only two models known to enable a steady bacterial
69 infection are the nine-banded armadillo (9) and the footpads of BALBc mice (10).

70 Hence isolation of monocytes from PBMCs and further differentiation of monocytes to macrophages *in*
71 *vitro* is a feasible method to investigate innate immune cell interactions with *M.leprae*. We therefore
72 employed and standardized a novel approach to investigate the interactions of monocytes and

73 macrophages with *M. leprae* by *in vitro* co-culture experiments utilizing *M. leprae* whole cell sonicate
74 as antigen.

75 L-Selectin, or CD62L, has been identified as an essential part of leukocyte migration, which includes
76 the extravasation of monocytes into tissue in order to develop into macrophages at the site of infection
77 or acute and chronic inflammation (11, 12). An extensive interplay between lack of L-Selectin and
78 increase in inflammation causing cardiovascular disease has been proven, of which particularly
79 arteriosclerosis is of interest in the context of *M. leprae* and *M. tuberculosis* infections, as in all three
80 pathologies we find a thoroughly changed macrophage phenotype due to massive incorporation of
81 low-density lipid droplets (13). The particular importance of L-Selectin in clearing inflammation has
82 been underlined by the discovery that anti-CD62L antibodies efficiently inhibit the recruitment of
83 monocytes and neutrophils to inflammatory sites (11). Observing the activity of L-Selectin in an
84 inflammatory disease such as leprosy is therefore of great interest.

85 Apart from fighting pathogens and threats by cancerous cells, macrophages also are responsible for
86 cleaning up the sites of inflammation after an immunological intervention. Of particular importance to
87 tissue repair is the M2 macrophage sub-population (14). It is, amongst other things, characterized by
88 CD163, whose expression is characteristically up-regulated by the anti-inflammatory IL10, while the
89 pro-inflammatory cytokines IFN-γ and TNF-α cause a decreased expression (15). It is therefore to be
90 assumed that during the course of an active infection upon stimulation by a pathogen M2 macrophage
91 activity, and thus CD163 expression, would decrease, and only increase after pathogenic threat had
92 been repelled. We find strong evidence for this in other publications about the closely to *M. leprae*
93 related *M. tuberculosis*: Matilla *et al.* proved that in tuberculous granulomas in macaques we find anti-
94 inflammatory macrophages expressing CD163 almost exclusively in the outer regions, adjacent to
95 uninvolved tissue, and not in areas of high bacterial load (16). *In vitro* studies with an artificial infection
96 of human monocytes by *M. tuberculosis* showed a moderate reduction of CD163 expression in
97 monocytes derived from tuberculosis patients, and no significant change at all in monocytes derived
98 from healthy controls (17). An increase in CD163 expression could not be observed in any case.
99 These results were supported by a study from the university of Bern, in which immunohistochemical
100 staining of patient samples derived from tuberculous granulomas revealed only a small number of
101 CD163-positive samples (21%), as opposed to CD68 (90%) (18). Intriguingly, the picture almost

102 reversed when compared to schistosomiasis-induced granulomas, where CD163 was expressed
103 about twice as much as in tuberculosis-induced granulomas.

104 We intended to find out whether the assumption that M2 macrophage activity, presented by CD163
105 expression, is decreased during active bacterial threat would hold true in the case of a *M. leprae*
106 infection.

107

108 **Methods**

109 Study population: We investigated monocytes and macrophages isolated from six healthy individuals
110 who gave their signed written consent. They had no previous history of leprosy and none of them was
111 on immune-suppressive medication during or four months prior to blood sampling.

112 *M. leprae* antigen: The whole cell sonicate (WCS), (Cat#: NR-19329) of *Mycobacterium leprae* for our
113 co-culture experiments was received with thanks from Biodefense and Emerging Infections Research
114 Resources Repository (BEI).

115 PBMC isolation: The peripheral blood was diluted 1:1 with RPMI 1640(PAA Laboratories GmbH,
116 Cölbe, Germany). Using Ficoll Paque™ Plus (GE Healthcare Europe, Frankfurt, Germany)cells were
117 separated via density gradient centrifugation as described earlier (19). Subsequently, peripheral blood
118 mononuclear cells (PBMCs) were isolated after centrifugation.

119 Monocytes isolation: Monocytes were negatively isolated by utilizing the Dynabeads® Untouched™
120 Human Monocytes Kit (Invitrogen, Karlsruhe, Germany). Purity of the isolated monocytes was
121 checked by fluorescence activated cell sorting (FACS) via CD14-FITC- antibody (BD Biosciences,
122 Heidelberg, Germany). The presence of CD62L and CD163 on the cell surface was also established
123 via APC- and PE-labeled antibodies respectively (BD Biosciences, Heidelberg, Germany).The isolated
124 monocytes were then incubated separately in a growth medium consisting of RPMI 1640 (PAA
125 Laboratories GmbH, Cölbe, Germany) with 5% fetal bovine serum (PAA Laboratories GmbH, Cölbe,
126 Germany), 1% L-glutamine (Invitrogen, Karlsruhe, Germany), and 1% Pen Strep (Invitrogen,
127 Karlsruhe, Germany), in a cell culture plates at a concentration of 0.2 $x10^6$monocytes/mL.

128 Monocytes harvest: Monocytes were harvested after three days of incubation by removing the
129 supernatant and applying Accutase™(eBiosciences, Frankfurt, Germany) following manufacturer's
130 protocol. In brief: the supernatant was removed and the adherent cells were washed with phosphate-
131 buffered saline (PBS). Then Accutase was added at a volume of 10 ml per 75 cm^2 surface area and
132 the cells incubated at 37°C for 10 minutes for cells to detach. The detached cells were washed once
133 with PBS.

134 Activation of Monocytes: The harvested monocytes were incubated with 10µg/ml WCS overnight
135 (approximately 18 hours), the other half was incubated without any activation and served as a baseline
136 for comparisons. Incubation steps were performed with similar concentrations as done before with
137 RPMI 1640 (PAA Laboratories GmbH, Cölbe, Germany) with 5% fetal bovine serum (PAA
138 Laboratories GmbH, Cölbe, Germany), 1% L-glutamine (Invitrogen, Karlsruhe, Germany), and 1% Pen
139 Strep (Invitrogen, Karlsruhe, Germany), in cell culture plates. After incubation, CD62L-expression on
140 both activated and non-activated monocytes (identified through a CD14-FITC antibody) was measured
141 by FACS (BD Biosciences, Heidelberg, Germany) and a CD62L-APC antibody (BD Biosciences,
142 Heidelberg, Germany).

143 Macrophages harvest and activation: In parallel to monocytes isolation and harvest, monocytes were
144 subsequently allowed to mature and differentiate to macrophages for six days. After incubation, the
145 remaining incubated cells were harvested as above. After washing the cells, a portion of them was
146 taken to check for maturation by FACS. Utilized markers were antibodies against CD14-FITC, CD62L-
147 APC, and CD163-APC (BD Biosciences, Heidelberg, Germany). Macrophages were harvested as
148 described earlier for monocytes harvest procedure. The harvested macrophages were further
149 incubated with 10µg/ml WCS for approximately 18 hours, the other half was incubated without any
150 activation and served as a baseline for comparisons. Activation status after incubation was tested with
151 the same antibodies as above (CD14-FITC, CD62L-APC, and CD163-APC).

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155 **Results**

156 Monocytes maturation to Macrophages:

157 After six days of incubation, $CD14^+$cells showed an increase in internal complexity and size in FACS.
158 Only approximately 4.5% of them were still $CD62L^+$when compared to $CD14^+$cells on day zero (Figure.
159 1).When monocytes were only incubated for 3 days and then stimulated with WCS overnight, CD62L-
160 expression across individuals increased by 30% (Figure 2).

