NOD-2 and TLR-4 Signaling Reinforces the Efficacy of Dendritic Cells and Reduces the Dose of TB Drugs against *Mycobacterium tuberculosis*

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Key Words

Mycobacterium tuberculosis · Immunomodulation · NOD-2 · TLR-4 · Isoniazid · Rifampicin · Dendritic cells

Abstract

Tuberculosis (TB) is one of the leading killer infectious diseases. TB patients are inflicted with devastating side effects and the toxicity of a lengthy drug regime, accentuating an urgent need to explore newer and safer treatment methods. Recently, an improved understanding of host-pathogen interaction has opened new avenues for TB treatment, including immunotherapy. This has emboldened us to devise a novel strategy to restrict *Mycobacterium tuberculosis* (*Mtb*) growth by activating dendritic cells (DCs) through the NOD-2 and TLR-4 molecules of innate immunity. Triggered DCs show a robust release of cytokines and nitric oxide, autophagy and improved migration towards the lymph nodes, and consequently impede the intracellular survival of *Mtb*. Of note, this approach enhanced the efficacy of TB drugs by reducing their dose to a 5-fold lesser concentration than recommended. In vivo administration of ligands of NOD-2 (NOD-2L) and TLR-4 (TLR-4L) substantially increased the pool of effector memory CD4 and CD8 T cells. Additionally, NOD-2L and TLR-4L, in conjunction with the reduced dose of isoniazid, substantially declined the *Mtb* burden in the lungs.

In the future, adjunct therapy involving NOD-2L, TLR-4L and TB drugs may have enough potential to reduce the dose and duration of treatment of TB patients. © 2015 S. Karger AG, Basel

Introduction

Despite the availability of highly effective drug regimens, tuberculosis (TB) continues to wreck public health worldwide. Furthermore, the threat is compounded by the emergence of the AIDS pandemic and drug-resistant strains of *Mycobacterium tuberculosis* (*Mtb*) [1]. The bacillus Calmette-Guérin (BCG) vaccine is available against TB, but its role is highly controversial due to its variable efficacy globally [2]. Hence, there is an urgent need to develop innovative strategies to treat TB. Since recently, novel therapies employing immunomodulators are being explored for treating various ailments such as cancer, autoimmunity and cardiac diseases [3–6]. Immunotherapy holds great promise for various diseases, but no serious effort has been undertaken in the case of TB.

Adaptive immunity plays a vital role against *Mtb* due to its specificity and memory response. However, in the last few decades, the focus has been generated more on innate immunity. Innate cells perform multiple functions

employing various pattern recognition receptors such as Toll-like receptors (TLRs), C-type lectin receptors and Nod-like receptors (NLRs) [7, 8]. Recently, the experimental model of TB has highlighted the importance of TLRs in protecting against *Mtb* [9]. TLRs contribute substantially in resistance against *Mtb*, but this attribute is not endowed to their function alone. Furthermore, the recognition of antigens by NOD-2 (nucleotide-binding oligomerization domain 2), a member of the NLR family also critically contributes in imparting immunity against bacteria or viruses [10–12]. This indicates that synergistic triggering through TLRs and NOD-2 may result in a stronger and enduring immune response that could restrict the growth of *Mtb*. The WHO has also recommended the use of immunomodulators as an adjunct therapy to the existing TB drugs [13].

Dendritic cells (DCs) are the most potent antigen presenting cells that activate naive T cells. Despite their key role in initiating cell-mediated immunity, DCs exhibit several deficiencies including inferior bactericidal activity and limited migration to the draining lymph nodes (DLNs) [14]. Noteworthy, DCs are the major players in bridging innate and adaptive immunity. Targeting DCs in order to improve their bactericidal activity could strengthen both innate and adaptive immunity. Taking into consideration the above-mentioned facts, we designed an innovative approach to bolster DC efficacy by delivering signals via NOD-2 and TLR-4 (N_2T_4). It is important to mention that NOD-2 is expressed in the cytoplasm. To ligate with receptors, a ligand for NOD-2 is internalized into acidified vesicles by endocytosis dependent on the clathrin and dyamin pathways [15]. We selected N-glycolyl muramyl dipeptide (MDP) as a NOD-2 agonist as it exhibits 10 to 100 fold more potent immunogenicity than the commonly studied N-acetylated MDP [16]. We used LPS as a source of TLR-4 ligand (TLR-4L). Recently, the FDA has approved the use of LPS in medicines, which has opened new avenues to harness its remedial potential [17, 18]. DCs activated by signaling through NOD-2 and TLR-4 (N_2T_4 .DCs) exhibited improvement in bactericidal activity. Further, N_2T_4 triggering afforded a favorable window for TB drugs to achieve an optimum immune response against *Mtb*. Importantly, N_2T_4 .DCs required a considerably lesser dose of the drug to kill the *Mtb*. Activated DCs also induced a better T cell response in vivo due to improved migration and augmented autophagy. This study may open new avenues of adjunct therapy employing drugs in combination with signaling through NOD-2 and TLR-4 in successfully treating TB.

