

Targeted delivery of doxycycline with nanoparticles mitigates cytokine storm and reduces immune cell infiltration in LPS mediated lung inflammation.

Shivani Arora¹ and Naren Vyavahare¹

¹Clemson University

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Abstract

Background and Purpose: Cytokine storm invoked during acute and chronic lung injury promotes alveolar damage and remodeling. The current study shows that degraded elastin-targeted nanoparticles releasing doxycycline (Doxy NPs) are potent in mitigating cytokines storm, migration of immune cells in the lungs, and inhibiting inflammasome pathways in the LPS mouse model. **Experimental Approach:** Cytokine storm and lung injury were induced using LPS and elastase in C57BL/6 mice (rodent model for emphysema). The mice were then treated with I.V. Doxy NPs, blank NPs, or Doxy a day before LPS administration. Cytokine levels, immune cell population, and MMP activity were analyzed in BALF 4 hours after LPS administration. Additionally, gene expression of IL-6, IL-1beta, MCP-1, NLRP3, Caspase 1, and MMPs were investigated in alveolar cells on day three after LPS administration. **Key Results:** Doxycycline NPs but not Doxycycline significantly decreased IL-6, TNF- α , and IL-23 and were significantly more effective in decreasing the percentage of immune cells in the BALF. This is the first in-vivo study to demonstrate that Doxycycline can effectively inhibit lung inflammasome pathways. **Conclusion and Implications:** IV administration of elastin antibody conjugated Doxycycline-loaded albumin Nps can effectively modulate the local immune environment in the lungs, which is not achieved by IV Doxycycline even at a 100-fold higher dose. This novel method of drug delivery can effectively lead to the repurposing of traditional Doxycycline as a potential adjunct treatment for managing the cytokine storm in the lungs in COPD and viral infections.

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Shivani Arora, Narendra Vyavahare

Affiliations:

Department of Bioengineering, Clemson University, Clemson, South Carolina, USA

Word Count:

Contact Information:

Narendra Vyavahare:

Hunter Endowed Chair and Professor

Department of Bioengineering

501 Rhodes Engineering Center

Clemson University, Clemson, SC 29634

Phone: 864-656-5557

narenv@clemson.edu

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ABSTRACT

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INTRODUCTION

Airway remodeling, a most apparent consequence of acute and chronic lung conditions like acute viral infections, including SARS-CoV-2 and emphysema, is a sequel of proinflammatory intra- and extracellular events in the architectural components of the respiratory system (Holtzman et al. 2005). These airway disorders cause epithelial cell apoptosis and inflammatory cellular infiltration. All these pathological events either lead to/or result from elastin damage in the alveoli, which is a critical components of breathing mechanics responsible for lung elastic recoil.

The proinflammatory cytokines; IL-6, IL-1 β , TNF- α , IL-23; and proteases; MMPs 2,9 &12; are the common orchestrators of inflammation-associated changes in lungs. They have been shown to decrease elastin mRNA expression or directly degrade elastin in the lungs. These cytokines are currently the prime targets for not only COPD drug discovery but also for acute lung injury occurring from viral infections in the lungs. FDA has approved two classes of IL-6 inhibitors for the treatment COVID 19, viz. a) anti-IL-6 receptor monoclonal antibodies (mAbs) (e.g., sarilumab, tocilizumab), and b) anti-IL-6 mAbs (i.e., siltuximab). However, these drugs have severe side effects, limiting their use to a generally healthy population.

A recent approach of targeted drug delivery has revolutionized the field of drug discovery. We have developed albumin-based nanoparticles (NPs) conjugated with an elastin antibody targeting only damaged elastin in tissues while sparing healthy elastic fibers (Parasaram et al., 2016 and 2018; Wang et al., 2018). These NPs target the site of elastin damage for prolonged and sustained delivery of drug locally and limiting the systemic side effects.

Previously, we have shown that a single i.v injection of elastin antibody conjugated-doxycycline loaded bovine serum albumin nanoparticles (Doxy NPs) can effectively target emphysematous lungs and result in sustained release of Doxycycline locally over four weeks leading to a decrease in Matrix Metalloproteinases (MMPs) activity as compared to Doxycycline IV (Parasaram et al. 2016). Doxycycline (Doxy) has been found to be a

useful drug in many conditions. In a recent report, both pre and post-treatment Vero E6 cells with Doxy were shown to effectively inhibit SARS-CoV-2 strain (IHUMI-3) in a dose-dependent manner (Gendrot et al. 2020). These studies, however, lack the In vivo data. Doxy's anti-inflammatory potential is being harnessed for treatment of chronic conditions including- Brucellosis spondylitis, traumatic brain injury, abdominal aortic aneurysms, where in it has been shown to decrease levels of systemic inflammation markers, IL-6, and inhibit chemokines like MCP-1 and MMPs (Yang et al. 2021, Marjani et al. 2021, Yu et al. 2017, Conforti et al. 2020).

Doxy acts in part by inhibiting protein kinase B (AKT) signaling pathway and mitogen-activated protein kinases (MAPKs) signaling proteins, including extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinases (JNK) in vitro in VSMCs (Yu et al. 2017). Its inflammasome inhibitory capacity was found to be beneficial in altering the tumor microenvironment in prostate cancer (PC3) and a lung cancer cell line (A549) (Alssadi et al. 2021). However, it is still unknown how Doxy inhibits inflammatory response, particularly in the lungs. We wanted to test if IV delivery of Flexibzumab conjugated doxycycline loaded bovine serum albumin nanoparticles (Doxy NPs) can prevent acute cytokine storm in the lungs in exogenous administration of endotoxin lipopolysaccharide (LPS) mouse model.

Material and Methods

Synthesizing Doxycycline loaded BSA nanoparticles (Doxy NPs) and BSA nanoparticles (Blank NPs)

Doxy Nps were prepared as per the procedure described by Parasaram et al. (2016). Briefly, 25 mg of Doxycycline Hyclate (Sigma Aldrich, St. Louis, MO) was dissolved along with 100 mg of bovine serum albumin (BSA) in 2 mL of water and was stirred at 500 rpm. Using an automated dispenser, ethanol (4 mL) was added to the Doxy and BSA solution at 1ml/min. Following alcohol addition, 8% glutaraldehyde (40µg/mg BSA) was used to crosslink the particles, and the mixture was stirred for additional 2 hours at room temperature at 500 rpm. The resulting particles were pelleted by centrifuging at 14000

rpm for 10 min. and washed three times with DI water and resuspended in either 1 ml of PBS (for conjugation) or in 1 ml of 5% sucrose (to calculate yield and release).

