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Inflammatory Kinetics and Efficacy of Anti-inflammatory Treatments on Human Nucleus Pulposus Cells

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Abstract

Study Design—Human nucleus pulposus (NP) cell culture study investigating response to tumor necrosis factor- α (TNF α), effectiveness of clinically available anti-inflammatory drugs, and interactions between pro-inflammatory cytokines.

Objective—To characterize the kinetic response of pro-inflammatory cytokines released by human NP cells to TNF α stimulation and the effectiveness of multiple anti-inflammatories with 3 sub-studies: Timecourse, Same-time blocking, Delayed blocking.

Summary of Background Data—Chronic inflammation is a key component of painful intervertebral disc (IVD) degeneration. Improved efficacy of anti-inflammatories requires better understanding of how quickly NP cells produce pro-inflammatory cytokines and which pro-inflammatory mediators are most therapeutically advantageous to target.

Methods—Degenerated human NP cells (n=10) were cultured in alginate with or without TNF α (10ng/mL). Cells were incubated with one of four anti-inflammatories (anti-IL-6 receptor/atlizumab, IL-1 receptor antagonist, anti-TNF α /infliximab and sodium pentosan polysulfate/PPS) in two blocking-studies designed to determine how intervention timing influences drug efficacy. Cell viability, protein and gene expression for IL-1 β , IL-6 & IL-8 were assessed.

Results—*Timecourse*: TNF α substantially increased the amount of IL-6, IL-8 & IL-1 β , with IL-1 β and IL-8 reaching equilibrium within ~72 hours (IL-1 β : 111 $\pm$ 40pg/mL, IL-8: 8478 $\pm$ 957pg/mL), and IL-6 not reaching steady state after 144 hours (1570 $\pm$ 435 pg/mL). Anti-TNF α treatment was most effective at reducing the expression of all cytokines measured when added at the same time as TNF α stimulation. Similar trends were observed when drugs were added 72 hours after TNF α stimulation, however, no anti-inflammatories significantly reduced cytokine levels compared to TNF control.

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Conclusion—IL-1 β , IL-6 and IL-8 were expressed at different rates and magnitudes suggesting different roles for these cytokines in disease. Autocrine signaling of IL-6 or IL-1 β did not contribute to the expression of any pro-inflammatory cytokines measured in this study. Anti-inflammatory treatments were most effective when applied early in the inflammatory process, when targeting the source of the inflammation.

Keywords

TNF α ; intervertebral disc degeneration; nucleus pulposus cells; alginate beads; infliximab; atilizumab; IL-1Ra; pentosan polysulfate

Introduction

Chronic inflammation is a key element of degenerative disc disease and failed clinical trials using anti-inflammatories for back pain^{1,2} are at least partly due to limited understanding of inflammatory kinetics and interactions of pro-inflammatory cytokines within the intervertebral disc (IVD). Back pain is the leading cause of global disability³ and is commonly associated with IVD degeneration and has few effective minimally invasive treatments. Pro-inflammatory mediators are correlated with degeneration and aging processes⁴⁻⁷ and strongly associated with the matrix breakdown and pain⁸⁻¹⁵ observed in disease. Pro-inflammatory mediators are an important clinical target for IVD degeneration with the potential to slow disease progression and alleviate pain^{8,16,17}. While the catabolic effects of pro-inflammatory mediators on IVD cell metabolism are well-characterized, many open questions remain regarding if primary IVD cells can meaningfully contribute to the development of the pro-inflammatory environment, how that pro-inflammatory environment develops over time, and whether targeted anti-inflammatory therapies can slow the inflammatory cascade.

Determining which inflammatory targets are most therapeutically advantageous to target in the IVD requires an improved understanding of how quickly the pro-inflammatory environment develops within the IVD and how inter-cytokine interactions affect cytokine expression. The lack of such knowledge helps explain why the clinical use of anti-TNF α therapies have had limited success at addressing radicular pain^{1,2,18,19}. Multiple studies demonstrated that pro-inflammatory cytokines can induce a positive feedback loop, where the expression of one cytokine drives more expression of that cytokine and others, perpetuating the inflammatory environment^{20,21}. For example, the treatment of nucleus pulposus (NP) cells with both TNF α and IL-6 amplifies the gene expression of IL-6²² suggesting that additional IL-6 expression may have promoted a feedback loop driving further expression. This self-perpetuating inflammatory response is particularly important in the IVD where slow transport kinetics and clearance rates increases the likelihood that multiple pro-inflammatory cytokines have extended residence times to act on IVD cells.