Material and Methods

Animals

C57BL/6 mice, 6–8 weeks of age, were procured from the institute's animal facility. All experiments were approved by the Institutional Animal Ethics Committee (No. 55/1999/CPCSEA), Ministry of Environment and Forests, Government of India.

Antibodies and Reagents

All standard chemicals and reagents used in the study were purchased from Sigma (St. Louis, Mo., USA), and antibodies and recombinant cytokines from BD Biosciences (San Diego, Calif., USA) unless stated otherwise. TLR-4L, i.e. LPS, and NOD-2 ligand (NOD-2L), i.e. N-glycolyl MDP, were procured from Invitrogen (San Diego, Calif., USA). Anti-mouse LC-3 and inducible nitric oxide synthase (iNOS) antibodies were obtained from Sigma and Abcam (Cambridge, UK), respectively.

Mycobacterial Strain and Antigens

Mtb strains (H37Rv and H37Ra) were provided by Dr. V.M. Katoch, National JALMA Institute for Leprosy and Other Mycobacterial Diseases, India, and *Salmonella typhimurium* was provided by Dr. Amitabha Mukhopadhyay, National Institute of Immunology, India. *Mtb* was cultured in Middlebrook 7H9 broth containing glycerol (0.2%) and Tween-80 (0.05%) supplemented with albumin, dextrose and catalase. The viability of the bacteria was determined by plating on Middlebrook 7H11 medium supplemented with oleic acid, albumin, dextrose and catalase, and then counting the number of colony-forming units (CFU). *S. typhimurium* was cultured in LB broth. Viability was determined by plating on LB agar medium.

Culture of Bone Marrow-Derived DCs and Their Stimulation via NOD-2 and TLR-4

Bone marrow-derived DCs were cultured according to Lutz et al. [19]. Briefly, bone marrow cells (BMCs) were flushed aseptically from femurs and tibias. For DC cultures, cells were grown in RPMI 1640 (Invitrogen, Life Technologies, Eugene, Oreg., USA) containing FCS (10%; Gibco, Grand Island, N.Y., USA) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (100 mM), granulocyte-macrophage colony-stimulating factor (GM-CSF) (2 ng/ml) and murine r-IL-4 (4 ng/ml) for 6 days. Cultures were maintained in a humidified atmosphere and 5% CO₂ at 37°C. The medium was replenished on day 3. DCs were then harvested, washed and stimulated with N-glycolyl MDP (10 µg/ml) and LPS (5 ng/ml), respectively, for 24 h.

For macrophages, BMCs were grown in RPMI 1640 + FCS and replenished with L929 (20%) supernatant, as a source of M-CSF. Later, macrophages were harvested, washed and stimulated with NOD-2L (10 µg/ml) and TLR-4L (10 ng/ml) for 24 h.

In vitro Infection with Bacteria and CFU Assay

Bacterial aliquots were rapidly thawed, washed 2× with PBS and resuspended in RPMI 1640 + FCS. To remove clumps, bacteria were passed through an insulin syringe. DCs were infected with the indicated multiplicity of infection (MOI). Cells were infected with *Mtb* for 4 h (MOI 1:5) and with *Mycobacterium smegmatis* for 3 h (MOI 1:5). After infection, cells were washed extensively to remove extracellular bacteria, and were then further incubated with NOD-2L and TLR-4L for 24 and 72 h with the mycobacteria

in the presence of amikacin (2 µg/ml). After 24 or 72 h, cell supernatants were removed, saponin (0.1%) was added to lyse the cells and plating was done with 10× serial dilution on a 7H11 agar plate. The colonies were enumerated at 3 days and 3 weeks for *M. smegmatis* and *Mtb*, respectively, after the incubation at 37°C in a humidified CO₂ (5%) atmosphere. Where indicated, isoniazid (INH; 25 µg/ml), rifampicin (RIF; 0.5 µg/ml) or the iNOS inhibitor N-monomethyl-L-arginine (1 mM) was added, along with NOD-2L and TLR-4L, for 24 h. For *S. typhimurium* experiments, DCs were infected with the bacterium (MOI 1:10) for 30 min. After infection, cells were extensively washed and incubated for 1 h in cell culture medium containing gentamicin (50 µg/ml). The cells were washed and cultures were maintained in medium containing gentamicin (10 µg/ml) for 16 h in the presence or absence of NOD-2L and TLR-4L. For enumeration of the bacteria, cells were lysed with Triton X-100 (0.1%) and serial dilution lysates were plated onto LB agar plates. To block autophagy, DCs were treated with the autophagy inhibitor wortmannin (for 2 h) prior to *Mtb* infection, but chloroquine (10 µM) was present throughout the culture (24 h).

Nitric Oxide Production/Release

Supernatants were harvested after 4 h of infection followed by stimulation via either NOD-2 or TLR-4 or N₂T₄ for 48 h. The nitric oxide (NO) level was measured by the Griess method. Briefly, supernatants (50 µl) were incubated with an equal volume of Griess reagent for 5 min at room temperature. Later, absorbance was measured at 595 nm.

Cytokine Assessment by ELISA

The cytokines IL-6 and IL-12 were detected in culture supernatants at an indicated time point by standard ELISA, according to manufacturer's instructions.

