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Communication

Potassium-Incorporated Titanium Oxide Nanoparticles Modulate Human Dendritic Cell Immune Response to *Mycobacterium leprae*

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Abstract: The two polar clinical forms of leprosy, termed tuberculoid and lepromatous, have polarized cellular immune responses with complex immunological distinctions. The predominance of DCs in tuberculoid leprosy has been reported, while the lepromatous pattern of illness is associated with weak activation of local populations of DCs. TiO_2 nanoparticles have previously been shown to induce maturation of these cells, leading to an inflammatory response similar to adjuvant usage in vaccine administration. We aimed to evaluate the effect of potassium-incorporated Ti oxide nanostructures, namely $KTiO_x$s, in the response of human monocyte-derived DCs to live *M. leprae*. Human monocytic cell line dual THP-1, which harbors two inducible reporter plasmid systems for transcription factor activation of NF-κB and interferon regulating factor (IRF), was treated with titanium control or with 1 mol/L KOH-treated Ti or 10 mol/L KOH for 24 h. Subsequently, cells were infected with *M. leprae*. $KTiO_x$ nanoparticles increase DC phagocytic activity without inflammation. $KTiO_x$ exposure of DCs led to an increase in IRF activation with modulation of the inflammatory response to live *M. leprae*. It also led to differential secretion of the critical components of innate immune response and the development of cell-mediated immunity against intracellular pathogens. This study demonstrates the effect of nanostructures of $KTiO_x$s and the usefulness of nanoparticle technology in the in vitro activation of human DCs against an infectious disease with a puzzling immune spectrum. Our findings may prompt future therapeutic strategies, such as DC immunotherapy for disseminated and progressive lepromatous lesions.

Keywords: titanium oxides; KOH treatment; dendritic cell; *Mycobacterium leprae*; leprosy

Citation: Warren, S.; Lee, S.Y.; Barragan, J.; Kositangool, P.; Yoshikubo, H.; Cervantes, J. Potassium-Incorporated Titanium Oxide Nanoparticles Modulate Human Dendritic Cell Immune Response to *Mycobacterium leprae*. *Microbiol. Res.* **2023**, *14*, 787–797. https://doi.org/10.3390/microbiolres14020055

Academic Editor: Michal Letek

Received: 2 April 2023
Revised: 30 May 2023
Accepted: 10 June 2023
Published: 13 June 2023

1. Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, which is still endemic in many parts of the world, including southern Texas where armadillos serve as natural reservoirs [1,2]. The disease presents clinically as a spectrum with two polar clinical forms of leprosy, termed tuberculoid and lepromatous [3]. Lepromatous leprosy represents the more severe of the two diseases, being a disseminated and poorly corralled infection which affects the skin, nerves, and internal organs. In this form, the disease is more contagious to others and presents a much greater disease burden for the patient.

The human immune response in leprosy is complex, involving different cell types and immunological distinctions in both clinical poles [3,4]. *M. leprae* infects macrophages, dendritic cells, and Schwann cells [4]. Dendritic cells (DCs) are the primary antigen-presenting cells in the immune system. Acting at the site of *M. leprae* invasion, DCs may be the first cells to encounter the bacilli and likely play a key role in modulating the early innate immune response to *M. leprae* [5]. Their presence in the epidermis and in areas of

infiltration and granuloma formation has shown that DCs participate in the development of an effective immune response against *M. leprae* [4]. The predominance of DCs in tuberculoid leprosy has been reported and is associated with increased local infiltration relative to the lepromatous pattern of illness, suggesting that weak activation of CD1 proteins may be related to a local population of DCs within tuberculoid leprosy lesions [6].

TiO_2 nanoparticles have previously been shown to induce maturation of these cells, leading to an inflammatory response similar to adjuvant usage in vaccine administration [7]. TiO_2 nanoparticles have been found to be biocompatible as well as suitable for intravenous injection [7]. Several studies have demonstrated the antimicrobial activity of titanium dioxide (TiO_2) with negligible cytotoxicity to the host, as well as a moderate pro-inflammatory response [8,9]. Up to now, nanostructured Ti-based materials have attracted attention due to their unique and diverse physico-chemical properties and their potential in biomaterials because of their superior biocompatibility, non-toxicity, and low cost [10,11]. In particular, nanostructured potassium-incorporated titanium oxide films (K-incorporated TiO_x = $KTiO_x$s) represent a promising candidate due to the possibility of tuning the morphology and properties by controlling K content [12,13].

We aimed to evaluate the effect of potassium-incorporated Ti oxide nanostructures, namely $KTiO_x$s, in the response of human monocyte-derived DCs to live *M. leprae*. Our findings suggest that human DC activation upon $KTiO_x$s exposure leads to a more efficient immune response to *M. leprae*.

