Activation of the NLRP3 Inflammasome by Titanium Dioxide Nanowires

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ABSTRACT

Titanium Dioxide Nanowires (TNW) exhibit many properties that make them a promising material for medical applications. Nanowire assemblies for medical implantable devices are especially promising. Many different particles activate the NLRP3 Inflammasome including uric acids crystals, asbestos, and silica crystals. However, the mechanism of NLRP3 Inflammasome activation is not clearly known. Our hypothesis is that TNW share a common mechanism with other fibers, including asbestos, and that the enzymes Cathepsin B and Syk are included in this mechanism. This mechanism involves phagocytosis of the fiber, the subsequent rupture or increased permeability of the lysosomal membrane, which in-turn releases the protease Cathepsin B, which activates the NLRP3 Inflammasome. It has been proposed that Syk, a tyrosine kinase, is intricately involved in phagocytosis of particles (Ng et al. 2008). Our approach used an in-vitro model with human THP-1 monocytes. NLRP3 Inflammasome activation was determined using an ELISA assay that measured IL-1β production. Caspase-1 activation was measured by Western Blot and fluorescent assay. The catalytic activity of the enzymes Cathepsin B and Syk was blocked using inhibitors. Our studies demonstrate that Cathepsin B is essential for TNW activation of the NLRP3 Inflammasome; while Syk may not be essential for phagocytosis of TNW. By knowing the mechanism in which the NLRP3 Inflammasome is activated, more effective treatments for gout, asbestosis, and silicosis could be developed; and more effective medical implantable devices could be constructed using TNW.

INTRODUCTION

Recent studies have indicated that activation of a protein complex termed the NLRP3 Inflammasome is critical for the development of inflammation mediated by IL-1β. Excessive release of IL-1β has been reported to be involved in ailments including: gout, asbestosis, and silicosis. By targeting the NLRP3 Inflammasome with effective drug design, ailments caused by the unabated release of IL-1β could be prevented.

The NLRP3 Inflammasome is composed of three subunits: NLRP3, an immune sensor; pro-caspase-1, which when cleaved is activated; and ASC, the bridge between NLRP3 and pro-caspase-1. Activated Caspase-1 cleaves pro-IL-1β, and pro-IL-18, producing the inflammatory cytokines IL-1β and IL-18. Activation of TLR4 by LPS initiates a signal cascade producing pro-IL-1β and pro-IL-18. Therefore, production of IL-1β involves the crosstalk of two independent and converging pathways.

Although the mechanism for the generation of the cytokine precursors is well known, the mechanism of NLRP3 Inflammasome activation remains unclear. Particulates that activate the NLRP3 Inflammasome include: uric acid crystals, asbestos fibers, silica crystals, and nanowires. Although the steps involved in NLRP3 Inflammasome activation are being deduced, fibers may activate the NLRP3 Inflammasome by not allowing the lysosomal membrane to fuse around long fibers causing the release of the protease Cathepsin B, which activates the NLRP3 Inflammasome. It also remains unclear how particulates enter the cell to initiate the whole activation process. Recently it was reported that uric acid crystals enter the cell in a receptor independent manner, involving direct membrane binding and lipid sorting which begins a signaling cascade that involves the tyrosine kinase Syk and its substrate PI3k. (Ng et al., 2008). Our hypothesis is that TNW cause lysosomal membrane disruption and thereby release Cathepsin B, a known activator of the NLRP3 Inflammasome; and that Syk and PI3k are involved in the phagocytosis of TNW.

REFERENCES

G. Ng, K. Sharma, S.M. Ward, M.D. Desrosiers, L.A. Stephens, W.M. Schoel, T. Li, C.A. Lowell, C.-C. Ling, M.W. Amrein and Y. Shi, *Immunity* **29** (2008), pp. 807ƹ818 this issue.

METHODS

Human cell lines:
The cell line used were human THP-1 monocytes. The THP-1 cells were cultured in 1640 RPMI media with 10% FBS. THP-1 cells were differentiated into macrophages like cells by adding phorbol ester (PMA @ 1 μg/mL) 24 hours prior to use.

Cytokine measurements:
Human IL-1β was measured by ELISA using a kit from R&D systems, according to manufacturer's protocol.