The timing of interventions significantly influences the success of the treatment in many disease processes. In IVD diseases, the use of anti-inflammatory treatments has been proposed immediately following surgery or via an intradiscal injection to ease pain. Multiple anti-inflammatory agents can reduce or prevent the amount of inflammatory mediators

released by IVD cells when added at or close to the time of stimulation (within 2 hours) by IL-1 β or TNF α ²³⁻²⁶. However, in the clinical situation, anti-inflammatory therapy would likely be introduced into a previously inflamed environment, given the strongly inflammatory component to IVD disease^{8,10,27}. It is therefore a priority to investigate how the efficacy of anti-inflammatory therapies is influenced by the existing inflammatory state as would be expected during disease progression.

The study aimed to (1) characterize the kinetic response of degenerated human NP cells following TNF α exposure, and (2) determine the efficacy of anti-inflammatory therapies and inter-cytokine interactions through the addition of multiple clinically available anti-inflammatory therapies in two blocking studies (Same-time blocking vs Delayed blocking). Human NP cells in 3D cell cultures were treated with TNF α and multiple anti-inflammatory drugs to assess the time-dependent changes in multiple pro-inflammatory cytokines on both gene and protein levels. TNF α treatment was used because is thought to be involved in the initiation of inflammatory responses within the IVD²⁰ and anti-inflammatories that inhibit the cytokine receptors for IL-1 β and IL-6 were used to probe the inter-cytokine interactions and their autocrine effects (where an expressed cytokine binds to the same cell and promotes further expression). A cell culture screening strategy was chosen since it involved a controlled micro-environment where drug transport was assured.

Materials and Methods

Nucleus Pulposus Cell Isolation

Nucleus Pulposus (NP) cells were obtained from ten surgery or autopsy samples (Table 1) with mean $\pm$ SD age (54 $\pm$ 21 years). IVD tissues were obtained from patients undergoing surgery for degenerative conditions of the spine following a protocol approved by the Institutional Review Board at the Icahn School of Medicine at Mount Sinai or from autopsy services with permission. IVDs were classified as Thompson grades 2-5²⁸. Cells were isolated from NP tissue that was clearly distinguished from the annulus and contained no herniation or other unidentifiable tissue as previously described²⁹. Briefly, tissue was diced, rinsed in phosphate buffered saline, and cells were enzymatically released using 0.2% protease for 1 hour followed by 0.2% collagenase for 4 hours.

Culture Conditions

Isolated cells were expanded in monolayer (5% CO₂, 37°C) using high glucose DMEM containing 10% fetal bovine serum (FBS), 50ug/mL ascorbic acid, and 1% penicillin/streptomycin. NP cells (passage 4) were suspended in alginate beads (2 $\times$ 10⁶ cells/mL, 1.2% low viscosity alginate) and allowed to re-differentiate for at least 2 weeks prior to the start of the experiment in hypoxia (5% O₂, 5% CO₂, 37°C). Samples were cultured in 12-well plates (11 beads/well) with 2mL media per well. Cells were cultured in serum free Basal culture medium (low glucose DMEM, 1% insulin-transferrin-selenium, 50ug/mL ascorbic acid, 1% penicillin/streptomycin) for at least four days prior to the start of the experiment. The experiments were organized in a repeated measures design with the cells of each patient separated into all of the groups. For all experiments, TNF α groups were cultured in Basal medium supplemented with human recombinant TNF α (Invitrogen, PHC3016, 10ng/mL).

TNF α was chosen because of its association with painful IVD degeneration and radiculopathy and is considered an initiator or a larger pro-inflammatory and catabolic cascade in the IVD²⁰.

Timecourse Study

To assess how NP cells released pro-inflammatory cytokines over time, NP cells (n=6/group) were placed into either a Basal Control or TNF α group and cultured for 6, 12, 24, 48, 72 or 144 hours (Figure 1A). At 72 hours, half the culture medium was replenished using Basal media without TNF α . Gene expression, cell viability, and enzyme linked immunosorbent assay (ELISA) for pro-inflammatory cytokines in the medium was measured at each timepoint.

