

Immunostaining of SARS Coronavirus Infected Lung Tissue

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Matthew S. Hayden

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APPROVED:

Daniel Libraty, M.D.

Center for Infectious Disease and
Vaccine Research
Umass Medical Center
Major Advisor

David S. Adams, Ph.D.

Professor, Biology and Biotech
WPI Project Advisor

ABSTRACT

This project focuses on SARS coronavirus infection. Immunofluorescence microscopy was used to locate several factors that could help in investigating SARS pathology. The locations of two cytokines were investigated in relation to SARS CoV infected cells. Infected cells were also stained to determine their cell type. SARS CoV infected cells did not stain positive for macrophage or epithelial cell markers. SARS CoV infected cells were also not producing TGF- β or TNF- α , two prominent cytokines involved with inflammation. These results will be used to design *in vitro* experiments to further examine SARS CoV infection.

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BACKGROUND

Late in the year 2002, Southern China began to see the emergence of an unknown acute respiratory syndrome. By February 2003, there were 305 confirmed cases and 5 deaths resulting from this unknown syndrome. The cases had spread through households and through healthcare workers. Over the next month, the disease began to spread rapidly throughout Southeast Asia. The unknown disease was termed “severe acute respiratory syndrome”, or as it is more commonly known now, “SARS”. The outbreak spread to several other countries through international travel, Canada being the closest to the United States, but the spread was limited. The World Health Organization and Center for Disease Control began researching, and this new disease became of high interest to many scientists around the globe. This MQP focuses on the pathology of SARS, especially the role of cytokines. The data obtained will eventually be used to develop further in vitro experiments with SARS CoV, with the overall goal of determining the pathophysiology underlying SARS CoV infection. With this knowledge it may be possible to rationally develop a vaccine against SARS CoV, preventing future outbreaks like that of 2003.

SARS Coronavirus

Shortly after the outbreak of SARS, the World Health Organization (WHO) setup a global association of laboratories to concentrate on identifying the causative agent for SARS. Early after the outbreak, several sources were cited for the possible reasons for SARS. Some scientists theorized that the SARS outbreak was related to the recent

outbreak of an avian influenza. This was ruled out due to lack of data, and the Chinese reported Chlamydia-like organisms in fatal cases of SARS. This was eventually rejected, as results could not be duplicated outside of China. At this point the WHO began its network of laboratories to search for the cause. The labs frequently met through telephone conferences and virtual communication. In late March of 2003, labs in the United States, China and Germany all had found evidence of a novel coronavirus in the blood of patients with SARS. In April, the WHO confirmed this virus as the cause for the disease.

SARS CoV Identification

To identify the cause of this disease, several tests were run on the blood of an index patient and two of his close contacts. As is standard practice in identifying unknown pathogens, the blood and respiratory specimens were tested by polymerase chain reaction (PCR) with specific primers for several known human pathogens. Some of these included human cytomegalovirus, adenovirus, parainfluenza virus of several strains, *Chlamydia pneumoniae* and several coronavirus strains. General primers for many other common viruses were also used (Drosten, et al, 2003). A random reverse transcriptase PCR (RT-PCR) reaction was run with extracted RNA as well. The results came back negative on all pathogens tested, with a few minor exceptions that were dismissed as possible causes. However, a cytopathic effect was seen on Vero-E6 cells incubated with sputum from the index patient. After one passage of these cells, nucleic acids were isolated from the supernatant. About 20 different distinct DNA fragments were amplified. Most of these turned out to match monkey chromosome sequences, as the Vero cells are obtained from monkeys. However three fragments did not match any

sequence in the database. The Basic Local Alignment Search Tool (BLAST) was used with the translated sequences of unknown origin. The sequence showed homology to the coronavirus amino acid sequences leading scientists to see that a novel coronavirus had been isolated (Drosten, et al, 2003). Two of the fragments were 300 nucleotides in length, and the third was 90 nucleotides (Figure 1).

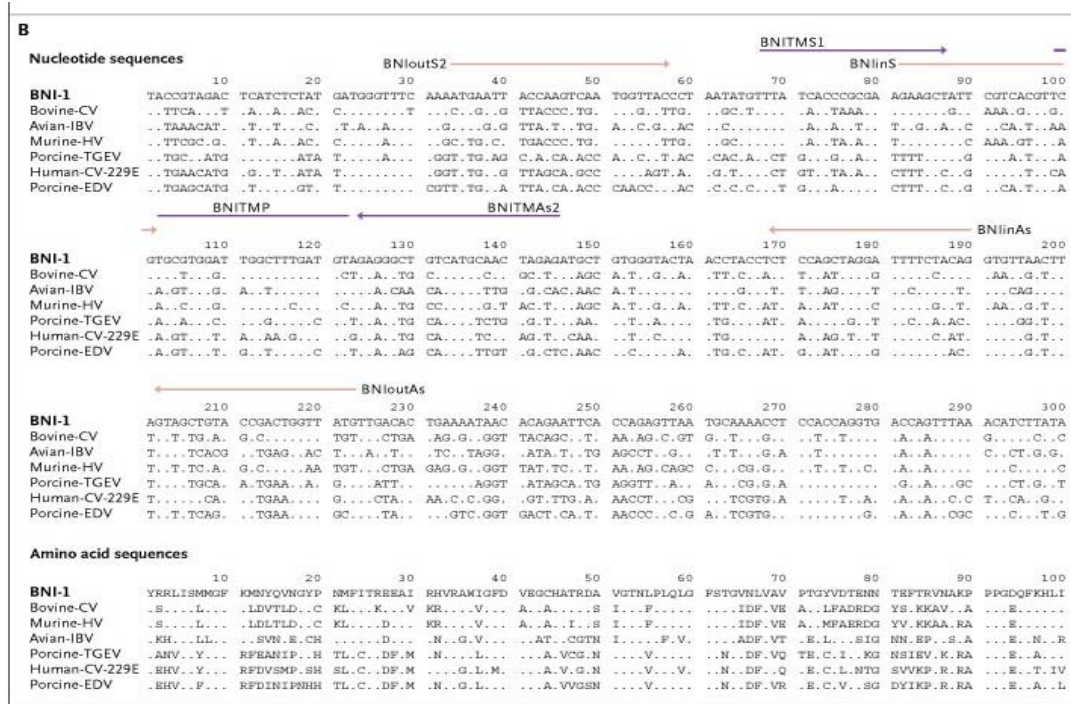


Figure 1: Nucleotide and Amino Acid Sequence Alignment of the Original SARS CoV Fragments with Other Coronaviruses. The top part of the figure shows the nucleotide alignment of fragment BNI-1 with other known coronaviruses. The bottom part shows the amino acid alignment of the same. The nucleotide sequence was translated into one-letter amino acid code. A dot indicates identical sequence to the reference sequence. The primers for RT-PCR (brown) and real-time PCR (blue) are shown and point in the direction of elongation (Drosten, et al, 2003).

Coronaviridae

Coronaviruses belong to the order *Nidovirales* and the family *Coronaviridae*. They are large, single positive-strand RNA viruses. They range in diameter from about 60-220 nanometers. Coronaviruses have the largest genome of any RNA virus, ranging from 27-32 kilobases. Before the identification of SARS coronavirus (CoV), there were

three groups of coronaviruses. Two of these infected mammals and caused anywhere from the common cold to a rare fatal pneumonia in humans. The third group infected birds and was of no harm to humans. However, when SARS CoV was discovered, a fourth group of coronaviruses needed to be added. Upon analysis of the newly discovered SARS CoV, it was seen that genetically, the new SARS virus was not related to the previous human coronaviruses, but rather more closely related to animal strains that infected Chinese civet cats. Civet cats are commonly eaten in Southern China, the proposed origin of the SARS outbreak. It is believed that the virus may have been passed from these cats into the human population, although it has not been scientifically proven.

SARS-CoV Genome

Once this coronavirus was officially declared as the cause of SARS, research began to sequence the genome of this new virus. A Canadian group was the first to sequence the SARS CoV (Marra, et al, 2003), followed two days later by the Center for Disease Control in the United States (Rota, et al, 2003). It was found that the SARS-CoV genome was comprised of 29,727 bases of polyadenylated RNA. The organization of the genome (Figure 2) was characteristic of most coronaviruses, having the following normal gene order: 5'-*replicase(rep)*, *spike(S)*, *envelope(E)*, *membrane(M)*, *nucleocapsid(N)*-3' (Rota, et al, 2003). The *rep* gene comprises two-thirds of the SARS CoV genome. There are four open reading frames (ORFs) downstream of the *rep* gene that encode the 4 structural proteins, S, E, M and N.

