Title
Characterizing the Structure and Formation of Organized Lymphoid Follicles in the Gut and Lung in Models of Disease and Chronic Inflammation

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CHARACTERIZING THE STRUCTURE AND FORMATION OF ORGANIZED LYMPHOID FOLLICLES IN THE GUT AND LUNG IN MODELS OF DISEASE AND CHRONIC INFLAMMATION

By

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A capstone project submitted for Graduation with University Honors

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University Honors
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Abstract

Organized lymphoid tissues in the gut, called Peyer’s Patches, are areas in which luminal pathogens are detected and effective immune responses are subsequently triggered. This process heavily relies on specialized epithelial cells, known as Microfold Cells (M cells), which aid in the majority of immune surveillance in the gut. M cells accomplish this by capturing and transcytosing microparticles across their apical and basal lateral surfaces and delivering them to antigen presenting cells, such as dendritic cells, which may then trigger an immune response. Thus, organized lymphoid tissues in the gut can be characterized by the identification of these specific cell types, in addition to a plethora of other cells and structural components. However, in cases of chronic inflammation, organized lymphoid follicles, or unorganized lymphoid aggregates, may be induced in the gut and even in the lung. Therefore, through our studies we aim to recognize identifying elements of these induced lymphoid structures in the guts of transgenic mice that act as models of Crohn's Disease. In addition, we aim to use these markers to identify the appearance of organized, or unorganized, lymphoid structures in the airways of the lung after an intranasal administration of silica nanoparticles.
Acknowledgments

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# Table of Contents

Abstract........................................................................................................................................... ii

Acknowledgments........................................................................................................................... iii

List of Figures ................................................................................................................................... v

Introduction....................................................................................................................................... 1

Methodology....................................................................................................................................... 5

Literature Review ............................................................................................................................. 9

Analytic Discussion: Gut .................................................................................................................. 11

Analytic Discussion: Lung ................................................................................................................ 14

Conclusion......................................................................................................................................... 18

Works Cited ....................................................................................................................................... 19
List of Figures

Figure 1.......................................................................................................................... 11
Figure 2.................................................................................................................................. 12
Figure 3 ................................................................................................................................... 14
Figure 4.................................................................................................................................. 16
Introduction

Due to its essential role in disease and infection, developing a further understanding of the immune system, and the manner in which it responds to the environment, is an essential component of understanding the overall health of an individual. This is particularly true with respect to mucosal immune surveillance since these sites are consistently exposed to potentially pathogenic microorganisms. Therefore, further understanding of the various factors that partake in defending and protecting these areas could potentially provide great therapeutic value for a plethora of diseases. In this project, the focus is specifically narrowed to the mucosal epithelium of the small intestine and lung in cases of chronic inflammatory disease. Thus, mouse models for Inflammatory Bowel Disease/Crohn’s Disease and Asthma were utilized in these studies so that we may better understand how mucosal surveillance may be affected by these conditions. The underlying premise that connects both cases stems from the idea that pro-inflammatory signals may also be involved in cell differentiation signaling (Patel et al., Parnell et al.). Thus, there is evidence that when chronic inflammation is present, an induction of organized, or un-organized, lymphoid aggregates can occur. Therefore, the ultimate goal of this project is to characterize the potential formation, and structure, of inflammation induced organized lymphoid follicles in comparison to constitutive organized lymphoid follicles.

Throughout the small intestines of healthy wildtype organisms, areas of constitutive organized lymphoid follicles are present and play a vital role in surveying luminal contents for potentially pathogenic agents. These mucosal immune surveillance
areas are known as Peyer’s Patches and are pivotal in the process of activating effective immune responses to pathogens present in the gut. This process is heavily aided by specialized epithelial cells that exist within the epithelial layer of the Peyer’s Patch in a checkerboard fashion. Known as Microfold Cells (M cells), they perform the majority of the immune surveillance that takes place within the gut. This is accomplished by capturing, and transcytosing, microparticles across the apical and basal lateral surfaces of the cell and delivering them to antigen presenting cells, such as dendritic cells, which may then trigger an immune response (Neutra MR et al.). Thus, organized lymphoid tissues in the gut can be characterized by the identification of these specific cell types, in addition to a plethora of other cells and components. In this project, various markers for normal Peyer’s Patches were identified and their staining patterns were noted. These could then be utilized to identify potential organized lymphoid follicles that form in response to chronic inflammation. This is important because the distinction between organized lymphoid follicles and unorganized lymphoid aggregates could play different roles in cases of chronic inflammation.