Blank nanoparticles were prepared similarly, except that no doxycycline was used during the process

Conjugation of elastin antibody to nanoparticles

We have created a new humanized elastin antibody that targets degraded elastin named Flexibzumab (Vyavahare et. al 2019). Albumin NPs were activated with heterobifunctional crosslinker α -maleimide- ω -N-hydroxysuccinimide ester poly (ethylene glycol) (Maleimide-PEG-NHS ester, MW 2000 Da, Nanocs Inc., NY, USA) to achieve a sulfhydryl-reactive particle system. Thus, 2.5 mg of Maleimide-PEG-NHS ester solution was added to 10 mg albumin NP dispersion. The reaction was continued for 1 hour at room temperature. Separately, Flexizumab was thiolated using Trout's reagent (34 μ l to 10 μ g of antibody in 400 μ l of HEPES and incubated at room temperature for one hr. After incubation, the Trout's reagent was removed using a 30 KDa filter. The thiolated antibody and pegylated nanoparticles were incubated at 4°C overnight. The conjugated particles were pelleted at 10,000 rpm for 10 min and resuspended in 5% sucrose by sonication followed by lyophilization for final use.

Characterization of Doxy Nps

The Doxy Nps made using the above method were analyzed for size, yield, loading percentage, and release profile. The size of the doxy particles was measured using 90Plus Particle Size Analyzer (Brookhaven Instruments Co, Holtsville, NY). The supernatant obtained from the washout during NP preparation was used to estimate the amount of free Doxy by measuring absorbance at 273 nm using a UV spectrophotometer (BioTek Instruments Inc., Winooski, VT). The difference between the initial Doxy used and Doxy in supernatant gave the amount of doxycycline encapsulated in NPs.

In vitro release

NPs (30 mg) were resuspended with sonication in 1 ml of PBS. After 24 hrs, NPs were centrifuged at 10,000 rpm for 10 min and 3 aliquots of 100 μ l of the supernatant were used

to measure Doxy at 260 nm. Pure doxycycline hyclate solution in PBS was used for making std curves. Blanking was performed with PBS, and for normalization, an equivalent amount of blank BSA NPs treated similarly was used.

Lung Injury using Elastase and LPS

All procedures involving mice were performed according to the IACUC standards following ethics approval by the animal committee at the Clemson University (Protocol#-AUP2019-040). 6 weeks old Male C57BL6/J mice were purchased from Charles River and were maintained on a chow diet, in pathogen-free cages, and on std. 12h light-dark cycle at 23°C throughout the study. All experiments on mice were performed at 8 to 10 weeks of age. On Day 1, the lungs of animals in all groups except for the sham group animals were treated with elastase (1.5U, I.T.), and the sham group animals received sterile PBS. On day 8, NP treatment was performed. The animals were randomly divided into four groups: control group (PBS), Doxy IV group (10 mg/kg Doxy), Doxy NPs treated group (10 mg/kg of antibody conjugated Doxy loaded BSA nanoparticles), and blank NPs (10 mg/kg antibody conjugated blank BSA NPs). The drugs were suspended in PBS to formulate IV injections and given via tail vein. One day after NP injections, (day 9) Lipopolysaccharides from *Escherichia coli* O111:B4 (LPS. Sigma, 1mg/kg I.T.) was administered to all the animals except for the animals in the sham group, which received sterile PBS.

Separate cohorts of 4 experimental groups; control group, Doxy IV group, Doxy NPs group, and blank NPs treated group; were set up for cytokine analysis (n=8 per group), MMP activity analysis for zymography (n=5 per group) in BALF and samples were collected 4 hrs after LPS administration on day 9. Separate cohort of experimental groups was set up for gene expression analysis (n=5 per group) in the alveolar tissue and immune cell population analysis in BALF (n=6), samples from these cohorts were collected on Day 12 i.e. 3 days after LPS administration. The timeline for the experiment is given in Fig. 1.

Sample collection for analysis

For sample collection, either after 4 hrs or on day 3, the animals were euthanized using carbon dioxide asphyxiation. The bronchoalveolar lavage fluid (BALF) and the cells were collected as per the protocol described by Hoecke et al. (2017). Briefly, after euthanizing the animal, the tracheas were exposed by making an incision in the neck, and the catheter (made with 23G needle) was sutured inside the trachea for performing the lavage. The lungs were lavaged with 3 ml of PBS containing 100 μ m of EDTA, and the lavage fluid was collected and centrifuged for 7 min at 400g and 4°C. Protease inhibitor was added to the supernatant and was stored immediately at -80°C for ELISA and zymography. The cell pellet obtained after centrifugation was treated with ACK lysis buffer at RT for 2 min, and after the ACK was immediately resuspended in PBS. These cells were used for immune population analysis.

Measurement of cytokine levels

Cytokine levels were measured in BALF samples collected 4 hours after LPS administration to perform cytokine level analysis. The levels of IL6, TNF- α , IL-23 and IFN- γ were measured using kits from Biolegend (Cat. No. 431304, 430904, 433704, 430804 resp.) following the manufacturer defined protocol.

Immune cell population analysis

Immune cell population analyses were performed in the samples collected after three days. The cells from the BALF were collected as described above and, after treatment with ACK lysis buffer, were washed and resuspended in PBS for counting. 1×10^6 cells were used for staining. Fig 3A describes the cell staining and identification strategy. Total cells were gated to exclude doublets; from the singlets, live cells were identified using Dapi and were defined as DAPI-ve; from the live cell population, Cd11C low cell population was further narrowed down to identify B cells defined as CD19+MHCII+; T cells; defined as CD3+MHCII-. The remaining cell population, i.e CD19-CD3-MHCII-; was further gated to identify eosinophils defined as, Cd11b+Ly6G-, and neutrophils defined as, Cd11b+Ly6G+.

In a parallel experiment the same sample and number of cells was used to identify dendritic cells macrophages. Total cells were gated to exclude doublets; from the singlets, live cells were identified using Dapi and were defined as DAPI-ve; from the live cell

population, Cd11Chi cell population was further narrowed down to identify Macrophages; defined as SiglicFHiMHCII- and SiglicFintMHCII-; and dendritic cells defined as SiglicF-MHCII+. Data was analysed using FlowJo software (v10.5.) and statistical analysis was performed between groups following one way-ANOVA using GraphPad Prism software. The results are represented as Mean $\pm$ SEM, and a P-value of < 0.05 was considered significant.

MMP activity analysis using Zymography

MMPs activity in the BALF harvested 4 hours after LPS treatment was measured using in-gel zymography, following protocol from Leber et al. (1997) with slight modifications. Briefly, BALF samples from each treatment group (containing 20 μ g of total protein) were loaded on gelatin-impregnated SDS gels (ZY00105, Fisher Scientific). Coomassie blue staining was used for visualizing the sites of proteolytic digestion.

