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Reprogramming diminishes retention of *Mycobacterium leprae* in Schwann cells and elevates bacterial transfer to fibroblasts

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SHORT RESEARCH ARTICLE

REVISED Reprogramming diminishes retention of *Mycobacterium leprae* in Schwann cells and elevates bacterial transfer property to fibroblasts [v3; ref status: indexed, <http://f1000r.es/2ae>]

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Abstract

Background: Bacterial pathogens can manipulate or subvert host tissue cells to their advantage at different stages during infection, from initial colonization in primary host niches to dissemination. Recently, we have shown that *Mycobacterium leprae* (ML), the causative agent of human leprosy, reprogrammed its preferred host niche de-differentiated adult Schwann cells to progenitor/stem cell-like cells (pSLC) which appear to facilitate bacterial spread. Here, we studied how this cell fate change influences bacterial retention and transfer properties of Schwann cells before and after reprogramming.

Results: Using primary fibroblasts as bacterial recipient cells, we showed that non-reprogrammed Schwann cells, which preserve all Schwann cell lineage and differentiation markers, possess high bacterial retention capacity when co-cultured with skin fibroblasts; Schwann cells failed to transfer bacteria to fibroblasts at higher numbers even after co-culture for 5 days. In contrast, pSLCs, which are derived from the same Schwann cells but have lost Schwann cell lineage markers due to reprogramming, efficiently transferred bacteria to fibroblasts within 24 hours.

Conclusions: ML-induced reprogramming converts lineage-committed Schwann cells with high bacterial retention capacity to a cell type with pSLC stage with effective bacterial transfer properties. We propose that such changes in cellular properties may be associated with the initial intracellular colonization, which requires long-term bacterial retention within Schwann cells, in order to spread the infection to other tissues, which entails efficient bacterial transfer capacity to cells like fibroblasts which are abundant in many tissues, thereby potentially maximizing bacterial dissemination. These data also suggest how pathogens could take advantage of multiple facets of host cell reprogramming according to their needs during infection.

Article Status Summary

Referee Responses

Referees	1	2	3
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v2 published 01 Nov 2013 REVISED	 report	 report	 report
v3 published 14 Nov 2013 REVISED		 report	 report

- 1 Maximiliano Gutierrez**, MRC National Institute for Medical Research UK
- 2 Yoshiko Takahashi**, Kyoto University Japan
- 3 Tom Gillis**, Louisiana State University School of Medicine USA

Latest Comments

No Comments Yet

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REVISED Changes from Version 2

We thank for reviewers for their additional constructive comments. Accordingly, the discussion section has now extended to address the relevance of the findings in the context of disease pathogenesis and bacterial spread *in vivo*. As recommended, a new figure illustrating the summary of the experimental procedure and results has also been added (the new Figure 1).

See referee reports

Introduction

The peripheral nervous system (PNS) is the preferred residence for one of the oldest bacterial pathogens known to mankind, *Mycobacterium leprae* (ML). It causes human leprosy, which is a chronic neurological disease and still remains a public health problem¹. The large gaps in our understanding of the infectious process have halted the progress towards developing effective early diagnostics and therapeutics for the management of nerve damage, the pathological hallmark of the disease. The distinctive peripheral nerve involvement is directly associated with the remarkable capacity of ML to invade the supporting glial cells of the adult PNS, Schwann cells². Because peripheral nerves are a privileged site and are therefore protected from immune cells due to blood nerve barrier, Schwann cells provide a safer niche for ML survival, propagation and initial colonization.

One of the primary functions of Schwann cells is to synthesize the myelin sheath around axons that deliver rapid nerve conduction³. Despite the terminal differentiation of Schwann cells to a highly sophisticated myelinated phenotype, these mature Schwann cells show unprecedented plasticity; they can switch off their myelin program in response to injury and acquire a de-differentiated state resembling an immature phenotype, but maintain Schwann cell lineage properties^{4,5}. ML appear to take advantage of this natural property and induce myelin damage (demyelination) as an adaptive mechanism to generate similar de-differentiated Schwann cells that are more favourable for bacterial colonization and manipulation⁶. We have recently shown that once infected these de-differentiated Schwann cells purified from adult nerves, they undergo a reprogramming process and convert to highly immature progenitor/stem cell-like cells (pSLC) that are more suitable for spreading infection to other tissues either by direct re-differentiation or through macrophages⁷. However, it is not clear if fibroblasts, which are ubiquitous in many tissues, could play an intermediary role by receiving ML and if so how pSLC differ from non-reprogrammed Schwann cells in terms of bacterial transfer.

In this study, using primary mouse fibroblasts as a model, we assessed the bacterial transfer capacity of Schwann cells before and after reprogramming. We showed that before reprogramming, lineage committed Schwann cells possess a high bacterial retention capacity. However, after reprogramming, which downregulated all Schwann cell lineage markers and myelin markers, Schwann cells lose bacterial retention and acquire an efficient bacterial transfer property to co-cultured fibroblasts. These findings show an example of how a bacterial pathogen could use an induced cell fate change to suit its own ends during different stages of infectious process.

Materials and methods

Preparation of primary Schwann cells from adult peripheral nerves

Adult CD-1 mice (4–6 week old, ICR strain code: 022) and 6–8 week old GFP mice that constitutively express eGFP (strain: C57BL/6-Tg [ACTB-EGFP]10sb/J, stock: 003291) were purchased from Charles River and Jackson Laboratories (Bar Harbor, ME). Animals were maintained at the Rockefeller University animal facilities in pathogen free cages. Institutional Animal Care and Use Committee (IACUC) at the Rockefeller University approved all animal procedures and ethical issues. For isolating Schwann cells, 6–8 mice were used and cells were then purified using magnetic cell sorting system and FACS sorting using anti-p75 antibody (AB1554, Millipore, USA) as described⁷.