Blocking Studies

NP cells were separated into 6 groups (Figure 1D); Basal Control, TNF α Control, and TNF α plus one of four clinically available anti-inflammatory drugs: anti-TNF α /Infliximab (100ng/mL, Janssen), anti-interleukin-6 receptor/Atlizumab (Anti-IL-6R, 100ng/mL, Roche), Interleukin-1 receptor antagonist (IL-1Ra, 100ng/mL, R&D Systems), and sodium pentosan polysulfate (PPS, 100 μ g/mL). The anti-inflammatories used in study are not labeled for the described use. The most prevalent anti-inflammatory in the IVD literature is IL-1Ra at a dose of 100ng/mL^{13,30-32}. The additional anti-inflammatories (Anti-IL-6R and Anti-TNF α) were chosen at the same dose in order to inform the relative efficacy. The PPS dose was chosen based on the literature³³ and on a pilot dose study where the lowest dose that was effective at reducing pro-inflammatory cytokine gene expression was used.

Drugs were added at two different times. The 'Same-time Blocking' study (Figure 1B) investigated inter-cytokine interactions and evaluated efficacy of anti-inflammatories in a best case scenario that might prevent an inflammatory cascade. The 'Delayed Blocking' study (Figure 1C) investigated if anti-inflammatories could inhibit a pro-inflammatory response following TNF α exposure (i.e., simulating a previously inflamed environment). For the 'Delayed Blocking' study, all treatment groups were pre-treated with TNF α for 72 hours. At 72 hours half of the culture medium (1mL) was removed and 1mL of Basal medium containing anti-inflammatories was added. Only half the medium was changed so that some pro-inflammatory cytokines produced in the first 72 hours would remain in the media to simulate pre-existing inflammation. For TNF α Control and Basal Control groups, half the medium (1mL) was replaced with Basal medium. Cell viability and ELISA on the media was assessed at each timepoint.

Gene Expression

RNA was isolated from cells as described²⁹. Briefly, cells were released from alginate using dissolving buffer and pelleted. RNA was extracted using the RNeasy Kit (Qiagen, CA) following manufacturer's instructions. cDNA was synthesized using the SuperScript[®] VILO kit (Invitrogen, Carlsbad, CA). Gene expression was quantified using human specific TAQMAN (Life Technologies, Grand Island, NY) primers for IL-1 β (Catalog #: bt03212741_m1), IL-8 (Catalog #: bt03211908_m1) and IL-6 (Catalog #: bt03211904_m1)

and housekeeping gene r18S (Catalog #: hs99999901_s1), and analyzed using the C_T method³⁴.

ELISA for Pro-Inflammatory Cytokines

An ELISA specific for human TNF α , IL-1 β , IL-6, IL-8 (MSD N45025B-1) was performed on media collected from all timepoints following manufacturer's instructions. These pro-inflammatory cytokines are associated with catabolism (TNF α , IL-6 & IL-1 β)^{13,14,22}, pain (IL-6)^{35,36} and macrophage recruitment (IL-8)^{37,38}. The amounts of pro-inflammatory cytokines released were compared to the known median effective dose (ED₅₀) of the same recombinant proteins commercially available from R&D Systems. The ED₅₀ is the protein concentration known to induce a desired effect in 50% of the exposed population and is commonly used in pharmacology to put a concentration into a biologic context.

Cell Viability

Cell viability was assessed via the LIVE/DEAD[®] Viability Kit (Life Technologies, NY, USA) using two beads from each group. Ten z-stack images (10 $\times$) were captured from each bead penetrating ~500-800 μ m using an inverted microscope (Axiovert 200M, Zeiss, Germany). The average percentage viability for each z-stack was assessed using Axiovision software to quantify calcein-AM (green, viable) and ethidium homodimer-1 (red, dead) cells at each timepoint.