RNA extraction and quantitative reverse transcriptase PCR

The expression of genes of interest was performed in lung samples harvested on day three after LPS treatment. The lung tissue was stored immediately after retrieval in the Trizol. The tissue was homogenized in Trizol using Powergen 125 (FS-PG125, Fisher Scientific) homogenizer, and RNA was extracted using the Zymogen total RNA microprep kit (Zymogen) according to the product instructions. A Nanodrop instrument was used for quantitative and qualitative analysis of the extracted RNA. cDNA synthesis was performed using 200ng of total RNA, iScript gDNA clear cDNA synthesis Kit (Cat# 1725034, Bio-Rad) was used for this. The total cDNA obtained was diluted five times before amplification with iTaq- Universal SYBR Green Supermix reagent (Cat# 172512, Bio-Rad) on Bio-Rad quantitative PCR platform (96-well format). Quantification was performed using the delta-delta C_T method, using HPRT or β -Actin as a housekeeping gene.

Quantification and statistical analysis

The number of biological replicates (n) for each experiment is indicated in the figure legend. Statistical analysis was performed between treatment groups using one-way

ANOVA as there were more than three groups in the study, with corrections for multiple testing. The results are represented as Mean $\pm$ SEM, and a P-value of < 0.05 was considered significant.

Results

The current study aimed to compare the efficacy of targeted Doxy NPs with standard doxycycline IV treatment in mitigating cytokine spurt as well as at evaluating the mechanism of action of Doxycycline by which it exerts its anti-inflammatory action in the lungs.

Doxycycline NPs but not Doxycycline IV mitigates cytokine spurt in BALF

We analyzed the levels of proinflammatory cytokines - IL-6, TNF- α , IL-23 and IFN- γ in the BALF, 4 hours post LPS treatment using ELISA (Fig. 2). Our results indicate that at the doses used in the study, Doxy NPs were significantly more effective in lowering IL-6, TNF- α , and IL-23 levels (with a p value <0.0001), whereas treatment with Doxy IV was ineffective in lowering the level IL-23 & TNF- α and only slightly decreased IL-6 levels (p=0.012) (Fig. 2 A-C). Levels of IFN- γ were significantly reduced by both Doxy NPs and IV Doxy (p=0.0074 and p <0.0001). However, with the Doxy NPs, we observed that they were maintained in a range closer to the control group (71.38 $\pm$ 9.72pg/ml vs. Control 59.80 $\pm$ 4.25pg/ml). In contrast, its level was significantly lowered compared to controls by Doxy IV treatment (19.95 $\pm$ 5.04pg/ml, with p <0.0001) (Fig. 2D). These results indicate higher efficacy of Doxy NPs over Doxy IV treatment.

Doxycycline Nanoparticles are equally effective in decreasing MMPs expression and Activity

Previously we showed that MMPs are inhibited with Doxy NPs in the elastase model of emphysema; we thus evaluated whether pretreatment with Doxy NPs could inhibit MMP activity compared to Doxy treatment in the LPS model.

We analyzed protein level activity in BALF (by gelatine zymography) and gene expression of MMP 2, 9 &12 in the lungs (by RT-PCR). Both Doxy IV and Doxy NPs treatments showed an 8-9 fold decrease in MMP-9 activity (Figure 3 A-B). We found that Doxy NPs treatment was slightly more efficient than doxy IV in inhibiting MMP-9 activity (with

p=0.0002, Fig. 3B) as well as expression of MMP-2, MMP-9 and MMP-12 in the lungs (Fig. 3C).

Doxy NPs decrease immune cell infiltration In BALF

Having seen a decrease in the cytokine levels and MMP activity in the BALF, we were further interested in evaluating whether or not doxycycline treatment can inhibit immune cell infiltration in the lungs. We analyzed the percentage of B cells, T cells, eosinophils, neutrophils, dendritic cells, and macrophages in the BALF using FACS (Fig. 4).

We observed that both Doxy IV and Doxy NPs brought down the percentage of B cells (from 17.04% in the blank NP treated group to 5.287% & 2.106% resp.) (Fig. 4B), T cells (from 33.84% in the blank NP treated group to 16.64% & 8.54% resp.) (Fig. 4C), neutrophils (from 23.13% in the blank np treated group to 16.29% & 9.2% resp.) (Fig. 4D) and Dendritic cells (from 4.15% in the blank NP treated group to 1.44% & 0.62% resp.) (Fig. 4I). However, the percentage of eosinophils increased significantly in Doxy IV treatment and Blank NPs (from 0.4% in controls to 11.38% and 7.88% resp.). This increased eosinophil count was mitigated only in the Doxy NPs treated group (2.84%). (Fig. 4E). We also observed that the overall percentage of macrophages was also brought down by Doxy NPs. Of particular interest here is the macrophage population defined as SiglicFHi that was particularly affected by doxycycline treatment. A significant decrease was seen in their population with Doxy IV and Doxy NPs treatment resp. (p<0.05 as compared to controls) (Fig. 4F-H).

Doxycycline Inhibits NLRP3 and Effectors of Inflammasome Pathway

To evaluate the mechanism of Doxycycline in lung inflammation, we focused on its ability to influence the expression of the effectors of inflammasome pathways. We performed qPCR for NLRP3, Caspase 1, IL-6, IL-1 β , and MCP-1 in the lung alveolar cells. We observed a significant decrease in IL-1 β (p=0.0029), and MCP-1 (p=0.015) expression in the Doxy NP treatment group where as Doxy IV treatment failed to cause any significant changes in their expression. However, both were equally effective in significantly lowering the expression of IL-6, NLRP3 and Caspase 1 (Fig. 5a-5e).

Effective Doxycycline dose in Doxy NPs vs. Doxy IV groups

We have previously established (Parasaram et al. 2016) that Doxy loading in BSA NPs is about 17%, and Doxy NPs release doxycycline slowly over 30 days. We confirmed in the current study (Supp Fig.1) that the amount of doxycycline released in 24 hrs. from the dose of 10 mg/kg of Doxy NPs is ~90 µg/kg. This is about 100 times lower dose than what is available for std. Doxy IV (10 mg/kg, with its half-life of 16-22hrs) used in the current study. Thus, Doxy NPs were proven to be significantly more potent in inhibiting proinflammatory cytokines, macrophage infiltration, and eosinophilia in lungs than std. Doxy IV administration at 100 fold lower concentrations.

Discussion

As much as there is encouraging data to support Doxycycline testing for inflammatory lung conditions, there are also some conflicting reports that indicate against doxycycline use. It has been reported that systemic administration of doxycycline hyclate aggravates granulomatous lung inflammation in a dose-dependent manner in *S. mansoni*-infection by causing eosinophilic infiltration and by downregulating Th2 effectors (Souza et al. 2021). In another clinical trial, doxycycline was found to be ineffective in lowering MMP-8 and 9, IL-6, and IL-8 concentrations and in improving lung function parameters; however, it did decrease systemic inflammation in COPD patients (Prins et al. 2016). The possible justification given for these contradictions is the variation in the percentage of neutrophil infiltration seen at the beginning of the study and the possibility that doxycycline has an altogether different mode of action in the lung as compared to the aorta or periodontal diseases.

