Cystatin 9: the key to effective treatment for bacterial lung disease?

Dr Tonyia D Eaves-Pyles' current research focuses on the role of cysteine proteinase inhibitors called cystatins in the control of immune inflammatory responses in the lungs. These inhibitors may hold the key to limiting the damage done by an overzealous immune response to pathogens in diseases such as pneumonia and influenza.

Lung infections can be devastating illnesses, and are particularly lethal to those who are already vulnerable, such as children, the elderly and those with already weak immune systems. Pneumonia and lung damage caused by infection with drug-resistant pathogens are also associated with high costs for healthcare services. In the US, pneumonia and influenza are the eighth most significant causes of mortality and in 2005 cost the US economy an estimated $40 billion plus $6 billion in indirect costs.

THE IMMUNE SYSTEM OVERREACTS WHEN REGULATION IS ABSENT

The immune system normally deals with pathogens via the process of inflammation, but without regulation the response can be too intense and can cause cell damage. In addition, for some pathogens, inflammation provides the perfect conditions for them to infect their hosts, therefore increasing the level of infection.

Pneumonia and influenza are associated with dysregulated inflammatory responses and the break-down of the pulmonary epithelial cell lining, such as the air sacs (alveoli) that exchange oxygen and CO_2 and oxygenate the blood. This causes the membranes to become more permeable and inflamed, and therefore more susceptible to disease. One of the factors that contribute to this over-reaction is caused by enzymes called matrix metalloproteinases (MMPs), which break down the cellular scaffolding present around cells known as the extracellular matrix (ECM).

CYSTATINS: THE REGULATORS

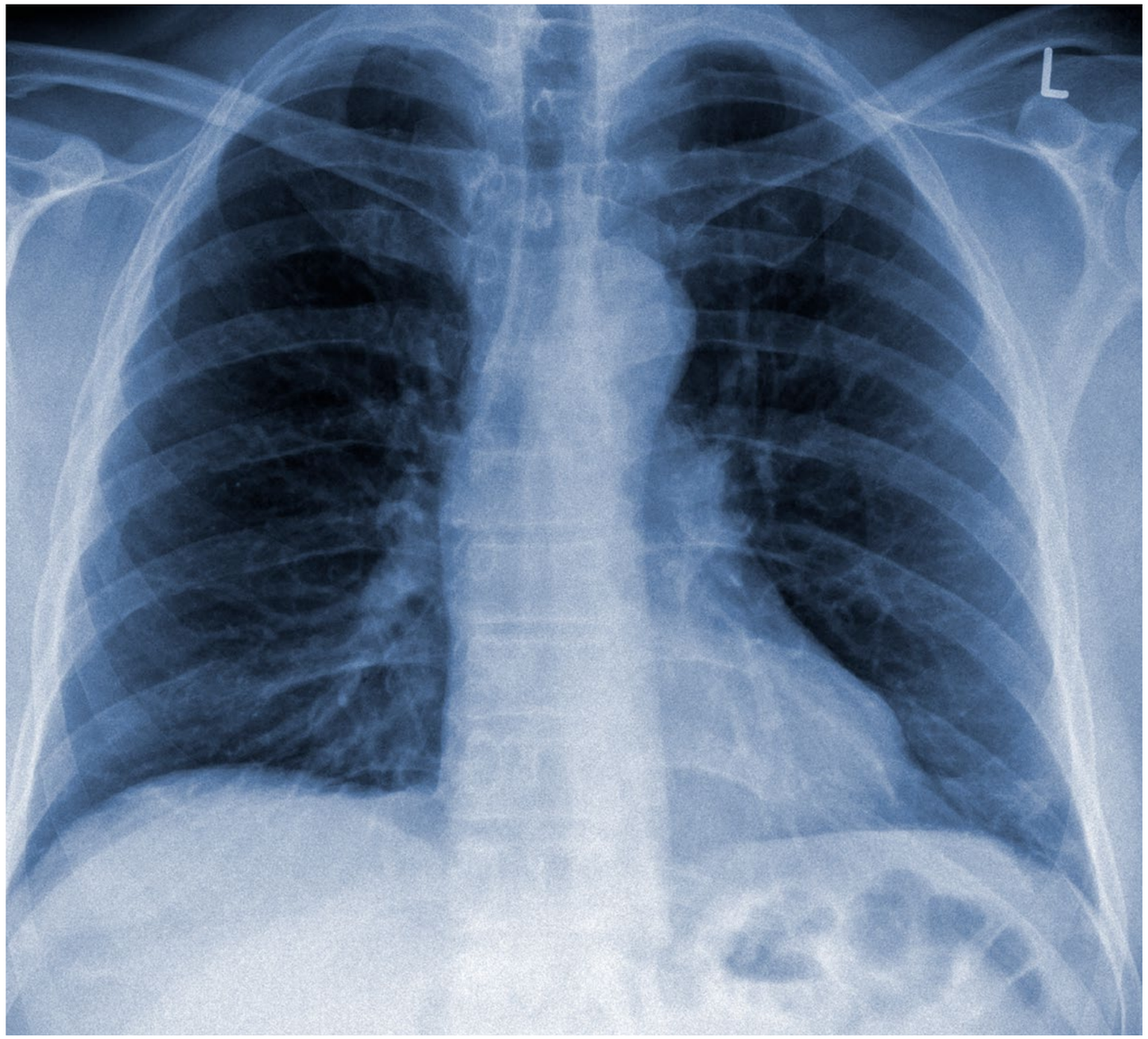
MMPs are enzymes that form part of the endopeptidase subfamily that hydrolyses collagens and elastin. Under normal circumstances, these enzymes control tissue remodelling such as cell propagation and differentiation. MMPs are under tight regulation by cysteine proteinase inhibitors known as cystatins.