2. Materials and Methods

2.1. Synthesis of Nanostructured $KTiO_x$s Nanoparticles

Ti particles (purity > 99.8% and diameter of 125–250 μm) (Sigma-Aldrich, Tokyo, Japan) were soaked in 5 mL KOH solution at concentrations of 1, 5, 10, 15, 20, and 25 mol/L at room temperature for 24 h. This KOH treatment is known as the wet corrosion process (WCP). After this KOH treatment, all particles were washed with deionized water and dried. These particles contained a Ti core and a shell of $KTiO_x$s nanowire forming a network.

Changes in the surface structure, shape, and size of the obtained nanostructured KTiOxs were observed by using a Field Emission Scanning Electron Microscope (FE-SEM; JSM-7610, JEOL Ltd.) which was operated at 15 kV. To investigate the structural property of the obtained product, Raman spectroscopy (Renishaw, inVia Raman microscope) was performed using 514.5 nm Ar laser radiation as the excitation source.

Surface area analysis was performed by using the NOVA 300E (Quantachrome), which estimates the surface area according to nitrogen adsorption behaviors. The surface area was calculated based on Brunauer–Emmett–Teller (BET) theory.

2.2. M. leprae

Live *M. leprae*, strain Lombardo-Pellegrino (ATCC 4243), were cultivated using Middlebrook 7H9 and OADC enrichment broth (Thermo Scientific).

2.3. Cell Assay

Dual THP-1 human monocytes (Invivogen) were differentiated into DCs for 6 days using commercially available human Mo-DC Differentiation Medium (Miltenyi Biotech) and maturated with recombinant human TNF-alpha (Miltneyi Biotech) for 24 h. Cells were then treated with titanium control or with 1 mol/L KOH-treated Ti or 10 mol/L KOH for 24 h. Subsequently, cells were infected with *M. leprae* (ATCC 4243) at an MOI of 10:1 for 24 h.

These cells harbor two inducible reporter plasmid systems for NF-κB and interferon regulating factor (IRF) transcription factor activation (Invivogen). Activation was measured according to the manufacturer's guidelines and expressed as a response ratio compared to the untreated/unstimulated conditions.

Phagocytosis was assessed using an immunofluorescence assay to observe mycobacteria internalized by DCs. Briefly, upon live *M. leprae* infection, cells were fixed using

fixation buffer (Cell Signaling) for 10 min, then washed with PBS, blocked with human IgG, and permeabilized with permeabilization buffer (Cell Signaling). Staining included the use of primary antibodies against *M. leprae* MLMA-LAM (bei-resources) and lysosomal LAMP-1 (DHSHB) overnight, followed by the use of secondary antibodies (Alexa 488, Green, to visualize mycobacteria, and Alexa 647, Red, respectively) (ThermoFisher). Images were acquired using a fluorescence microscope (Motic, Texas) and processed with ImageJ (NIH, Bethesda, MD). A phagocytic index, i.e., the number of cells containing internalized mycobacteria, as well as the number of mycobacteria per cell were calculated.

2.4. Multiplex ELISA

Secreted human cytokines were measured in the culture supernatants by a multiplex ELISA system (Milliplex Map Human Cytokine/Chemokine Magnetic Bead Panel (Millipore, Burlington, MA, USA). The system provides simultaneous analysis of multiple cytokine and chemokine biomarkers using bead-based multiplex assays and Luminex technology (sCD40L, EGF, Eotaxin/CCL11, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AB/BB, RANTES, TGF-α, TNF-α, TNF-β, and VEGF).

3. Results

3.1. Synthesized the Nanostructured Ti Nanoparticles after KOH Treatment

Successfully nanostructured Ti nanoparticles were obtained after various KOH treatments (Figure 1). Ti particles which had been treated using the KOH solution yielded elongated nanostructures. When the concentration of KOH aqueous solutions was below 10 mol/L KOH solution, shorter and thicker nanowires with a diameter ranging from 20 to 150 nm were obtained. Longer and thinner nanowires were obtained with the 10 mol/L KOH solution treatment, ranging from 10 to 100 nm in diameter and several tens of micrometers in length. This indicated that the morphology of nanostructures is governed by the KOH concentration.