Morphological lung changes precipitated by either viral or chronic autoimmune disorders incite inflammatory responses. IL-6, one of the most prominent proinflammatory cytokines unleashed by either acute or chronic lung injury, was examined in the study. It participates at multiple levels to induce cellular damage. A) It activates multiple downstream signaling pathways, including Janus/kinase signal transducers and activated transcription JAK-STAT P13K-AKT(STAT 1,3 and 5) Rapidly accelerated fibrosarcoma RAS-RAF, SRC-yes associated protein (YAP)-Neurogenic locus notch homolog

(NOTCH), via classical signal transduction. B) IL-6 not only causes recruitment of immune cells in the local tissue environment but also helps in the direct proliferation of B cells, activates cytotoxic T cells, prompts helper T cells, and causes induction of proinflammatory Th-17 cell responses. In the present study with targeted delivery of small Doxy with NPs, we were able to mitigate IL-6 levels in BALF and turn down its expression in the lungs.

IL-23 is another proinflammatory cytokine of interest that was investigated in the current study. It is involved in the differentiation of Th17 cells in a proinflammatory context and especially in the presence of TGF- β and IL-6. Activated Th17 cells produce IL-17A, IL-17F, IL-6, IL-22, TNF- α , and GM-CSF. Inflammatory macrophages express IL-23R and are activated by IL-23 to produce IL-1, TNF- α , and IL-23 itself. These effects identify IL-23 as a central cytokine in autoimmunity and a highly promising treatment target for inflammatory diseases (Duvallet et al. 2011). We observed a significant decrease in IL-23 levels in the lavage fluid after Doxy NP treatment. IFN- γ is responsible for macrophage activation in alveolar tissue and inducing autophagy. However, increased levels of IFN- γ are considered beneficial when it comes to countering viral attack. Hence, a therapy that could fine-tune its level would be more appropriate in lung pathology. In our study, we observed that Doxy NPs did bring down the levels of IFN- γ to normal levels but did not mute it entirely as Doxy IV group. TNF α is another cytokine well established for its role in promoting lung inflammatory conditions. It is responsible for the activation of NF- κ B and Activator Protein 1 (AP1) pathway and the production of ICAM-1, VCAM-1, and RAGE (Mukhopadhyay et al. 2006). In this study with Doxy NPs treatment, we observed a significant decrease in the TNF- α levels.

In the current study, we modified the pre-established method of inducing lung injury to induce a cytokine storm. Lungs of C57BL/6 mice were first treated with elastase (I.T.), and seven days after cytokine infiltration was induced by IT LPS. It mimics compromised lung conditions for COPD patients. The priming of lungs with elastase not only induces damage to the elastin rich connective network of the lungs but also causes infiltration of

neutrophils (Sandhaus and Turino 2013). The second hit with LPS was used to aggravate the condition further so that significantly higher levels of proinflammatory cytokines and immune cell infiltration could be achieved, as is seen clinically during asthmatic attack or in acute viral infections. This model has been previously used by many researchers, including us (Sajjan et al. 2009, Ganasan et al. 2010, 2012, Parasaram et al. 2016, De Olivers et al. 2019), to mimic the pathology of emphysema. Using this approach, we were not only able to achieve very high concentrations of proinflammatory cytokines IL-6 (up to 805.74 ± 72.06 pg/ml), TNF- α (246.77 ± 43.31 pg/ml), IL-23 (47.70 ± 4.91 pg/ml) and IFN- γ (115.05 ± 20.88 pg/ml) but also significantly higher immune cell infiltration (B cells, T cells, neutrophils, macrophages and eosinophils) as compared to control group (all at $p < 0.0001$).

Having established the model, we further analyzed the mechanism of action, as well as the efficacy of elastin-antibody conjugated Doxycycline NPs, to evaluate their efficacy for alleviating cytokine storm over Doxycycline IV to mimic acute and chronic inflammatory lung disorders, including COPD and SARS CoV 2 infections. We compared the efficacy of Doxy NPs with standard Doxycycline IV. Of note, we have already shown using dye loaded nanoparticles, that elastin antibody targeted nanoparticles specifically target damaged alveolar lung elastin when given i.v. and are retained in lungs over a period of 2-4 weeks (Parasaram et. al 2016). We found that reformulating Doxycycline to nanoparticles not only helps in targeting it to the desired site of action but also makes the drug more effective in mitigating immune responses at 100 times lower concentrations. In the current study, we observed that although Doxycycline in either of the formulations (std IV or NPs) was capable of decreasing dendritic cell, B Cell, and T cell population, However only when targeted in the form of Doxy NPs, was it capable of mitigating macrophage, neutrophil and eosinophil count as well as alleviate the cytokine storm in lungs. This selective decrease in macrophage, neutrophil and eosinophil population caused by Doxy NPs could be accounted responsible for its substantial response against cytokine spurt. Many novel therapeutic strategies are also focusing on alleviating eosinophilia during COPD. Mepolizumab monoclonal antibodies directed against IL 5 to mitigate eosinophil population in lungs is currently under phase 3 clinical trial for the

treatment of COPD. Although using MABs is an attractive strategy, it has several limitations that restrict its use in a limited population (Pavord 2017, ClinicalTrials.gov numbers, NCT02105948 and NCT02105961, Xia et al. 2018). Targeted delivery of a pre-approved drug (Doxycycline) to lungs to check eosinophilia and cytokine spurt offers an attractive opportunity to fast-track the translation to the clinic.

We also found that high doses of Doxy IV can (10 mg/kg IV) inhibited MMPs activity and recruitment of immune cells but was significantly less effective in lowering cytokine levels as well as immune cell infiltration, in the LPS primed lungs as compared to Doxy NPs, that were specifically targeted to damaged elastin in lungs. In a recent report by Sousa et al. in 2021, IV treatment with doxycycline hyclate was reported to cause dose-dependent eosinophilia by downregulating Th-2 responses during *S. mansoni*-infection. In vitro studies with low doses of doxycycline (20 to 40 mg) were shown to inhibit IL-6, but an inhibitory effect on MMPs was only observed when it was used in higher doses of 100-120 mg (Caprio et al. 2015). The results of our study are in line with previous findings (Dalvi et al. 2011) and provide a unique method for achieving a slow, sustained release of a low dose of doxycycline that not only inhibits inflammatory response but also lowers MMP expression.