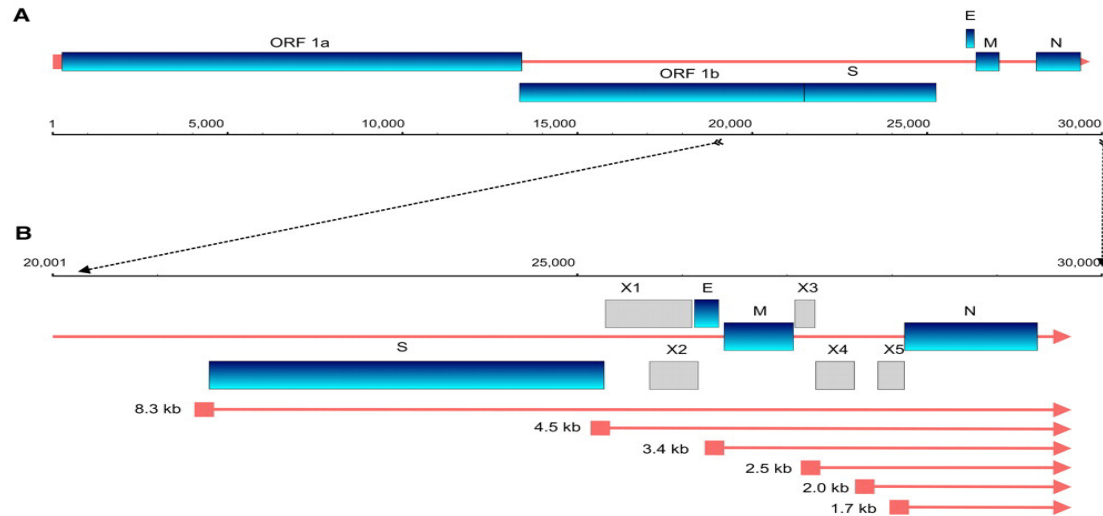


Figure 2: Genome Structure of SARS CoV – This figure shows the organization of the SARS CoV genome. The fragments labeled X represent nonstructural proteins of unknown function (Rota, et al, 2003).

Through more detailed analysis, the SARS CoV genome was determined to be phylogenetically different from the other known coronaviruses. The five major proteins in the genome were compared phylogenetically to the other known proteins for the known coronaviruses. In all cases, the SARS CoV separated into distinct, well-defined branch on the tree. Bootstrap analyses were performed and values above 90% were recorded (Rota, et al, 2003). It was seen that there was greater conservation of the enzymatic proteins like polymerase and helicase, and less conservation of the structural proteins. This information shows that SARS CoV forms its own distinct group within the genus *Coronavirus*. It was also seen that there was very little recombination in the SARS CoV and that it also wasn't a mutant of the previously known strains.

SARS CoV Structure and Function

The SARS CoV is similar in structure to most other coronaviruses (Figure 3). The essential structural proteins, S, E, M, and N all exist and are basically similar. There

are, however, some differences that make the SARS CoV different. These differences are the focal point of research in finding a cure, and even a vaccine for the disease.

The replicase gene of SARS CoV makes up two-thirds of the entire SARS CoV genome. Two ORFs exist that produce two major polyproteins that undergo proteolytic processing to yield the enzymes and proteins needed for viral replication. One of these polyproteins is a chymotrypsin-like protease that is involved in the proteolytic cleavage to yield the resulting replication enzymes. This protease has become a target for possible anti-SARS CoV agents. If this protein is altered or inhibited, the viral replication cannot commence, halting infection.

The nucleocapsid protein of the SARS CoV is another protein that shares very little with other known coronaviruses, in the area of 20-30% sequence homology. In other coronaviruses, the N protein is involved with transcription of the viral genome and packaging of the viral RNA, but these roles have not been defined in the SARS CoV. The N protein also elicits a humoral response in infected patients.

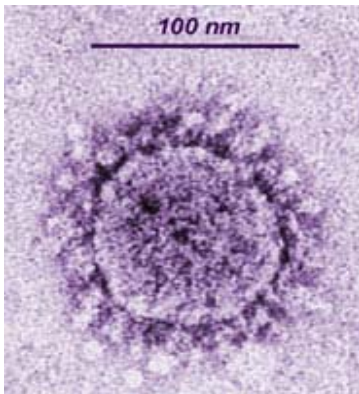


Figure 3: EM Micrograph of the SARS Coronavirus. This electron micrograph shows the coronavirus particle, including the S protein forming the halo, or “crown” on the exterior. Picture from The Big Picture Book of Viruses, http://www.virology.net/Big_Virology/BVRNAcorona.html.

The spike protein of the SARS CoV is the other protein that shows distinct differences from other coronaviruses. The S protein is divided in two subunits, S1 and S2. S1 is responsible for cellular receptor recognition, and the S2 subunit is responsible

for cellular fusion, or virulence. The S protein is very little conserved compared the other coronaviruses, about 20-27%, as seen in Figure 4 (Rota, et al, 2003). Due to this fact, the comparison of primary amino acid sequences gives very little information on the receptor binding specificity or the antigenic properties of SARS CoV. However, the fact that the SARS CoV S protein is known to be the primary protein responsible for viral entry, experiments were conducted to determine possible cellular receptors. Analysis of protein isolated by immunoprecipitation with SARS CoV infected Vero E6 cells showed sequence analysis with human angiotensin-converting enzyme 2, or ACE2 (Li, et al, 2003). This protein's cellular localization and distribution made it an appropriate receptor for the SARS CoV.

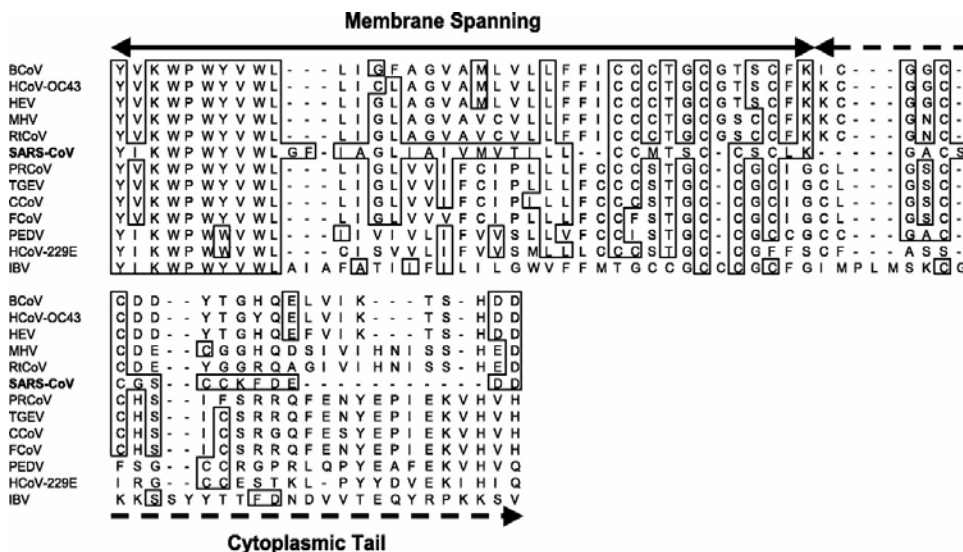


Figure 4: Conserved Regions of the SARS-CoV S Protein. This figure shows the regions of the S protein of SARS-CoV compared with other known coronaviruses. It can be seen that the membrane spanning region is fairly similar, but the cytoplasmic regions differ greatly. Rota et al, 2003.

Clinical SARS

SARS is very difficult to identify, especially in the winter months due to the rise in flu and common cold cases. Some of the symptoms include fever, chills, headache, malaise, cough and other fairly common symptoms. However, as the SARS CoV infection progresses, the symptoms worsen and intensify, leading to leukopenia, or low

white blood cell count, T-Lymphopenia, or low T-cell count and even acute respiratory distress, where the lungs become inflamed and fluid collects in the alveoli.

Several factors occur in the pathology of SARS. One large event is exudation of protein containing fluid in the lungs. The lungs become “leaky” with an inflammatory liquid that threatens pulmonary gas exchange. The pathology is that of typical diffuse alveolar damage (DAD). There are three main phases of DAD: the exudative phase, proliferative phase and the fibrotic phase. The exudative phase begins 4 to 7 days after onset, leading the lungs to become heavy and the pulmonary capillaries become congested. The proliferative phase, beginning 1-3 weeks post onset, shows organized inflammation and early fibrosis. Lastly, the fibrotic phase, 3-4 weeks after onset, shows complete lung remodeling by sparsely cellular collagenous tissue.