DC and T Cell Coculture

N₂T₄-DCs (C57BL/6) were cocultured with magnetic-associated cell sorted (98%) purified CD4 T cells (BALB/c) at a ratio of 1:5. After 72 h, ³H-thymidine (0.5 µCi/well) was added to the cultures. The plates were harvested after 16 h and the radioactivity incorporated into the cells was measured as counts/min (cpm) by scintillation counting. In parallel, cultures were set for 48 h for cytokine assessment (IFN-γ) by ELISA and intracellular staining. For intracellular staining, cells were treated with PMA and ionomycin for 3 h followed by brefeldin for 2 h and then stained for IFN-γ.

RT-qPCR for the Quantification of IFN-γ and iNOS

Total RNA was isolated by Trizol reagent from *Mtb*-infected DCs stimulated via NOD-2, TLR-4 or N₂T₄ for 6 h or from the lungs of *Mtb*-challenged mice, and followed by immunotherapy, according to the manufacturer's instruction (Invitrogen, Carlsbad, Calif., USA). RNA was quantified with the help of a NanoDrop spectrophotometer. A260/A280 ratio of all samples was in the range of 1.90–2.00. Integrity of RNA samples was determined with the help of formaldehyde denaturing agarose gel-electrophoresis. DNA contamination from RNA samples was removed by amplification grade DNase. Briefly, RNA samples (1 µg) were incubated with DNase (1 U) for 15 min in the reaction buffer. After the incubation, DNase activity was terminated by stop solution. The samples were then heated to 70°C for 10 min to inactivate DNase. Results are represented in the form of relative expression (fold) relative to untreated controls and placebo. Analysis was

done by comparative Ct method, with Ct values normalized against housekeeping control actin. Using the comparative Ct method, relative gene expression was calculated as $2^{(-\Delta\Delta Ct)}$, where $\Delta Ct = Ct$ (gene of interest) – Ct (normalizer = β -actin) and the $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (calibrator). The calibrator was total RNA from placebo-treated lungs. RT-qPCR and data analysis were done with a Realplex Mastercycler (Eppendorf, Hamburg, Germany).

IFN-γ: fwd 5'-CTAAGCAAGGACGGCGAAT-3'; reverse 5'-TTCCACACTGCACCCACTT-3'

β -actin: fwd 5'-AGAGGGAAATCGTGCGTGAC-3'; reverse 5'-CAATAGTGATGACCTGGCCGT-3'

iNOS: fwd 5'-AACGGAGAACGTTGGATTG-3'; reverse 5'-CAGACAAGGGGTTTCTT-3'.

Propidium Iodide and Annexin V Assays

DCs were stimulated through NOD-2, TLR-4 or N₂T₄ at 37°C and 5% CO₂ for 24 h. This was followed by resuspension of the cells in binding buffer (0.01 M HEPES, pH 7.4, 0.14 M NaCl and 2.5 mM CaCl₂). FITC-conjugated annexin V (5 µl/tube) and 5 µl of propidium iodide (50 µg/ml) were added to the cells and incubated in the dark for 15 min at room temperature. Later, binding buffer (400 µl) was added and cells were acquired immediately, employing a BD FACS Calibur flow cytometer; analysis was done using BD DIVA software.

Western Blotting

Mtb-infected DCs were stimulated through NOD-2, TLR-4 or N₂T₄ for 2 h. Later, cells were harvested, washed and lysed in lysis buffer (RIPA buffer, protease and phosphatase inhibitor cocktail). Proteins in the supernatants were estimated and an equal concentration was subjected to SDS-PAGE. After transfer to the nitrocellulose membrane and subsequent blocking, the membranes were immunoblotted with antibodies against LC3-I/LC3-II and actin as a loading control. Blots were developed using a chemiluminescence kit (Amersham Pharmacia Biotech, Amersham, UK). Blots were scanned with the help of a phosphorimager (Fujifilm, Tokyo, Japan) and image analysis was performed with MultiGauge software. For iNOS, *Mtb*-infected DCs were stimulated through NOD-2, TLR-4 or N₂T₄ for 16 h and the expression was analyzed by Western blotting.

Flow Cytometric Analysis for the Expression of Activation Markers

Mtb-infected or uninfected DCs stimulated with NOD-2 and TLR-4 in the presence or absence of IL-12-neutralizing antibodies for 24 h were harvested and resuspended in staining buffer (2% FCS, 2 mM NaN₃ in PBS). To block nonspecificity, cells were first incubated with Fc block (anti-CD16/32 Ab) for 25 min at 4°C. The cells were washed and then stained with fluorochrome-conjugated antibodies specific for CCR7, and the control cells with isotype-matched antibodies for 30 min at 4°C. Cells were washed and fixed with paraformaldehyde (1×). Data were collected using FACS ARIA II and analyzed with BD DIVA software.

siRNA Knockdown of NOD-2

DCs were incubated with NOD-2 siRNA (1 µM) in Accell siRNA delivery media, as per the manufacturer instructions (Thermo-Scientific Dharmacon). After 48 h, inhibition in the expression of NOD-2 was detected at mRNA level by RT-qPCR.

