7-2010

Small Molecule Inhibition of Macrophage TNF-a Release

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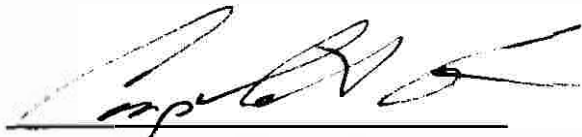# Small molecule inhibition of macrophage TNF-α release

By

Razwana Wahdat

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences from the Department of Biological Sciences, Seton Hall University
July 2010

APPROVED BY

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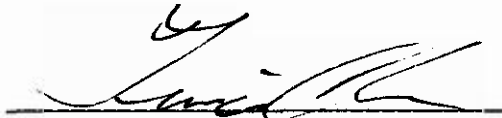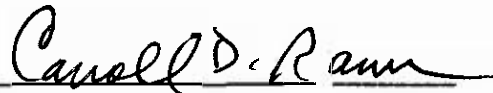Dr. Allan Blake

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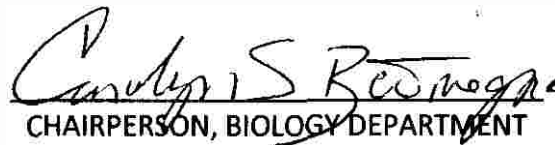
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AKNOWLEDGEMENTS

I would like to extend my gratitude to the following people:

Dr. Allan Blake, my mentor, who led me through this research project. Dr. Blake's guidance and encouragement has permitted me to acquire invaluable knowledge and laboratory skills that have peaked my interest in the field and opened my eyes to the value of research. My experiences both inside and outside of the laboratory have allowed me to grow not only as a student but as a person; for this I am forever indebted. I would like to extend my deepest gratitude to Dr. Blake for granting me the honor of working in his laboratory and allowing me to learn from such a brilliant mind.

Dr. Angela Klaus, for her unwavering willingness to help on any occasion and sitting on my defense committee.

Dr. Tin-Chun Chu, for sitting on my defense committee and aiding in the completion of my degree.

Johnathan Bartee and Jennifer Peek, fellow graduate students, for their assistance in completing this project and their unrelenting support and friendship throughout my tenure at Seton Hall University.

Dr. Carolyn Bentivegna, the chair of the Department of Biological Sciences, for affording me the opportunity to do research and serve as a teaching assistant for the department.

The biology department faculty and staff, for establishing a solid support system and positive learning environment for students.

My family and friends, who have always been there for me; words are too feeble to describe how much their infinite support and encouragement mean to me.

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ABSTRACT

Tumor Necrosis Factor Alpha (TNF-α) is a vital pro-inflammatory cytokine produced in response to the activation of the innate immune response via the Toll-like receptor signal transduction pathway. This thesis explores the release and inhibition of TNF-α from RAW 264.7 mouse macrophages and human U937 cells that have been exposed to a potent activator of innate immunity. Using lipopolysaccharide (LPS), a Gram negative bacterial endotoxin, in the presence or absence of proinflammatory cytokine interferon γ (IFN-γ) as activators of the TLR-4 pathway, a robust release of TNF-α was observed. Co-incubation of stimulated macrophages with the tyrosine phosphatase inhibitor, sodium vanadate, resulted in a time and concentration dependent inhibition of stimulated TNF-α release. These results demonstrate for the first time that soluble TNF-α release can be inhibited by a small molecule tyrosine phosphatase inhibitor, and indicates that protein tyrosine phosphorylation is critical to macrophage TNF-α release. Since the inappropriate expression of TNF-α occurs in the pathogenesis of many chronic inflammatory disorders, these results provide a potentially novel mechanism for regulating macrophage TNF-α release.

INTRODUCTION

In our everyday lives there exists a constant exposure to harmful pathogens that challenge our innate immune system and prime its response to suppress potential pathogens. One critical sentinel of innate immunity is the monocyte/macrophage. When monocytes are acutely exposed to pathogens such as the Gram-negative bacterial endotoxin, lipopolysaccharide (LPS), they respond by producing pro-inflammatory cytokines which serve to neutralize the immune challenge. On the macrophage's cell surface, Toll-like receptors (TLR) initiate the cellular response. Upon LPS binding to CD14 and stimulation of the TLR receptor, intracellular adaptor proteins are recruited, resulting in the activation of protein kinase cascades, such as the IkappaB kinase (IKK) and mitogen-activated kinase (MAPK) pathways (Guha and Mackman, 2001).

The initiation and propagation of the cellular response activated by LPS relies on a tightly orchestrated series of enzymatic reactions. The structural components of LPS, especially its lipid A portion is vital for recognition and thus activation of the inflammatory pathway. Once LPS is introduced into a system it binds a serum protein, LPS-binding protein (LBP), which facilitates its delivery to the cell surface receptor CD14. CD14 is bound to the cell surface via glycoplipid linkages but requires additional proteins to carry out transmembrane signaling. The newly formed CD-14/LBP/LPS complex activates the receptor, TLR-4 and its accessory protein, MD-2. TLR-4 and MD-2 form a

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complex that initiates an intracellular signaling cascade. At the extracellular surface,

MD-2 associates with TLR-4 through its leucine-rich repeats (LRR) and binds LPS directly.