Several methods are used to determine if the SARS CoV is the cause of infection. One is the use of RT-PCR to detect viral mRNA in the nasal aspirate of patients. This procedure is effective, although it requires some time. The easier method of determination is serological assays. Recently, antibodies have been characterized against the S and N proteins of the SARS CoV (Tan, et al, 2004). Some data from this paper show that all convalescent-phase sera showed reactivity with the S protein antibody. However, sera from the acute phase (2-9 days post infection) showed no reactivity, suggesting that the N protein antibodies are present earlier in the humoral response (Tan, et al, 2004). It has been seen that DNA immunization with N protein from a porcine coronavirus induced immunity by T-cell activation and memory (Liu, et al, 2001). This data shows that not only can these antibodies be used to detect SARS CoV infection earliest, but can also be a method of building a strong, early immune response.

The Immune System

To better understand the way SARS CoV affects the human body, one must understand the human immune system. This system is very complex with the main goal of destroying invading pathogens to prevent illness. There are two systems involved in response to an invading pathogen: innate immunity and adaptive immunity.

Innate Immunity

Innate immunity is what every human is born with as a first line of defense for preventing illness. On the large scale there are several barriers in place to prevent entry of foreign pathogens. First and foremost, the skin cannot be penetrated unless it has an opening, such as a nick, cut or scratch. Secondly, the natural actions of sneezes and coughs are methods of expelling living and non-living things from the respiratory system. Mucus is another line of defense, catching bacteria and pathogens in the nasal and respiratory tracts. Lastly, many of the body's secretions and fluids are acidic and prove to help destroy bacteria that are not welcome on the skin and in certain areas in our body. Sweat, tears, saliva and stomach acid are a few fluids that help protect from invading bacteria and pathogens.

On a smaller scale, there are many cells inside our body whose sole purpose is to destroy foreign material. Every cell in our body has a marker called the major histocompatibility complex. This is the body's way of determining self and non-self. Any cell that does not display this marker is considered non-self and attacked. The innate immune system has no specific memory and does not process foreign antigens in what is

a rapid early response to infection. Cells called phagocytes are responsible for adhering, engulfing and ingesting foreign bodies. Monocytes, or macrophages, flow through our blood stream and can even exit the blood to move towards an infection site to destroy invading bacteria. Other cells called natural killer cells, or NK cells, flow through the blood and lymph systems destroying cancerous and viral containing cells. Neutrophils are short-living white blood cells that are usually the first to arrive at an infection site.

Adaptive Immunity

Adaptive immunity is a complex system with a main goal of mounting a specific response against specific antigens. The two major cells involved with adaptive immunity are the T-cell and the B-cell. Both these lymphocytes flow through the blood and lymph systems. They perform two types of immunity: cell-mediated and humoral immunity.

Cell-mediated immunity involves macrophages and other antigen-presenting cells like dendritic cells and even B cells. The macrophage will engulf foreign antigen through endocytosis. The antigen will then be inactivated through enzymatic processing. Enzymes will then move a small part of the antigen to the macrophage surface, presenting it to the T-cells. This sensitizes the T-cell to recognize these antigens and mount a memory response. Other cells called cytotoxic or killer T-cells release lymphotoxins that cause cell lysis. Helper T-cells mediate this process by directing the immune response.

The humoral response is a bit more complicated. When an immature B-cell is presented an antigen, it becomes sensitized and undergoes clonal selection. It divides and most of them become plasma cells. After an initial lag, these cells begin to produce

antibodies at a rate of up to 200 per second for 4-5 days. Antibodies are soluble proteins that inactivate antigens by several methods: attaching to antigens and causing cell lysis, blocking antigen-binding sites, causing aggregation and precipitation. There are many forms of antibodies and their exact methods have been detailed, although they are not a major point of this paper.

B-cells that do not become antibody-releasing cells can become long-lived memory cells. These cells, along with the memory T-cells form the main point of the adaptive immunity: immune memory. If by chance the same antigen is found in the body after an initial infection, the second response will be much faster and much stronger than the initial response. This is a quality unique to the adaptive immune system.

This system provides the basis for prophylactic vaccination. The point of vaccines is to introduce an inactivated, attenuated antigen to the body. By doing this, the body will mount an immune response to this antigen. Therefore, when the true infectious antigen enters the body, it will elicit a much quicker immune response and the infectious agent will be destroyed quickly. In theory, this concept seems like a “cure-all”, but in reality, the formation of an effective vaccine proves to be a difficult task.

Cytokines

The cellular components of the immune response are not the sole messengers during infection. Small, secreted proteins called cytokines are used as messengers during the reaction to an infection. These proteins are responsible for the regulation of immunity, inflammation and hematopoiesis. Cytokines act by binding to specific membrane receptors and, with the help of secondary messengers like tyrosine kinases,

alter the gene expression of the target cell. Some responses to cytokines include an increase or decrease in certain protein production, proliferation of the cell or secretion of effector molecules. There are many types of cytokines, each triggering different responses from the target cells. However, more than one cytokine can elicit the same response from a cell and also the same single cytokine can exhibit pleiotropism, meaning it can have many functions. They also often work in a cascade, with one cytokine triggering the response of more cytokines and so on.

There are many different types of cytokines. The interleukin class of cytokines is made by leukocytes and act on other leukocytes. The interferon class, more specifically IFN α and IFN β act on viral infected cells, halting viral replication. Two cytokines are the principal focus in this experiment. They are the transforming growth factor beta (TGF- β) and tumor necrosis factor alpha (TNF- α).

Transforming Growth Factor Beta

Transforming growth factor-beta, or TGF- β , is a gene superfamily of structurally and functionally related proteins. There are three different isoforms, the effect of each dependent on the conditions of the target cell and other growth factors present. It's functions include signaling cellular proliferation *in vitro*, but is also generally considered as an inhibitor of proliferation and promoter of differentiation (Choi et al, 1993). It has also been shown to block or initiate cellular differentiation and migration, depending on the surrounding conditions and the conditions of the target cell. As one can see, the roles of TGF- β are very broad and are all dependent on surrounding conditions.

One function of TGF- β is the role it plays in vascular remodeling. TGF- β 1, which is the predominant isoform, can inhibit the activity of other angiogenic factors in endothelial proliferation, and can stimulate the production of extracellular matrix proteins and proteinase inhibitors (Pepper, 1997). However, the cytokine shows a biphasic effect on angiogenesis. When TGF- β concentration is low, there is an increase in the vascular invasion of cultured endothelial cells that were stimulated by other angiogenic factors. When the concentration of TGF- β is high, there is a decrease in such invasion (Pepper, et al, 1993).

The restoration of an epithelial layer after damage is important to maintain a healthy respiratory tract. TGF- β 1 and TGF- β 2 have been studied in relation to their role in this repair. It has been seen that both isoforms were converted to their active forms 2 hours after wounding, and TGF- β 1 speeds up the process of epithelial repair (Howat, et al, 2002).

Human carcinomas also have ties to TGF- β . Cancer cells lack the signal to divide or die when they become too large. The growth is uninhibited. TGF- β is one of the cytokines that signal a cell to stop growing or to lyse. This is why most carcinomas are characterized by their lack of normal growth-inhibiting and apoptotic responses to TGF- β (Reiss, 1997). The roles of TGF- β are many, and as the molecular and cell biology is relatively well known, the signaling pathways are just beginning to be discovered.

Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha, or TNF- α , is also a cytokine that is produced in a variety of cells, but especially by macrophages. Like TGF- β , this cytokine has many

functions in the body, most not fully understood yet. TNF- α is released by stimuli such as exposure to bacterial toxins, viruses such as HIV and influenza, fungi, parasites and other cytokines. TNF- α also appears to possess a self-regulating ability as well. The scope of its functions is too broad to cover completely, so instead a few main functions will be discussed.

Strictly by seeing its name alone, one can realize that this cytokine has a tumor suppressing quality. In fact, it was first isolated in 1975 during an attempt to identify the cause for tumor necrosis in the sarcoma Meth A (Carswell, et al, 1975). However, in some conditions, TNF- α been known to promote growth in certain tumor cells. High levels of TNF- α have been linked to increased mortality in tumor patients in some circumstances (Rink and Kirchner, 1996). There are also other functions of this cytokine that do not involve tumors or cancer.

One negative aspect of TNF- α is its involvement in septic shock syndrome. Sepsis is the presence of bacteria or other invading organism and the toxins that are involved with the infection. It was once believed that solely the invading organisms and their toxins caused the syndrome. Now it is seen that the cause of the syndrome may be all due to the overproduction of cytokines, primarily TNF- α . This is because TNF- α is acutely overproduced during extreme sepsis and administration of TNF- α itself, regardless of sepsis, causes shock and tissue injury (Tracey and Cerami, 1994). This and other data show that septic shock can be associated to TNF- α and not sepsis. When TNF- α is removed from the system, septic shock does not ensue even with the existence of bacteria in the bloodstream (Tracey, et al, 1987).