For functional assays, DCs incubated with NOD-2 siRNA for 72 h were washed and restimulated with N₂T₄L in RPMI + FCS for 24 h. Later, IL-6 production was detected in supernatants by ELISA.

Migration of DCs in vivo

DCs were infected with *Mtb* followed by stimulation through N₂T₄ for 24 h. Later, N₂T₄.DCs (3 × 10⁶ cells) were labeled with CFSE (5 μM) and adoptively transferred into mice. After 72 h, the mice were sacrificed, the DLNs were isolated and a single-cell suspension was prepared. The frequency of CFSE-positive cells was quantified employing flow cytometry.

Therapeutic Strategy

Mice were aerosol-challenged with *Mtb*, and 100 CFU were deposited in the lungs. Later, the animals were injected twice subcutaneously with a gap of 15 days with N₂T₄L and the controls with NOD-2L or TLR-4L. The dose of NOD-2L versus TLR-4L for an *in vivo* assay was selected on the basis of the reduction in CFU in the lungs of *Mtb*-challenged animals treated with different doses of NOD-2L (3, 30 and 300 μg/100 μl PBS) or TLR-4L (0.1, 1 and 10 μg/100 μl PBS) alone or in combination, twice with a gap of 15 days. After the last injection, the animals were sacrificed and CFU were determined in the lungs. Total CFU per gram of lung were calculated. The optimum reduction in CFU was noticed with 3 μg/100 μl of NOD-2L and 0.1 μg/100 μl of μl TLR-4L, so these doses were selected to perform all *in vivo* experiments.

Isolation of Lymphocytes

Mice were aerosol-challenged with *Mtb* followed by treatment with NOD-2L, TLR-4L or N₂T₄L. After 45 days of aerosol challenge with *Mtb*, the mice were sacrificed. The lungs were perfused and the spleen and lymph nodes (mediastinal) were harvested and a single-cell suspension was prepared. Briefly, lymphocytes from the spleen were prepared by lysis of red blood cells with ACK lysing buffer (0.15 M NH₄Cl, 10 mM KHCO₃ and 88 mM EDTA), washed 3× with PBS and resuspended in RPMI 1640 + FCS. Viability of the cells was assessed by the trypan blue dye exclusion method.

Staining of the T Cell Surface Markers and Intracellular IFN-γ Expression

Cells were incubated with Fc block for 25 min and stained for the expression of CD4, CD8, CD69, CD44 and CD62L by their respective antibodies. Cells were fixed in 1% paraformaldehyde. For intracellular expression, cells stained for the surface CD4 and CD8 molecules were fixed with 4× paraformaldehyde for 30 min followed by permeabilization with 0.01% saponin in PBS for 30 min at 4°C. Later, cells were incubated with anti-IFN-γ antibody in a staining buffer containing 0.01% saponin. The usual steps of washing were performed after each incubation. The control cells were stained with the respective isotype-matched antibodies. Cells were acquired in FACSaria II and analyzed with DIVA software.

T Cell Proliferation

Lymphocytes (2.5 × 10⁵ cells/well) were cultured in round-bottom, 96-well plates, in 200 μl of RPMI 1640 + FCS 10% and purified protein derivative (PPD; 6 μg/ml) for 48 h. Cells cultured with the medium alone (without antigen) were used as a control. Later, cultures were pulsed with 0.5 μCi of ³H-thymidine (Amersham Pharmacia Biotech). The plates were harvested after 16 h onto glass-fiber filter mats using a Tomtec Harvester96 (Tomtec, Hamden,

Conn., USA). Radioactivity incorporated into the cells was measured by liquid scintillation spectroscopy utilizing Wallac 1450 MicroBeta TriLux (Perkin Elmer, Waltham, Mass., USA).

Histopathology

Mice were sacrificed and lung tissues were fixed in buffered formalin (10%). Histological sections were stained using hematoxylin and eosin. Photomicrographs were captured on an Olympus IX71 microscope and displayed at ×40 magnification.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) with the post-Tukey-Kramer multiple-comparison test by using GraphPad Prism software.

Results

Signaling through N₂T₄ Enhances the Activation of DCs

This study focused on improving DC efficacy by exploring their potency through N₂T₄ signaling. We observed that DCs triggered through TLR-4 showed substantial production of IL-6 (p < 0.001) and IL-12 (p < 0.001; fig. 1a, b). This effect was observed to be dose dependent. In contrast, triggering through NOD-2 failed to activate DCs, demonstrated by a lesser release of IL-6 and IL-12. However, it was quite interesting to note that signaling of NOD-2 in conjunction with TLR-4, i.e. N₂T₄, significantly enhanced the yield of both IL-6 (p < 0.01) and IL-12 (p < 0.01; fig. 1a, b). We observed the optimum release of IL-6 and IL-12 with 10 μg/ml of NOD-2L and 5 ng/ml of TLR-4L. In contrast, 80 ng/ml of TLR-4L on its own induced a significantly lower release of IL-6 (p < 0.01) and IL-12 (p < 0.01). Hence, in all subsequent experiments, a dose of 10 μg/ml NOD-2L and 5 ng/ml TLR-4L was selected. We also confirmed and validated the specificity of our results in DCs by knocking down the NOD-2 expression through siRNA (N₂KD; online suppl. fig. S1; see www.karger.com/doi/10.1159/000439591 for all online suppl. material) and, with its inhibitor CLI-095, blocking TLR-4 signaling (T_{4i}). Interestingly, compared to wild-type DCs, N₂T₄ signaling showed no release by N₂KD T_{4i}.DCs (fig. 1c).