ML infection and reprogramming of mouse Schwann cells

Purified Schwann cells were grown in collagen coated T25 or T75 flasks (BD Biosciences, NJ, USA) and infected with ML and reprogrammed cells were generated according to our previous protocol⁷. *In vivo*-grown viable ML derived from nude-mouse footpads were prepared as described previously⁸. Briefly, p75+/Sox10+/Sox2+ Schwann cells purified from adult GFP-mice and wild type were infected with ML and maintained in Schwann cell media as described in details⁷. At day 3 post-infection, Schwann cells maintain p75+/Sox10+/Sox2+ and all other Schwann cell phenotype, and these infected cells were used in this study. In parallel, infected cells were incubated for four weeks and then FACS sorted for p75-cells; their phenotypes were confirmed as p75-/Sox10-/Sox2+, and reprogrammed cells were isolated from this population based on their ability to grow in mesenchymal stem cell media (StemCell Inc, Vancouver, Canada) and were termed as progenitor/stem-like cells (pSLC)⁷. pSLC were transduced with CopGFP-CDH-MSCV-cG reporter vector⁹ obtained from System Biosciences (CA) and stable expression of copGFP in pSLC was performed as reported earlier⁷. GFP+pSLC were re-infected with ML in order to maintain similar bacterial load as with Schwann cells at day 3 post-infection, since reprogramming process that accompanies long incubation and cell proliferation causes bacterial dilution.

Preparation of primary neural and skin fibroblasts

Neural fibroblasts were prepared from adult CD-1 male mice (4–6 week old, ICR strain code: 022) at the same time when Schwann cells were prepared from these mice¹. In brief, peripheral nerve tissues were isolated in MEM (Invitrogen) and then digested with 0.125% trypsin (Invitrogen)/0.05% EDTA and 0.1 mg/ml collagenase I (Worthington Biochemical) and passed through 100 micron mesh nylon filter (BD Falcon). Cells were collected and seeded on T25 flask cultured in 10% FCS medium. Propagated cells negative for p75NGFR Schwann cell surface marker were separated by magnetic cell sorting system followed by FACS sorting using anti-p75 antibody (AB1554, Millipore, USA). The purified p75-negative cells, which were also negative for Sox2 and Sox10 were used for co-culture experiments. Skin fibroblasts were prepared from adult wild-type mouse skin using a similar protocol described in detail previously¹⁰.

Bacterial retention and transfer determination and microscopy

Figure 1 summarizes the experimental scheme described below. Day 3-infected GFP+Schwann cells and re-infected GFP+pSLC

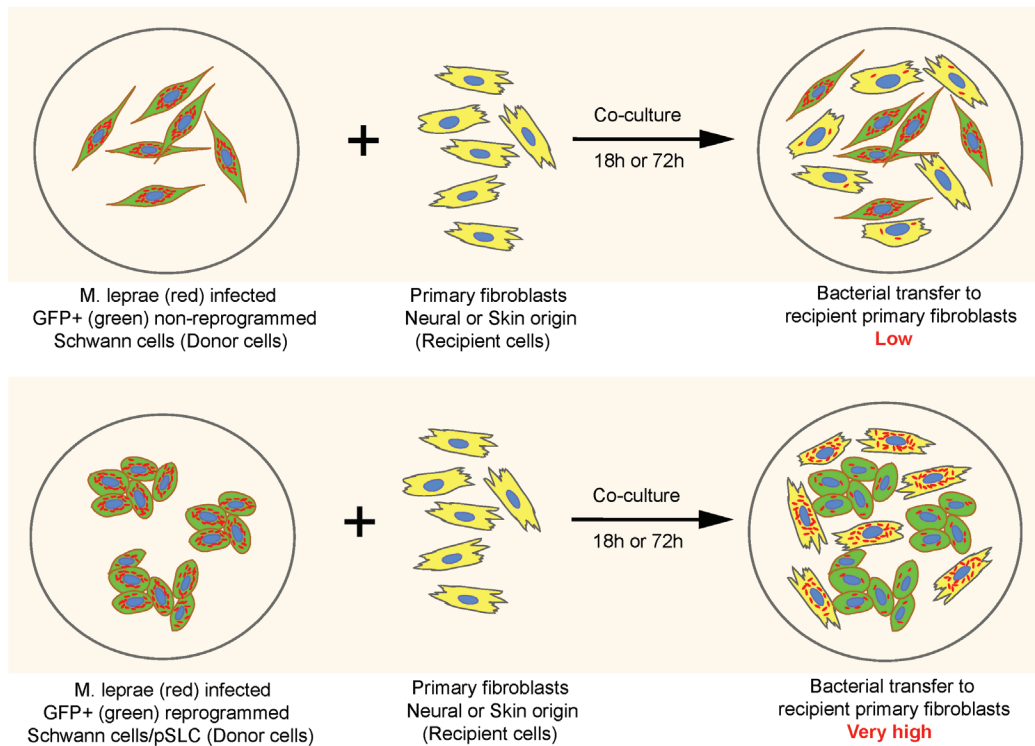


Figure 1. Schematic illustrating the summary of the experimental approach and the results. GFP+ non-reprogrammed and reprogrammed Schwann cells (pSLC) that are infected with ML (red bacteria in green cells) are co-cultured with exogenously added primary fibroblasts (yellow) derived from adult peripheral nerves or skin. Evaluation of bacterial transfer to recipient fibroblasts from GFP+ cells after 18h and 72h revealed an effective bacterial retention in non-reprogrammed cells (top) and a significant ML transfer to fibroblasts from reprogrammed Schwann cells (bottom).

that carry >50–100 ML per cell (infection efficiency is >90%) were first washed to remove any extracellular ML and then co-cultured with GFP-negative fibroblasts for 18 hours, 3 and 7 days. Pre-evaluated culture medium containing DMEM with 6% serum (HyClone, USA) that suits short-term co-cultures, Schwann cell-fibroblasts and pSLC-fibroblasts co-culture combinations were selected. Although we used this medium for short-term cultures for quantification, both mesenchymal media without supplements and Schwann cell media were also supported cell growth for a short period with no apparent phenotypic change in Schwann cells and pSLC.