One positive affect of TNF- α is its role in the inflammation response. It has been shown that TNF- α activates leukocytes, enhances the adherence of neutrophils and monocytes to the endothelium, induces fibroblast proliferation and triggers the release of other pro-inflammatory cytokines. These beneficial functions contribute to the localization of infection and the increase of tissue remodeling. It can be presumed that these features, not the negative ones that have been mentioned, led to the conservation of the TNF gene through evolution (Tracey and Cerami, 1994).

TGF- β and TNF- α in viral replication

TGF- β is also known to affect viral replication, although it has never been tested on SARS CoV specifically. As an anti-inflammatory cytokine, TGF- β acts by inhibiting inflammation of tissue, and therefore would help the virus by not clearing it from the system. This would be a positive affect on viral replication. However, a study was conducted on tuberculosis-induced HIV infection and it was shown that *in vitro*, TB induced HIV showed high levels of pro-inflammatory cytokines like interleukins 2, 6 and 8 (Goletti, et al, 1997). It was then found that the addition of anti-inflammatory cytokines like IL-10 and TGF- β inhibited TB induced HIV replication. In this model, TGF- β inhibited the viral replication by decreasing cellular proliferation, and by decreasing the endogenous pro-inflammatory cytokines (Goletti, et al, 1997). This is proof that the effect of cytokines on viral replication is dependent on circumstances regarding the specific virus.

TNF- α affects viral replication in different ways. This cytokine has several affects on viral replication and could be looked at as a “dual-edge sword”. This means

that TNF- α can affect viral replication in a positive or negative way, depending on the circumstances during replication. As a pro-inflammatory cytokine, TNF- α promotes inflammation of tissue and promotes the clearance of virus (Haagmans, et al, 1994). However, it has been shown that TNF- α can also promote viral replication of rat CMV under certain conditions (Haagmans, et al, 1994). This shows the pleiotropism of TNF- α and its affects on viral replication.

TGF- β and TNF- α in Acute Lung Injury

Acute lung injury, or ALI, is a devastating disorder with a mortality rate of 30-40%. There is no known cure. The disorder is characterized by flooding of the alveolar spaces with a protein-rich exudate that impair the pulmonary gas exchange. This pathology is the beginning of diffuse alveolar damage, as mentioned before. ALI can follow many complications like sepsis, toxic inhalation and even SARS.

TGF- β has been shown to play a critical role in the resolution of tissue injury in many organs, including the lungs. It has been most evaluated in the late phases of ALI where it plays a major role in pulmonary tissue fibrosis. However a study showed that TGF- β may be active earlier, even up to 2 days after the induction of the injury. It was reasoned that TGF- β may be active much earlier and play a larger role in the development of the pulmonary edema that distinguishes the disorder (Pittet, et al, 2001). Results showed that TGF- β was activated by an integrin (α V β 6) in the lungs and skin. Transgenic mice that lacked this integrin were protected from pulmonary edema in a model of ALI. The results from this study show that the integrin-mediated activation of TGF- β is crucial in the formation of the pulmonary edema in ALI. Blocking the TGF- β

or its activation could protect from the formation of the edema and overall the induction of ALI (Pittet, et al, 2001). Though TGF- β is generally considered to be critical in the resolution phase of lung tissue injury, inhibiting inflammation and enhancing scarring, it also was discovered that it also may play a role in alveolar flooding, a pathology of ALI (Pittet, et al, 2001). By inactivating TGF- β , complete attenuation of alveolar flooding was achieved in a murine model, which could be insight into possible therapeutic devices for ALI (Pittet, et al, 2001).

Acute lung injury after hemorrhagic shock (HS) has long been associated with TNF- α levels in the lung. However the exact role the cytokine played was unclear. To establish a clearer view of TNF- α function during ALI after HS, a study was performed to determine the relationship between pulmonary TNF- α and neutrophil accumulation and lung leak. Upon induction of HS in mice by removal of 30% of their total blood volume, a slight increase was seen in TNF- α (Song, et al, 2001). However, neutrophil accumulation preceded this increase. Normally, the neutrophil increase would follow an increase in TNF- α , as the cytokine is an inducer of neutrophil activity. A knockout mouse was produced that lacked TNF- α . The neutrophil buildup and lung leak were eliminated completely, but restored in a knockout mouse supplied with a recombinant TNF- α plasmid (Song, et al, 2001). The lung leak and neutrophil accumulation were also prevented in mice that lacked the p55 TNF- α receptor (Song, et al, 2001). Even with the small amounts of TNF- α restored by plasmids, the neutrophil buildup and lung leak, which are key components of ALI, were again detected. These results show that a low level of pulmonary TNF- α is adequate to induce ALI during hemorrhagic shock.

SARS and Cytokines

Severe acute respiratory syndrome has proven itself a very dangerous viral infection. It can spread very quickly, similar to the flu, through close contact with an infected individual. It also can cause death if not treated promptly and correctly. The containment of the outbreak in late 2002 and early 2003 was crucial in stopping the spread of this virus. Now, we have more time to study this new virus and determine what exactly occurs with an infection. This information has not been clearly established and is of much interest to many people around the world.

One direction in studying the pathology of SARS is to observe cytokines and determine their function during infection. As has been established in the previous sections, cytokines are key mediators in immune responses. Determining the roles of cytokines like TNF- α and TGF- β in a SARS CoV infection could lead to the rational development of therapeutic interventions or vaccine strategies. It has been established that during a SARS CoV infection, the levels of TGF- β and TNF- α in the blood do fluctuate more than normal (Zhang, et al, 2004). With that and the previous information given, it can be presumed that these cytokines do play large roles in a SARS CoV infection. If we can look at the cytokine patterns and accumulation *ex vivo* in autopsy lung tissue, then experiments can be designed for use *in vitro*. Then, if the effects of TNF- α and TGF- β can be assessed *in vitro*, an area of focus could be established for experiments *in vivo*.

PROJECT PURPOSE

The background information given above now allows a statement of the purpose of this project. The overall goal of biologists studying SARS CoV is to create a vaccine or an effective cure that will either eliminate the virus from the human population, or make it easier to detect and cure it. The biggest problem with this goal is that the pathogenesis of SARS CoV infection is highly unclear. We must first investigate the SARS CoV infection to find points of interest for vaccines. In other words, we must start at the bottom of the ladder and work upwards. This MQP project started at the bottom of the ladder, and investigated the levels of two cytokines (TNF- α and TGF- β) and their location in lung tissue from two fatal cases of SARS. The project also investigates what types of cells the SARS virus is infecting. Immunofluorescence microscopy was used to show where these cytokines are located in relation to SARS CoV infected cells, as well as what types of cells the SARS CoV was infecting. With this information, experiments can be created for use *in vitro* with live virus applications to move further towards a vaccine or cure.

METHODS

Lung Samples

The human lung tissue sections used in this project were from two fatal cases of SARS from the Philippines. The sections were cut at 4-5 microns and fixed in formalin prior to their use in this study. They were then embedded in paraffin and mounted on slides.

Immunoperoxidase Staining

To visualize which cells contain TGF- β and TNF- α in the fixed tissue, immunoperoxidase staining was used. The general procedure from the Vectastain ABC kit for immunohistochemical staining was followed. The slides were deparaffinized in xylene and rehydrated in a graded alcohol series. The slides were then immersed in 0.01 M citrate buffer solution and heated for 10 minutes as an antigen retrieval step. The primary antibody used was a mouse derived anti-human TGF- β from Serotec. The control primary antibody used was a mouse purified IgG isotype control. The anti-cytokine and isotype control were diluted in phosphate buffered saline (PBS), to a final concentration of 0.33 $\mu\text{g/mL}$. Slides were incubated for 2 hours in primary antibody, followed by a 30-minute incubation in a biotinylated secondary antibody supplied in the ABC kit. To visualize, a diaminobenzidine (DAB) solution was used, and slides were incubated for 7 minutes with the DAB. The slides were then counterstained with hematoxylin. A graded alcohol series was used to dehydrate the slides, and then a xylene based mounted media was used to mount for viewing.

Single Color Immunofluorescence Staining

The procedure for immunofluorescence staining was derived from several sources, including personal communication with researchers who have performed the procedure before. As in immunoperoxidase staining, the slides were deparaffinized, rehydrated and treated with the citrate buffer solution to bring antigens to the surface of the cells. The slides were then incubated for 20 minutes in diluted normal serum, derived from a goat, the same species from which the secondary antibody was derived. The purpose of this step is to prevent any non-specific staining. The slides were then incubated for 2 hours in primary antibody. This was followed by a 30-minute incubation in a secondary antibody which was conjugated to a fluorophore. Several washes in PBS were performed between each step in the procedure to wash away previous solutions. The slides were then mounted with an aqueous mounting media for viewing.