N₂T₄-Elicited DCs Showed Enhancement of T Cell Proliferation and IFN-γ Production

N₂T₄.DCs acquired a significantly (p < 0.001) higher capacity to induce IFN-γ production by allogeneic CD4 T cells, as corroborated by the ELISA and flow cytometry results (fig. 2a, b). Further, the improved performance of N₂T₄.DCs was further authenticated by the substantial

Fig. 1. Cumulative signaling through N₂T₄ induces the maturation and activation of DCs. The bone marrow-derived DCs were stimulated with different doses of TLR-4L or NOD-2L. Later, culture supernatants were assessed for IL-6 (**a**) and IL-12 (**b**) cytokines by ELISA. The x-axis shows the concentration of TLR-4L (ng/ml) and NOD-2L (µg/ml); (5/5) indicates the concentrations 5 µg/ml NOD-2L and 5 ng/ml TLR-4L, and (10/5) indicates 10 µg/ml

NOD-2L and 5 ng/ml TLR-4L. **c** DCs were knocked down for NOD-2 through siRNA. N₂KD.DCs were stimulated with N₂T₄ and cultured in the presence or absence of TLR_{4i} for 24 h. Later, IL-6 was detected in culture supernatants. Data represented as mean ± SD are of 3 experiments, each assayed in triplicate wells. ** p < 0.01, *** p < 0.0001, one-way ANOVA.

(p < 0.0001) proliferation of T cells (fig. 2c). These observations demonstrate that signaling of DCs through N₂T₄ has a strong bearing on improving the activation of T cells.

Signaling of DCs through N₂T₄ Restricted the Intracellular Growth of Bacteria

Based on the above results, we subsequently monitored the bactericidal efficacy of the N₂T₄.DCs. DCs infected

with the H37Ra strain of *Mtb* on stimulation with N₂T₄ for 24 h (p < 0.01) and 72 h (p < 0.05) significantly inhibited the intracellular growth of the bacterium (fig. 3a). Further, this phenomenon was corroborated by demonstrating the effect on a virulent strain of *Mtb* (H37Rv; fig. 3b). Furthermore, it appeared that N₂T₄ signaling of DCs could be a unique strategy to inhibit the growth of an array of intracellular pathogens, since it not only retarded the growth of

Fig. 2. Stimulation of DCs via N₂T₄ bolsters their ability to enhance T cell proliferation and IFN- γ production. N₂T₄.DCs were cultured with allogeneic CD4 T cells for 48 h at a ratio of 1:5. **a** IFN- γ was detected in the supernatants by ELISA. **b** Intracellular expression on flow cytometry. The data in the insets are the percentages

of CD4/IFN- γ -positive T cells. **c** T cell proliferation was monitored by ³H-thymidine, and results are expressed as counts/min (cpm). Data represented as mean $\pm$ SD are of 3 independent experiments. n.d. = Not detected. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA.

Mtb (fig. 3a, b) but also *M. smegmatis* ($p < 0.01$) and *S. typhimurium* ($p < 0.05$; fig. 3c; online suppl. fig. S5). It is important to mention here that the reduction in bacterial burden is not due to a differential uptake of *Mtb* by DCs. The bactericidal activity of DCs was demonstrated by first infecting DCs with bacteria for 4 h. Later, infected DCs were stimulated through N₂T₄. It was further confirmed by plating cell lysates of infected DCs at the initial time point (after 4 h) of infection, which showed no difference in the uptake of *Mtb* (online suppl. fig. S2).

N₂T₄ Triggering of DCs Potentiated RIF and INH Mediated the Killing of *Mtb*

We also discerned that signaling of *Mtb*-infected DCs through N₂T₄ considerably boosted the killing efficacy of the anti-TB drugs RIF ($p < 0.01$) and INH ($p < 0.01$), when

compared with the drugs alone (fig. 3d, e). Interestingly, N₂T₄L + RIF and N₂T₄L + INH showed a 75 and 60% reduction, respectively, in the bacterial burden when compared to RIF (37%) or INH (46%) alone. It may be concluded from these results that the adjunct therapy involving NOD-2L and TLR-4L in conjunction with INH and RIF may have an important implication in considerably improving the efficacy of anti-TB drugs to restrict the growth of *Mtb*.