161 Interaction of monocyte derived macrophages with *M.leprae* WCS:

162 After incubation with WCS, the matured macrophages showed a significantly different phenotype when
163 compared to non-activated macrophages (Table1). Both size and internal complexity, represented by
164 the FSC and SSC values respectively, changed upon overnight stimulation with WCS (Figure 3).Upon
165 activation with WCS, macrophages showed an up-regulation of CD163 that was more than 20% higher
166 than that of non-stimulated controls (Table1, Figure 4).

167 **Discussion**

168 The nature of *Mycobacterium leprae* as a pathogen that cannot be grown *in vitro* and only to certain
169 extents *in vivo* makes it essential to achieve an activation of cells of interest that is as close as
170 possible to real pathogen-host interactions. We believe an activation via *Mycobacterium leprae* WCS
171 achieves this.

172 We utilized CD62L, or L-Selectin, as a marker of increased monocyte mobility and of maturation of
173 monocytes into macrophages. L-Selectin is a cell adhesion molecule that plays an important role in the
174 migration of leukocytes from the blood stream into tissues (12, 20, 21). Specifically, L-Selectin binds
175 free-flowing monocytes to the endothelium and mediates extravasation into the tissue (22). Therefore
176 a reduction in L-Selectin expression on the surface of the cell is an indicator of the change from a free-
177 flowing monocyte phenotype towards a tissue-bound macrophage phenotype. After three days of
178 incubation, our cells showed a marked increase of L-Selectin expression by 30% on their surface
179 when stimulated with WCS. This proved that three days of incubation are not enough to turn
180 monocytes into macrophages without additional cytokine-stimulus. Furthermore it also represents the
181 ability of WCS to greatly increase monocyte mobility by directly interfering with genetic expression of

182 the host's monocytes, as an increase in L-Selectin presentation is an indicator for a greater readiness
183 to emigrate into tissue towards the site of infection.

184 The monocyte-derived macrophages (MDMs) achieved after six days of incubation of monocytes
185 showed minimal amounts of L-Selectin, which is an indicator for successful monocyte-maturation. The
186 MDMs achieved in this manner were then incubated for 18 hours with WCS. Changes in morphology
187 and CD163-expression were observed. We found a notable increase of internal complexity,
188 represented by the SSC value in FACS and a tendency towards a slight decrease in size, represented
189 by the FSC-value, when comparing activated with non-activated macrophages. The increase of
190 internal complexity can be attributed towards an increased number of phagosomes. Whole cell
191 sonicate consists of fragments of formerly whole cells that were treated with ultrasound, it is therefore
192 to be expected that macrophages would increase their phagocytic activity in the presence of this
193 antigen. The slight variation in size observed is not in keeping with any general knowledge about
194 macrophages at the moment and may simply be attributed towards the small amount of individuals in
195 this study.

196 We also observed a marked increase of around 20% in CD163 on the surface of our monocyte-
197 derived macrophages after activating them with WCS when compared to non-activated MDMs. CD163
198 belongs to the scavenger receptor cystine rich family type B and is a marker of alternatively activated
199 M2 macrophages (23, 24). As described in the introduction, M2 macrophages are a diverse sub-
200 population that is predominantly occupied with wound healing and clearing of inflammation as well as
201 tissue repair, but also participates in parasite clearing (25, 26). A recent study that established an *in*
202 *vitro* model for granuloma formation in leprosy observed the same up-regulation of CD163 after
203 activation with live *M. leprae* bacilli incubated with PBMCs (27). Recently it has also been
204 hypothesized that CD163 functions as an alternative route of entry for *M. leprae* and that the observed
205 up-regulation under influence of the bacteria is a mean of supporting the infection via facilitating the
206 entry into host cells (28). These results were achieved with cells directly isolated from leprosy patients,
207 observing 41.7% of isolated macrophages to be CD163 positive. Our observed up-regulation of
208 21.1±5.6% may be due to our *in vitro* approach and activation through WCS as opposed to whole
209 cells.

It is of particular interest that, in the case of CD163 expression, cells under threat by *M. leprae* show an opposite reaction to cells under threat by *M. tuberculosis*. While in the latter case we find an expected decrease or stagnation in expression during active threat (15, 17, 18), *M. leprae* causes an increase. A strong hint on why this might be comes from Strebel *et al.* (18), as they found an up-regulation of CD163 expression in Schistosomiasis-induced granuloma. The ability of Schistosoma-species to circumvent and influence the immune-system is well-documented (18, 29, 30). It is possible that *Mycobacterium leprae* enhances M2-anti-inflammatory activity in order to escape immunological clearance. This is supported by findings that find exactly this enhancement in the case of patients suffering under lepromatous leprosy (31).

In conclusion, we have observed that *Mycobacterium leprae* whole cell sonicate induces activating properties in monocytes and macrophages, up-regulating mobility in the former, represented by an increase of L-Selectin expression, and activating the M2 portion of the later, possibly as part of an evasion of the cellular immune answer. Future studies to support our hypothesis with greater numbers of individuals should be able to validate our results, which would help to develop an adequate *in vitro* macrophage activation model for leprosy as well as give intriguing implications on the activity of M2 macrophages in bacterial disease.

Disclosure

There is no conflict of interests.

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Figure legends

Figure 1: **Reduction of CD62L in monocytes after six days:** Expression of CD62L (APC) and CD14 (FITC) on incubated monocytes on day zero (A – Q2) and after six days of incubation (B – Q2-1). The gating is different due to the measurements being taken at two different time points. It was, however, performed utilizing the appropriate isotype controls.

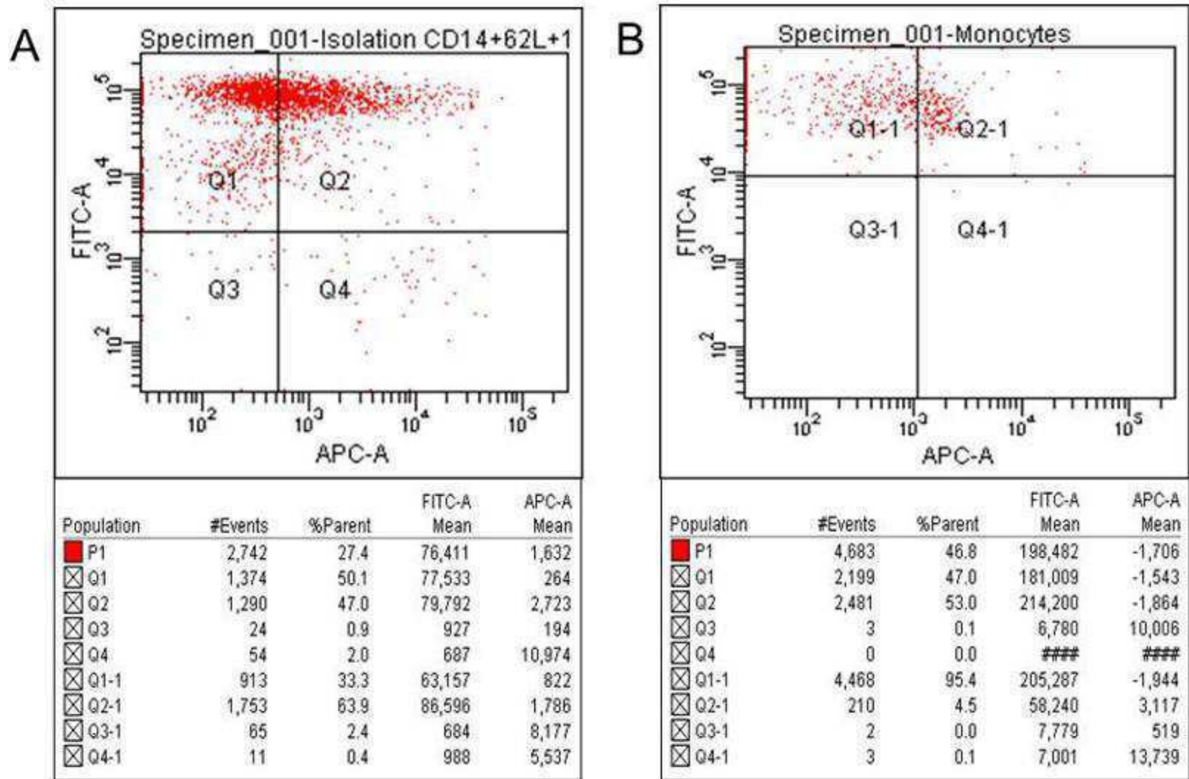
Figure 2: **Activation of monocytes with WCS after three days of incubation:** Comparison of CD62-L (APC, Q2) expression of day 3 monocytes without (A) and with (B, Q2) activation by WCS. Expression in B in this case is 34,8% higher.