Some infections cause an imbalance between MMPs and cystatins resulting in excessive tissue breakdown and immune cell activation at the site of infection. This can lead to unrestrained inflammation resulting in acute lung injury, acute respiratory distress syndrome, and multiple organ failure. Sometimes the inflammation allows pathogens to infect healthy tissue, therefore increasing infection throughout the body.

The restoration of normal cystatin levels, however, has a powerful modulating effect, down-regulating the breakdown of cells and tissues by cysteine proteinases and regulating the immune response so that it does not become over-zealous and trigger excessive inflammation.

CST9

Dr Eaves-Pyles, in conjunction with collaborators Drs Rick B Pyles and Bernard Arulanandam, has been investigating the immunomodulatory effects of cystatin 9, also known as CST9. They have identified it as an effective tool to control the inflammatory process to ensure that it is strong enough to combat infection but not so rigorous that it disrupts tissues.



The team has shown that CST9 not only modulates host immune responses but can directly affect the viability of deadly human pathogens such as *Francisella tularensis*, which causes pneumonic tularemia. Specifically, CST9 disrupts various metabolic pathways of *F. tularensis* affecting its ability to thrive and replicate in the lungs resulting in enhanced killing by macrophages (immune cells that engulf and destroy foreign cells).

To do this, they have employed a variety of techniques. They carried out tests *in vitro* to study the effects of CST9 on macrophages, as well as *in vivo* using various mouse models of infection. In mice treated with CST9, Dr Eaves-Pyles observed modulation of inflammation, decreased lung damage and a lower bacterial load in vital organs following a pulmonary *F. tularensis* infection resulting in improved mouse survival. This suggests that the administration of cystatin 9 can restore crucial immunomodulatory capabilities to the body's immune responses to fight against invading bacteria.

Further work involves the use of a novel human lung model developed by Dr Joan Nichols, which uses rat lungs removed of all rat cells then re-populated with human pulmonary cells. This method provides unique insight into the interaction of CST9 in a human context, rather than in rats or mice. This therefore allows further exploration of the role of CST9 in a more complex environment that is closer to that of the human body.

> **In the US, pneumonia and influenza are the eighth most significant causes of mortality"**

USING CST9 IN TREATMENT

Eaves-Pyles's work has shown that the pathways that mediate inflammation in the immune system are complex. However, she hopes to develop CST9 into a useful therapeutic intervention that will improve the survival of patients suffering from deadly lung infections and diseases.

The restrained inflammatory response mediated by CST9 may be useful therapeutically because it regulates inflammatory responses through key proteins and pathways in a variety of cells such as macrophages. The identification of these key proteins modulated by CST9 could be harnessed to further temper damaging inflammation and used to prevent death from infection and/or sepsis.

FUTURE APPLICATIONS

Other cystatins have been demonstrated to have anti-tumour properties (cystatins C, B and E/M) and protective properties against neurodegenerative diseases such as Alzheimer's (cystatin C). However, less is currently known about the role of cystatins in combatting bacterial infection. Dr Eaves-Pyles' work is therefore extremely pertinent.

Dr Eaves-Pyles' studies have shown that cystatin 9 is a strong candidate ideally suited to develop as a promising therapeutic treatment for deadly lung diseases, which have a considerable health impact worldwide. Her results demonstrate the diverse impacts of CST9 on inflammation and bacterial virulence (e.g., multi-drug resistant *K. pneumoniae*, as well as various other deadly bacterial pathogens) that may prove to be a novel intervention against pulmonary pathogens.