N₂T₄.DCs Limited the Intracellular Survival of *Mtb* by Enhancing Autophagy and NO Production

It was important to elucidate the mechanism involved in restricting the survival of *Mtb* by N₂T₄.DCs. Notably, we observed that inhibition of the growth of *Mtb* by N₂T₄.DCs was operating through autophagy. This was

Fig. 3. Activation of DCs through N₂T₄L inhibits the replication of *Mtb* and potentiates the RIF- and INH-mediated killing. DCs were infected with H37Ra (**a**), H37Rv (**b**) and *M. smegmatis* (**c**) and then stimulated with N₂T₄L for 24 and 72 h. The control DCs were cultured with TLR-4L or NOD-2L. The cells were lysed, and CFU were enumerated on day 3 or day 21. **d, e** DCs infected with *Mtb* were cultured with either N₂T₄L alone or in conjunction with RIF

(N₂T₄L + RIF) or INH (N₂T₄L + INH). Later, cells were lysed and CFU were enumerated on day 3 or day 21 and expressed as a percentage decrease. Decline in *Mtb* growth was monitored in response to RIF and INH. The bar diagram represents the mean $\pm$ SEM of 3 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA.

demonstrated by the conversion of LC3-I to LC3-II (fig. 4a), which is a prominent indicator of autophagy [20]. This phenomenon was further confirmed by blocking autophagy, using its inhibitors wortmannin ($p < 0.01$) or chloroquine ($p < 0.05$), which significantly restored the survival of *Mtb* in N₂T₄.DCs (fig. 4b).

We also discerned augmentation in the production of NO by *Mtb*-infected N₂T₄.DCs compared to control NOD-2L- or TLR-4L-treated DCs or untreated DCs (uDCs; fig. 4c). NO is an established indicator of bactericidal activity [21]. Furthermore, this finding was proved by quantifying the expression of iNOS by Western blotting and RT-qPCR (fig. 4d, e). It is important to mention here that NO release by N₂T₄.DCs inhibited the survival of *Mtb*. This information was confirmed by the CFU data. N₂T₄.DCs failed to restrict *Mtb* growth in the presence of the iNOS inhibitor N-monomethyl-L-arginine (fig. 4f). It

can be concluded that N₂T₄ triggering enhances the bactericidal activity of DCs by augmenting autophagy and NO release.

Signaling through N₂T₄ Enhances the Ability of DCs to Migrate

It is important that the activated DCs should capture antigens from the site of infection and migrate to the DLNs to activate naive T cells. Therefore, next, we studied the migratory capacity of N₂T₄.DCs. *Mtb*-infected DCs activated through N₂T₄ were CFSE-labeled and adoptively transferred into mice. It was observed that the frequency of *Mtb*-infected CFSE-positive N₂T₄.DCs in the DLNs was significantly ($p < 0.01$) higher than unstimulated counterparts (fig. 5a). Similar results were noted in the uninfected N₂T₄.DCs.

Fig. 4. Triggering DCs through N₂T₄ induces autophagy and enhances the NO secretion. **a** DCs stimulated through N₂T₄, TLR-4 or NOD-2 were monitored for the induction of autophagy through conversion of LC3I to LC3II. **b** DCs infected with *Mtb* were triggered through N₂T₄ in the presence or absence of the autophagy inhibitors wortmannin and chloroquine for 24 h. Later, bacterial burden was determined by CFU plating. DCs stimulated through N₂T₄, TLR-4 or NOD-2 were monitored for NO in supernatants (**c**), iNOS by Western blotting (**d**) and iNOS by RT-qPCR (**e**). The

graph depicts the fold change in the expression of iNOS compared to uDCs. The number in parentheses indicates the concentration of NOD-2L (μg/ml) or TLR-4L (ng/ml). **f** DCs infected with *Mtb* were stimulated through N₂T₄ in the presence of iNOS inhibitor (N-monomethyl-L-arginine) for 24 h. Later, the bacterial burden was determined through CFU plating. Data depicted as mean ± SD or Western blots are representative of 2–3 independent experiments. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001, one-way ANOVA.

DCs stimulated through N₂T₄ in vitro displayed up-regulation of CCR7 (fig. 5b). CCR7 is responsible for the migration of DCs [22, 23]. IL-12 is known to upregulate the expression of CCR7 [24]. We demonstrated earlier that N₂T₄.DCs release greater amount of IL-12 (fig. 1b).

Importantly, we observed that neutralization of IL-12 by using anti-IL-12 antibody reduced the expression of CCR7 on N₂T₄.DCs (fig. 5b). These data suggest that N₂T₄-driven production of IL-12 by DCs upregulates the CCR7 expression on N₂T₄.DCs.

Fig. 5. N₂T₄.DCs acquire enhanced in vivo migratory capability and capacity to prime naive T cells. DCs infected with *Mtb* were stimulated through N₂T₄ for 24 h. **a** The cells were labeled with CFSE prior to adoptive transfer into the mice. After 3 days, animals were sacrificed and N₂T₄.DCs were tracked through flow cytometry in the DLNs. Data are expressed as dot plot and scatter graph for augmented migration (%). **b** Elevated expression (MFI) of

CCR7 was assessed on N₂T₄.DCs cultured in vitro for 24 h. The results also show IL-12-mediated expression of CCR7, since neutralization of IL-12 with anti-IL-12 Abs reduces the expression of CCR7 on DCs. Each dot in the scattered plot indicates 1 mouse. Data represented as mean ± SD are of 2 independent experiments with 3 mice/group. Inf. = DCs infected with *Mtb*. * $p < 0.05$, ** $p < 0.01$.

