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Effects of Sodium Orthovanadate on LPS-Induced Senescent RAW 264.7 Cells

Andrew Pugliese
andrew.pugliese@student.shu.edu

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By

Andrew Pugliese

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Andrew Lee Pugliese has successfully defended and finalized the text of his master's dissertation for the M.S. during this Spring Semester 2022.

Approved By

MENTOR

Dr. Jessica Cottrell

COMMITTEE MEMBER

Dr. Daniel B Nichols

COMMITTEE MEMBER

Dr. Constantine Bitsaktsis

DIRECTOR OF GRADUATE STUDIES

CHAIRPERSON,

DEPARTMENT OF BIOLOGICAL STUDIES

Dr. Heping Zhou

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Abstract

Cellular senescence is considered a signal transduction process where damaged or stressed cells arrest proliferation. Exogenous stressors including bacterial infection, chemotherapy, and irradiation can drive normal cells to senescence. Sodium orthovanadate (Na_3VO_4) is a phosphatase inhibitor known to mediate the release of the proinflammatory cytokine, TNF-α. We hypothesized that $NaVO_4$ treatment will reduce proinflammatory expression from senescent cells. In our study we illustrate that prolonged exposure to LPS caused RAW 264.7 cells induced morphological changes indicative of senescent cells including the formation of giant multinucleated cells (GMCs). Furthermore, we found that RAW 264.7 GMC cells stain positive for senescence and increase expression of key proinflammatory cytokines associated with the senescent phenotype including IL-10 and TNF-α. Our data demonstrate that 100uM and 1000uM sodium orthovanadate treatment reduced cell proliferation. The reduction in proliferation was concomitant with changes in pro-inflammatory cytokines, IL-10, and TNF-α (Figure 9B &C at 24hrs). IPA analysis confirmed connections between these proinflammatory cytokines, senescence, and disease progression.

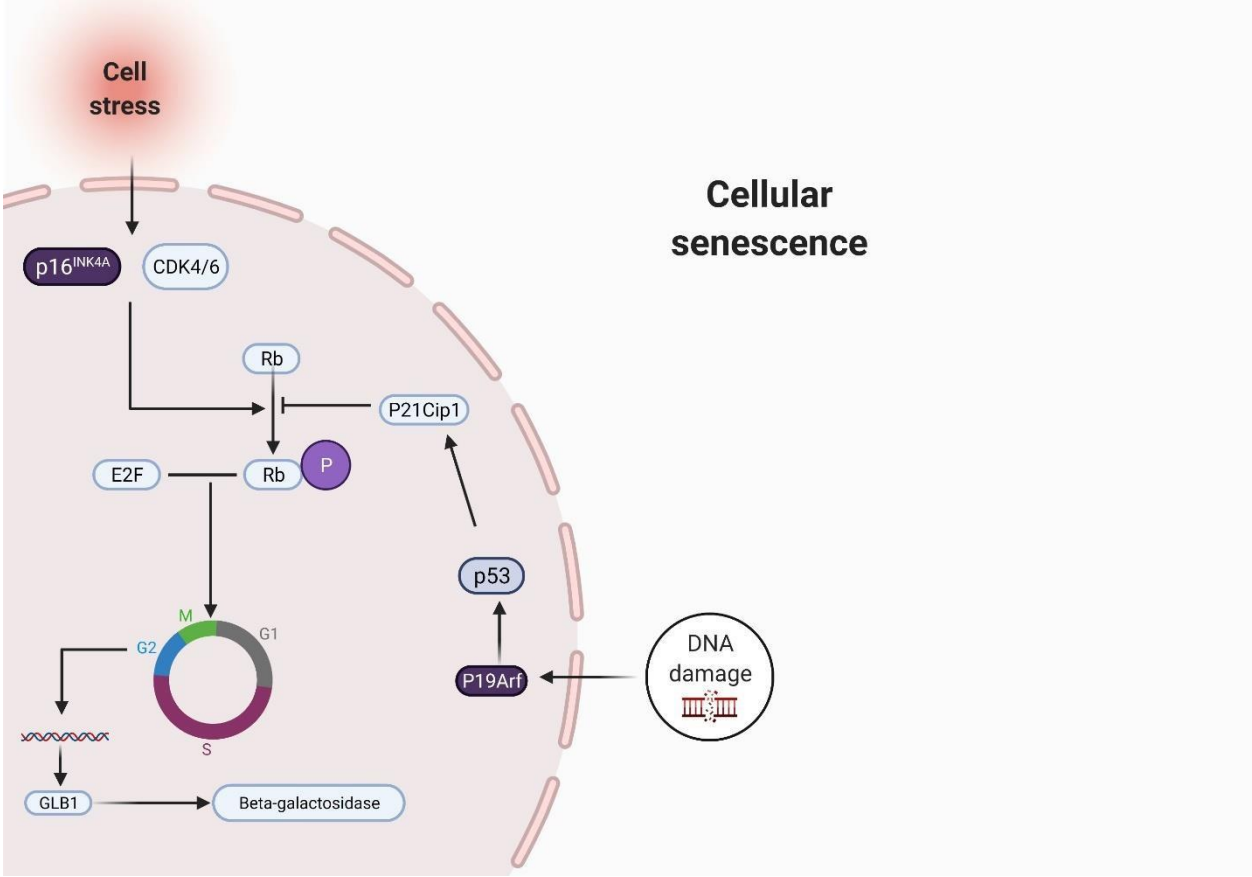
Introduction

Cellular senescence was first described by Leonard Hayflick and Paul Moorhead in 1961 [1]. This phenomenon is now considered a signal transduction process where cells, once they are damaged, can establish a stable proliferation arrest. Cells which become senescent are characterized by various metabolic and morphological changes including chromatin reorganization, gene expression alteration, and the development of a proinflammatory phenotype [2]. Biologically senescence can play both an advantageous and deleterious role. For example, cellular senescence can be a way in which cells can avoid malignant transformation[3]. Cellular senescence is a mechanism of tumor suppression and can act as an impediment for cancer development [2]. It is well documented that senescent cells are often present within tumor microenvironments [4]. It has also been documented that the proinflammatory phenotype associated with senescent cells alters the cellular microenvironment and can promote cancer progression [2, 4, 5].

Cells can enter nondividing states of rest. Quiescent cells are in a reversible non-dividing state of rest while senescent cells are in an irreversible nondividing state. These two phases or states are often distinguished by various characteristics and the causative nature in which the cells delineated to the non-dividing state [6]. Often cells enter into senescence from a multitude of factors including excessive cellular replication, irreparable DNA damage, activation of particular oncogenes, oxidative stress, particular chemical agents, and mitochondrial dysfunction. Once the cell is damaged it has the potential to induce apoptosis, repair the damage, or enter into a stable cell cycle arrest [7]. The stable cell cycle arrest is mediated by the activation of various signal transduction pathways such as the p16INK4a-pRb and p53 pathways that regulate the process of the cell cycle (Figure 1) [7].