Also, a possible release of bacteria to the culture media from infected pSLC was determined by using pooled culture media from day 2-cultured pSLC. Cell supernatants from infected pSLC were centrifuged at 15,000 rpm for 20 minutes, and pelleted ML were resuspended in 50 μ l PBS, placed on glass coverslips and fixed and immunolabelled with anti-PGL-1 antibody as described below. As a positive control for ML, a suspension of ML in DMEM (1×10^6 ML/ml) was used. Whereas ML suspension showed numerous PGL-1+ ML, hardly any bacteria were detected from supernatants from pSLC.

Bacterial transfer to non-GFP fibroblasts from GFP+ cells or bacterial retention within GFP+ cells were evaluated by immunolabeling of fixed cultures using anti-PGL-1 antibody⁷ against GFP+/- detection. PGL-1/ML+ cells, both GFP+ and non-GFP cells,

containing >50 ML per cells were quantified 18 hours, 3 and 5 days after addition of fibroblasts. Data were analyzed for statistical significance using Student's t test or by regression analysis (Sigma-Plot). Paired sample analysis with P values <0.01 were considered as significant.

Immunofluorescence was performed as described⁷. In brief, cells were fixed with 4% formaldehyde (Sigma) for 10 min at room temperature and 100% methanol (Sigma) for 10 minutes at -20°C. The samples were washed with PBS, blocked with 10% goat serum and then incubated with primary antibody followed by secondary antibody conjugated with Alexa Fluor 488 or 594 (Invitrogen) as described; details of anti-p75 and anti-PGL-1 and secondary antibodies have been described previously^{7,14}. Assays for apoptotic cells in co-cultures were performed using TUNEL assay kit (R&D Systems) according to manufactures instructions as described previously^{7,15}. Images were captured with Nikon Eclipse 2100 microscopy.

Gene-Expression analyses

Gene-Expression Analyses were performed using Affymetrix mouse gene chips according to Affymetrix protocol as described previously⁷. In brief, total RNA was isolated from uninfected/control Schwann cells, 3 days post-infection and pSLC derived from day 28-infected Schwann cells using RNeasy columns (QIAGEN).

Affymetrix Test3 arrays and mouse genome MG-430A2 arrays were probed with the cRNA prepared by reverse-transcription of the total RNA. Microarray data were processed by the Robust Multichip Average (RMA) method¹¹. The R statistical programming language was used (version 2.15.2), in tandem with the Bioconductor Analysis suite (version 2.12)¹². The resulting probe mRNA detection data was searched and selected for markers relating to Schwann cell lineage/myelination, based on published literature³⁻⁷. This list of probes contained duplicate probes for the same gene symbol and so was trimmed to include only one representative probe per gene symbol, both for clarity and due to space constraints. The data were presented in the form of heatmap representing expression of the genes (absolute log₂ expression values) associated with these probes in each replicate at each time point. Genes were clustered by Euclidean distance and average linkage. Individual values and probes are shown in [Supplemental table 1](#). Also, differentially expressed lineage marker genes (from means of replicates) were calculated as relative to control/uninfected cells in both day 3-infected cells and pSLC state.

Results

Properties of infected non-reprogrammed and reprogrammed Schwann cells

Schwann cells purified from mouse adult peripheral nerves maintained Schwann lineage and myelin markers and are highly susceptible to ML infection⁷. In this study, we used Schwann cells which were infected with ML for 3 days, and previously described pSLC⁷ which were derived from reprogrammed Schwann cells after day 28 infection. Both day 3-infected Schwann cells and pSLC showed high level of infection and strict confinement of ML to the cytoplasm ([Figure 2A and B](#)). We found no evidence for bacterial leakage into the surrounding media when either cell type is cultured on its own. Supernatants collected from these cells after infection showed no evidence of ML in the media (data not shown). We next determine if ML infection at day 3 changed Schwann cell lineage marker expression when compared with pSLC, which are known to be reprogrammed cells exhibiting the loss of Schwann cell lineage markers⁷. Comparative analyses revealed that infected Schwann cells at day 3 express a similar profile of Schwann cell lineage/myelination-related genes as compared to uninfected controls ([Figure 2C-a](#)). In contrast, pSLC showed a striking downregulation of the same markers. Absolute expression profiles of Schwann cell lineage/myelination-related genes are shown in [Figure 2C-a](#). Differential expression of day 3-infected and pSLC as compared to uninfected control cells further revealed that there is almost no change in Schwann cell identity in cells at day 3 post-infection as compared to the marked downregulation of the same genes in pSLC ([Figure 2C-b](#)). Since pSLC, but not cells infected for 3 days, lost Schwann cell identity, we refer to pSLC and infected Schwann cells at day 3 as reprogrammed and non-reprogrammed Schwann cells respectively ([Figure 1C a, b](#)).

Non-reprogrammed Schwann cells possess high bacterial retention capacity in the presence of fibroblasts

The difference between non-reprogrammed and reprogrammed Schwann cells at the mRNA level correlated with their capacity to maintain or transfer ML when primary fibroblasts were introduced to these cell types ([Figure 1](#)). GFP+ Schwann cells purified from

GFP mice show high susceptibility to ML infection. When GFP+ Schwann cells at day 3 post-infection were co-cultured with non-GFP fibroblasts we found intracellular ML to be retained within the cytoplasm of Schwann cells after 3 and 5 days ([Figure 3A](#)). Bacterial transfer to GFP negative fibroblasts was minimal even after 5 days of co-culture. Identical results were obtained regardless of the tissue type (neural or skin) from which fibroblasts were isolated and the media used for co-culturing the cells. These data suggest that non-reprogrammed Schwann cells, which preserve Schwann cell identity, expressing the full spectrum of Schwann cell lineage functional markers ([Figure 2C](#)), retain ML in large numbers.