Autofluorescence Control

To solve autofluorescence in the tissue at emissions around 488 nanometers, a 30 minute incubation in 0.25 % Sudan Black B solution was used. This step was inserted in the general procedure for immunofluorescence staining immediately before the mounting step. This incubation significantly reduced autofluorescence in the tissue when observed at 488 nanometers (Baschong et al, 2001).

Antibodies and Dilutions

When staining for the S-protein of the SARS coronavirus, an antibody raised in rabbit was used and normal rabbit serum was used as an isotype control, both obtained from a local lab at UMass Medical School. Both antibodies were used at a 1:500 dilution. To detect TNF- α , a goat IgG biotinylated anti-TNF- α was obtained from R+D Systems. This antibody was used at a concentration of 7.5 micrograms/mL. The anti-TGF- β antibody was raised in mouse and obtained from Serotec. This antibody was used at a concentration of 0.33 micrograms/mL. All cell surface marker antibodies (CD68, EMA) were raised in mouse and obtained from Dako. The antibodies against CD68 and EMA were used at a 1:25 dilution.

Secondary antibodies conjugated to different fluorophores were obtained from Molecular Probes Inc., all of the AlexaFluor variety. AlexaFluor goat anti-rabbit 488 (green) was used to visualize the S-protein. AlexaFluor streptavidin 647 (deep red) was used to view the biotinylated TNF- α and TGF- β . All secondary fluorophore antibodies were used at a dilution of 1:250, which was obtained through performing a dilution series of the secondary antibody and determining the lowest dilution that gave no background staining.

Two Color Immunofluorescence Staining

When two color immunofluorescence staining was performed, the same protocol as single color staining was followed with one slight alteration. During the primary and secondary antibody incubations, the antibodies for all three desired antigens, S-protein, cytokine and cell surface marker, were mixed together in the same solution. Since all

antibodies were raised in different species, there was no foreseen interaction in the incubating solution.

RESULTS

The first step in determining the relationship between SARS CoV infected cells and the cytokines in question was to prove that these cytokines exist in the infected tissue. Once that fact was established, immunofluorescence staining was used to show the location of SARS CoV infected cells in relation to the cytokines. Lastly, the tissue was stained for cell surface markers to add a third aspect of location.

To determine the existence of TGF- β and TNF- α in the infected tissue, simple immunohistochemical staining was used. First, negative control slides were run using a purified mouse IgG isotype control antibody (Figure 5 A-D). As expected, the resulting slides showed no positive staining, with all tissue and cells staining blue.

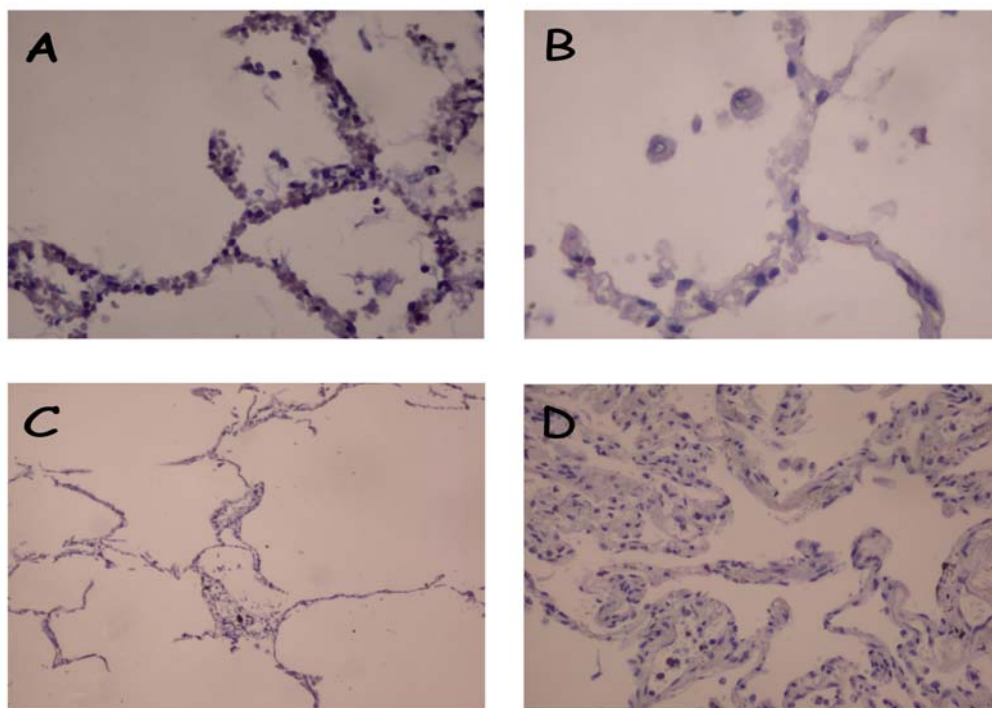


Figure 5 – Negative Controls for TGF- β and TNF- α – Panels A and B are the controls for Case A TGF- β and TNF- α , respectively, at 400x magnification. Panels C and D are the controls for Case B TGF- β and TNF- α , respectively. Panel C is at 100x magnification and Panel D is 400x magnification. There is no positive brown stain in any of the tissue or cells.

The tissues were then labeled with anti-human TGF- β and TNF- α antibodies and visualized using peroxidase staining and DAB (Figure 6). The resulting positive stain is brown against the counterstain color of blue. For both cytokines, there was definitive positive staining in the tissue and some isolated cells (Figure 6). It is clear which cells have stained brown, as the blue counterstain can be seen in some tissue and cells which are negative for cytokine.

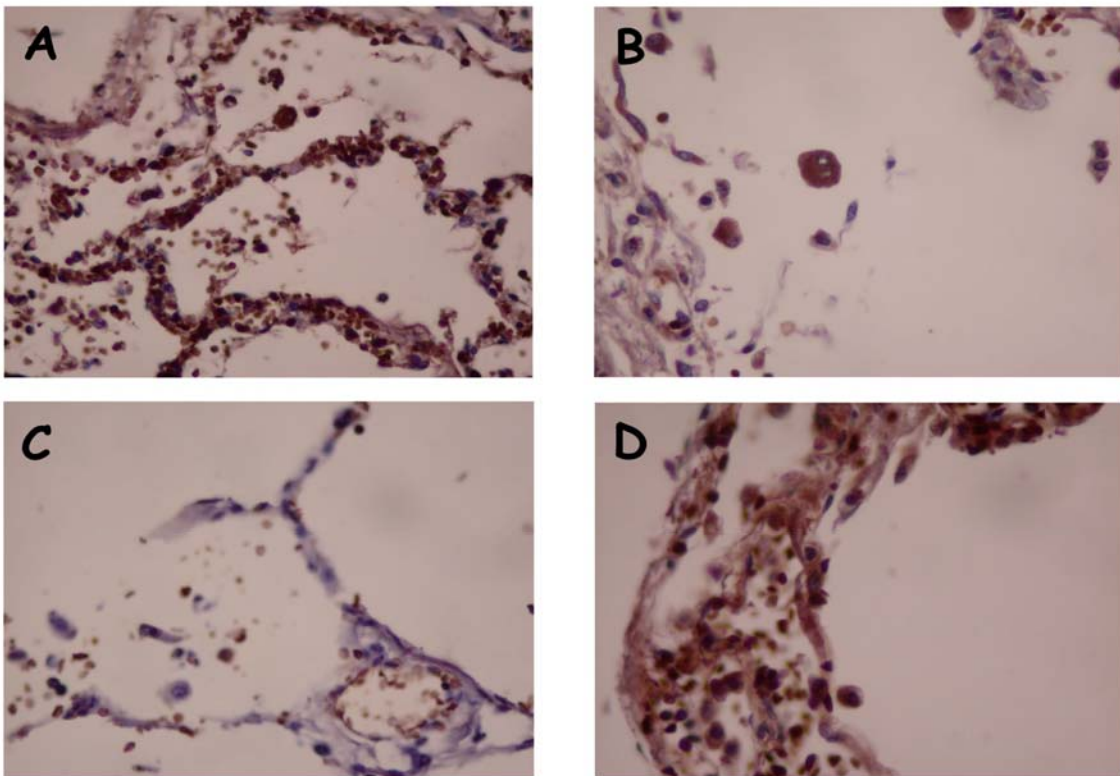
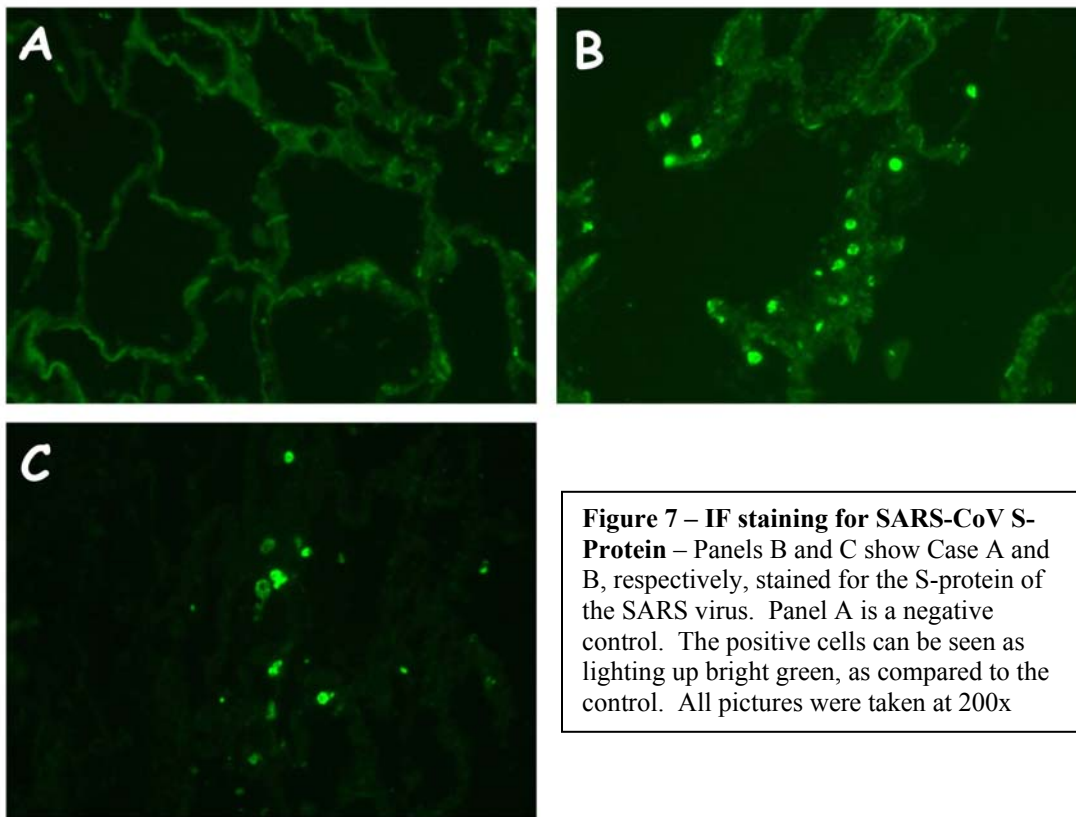