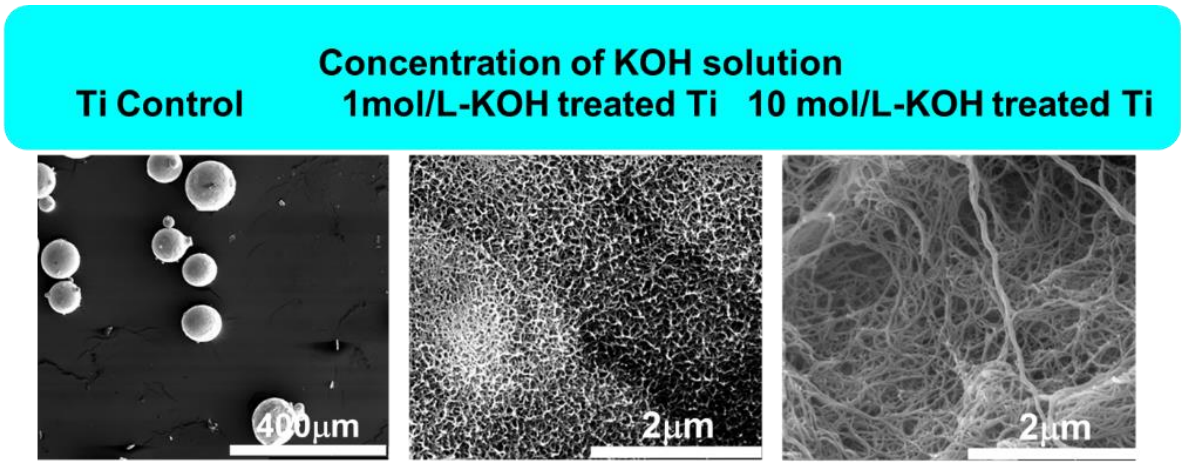

Figure 1. SEM observations of the morphology of the synthesized nanostructured Ti oxide after various KOH treatments.

To visualize the synthesized nanostructures, the obtained products were examined through Raman spectroscopy. As we previously reported, nanostructures originating from Ti particles via the KOH treatment consisted of two components, namely TiO_2 phase containing Ti–O bonds and K-incorporated Ti–O–K bonds, namely $KTiO_x$s [12]. Both were generated by rearrangement of the atoms in the Ti metal during the WCP [12]. The K-incorporated Ti–O–K bonds give rise to a new peak in the Raman spectrum close to 280 cm^{-1}, which is present in the Raman spectra of KOH-treated samples (Figure 2).

Consequently, it can be concluded that the synthesized Ti-O-K bonds are K-incorporated TiO_2; thus, we decided to call these nanostructures $KTiO_x$s.

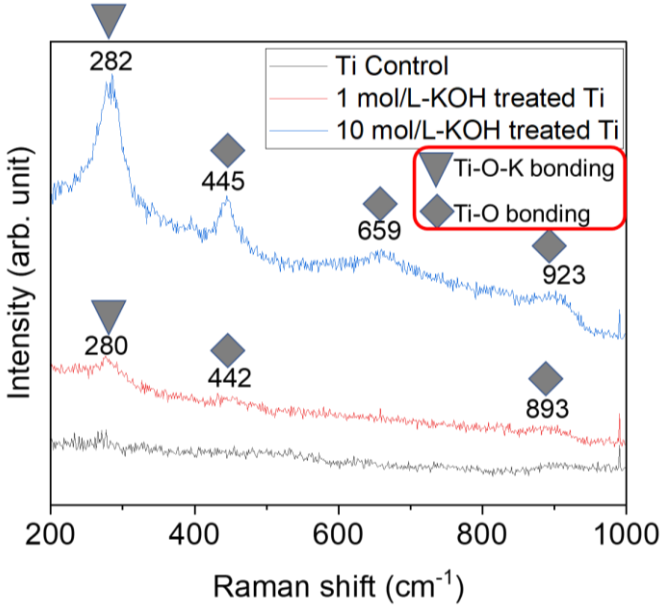

Figure 2. Raman spectra of the surfaces of the Ti control and the 1 and 10 mol/L KOH-treated Ti nanoparticles.

To evaluate the effect of the nanostructures on surface area, BET analysis was performed. The surface area gradually increased with the KOH concentration in agreement with the SEM results (Figure 3), where the structural evolution from individual thick and short nanostructures to a three-dimensional network of thin and elongated nanowires was observed. Accordingly, we can conclude that the KOH treatment induced strong morphology changes with the KOH concentration. These changes effectively go together with enlargement of the surface area. On the basis of this result, it can be expected that the increased surface area influences the activity of DCs.

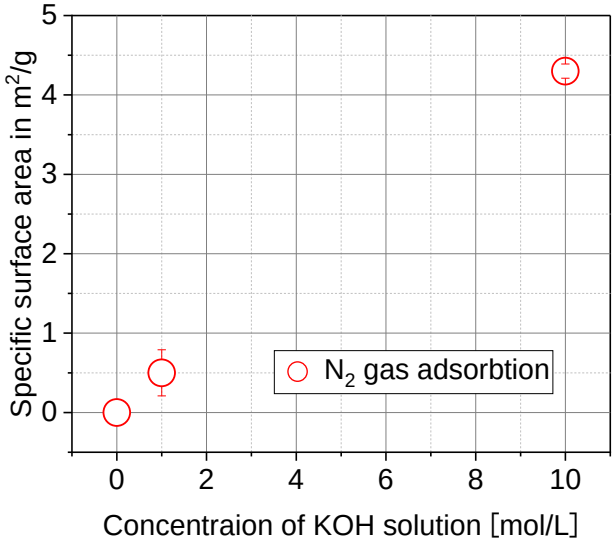

Figure 3. Change in the specific surface area of the obtained nanostructured $KTiO_x$s according to BET analysis. The surface area profile is presented as a function of the concentration of the KOH solutions.