Statistical Methods

For all protein measurements, the concentrations were transformed via a natural logarithm in order to stabilize the variability and parametric analyses were conducted on the transformed values. Continuous measurements such as protein and gene expression were modeled via the PROC MIXED procedure in SAS (SAS Institute Inc., Cary, NC). This is similar to an Analysis of Variance procedure for repeated measurements. Due to the fact that repeated measurements within patients may be correlated, this procedure allows one to model this "correlation structure", commonly referred to as a **covariance pattern**. This accurate estimate allows for improved estimates of the standard errors of measurement, and therefore more powerful tests. There are a number of various covariance structures to choose from. Three of the more common covariance structures include "compound symmetry" (**cs**), for correlations that are constant for any two points in time, "auto-regressive order one" (**ar1**), for correlations that are smaller for time points further apart, and "unstructured" (**un**), which has no mathematical pattern within the covariance matrix.

The procedure known as Akaike's information criterion (AIC)³⁹ was used to discern which covariance pattern allowed for the best fit. For the time course analysis, a p value < 0.05 for the group by time interaction term indicated a significant difference between the two treatment means for the particular measure over time. We also used PROC MIXED to discern differences among the six (6) treatment arms in the Same-time and Delay studies, since the same patient was tested for each of these treatments. The PROC MIXED procedure also allowed for the adjustment of other covariates such as age, gender, passage, source, region and Degenerative Grade for both type of analyses. All values are reported as mean $\pm$

SEM with $p < 0.05$ (*) significant and analysis was conducted using SAS system software, Version 9.3.

Results

Cell Viability

All samples retained high viability at all timepoints ($>89.7\%$) with no significant differences between groups demonstrating that doses of TNF α and anti-inflammatories were not cytotoxic (Table 2).

Timecourse Study

TNF α induced a significant up-regulation of the protein and mRNA of all measured pro-inflammatory cytokines (Figure 2). Protein levels reached equilibrium for IL-1 β and IL-8 within 72 hours however IL-6 protein continuously increased beyond 72 hours (Figure 2A,C,E). There were large differences in the amounts of protein released between the different pro-inflammatory cytokines at 72 hours (IL-1 β : 111 ± 40 pg/mL, IL-6: 1200 ± 409 pg/mL, IL-8: 8478 ± 957 pg/mL). Interestingly, there was no lag between increased mRNA expression and protein production (Figure 2B,D,F). After 144hrs of TNF α exposure all measured cytokines were elevated above previously published ED₅₀ values (Table 3).

Blocking Studies

Negligible amounts of pro-inflammatory cytokines were detected in Basal samples in both blocking studies (72hrs – IL-1 β : 2 ± 0.3 pg/mL, IL-6: 13 ± 4 pg/mL, IL-8: 482 ± 366 pg/mL).

Same-time Blocking—Anti-TNF α significantly inhibited protein expression of IL-1 β and IL-6 compared to the TNF α control (Figure 3A-D). Anti-IL-6R and IL-1Ra treatments did not influence the expression of any pro-inflammatory cytokine measured following TNF α exposure.

Delayed Blocking—All anti-inflammatories were less effective at reducing the amount of pro-inflammatory cytokines released when they were introduced to cells that were previously stimulated with TNF α (Figure 4A-D). Anti-TNF α and PPS had the largest reductions in pro-inflammatory cytokine levels, although no drug significantly changed the amount of any cytokine relative to the TNF control.

Discussion

Painful spine conditions are associated with increased pro-inflammatory cytokine levels and mixed outcomes of anti-inflammatories in back pain clinical trials motivate additional fundamental investigations. The first aim of this study characterized the kinetic gene and protein response of IL-1 β , IL-6, and IL-8 by degenerated human NP cells because of their known roles in catabolism (TNF α & IL-1 β), pain (IL-6 & IL-8) and macrophage recruitment (IL-8). The second aim of this study investigated the efficacy of anti-inflammatory therapies and inter-cytokine kinetics through two-blocking studies. NP cells produced IL-1 β , IL-6, and IL-8 in response to TNF α at dramatically different rates and magnitudes, suggesting

different roles for these cytokines in disease pathology. The blocking studies suggested that autocrine signaling of IL-6 and IL-1 β does not contribute to the expression of pro-inflammatory cytokines following TNF α exposure. Inhibition of TNF α was most effective at reducing pro-inflammatory mediator expression and results suggested that the broad based anti-inflammatory (PPS) may also be effective. Results also suggested that the drug target and timing of the intervention are crucial in the overall success of the therapy as all anti-inflammatories were less effective when introduced in an environment primed with pro-inflammatory mediators with non-significant reductions in pro-inflammatory cytokine levels observed.