Q&A

What are the relative advantages and disadvantages of the methods you used to investigate the immune response?
Advances in technology combined with a better understanding of the innate immune system have made it possible to dissect different parts of the immune responses, their pathways and mechanisms that are mobilised to fight against bacterial infections. We measure cytokine secretion from infected lung cells using a human and mouse inflammatory cytokine array, which can detect up to 43 different chemokines and cytokines. This type of protein multi-plex array is highly sensitive, quantitative and offers a rapid, high-throughput system that can identify proteins to pursue studies of cell signalling pathways, cell migration, and cell–cell communication and potential therapeutic targets. For our purposes, the mulit-plex array has been a crucial tool in addressing which chemokines and cytokines are modulated by cystatin 9 during infection. We also use flow cytometry in our *in vivo* studies to detect and quantify what population of immune cells infiltrates the lungs following cystatin 9 treatment of bacterial pneumonia and helps track the course of residential infection. The level of immune cell infiltration into the lungs can correlate with inflammatory responses (cytokine array) and tissue damage (lung histology).

However, even with all the advances in technology, one of the best, most straightforward methods to measure the amount of bacteria load is old-fashioned plating to determine colony forming units. Bacterial loads and immune responses can be determined at various time points after cystatin treatment and infection by culturing a portion of the organ homogenates on agar plates and analysing the remainder by cytokine array.

Because of technological advances we are able to generate large amounts of raw data, which can be a challenge, raising more questions than it answers. It is the analysis and interpretation of the data that takes time to allow the data to lead to the next logical step(s). The methods that we utilise in our studies to evaluate the immunomodulatory effects of cystatin against deadly bacterial pathogens allows us to observe individual immune events in a single cell population as well as to elucidate the features of inflammation in multiple cell types that interact and communicate to form responses that are beneficial to the host. It is like pieces of a puzzle; each piece of the puzzle works together to form a complete picture. And although there are limitations and challenges when using experimental *in vitro* and *in vivo* models, such as discordance between findings in experimental models and translation to results in humans; these models are crucial to bridge the gap from bench (basic science) to bedside (clinic).

How does the body fine-tune the inflammatory response to get it just right?
Wow, I wish I had the answer to this question!! This is a simple question but the answer is very complicated and continually evolving. The inflammatory response is a complex system of checks and balances that is a functional network involving numerous components. Therefore, fine-tuning the inflammatory response involves complex interplay between host cells and the products they produce leading to stimulatory and inhibitory signals that require tight regulation. There are many systemic cell types (neutrophils, monocytes), tissue host factors (Toll-like receptors, resident immune cells) and dietary components involved in balancing inflammatory responses.

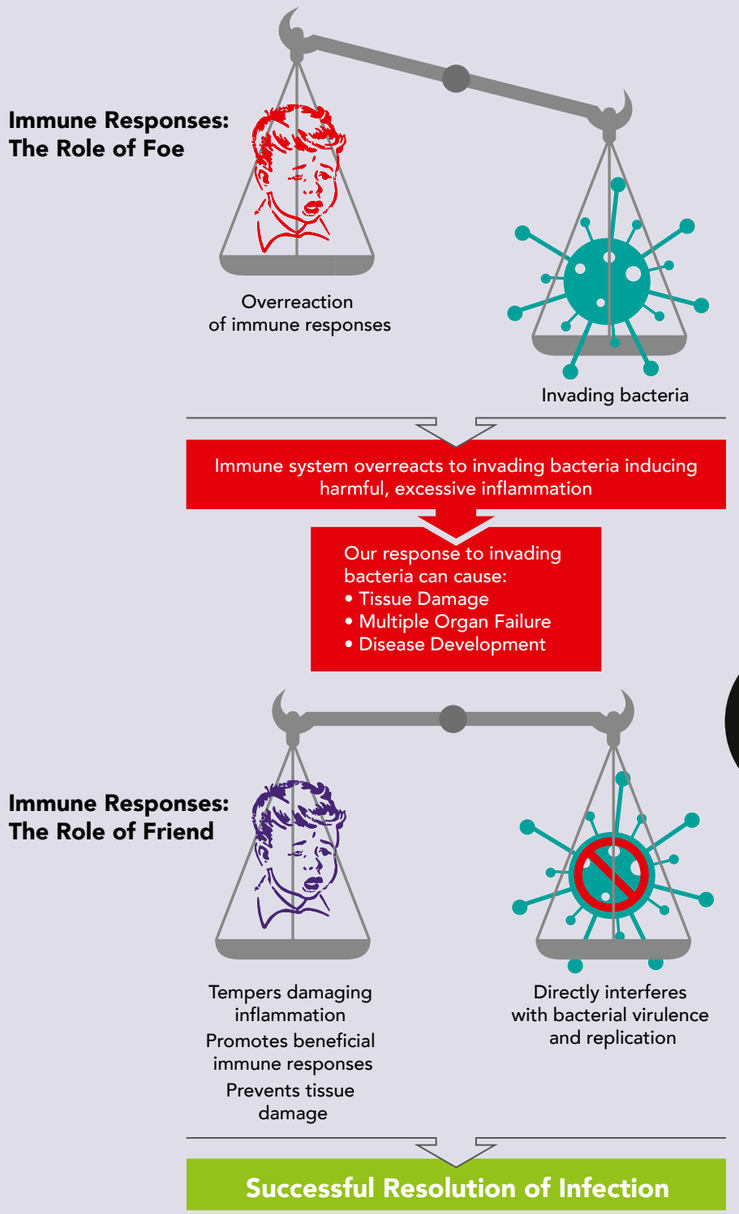
The problem is that invading bacterial pathogens can cause an overactive, prolonged inflammatory response that is destructive to tissues and organs. In fact, typically we are in more danger from our responses to the invading bacteria than the bacteria itself. Therefore, I am striving to harness specific features of the immune response that will prevent excessive inflammation that destroys healthy tissue, and will maintain a balanced, active immune response that can effectively eliminate harmful invaders and preserve tissue/organ integrity. This may represent a realistic opportunity to fine-tune immune responses and design new immunotherapeutic interventions that could profoundly impact various disorders and diseases.