Figure 6 – Positive Stain for TGF- β and TNF- α – Panels A and B are Case A tissue stained for TGF- β and TNF- α , respectively. Panels C and D are Case B stained for TGF- β and TNF- α , respectively. All panels are 400x magnification. The brown stain can be seen in all samples, showing that the cytokines are present in the infected tissue.

TGF- β staining (left panels) will stain TGF- β positive cells, as well as blood platelets and red blood cells. Some of the positive staining observed in Figure 6 is this blood factor staining, but definitive large cells, most likely macrophages, are staining

positive as well as positive stain in the interstitium. The staining for TNF- α (right panels) also shows distinct, round cells staining positive as well as TNF- α in the tissue.

The tissue was ready to be analyzed with immunofluorescence microscopy using one, two and three colors. First, an anti-human antibody against the SARS CoV S-protein, raised in rabbit, was obtained and the tissue was stained (Figure 7).



To visualize the S-protein, an anti-rabbit secondary antibody that was conjugated to a fluorescent dye was used. The Alexa 488 dye was similar to fluorescein isothiocyanate (FITC) and exhibited a bright green color (panels B and C) under the correct filter of fluorescent light. Therefore, the positive cells would light up green under the microscope. There was a problem with autofluorescence of the lung tissue itself. An incubation in Sudan Black B dye was used to lessen the autofluorescence, but the tissue

still stained green. The positive cells, however, glowed a much brighter and more intense green; so distinguishing positive from negative was relatively easy. It was seen that in both Case A and Case B tissue, there was positive S-protein staining (Figure 7). Case A tissue was well intact structurally (Figure 7 Panel B), but Case B showed a deteriorated lung structure, so some of the pictures are less clear (Figure 7 Panel C). This case was much more hemorrhagic in nature and caused more lung damage than Case A.

The next step in the process was a double stain for the SARS CoV S-protein and the cytokines. This localizes the cells positive for SARS CoV and the cells that were producing cytokines. First a stain was performed using the S-protein antibody and a mouse anti-human TGF- β antibody. The S-protein was labeled with green again (Figure 8, B and D). and the TGF- β was labeled with Alexa 647, a deep red color (Figure 8, A and C). The TGF- β isotype mouse control (Figure 8 A and B) showed no staining. When stained alone, the TGF- β produced an expected signal (panel C). The same frame for the single TGF- β stain was also used for the S-protein picture. These two pictures were then digitally combined to give a two-color picture showing both objects of interest (Figure 8 D). Using both pictures, it can be seen that most of the SARS CoV infected cells do not show positive for TGF- β .

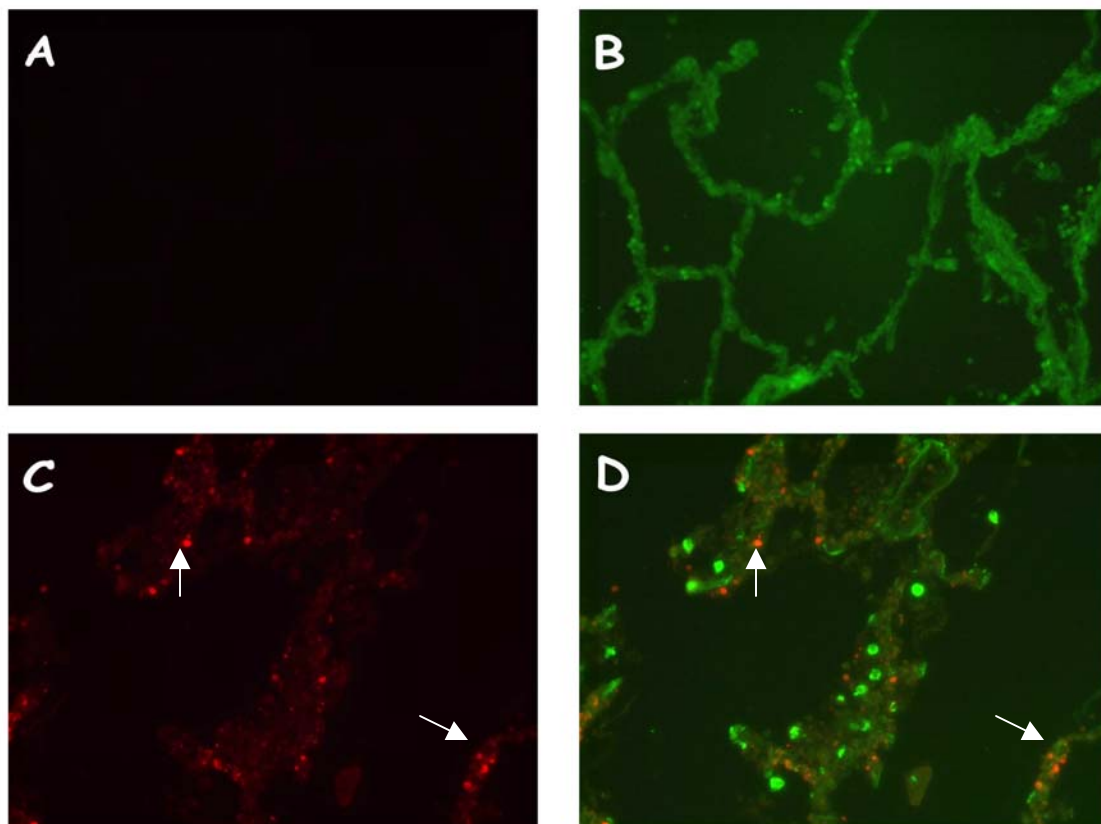


Figure 8 – IF double color stain for S-protein and TGF- β on Case A – Panels A and B are the negative controls for both mouse (red, TGF- β) and rabbit (green, viral S-protein). Panel C shows TGF- β stain only. Panel D is the double color showing both S-protein and TGF- β . The arrows indicate a special subset of viral infected cells that express TGF- β .