Figure 3: **Change in size and internal complexity in macrophages caused by WCS:** Size (FSC-value) and internal complexity (SSC-value) in Macrophages without stimulation (A) and after 18 hours of incubation with WCS (B).

Figure 4: **CD163-expression in macrophages after activation with WCS:** Non-activated (A, P2) and with WCS for 18 hours activated (B, P2) macrophages present different fluorescence after treatment with CD163-PE antibodies. Expression increases by 21.1%.

Figures

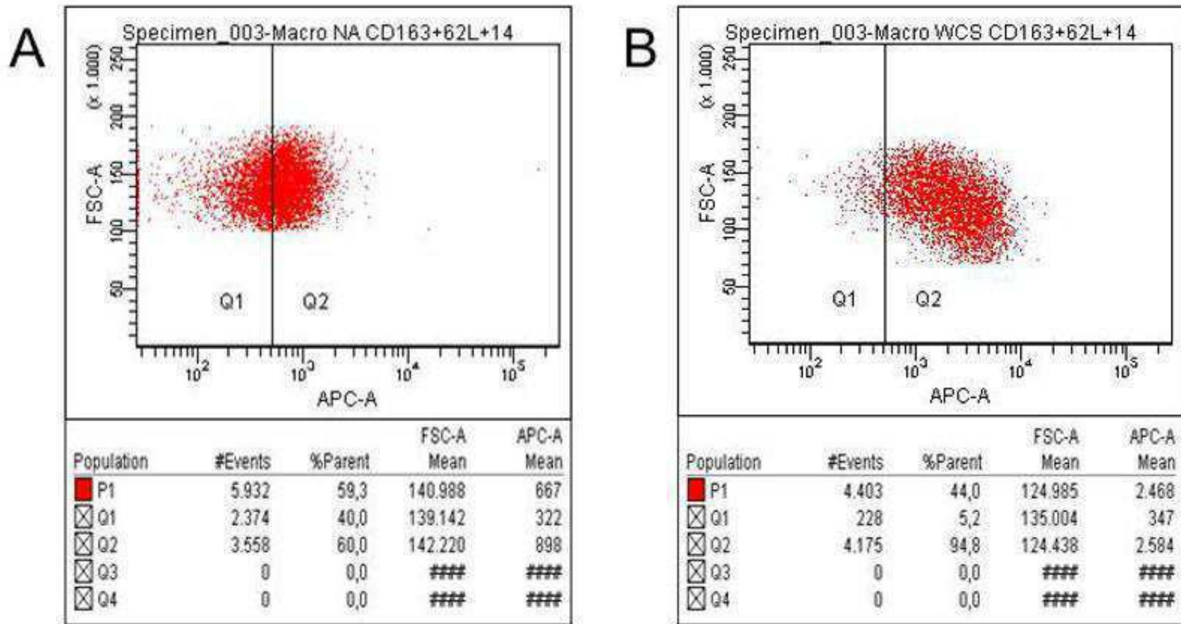
Figure 1



Population	#Events	%Parent	FITC-A Mean	APC-A Mean
P1	2,742	27.4	76,411	1,632
Q1	1,374	50.1	77,533	264
Q2	1,290	47.0	79,792	2,723
Q3	24	0.9	927	194
Q4	54	2.0	687	10,974
Q1-1	913	33.3	63,157	822
Q2-1	1,753	63.9	86,596	1,786
Q3-1	65	2.4	684	8,177
Q4-1	11	0.4	988	5,537

Population	#Events	%Parent	FITC-A Mean	APC-A Mean
P1	4,683	46.8	198,482	-1,706
Q1	2,199	47.0	181,009	-1,543
Q2	2,481	53.0	214,200	-1,864
Q3	3	0.1	6,780	10,006
Q4	0	0.0	####	####
Q1-1	4,468	95.4	205,287	-1,944
Q2-1	210	4.5	58,240	3,117
Q3-1	2	0.0	7,779	519
Q4-1	3	0.1	7,001	13,739

367 Figure 2



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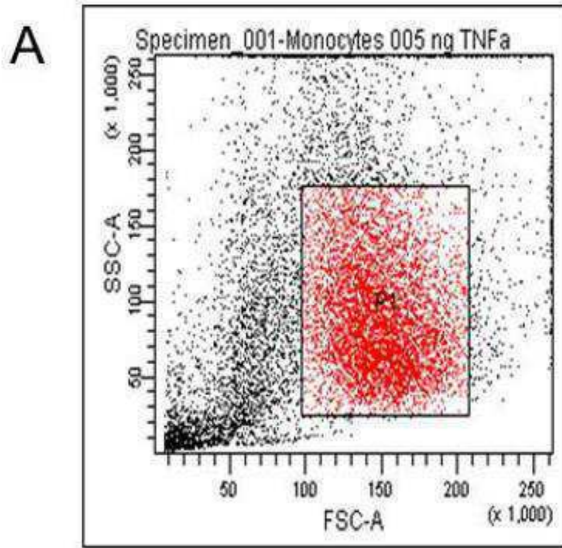
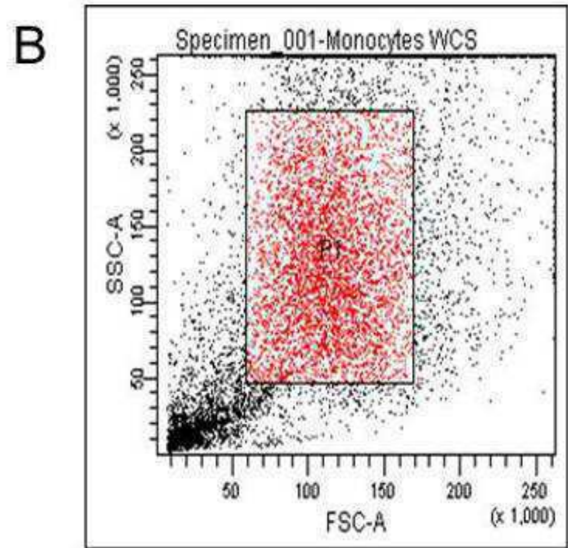
382 Figure 3