Reprogramming converts Schwann cells to a stem-like cell type with effective bacterial transfer capacity

When infected GFP+pSLC were co-cultured with primary mouse fibroblasts we found that most of the ML present within pSLC were transferred to non-GFP fibroblasts within 24 hours. [Figure 3B](#) shows the transfer of PGL-1+ ML to non-GFP fibroblasts when they co-cultured for 18 hours or 3 days. [Figure 1](#) illustrates the experimental scheme and summary of the results described below. Regardless of the origin of fibroblasts, whether they are neural fibroblasts (isolated from peripheral nerves) or dermal fibroblasts (isolated from adult skin), and regardless of choice of media for co-culture, mesenchymal media, DMEM with 6% serum or Schwann cell media, bacterial transfer assays from pSLC showed similar results. Unlike macrophages, fibroblasts are not professional phagocytic cells equipped with highly potent immune mediators capable of killing bacteria and their host cells, and thus bacterial residence in fibroblasts may provide an immune-evasion strategy for ML with a decayed genome¹³. As expected, we could not detect any apoptotic GFP+pSLC debris phagocytosed by these fibroblasts in co-cultures. Therefore, ML transfer to fibroblasts from pSLC is unlikely to occur by an apoptosis-mediated mechanism, but mainly by a cell-to-cell transfer mechanism.

Discussion

Results presented in this study show an example of how a bacterial pathogen could use cell fate change of its preferred host cellular niche to its own advantage during different stages of the infectious process, from bacterial colonization to bacterial transfer to fibroblasts that could facilitate the complex process of dissemination of infection. We have frequently observed that primary Schwann cells as mono-cultures retained intracellular ML for a long period within Schwann cells, regardless of their species origin, rat, human or mouse^{1,14,15}. Intriguingly, intracellular ML maintained Schwann cells without causing any apoptosis; this anti-apoptotic property is a defining feature of ML as compared to other pathogenic bacteria^{15,16}. Co-culture of infected GFP+ Schwann cells with high bacterial load with fibroblasts failed to produce a significant bacterial transmission even after 5 days ([Figure 3](#)). Such bacterial retention capacity in adult Schwann cells may also be of functional significance during human infection, since Schwann cells in leprosy patients are known to harbour ML for an extensive period, which may be critical for bacterial expansion within this privileged niche^{3,17}. For this purpose, initial bacterial retention within Schwann cells is critical so that ML replication and colonization can be ensured. On the other hand, following sufficient intracellular propagation of ML within Schwann cells, the next step of the infectious process, as in many bacterial infections, is to transfer their progeny to a secure host cell type,