Figure 1: The Cellular Senescence Signaling Pathways.
(Created using BioRender Software)

(**Abbreviations:** $p16^{INK4A}$: cyclin-dependent kinase inhibitor 2A, CDK4/6: cyclin dependent kinase 4/6, Rb: Retinoblastoma, P: Phosphorylation, P21Cip1: cyclin-dependent kinase inhibitor 1, p53: tumor protein p53, P19Arf: ARF tumor suppressor, E2F: Transcription factor E2F, GLB1: galactosidase- β 1 protein)



Once a cell enters into senescence it begins to exhibit morphological and metabolic changes that differ from their non-senescent counterparts [2]. Morphologically, senescent cells can appear as large, flat, refractile, multinucleated cells. Senescent cells can also display

extensive vacuolization brought on by external stressors. These changes are often biomarkers used to determine senescence [8]. The most widely used and defined biomarker for cellular senescence and aging cells is senescence-associated β-galactosidase (SA- β -Gal) activity. Most cells express lysosomal β-galactosidase (β -Gal) at a pH of 4, however only senescent cells will express β -Gal at a pH of 6. This activity of β -Gal at the pH of 6 is termed SA- β -Gal [8]. It is thought that this activity is linked to the increase lysosome content of senescent cells, suggesting that lysosomal β-galactosidase activity increases in senescent cells [8].

The senescent phenotype is not limited only to metabolic and morphological variations. When senescent cells undergo metabolic and morphological changes, they also involve genetic changes of various secretory proteins. When these changes occur, the senescent cell develops what is known as the senescent associated secretory phenotype (SASP) [2, 9, 10]. This particular phenotypic presentation of senescent cells is associated with secretion of inflammatory cytokines, immunomodulators, growth factors, and various proteases. The secretion of these various factors into the extracellular matrix augments the microenvironment [11-14]. When this occurs, the senescent cells can propagate the stress response as well as communicate with adjacent cells through this SASP. The SASP can allow for numerous amounts of beneficial processes such as embryonic development, wound healing, tissue regeneration and tumor suppression[4, 9-11, 15]. However, more often than not the SASP has detrimental effects particularly as the cell or organism begins to age including tissue degeneration, chronic inflammation, and tumor promotion (Figure 2).

Figure 2: Balance of Physiological Effects based on Cytokine Release
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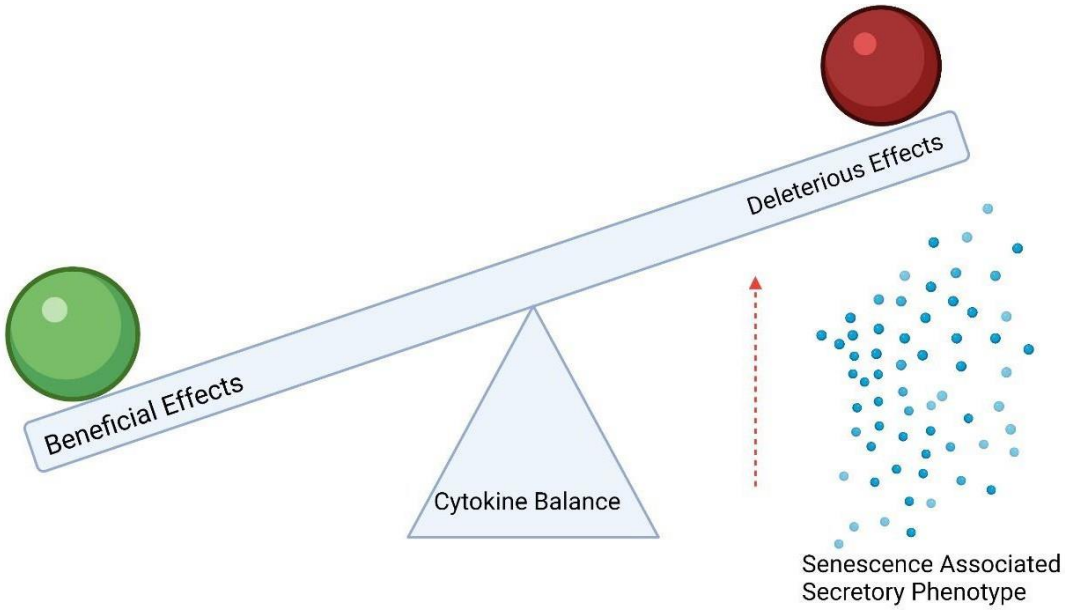

The SASP causes major changes in inflammatory cytokines, immunomodulators, growth factors, and various proteases (Figure 2) which alter signal transduction pathways and affect surrounding cells within the microenvironment [5, 9, 10, 15, 16]. For example, the microenvironment of a cancerous tumor, or an arthritic joint would be home to local cells as well as senescent cells, thus cancer and osteoarthritis are beneficial disease models for studying senescence. Within the disease model of cancer, SASP secrete growth factors that allow sustained growth of malignant cells and help them to invade the immune system. The SASP can also enhance cellular proliferation and promote an epithelial to mesenchymal transition, a

hallmark of metastatic cancer [4, 5, 11-15, 17]. Osteoarthritis is another illustrative model of the negative effect the SASP can have on the cell and tissue's microenvironment. Cells within the knee joint such as fibroblasts, macrophages, adipocytes, osteoblasts, and osteoclasts all become senescent because of a multitude of factors including aging and traumatic events. When these cells become senescent and develop an SASP they create an inflammatory response in the damaged joint, which ultimately causes loss of function [18].

Multinucleated giant cells are a prime cellular model to study senescence and are found in both osteoarthritis and cancer. Previous studies have provided and illustrated the cellular biology of multi-nucleated giant cell (GMC) formation[19]. In a recent study, gram-negative bacterial endotoxin lipopolysaccharide (LPS) stimulation of RAW 264.7 cells was found to upregulate TLR4 pathway and the NF-kβ pathway, important stress response pathways [19]. This study and many others demonstrate that LPS-stimulated RAW264.7 cells are a good in-vitro model for developing multi-nucleated giant cell formation when a stress response is triggered [20]. The role of LPS-induced GMCs on the p16INK4a-pRb and p53 pathways have also been documented. Feng et al. illustrated that when stimulated with LPS, senescent dental pulp stem cells, showed that the TLR4/MyD88- NF-kβ -p53/p21 signaling pathway becomes active, as well as the p16INK4a signaling pathway when repeated stimulation of LPS occurred [21]. Most of these studies conducted with GMC formation have illustrated the production of proinflammatory cytokines consistent with the SASP, most notably TNF-α, IL-6 and IL-10. For example, Trias et al. illustrated that multinucleation and release of inflammatory cytokines are associated with the senescence of microglial cells which showed positive labeling of p16INK4a [22]. Furthermore, these studies illustrated how GMCs are induced by cellular senescence pathways, and how such multinucleated senescent cells can be deleterious. Mirzayans et al. illustrated the increased

occurrence of GMCs within the tumor microenvironment following anticancer treatment. These GMCs were induced via senescent pathways under genotoxic stress. These cell types were shown to cause an increase in metastasis and resistance to anticancer treatments[23].