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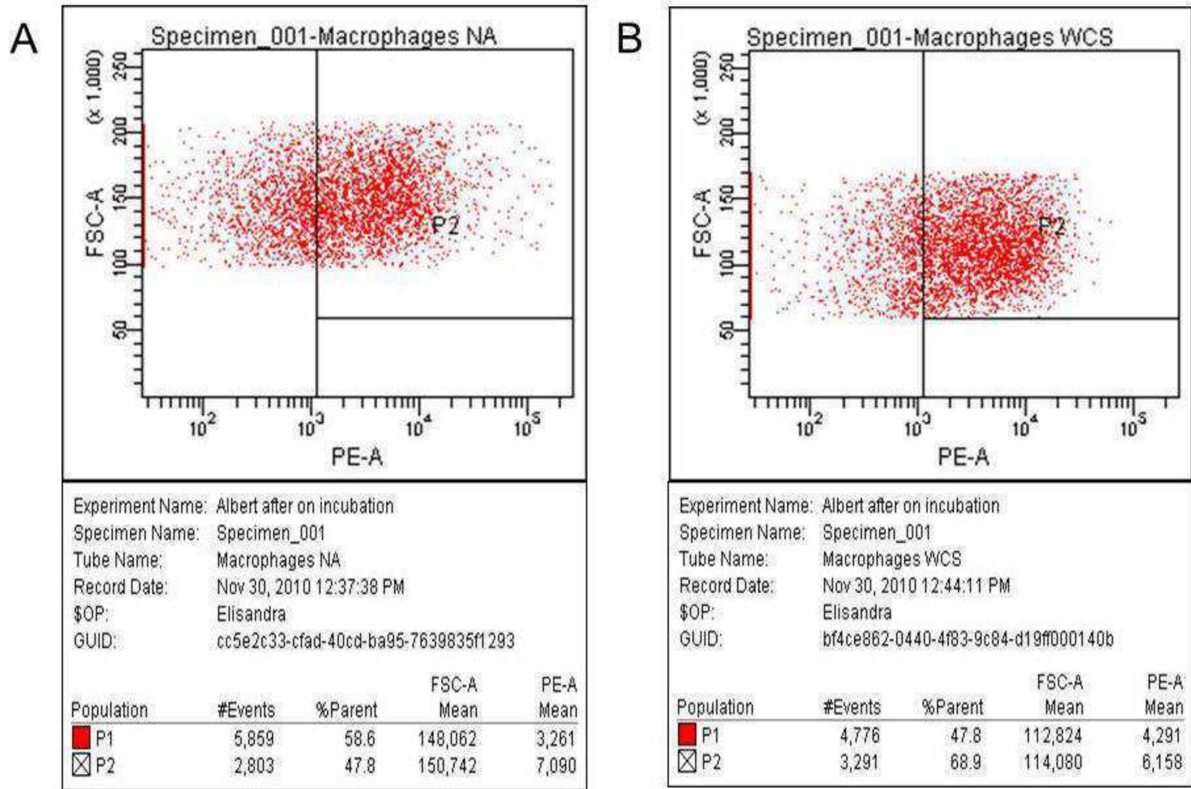
398 Figure 4



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Transcriptome profile of natural killer cells in patients treated with multi-drug-therapy

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Running Title: Gene expression of natural killer cells in *Mycobacterium leprae*-infection

Abstract

Background: Leprosy is one of the most neglected tropical diseases of the skin and the nerves caused by the intracellular pathogen *Mycobacterium leprae.* Natural killer cells, at early stages contribute towards establishment of the infection and subsequent adaptive immune responses. This study investigated the differential expression of natural killer cells transcriptome in paucibacillary and multibacillary leprosy patients.

Methods: Peripheral blood mononuclear cells were extracted from ten leprosy patients (five paucibacillary and four multibacillary) and from three healthy, ethnically matched controls. The purified population of natural killer cells (>90%) were isolated from patients and controls. The mRNAs of natural killer cells were subsequently used for microarrays and gene expression in leprosy patients were compared to healthy controls.

Results: The LCN2 and APOBEC3 genes were predominantly upregulated in paucibacillary patients. A comparison of paucibacillary and multibacillary patients with controls as a baseline revealed that multibacillary patients generally showed a higher transcription of genes associated with cell communication, such as SIRPG and various defensins. However, paucibacillary patients showed a down-regulation in comparison with healthy controls. Further, multibacillary patients also showed an upregulation in gene products involved in immunity.

Conclusion: Differential transcriptome profile were observed between the investigated pauci- and multi-bacillary leprosy patients.

Keywords: Leprosy, *Mycobacterium leprae*, natural killer cells

Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. Despite an effective antibiotic therapy to control the infection, number of new case detection and registered cases remain high in 130 countries worldwide [1]. The spectrum of leprosy presentation can be classified in between two extreme poles: paucibacillary (PB) and multibacillary (MB) [2]. While the paucibacillary (or tuberculoid) presentation is categorized as a successful immune response, owing to the little presence of bacilli and less than five lesions, the multibacillary (or lepromatous) form is considered a failure of immunity. According to the classical Ridley and Jopling scale based on the increasing severity of symptoms, the medical classification is further divided into tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL), and lepromatous (LL). Generally, TT and BT individuals are considered part of the paucibacillary side of the spectrum, while BB, BL, and LL individuals are counted towards the multibacillary side.

The clinical spectrum in leprosy is associated with host immune responses [2, 3], individual variability in host responses therefore can significantly influence the clinical outcome. The variety in immune response is caused directly by the ability of immunity to fight off *M. leprae* infection. The latter occurs most commonly because instead of cellular immunity, an antibody-based response is up-regulated. In addition, the bacterial load, which is the base of the multibacillary or paucibacillary diagnosis, is not strongly correlated with the severe lepromatous. Therefore, the gene expression profile of patients will provide a useful basic to understand their particular representation of the disease [4]. The failure of treatment can be traced back to immunogenetic properties of patients and is therefore a possible reason for the observed relapse during or shortly after treatment [5-7].

The primary targets of *M. leprae* are human macrophages and Schwann cells. Particularly, macrophages have been extensively studied in the context of leprosy due to their dual role as host cell and major player of innate immunity. Studies have shown that *M. leprae* was associated with massive modifying of infected macrophage functions such as inducing import

of low density lipids or influencing the immune response, particularly via modulating inflammation [8] [9]. Accordingly, the effect of the bacteria on the transcriptome of macrophages and Schwann cells has been studied [10] [11]. Transcriptomic analysis was employed to study leprosy's type I, a well-known phenomenon resulting in localized inflammation of skin and nerves after immunity in MB patients has been reinstated [12, 13]. Studies have also demonstrated the important role of natural killer (NK) cells in murine leprosy both *in-vivo* and *in-vitro* [14]. The macrophages heavily infected with *M. lepraemurium* were susceptible to be lysed by activated NK cells *in-vitro*, whereas control of monoclonal antibodies against NK cells enhanced susceptibility to mouse leprosy [14]. Also, treatment of BALB/c mice in the hind footpads with irradiated *M. leprae* led to a significant enhancement of natural killer activity [15].

Based on these promising findings and the important roles of both NK cells and macrophages in innate immunity, we aim to investigate the role of NK-cells in leprosy and their activation during *M. leprae* infection by transcriptome analysis.

Materials and Methods

Sampling and ethics

The patient cohort consisted of individuals from the state of Andra Pradesh, India that had been diagnosed as either paucibacillary or multibacillary by the medical staff of the Blue Peter Public Health Research Centre (BPHRC). Diagnosis was made based upon clinical signs (less or more than five lesions, sensual impairment within the lesions, thickening of nerves) and histological presence of bacteria in skin slit smears. All of the patients had, without inflammatory episodes, completed multi-drug treatment according to WHO standards for their respective presentation of the disease (http://www.who.int/lep/mdt/regimens/en/index.html).

Sampling occurred from February up until April 2013, including 24 patients altogether. As only 20 milliliters of venous blood could be used for NK cell and consecutive RNA isolation as per the ethical agreement, only seven samples of the highest quality and yield of RNA were chosen for microarray analysis. A summary of patient parameters is presented Table 1.

Classification of leprosy was carried out according to Ridley Jopling. Paucibacillary (PB) patients were identified as having one to five well-defined lesions, none or only one thickened/tender nerve trunk, and they can be smear-negative. The classification as a multibacillary (MB) case was done as follows: Six or more lesions, more than one thickened/tender nerve trunk, a bacterial index greater than three, and smear positivity. We grouped patients based on their bacterial load rather than clinical representation, as the former is a quantifiable parameter.

Three controls that were matched for ethnical background and came from the state of Andra Pradesh were included. All patients provided written informed consent before participating in this study. The standards of sampling followed the guidelines of the ethical committee of the BPHRC as specified in the context of the cooperation.