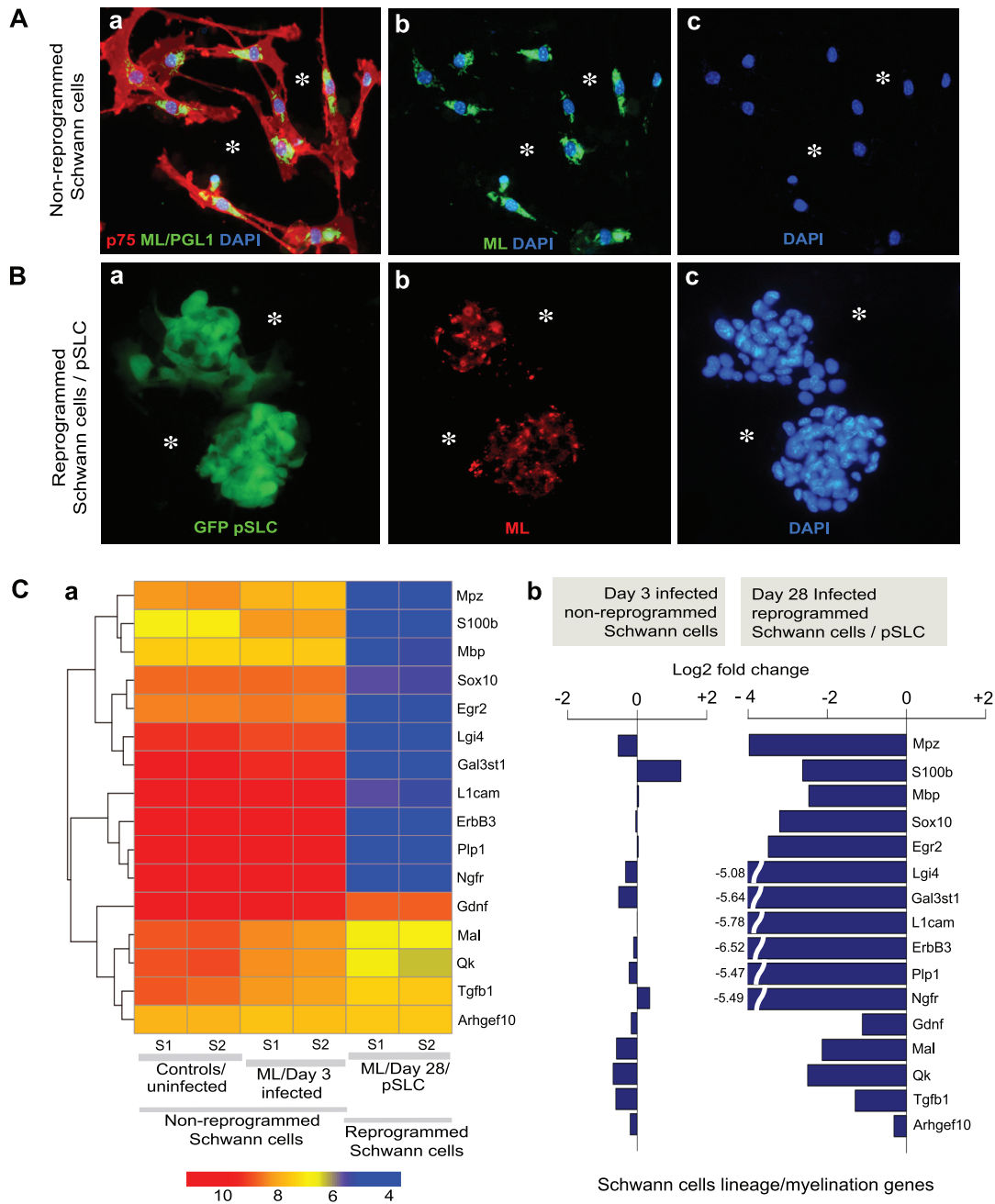


Figure 2. Properties of ML infected non-reprogrammed and reprogrammed Schwann cells. (A) Purified adult de-differentiated Schwann cells infected with ML for 3 days and labelled with antibodies to p75NTR (red) and ML-specific PGL-1 (green), counterstained with DAPI for nuclei (blue). (B) GFP pSLC (green) derived from day 28 infected Schwann cells labeled with anti-PGL1 and counterstained with DAPI (blue). Asterisks denote the absence of ML outside infected cells; ML strictly retain within the cytoplasm and no evidence of bacterial leakage to the surrounding when maintain as monocultures. Magnification: (A-a, b, c to B-a, b, c) 20x. (C) (a) Expression levels of known Schwann cell lineage/myelination genes, inferred by mRNA detection by Affymetrix Mouse microarray, from 2 samples (S1 and S2) each from control/uninfected Schwann cells, infected Schwann cells for 3 days (non-reprogrammed cells preserving Schwann cell identity) and pSLC-derived cells from day 28-infected Schwann cells (reprogrammed cells with loss of Schwann cell identity). Genes are clustered by Euclidean distance and average linkage for clarity. (b) Differential expression (log2 fold change) of known Schwann cell lineage markers, shown as relative to control for both day 3 infected cells (left) and 28-day-derived pSLC-state cells (right). The mean of both replicates for each of the three time points was used when calculating log2 fold change relative to control. Note that both absolute (a) and differentially expressed (b) patterns show a high degree of similarity between control/uninfected and day 3-infected cells, as compared to marked downregulation of Schwann lineage/in pSLC. Robust Multichip Average (RMA) values representing, on a logarithmic scale (base 2), the relative abundance of an mRNA transcript for a given gene, shown as a colour scale from highest (around 10) to lowest (around 4). Colour scale for heatmap ranges between minimum and maximum detection of selected genes, while full array's range was 2.4 to 14.2.