Many studies have illustrated a mechanism which can attenuate the release of such proinflammatory cytokines consistent with the SASP. These compounds are termed “senolytics” and can help eliminate the SASP by reducing the release of proinflammatory cytokines or eliminating the senescent cells themselves. By reducing the cytokines released in the SASP it is hypothesized that a normal phenotype would be restored. Sodium orthovanadate, is a type of senolytic which functions as a tyrosine phosphatase inhibitor. Previous studies have demonstrated that treatment with sodium orthovanadate can inhibit the release of TNF-α in LPS stimulated RAW 264.7 cells [24]. Other data shows that other tyrosine phosphate inhibitors can negatively regulate signaling molecules such as IRAK-M, RP105 and A20, which in turn inhibits the NFκβ signaling pathway. Ultimately, downregulation of the NFκβ signaling pathway reduced the production of various pro-inflammatory cytokines [24]. Together, the literature suggests that RAW 264.7 murine macrophages under prolonged LPS induced stress develop into a morphologically different phenotype that resembles senescence cells. The purpose of this research was to 1) characterize that LPS-induced RAW 264.7 cells SASP and 2) determine if sodium orthovanadate treatment could inhibit SASP pro-inflammatory secretion.

Materials and Methods

RAW 264.7 Cell Culture

The mouse macrophage cell line, RAW 26.47 (gift from Dr. Pat O'Connor at Rutgers), was grown and propagated in suspension as described by (Dos Santos et al. 2007). Briefly, the cellular suspension were maintained in 100 mm^2 culture dishes in Roswell Park Memorial Institute (RPMI) media. The media was supplemented with heat inactivated, endotoxin tested, 5% fetal calf serum, and GlutaMAX™, high glucose, contained 1 % streptomycin and penicillin. The culture was kept in a humidified atmosphere with 5% CO_2. All solutions and plastics ware were used were certified cell culture grade, endotoxin free. Cells were routinely passaged at 70% confluence and split 1:3 (dish: dish ration). Cells used in experiments were allowed to recover for 48 hours before experimentation.

Giant Multinucleated Cell Formation

RAW 264.7 cell monolayers were cultured, plated, and treated as described by (Bartee J., 2011). Briefly cells were plated in 24-well tissue culture grade plastic dishes at a seeding density of 100,000 cells/ well and a total of 1 mL media per well. After 48 hours in culture, the cells were rinsed with PBS to remove growth media and then were treated with LPS supplemented growth media. Media was supplemented with 10 micrograms/mL LPS (Invitrogen, Waltham, MA). The culture was then incubated in a humidified atmosphere with 5% CO_2 for 96 hours. GMC formation was documented using with (PS-MC5UW camera, PROSCOPE, Oregon City, OR). The monolayers were designated as outlined in Table 1.

Table 1: Summary of Treatment Groups

Non-senescence Groups n=3		**Senescence Groups n=3**			
-GMC	-GMC/+SV	+GMC	+GMC/+LPS	+GMC/+SV	+GMC/+SV/+LPS
Vehicle	+Na_3VO_4	Vehicle	+LPS	+Na_3VO_4	+ Na_3VO_4 + LPS

Table 1: Summary of treatments groups. RAW 264.7 cells were treated with or without LPS 10ug/mL for 96 hours to establish senescence (+/- GMC). After 96 hours the groups were treated with vehicle, 100uM Na_3VO_4, 10ug/mL LPS or neither. (GMC: Giant Multinucleated Cell, SV/ Na_3VO_4: Sodium Orthovanadate, LPS: Lipopolysaccharide)

Senescence Associated β-Galactosidase Staining Assay

To confirm that GMCs formed via prolonged exposure to LPS were indeed senescent, a commercially available staining kit was purchased (Cell Signaling, Danvers, MA). Following the manufacture protocol, the cells were stained and confirmed for senescence. First, the media was removed from the cellular monolayers. The plates were then rinsed with 1X PBS and 500 uL of fixative solution was placed and the cells were allowed to fix for 13 min at room temperature. The plate was then rinsed twice with 1X PBS, and 500 uL of β-Galactosidase staining solution at a pH of 6 was added to each well. The plates were wrapped and covered in parafilm and allowed to incubate overnight in a dry incubator (without CO_2). Following incubation, development of blue pigmentation, indicating a positive senescent cell, was viewed by light microscopy with (PS-MC5UW camera, PROSCOPE, Oregon City, OR). Quantification was done by manually counting positive senescent cells of the various treatment groups in triplicate.

Sodium Orthovanadate Treatment Assay

Once senescence was established, cells were treated as either a control (non-senescent cell) or a treatment group (senescent). These groups were then treated with or without sodium orthovanadate (100 μM Na_3VO_4). The cells were allowed to incubate with their various treatments for 4hrs, 6hrs, and 24 hrs. The cells were then stained with β-galactosidase stain, viewed via light microscopy, and quantified by manually counting positive senescent cells of the various treatment groups in triplicate.

Cytokine Expression Assay

Cytokine expression levels of the various experimental groups were analyzed using a premade Luminex™ kit from R&D systems. Briefly the cell supernatants were collected at 0, 4, 6, and 24 hrs and stored at -80°C. Samples were thawed on ice and assayed using the manufacturers standard protocol (Luminex discovery Assay, Minneapolis, MN). The cytokines analyzed included TNF-α , IL-1β, IL-6, CCL8, IL-1α, IL-10, and IGF-1 which were identified using Qiagen's IPA (Figure 3).

Ingenuity Pathway Analysis

QIAGEN ingenuity pathway analysis is for modeling, analyzing, and understanding complex omics data, it allows individuals to perform various analysis on data to develop an understanding of various interactions of molecular pathway and disease processes. IPA contains many various features, such as the Molecular Activity Predictor tool (MAP) which allows an individual to connect specific disease states and molecules and illustrate how expression of one such entity can alter expression of the connected entity. IPA's BioProfiler allows for the

identification of various molecules associated within a given disease state or gene of interest. In this present study both MAP and BioProfiler were used to illustrate connections and activity of secreted factors and the outlined disease states.

MTT Proliferation Assay

A MTT assay was used to measure cellular proliferation after treatment of sodium orthovanadate to determine if the compound is cytotoxic. Briefly RAW 264.7 cells were plated at a seeding density of 50,000 cells/ well. The cells were then treated with increase concentrations of sodium orthovanadate (10 µM, 100 µM, 1000 µM). The cells were allowed to incubate for a period of 24 hours. The MTT reagent was prepared to a final concentration of 5mg/mL, which was then added to each well and allowed to incubate at 37˚C for 2-4 hours. DMSO was then added to each well, the samples were then incubated at 37˚C for 2-4 hours. The absorbance of the samples was then detected utilizing a 96- well plate reader (spectraMAX M5, molecular devices, San Jose CA) at an absorbance of 590 nm.

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 ANOVA and t-test with posttest. Statistical comparisons and analysis were performed with Sigma Plot and statistical significance was defined as $p<0.05$.