Upon ligand stimulation, TLR-4 is oligomerized and an adaptor protein, myeloid

differentiation factor 88 (MyD88), associates with TLR-4 via the intracellular Toll/IL-1

receptor (TIR) domain. MyD88 possesses two domains: a TIR domain at the C-terminal

end and a death domain at the N-terminal end. MyD88 also associates with TIR domain-

containing protein (TIRAP). Because TIRAP contains a phosphatidylinositol 4,5-

bisphosphate (PIP2) binding domain, it facilitates MyD88 delivery of the activated TLR-4

to the membrane (Tanimura et al, 2008).

MyD88 then recruits IL-1 receptor-associated kinase 1 (IRAK-1) to TLRs by

interaction of death domains of the two molecules. This allows the subsequent

association of IRAK-4 which serves to phosphorylate and thus activate IRAK-1. Once

activated IRAK-1 partners with TNF receptor associated factor 6 (TRAF-6). The

oligimerization of TRAF-6 activates its ligase activity, leading to polyubiquitination of

target proteins such as TRAF-6 itself (Chen et al, 2008). This ubiquinated TRAF-6/IRAK-1

complex will then activate TAK-1 binding protein (TAB-1) and TGF-β-activated kinase 1

(TAK-1) (Takeda and Akira, 2004). TAK-1 and TAB-1 are responsible for triggering IκB

kinase (IKK) and mitogen-activated protein kinase (MAPK) pathways.

These pathways are further broken down into their components. IKKα, IKKβ and

IKKγ of the IKK pathway associate with one another to form a complex that is

responsible for phosphorylating IκB proteins. IκB proteins serve as inhibitors of the κ

light chain gene enhancer in B cells(Lu et al, 2008), which contain a number of copies of

ankyrin repeat sequences which actually mask the nuclear localization signal (NLS) of

NF-κB proteins (Jacobs and Harrison, 1998). The masking of the NLS results in

maintenance of NF-κB proteins in its inactive form and thus the form found sequestered

in the cytoplasm. Phosphorylation of this inhibitory protein, IκB, leads to its

ubiquitination which then leads to its degradation by proteosomes (Jacobs and Harrison,

1998). With IκB degraded, the transcription factor, NF-κB, is no longer restricted and

moves into the nucleus where it activates the transcription of pro-inflammatory

cytokines and many other immune associated genes. LPS exposure also triggers the

MAPK pathway which is activated along with the IKK pathway. The MAPK pathway also

ultimately leads to the activation of the AP-1 transcription factor which is fundamental

to the expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Trahn-Thi,

1995).

While this complex TLR-4 signal pathway is essential in amplifying the signal

initiated by LPS, numerous levels of regulation exist. For example, there are protein

inhibitors at the cell surface such as radioprotective 105 (RP105) and MD-2 homolog,

MD-1. RP105 and MD-1 form a complex that interacts with the TLR-4/MD-2 complex to

directly inhibit its association with LPS (Lu et al, 2008). Also, once activation has already

begun there are a number of additional negative regulators that serve to regulate the

pathway such as triad domain-containing protein 3 variant A (TRIAD3A) and suppressor

of cytokine signaling-1 (SOCI-1). TRIAD3A and SOCI-1 promote the degradation of many of the key pathway components such as TLR-4 and TIRAP (Lu et al, 2008). Acting even further downstream are the negative regulators IL-1 receptor-associated kinase M (IRAK-M)and A20. Previous studies suggest that despite its name IRAK-M lacks kinase activity and works in inhibitory manner by preventing the dissociation of MyD88 from IRAKs (Wesche et al, 1999). A20 is an enzyme the de-ubiquinates thereby removing ubiquitin moieties from TRAF-6 (Lu et al, 2008). As noted earlier, the non-ubiquinated form of TRAF-6 will not be able to complex with IRAK-1 to recruit TAB-1 and TAK-1 thus terminating the downstream signaling events (Figure 1).

While protein kinases are known activators of macrophages TNF-α release, the role of protein phosphatases, which regulate kinase function, remains unclear. Analogous to protein kinases, protein phosphatases fall into three general categories: serine/threonine phosphatases, tyrosine phosphatases and dual specificity threonine/tyrosine phosphatases (Barford, 2002) Protein phosphatases are generally categorized by functional inhibitors, for example while serine/threonine phosphatases are differentially inhibited by natural products, such as okadaic acid, tyrosine phosphatases can be inhibited by a range of products such as small molecules and synthetic chemical compounds (reviewed in Heneberg, 2009). Protein tyrosine phosphatase (PTP) inhibition leads to an up-regulation of kinase activity (Hooft van Huisduijnen et al, 2002). In the current study, we examine the effects of a broad spectrum tyrosine phosphatase inhibitor, sodium vanadate, on endotoxin stimulated

macrophage responses. We find that sodium vanadate inhibits macrophage TNF-α

release in a concentration and time-dependent manner. Our results provide the first