PBMC processing

PBMCs were isolated from fresh blood as previously described [16]. Briefly, 20 ml of fresh venous blood were taken utilizing 9 ml Monovette© ammonium-heparin-vacutainers by Sarstedt. The blood was mixed 1:1 with RPMI 1640 and the resulting solution was utilized in a Ficoll desnity gradient separation. After separation the PBMC-layer was taken off and washed twice in adequate medium (RPMI 1640 + 2% fetal bovine serum). The cells were transferred into growth medium (RPMI 1640 + 5% fetal bovine serum + 1% PenStrep + 1% L-Glutamine) at a concentration of $1.2x10^6$ cells/ml and incubated in Corning® Costar® 24-well plates for 18 hours.

Cultured PBMCs were harvested from wells by vigorous pipetting. Harvested cells were treated with the Human untouched NK cell isolation kit by Miltenyi in order to achieve a purity of greater than 93%. The provided kit protocol was followed exactly. The achieved purity was tested by flow cytometry, identifying the NK cell population as $CD56^{+}CD3^{-}$. The utilized FACS buffer was made from 1x PBS plus 1% fetal bovine serum.

After isolation, if a purity >93% had been achieved, RNA was isolated from the cells utilizing the RNEasy Mini Kit following the instructions of the manufacturer (Qiagen, Hilden, Germany).

Microarray procedures

Microarray procedures were performed in Hyderabad, India. RNA fidelity and concentration were estimated using the Bioanalyzer system (Agilent, Santa Clara, USA). The transcriptome analysis was carried out via the whole-genome cDNA-mediated Annealing, Selection, and Ligation (WG-DASL) assay (Illumina San Diego, USA).

Statistical analysis

Raw data was background corrected with standard Ilumina software. Hereafter all further processing and analysis was done with the software package R 2.13.0 (http://www.r-project.org/). Two arrays were excluded from further analysis since they show a striking different correlation pattern to the other arrays detected by a correlation plot. This observation was further confirmed by hierarchical clustering using the average linkage method with Euclidian distance.

The data was log2 transformed and normalized using quantile normalization as implemented in the software package Lumi. Sex chromosome specific genes (813 genes for the X chromosome, 102 genes for the Y chromosome) were excluded from further analysis as well as genes that have a non-significant detection call (P>0.01, 12206 genes) leaving 11794 genes for further analysis. Differential expression was detected with the Limma package.

Since we used only two arrays for each comparison none of the genes have a significant P value (FDR<0.05). Therefore we decided to focus on genes showing at least a fold change of 3 between two conditions.

Results

Highly pure NK cell isolates could be achieved

Out of 24 patient samples, sufficient amount of NK-cells at a purity of 93% or higher were isolated from 10. The increase in NK cell concentration, identifiable by presence of CD56 and absence of CD3 on the surface, is exemplified by the results of a patient isolation (Figure 1), where a purity of 96.4% was achieved.

Finally, the RNA of seven out of ten samples with sufficient quantity and purity of NK cells was considered for microarray analysis (Table 1). The three remaining samples presented RNA of insufficient quality after concentrating it in preparation for hybridization onto the DASL chip and were therefore not included in further analysis.

The cluster dendrogram of leprosy patients reveals three groups

When comparing the expression profiles of the remaining patients (NK 2, 4-6, 8, and 11-12) with each other and with the healthy controls we found three groups emerging (**Fig. 2**): NK2 and NK11 are both member of the paucibacillary (PB) group, while NK4 and NK6 both are multibacillary (MB) patients. NKK1 and NKK3 both belong to the control group. It is notable that we observed a number of outliers that couldn't be included in any of the mentioned groups based on their expression, even including one of the controls. All of the outliers were excluded from further analysis.

Comparison on single patient level reveals the importance of communication for natural killer cells in leprosy

When comparing the remaining single patients with each other and with the single controls (**Fig. 3**), we found a number of genes up-regulated in infected individuals when compared to healthy controls (NKK samples). Of particular prominence were the genes for lipocalin 2 (LCN2) and apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3), which both were less up-regulated, or even down-regulated, in multibacillary patients than in paucibacillary ones.

We also found a number of Y- and X-chromosomally coded genes differentially expressed, such as protein kinase, Y-linked (PRKY) and kelch-like 4 (KLHL4) (not pictured). Those weren't included into the results, though they were adequately expressed in different genders, with PRKY, for example, being only up-regulated in males. This serves as an additional quality control for the microarray data.

The multibacillary patients differ from the control group

Comparison of gene-expression in the MB group with the control group as a baseline revealed three clusters of interest in a heat map of flad-changes equal to or greater than $\log_2(3)$ (**Fig. 4**).

The first cluster is dominated by down-regulated gene-products that play a role in immunity (CD70, phospholipase A2, group IVC (cytosolic, calcium-independent) [PLA2G4C], and tumor necrosis factor receptor superfamily, member 9 [TNFRSF9]).

The second, larger gene-cluster consists of strongly up-regulated genes. Many of the genes play no known role in immunity. However, this group also contains the NK-cell-activity specific gene-products of Killer Cell Immunoglobulin-Like Receptor, Two Domains, Short (KIR2DS3) and CD93.

The third and largest cluster of gene-products includes many proteins involved in general cell-cell-communication, like signal-regulatory protein gamma (SIRPG), or pro-platelet basic

protein (chemokine (C-X-C motif) ligand 7) (PPBP). The gene-products in this cluster are moderately up-regulated when compared to the baseline.

The paucibacillary patients present different clusters of gene-activity

Observing **figure 4** once more reveals that the gene expression of the patients belonging to the paucibacillary end of the spectrum is quite different from the multibacillary patients. Here too we observe three clusters for mostly the same genes as in the multibacillary cases, but the expressions are partially the diametrical opposite.

The first cluster consists of four strongly up-regulated gene-products. They derive from NDUFA7 (NADH Dehydrogenase (Ubiquinone) 1 Alpha Subcomplex, 7), RMRP (RNA Component of Mitochondrial RNA Processing Endoribonuclease, also known as NME1), NME2 (NME/NM23 Nucleoside Diphosphate Kinase 2), and GDPD1 (Glycerophosphodiester Phosphodiesterase Domain Containing 1). In MB patients, they present an expression on par with the healthy controls.

Cluster 2 consists almost exclusively of genes whose expression is only slightly or not at all up-regulated when compared with the healthy controls. It consists of the genes that form clusters one and two in the MB group.

The last cluster in the PB group presents a mirror image to the third cluster in the MB group: The same genes that are moderately or strongly up-regulated in the former present a moderate or strong down-regulation in the latter.

Quantitative differences between the MB and PB groups

Table 2 presents the quantitative differences in the genes shown in the heat map **figure 4**, sorted by how far up- or down-regulated they are in comparison to the controls as well as how different the gene is in each group. CD70, for example, has a fold-change of -2.815 in the MB group and of 0.377 in the PB group, which is the greatest absolute distance in all of

the displayed genes. We note that in the ten genes that differ the most in expression in the two groups we find a great number of genes with little to no known immunological activity, such as OXER1 or CCDC144A.

Notable differential expression by a number of important genes

Beyond the clusters presented in **figure 4**, a number of important genes were found to have notable differential expression when compared to healthy controls. The LY96 (also known as MD2) gene product, for example, was down-regulated by -1.85 in MB while being up-regulated by 2.05 in PB cases.

Both alpha 1 and beta defensin production was differential in MB and PB patients. DEFA1, encoding for alpha 1 defensin, was transcribed with fold-changes of 2.56 in MB patients and -3.17 in PB individuals. DEFA1B (alpha defensin 1B) presented comparable values: 2.37 in MB and -3.14 in PB cases. It is notable that DEFA3 presented a similar expression pattern, 2.31 fold-change in MB and -2.98 in PB individuals.