Although it has been previously established that doxycycline inhibits proinflammatory responses by acting via the mTOR pathway in cancer cell lines, its impact on the inflammasome pathway, particularly in the lungs, has not yet been evaluated. There have been only a few in-vitro studies conducted that report the inflammasome inhibiting potential of doxycycline. Xu et al. in 2020 have tested its inflammasome inhibiting potential in-vitro against *P. gingivalis*, the causative organism for gingivitis, and Alassadi et al. 2021, have reported that in cancer cell lines, doxycycline is capable of suppressing NLRP3 derived inflammatory signals due to which it attenuates cancer cells growth. From these studies, it is indicated that the anti-inflammatory activity of doxycycline could be partly attributed to its inflammasome pathway inhibition. However, a definite in-vivo study to evaluate its activity in this particular context is lacking to date.

The use of NLRP3 inhibitors for treating chronic inflammatory conditions in the lungs has been theorized by many groups (Theofani et al. 2019, Wei et al. 2020). NLRP3 mediated inflammasome formation activates caspase-1 mediated programmed cell death and is responsible for not only increased neutrophil infiltration but the upregulation of chemokines and proinflammatory cytokines via this pathway also leads to eosinophilia (Lin et al. 2016) as well as increased housing of macrophages, B cells and T cells in lungs. Inhibition of NLRP3 by doxycycline in our study explains how it inhibits infiltration of immune cells and mitigates the cytokine storm.

The current study is the first of its kind to evaluate the action of doxycycline on the inflammasome pathway in vivo in the lungs. We evaluated the effect of doxycycline on the inflammasome pathway three days after induction of injury and four days post-treatment with either Doxycycline IV or Doxy NPs. We observed a significant upregulation of IL-6, MCP1, IL-1 β in the injured group. MCP1, IL-1 β levels significantly decreased with doxycycline NP treatment, where as IL-6 levels were effectively brought down by both std. doxy and Doxy Nps. This inhibition of IL-6, MCP1, IL-1 β at the alveolar tissue level also explains the improvement in immune cell infiltration seen with Doxy NP treatment. Of note, Doxy NPs only contained a 17% loading of doxycycline and only released ~90 μ g/kg in mice, which means the dose use was 100 times lower than Doxy IV (10 mg/kg) and showed better results, clearly suggesting that targeted therapy is highly effective. This study is the first of its kind to evaluate the inhibitory effect of doxycycline on the Inflammasome pathway in lungs, further studies using a genetic model for NLRP3 K/O mice, and NLRP3 agonists are warranted.

Limitations

The study has a few limitations. First, in the current study, we used a low dose of elastase (1.5U/kg) to induce lung injury; at this dose, neither is it possible nor did we perform a lung compliance study to evaluate the physiological outcome. Second, although the intervention was performed seven days after elastase treatment, it was done 24 hrs prior to LPS treatment. This implies that treatment with Doxycycline NPs were effective against sudden exacerbation of inflammatory response in the lungs with preexisting mild

inflammatory conditions. However, for better clinical extrapolation of the results from the current study, using a more robust COPD model with a lower dose of std doxycycline IV is recommended. We also did not measure actual concentration of Doxy in BALF or serum but blank NPs that were targeted with elastin antibody did not show any effects, clearly showing Doxy delivered and caused the changes. Third, as Doxy NPs release the drug over 30 days; albeit at miniscual quantities, the long-term effect of this therapy needs to be evaluated including nanoparticle clearance.

Conclusion

We show that degraded elastin-targeted nanoparticles can deliver doxycycline to the lungs and prevent cytokine storm and inflammatory cell infiltration at 100-fold lower concentration than Doxy IV treatment. This approach can lead to fast-track target validation via drug repurposing. It can considerably decrease the time lag between bench to bedside therapy to deal with emergencies that develop rapidly across the globe, as the world has recently seen with the novel COVID 19 outbreak. In the future, we will test if one dose of targeted Doxy NP therapy would keep the cytokine storm in check for more extended periods.

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Conflict of Interest: Elastrin targeted nanoparticle system is licensed to Elastrin Therapeutics Inc. and NRV holds significant equity in the company.

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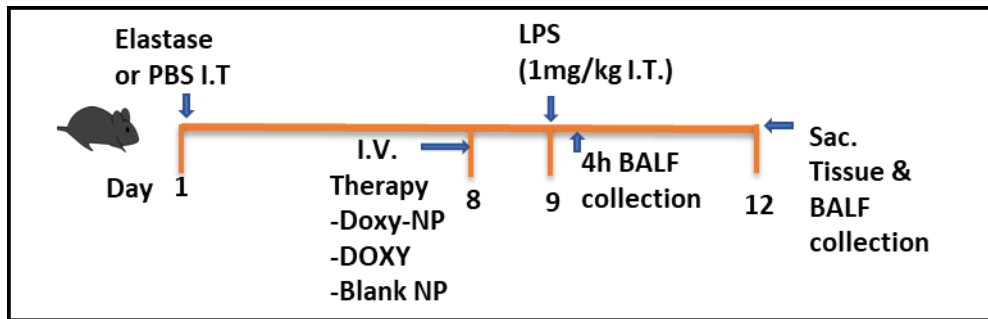


Figure 1: Experimental Timeline

Separate cohorts of C57 bl/6 mice were used for the study. On day 1, animals in the control group received PBS, whereas all other treatment group received elastase intratracheally. On day 8, therapy was given intravenously (tail vein), and on day 9, LPS was administered intratracheally. The first cohort of animals was sacrificed for sample collection 4 hrs after LPS administration, and the animals in the second cohort were euthanized 3 days after LPS treatment (at Day 12).