3.2. $KTiO_x$ Nanoparticles Increase DC Phagocytic Activity without Inflammation

Cell activation in response to an invading pathogen may be observed as an increase in phagocytosis. In our system, we observed this as a higher number of DCs with phago-

cytized mycobacteria and a higher number of mycobacteria inside each of these cells. We noted a marked increase in the phagocytic activity and number of internalized mycobacteria per cell in the 1 mol/L KOH-treated Ti and 10 mol/L KOH-treated groups when compared to our Ti control and unstimulated groups (Figure 4).

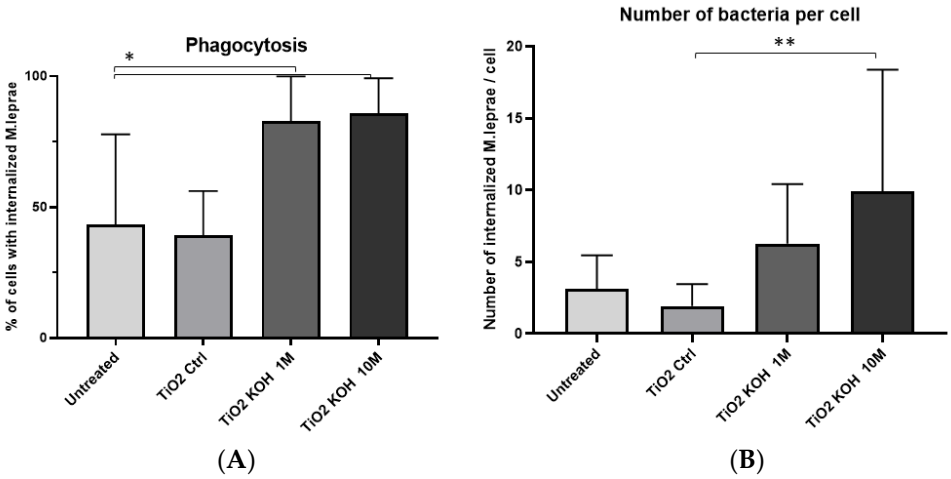

Figure 4. $KTiO_X$ exposure increases the phagocytic activity of human DCs in response to live *M. leprae*. Mature DCs exposed to Ti control, 1 mol/L KOH-treated Ti, or 10 mol/L KOH-treated Ti were stimulated with live *M. leprae*. (**A**) Increased phagocytosis per cell as well as (**B**) an increased number of mycobacteria internalized per DC was observed in the 1 mol/L KOH-treated Ti and 10 mol/L KOH-treated Ti versus the Ti control and untreated samples. * $p < 0.05$ *t*-test. ** $p < 0.05$ ANOVA trend analysis.

We noted decreased NF-κB activation in 1 mol/L KOH-treated Ti and 10 mol/L KOH-treated Ti versus our Ti control group (Figure 5). Simultaneously, we noted a moderate increase in IRF activation for the 10 mol/L KOH-treated Ti versus all other groups, although this was not statistically significant.

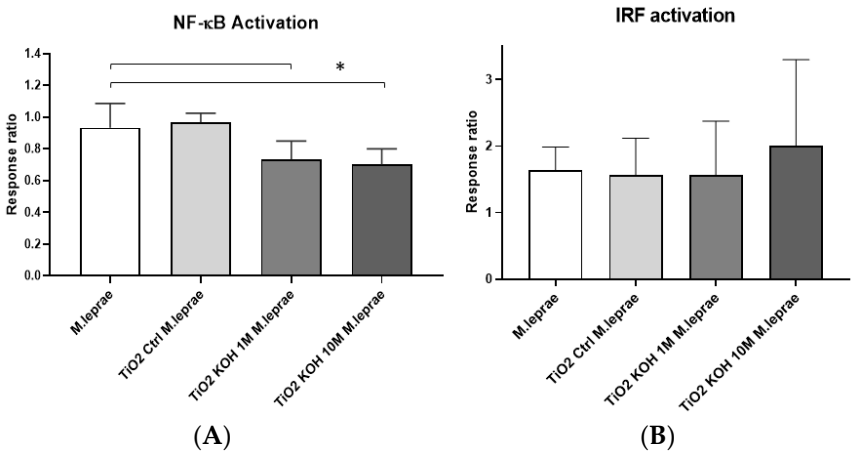

Figure 5. $KTiO_X$ exposure in DCs leads to an increase in IRF activation with modulation of the inflammatory response to live *M. leprae*. (**A**) A slight decrease in NFk-B activation upon *M. leprae* stimulation was observed in cells exposed to KOH-treated Ti. (**B**) A slight increase in IRF activation upon *M. leprae* stimulation was observed when cells were treated with 10 mol/L KOH-treated Ti. * $p < 0.05$ *t*-test.