Results

Network Analysis of Key Cytokines in Senescent Cells

A preliminary analysis of the senescence pathway revealed a link between, SASP, inflammation, and tumor progression. Furthermore, an additional link was noted from the SASP to cellular senescence via paracrine senescence. This analysis also illustrated that cellular senescence can be linked to oxidative stress which induces DNA damage (Figure 3). The data demonstrates that increased levels of TNF-α is correlated with decrease cell proliferation of tumor cell lines and will increase senescence of cells (Figure 4). Additionally, it was found that IL-10 inhibited TNF-α expression while increased levels of IL-10 are also associated with increased levels of tumor cell proliferation. IL-1β and IGF-1 both have connections to inflammation, increased tumor cell proliferation, and senescence of cells. IL-1α and IL-6 have connections inflammation, cell proliferation of cell lines, senescence of cells and the senescence pathway.

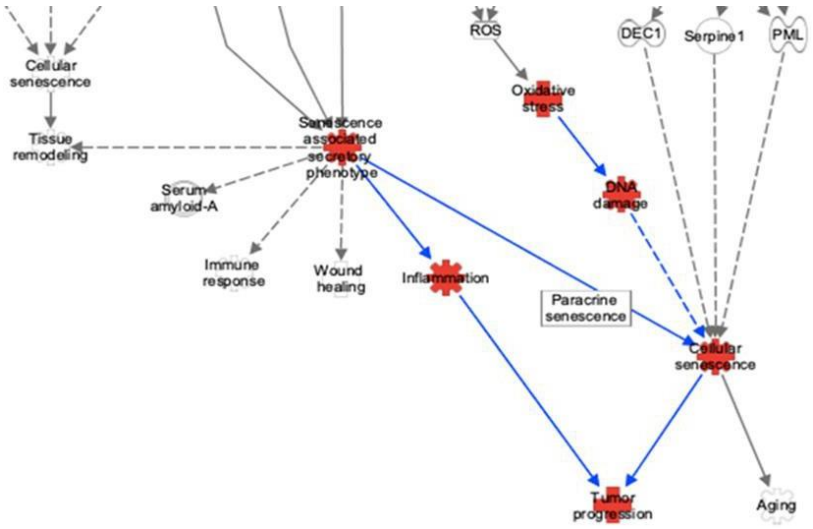

Figure 3: Ingenuity Pathway Analysis Pathway Tool Illustrating the Role of SASP and its Role in Paracrine Senescence. The above illustration was generated using IPA pathway tool. The red highlighted areas are important connections of the senescence associated secretory phenotype including inflammation and tumor progression. The various nodes of the pathway are represented by various symbols. The distinction of the symbols depends on their relationship to the pathway, for example the node tumor progression is denoted by a "t" symbol which represents a disease state, while inflammation is denoted by a "*" symbol which represents a cellular state. The lines within the pathway are also representative, a dashed line represents a possible connection, while a solid line represents a definitive connection.

The network were generated through the use of Ingenuity pathway analysis tool. [25]

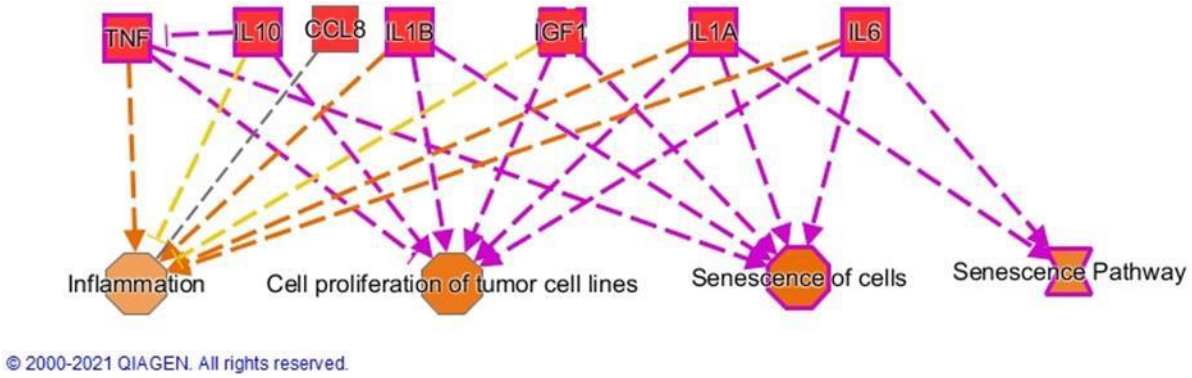

Figure 4: Ingenuity Path Analysis connect Feature Highlighting Roles of Cytokines involved in Senescence. The above figure illustrates the connection between the various cytokines and their connection to inflammation, cell proliferation of tumor cell lines, senescent cells, and the senescence pathway. The molecule activity predictor tool was utilized to see the effects of increasing concentrations of particular molecules. A red color indicates an increased level of the molecule while orange shows an increase effect.
The network were generated through the use of Ingenuity pathway analysis tool.[25]

Sustained LPS Treatment Induces Senescence in Raw 264.7 Cells.

Figure 5 and 6a illustrates that the prolonged exposure of LPS drive RAW 264.7 cells to a morphologically GMC distinct phenotype that includes enlarged flattened shape, along with distinct multinucleation. Our data is supported by Yangashita et al previously work on RAW 264.7 cells [22]. Figure 6a illustrates the difference in morphology and staining of a senescent GMC compared to a healthy normal cell. LPS treatment caused a 92% (P=<0.016) increase in the number of senescent cells overtime (Figure 6b).

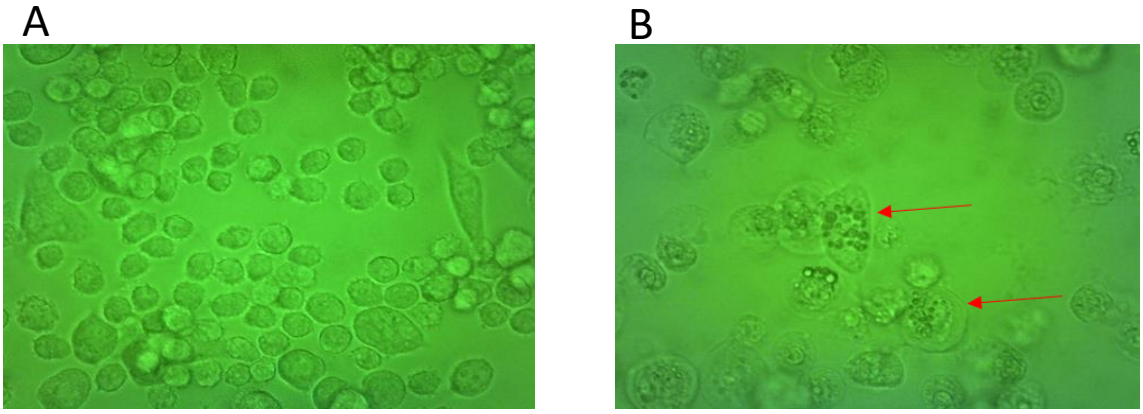

Figure 5: LPS-Treated RAW 264.7 cells 5 days post-Incubation. A) Control group, no morphologically distinct cells are observed, B) LPS treated cells cause GMC induction including flatten enlarged shape and multinucleated morphological changes (red arrows).