experimental evidence that tyrosine phosphatases are critically involved in TNF-α
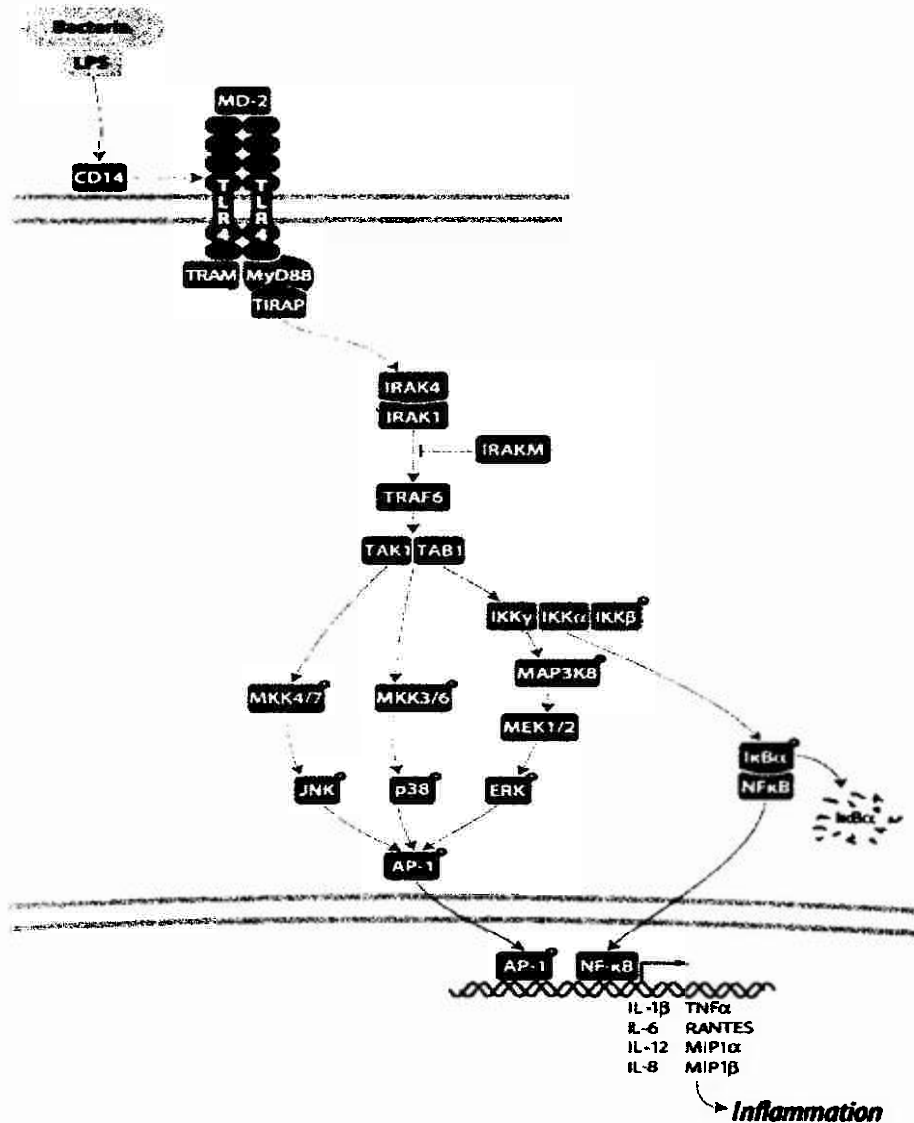
release.

Figure 1. *Toll-Like Receptor (TLR) Pathway.* The modified image demonstrates the LPS activated TLR-4 pathway, illustrating adaptor proteins, kinases, transcription factors and cytokines produced during an inflammatory response. Modified from Invitrogen Corporation.

MATERIALS AND METHODS

Cell Culture

The human leukemic monocyte lymphoma cell line (U937) was obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained with Roswell Park Memorial Institute (RPMI) growth medium (Invitrogen; Carlsbad, CA) plus 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were grown at 37°C in a humidified atmosphere in the presence of 5% CO_2. Cells were maintained in T-75 cm^2 flasks and 24-well cell culture dishes (Corning; Corning, NY). The mouse macrophage cell line, RAW 264.7, was obtained from the ATCC and was propagated in culture as described (Dos Santos et al, 2007). RAW 264.7 cells were maintained in 100 mm^2 culture dishes in RPMI medium with 5% fetal calf serum in a humidified atmosphere with 5% CO_2. All solutions and plastic-ware were cell culture grade, endotoxin free. Cells were routinely passaged at 70% confluence and split 1:10 (dish:dish ratio). Cells used in experiments are allowed to recover for 48 hours prior to experimentation.

Enzyme Linked Immunosorbent Assay

Soluble TNF-α levels were quantified using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Biolegend; San Diego, CA). U937 and RAW 264.7 cells were cultured separately in 24-well cell culture plates (Corning; Corning, NY) at a density of 40,000 cells/ml for 48 hours or until 90-100% confluency was achieved. After this incubation period, cell were washed with Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-Aldrich; St. Louis, MO) and treated with Opti-MEM, a serum-free cell culture media (Invitrogen; Carlsbad, CA) .

After 12-18 hours of incubation with Opti-MEM, the media was removed and the cells were treated with lipopolysaccharide (LPS), interferon gamma (IFN- γ) and sodium vanadate. One control and six experimental groups were used (Table 1). The cells were treated with 10 μg/ml LPS (Invitrogen; Carlsbad, CA) in the presence or absence of 10ng/ml IFN-γ (Sigma-Aldrich; St. Louis, MO). TNF-α inhibition experiments were carried out in the presence of 100 μM sodium vanadate (Sigma-Aldrich; St. Louis, MO). The treated cells were incubated at 37°C and the supernatants were collected after 2 hours. For time courses the same steps were taken up until sample collection. Instead, samples were collected at 0 minutes, 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 6 hours. Each sample was frozen at -80°C upon collection before assay.