Activity Assay:
Caspase-1 activity was measured using an Carboxyfluorescein FLICA Apoptosis Detection Kit Caspase Assay from AbD serotec. Cathepsin B activity was measured using an InnoZyme Cathepsin B Activity Assay Kit from Calbiochem. Both Manufacturer's protocols were followed.

Cell Proliferation Assay:
Cell viability was measured using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) from Promega, according to the manufacturers protocol.

Inhibitors:
Syk Inhibitor: Piceatannol from Bachem. PI3k inhibitor: LY294002 from Gibco. Cathepsin B Inhibitor: CA-074 Me from PEPTIDE INSTITUTE, INC.

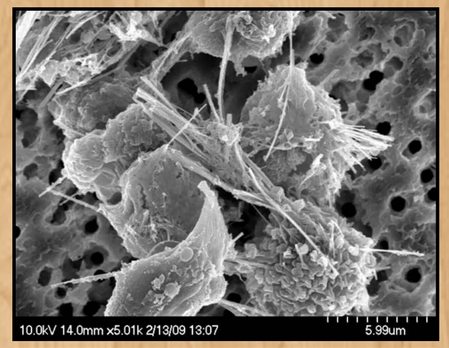

Figure 5: Several macrophages interacting with TNW (100 μg/mL) after one hour of incubation. Notice the changed surface dynamics of the macrophages. Picture taken by a scanning electron microscope.

RESULTS

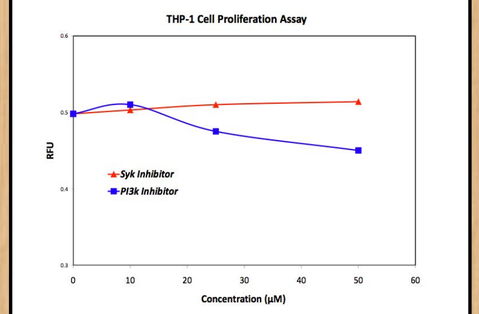

Figure 1: THP-1 cells were incubated with the inhibitors for 24 hours. Cell viability was measured relative to the baseline RFU value of 0.5. Therefore values smaller than 0.5 are indicative of cell distress, and the smaller the RFU value the more indication of cell distress.

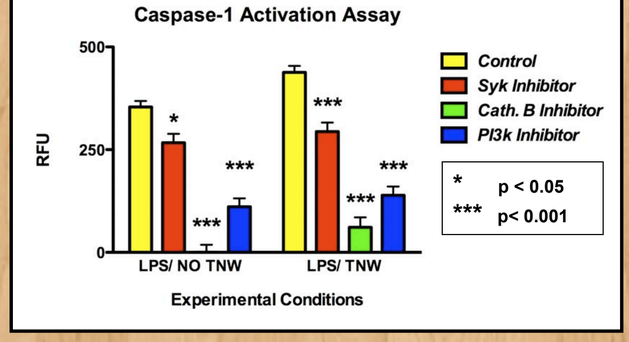

Figure 3: THP-1 cells were stimulated with LPS (0.5 μg/mL) and TNW (50 μg/mL) for a 5 hours incubation. Supernatants were collected and were assayed for Caspase-1 activation by FLICA Caspase-1 Assay. The Inhibitors were used at the following concentrations: Syk (10 μM), Cathepsin B (10 μM), and PI3k (5 μM). The high baseline activation of Caspase-1 was a result of treating the THP-1 monocytes with phorbol ester, which was used to differentiate them into macrophages.

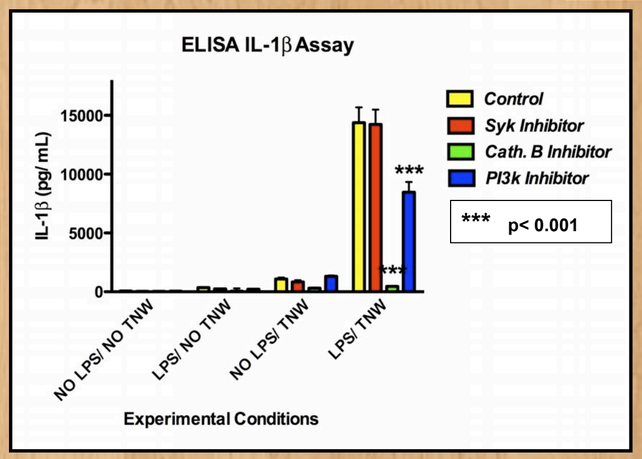

Figure 2: THP-1 cells were stimulated with LPS (0.5 μg/mL) and TNW (50 μg/mL) for a 24 hour incubation. Supernatants were collected and were assayed for IL-1β by ELISA. The Inhibitors were used at the following concentrations: Syk (10 μM), Cathepsin B (10 μM), and PI3k (5 μM).