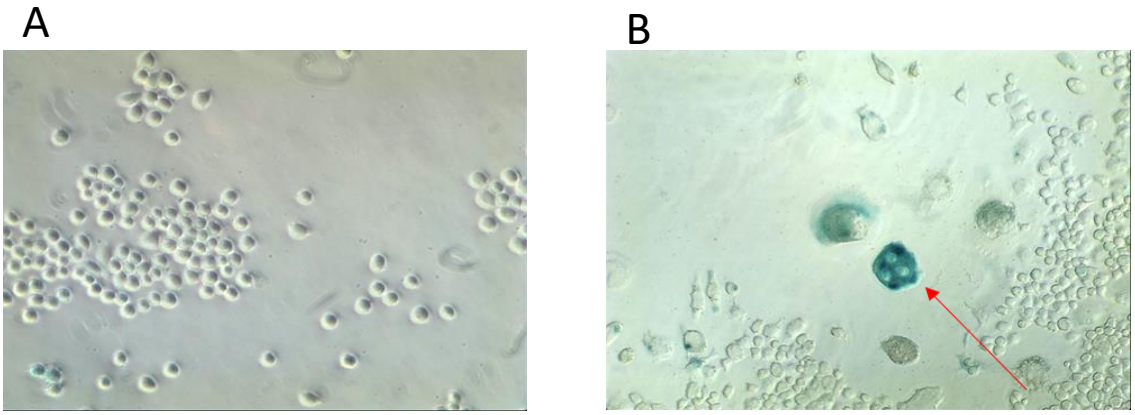

Figure 6A: LPS-Treated RAW 264.7 cells 5 days Post-Incubation Stain Positive for Senescence. A) Control group (-LPS), no morphologically distinct or positively stained cells are observed, B) +GMC cells (LPS treated), morphological changes and stain positive with β-galactosidase stain (blue, red arrows).

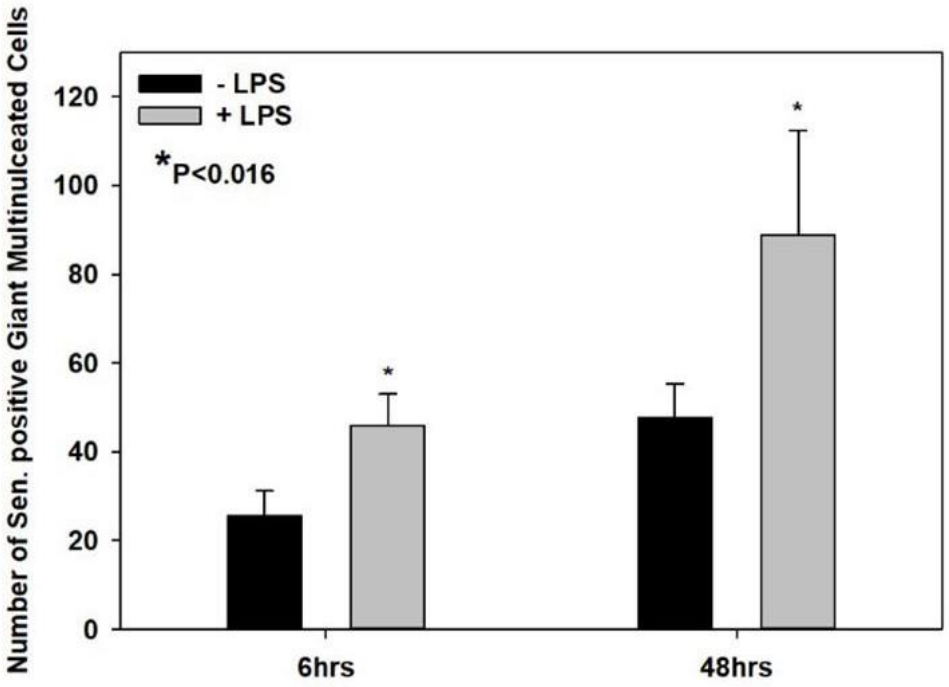

Figure 6B: Comparative Analysis of Senescent Positive GMCs after 48 hours of LPS Treatment. Cells were manually counted and confirmed to be senescent via β-galactosidase stain at the indicated time intervals

Sodium Orthovanadate Treatment Reduces Senescence

Figure 7 illustrates that non-senescent cells (-GMC) are unaffected by sodium orthovanadate treatment overtime (P= 0.350). Continual treatment of LPS appears important to maintaining senescence, as its absence leads to a significant reduction in senescent positive cells in +GMC cell group overtime (P<0.050) while senescence is maintained in the +GMC+LPS group. Further, senescent cells (+GMC) cell numbers are reduced at 4, 6, and 24 hrs by greater than 18% when treated with sodium orthovanadate treatment (+GMC+SV) P<0.050). The +GMC+LPS+SV group also shows that sodium orthovanadate significantly reduces senescence at 6 and 24hrs after treatment (35%, P<0.050)

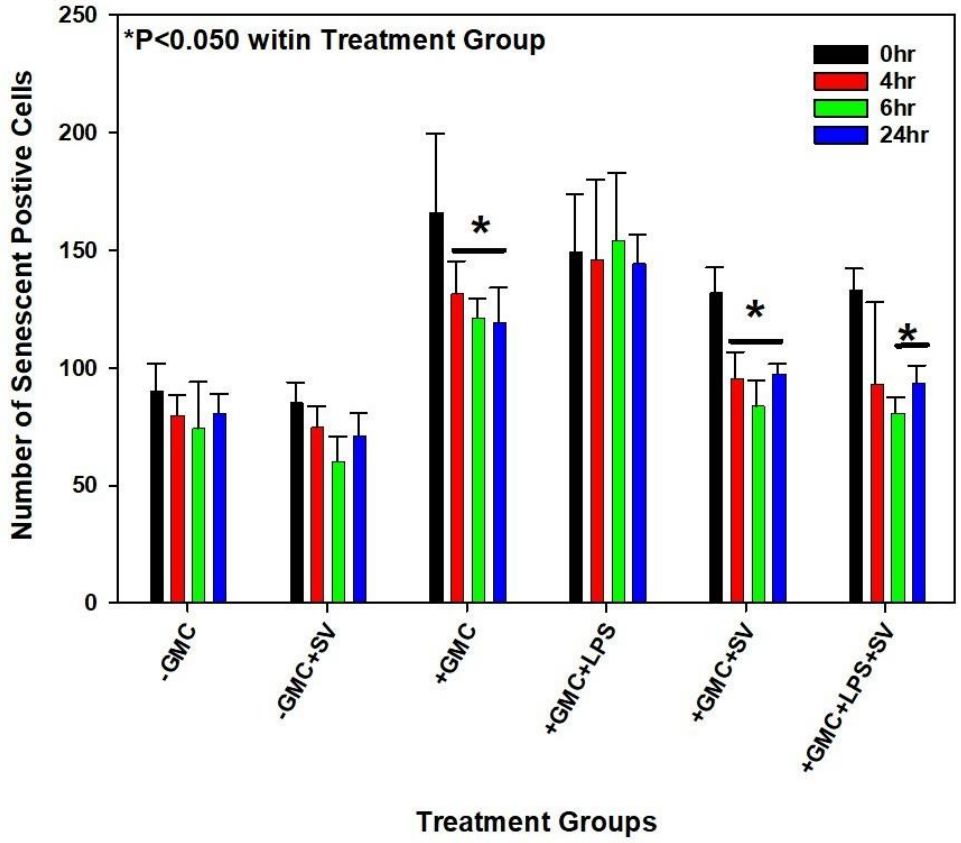

Figure 7: Comparative Analysis of Senescent Positive GMCs Post Sodium Orthovanadate Treatment. Cells were manually counted and confirmed to be senescent via β-galactosidase stain over time. *(P<0.050)