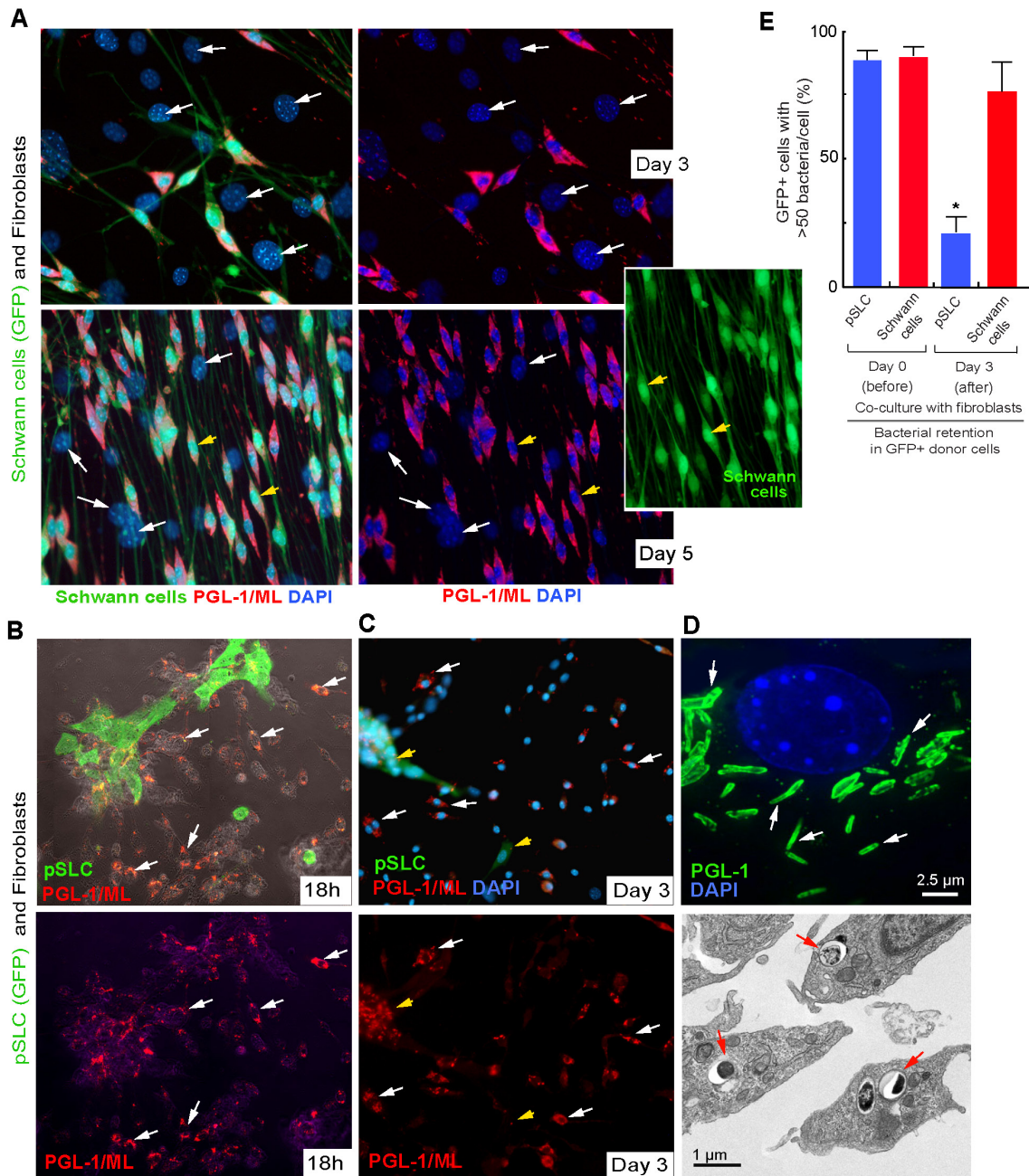


Figure 3. Reprogramming significantly reduces ML retention capacity of adult Schwann cells and elevates bacterial transfer property to fibroblasts. (A) De-differentiated Schwann cells purified from adult peripheral nerves from GFP mice were infected with ML for 3 days and incubated with fibroblasts (isolated from wild type/non-GFP peripheral nerves) for another 3 (top panel) and 5 (bottom panel) days. Fixed co-cultures were labelled with antibody to ML-specific PGL-1 (red) and nuclei were counter stained with DAPI (blue). White arrows show non-GFP fibroblasts with very few ML transferred from GFP+ Schwann cells. Note that almost all GFP+ Schwann cells carry high number of ML (yellow arrows). Inset shows GFP+ Schwann cells with typical bipolar morphology with content of ML (same as in bottom panel). (B, C) GFP+ reprogrammed Schwann cells, pSLC effectively transfer ML to exogenously added fibroblasts. GFP-pSLC were co-cultured with fibroblasts and fixed after 18h (B) and 3 days (C) and labelled with anti-PGL1 antibody. In B (top) shows the phase-contrast image and in C (top) cells are counterstained with DAPI (blue). Note that numerous ML were transferred to fibroblasts within 18h; arrows in B, C show PGL-1+ ML in non-GFP fibroblasts. Yellow arrows mark the GFP+pSLC (B, C). Magnification: (A, B, C) 20x. (D) Representative high-resolution confocal image (top) showing PGL-1 antibody-reactive intact rod-shaped ML (green; arrows) within fibroblasts (nucleus is labelled by DAPI; blue), and electron micrograph illustrating whole ML with electron transparent outer lipids (red arrows) in the cytoplasm of fibroblasts (bottom). (E) Quantitative analysis of bacterial retention in GFP+ non-reprogrammed Schwann cells as compared to GFP+ reprogrammed Schwann cells/pSLC in the presence of fibroblasts. * <math>p < 0.01</math>.

which could serve as either mediator cells or vehicle that can spread the infection locally or systemically¹⁸. Tissue fibroblasts could serve as non-immune mediator cell types for spreading the infection, as they are ubiquitously present in many tissues whereas macrophages, which come to action following inflammatory responses, are known to serve as a vehicle for bacterial dissemination both locally and systemically¹⁹.

Once colonized, reprogramming of infected Schwann cells may be necessary for the conversion of bacterial retention capacity of parent Schwann cells to a bacterial transfer property of reprogrammed Schwann cells for effective dissemination. We have recently shown that pSLC, but not Schwann cells, effectively transfer ML to macrophages *in vivo* under inflammatory conditions⁷. In this study, we showed that non-immune tissue cells like fibroblasts, which are much safer for ML survival than macrophages and are widely distributed (in the absence of inflammation) in peripheral nerves and skin, two preferred tissue niches for ML²⁰, are a likely target for mediating bacterial dissemination. Effective ML transfer to neural fibroblasts is of particular significance, since neural fibroblasts, which are present in the peripheral nerve microenvironment could serve as an immediate target for ML once they colonized Schwann cells and subsequently undergo reprogramming. Thus, the reprogramming of Schwann cells provide ML with ample advantages – first to colonize intact Schwann cells and then to gradually change the fate of Schwann cells to the pSLC stage, promoting transfer of bacteria to fibroblasts or perhaps to other surrounding tissue cell types. Such a strategy suggests the intriguing possibility of effective bacterial spread to a wide range of tissues via pSLC as the reprogrammed form of infected Schwann cells also acquired other essential features such as re-differentiation, and migratory and immunomodulatory properties that are highly advantageous for bacterial dissemination. Therefore, we propose that the effective ML transfer capacity of the reprogrammed form of Schwann cells to fibroblasts could be a functionally-important event during ML dissemination.

Ubiquitous distribution of fibroblasts in almost all body tissues types suggests that pathogens are most likely to take advantage of these cells in order to reach or exit from their specific tissue niches. Neural fibroblasts are abundant in peripheral nerve tissues and ML may also use these cells during the exit from Schwann cells after colonization or simply use as a safe reservoir for bacterial survival during human infection^{21,22}. Present studies showed that primary neural fibroblasts derived from peripheral nerves could indeed serve as a susceptible recipient cell type for ML when these fibroblasts contact with infected reprogrammed Schwann cells (pSLC). The new data from our study can be extended to the conditions associated with neuropathogenesis in leprosy patients, as neural fibroblasts in leprosy patients are known to secrete and deposit extracellular matrix components, particularly collagen and causes fibrotic conditions, contributing to the irreversible nerve damage observed in leprosy^{21–23}. Under such conditions, fibroblasts harbouring ML could serve as an additional niche for bacterial survival, persistence and spread. Indeed, in leprosy patients and nine-banded armadillos infected with ML, neural fibroblasts carrying high

number of ML has been clearly demonstrated mostly in perineurial compartment^{22–24}. Such bacterial persistence within neural fibroblasts may further perpetuate the nerve injury process by increased fibrosis and inflammation. Further molecular studies on this new role of fibroblasts in ML infection will provide novel insights into neuropathogenesis of ML infection and perhaps developing new strategies for preventing fibroblast-mediated bacterial spread from neural compartment to non-neural tissues.

The underlying mechanisms of effective bacterial retention in non-reprogrammed Schwann cells and rapid bacterial transfer capacity of pSLC are currently unknown. Based on the transcriptomic evidence from gene expression data it is possible that preserved Schwann cell identity in non-reprogrammed cells and loss of Schwann cell identity in reprogrammed cells are associated with these two distinct functional properties. It is also intriguing that both non-reprogrammed and reprogrammed Schwann cells retain bacteria in the cytoplasm in the absence of exogenously added cells (Figure 1A and B). However, rapid transfer of bacteria to exogenously added fibroblasts occurs only when pSLC interact with fibroblasts or macrophages⁷, suggesting that signals received from recipient cells or interacting cells following cell-to-cell interaction could trigger the signals necessary for bacterial transfer process. Since apoptotic events are minimal or not detected in these pSLC and fibroblast co-culture conditions, bacterial transfer from pSLC to fibroblasts is likely mediated by non-apoptotic and cell-to-cell transfer mechanisms. Although mechanisms involving such cell-to-cell bacterial transfer process appear to be highly complex, identification of details allow for the development of strategies to ablate bacterial spread at the early stage of infection.