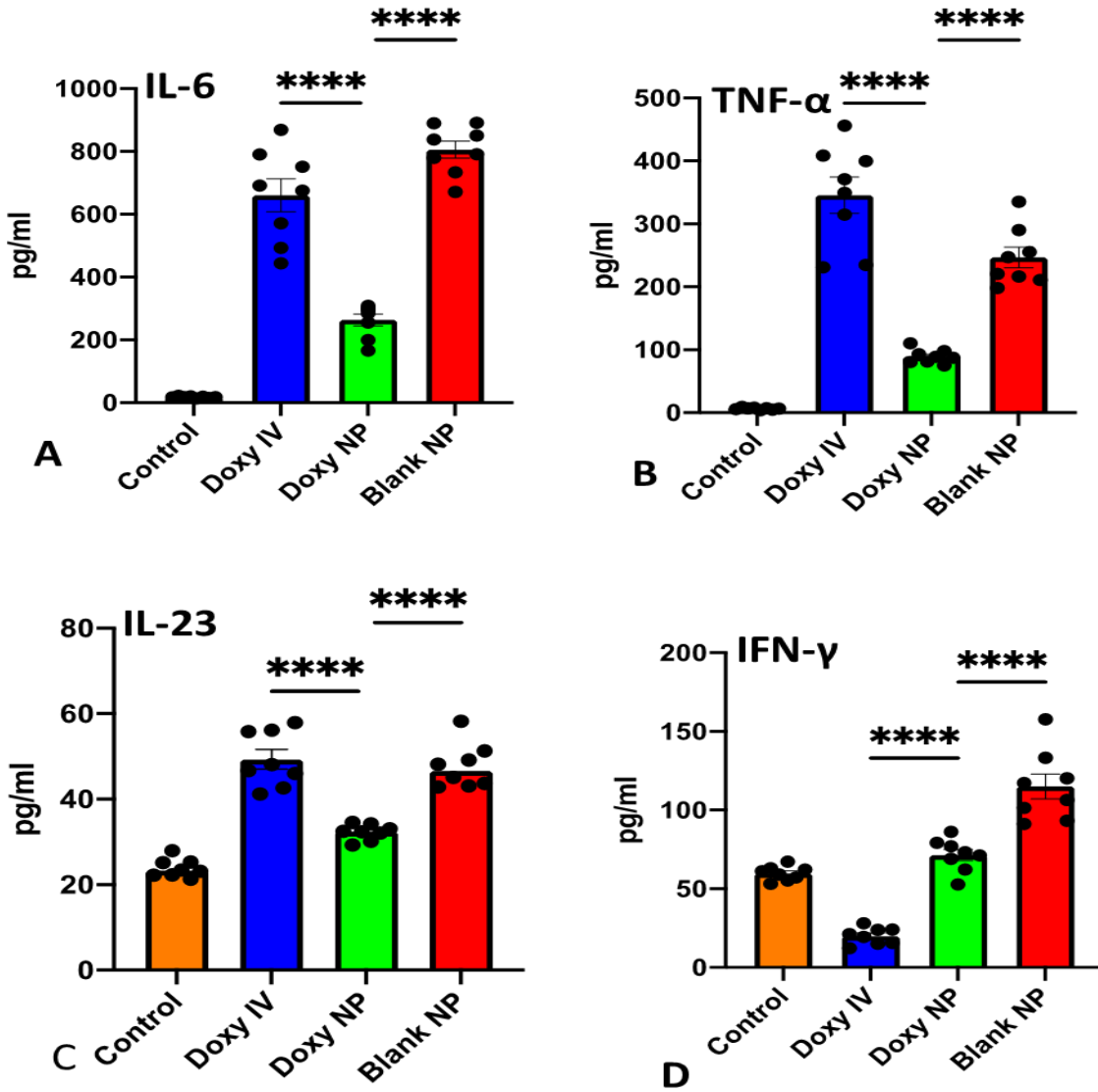


Figure 2- Doxycycline Nanoparticles More Effectively Mitigate Cytokine Storm

A) Elastase and LPS were administered I.T, and the drug treatment was given by the IV route. 4 hours after LPS administration BALF and lungs were harvested for analysis. (B) to (D) **ELISA** of IL-6, TNFα, IL-23 and IFN-γ resp. in BALF, showing Mean± SEM of n = 8 mice per group, *p < 0.05, one-way ANOVA.

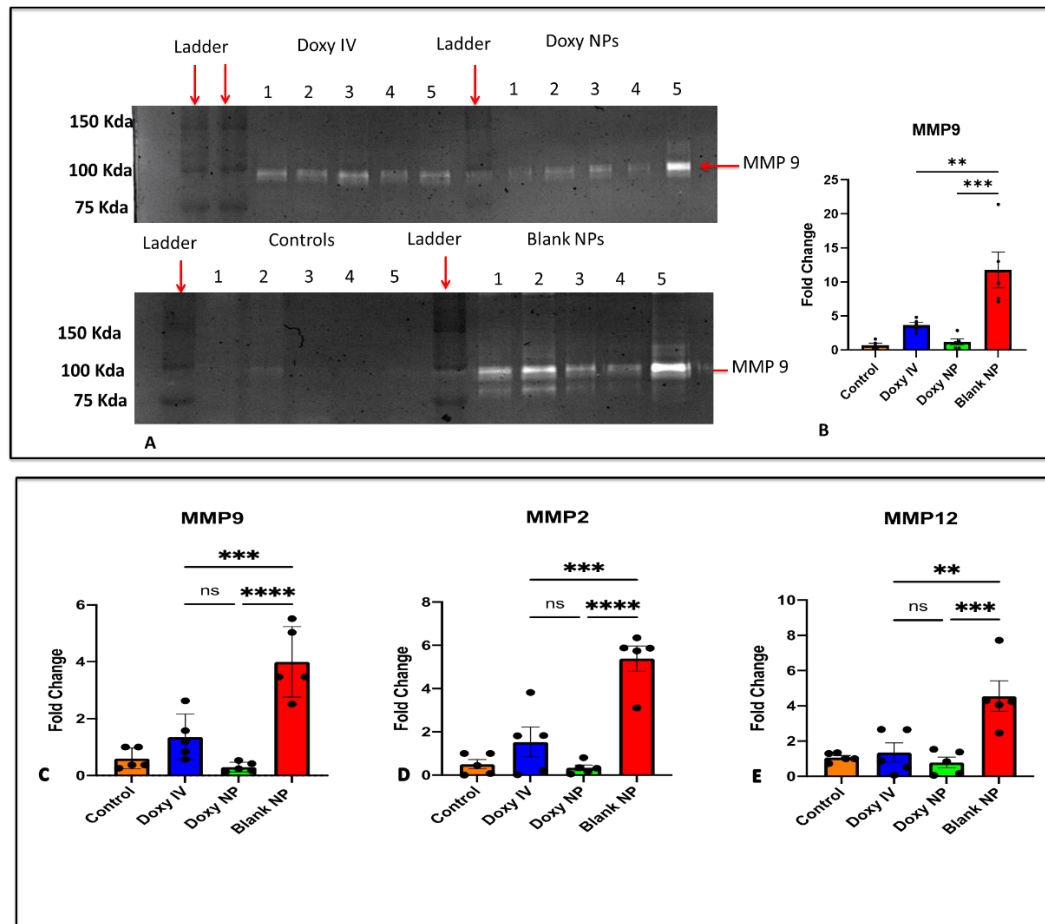


Figure 3- Doxycycline Nanoparticles Effectively inhibit MMP 2 & 9

A) In-Gel Zymography was performed to detect MMP activity in the BALF samples.(B) Quantification of the enzymatic activity was done using Image J software, results represented as Mean± SEM; n= 5 mice per group; *p < 0.05, as analyzed by one-way ANOVA. (C)-D)qPCR based relative quantification of MMPs expression in the lungs harvested at day 12 from each treatment group; HPRT was used as housekeeping gene; results showing Mean± SEM of n = 5 mice per group, *p < 0.05, **p < 0.01, as analyzed by one-way ANOVA.