Inflammatory Cytokines Increase During Cellular Senescence

Figure 8 illustrates a significant increase in the expression of cytokines associated with the SASP. Most notably IL-10, TNF-α and IL-6 displayed a 256%, 503% and 136% statistically significant increase (P<0.001), respectively when comparing non-senescent cells to senescent cells. Likewise, IL-1α and IL-1 β also displayed a 32% and 11% statistically significant increase when comparing the two groups (P<0.001). Conversely, the level of IGF-1 expression showed a 53% significant decrease when comparing non-senescent cells to senescent cells (P<0.001)

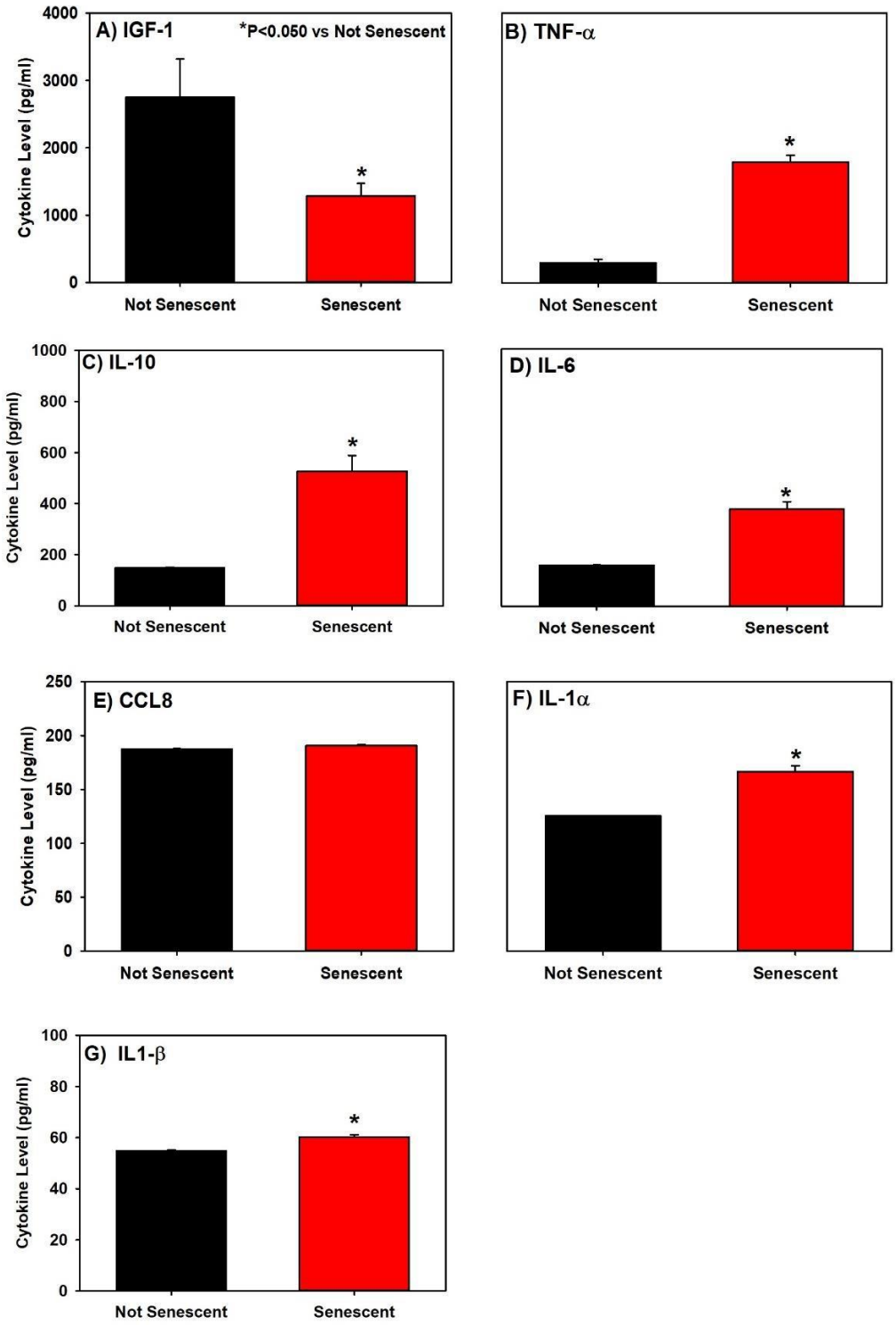

Figure 8: Comparative Cytokine Profile Analysis of Cell Populations. Not Senescent Cells- Raw 264.7 cells left untreated; Senescent: Raw 264.7 cells treated with LPS for 96 hours. Cell supernatants were collected and analyzed via Luminex for cytokines, n=3 (A) IGF-1, (B) TNF-α, (C)IL-10, (D) Il-6, (E) CCL8, (F) IL-1α, (G) Il-1β, *= (P= <0.001) vs Non-Senescent Cells