Is cystatin 9 the only cysteine proteinase inhibitor that has this effect?
We have discovered that another of the type 2 cystatin superfamily, alone and in combination with cystatin 9, affords unprecedented protection against multi-drug resistant bacterial pneumonia as well as other deadly pulmonary pathogens. Interestingly, the two cystatins differentially modulate various features of the innate immune system and in combination; they work synergistically to improve morbidity and mortality outcomes in an *in vivo* model of pneumonia.

How easy is it to separate the effects of specific actors in the immune system from one another?
Investigating the immune system in a laboratory setting inevitably presents certain benefits as well as challenges. One model that we employ to investigate specific features of the innate immune response is a respiratory tissue culture model using human lung epithelial and/or immune cells. This model gives us an opportunity to examine the bacterial-induced immune responses and intracellular communication networks of individual cell types in the lungs. We can analyse the signalling pathways and secretions from the same cell type to identify what types of inflammatory cytokines/factors are produced as well as how much they are producing. The level of bacterial-induced inflammation can be correlated with cell damage by microscopy analysis of cell histology. This will allow us to pinpoint harmful, damaging inflammation then attempt to develop therapeutic approaches to restrain it. However, in the intact biological system, cells do not typically function independently of one another but in a complex multi-level interaction network. In other words, it is a team effort to fight against invading pathogens. As such, the "first responder" cells will secrete inflammatory cytokines that can stimulate and activate surrounding cells and call in circulating immune cells to the lung to rise up and fight.

To mimic this cell–cell communication as it occurs in the immune system network, we utilise a co-culture model where immune cells such as human alveolar macrophages, dendritic cells or neutrophils, are added at physiological levels to respiratory epithelial cells during infection. The co-culture system allows us to better understand the biological function and cross-talk between cells in the event of a bacterial invasion.

The advantage of this *ex vivo* human lung model system is that it is controlled and reproducible, revolutionising the ability to test new and existing therapeutic approaches to treat lung dysbiosis caused by pathogenic bacteria.



What is the next step for your research?
The next step of our research is to translate the protective effects of cystatin 9 against pneumonia from our experimental *in vivo* model of pneumonia to a human bioengineered lung model (as developed by our collaborator, Dr Joan Nichols) that will allow translation to drug development in patients.

Detail

RESEARCH OBJECTIVES
Dr Tonyia D Eaves-Pyles' work focuses on pathogen-induced inflammatory responses that cause damage and/or death to the host. Her research investigates ways in which unrestrained immune responses can be moderated to successfully fight against deadly pathogens and avoid damage from excessive inflammation.

FUNDING
NIH: NIAID

COLLABORATORS
- Dr Rick B Pyles (Professor at University Texas Medical Branch, Galveston, TX)
- Dr Bernard Arulanandam (Professor, Assistant Vice President for Research and Director of the South Texas Center for Emerging Infectious Diseases at University of San Antonio, TX)
- Dr Joan Nichols (Professor at University Texas Medical Branch, Galveston, TX)

BIO
Dr Tonyia Eaves-Pyles received her PhD in microbiology from the University of Cincinnati School of Medicine. Currently, she is a tenured Associate Professor in the Department of Microbiology and Immunology at the University of Texas Medical Branch and adjunct faculty at Shriners Hospitals for Children, Galveston, Texas.

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