Cell supernatant samples were then thawed on ice to avoid TNF-α degradation. Once the samples were thawed, there were analyzed using ELISA MAX[TM] Deluxe Sets to quantify levels of soluble TNF-α. One day prior to running the ELISA, depending on the cell type being analyzed, human or mouse TNF-α capture antibody is added to the NUNC Maxisorp 96 MicroWell Plates and left to incubate at 4 °C overnight. The plate was then washed 4 times with 300 μL wash buffer and blotted dry each time. Because the capture antibody does not cover the surface of the high-affinity binding plate in its entirety, there are spaces left empty where protein may attach and in turn, cause a false reading. In order to block non-specific binding to these empty spaces, 200 μL of assay diluent was added to each well and allowed to incubate for one hour at room temperature. The assay diluent contains proteins that will attach to the surface of the plate thereby minimizing non-specific interaction of other proteins such as the antigen to the surface of the plate. The blocking step was then followed by four washings.

Next 100 μL of each sample was loaded into each well. A TNF-α standard curve was generated for each ELISA plate and all treatments were performed in triplicate. The standard curve was made up of the following TNF-α concentrations: 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 0 pg/mL. All sample dilutions were carried out with assay diluents. Loading of the samples into the wells was followed by a 2 hour incubation period at room temperature with gentle shaking. After, the plate was washed once again four times with wash buffer, and then treated with 100 μL of TNF-α detection antibody. This was followed by a 1 hour

incubation period at room temperature with shaking and subsequent washes. Next, 100 μL of Avidin-HRP (Horse Radish Peroxidase) was added and let to incubate for 30 minutes at room temperature with shaking. This was followed by four washes but in an effort to minimize background the last wash was prolonged allowing the wash buffer to soak the wells.

Next, 100 μL of a fresh solution of tetramethylbenzidine (TMB) substrate solution was added to each well and left to incubate in the dark for 15 minutes at room temperature. TMB must be handled in the dark because it is a photosensitive chromogenic substrate and exposure to light may nullify results. Finally, 100 μL of stop solution (sulfuric acid) was added to each well causing immunoreactive positive wells to change color from yellow to blue. Using the SPECTRAmax250 plate reader (Molecular Device; Sunnyvale, CA), TNF-α levels were determined using the cleavage of TMB substrate to its oxidized form indicating the concentration of TNF-α present in each well (Abs 450 nm and 570 nm).

Treatment Groups						
Basal	LPS	sodium vanadate	IFN- γ	LPS+ IFN- γ	LPS + sodium vanadate	LPS+ IFN-γ + sodium vanadate

Table 1. *Treatments used for ELISA.* The U937 and RAW 264.7 cells were treated in the above manner. Cells were treated with 10 μg/ml LPS, 10 ng/ml IFN- γ and 100 μM sodium vanadate. Stock solutions were diluted in Opti-MEM.

Statistical Analysis

All results were calculated and presented as the mean ± SEM for each control or treatment group. Statistical comparisons between the means of different groups were performed by multiple ANOVA with a Neuman-Keuls multiple comparison post-test. Statistical comparisons and analysis were performed with GraphPad Prism 4 and statistical significance was defined as $p < 0.05$.

Soluble TNF-α Secretion

TNF-α is a critical pro-inflammatory cytokine. In our studies, U937 and

RAW264.7 cells were used as a monocyte model system to assess the effects of LPS, IFN-

γ, and sodium vanadate on soluble TNF-α levels which were stimulate by the Gram

negative bacterial endotoxin, LPS. LPS activates a robust and sustained TNF-α response

in our cell models, thereby making these cells a valuable model for studying pro-

inflammatory cytokine release (Guha and Mackman, 2000). Untreated U937 cells

demonstrated basal release of TNF-α at a level of 105 pg/ml (see Fig 1). Treatment of

U937 cells with 10 μg/ml LPS increased TNF-α levels to 560 pg/ml translating into an

increase by 455 ± 40 pg/ml from basal (see Fig 2, **p<0.001 basal versus LPS treated).

Treatment with 10ng/ml IFN-γ stimulated the release of TNF-α to 375 pg/ml thus

increasing by 270 ± 55 pg/ml from basal (see Fig 2, *p<0.01 basal versus IFN-γ treated).

When cells were treated with both LPS and IFN-γ together 515 pg/ml TNF-α was

released increasing by 410 ± 35 pg/ml (see Fig 2, **p<0.001 basal versus LPS + IFN-γ

treated). All three of these experimental groups caused a significant increase in TNF-α

levels. On the other hand, treatment of U937 cells with sodium vanadate, a broad

spectrum protein tyrosine phosphatase inhibitor, left TNF-α levels at 108 pg/ml, a value

very close to that of basal levels. Sodium vanadate suppressed LPS stimulated TNF-α

from 560 pg/ml to 130 pg/ml when cells were treated with LPS in the presence of

sodium vanadate. Similarly, when cells are treated with IFN-γ and LPS in the presence of

sodium vanadate there is a decrease to 185 pg/ml TNF-α from the 375 pg/ml seen with