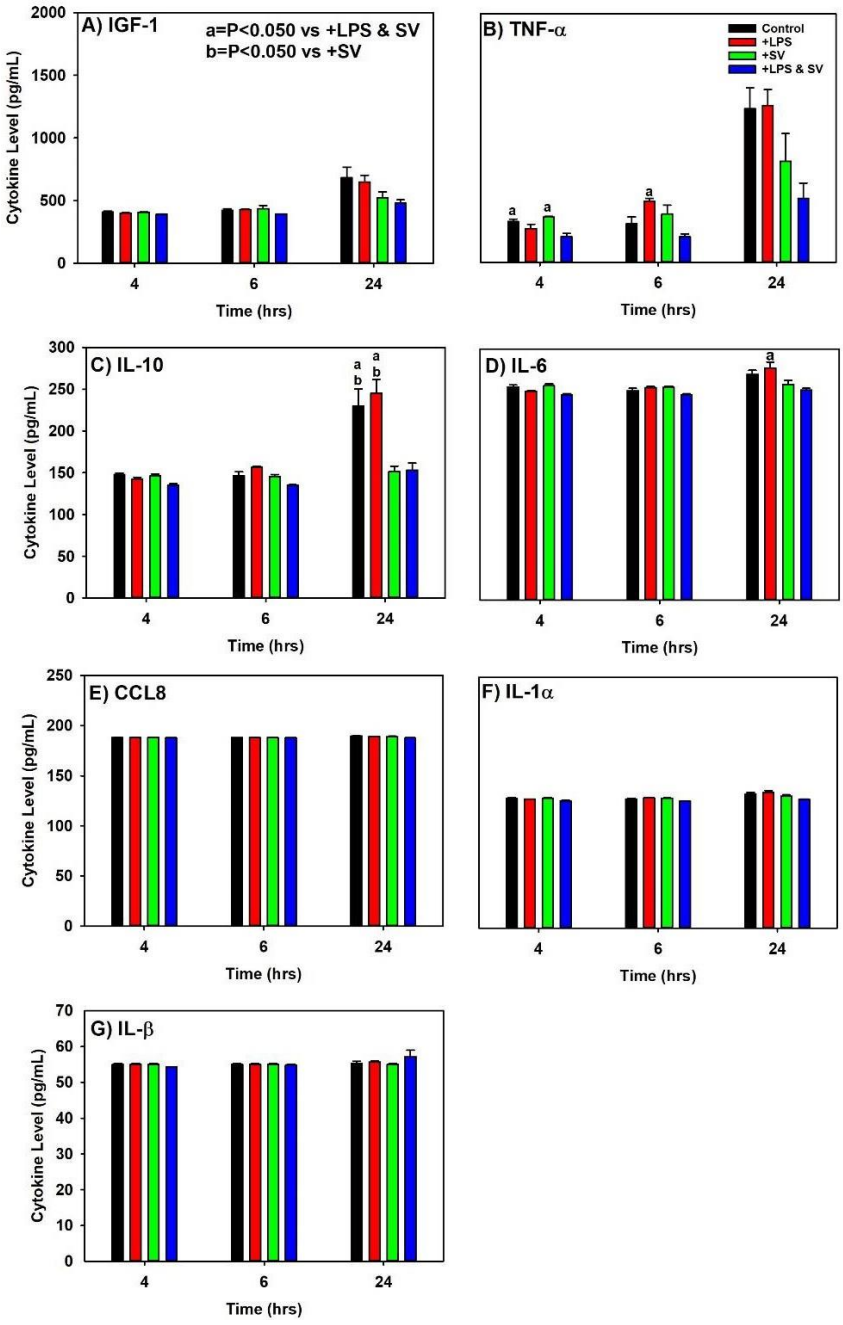

Figure 9: Comparative Cytokine Profile Analysis of Senescent Cells Post Sodium Orthovanadate Treatment. n= 3. Cell supernatants were collected and analyzed via Luminex for cytokines. (A) IGF-1, (B) TNF-α (P<0.050), (C) IL-10 (P<0.050), (D) Il-6, (E) CCL8, (F) IL-1α, (G) IL-1β, compared via a One-Way ANOVA of the treatment groups at each timepoint. Control: untreated; +LPS treatment with liposaccharide; +SV: sodium orthovanadate treatment; LPS&SV: Liposaccharide & Sodium orthovanadate combined.

Sodium Orthovanadate Treatment Showed Reduced Expression of TNF-α and IL-10 within 24 hours.

Figures 9 illustrate CCL8, IL-1α, and IL-1β where unaffected by sodium orthovanadate treatment within the first 24 hours. The release of TNF-α and IL-10 was found to increase by 71% ($P<0.050$), and 73% ($P<0.050$), when restimulation of senescent cells (SEN + LPS) occurred. When treated with sodium orthovanadate (SENS+LPS+SV) it was seen that at 24 hours post treatment IL-10 levels decreased by 51% ($P<0.050$), TNF-α showed a 1.4 % decrease in expression, however, began to increase after 24 hours, when compared to the treatment group (SEN +LPS). Sodium orthovanadate (SENS+LPS+SV) showed to attenuate the release of TNF-α, when comparing the two groups it showed to have an overall 60% ($P<0.050$), decrease in TNF-α release. Likewise, IL-6 was found to decrease in the sodium orthovanadate treatment groups at 24 hours, but the difference was not found to be significant.

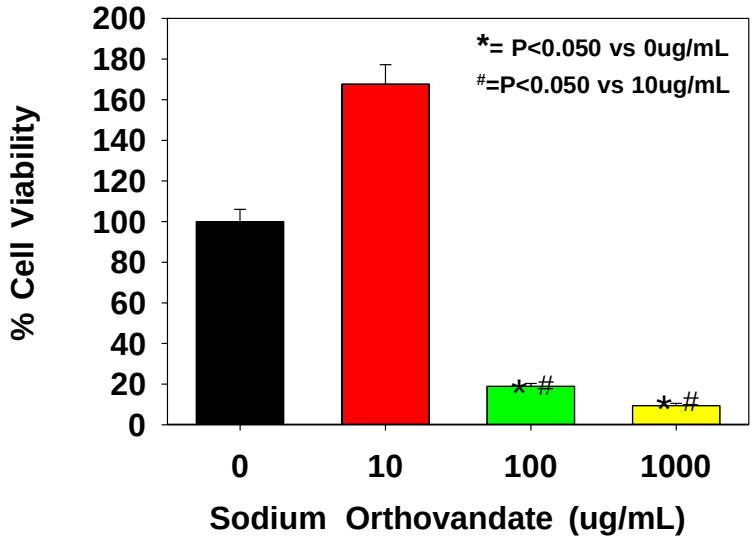

Figure 10: Comparative Analysis of Percent Cell Proliferation Post Sodium Orthovanadate treatment at 24hrs post-treatment. RAW264.7 cells were treated with increasing concentrations of sodium orthovanadate. A MTT proliferation assay was conducted, and percent absorbance was obtained using automated plate reader (spectraMAX M5, molecular devices, San Jose CA) at an absorbance of 590 nm. Percentage greater than control indicates cell proliferation, percentage less than control indicates inhibition of cell proliferation.

Sodium Orthovanadate Treatment Showed a Decrease in Cellular Proliferation at High Concentrations.

Figure 10 shows the effect of sodium orthovanadate on the proliferation of RAW264.7 cells. It can be seen that when treated with 100 µM and 1000 µM there is a reduction in cellular proliferation. Respectively there was an 82 % reduction when treated with 100 µM ($P<0.050$) and 91% reduction when treated with 100 µM ($P<0.050$). When treated with 10 µM there appears to be an increase in cellular proliferation, but this was not found to be statistically significant.

Discussion

Cellular senescence occurs when stressed cells enter an irreversible proliferation arrest. In this state, senescent cells develop the SAS phenotype, become resistant to cell death, and are often associated with disease progression [2, 5, 18, 26]. As part of the SAS phenotype, cells secrete signaling mediators like TNF-α, IL-1α, IL-1β, IL-6, IL-10, CCL8, and IGF-1. Through paracrine signaling these mediators are thought to drive surrounding healthy cells to senescence, perpetuate local inflammation, and lead to tissue damage and/or disease progression [5]. In many cases, the ability to eliminate senescent cells or attenuate their release of proinflammatory cytokines are advantageous to reduce disease progression [27]. Substances that can attenuate the secretory phenotype by selectively inducing apoptosis of senescent cells are termed senolytics. The compound, sodium orthovanadate, has been shown to decrease TNF-α in the RAW 264.7 macrophage cell line [19] [24]. Here, we investigate sodium orthovandate as a possible senolytic in LPS induced senescent cells.