Lastly, we looked to the actual secretion of inflammatory mediators and observed the secretion of IFNa2, TNF-b, and IL-1a. IL-1b, along with its receptor IL-1RA, was increased in response to *M. leprae* in cells treated with $KTiO_X$ in a dose dependent manner (Figure 6A). Interestingly, IL-4 also followed this trend as well. On the other hand, IFN-g, eotaxin,

G-CSF, GM-CSF, and IL-10 secretion levels decreased in a dose dependent manner with treatment of 1 mol/L KOH-treated Ti and 10 mol/L KOH-treated Ti (Figure 6B). Other major pro-inflammatory cytokines, such as IL-8, IP-10, MCP-1, TNF-a, MIP-1a, and MIP-1b, showed no difference with KTiOx treatment, although the levels of secreted IL-8 and MIP-1a were higher upon *M. leprae* infection (Figure 6C).

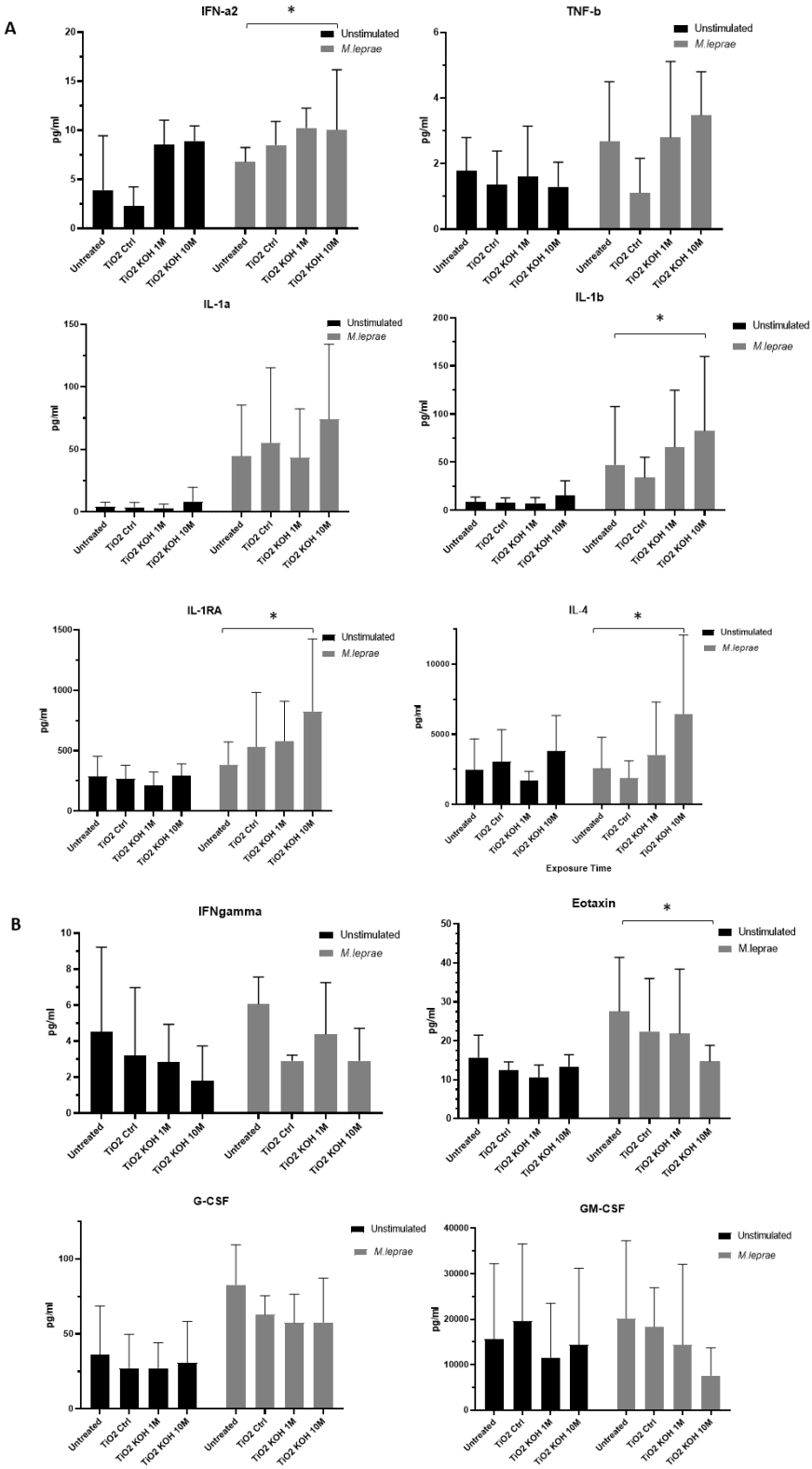

Figure 6. *Cont.*

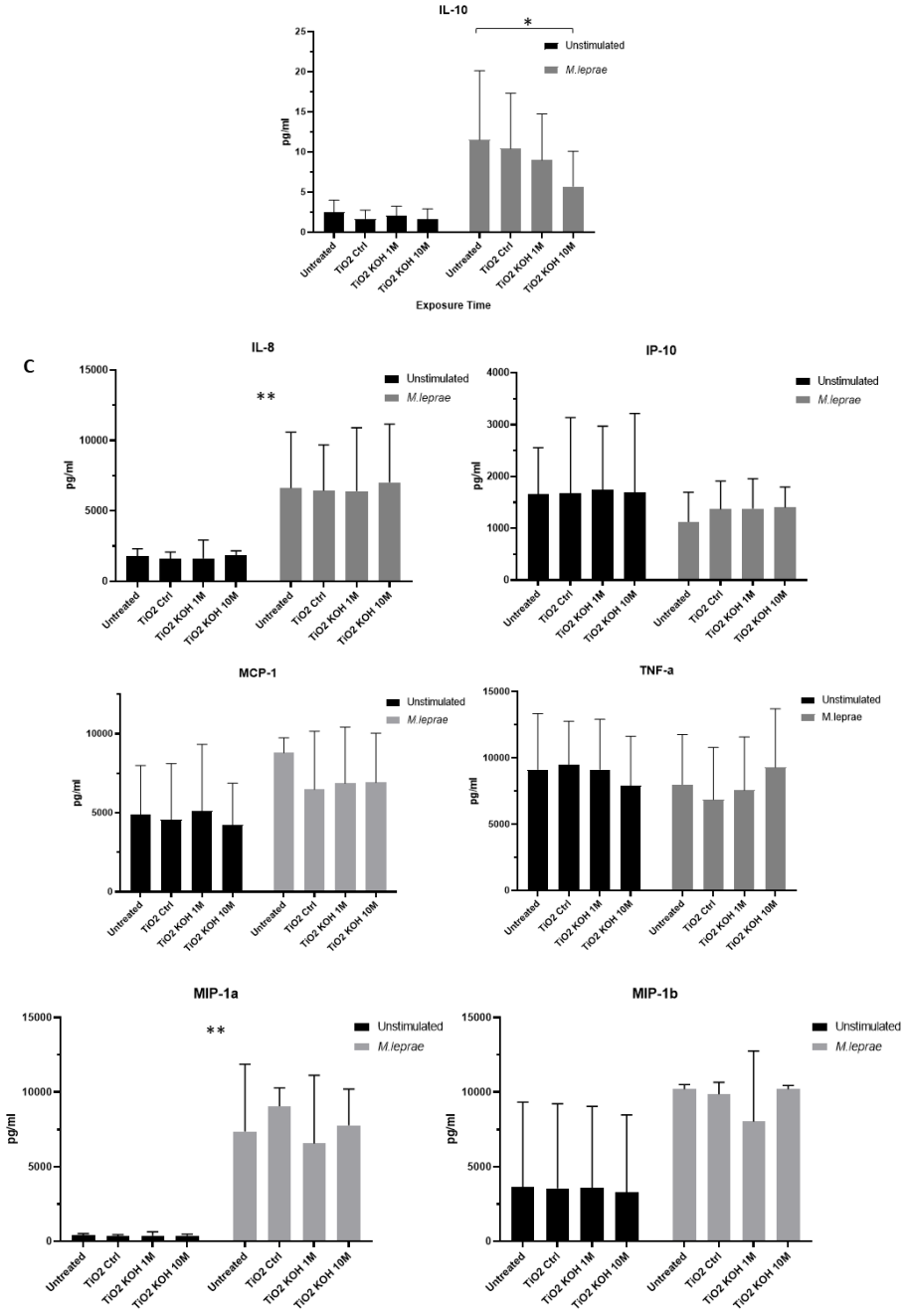

Figure 6. Exposure of DCs to $KTiO_x$ leads to differential secretion of inflammatory mediators in response to live *M. leprae*. (**A**) Inflammatory mediators increasing or (**B**) decreasing with KTiOx in *M. leprae*-stimulated human DCs. (**C**) Markers that showed no change compared to untreated DCs. Cells were exposed to Ti control, 1 mol/L KOH-treated Ti, and 10 mol/L KOH-treated Ti. * $p < 0.05$ ANOVA trend analysis. ** $p < 0.005$ ANOVA group analysis.

4. Discussion

Excessive inflammation versus a lack of an adequate inflammatory response underlies the complex processes behind the clinical spectrum of leprosy. Cutaneous infection is a hallmark of the disease, while nerve and tissue damage are notable clinical findings related to the most aggressive form, lepromatous leprosy [1]. Evidence has shown that macrophage phagocytosis and the inability of macrophages to destroy ingested mycobacteria leads to localized inflammation. When this occurs along the myelin sheath of nerve tissue it can

lead to catastrophic destruction of nerve function and the hallmark loss of sensation in leprosy infections [14].