The pictures for TNF- α (Figure 9) show similar results. The TNF- α was stained using a mouse anti-human TNF- α antibody. This was then visualized using AlexaFluor 555 secondary antibody. The negative control for TNF- α (Figure 9 A) showed no staining as expected. The TNF- α stain alone (Figure 9 B) showed positive stain in the tissue as well as some cells, as expected based on the IHC results. The S-protein staining showed definitive positive cells, as in previous results (Figure 9 C). The double color stain (panel D) showed that most SARS CoV infected cells did not have positive stain for TNF- α . The white arrows (Panels B and C) show cells that stain positive for TNF- α but

not for S-protein. The yellow arrows in the same panels indicate cells positive for S-protein, but not TNF- α .

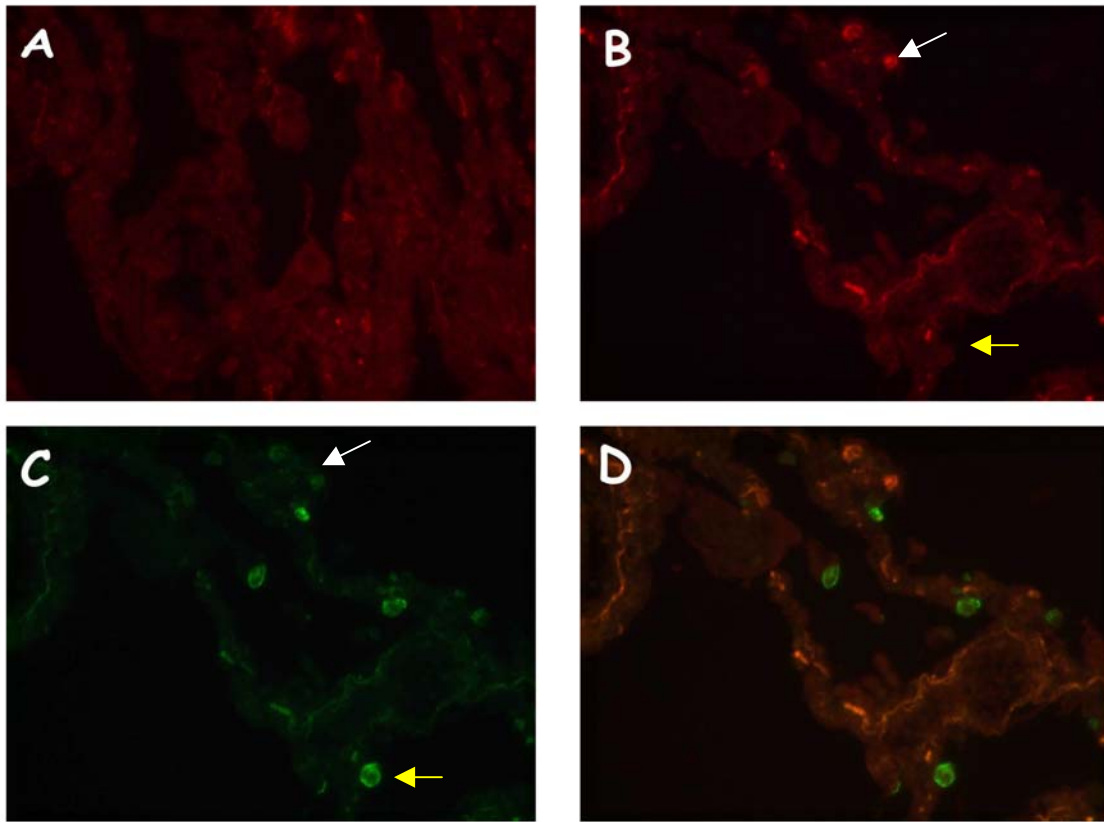


Figure 9 – IF double color stain for S-protein and TNF- α on Case B – Panel A is the negative control for goat IgG (red, TNF- α). The isotype control for rabbit IgG also showed very little background (data not shown). Panel B is a TNF- α stain only. Panel C is a stain for S-protein only. Panel D is the double color stain for TNF- α and the S-protein. The white arrow indicates cells positive for TNF- α and negative for S-protein. The yellow arrow indicates a large cell positive for S-protein, but not TNF- α .

To more clearly illustrate where the S-protein is being detected, an antibody against cell surface markers was used. The assumption was that the S-protein, and therefore SARS CoV, would be most prominent in the macrophages of the lung. To detect macrophages, an antibody against the marker CD68 was used (Figure 10). A mouse control antibody yielded no positive staining (Figure 10 A). There was a certain amount of background fluorescence, but the positive stain could be clearly distinguished. The rabbit control used for the S-protein also showed no positive stain, with a small

amount of background fluorescence (data not shown). First, a picture showing just CD68 positive cells staining blue was taken. A few cells were found that were illuminated more brightly than background, and upon further investigation, were determined to be macrophages (Figure 10 B). Next, the same frame was used to take a picture showing only S-protein positive cells, staining green as before (Figure 10 C). The two were then combined to show both interests in the same frame (Figure 10 D). By comparing the two single color pictures, it is clear that no cells show two colors. In other words, one color is not covering another in the combined color picture. This pattern held true throughout all the slides examined with this stain. This data shows that the S-protein, therefore SARS CoV, is not contained in macrophages.

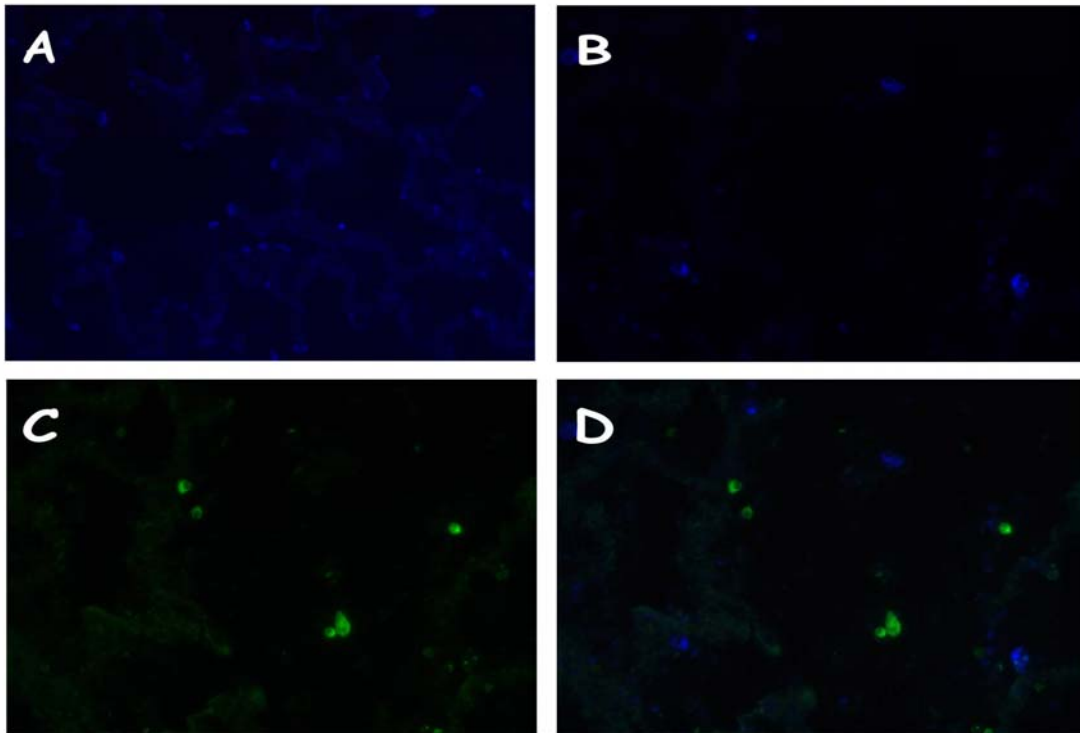


Figure 10 – Double color stain for S-protein and CD68 on Case B– This figure shows staining for the SARS CoV S-protein and the cell surface marker CD68. Panel A is a negative control slide for the mouse isotype antibody. Panel B shows positive staining for the CD68 cell marker, with positive cells staining blue. Panel C shows staining for the SARS CoV S-protein, as has been shown before. Lastly, Panel D shows both previous pictures combined into one, showing both colors in the same frame. There is no overlapping colors, therefore no positively stained macrophages expressing S-protein.

Since the S-protein positive cells were not staining positive for CD68, it was theorized that the SARS CoV infected cells were epithelial cells. To investigate this idea, the same procedure was used as for CD68 staining, substituting an antibody against epithelial membrane antigen (EMA) for the CD68 antibody (Figure 11).