Preliminary analysis illustrated that LPS stimulated RAW 264.7 cells have the ability to fuse and develop into giant multinucleated cells[19]. Our studies found LPS-treatment caused a 30% increase in GMC formation of which 100% were β-galactosidase senescent positive cells. These results support the hypothesis that LPS exposure promotes the generation of GMCs and cellular senescence. Interestingly, Bartee et al. illustrated that proinflammatory cytokines, most notably TNF-α contributed to the formation of these cells [19]. Weiler et. al had also previously shown that the proinflammatory cytokine TNF-α is a strong inducer of cell fusion in human M13SV1-Cre breast epithelial cells and human MDA-MB-435-pFDR1 cancer cells. In follow up studies, they noted these fused cells contributes to malignancy in perpetuate a hostile tumor

microenvironment [28]. Similarly, our data showed elevated TNF-α levels when senescent-GMC cells were compared to non-senescent cells.

To characterize the cytokine profile that were being expressed by these senescent cells, a Luminex multiplex assay was performed targeting the seven most common cytokines associated with the SAS phenotype identified through IPA analysis (Figures 3&4). Of the inflammatory cytokines analyzed TNF-α, IL-10, IL-6, IL-1α, and IL-1β were all found to be significantly increased in senescent cells (Figure 9) [8, 10, 29]. Data from Zhang et al supports our findings, by demonstrating that the presence of proinflammatory cytokines in conjunction with a positive β-galactosidase staining is indicative of cellular senescence [29]. For example, Su et al. illustrated that ionizing radiation led to an increase in senescent macrophages, which coincided with increased levels of cytokines IL-1α, IL-6, and TNF-α. These scientists hypothesized that the SAS phenotype dysregulated the cytokine profile expression of macrophages and let negative effects in radiation-induced pulmonary fibrosis [30]. Other data completed by Coppé et al. compared cytokine profiles of senescent and pre-senescent cells in fibroblast and epithelial cell lines. Their data found that the repertoire of senescence specific cytokines released under stress is cell type dependent [31]. Data from Demaria et al. effectively showed that treatment induced senescent (TIS) cells releasing cytokines associated with the SASP led to cancer relapse. Their data also found that removal of senescent cells improve cancer relapse outcomes[32]. As such it is well documented that the release of such cytokines can drive an increase in inflammation within the tumor microenvironment and drive tumor progression [32].

Next, we studied the effect of sodium orthovanadate on nonsenescent and senescent RAW 264.7 cells. Cell viability studies found that sodium orthovanadate decreased metabolic activity at >100uM in nonsenescent RAW 264.7 cells. Therefore, we hypothesized that

treatment with 100uM sodium orthovanadate would be sufficient to reduce the SAS phenotype in senescent RAW 264.7 cells. Our data found that nonsenescent cells treated with sodium orthovanadate led to an increase in cytokine expression (FigX). These results in conjugation with the reduced cell proliferation suggest that at this concentration sodium orthovandate induces inflammation in nonsenescent cells, which could lead to a reduction in cellular metabolism, and/or viability. When senescent cells treated with sodium orthovandate were compared to untreated cells, reductions of TNF-α and IL-10 levels were found (Fig XX). Since cell proliferation/viability was not studied on the senescent cell population, we do not know if the reduction in this cytokine expression is a result of reduced cell viability.

But previous research has speculated on the mechanism by which sodium orthovanadate exerts its effect on cell including post-translational modification and interference with cell toxicity. Whadat et al. suggested that reduced TNF-α expression was likely acting at the post transcriptional level rather than inhibiting a distinct pathway[24]. At the post-transcriptional level, sodium orthovanadate could be interacting with soluble N-ethylmaleimide- sensitive factor attachment protein receptors (SNARE) proteins to release cytokines. SNARE proteins are a family of membrane fusion proteins and have a direct effect on cytokine release [33]. Previous studies have illustrated that vanadium compounds such as sodium orthovanadate have negative effects on exocytosis and vesicular transport via interaction through SNARE proteins [34] which may contribute to the reduction in cytokine expression. Delwar et al. illustrated that sodium orthovanadate could be cytotoxic and a result would be a useful therapeutic for cancer [35]. This research focused on the treatment of gliomas with sodium orthovanadate and found it had antineoplastic effects on cancer cells. Other research found that sodium orthovanadate treatment also negatively impacted the growth of particular epithelial cancers such as lung, kidney, and

prostate [36]. Further research using flow cytometry or other methods would be useful to determine if the reduction in cytokine activity is a result of senescent cell death, downregulation of cytokine expression, or other factors[27].

Connections between sodium orthovanadate, senescence, and the SASP can be found in the literature (Figure 3). Paracrine senescence is reinforced by the SASP and ensures senescence growth arrest [7]. Our data found increased expression of TNF-α, IL-10, IL-6, IL-1α and IL-1β was found in our senescence cells and may play a role in paracrine senescence and disease progression. Huang et al's work on hepatic stellate cell senescence supports this idea. Their data showed IL-10 upregulated the expression levels of p53 and p21, genes commonly associated with disease progression [37]. In addition, their data also illustrated that IL-10 plays a regulatory role in increasing levels of TNF-α. Further, additional data has shown that attenuation of TNF-α effects the SASP and decreases disease progression [38]. In this study, MCF-7 breast cancer cells treated with adalimumab, a monoclonal antibody directed against tumor necrosis factor-α, reductions in migration rate and attenuation of senescent cytokine secretome were found. These changes were concomitant with decreases in pro-tumorigenic and pro-metastatic behavior.

Regardless of the mechanism of action that sodium orthovanadate possesses in regard to cytokine release, it was shown that it has the capabilities to attenuate the release of cytokines associated with the SAS phenotype. A molecule with such capabilities would be beneficial for further investigation into its effects on senescent cells given the relationship that exist with inflammation, paracrine senescence, and progression of disease states such as tumor progression. As a result, this molecule could aid in and serve as a potential synergistic treatment to prevent tumor progression and recurrence.

Conclusion

In this study we illustrated the ability to induce formation of giant multinucleated cells via prolonged exposure to LPS. It was illustrated that these GMC are senescent and have increased levels of pro-inflammatory cytokines associated with the SASP. All cytokines tested except for 1 (IGF-1 and CCL8) showed a statistically significant increase, when comparing non-senescent cells to their senescent counterparts. Furthermore, we illustrated that the small molecule sodium orthovanadate could possess the capabilities of attenuating the release of TNFα and IL-10. This attenuation could be cause by different mechanisms, which should be further researched. The capabilities of sodium orthovanadate to attenuate proteins from the SASP could provide a beneficial treatment for chronic inflammation and other senescence associated diseases such as cancer recurrence.

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