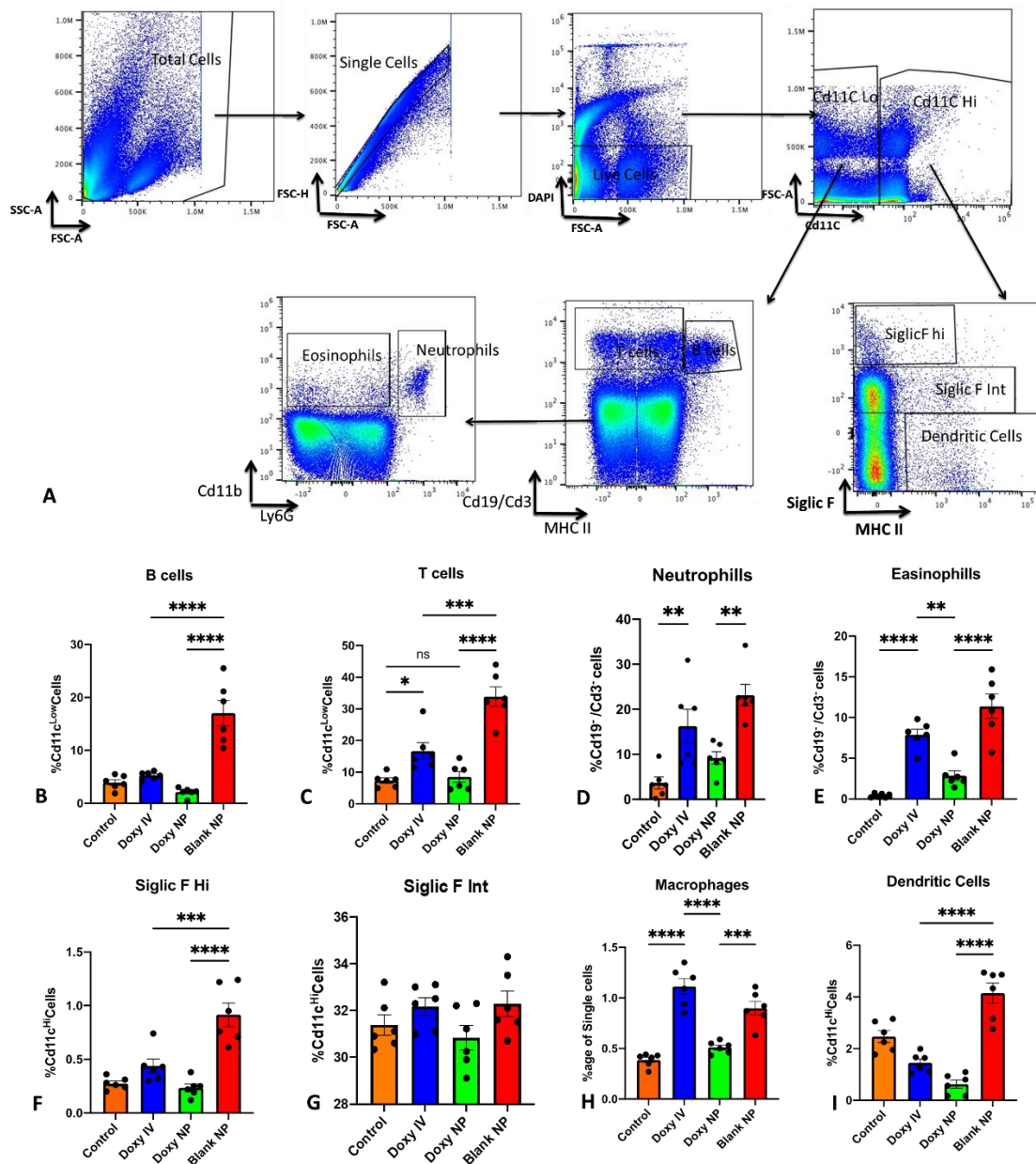


Figure 4- Doxycycline Nanoparticles More Effective In Mitigating Immune Cell Recruitment To Lungs

(A) Representative dot plots for FACS based analysis of immune cell population in BALF, (B-I) Quantification of B cells, T cells, Neutrophils, eosinophils, SiglicF^{hi} macrophage population, SiglicF^{int} macrophage population, total macrophages, and dendritic cells in all treatment groups. Results showing Mean± SEM of n = 6 mice per group, *p < 0.05, **p < 0.01, ***p < 0.001 one-way ANOVA.