Granuloma formation in leprosy is the host's most typical way of slowing the infection, and there is significant interplay amongst DCs and macrophages in the formation of leprosy granulomas. In fact, epithelioid cells exhibit markers for both cell types [6], while fully mature DCs are predominant in tuberculoid leprosy [15]. M1 and M2 macrophage populations coexist with a wide diversity of DC subsets in the skin of lepromatous patients that develop reversal reactions amidst the inflammatory environment. Reversal reactions are acute inflammatory processes in the skin and nerves in response to *M. leprae* that may occur throughout the whole clinical spectrum, except the tuberculoid form. A reversal reaction is defined by immature and loose epithelioid granulomas, in contrast to the mature epithelioid granulomas seen in the tuberculoid form [6]. Defective antigen-presenting ability in antigen-presenting cells may lead to more severe leprosy [16]. DC and macrophage infection with *M. leprae* is shown to induce IL-10 production towards the lepromatous pole of the disease. This increased production of IL-10 by both cell lines contributes to the blockade of antimicrobial pathways [6]. This complex story of interplay amongst these two cell types appears to be quite pertinent to elimination of the disease from the host body in an effective manner.

Dicationic oxides, such as titanium dioxide nanoparticles, are widely used in topical skin care products. The concept of using nanotechnology in medical research and clinical practice is known as nanomedicine [17]. Nanostructured $KTiO_x$s is a promising candidate for high-performance biomaterials. Since nanostructures have a high surface area, this can lead to the activation of a greater number of biomolecules for them to react with. Nanostructure fabrication, however, has generally involved complicated processes, low reproducibility, and high costs for well-controlled chemical modification [18]. A simple method to synthesize and tune the desired morphology and properties is strongly desirable. In this study, we also demonstrate the usage of the WCP as a simple one-step method for nanostructure fabrication using KOH solution treatment [12,13].

We noticed variability in the immune response to *M. leprae* infection in cells exposed to varying KOH-treated TiOx concentrations. While there was a decrease in transcription factor NF-κB, IRF activation was increased. NF-κB activation is a mediator of downstream response in conventional type I DCs, which will in turn recruit and activate cytotoxic T cells [19]. Such inflammatory immunomodulation could translate into beneficial effects clinically, preventing tissue damage in leprosy.

IRF activation is understood to guide DC differentiation function [20]. IRFs are major transcription factors for the induction of type I IFN transcription. Type I IFN IFNa2 is a critical component in innate immune response, with antiviral, antiproliferative, and immunomodulatory effects [21]. IFNa2 and its downstream gene, IL-10, are preferentially expressed in disseminated and progressive lepromatous lesions [22], as well as by monocyte-derived DCs from lepromatous leprosy patients [23]. Our KTiOx-treated DCs exhibited increased secretion of IFNa2 (Figure 7), which, through a paracrine or autocrine effect, could lead to alpha-type-1 polarized DCs, which are mature non-exhausted DCs with potent immune-stimulatory activity [24].

We observed a decrease in IL-10 secretion in *M. leprae*-infected cells upon $KTiO_x$ treatment. IL-10 is identified as playing a part in initial disease development after infection [25]. This cascade of genetic activation in certain individuals seems to drive the development of lepromatous leprosy [26]. DCs increased the secretion of IL-1a and IL-1b, possibly following the decrease in IL-10 secretion in KTiOx-treated DCs [26] (Figure 7), helping counteract the tolerogenic state induced by *M. leprae* infection [27,28].