cells treated with IFN-γ alone (see Fig 2).
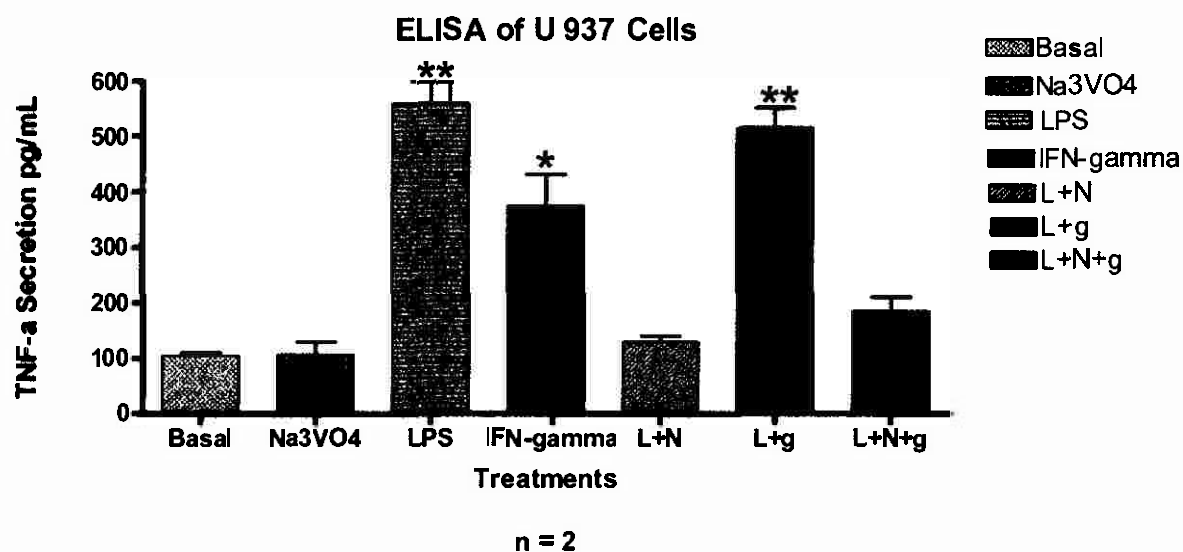
ELISA of U 937 Cells

Figure 2. *ELISA reveals levels of soluble TNF-α secretion from U937 cells.* U937 cells were treated with [10 μg/ml] LPS and [10 ng/ml] IFN-γ in the presence or absence of [100μM] Na_3VO_4. Cell supernatants were collected after 2 hours of incubation and soluble TNF-α quantified by ELISA. Data are means +SEM from two independent experiments, each performed in triplicate. Data were analyzed by multiple ANOVA with Newman-Keuls post hoc test. Using GraphPad Prism statistical significance was defined as $p < 0.05$. *$p < 0.01$; **$p < 0.001$. L: LPS; N: Na_3VO_4; g: IFN-γ.

Quantifying TNF-α release from RAW 264.7 cells in response to LPS +/- IFN-γ

resulted in similar effects as those observed with U937 cells, although we noted a

substantial difference in the total amount of TNF-α released (see Fig 3). Basal release of

TNF-α in RAW 264.7 is 442 pg/ml. There is an increase from basal levels to 26,200

pg/mL TNF-α observed when RAW 264.7 cells are treated with 10 µg/ml LPS (an

increase by 25,800 ± 3,100 pg/ml from basal) and an increase to 20,600 pg/ml when

treated with 10 ng/ml IFN-γ (an increase by 20,200 ± 1,500 pg/ml from basal). LPS and

IFN-γ together increase TNF-α levels to 31,000 pg/mL TNF-α (an increase by 30,600 ±

5,000 pg/ml from basal). Cells treated with sodium vanadate alone have levels of TNF-α

that remain in the range of basal release; in this case, 528 pg/ml. Once cells are treated

with sodium vanadate in the presence of LPS and/or IFN-γ there is an inhibition of TNF-α

release. When RAW 264.7 cells are treated with LPS in the presence of sodium

vanadate TNF-α levels drop to 4,040 pg/ml. Similarly, when cells are treated with LPS

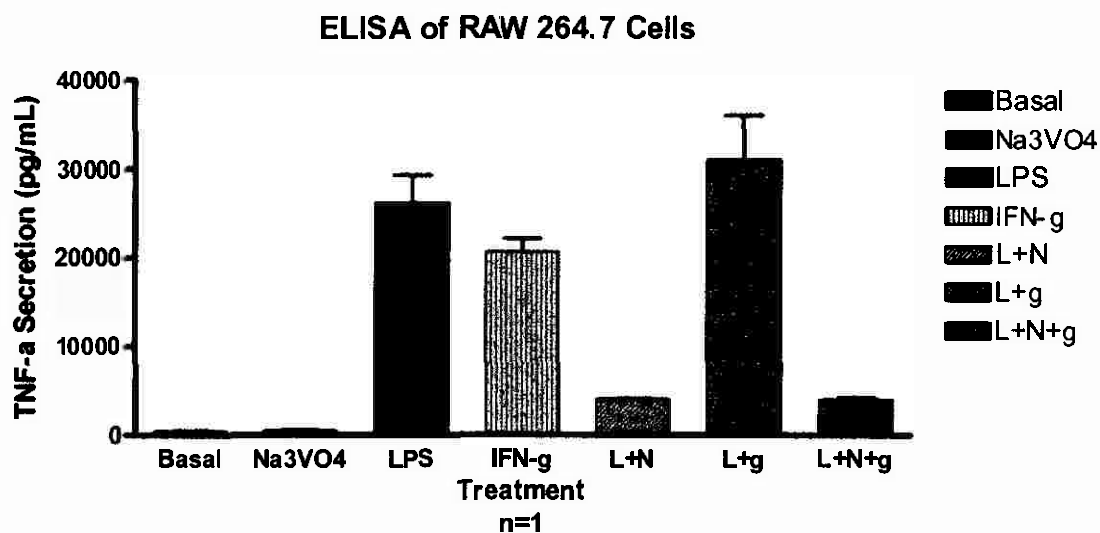and IFN-γ in the presence of sodium vanadate TNF-α level drop again to 4,020 pg/ml

(see Fig 3).