LILRA2's expression was lower than in the controls for bot MB and PB patients, at -2.28 and -2.79, respectively. Production of IL8 was down-regulated by -1.6 in both MB and PB patients. The anti-microbial CTSG gene-product presented a fold-change of -2.08 in MB and of 2.46 in PB patients when compared to controls.

Discussion

It is important to note that a substantial number of our initial patient samples couldn't be included in the further analysis, as their expression patterns were too different from the emerging groups. This may be due to the great variation in the immune answer that is typical in leprosy. It may also be an explanation why the sample NK12 actually groups with the

control sample NKK2. NK12 may actually be the only individual that due to treatment reached expression levels that are comparable to a healthy control again, in this case NKK2.

When observing the comparison of the remaining single patients amongst each other, we found a number of genes with varying importance in human immunity. One example is secreted phosphoprotein 1 (**SPP1**), which acts as a cytokine that up-regulates interferon gamma and interleukin 12 production, both important players in type 1 immunity. It is logical that such a cytokine would be up-regulated in a leprosy patient when compared to a healthy control. On the other hand we observed lipocalin 2 (**LCN2**) to be differentially expressed in the patients. While the paucibacillary patients showed a clear up-regulation of the protein production, the multibacillary cases were rather unchanged or even down-regulated. Lipocalin has been indicated in anti-bacterial activity [17-19], a reduced activity in MB patients, who are classified as having failed to mount a successful immune answer, is therefore remarkable. The same goes for the apparent down-regulation of apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 (**APOBEC3**), which is normally associated with fighting off retroviral infections [20-22].

Comparing the patient groups with the control group as baseline revealed clusters of differentially expressed genes. As the remaining number of individuals (two for each group) was not high enough for statistically significant results, we opted to only include such genes that showed a fold-change equal to or greater than log3. Doing so and ordering the genes according to how much they differ from each other in the PB versus the MB group, we found that the gene that had the greatest difference in natural killer cells of leprosy patients is **CD70**. The gene product belongs to the tumor necrosis factor (TNF) ligand family and is a ligand for CD27 on T-cells, where the CD70/CD27 interaction is critical for the cell's expansion and survival [23]. Furthermore, it has been proven that CD70 directly enhances the direct cytotoxic activity of natural killer and T-cells [24, 25]. The negative fold-change of -2.8 in multibacillary cases is therefore of great interest.

Opposed to this we found **SIRPG** (signal-regulatory protein gamma) with a slight up-regulation (0.9) in the MB group and a strongly negative fold-change of -2.2 in the PB group. As the gene product of SIRPG is indicated in regulatory processes, especially in its interaction with CD47 [26], a strong variation in accordance to a differing representation of disease should prove to be a substantial change.

Another gene product that is strongly indicated in regulatory processes and was also up-regulated by a fold-change of 2.1 in the PB cohort of our study is **RMRP** (RNA component of mitochondrial RNA processing endoribonuclease), which has been proven to play a role in cell growth and division [27].

RMRP belongs to a cluster of four genes whose transcription was greatly up-regulated in PB patients, but on the same level as the baseline in MB individuals. The three additional genes were: **NDUFA7** (NADH Dehydrogenase (Ubiquinone) 1 Alpha Subcomplex, 7), **NME2** (NME/NM23 Nucleoside Diphosphate Kinase 2), and **GDPD1** (Glycerophosphodiester Phosphodiesterase Domain Containing 1). NDUFA7 is part of the electron transport chain in human mitochondria. NME2's gene-product is integral in the production of nucleoside triphosphates and is known for playing a role in negatively regulating cell-proliferation and therefore hindering cancer development [28-30]. GDPD1 has only been poorly researched up to this point in time, though a study has identified the gene-product as a part of the general NK-cell membrane proteome [31]. Why any of the four genes in the strongly up-regulated cluster of PB patients present themselves in this manner will have to be investigated further in future experiments.

Also of interest is the small 0.6 up-regulation of **PPBP** (pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) in the MB patients compared with the down-regulation of -1.7 in the PB cohort. Derivatives of PBP (platelet basic protein), such as PPBP, have demonstrated an anti-microbial activity in monocytes [32]. Also, the PPBP gene-product works as a chemo-attractant for neutrophils [33]. It is therefore curious why there should be such a substantial down-regulation of a gene product with importance in innate immunity in the paucibacillary

group. The paucibacillary pole of the leprosy spectrum is generally considered a representation of a working immune answer, for which an adequate innate immunity is paramount.

Another gene whose transcription is down-regulated in the PB group is **EMR4**. The gene product is a member of the EGF-TM7 receptor gene family, though sequencing results indicates that it may be soluble instead of membrane-bound in humans. In this regard it becomes interesting as a possible mean of G-protein-mediated cellular signaling. This also makes the down-regulation of the **FPR1** gene product in the PB group noteworthy. The negative fold-changes of *Emr4* vs. *Fpr1* (-1.9 vs. -1.8) are very similar, and *Fpr1* is a member of the G-protein coupled receptor family 1. *Fpr1* plays a role in natural killer cell-mediated cytolitic activity [34] and in anti-inflammatory activity [35].

KIR2DS3 is a member of the killer cell immunoglobulin-like receptor family and transfers activating signals into the natural killer cell. This makes the fold-change of roughly 1.9 observed in the MB group interesting, especially as the gene also plays a role in other pro-inflammatory diseases [36, 37].

Another gene of interest due to its importance in immunity is tumor necrosis factor receptor superfamily, member 9 (**TNFRSF9**). The gene product is known for part-taking in the recognition of antigen presenting cells by T- and B-lymphocytes [38]. It has also been implicated in increasing dengue virus-mediated apoptosis in vitro [39]. Finally, **CD93** may also be of interest, as it is known to be involved in cell-adhesion and phagocytosis [40, 41].

Observing expression outside of the clusters seen in **figure 4** revealed a number of differentially expressed genes that are notable for their roles in host immunity (not pictured). Of particular interest is the expression of the **DEFA1**, **DEFA1B**, and **DEFA3** gene products. All three genes code for variations of defensin alpha, an enzyme with anti-microbial properties predominantly produced in neutrophils and NK-cells [42, 43]. Our results presented a stronger expression in multibacillary cases and a weaker expression in

paucibacillary cases when comparing them to healthy controls. Considering the anti-microbial role of defensins this result is counter-intuitive. This may indicate an attempt of NK-cells to compensate for the failure in effective host-defense in multibacillary cases.

LY96, also known as MD2, codes for lymphocyte antigen 96. This enzyme interacts with toll-like receptor 4 in the innate immune response, particularly to lipopolysaccharide [44, 45] and in inflammatory reactions [46]. As the gene-product was observed to be down-regulated in MB patients and up-regulated in PB patients, this may hint at a role in the defense against *Mycobacterium leprae*.

Another gene-product we found to be down-regulated in MB cases and up-regulated in PB cases was that of **CTSG**. The gene codes for cathepsin G, an enzymatic protein belonging to the protease families mostly known for its involvement with neutrophilic cells. It conveys bactericidal activity and modulates inflammation [47]. In the context of our study it is notable that cathepsin G was found to contribute to lung-protective immunity against mycobacterial threat in a mouse model [48] as well as enhancing NK-cell cytotoxicity [49]. Given the involvement in immunity, the observations made in regards to MB and PB patients could indicate cathepsin G as a building block in mounting the host's defense against leprosy.

Finally, the **LILRA2** gene product was down-regulated in both MB and PB cases. The gene encodes a leukocyte immunoglobulin-like receptor (subfamily A) which can suppress innate immune responses [50]. As innate immunity plays a big role in leprosy, it stands to reason that a receptor which reduces this activity would show decreased use in this disease.

This is the first study that analyzes the whole transcriptome of natural killer cells in leprosy patients and compares the two opposing representations of the disease based on this context. Future studies will have to include greater patient cohorts, but the presented manuscript gives intriguing first hints as to why the disease can have such a great variety of representations in different humans and what role the natural killer cells may be playing in leprosy.