Author contributions

AR, TM and JQ conceived the study and designed the experiments. JQ and TM carried out the experiments. AM and SRT performed the bioinformatics of the gene expression analyses. AR wrote the manuscript with contribution from all authors. All authors have agreed to the content of the manuscript.

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplementary table

Table S1. Microarray data for Figure 1C. Robust Multichip Average (RMA) values for selected Schwann cell lineage markers, representing absolute gene expression as calculated across 6 microarrays, from 2 samples (S1 and S2) each from control/uninfected Schwann cells, infected Schwann cells for 3 days (non-reprogrammed cells preserving Schwann cell identity) and pSLC-derived cells from day 28-infected Schwann cells⁷.

Gene and Probe ID	“Control (1)”	“Control (2)”	“Day 3 (1)”	“Day 3 (2)”	“pSLC (1)”	“pSLC (2)”
“Tgfb1 (1420653_at)”	8.875085087	8.750895157	8.240683025	8.151701667	7.441773105	7.59125912
“ErbB3 (1434606_at)”	10.37548022	10.32681125	10.3069489	10.18805898	3.557050331	4.095306358
“Sox10 (1424985_a_at)”	8.771069925	8.756508626	8.740748716	8.70056877	5.645300737	5.467906393
“Plp1 (1425468_at)”	10.49937908	10.52663114	10.21998482	10.33987413	5.077788336	4.995512713
“Ngfr (1454903_at)”	10.42934191	10.26776964	10.75528146	10.66659223	4.974116673	4.74094638
“Lgi4 (1434121_at)”	9.391434237	9.35234632	9.014339856	9.056992656	4.786829187	3.791313289
“Mal (1432558_a_at)”	8.920254485	8.986191443	8.414628435	8.297979346	6.728140535	6.916327294
“Mpz (1423253_at)”	8.256161295	8.381011338	7.845112469	7.694736612	4.295568611	4.391234401
“S100b (1434342_at)”	6.899266755	7.029193803	8.242286924	8.204423493	4.173923744	4.505750456
“Qk (1425597_a_at)”	9.012841737	9.022546403	8.359367418	8.287088133	6.721881849	6.317931293
“Arhgef10 (1452302_at)”	7.921453062	7.896197547	7.64605098	7.766296023	7.576333594	7.610272438
“L1cam (1450435_at)”	11.22766628	11.33330355	11.21190865	11.35254211	5.658247758	5.340342385
“Egr2 (1427683_at)”	8.522475217	8.506068104	8.57181597	8.524460792	4.891965037	5.151886088
“Mbp (1419646_a_at)”	7.517747967	7.436965949	7.489265636	7.554494302	4.718879815	5.300037843
“Gal3st1 (1454078_a_at)”	10.04534115	9.950345605	9.470080807	9.457810701	4.324986565	4.37275971
“Gdnf (1419080_at)”	9.972677432	9.918438134	9.802476063	9.741177444	8.832869717	8.826992706

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Current Referee Status:

Referee Responses for Version 3



Tom Gillis

Departments of Microbiology, Immunology and Parasitology, Louisiana State University School of Medicine, New Orleans, LA, USA

Approved: 20 November 2013

Referee Report: 20 November 2013

doi:[10.5256/f1000research.2966.r2509](https://doi.org/10.5256/f1000research.2966.r2509)

The authors have improved the manuscript by addressing my concerns regarding their model of neuropathogenesis and how it relates to current clinical and histopathological findings in leprosy.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.



Yoshiko Takahashi

Department of Zoology, Kyoto University, Kyoto, Japan

Approved: 19 November 2013

Referee Report: 19 November 2013

doi:[10.5256/f1000research.2966.r2502](https://doi.org/10.5256/f1000research.2966.r2502)

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Responses for Version 2



Tom Gillis

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Approved with reservations: 06 November 2013

Referee Report: 06 November 2013

doi:[10.5256/f1000research.2880.r2338](https://doi.org/10.5256/f1000research.2880.r2338)

The studies described herein by Masaki *et al.*, extend earlier *in vitro* modeling of *M. leprae* - Schwann cell interactions by this group that may be at the crux of neurological changes and various pathogenic elements in leprosy. Specifically, this work describes phenotypic and transcriptome changes associated with SC reprogramming following long-term *M. leprae* (ML) infection that are associated with retention, or loss of retention, of ML in coculture with fibroblasts. The loss of retentive behavior is measured by the accrual of ML in fibroblasts placed in coculture with ML-infected normal or reprogrammed SC's.

These studies attempt to recapitulate events occurring *in vivo* and must always be appreciated within the view of what is observed in the disease both at the clinical and histopathological level. To that end it would be helpful for the authors to:

1. Place the phenomenon of "fibroblast infection" within the context of what is seen in clinical disease with a few pertinent references.
2. Broaden the Discussion section to include how the author's hypothesis of early dissemination of ML from infected pSLC (intra-neural compartment) fits with histopathological observations in lepromatous leprosy infections (man and armadillo) which demonstrate a much higher bacterial burden in the extra-neural compartment as compared to the intra-neural compartment.

Finally, since fibroblasts can take up *M. leprae* in culture and infected fibroblasts have been demonstrated in tissues of infected individuals and animals, I'm wondering whether the investigators did studies with ML and fibroblasts alone. Depending on the outcome of these studies, some light might be shed on whether the transfer to fibroblasts is actually an active process (e.g., cell-to-cell contact required) or passive in nature. If data of this nature is available, it would enhance the investigator's assertion that the reprogramming of SC's and loss of bacterial retention is an acquired property that leads to efficient bacterial transfer to fibroblasts. Please expand on your position in the discussion to clarify.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.