Multiple pro-inflammatory mediators were released in response to TNF α , yet at different rates and amounts. IL-8 elicited the largest and quickest response to TNF α stimulation reaching its 'equilibrium' value of 8.5-9ng/mL within 48 hours of stimulation. The baseline levels of IL-8 expression observed in the Basal group (482 $\pm$ 366pg/mL) were similarly low in a prior study⁴⁰. While there is a limited understanding of the role that IL-8 plays in IVD pathology, recent studies demonstrated that AF cells from degenerated IVDs spontaneously expressed "considerable" amounts of IL-8⁴¹ and IL-8 expression is significantly increased when IVD cells are co-cultured with macrophages⁴⁰. Additionally, the presence of IL-8 (which is also known as chemokine (C-X-C motif) ligand 8, CXCL8) is up-regulated in degenerated IVD tissue compared to non-degenerate controls and positively correlates with the degree of degeneration⁴². IL-8 is a potent chemo-attractant of immune cells^{37,38} and the large and rapid increase in IL-8 in response to TNF α suggests that the native IVD cells may attract immune cells into the IVD which have been shown to be elevated in painful degenerated IVDs^{27,43,44}.

IL-1 β induces matrix catabolism through the stimulation of enzymatic degradation in IVD degeneration¹³. In this study, IL-1 β protein rapidly increased and reached steady state within 72 hours. While the IL-1 β equilibrium value was much lower than that of the other pro-inflammatory cytokines it was ~8.5 times greater than the ED₅₀ dose (<12pg/mL) known to induce proliferation in 50% of exposed mouse helper T-cells (Table 3). While the ED₅₀ dose varies based on the observed effect and the cell type, it serves as a benchmark to place a cytokine concentration into a biologic context. The relatively high amount of IL- β compared to its ED₅₀ together with the well-known catabolic effects of IL-1 β ^{13,45} suggests that the native IVD cells can produce biologically significant amounts of IL-1 β and are an important source of pro-inflammatory cytokines. A recent study demonstrated that TNF α can penetrate an intact IVD under dynamic loading and induced substantial matrix breakdown leading to alterations in the biomechanical behavior of the IVD⁴⁶. This finding together with the known catabolic effects of IL-1 β ¹³, and the rapid release of IL-1 β following a short exposure to TNF α (72 hours) observed in this study, suggests that IL-1 β may contribute to the matrix breakdown which can rapidly impact the mechanical behavior of the IVD.

IL-6 can function as a pro-inflammatory or anti-inflammatory cytokine, however, persistent IL-6 production plays an important role in multiple chronic inflammatory diseases^{47,48}. In this study, large amounts of IL-6 were released following TNF α exposure and never reached an equilibrium point. This large and continuous release of IL-6 together with IL-6's association with painful IVD conditions^{35,36} and its ability to enhance matrix degradation

induced by TNF α and IL-1 β ^{22,49} suggests that IL-6 functions in pro-inflammatory role in IVD degeneration. The pro-inflammatory role of IL-6 is also suggested as IL-6 is up-regulated in multiple injury models^{50,51}, and is more strongly expressed by degenerated IVD cells than non-degenerated cells in response to lipopolysaccharide⁵². Additional characterization of the IL-6 kinetics, in particular whether it reaches equilibrium or if it continues to increase while TNF α is present may further help inform the role that IL-6 plays in IVD pathology.

The anti-TNF α therapy was the most effective anti-inflammatory in this study, however, the timing of the intervention did influence drug effectiveness. In the Same-time blocking study, anti-TNF α significantly decreased the expression of IL-1 β and IL-6, while in the Delayed blocking study no treatment, including anti-TNF α , significantly reduced cytokine levels compared to TNF control. This suggests that the timing of the intervention, and whether a previously inflamed environment is present, may be an important factor in the therapeutic success. The finding that anti-TNF α treatment did not significantly decrease IL-8 expression is consistent with a prior study that similarly observed that an anti-TNF α treatment did not decrease the expression of IL-8 in a macrophage / IVD cell co-culture model⁴⁰. The inability to slow the inflammatory cascade is also consistent with multiple studies which found that a transient exposure to TNF α had lasting effects of IVD cell metabolism and mechanical sensitivity^{9,53} and suggests more active interventions that promote anabolism are required once an inflammatory environment develops. Therefore, treating IVD's with pre-existing inflammation may have limited efficacy, or may require longer durations or greater doses of treatment.