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Conflict of Interests

Authors have no conflict of interests.

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Tables:

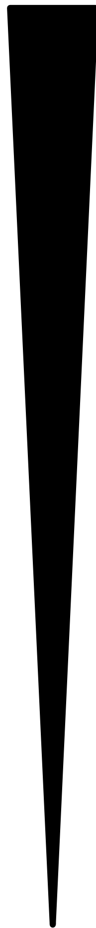
Table 1: Summary of included patients

Sample	Age	Sex	Lepromatous or Tubeculoid	Classification	on Steroids in the past	on MDT	MDT duration
NK2	19 y/o	f	tuberculoid	PB		Yes	2 months
NK4	18 y/o	m	lepromatous	MB	Yes	Yes	ongoing
NK5	34 y/o	m	lepromatous	MB		Yes	ongoing
NK6	43 y/o	m	lepromatous	MB		Yes	ongoing
NK8	12 y/o	m	tuberculoid	PB		Yes	5m (child)
NK11	53 y/o	f	tuberculoid	PB		Yes	ongoing
NK12	28 y/o	f	tuberculoid	PB		Yes	ongoing

Table 2: Quantitative difference in expression of genes

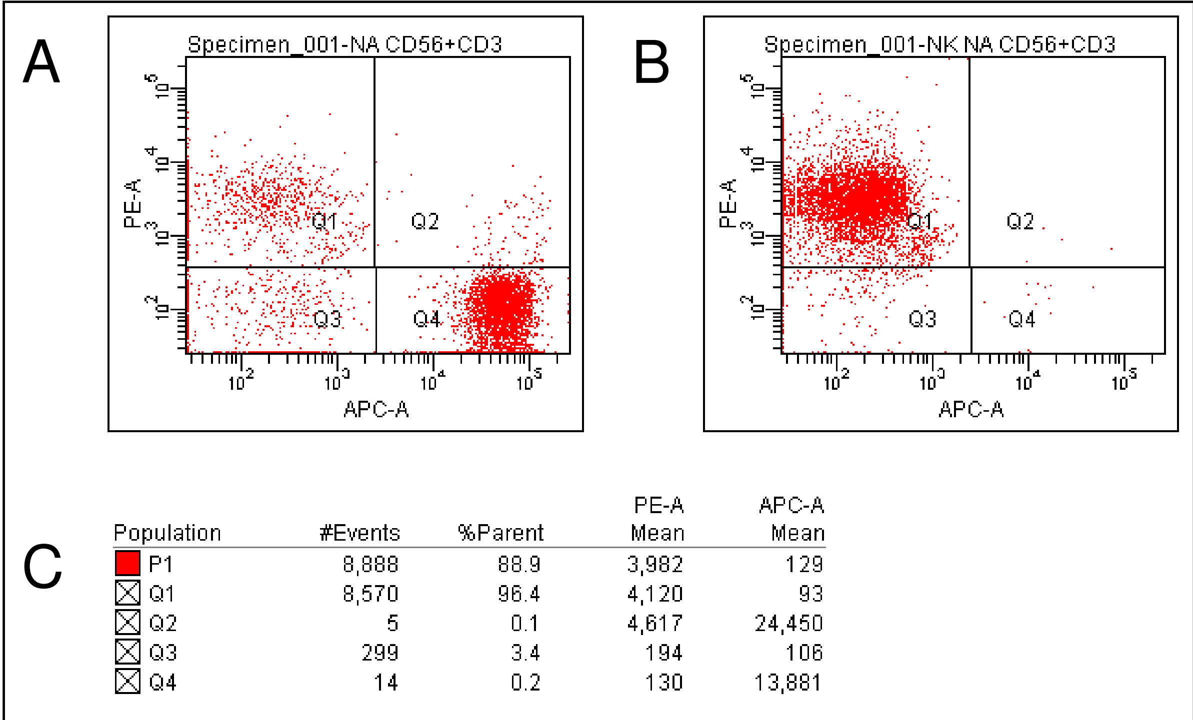
Gene	MB	PB
CD70	-2,815	0,377
HYDIN	2,183	-1,006
KRT72	0,747	-2,386
CCDC144A	2,343	-0,687
SIRPG	0,859	-2,155
KRT73	0,962	-1,928
TEX101	0,311	-2,568
SH3BGRL	0,319	-2,347
NDUFA7	-0,406	2,258
OXER1	0,175	-2,348
C17orf97	2,181	-0,339
RASGRP4	0,468	-1,977
AURKB	2,020	-0,378
TDRD9	2,142	-0,189
RMRP	-0,248	2,070
PPBP	0,559	-1,729
TMEM22	-1,871	0,409
SIGLECP3	1,861	-0,372
CHST13	0,442	-1,777
RPS23	1,780	-0,390
NME2	-0,324	1,827
LOC44034	1,885	-0,221
EMR4	0,163	-1,941
FPR1	0,249	-1,841
PACSIN1	0,324	-1,747
RNF17	1,734	-0,309
RTKN2	0,155	-1,882
NANOS1	-1,688	0,345
COL9A2	0,096	-1,880
ANO9	0,286	-1,679
KIR2DS3	1,855	-0,073
EMR4P	0,052	-1,859
GDPD1	-0,243	1,645
DYSF	1,775	-0,073
TNFRSF9	-1,664	0,078
MS4A6A	0,025	-1,656
CD93	1,591	-0,050
PLA2G4C	-1,596	0,023

Difference in expression



Quantitative difference in expression of genes with a fold-change greater or smaller than $\log_2(3)$, respectively $-\log_2(3)$, in either the paucibacillary (PB) or multibacillary (MB) group.

Figures:



Population	#Events	%Parent	PE-A Mean	APC-A Mean
P1	8,888	88.9	3,982	129
Q1	8,570	96.4	4,120	93
Q2	5	0.1	4,617	24,450
Q3	299	3.4	194	106
Q4	14	0.2	130	13,881

Figure 1: Example of success of natural killer (NK) cell isolation. NK cells are CD56-PE^{+} and CD3-APC^{-}. A: Before isolation. B: NK cell enrichment after isolation. C: Numerical representation of NK cell enrichment post isolation, Q1 is the representation of the CD56-PE^{+}/CD3-APC^{-} NK cell population and contains 96.4% of all cells in the suspension (up from ~15% before isolation, not pictured).

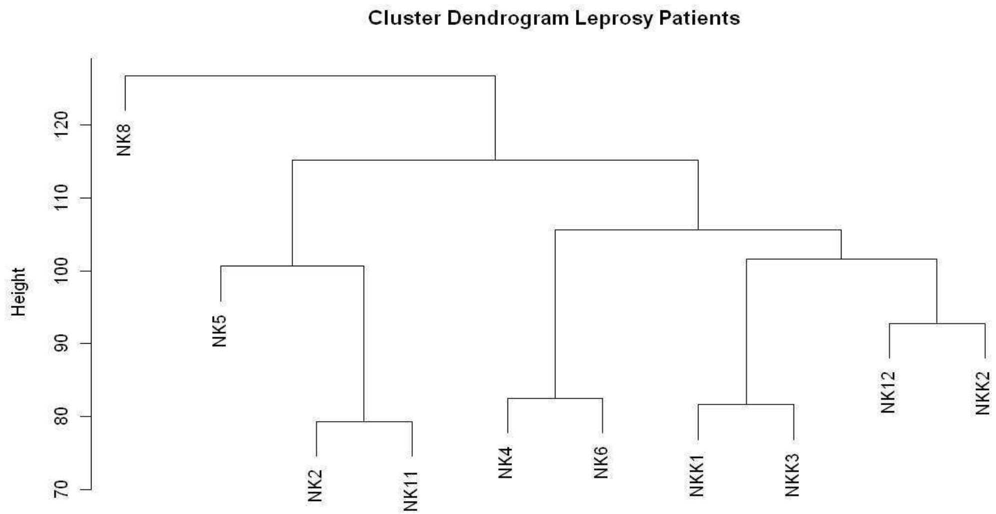


Figure 2: Cluster dendrogram comparing the expression patterns of the patient and controls samples and grouping them accordingly. NK2 and NK11 are both paucibacillary, NK4 and NK6 are both multibacillary. NKK samples are healthy controls. Outliers: NK8 (PB), NK5 (MB), NK12 (PB), and NKK2 (healthy control).