ELISA of RAW 264.7 Cells

Figure 3. *ELISA reveals levels of soluble TNF-α secretion from RAW 264.7 cells.* RAW 264.7 cells were treated with [100 µm] Na_3VO_4, [10 µg/ml] LPS, and [10 ng/ml] IFN-γ. Cell supernatants were collected after 2 hours of incubation and soluble TNF-α quantified by ELISA. Results are the mean from a single experiment performed in triplicate. LPS and IFN-γ induced TNF-α release was decreased from cells treated with Na_3VO_4. L: LPS; N: Na_3VO_4; g: IFN-γ.

Time Course of TNF-α Secretion

The TLR signal transduction pathway of the innate immune response elicits a sequential pro-inflammatory cytokine response. In U937 cells, basal levels of TNF-α increases approximately three-fold over a 6 hour time course ranging from 68 pg/ml at 0 minutes to 210 pg/ml at 360 minutes increasing by 142 ± 16 pg/ml from 0 minutes (see Fig 4). LPS treated U937 cells exhibited an elevated time-dependent increase in TNF-α levels increasing from 187 pg/ml at 0 minutes to 1,580 pg/ml at 360 minutes (see Fig 4; an increase by 1390 ± 13 pg/ml from 0 minutes). IFN-γ treated cells also demonstrated in increase in TNF-α over time, elevating from 120 pg/ml at 0 minutes to 1,230 pg/ml at 360 minutes (see Fig 4; an increase by 1110 ± 25 pg/ml from 0 minutes). Again, when cells were treated with both LPS and IFN-γ an increase in TNF-α is seen over time; levels increase from 137 pg/ml at 0 minutes to 1,170 pg/ml (see Fig 4; an increase by 1,000 ± 33 pg/ml at 0 minutes). However, cells treated with sodium vanadate showed no significant increase in TNF-α throughout the 6 hour period. Cells treated with LPS in the presence of sodium vanadate demonstrated a sustained inhibition of TNF-α over time ranging from 120 pg/ml at 0 minutes to 140 pg/ml at 360 minutes. Similarly cells treated with both LPS and IFN-γ in the presence of sodium vanadate exhibited a sustained suppression of TNF-α release ranging from 120 pg/ml at 0 minutes and 130 pg/ml at 360 minutes (see Fig 4).
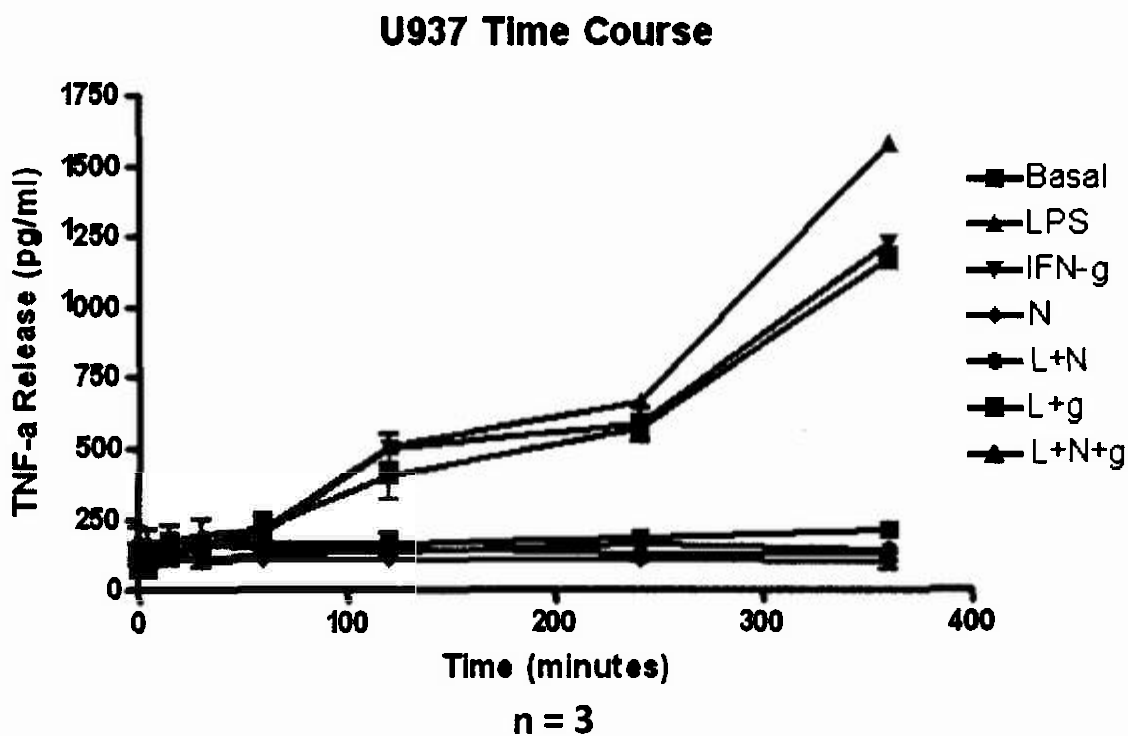
U937 Time Course