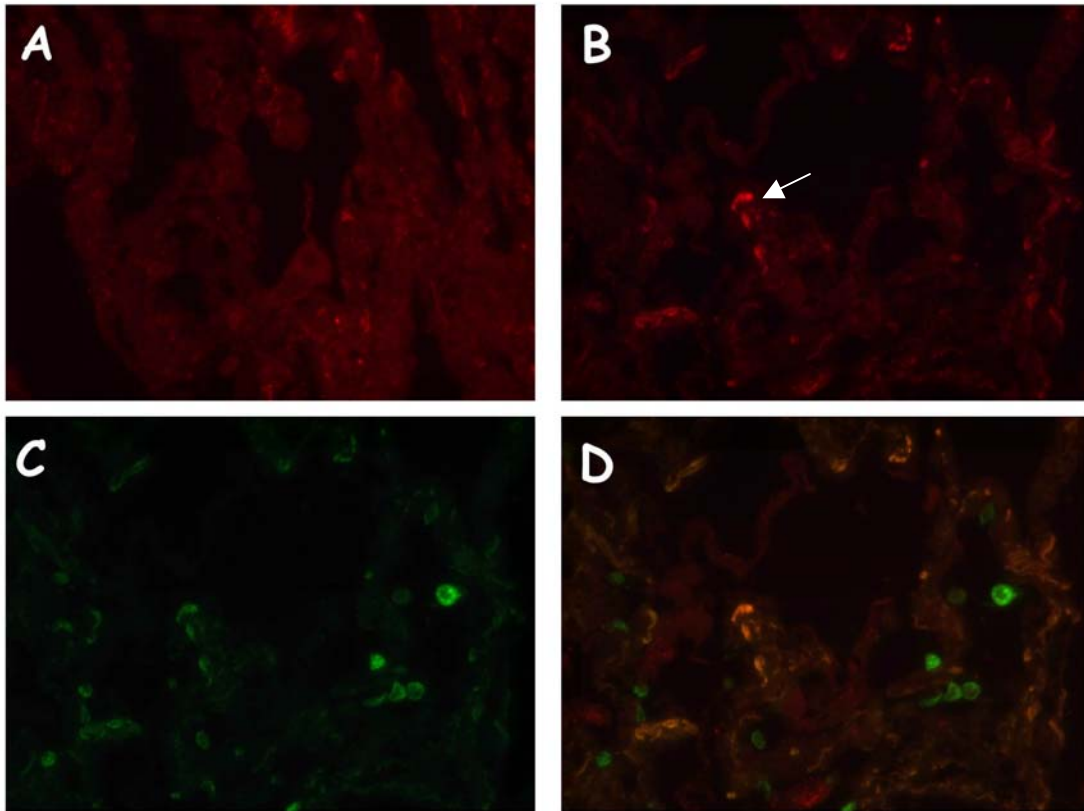


Figure 11 – Double stain for S-protein and epithelial membrane antigen on Case B– This figure shows the double stain for the SARS CoV S-protein and CD68. Panel A is the mouse IgG isotype control showing no positive staining, with some background fluorescence. A control for rabbit IgG was also run, showing no positive staining and limited background fluorescence (data not shown). Panel B is the stain for EMA alone. The arrow indicates probable positive staining. Panel C is positive S-protein containing cells, as seen previously. Panel D is the combination of Panels B and C, showing both EMA and S-protein locations.

The anti-EMA antibody was also a mouse-derived monoclonal antibody. As before, a mouse IgG isotype control was used and the result was no positive staining, although some background staining did exist (Figure 11 A). From there, a picture was taken of

EMA staining alone. There was some probable positive signal, indicated by the arrow in the picture (Figure 11 B). Again, the anti-S-protein antibody was used and a bright, positive signal was produced (Figure 11 C). The two photos were then combined to show the two desired aspects in the same frame (Figure 11 D). It can be seen that no cells that stain positive for S-protein also stain positive for EMA, as well as the converse statement (Figure 11 B, C, D). The bright cells that stain positive for S-protein in Panel C do not show the faintest signal in the EMA stain, Panel B.

DISCUSSION

To begin analyzing the results of this study, it would be beneficial to review them. Starting with immunohistochemistry, it was shown that TGF- β and TNF- α were both present in SARS CoV infected lung tissue. Next, immunofluorescence was used to show cells positive for the SARS CoV S-protein. These two factors were then combined into a two-color immunofluorescence stain. It was seen that cells containing SARS CoV S-protein did not stain positive for either TGF- β or TNF- α in the vast majority of circumstances. Staining for cell surface markers was performed next. Cells that were positive for S-protein did not stain positive for CD68 or EMA, leading to the fact that these viral infected cells were neither macrophages, nor epithelial cells. These results were analyzed and several inferences were made.

Several facts can be deduced from the results regarding the cytokines in question. It appears as if the cells with virus are not producing either of these cytokines. This could be due to several factors. The virus may be indeed down-regulating the production of these cytokines. The results are consistent with this theory, in that the cells that show bright staining for S-protein do not show the faintest hint of positive signal for either of the cytokines. In the case of TGF- β , this may be a reasonable finding. TGF- β was shown to decrease viral replication in TB induced HIV (Goletti, et al, 1997). The virus would be at an advantage by down-regulating TGF- β production. However, in the case of TNF- α , this is a strange finding. TNF- α was shown to be up regulated in the previously mentioned study. However, that paper was only one study conducted on HIV, not SARS CoV. A simple explanation is that SARS CoV acts differently than HIV in cytokine regulation. This theory of cytokine down-regulation cannot be justified with

immunostaining alone, but rather this result could lead to *in vitro* experiments that could further prove the fact.

The other results obtained regarding the cell markers and S-protein containing cells provide insight into where SARS CoV is infecting. Original presumptions, drawn from immunohistochemistry, were that the SARS CoV was residing in pulmonary macrophages and epithelial cells. Upon staining for CD68, this was shown to be not true. The thought then was that these S-positive cells were epithelial cells. However, when the tissue was stained for epithelial membrane antigen, the results showed again that this was not true. There are three main conclusions that can be drawn from this. The first

conclusion is that these are true findings. This would mean that the SARS CoV infected cells are neither pulmonary macrophages, nor epithelial cells. This provides a difficult question as to what cells these S-positive cells are. Macrophages would be the natural assumption as to which cells contain the most virus since the main function of macrophages is to remove foreign antigens from the body, so naturally they will contain viral particles. Since this fact was shown to be false, epithelial cells would be the next guess. This was also shown to be not true, it is difficult to say what other cells would contain this much virus. This conclusion may be less acceptable due to this fact.

The second conclusion is that the CD68 and EMA markers are somehow being altered in a fashion that would cause them to be less recognized by the detection antibodies. One theory is that the virus is altering or down-regulating the surface markers when it replicates inside the cell. Another idea is that the cells are dying and their surface markers are being altered or down regulated. This would explain why none of the S-

protein containing cells are showing positive for CD68 or EMA, when there are definite positive cells staining for these two markers elsewhere in the tissue.

The third conclusion, which can also be applied to the cytokine antibody tests, is that there is some technical aspect that is preventing the two different antigens to be shown in the same cell. The procedure used requires the antibodies to be prepared in the same solution when performing the double color stain. There may be some sort of interaction with this method of antibody application. There may be competition between antibodies to bind a certain cell, and the S-protein antibody may have more affinity to the cell, preventing any further antibodies from binding. This would explain the lack of overlapping colors in the two color trials.

The most useful step in this study would be to attempt a three-color stain, consisting of the antibodies for S-protein, one of the cell surface markers and one of the cytokines. This was a goal for this paper and several attempts at it were made. Unfortunately, none were successful. The problem with a three-color stain is to effectively coordinate all the antibodies, taking into account their host species. One primary antibody cannot have the same host species as another. If this were to happen, the secondary antibody would stain both these antibodies the same color, not giving any distinction to the two desired antigens. This was the largest problem encountered with this study. The use of biotinylated primary antibodies was implemented, but the antibodies were not useful for immunohistochemistry on paraffin embedded, formalin fixed tissue. To set up a plan for an effective three-color stain would be the next logical step for this study.

The results obtained in this study can be used to formulate studies that can be performed *in vitro*. A study could be designed to assess the cytokine production of live cells infected with SARS CoV. This could further clarify the finding that the SARS CoV infected cells are not producing TNF- α or TGF- β . Analysis of SARS CoV infected macrophages and epithelial cells could provide insight into the finding that the SARS CoV infected cells were neither of the two cells expected.

The results of this study as of now show some progress on the investigation of the pathology of SARS. More intensive study into antibodies and their target molecules could be used to provide further pictures, including the three-color stain that was unsuccessful in this project. This is the direction that needs to be followed to determine a detailed description of SARS CoV and its method of infection.

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