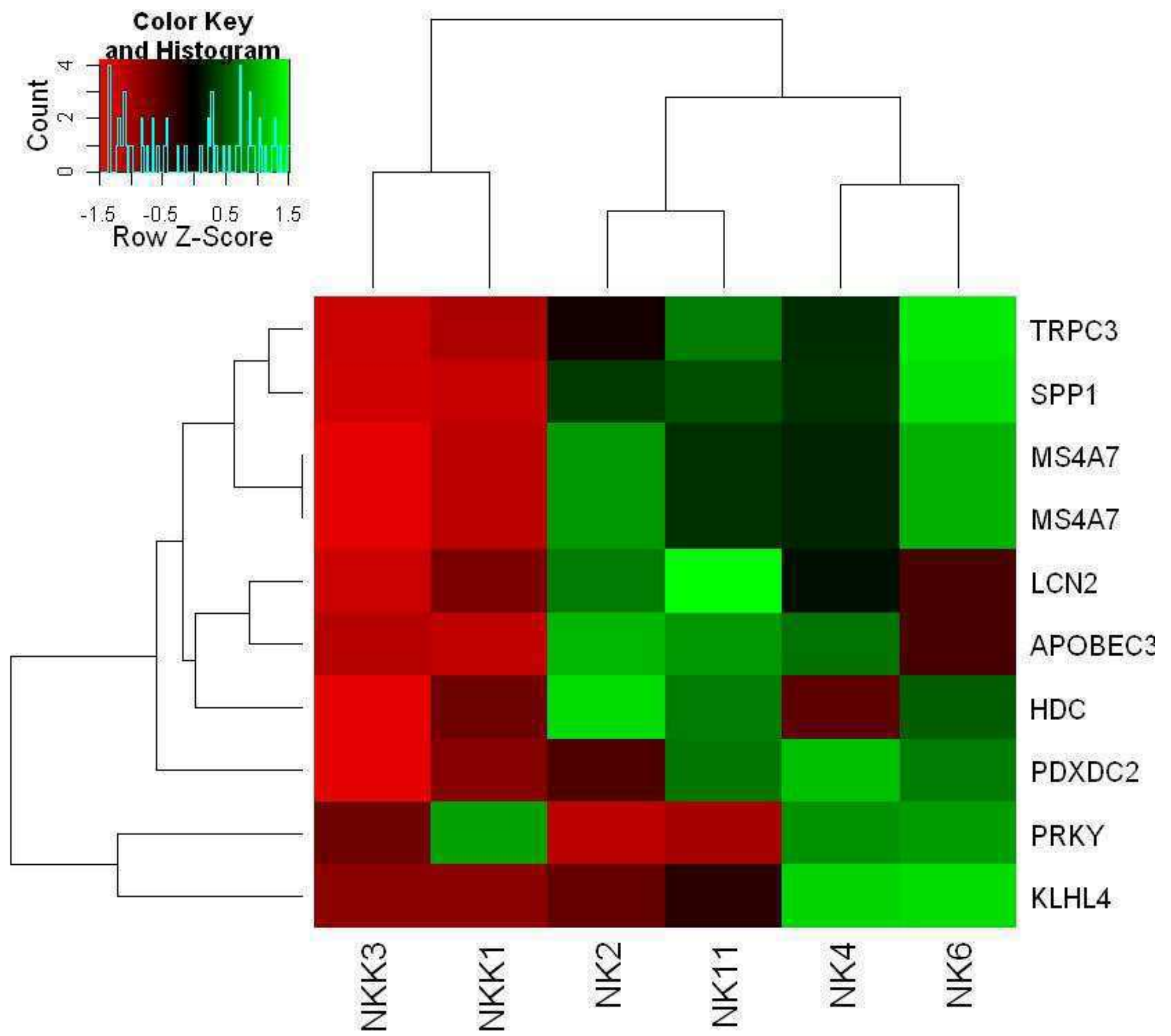


Figure 3: Comparison of differentially expressed genes in leprosy patients and healthy controls (NKK3 and NKK11). NK2 and NK11 are paucibacillary patients, NK4 and NK6 are multibacillary.

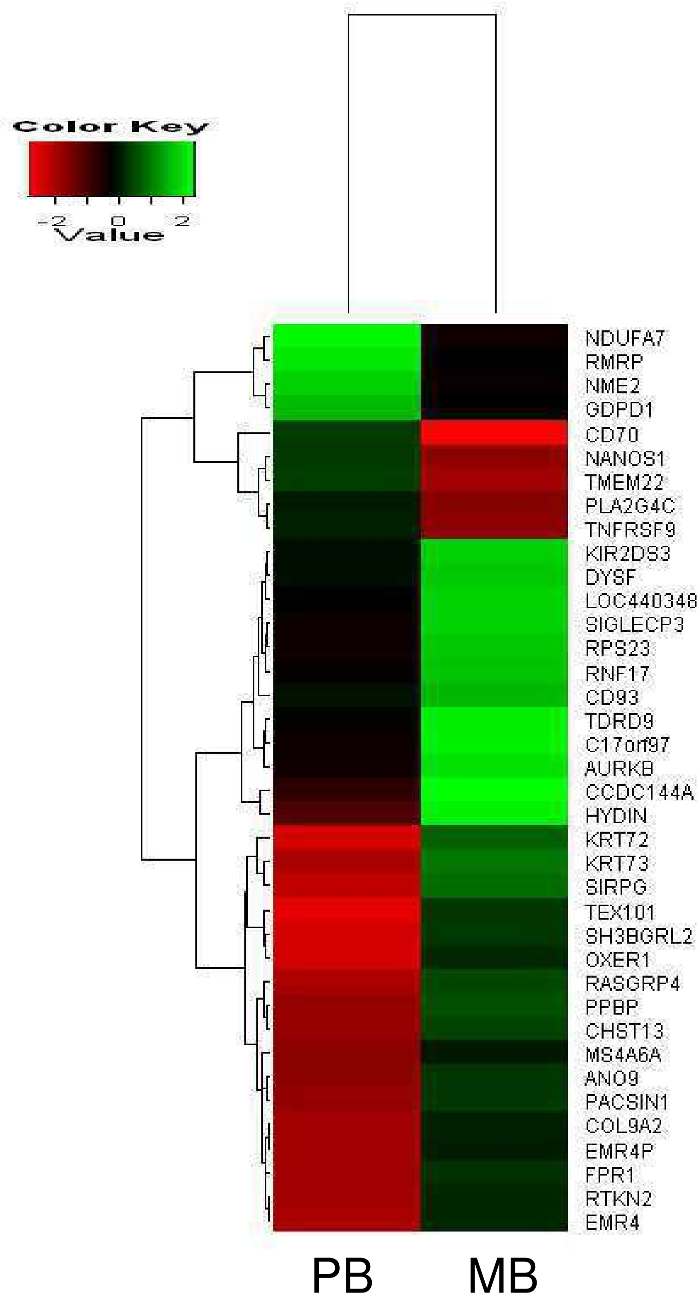


Figure 4: Differentially expressed genes in the paucibacillary (PB) and multibacillary (MB) patient groups with healthy individuals as a baseline. All genes have a fold-change greater or smaller than $\log_2(3)$, respectively $-\log_2(3)$, in either the paucibacillary (PB) or multibacillary (MB) group.

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Patrick Marcinek