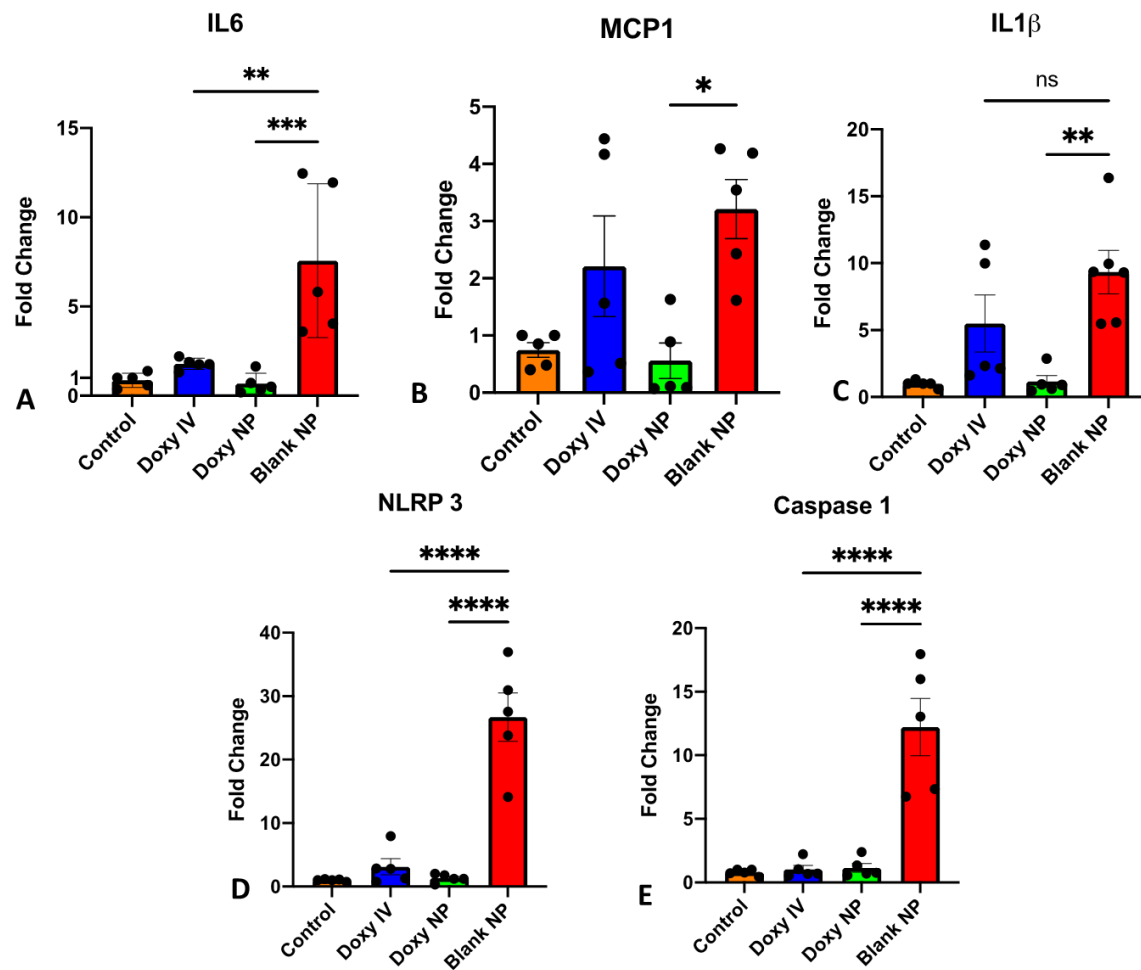


Figure 5: Doxycycline Nanoparticles inhibit Inflammasome formation

A)-D) qPCR based relative quantification of IL-6, MCP-1, IL1 β , NLRP3 and Caspase 1 expression resp. in the lungs harvested at day 12 from each treatment group; HPRT was used as housekeeping gene; results showing Mean $\pm$ SEM of n=5 mice per group, **p < 0.01, ****p < 0.0001 as analyzed by one-way ANOVA.

Supplement figures:

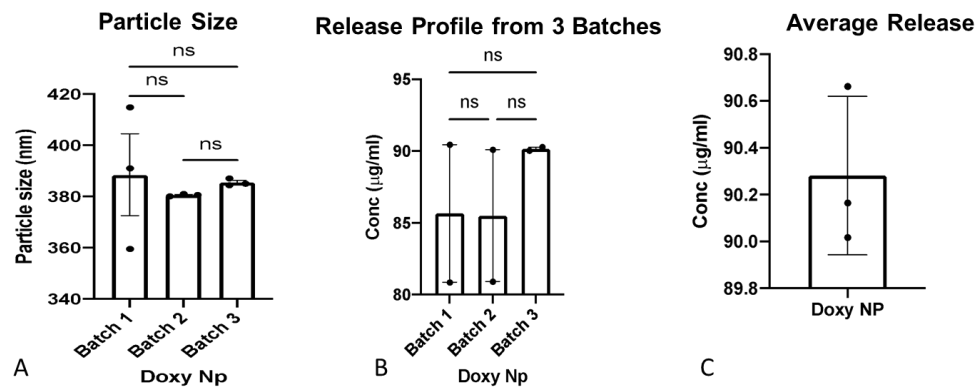


Fig. S1: Characterization of Doxy Nps

- A) Particle size analysis from 3 different batches of Doxy Nps
B) Quantification of Doxycycline released from the Doxy Nps in 24 hrs
C) Average amount of Doxycycline released from 3 different batches of Doxy Nps

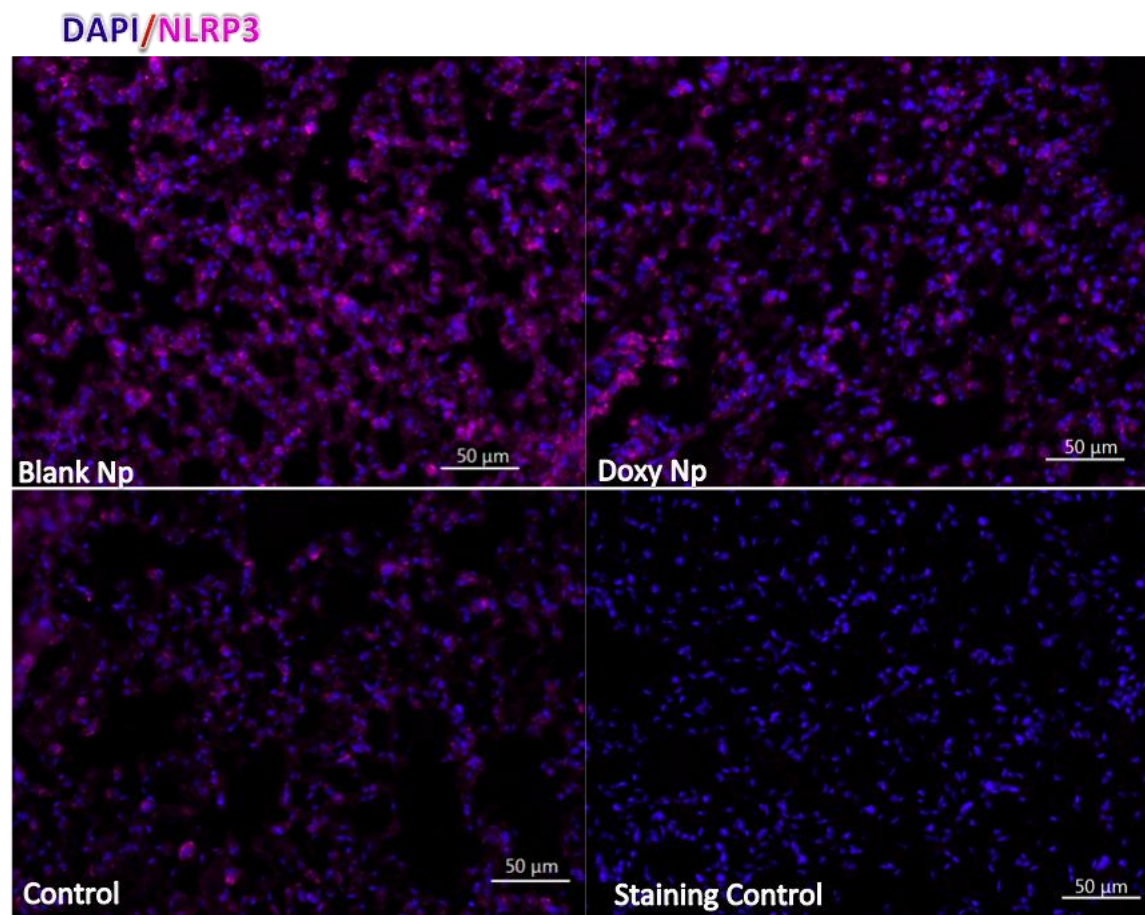


Fig. S2: NLRP3 Staining in Lungs

Representative IHC image to quantify the expression of NLRP3 protein in the lungs harvested on day 12.