Figure 4. *Time course of soluble TNF- α release from U937 cells.* The results demonstrate a time-dependent increase in LPS and IFN-γ TNF- α release. Na_3VO_4 blocked TNF-a release from these cells. The results shown are the mean+/- SEM for three experiments, each performed in triplicate. Data were analyzed by multiple ANOVA with a Newman-Keuls post hoc test. Statistical significance was defined as *p<0.05. L: LPS; N: Na_3VO_4; g: IFN-γ.

DISCUSSION

The introduction of foreign pathogens into the body elicits an immediate innate

immune response and triggers an array of immune cells to synthesize critical

inflammatory mediators and cytokines. The release of cytokines is fundamental for

intercellular communication in the immune response. Despite being essential in acute

inflammation, chronic expression of certain pro-inflammatory cytokines are an

underlying cause for numerous chronic inflammatory and autoimmune disorders. For

this reason, understanding the signal transduction and trafficking pathways of cytokines

such as TNF-α and IFN-γ is a vital area of study.

Activation of the TLR signal transduction pathway initiates an intracellular kinase

cascade that leads to the activation of transcription factors responsible for producing

inflammatory cytokines such as TNF-α. Protein kinases are crucial to cytokine release

and when these enzymes are selectively activated they serve to enable this pathway.

LPS, a potent Gram negative bacterial endotoxin specifically triggers the TLR-4 pathway

leading to an activation of a protein kinase cascade that ultimately up-regulates the

transcription of TNF-α (Aderem and Ulevitch, 2000) by NF-κB.

In our studies we demonstrate that LPS and IFN-γ stimulate the release of

soluble TNF-α from both a human monocyte cell line, U937 cell, and a mouse monocyte

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cell line, RAW 264.7 cells. Monocytic responses to LPS are well documented (Guha and Mackman, 2001) and the cellular events that culminate in TNF-α release are well known (see Fig 1). Although monocytes also have molecular components that suppress the TLR pathway, the availability of a small molecule chemical inhibitor of TLR-induced TNF-α release could prove invaluable for experimental studies. Small molecule inhibitors are known to exist, but these act predominately through the elevation of intracellular cyclic nucleotide levels (Essayan, 1999) or through transcriptional suppression of cytokine genes (Gupta et al, 2010). A pathway inhibitor that affects protein phosphatase activity has not been explored.

In this thesis, we demonstrate that the treatment of cells with the pro-inflammatory mediators, LPS and IFN-γ in the presence of sodium vanadate inhibits stimulated TNF-α levels. Based upon our knowledge of protein tyrosine phosphatase inhibitors, such as sodium vanadate, an inhibitor would suppress the effects of phosphatases thereby enabling kinases. Because we are studying a kinase enabled pathway, the treatment of cells with a phosphatase inhibitor would be thought to lead to an up-regulation of the transcriptional products. However, what we observe from the ELISA results shown above is that there is an extreme inhibition in soluble TNF-α levels when treated with LPS and LPS+IFN-γ in the presence of sodium vanadate in contrast with LPS or LPS+IFN-γ alone.

Although the mechanisms for sodium vanadate 's actions have not yet been established, numerous potential molecular targets exist. The TLR-4 signaling pathway is a tightly regulated cycle employing negative regulators such as IRAK-M, RP105 and A20 to shut off the pathway once the signal is no longer needed (Kobayashi et al, 2002). One speculation is that sodium vanadate may be acting to up-regulate negative regulators such as those listed above. Such an up-regulation would lead to an inhibition of the players involved in activating NFκb. NFκb is the transcription factor responsible for producing TNF-α therefore and inhibition of NFκb will lead to an inhibition of TNF-α production.

TNF-α is an early response cytokine that acts in the recruitment and activation of additional immune cells to the site of invasion. Because the ELISA is measuring soluble TNF-α, there is a possibility that sodium vanadate may not be interfering with the TLR-4 signaling pathway but rather acting at a post-transcriptional level. Once transcription is achieved in the nucleus, its products, such as TNF-α, must still follow a trafficking pathway before they can be secreted out of the cell to carry out cell to cell communication. Newly transcribed TNF-α move from the nucleus to the Golgi complex and from golgi complex to the recycling endosomes where it finally moves out towards the phagocytic cup (Figure 5). Once the TNF-α is at the phagocytic cup the ectodomain is cleaved by TNF-α converting enzyme (TACE) resulting in a now soluble cytokine that is released by the cell (Stow et al, 2009). If sodium vanadate acts as a TACE inhibitor, soluble TNF-α will not be formed and will not be secreted by the cell. If sodium

vanadate is indeed a TACE inhibitor it may serve as another explanation for the decreased levels of TNF-α shown by the above ELISA quantifications.