This study provided little evidence that autocrine signaling leads to additional expression of other pro-inflammatory cytokines as there was no effect of anti-IL-6R or IL-1Ra on the expression of any cytokine measured in either blocking study. Both of these drugs target the receptors (Figure 5), suggesting there was minimal autocrine signaling and that neither IL-6 nor IL-1 β contributed to the expression of other pro-inflammatory cytokines. While these results demonstrated that inhibition of the receptor for IL-6 or IL-1 did not influence the expression of pro-inflammatory cytokines, they may still be of value for preventing any downstream catabolism. Previous studies have demonstrated that together TNF α and IL-6 synergistically inhibit proteoglycan synthesis and promote glycosaminoglycan degradation^{22,49} and suggest that inhibition of IL-6 may slow matrix breakdown.

Many pro-inflammatory cytokines are thought to contribute to the progression of IVD degeneration^{8,10} however it remains unclear how they arise during disease. Three potential sources for pro-inflammatory cytokines have been proposed (i) the presence of immune cells, such as macrophages or mast cells^{43,44}, (ii) inward transport from surrounding tissues⁴⁶ and (iii) the native IVD cells. Substantial injury and detrimental mechanical loading have been shown to cause IVD cells to increase protein expression of inflammatory mediators released by IVD cells^{54,55}. The results from this study together with the literature further suggest that the native NP cell population may be a source of pro-inflammatory cytokines, as NP cells very quickly released large amounts of pro-inflammatory cytokines

The results of this study must be interpreted within the context of the limitations of the culture system. Many pro-inflammatory cytokines are relevant to the pathogenesis of IVD degeneration (IL-1 β , IL-6, IL-8, IL-17)⁸ and the stimulation with more pro-inflammatory cytokines other than TNF α would improve understanding of the IVD cells contribution to degeneration. We chose to stimulate with TNF α because its presence increases with degeneration⁵, it is associated with IVD injury⁵⁴ and is thought to be involved in the initiation of inflammatory responses within the IVD²⁰. The passage of the cells used varied and was a consequence of differences in the amount of cells that could be initially isolated. To address the potential influence that passage may have on cellular phenotype, cells were allowed to 're-differentiate' within the alginate beads for at least two weeks prior to the start of any experiments. This re-differentiation period has previously been shown to restore expression of phenotypic markers^{22,56}. Additionally, all statistical analyses were adjusted for demographic information, including passage number, and none of the factors were found to have a significant influence on the outcome. Future work investigating the inflammatory response of the other IVD cell types (annulus fibrosus & cartilaginous endplate) would provide a more complete understanding of the IVDs response to inflammation.

We conclude that native NP cells have the capacity to be a source of biologically significant amounts of pro-inflammatory cytokines because the amount of pro-inflammatory cytokines that were released in response to TNF α were all at least 2 $\times$ above the ED₅₀. The timing of treatment and the pro-inflammatory target were both important factors in determining the success of a treatment because the addition of anti-inflammatories in the Same-time study significantly reduced both IL-6 and IL-1 β while the addition of the same anti-inflammatories in the Delayed study did not significantly reduce the expression of any cytokines. Overall, the speed with which the NP cell mediated inflammatory response comes to equilibrium together with the reduced effectiveness of anti-inflammatories in a previously inflamed environment may help explain the limited efficacy of anti-inflammatory back pain clinical trials.

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Figure 1. Study Design
(A) Timecourse study design to investigate how the amount of pro-inflammatory cytokines expressed changes over time. (B) Same-time blocking study design to investigate interactions between pro-inflammatory cytokines and the ideal situation of blocking at the same time of initiation of inflammation. (C) Delayed blocking study design to investigate the efficacy of using different anti-inflammatories introduced into an environment with pre-existing inflammation. (D) The different groups and dose of anti-inflammatories used in the Same-time and Delayed blocking studies.