Immunization with NOD-2L and TLR-4L Bolsters the Immune Response and Reduces *Mtb* Burden

It was crucial for us to demonstrate the effectiveness of NOD-2L and TLR-4L in vivo to bolster the immune response and reduce the *Mtb* burden in the infected animals. It was observed that animals inoculated with N₂T₄L expressed significant enhancement in the CD69^{hi}/CD44^{hi}/CD62L^{lo} phenotype on CD4 and CD8 T cells (fig. 6a, b), consequently supporting the generation of effector memory T cells. These animals also exhibited a better ex vivo

percentage of IFN- γ ⁺ CD4 ($p < 0.01$) and CD8 ($p < 0.05$) T cells (fig. 6c). Furthermore, the elevated yield of IFN- γ was confirmed by RT-qPCR in the lungs of N₂T₄L-treated mice (fig. 6d). Similarly, *in vitro* stimulation of cells with PPD showed a considerable enhancement of IFN- γ ($p < 0.001$) production and displayed TNF- α ⁺ CD4 T cells and T cell proliferation ($p < 0.001$; fig. 6e–g).

N₂T₄L-treated animals manifested better protective efficacy against *Mtb*, as evident in the significant decline ($p < 0.001$) of the *Mtb* burden in the lungs (fig. 7a). Fur-

Fig. 6. Immunotherapy employing N₂T₄L chiefly evokes a memory Th1 response. *Mtb*-infected animals were treated with N₂T₄L and control groups with NOD-2L, TLR-4L or placebo. After 45 days, lymphocytes were isolated and stained for the surface phenotype of CD69, CD44 and CD62L on CD4 T cells (**a**) and CD8 T cells (**b**). Dot plots depict percentages of cells (left panels) and bar diagrams illustrate the fold changes relative to placebo (right panels). **c** Intracellular expression of IFN- γ by CD4 and CD8 T cells. Dot plots (left panels) and bar diagrams (right panels) depict percentages of IFN- γ -positive cells. **d** IFN- γ expression was quanti-

fied in the lungs of *Mtb*-challenged mice by RT-qPCR, and mRNA expression was normalized with control actin. For an antigen-specific response, lymphocytes were stimulated in vitro with PPD for 48 h. Later, cells were monitored for the release of IFN- γ in supernatants by ELISA (**e**) and the intracellular expression of TNF- α by flow cytometry on CD4-gated T cells (**f**). Numbers above insets indicate the MFI/percentage of TNF- α -positive CD4 T cells. **g** Proliferation by ³H-thymidine incorporation. Data (%) represented as bar diagrams are mean $\pm$ SEM from 2 independent experiments with 6 mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(For figure 6b–g see next pages.)

ther, N₂T₄L treatment displayed a substantial decline in granulomatous lesions and perivascular and peribroncholar cuffing in the histopathological samples. The lungs were less consolidated with more normal alveolar structures (fig. 7b). Excitingly, lungs resected from the mice administered with N₂T₄L revealed significant ($p < 0.001$)

augmentation in *Beclin* and *iNOS* (fig. 7c, d), also showing the involvement of autophagy and NO (fig. 4) as a mechanism of the reduction of bacterial burden upon stimulation through NOD-2 and TLR-4. It is important to mention that the *Mtb*-challenged mice treated with N₂T₄L were kept for 120 days, in order to study the sur-

vival of the treated mice. We did not observe any mortality in this group. This shows that the dose of N₂T₄ used in the experiments elicited a response that was not lethal for animals that could lead to their death.

*N₂T₄-Drug Adjunct Therapy Significantly Boosts in vivo Drug Efficacy in Restraining the Growth of *Mtb**

Finally, we demonstrated that a combination therapy employing N₂T₄L significantly ($p < 0.001$) potentiated the efficacy of INH in reducing the *Mtb* load in the lungs of infected mice (fig. 7e). Excitingly, adjunct therapy involving N₂T₄L caused a 5-fold reduction in the quantity of INH, demonstrated by being able to curtail the dose of INH from 25 to 5 mg/kg of body weight (BW). It is worth mentioning that administration of 5 mg of INH in conjunction with N₂T₄L imparted a considerably ($p < 0.001$) greater decline in CFU than 25 mg of INH only. These results establish the critical role of N₂T₄ in not only reducing the dose of anti-TB drugs but also markedly increasing their killing efficiency.

Discussion

Despite the availability of highly effective drug regimens against TB, many patients fail to comply with the treatment [25]. The threat posed by TB is further compounded by the emergence of the AIDS pandemic and multidrug-resistant, extensively drug-resistant and totally drug-resistant *Mtb* [1].

Innate immunity plays a pivotal role in imparting protection against pathogens [26, 27]. It may also reinvigorate host immunity suppressed by anti-TB drugs [28–30]. Thus, we designed a simple and elegant approach of strengthening host immunity by potentiating DC function by delivering signals through N₂T₄. We studied its role in vitalizing the potency of TB drugs to kill *Mtb*.