Yoshiko Takahashi

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Approved: 06 November 2013

Referee Report: 06 November 2013

doi:[10.5256/f1000research.2880.r2333](https://doi.org/10.5256/f1000research.2880.r2333)

This study is a follow up of their previous discovery published in *Cell*, 2013. The authors have described differences between non-programmed and programmed Schwann cells in their ML-retaining properties. Unveiling these differences is an important step toward understanding how ML disseminates in the body. The experiments were nicely done and the conclusion the authors tried to draw is justified.

I have one comment that must not be a big burden for the authors. This study deals with several different types of cells, donor primary fibroblasts, non-programmed Schwann cells, programmed Schwann cells, recipient fibroblasts derived from multiple origins etc. In addition, Schwann cells (either non-programmed or programmed) are considered either "recipient" or "donor" dependent on which way ML bacteria

translocate. So, the story is a bit confusing for readers. My strong suggestion is that the authors might be willing to put one more figure in which the experimental scheme is summarized in a simple diagram. In this way, fewer readers would need to repeatedly read the same sentences before getting points of what authors want to describe.

The authors have properly addressed the comments in the previous report, which has improved the paper.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.



Maximiliano Gutierrez

MRC National Institute for Medical Research, London, UK

Approved: 05 November 2013

Referee Report: 05 November 2013

doi:[10.5256/f1000research.2880.r2293](https://doi.org/10.5256/f1000research.2880.r2293)

I thank the authors for addressing and clarifying my specific comments. I appreciate the details that are now provided in the new version and I do not have further comments.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Responses for Version 1



Maximiliano Gutierrez

MRC National Institute for Medical Research, London, UK

Approved with reservations: 10 October 2013

Referee Report: 10 October 2013

doi:[10.5256/f1000research.2381.r1922](https://doi.org/10.5256/f1000research.2381.r1922)

This paper reports an intriguing aspect of the complex pathogenesis of the obligatory intracellular bacteria *M. leprae* (ML). The authors report that ML infecting Schwann cells are not able to disseminate to fibroblasts but the cells re-programmed by the bacteria in stem-cell like cells are efficiently transferred to the same cells *in vitro*. These observations are very interesting and contribute to our understanding of the phenomenon of mycobacterial cell-to-cell transfer.

There are only three technical points I think that authors could consider in this work, to unambiguously claim that the putative cell-to-cell transfer mechanism is a non-apoptotic, non-lytic one:

1. The authors stated as data not shown that there is not evidence of bacteria in supernatants. It would be important to show this information and indicate in the methods how it was performed.
2. The authors mentioned that they could not detect any apoptotic GFP+pSLC debris. It would be necessary to show these experiments as well, to exclude e.g. efferocytosis. They should indicate in the methods how apoptosis was detected (markers, staining etc...).
3. The bacterial transfer to fibroblasts is measured primarily by detection of one lipid, PGL-1. It is known that lipids from *M. tuberculosis* can be transferred from infected cells to “bystander” cells (Beatty WL et al. 2000). I understand that the system with ML is much more complicated, but would it be possible to confirm that the fluorescent signal represent ‘intact’ bacteria e.g by electron microscopy?

A minor comment: the authors refer to “strict confinement of ML to the cytoplasm” (results) or “retain bacteria in the cytoplasm” (discussion). Do they mean intracellular?

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

1 Comment

Author Response

Anura Rambukkana, University of Edinburgh, UK

Posted: 17 Oct 2013

We thank the reviewer for the constructive and valid comments. We note here that because of the short nature of this article we did not describe all negative results and present additional data in the original version. Here we address and clarify these issues:

1. Evidence for the absence of bacteria in the supernatant and how it was performed is now elaborated in the methods section of the revised manuscript.
2. As described in our previous reports (Masaki et al., 2013), (Tapinos and Rambukkana, 2005) apoptosis was measured using TUNNEL assay. It is known that *M. leprae* infection does not induce apoptosis (Lahiri et al., 2010), and in the present study no increase in *M. leprae* TUNNEL-positive cells were detected in infected co-cultures as compared to uninfected cells.
3. Phenolic glycolipid-1 (PGL-1), particularly its sugar moieties, is very unique to *M. leprae* and is present in high quantity in the *M. leprae* cell wall. The latter permits the detection of intact *M. leprae* using antibodies against PGL-1. We have used a well-characterized antibody (IgG) against the native sugar/lipid moieties of PGL-1 that has been widely used for detecting whole/intact *M. leprae* in infected cells and tissues (Ng et al., 2010; Masaki et al., 2013). PGL-1 antibody activity has also been shown to correlate with acid-fast labeling (Fite’s staining) that specifically stains intact mycobacteria in infected tissues (Masaki et al., 2013). Considering the specificity and the detection of rod-shaped bacteria in high numbers

in non-GFP fibroblasts co-cultured with pSLC, it was concluded that anti-PGL1 antibody detect the intact *M. leprae*, but not free lipids. Moreover, high load of *M. leprae* in non-reprogrammed Schwann cells (Fig. 2A), reflects the potential high content of bacterial lipids in the cytoplasm, but we failed to detect any significant PGL positivity in fibroblasts co-cultured with non-reprogrammed Schwann cells (Fig. 2A). This further suggests a lack of active (or significant) bacterial lipid transfer from one cell to another under these experimental conditions.

Nevertheless, to further clarify this point the updated version includes a representative high-resolution fluorescence image of intact rod-shaped bacteria in fibroblasts detected by anti-PGL-1 antibody. In addition, we also include a representative electron micrograph showing the presence of intact *M. leprae* in fibroblasts (Fig. 2B). It should be noted that infected fibroblasts are distinguishable from pSLC under the electron microscopy, since pSLC, but not fibroblasts, form cell aggregates/clusters (Fig. 1a).

4. Minor comment: here we emphasize that all *M. leprae* detection is strictly intracellular.

Competing Interests: No competing interests were disclosed.