Figure 2. Timecourse Study

Time-matched changes in the protein expression and corresponding mRNA for pro-inflammatory cytokines (A&B) IL-1 β , (C&D) IL-6, (E&F) IL-8 for Control and TNF α (10ng/ml) treated human NP cells over 144 hours. *p<0.05, ‡ p<0.1

Figure 3. Same-time Blocking Study

TNF α and anti-inflammatories were added at the same-time. The amount of (A) IL-1 β , (B) IL-8 and (C) IL-6 protein released into the culture medium at 72 hours for all groups. (D) Schematic of the expected result over time for the possible different outcomes. Successful blocking is expected to partially or fully reduce the pro-inflammatory cytokine levels, depending on the drug's efficacy. Different letters = $p < 0.05$.

Figure 4. Delayed Blocking Study

Cells were pretreated with TNF α for 72 hours prior to the addition of anti-inflammatories to simulate the addition of an anti-inflammatory into an environment with pre-existing inflammation. The amount of (A) IL-1 β , (B) IL-8 and (C) IL-6 protein released into the culture medium at 144 hours for all groups. (D) Schematic of the expected result over time for the possible different outcomes, including the change of half the medium at 72 hours. Different letters = $p < 0.05$.

Figure 5. Mechanism of Action

Schematic demonstrating that IL-6, IL-1 β , and IL-8 are released following TNF α exposure and where in the signaling cascade the anti-inflammatories target. Anti-TNF α directly targets both soluble and membrane bound TNF α . Both Anti-IL-6R and IL-1Ra inhibit their associated receptors and would prevent any autocrine signaling that may contribute to further cytokine expression. The mechanism of PPS action as an anti-inflammatory is currently unknown.

Table 1
Specimen Information

Gender	Age	Source	Level	Grade	Passage	Diagnosis
F	81	Autopsy	Lumbar	3	P2	-
M	93	Autopsy	Lumbar	4	P4	-
F	54	Autopsy	Lumbar	4	P2	-
M	50	Surgery	Cervical (C4/5, C5/6)	4	P3	Cervical Spondylosis
F	53	Surgery	Lumbar (L4/5, L5/S1)	3	P2	Degenerative Disease and Radiculitis
M	28	Surgery	Cervical (C4/5)	3	P3	Cervical Disc Degeneration
M	49	Surgery	Cervical (C5/6 & C6/7)	4	P3	Cervical Disc Herniation-Stenosis
M	39	Surgery	Cervical (C6/7)	3	P3	Cervical Disc Herniation
M	62	Surgery	Cervical (C5/6)	5	P3	Cervical Disc Herniation
F	29	Surgery	Lumbar (L4/5)	2	P3	Anterior Lumbar Fusion

Table 2**Cell Viability**

Timecourse		
	Basal Control	TNF α
144 hrs	95.3 $\pm$ 2.2 %	90.4 $\pm$ 10.9 %

Blocking Studies		
	Same-time	Delayed
Basal	90.6 $\pm$ 6.6 %	92.5 $\pm$ 5.1 %
TNF α Control	91.9 $\pm$ 7.8 %	93.4 $\pm$ 5.1 %
Anti-IL-6R	90.6 $\pm$ 9.9 %	92.3 $\pm$ 5.6 %
IL-1Ra	89.7 $\pm$ 6.5 %	90.2 $\pm$ 7.1 %
Anti-TNF α	94.5 $\pm$ 3.1 %	94.5 $\pm$ 5.2 %
PPS	89.9 $\pm$ 9.2 %	93.7 $\pm$ 5.1 %

Table 3
Cytokine Expression compared to the cellular Median Effective Dose (ED₅₀)

	Concentration at 144hrs (pg/mL)	Median Effective Dose - ED ₅₀ * (pg/mL)	Fold Change above ED ₅₀ at 144hrs
IL-1 β	105 $\pm$ 37	<12	8.7
IL-6	1571 $\pm$ 436	200-800	2.0
IL-8	9084 $\pm$ 337	500-2500	3.6

[Catalog #: IL-1 β : 201-LB, IL-6: 206-IL, IL-8: 208-IL]

*
ED₅₀ of recombinant human proteins produced by R&D Systems