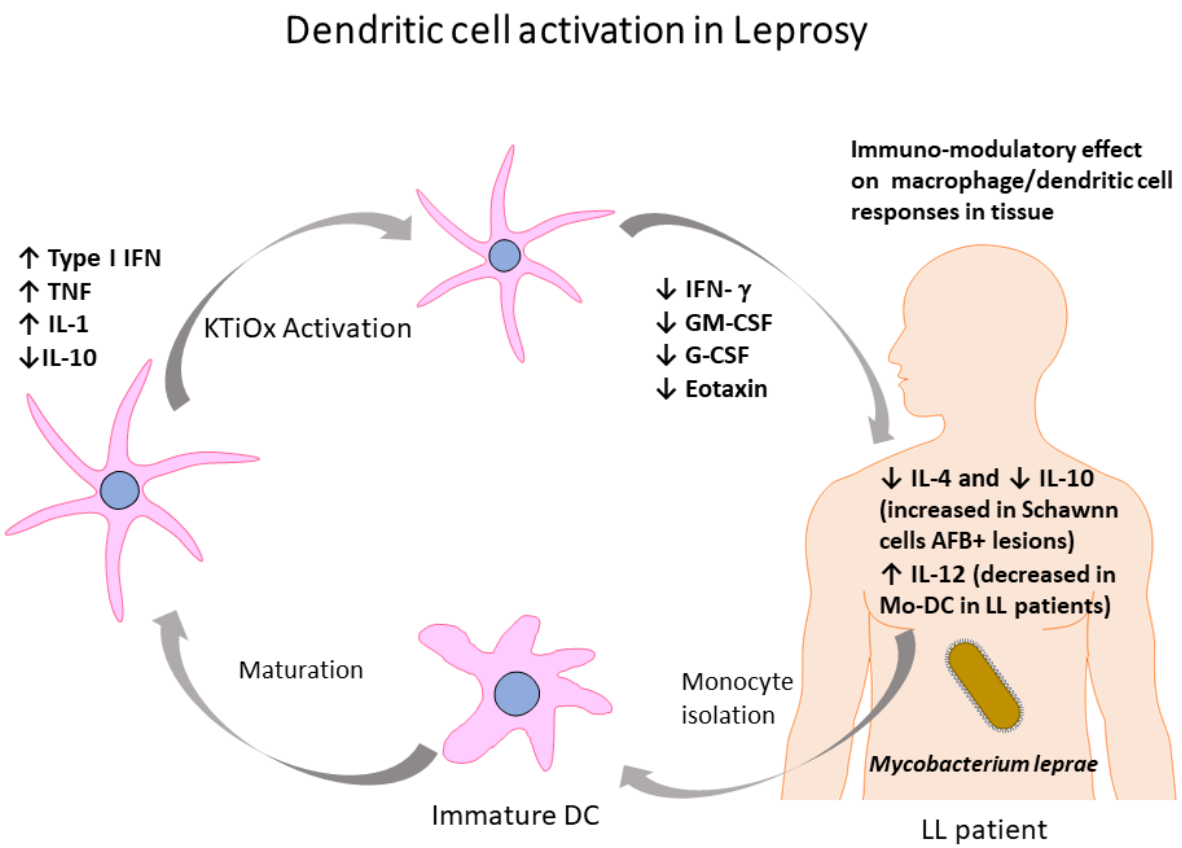

Figure 7. A plausible model for dendritic cell activation by KTiOxs in vitro and its potential use in leprosy.

IL-4 and IL-10 are upregulated in *M. leprae*-containing neural leprosy lesions [29]. A local Th2 (IL-4 and IL-10) T cell response, as well as its associated M2 macrophage polarization, leads to CD-209-positive human macrophage and Schwann cell activation by these cytokines, which helps in the binding and uptake of *M. leprae* by these cells [29]. Furthermore, monocyte-derived DCs under the influence of IL-4 and GM-CSF are very effective presenters of *M. leprae* antigens [5].

We also observed increased TNF-β secretion upon $KTiO_x$ exposure. Also known as lymphotoxin-α, TNF-β is a cytotoxic protein with antiproliferative activity [30] that is implicated in the development of cell-mediated immunity against intracellular pathogens. T cells from TNF-β-deficient mice fail to properly co-localize with granuloma macrophages, resulting in abnormal granulomas with elevated mycobacterial load [31]. Although TNF-β is not essential for controlling the growth of *M. leprae* during early infection, it may influence survival in the later stages of *M. leprae* infection [32]. The TNF-β gene is identified as a major risk factor for early-onset leprosy [33].

The increased surface area of greater molar concentrations seemed to induce greater immune response. This can be explained by the wider surface area of the 10 M preparation compared to 1 mol/L KOH-treated Ti. It is possible that this wider $KTiO_x$s area can activate more DCs due to an increase in contact area. This could translate into the production of certain inflammatory mediators, such as IL-12, which appear to be decreased in Mo-DCs from LL patients [23] (Figure 7).

This study demonstrates the effect of nanostructures of $KTiO_x$s and the usefulness of nanoparticle technology in the in vitro activation of human DCs against an infectious disease with a puzzling immune spectrum. Our findings may prompt future therapeutic strategies, such as DC immunotherapy for disseminated and progressive lepromatous lesions. DC activation in vitro has been used successfully as a form of immunotherapy for various forms of cancer [34,35].

Further research may focus on the risk of cellular harm from exposure to $KTiO_x$s nanoparticles and further evaluation of downstream immune responses resulting from increased dendritic cell activation in this manner. Previous research has demonstrated that TiO_2 exposure does not typically produce significant complications [36]. Honing in on a specific dose that promotes optimal activation and then further exploration of its potential therapeutic utility would be an end goal from our perspective.

Author Contributions: Conceptualization, J.C.; methodology, S.W., J.B., S.Y.L. and J.C.; formal analysis, J.C.; investigation, S.W., J.B., P.K., H.Y. and S.Y.L.; resources, J.C. and J.B.; data curation, S.W., P.K. and J.C.; writing, S.W., S.Y.L. and J.C.; project administration, J.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Choose not to share.

Conflicts of Interest: The authors declare no conflict of interest.

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