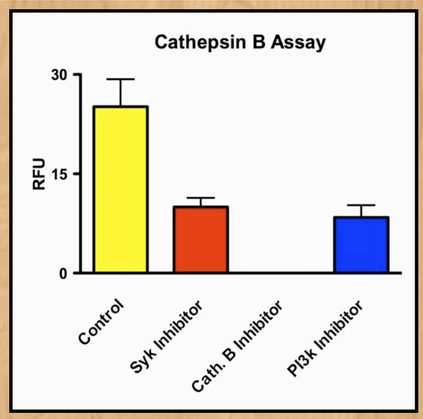

Figure 4: THP-1 cells were incubated for 4 hours with the various inhibitors. Supernatants were collected and were assayed for Cathepsin B activity. The Inhibitors were used at the following concentrations: Syk (10 μM), Cathepsin B (10 μM), and PI3k (5 μM).

DISCUSSION

Our hypothesis was that TNW cause lysosomal membrane disruption and thereby release Cathepsin B, a known activator of the NLRP3 Inflammasome; and that Syk and PI3k were involved in the phagocytosis of TNW. The combination of TNW and LPS induced robust IL-1β production (see Figure 2). The Cathepsin B inhibitor significantly reduced TNW ability to induce IL-1β production and activate Caspase-1 (see Figures 2 and 3). Therefore, Cathepsin B is crucial for TNW activation of the NLRP3 inflammasome. The Syk and PI3k inhibitors had minimal effects on IL-1β production. However, the Syk and PI3k inhibitors partially inhibited Caspase-1 activation, and significantly inhibited Cathepsin B activation (see Figures 3 and 4). These results support the notion that Syk and PI3k are involved upstream of Cathepsin B release from lysosomes. Therefore, it remains possible that Syk and PI3k are involved in the process of phagocytosis as previously reported for uric acid crystals. One possible explanation why the Syk and PI3k inhibitors did not significantly inhibit IL-1β production, like the Cathepsin B inhibitor, is that the production of pro-IL-1β occurs at a much slower rate than NLRP3 Inflammasome activation and thereby determines the rate of IL-1β production. The PI3k inhibitor was much more effective than the Syk inhibitor at inhibiting IL-1β production, caspase-1 activation, and Cathepsin B activation. Possible explanations include: that PI3k is not only activated by Syk but rather is activated in additional ways, or that the inhibitors effectiveness were not equal.

CONCLUSIONS

1) TNW activate the NLRP3 Inflammasome
2) Cathepsin B is fundamental for TNW activation of the NLRP3 Inflammasome
3) Syk and PI3k are involved in Caspase-1 activation
4) Syk and PI3k are upstream of Cathepsin B activity.
5) Syk and PI3k are likely involved in phagocytosis of TNW

PROPOSED PATHWAY

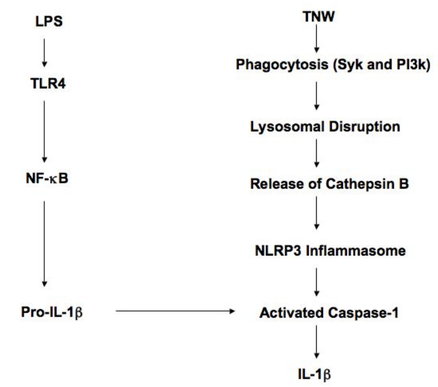

Figure 6: The TNW are phagocytosized in a manner that involves the enzymes Syk and PI3k. Once inside the cell the TNW induce lysosomal disruption which causes the release of Cathepsin B. Cathepsin B activates the NLRP3 Inflammasome to cleave Caspase-1, which in-turn cleaves pro-IL-1β.

ACKNOWLEDGMENTS

This work was funded by grant R25ES16247 from the NIH. I would like to thank the CEHS and the University of Montana for giving me this wonderful opportunity.