The simplistic route of TNF-α movement described above involves many different proteins such as adaptors, coat proteins, lipids, small GTPases and golgins to succeed in efficient trafficking of cytokines. One of the key proteins involved are members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) family of membrane fusion proteins. In general, Q-SNAREs complex with R-SNAREs on opposing membranes to allow for fusion of vesicles carrying cytokines such as TNF-α. In immune cells the specific components of the abovementioned SNAREs responsible for forming the complexes have been identified as syntaxin4 and SNAP23 from the Q-SNARE family and VAMP8, VAMP7 and VAMP2 from the R-SNARE family (Stow et al, 2009). These SNARE proteins are involved in every step of the way from the Golgi to the recycling endosome and from the endosome to the cell surface. For this reason the levels and concentrations of SNARE proteins have a direct effect on the volume and efficiency of cytokine trafficking. Previous studies have shown that siRNA knockdown of VAMP3, an R-SNARE protein involved in TNF-α trafficking, led to a reduced secretion of cytokines (Manderson et al, 2007). Therefore, sodium vanadate interference with the formation of SNARE complexes can serve as an explanation for decreased levels of soluble TNF-α.

Whatever the molecular target may be, because the presence of sodium vanadate has a sustained inhibitory effect on soluble TNF-α that remains unchanged over time, it may serve as a treatment for chronic inflammatory and autoimmune disorders. Current treatments include protein biologics that use monoclonal antibodies specific for TNF-α to remove already secreted TNF-α. However, the treatment of cells with sodium vanadate proposes a way to treat these conditions by preventing elevated levels of TNF-α before it is even released from the cell.
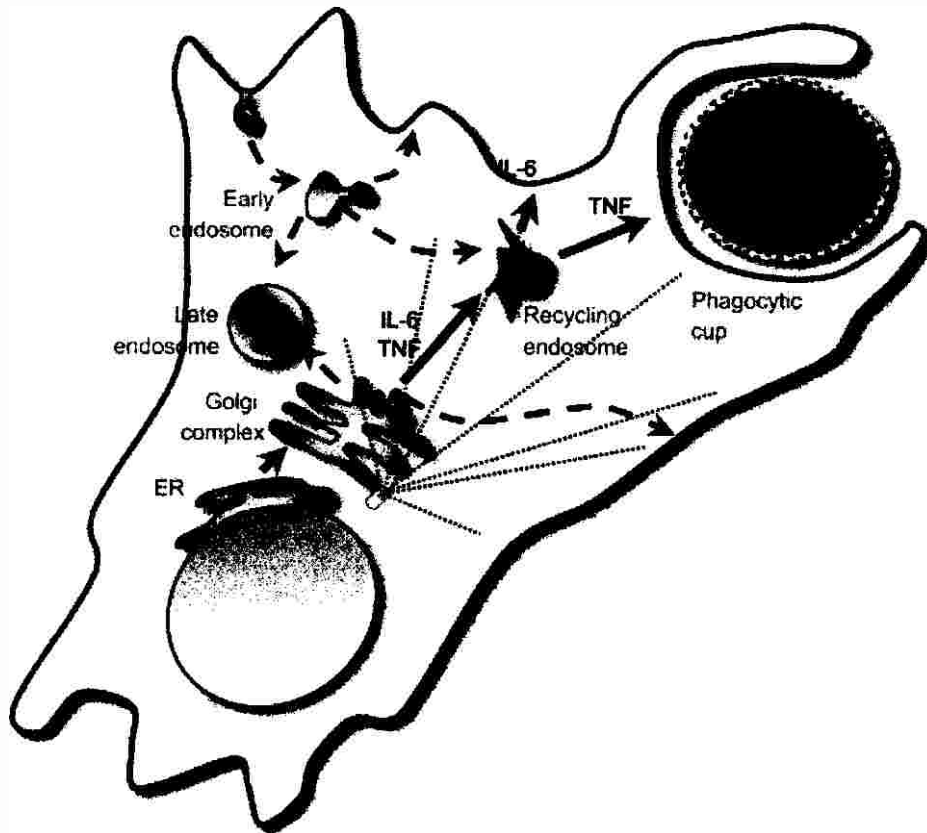
Figure 5. *TNF-α trafficking and secretion in macrophages.* Proposed model for TNF-α release. Once TNF-α leaves the endoplasmic reticulum, it is transported in the Golgi complex. From the Golgi, TNF-α is packaged into vesicles to move the cytokine into the recycling endosome and out to the surface of the cell, with the dominant release of TNF-α occurring at the phagosome. Taken from Stow et al. Cytokine secretion in macrophages and other cells: pathways and mediators. *Immunobiology* 214: 601-612 (2009).

CONCLUSION

In this study, we reveal the stimulatory effects of LPS and IFN-γ on soluble TNF-α levels in mouse and human monocyte cell lines, U937 and RAW 264.7. We have demonstrated that the presence of sodium vanadate in combination with the aforementioned stimulants is responsible for an inhibitory effect on soluble TNF- α levels. Although LPS and IFN-γ are causing a time dependent increase of TNF- α, once cells are treated with LPS and IFN-γ in the presence of sodium vanadatethere is a sustained inhibition of TNF- α. The use of two different cell lines has allowed us to make the conclusion that the effects are conserved across at least two different species.

This novel inhibitory effect of sodium vanadate on the cells of the immune system may lead to the development of therapeutic agents used to control chronic inflammatory and autoimmune disorders. In order for this to occur, a molecular target must first be uncovered. Potential molecular mechanisms/targets proposed include an up-regulation of negative regulators in the TLR signaling pathway, interruption in the formation of SNARE complexes, and mimicry of an inhibitor of TACE.

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