Delivering signals through N₂T₄ led to the emergence of the following major findings: (1) the enhanced activation of DCs, (2) the augmented ability of DCs to help T cells, (3) enhanced autophagy, NO release and a decline in the survival of the bacteria, (4) improved migration to-

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wards lymph nodes due to increased CCR7 expression, (5) increased in vivo effector T cell memory response and release of IFN- γ and TNF- α and (6) a reduction in the drug dose and an improvement of the in vitro and in vivo potency of the drugs in killing *Mtb*. DCs are a bridging component of innate and adaptive immunity [31]. Consequently, we thought that targeting DCs through N₂T₄ might be an appropriate immunotherapeutic approach in evoking host immunity against *Mtb*.

We observed that N₂T₄.DCs showed robust production of the cytokines IL-6 and IL-12. Mice deficient in IL-12p40 are highly susceptible to *Mtb* infection [24, 32].

N₂T₄.DCs also showed enhanced competence in activating T cells. Furthermore, migration of DCs to lymph nodes is a crucial step for initiating the activation of T cells. N₂T₄ treatment showed substantial increase in the pool of *Mtb*-loaded DCs in the DLNs. Importantly, N₂T₄ triggering had no adverse effect on the viability of DCs (online suppl. fig. S3). It is important to mention that N₂T₄ triggering enhances the bactericidal activity of macrophages as well (online suppl. fig. S4).

T cells require cognate interaction with DCs for their activation, proliferation and differentiation. However, recently, the contribution of innate receptors has been

Fig. 7. Immunization with N₂T₄L successfully imparts protection against *Mtb* and significantly bolsters INH potency to kill the *Mtb*. Mice infected with *Mtb* were treated with N₂T₄L and control groups with NOD-2L, TLR-4L or placebo. **a** Later, animals were sacrificed, and the *Mtb* load in the lungs was enumerated by CFU plating. Data are represented as CFU/g of lung ($n = 6$ animals/group). **b** Photomicrographs of stained lung sections (arrows indicate granulomas). HE. $\times 40$. **c, d** *Beclin* and *iNOS* in the lungs of

Mtb-challenged mice were quantified by RT-qPCR, and mRNA expression was normalized with control actin. **e** *Mtb*-infected mice were treated with N₂T₄L along with adjunct therapy using INH. The control groups were administered NOD-2L, TLR-4L, NOD-2L + INH, TLR-4L + INH or placebo. Figures in parentheses indicate 5 and 25 mg/kg BW of INH administered bimonthly to mice. Data are representative of 2 independent experiments ($n = 3$ mice/group) and expressed as mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$.

reported in clonal expansion of the effector T cells, independent of the occupancy of T cell receptors by the MHCII peptide [33]. Importantly, *Mtb*-challenged mice treated with N₂T₄L showed substantial T cell proliferation, with an abundant release of IFN- γ and TNF- α [34, 35]. In addition, it augmented the effector T cell memory response. Furthermore, the growth of *Mtb* was meticulously restricted even after 45 days of infection.

Autophagy has been reported as exhibiting a dual role in *Mtb* protection. It targets the antigen to lysosomes for degradation and delivers antimicrobial peptides to *Mtb*-harboring compartments. Simultaneously, it prevents an excessive inflammatory reaction in the host [36, 37]. In addition, it enhances the antigen-presenting ability of DCs to T cells [38]. Importantly, N₂T₄.DCs showed an augmentation of autophagy and NO release and a signif-

icant contribution to the reduction of the intracellular survival of *Mtb*. The expression of *Beclin* and *iNOS* in the lungs of *Mtb*-challenged mice treated with N₂T₄L also suggests autophagy and NO release to be a mechanism for conferring protection against *Mtb*.

The current TB regime is extremely efficient, but the major impediment is the long duration of 6 months, which inevitably gives rise to several side effects [39]. Still, the remarkable potency of TB drugs cannot be ignored. Henceforth, very pragmatically, we targeted N₂T₄ to reinvigorate host immunity, and the outcome was some control of the survival of *Mtb*. Intriguingly, a significant increase in mycobacterial mortality was noted when *Mtb*-infected DCs were treated with RIF in conjunction with N₂T₄ agonists. We further confirmed these results by administering INH in combination with N₂T₄L in *Mtb*-infected mice in vivo. Strikingly, animals inoculated with a 5-fold lesser dose (5 mg/kg BW) of INH bimonthly in conjunction with N₂T₄L showed a significant reduction in the bacterial load in the lungs, compared to the recommended daily dose of 25 mg/kg BW of INH only [40]. So

this approach significantly reduced the dose of the drug. There is another fundamental benefit to INH-N₂T₄ adjunct therapy, since the drug would kill the replicating *Mtb* and N₂T₄L would enhance host immunity for eliminating a nonreplicating, quiescent mycobacterium. So the INH-N₂T₄ strategy may not only be advantageous in overcoming the side effects because of the lower drug dosage, it also alerts the host immunity to restrain the emergence of drug resistance in *Mtb*. Implementing this innovative drug + N₂T₄ therapy may be a prudent approach in the future to effectively treat TB patients.

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