When an organized lymphoid aggregate is present, the passing of potentially pathogenic agents through the epithelial layer into the lamina propria is beneficial to the organism since it results in an amplified immune response. However, this could also be harmful if pathogens utilize M cells’ abilities of uptake and transcytosis to facilitate infection (Kraehenbuhl et al.) Therefore, in cases in which M cells are induced, without the simultaneous induction of an organized lymphoid follicle, their presence may be harmful rather than beneficial. As a result, if unorganized lymphoid aggregates form
(structures that contain some, but not all of the components present in organized follicles), and these structures contain M cells, then pathogenic agents could potentially pass into the lamina propria though the aggregate lacks the elements necessary to trigger an effective amplified immune response. As a result, the area would become even more susceptible to inflammation, causing a scenario in which chronic inflammation induces more inflammation (Parnell et al.).

Thus, we suggest that, in both of these models of disease, the natural inflammatory response mechanisms of the body can become detrimental by promoting excessive inflammation and thus causing tissue and cell damage. Therefore, we aim to show that this occurs due to the development of organized lymphoid follicles, and inflammation induced M cells, that contribute to the process of causing a more exaggerated inflammatory response. To better understand this, it must be noted that one pathway by which inflammation is mediated is known as the nuclear factor kappa-B signaling pathway (NFkB). Yet, this signaling pathway is not fully understood because it is activated by multiple receptors and ligands, which then results in the regulation of a plethora of genes that possess NFkB transcription factor binding domains. Therefore, since the NFkB pathway has such variation, it makes sense that it may be involved in more than just pro-inflammatory signaling. In fact, it is also thought to play a role in cell differentiation, however, the mechanistic distinction between inflammatory regulation and differentiation is uncertain (Parnell et al.). Thus, we aim to make these connections somewhat clearer through our studies of chronic inflammation, and the subsequent induction of organized lymphoid follicles.
Therefore, chronic inflammation in the gut and lung is an essential precursor to the induction of organized lymphoid follicles. To study this in the gut, transgenic mice that are deficient in the chemokine receptor CXCR2 will be utilized. This receptor is essential for neutrophil migration and tracking capabilities. This implies that in knockouts of this gene the neutrophils would be incapable of efficiently traveling to sites of infection. In turn, this could mean that the mice would respond differently to infection and chronic inflammation, potentially making them a good model to study Inflammatory Bowel Disease and Crohn’s Disease (Eash KJ et al.). On the other hand, the second model of chronic inflammation used in this project, Asthma, relies on the exposure of mice to allergens for an extended period of time. This is done by either an intranasal administration of the allergen or by placing them inside of an environmental chamber in which the allergens are aerosolized.
Methodology

Mice

All experiments were done using tissues collected from male and female 2-3 month old mice bred in the University of California, Riverside vivarium under specific pathogen-free (SPF) conditions. The handling of these organisms was completed in accordance with the guidelines and requirements of the Institutional Animal Care and Use Committee and the National Institutes of Health. While wildtype C57BL/6 mice were used, transgenic animals were also utilized in these studies. These include TNFR1KO X PGRP-S-dsRed and TNFR2KO X PGRP-S-dsRed in which the Tumor Necrosis Factor receptor gene (1 or 2) is knocked out, in addition to the PGRP-S-dsRed reporter transgene being present. This reporter results in red fluorescent cytoplasm in M Cells and Neutrophils. Additionally, transgenic mice with the CX3CR1-EGFP reporter express GFP in their macrophages, resulting in these cells being fluorescently marked green. Thus, CX3CR1-EGFP X PGRP-S-dsRed animals possess green fluorescently labeled macrophages and red fluorescently labeled neutrophils and M cells. To reduce the probability of chlorophyll-related tissue autofluorescence that may hinder the ability to visualize these markers, all animals were fed ad libitum with an alfalfa-free diet. Though not yet completed, future studies will involve the use of CXCR2KO mice. These will be bred in the vivarium at the University of California, Irvine, by our collaborator Dr. Manuela Raffatellu. Frozen O.C.T blocks (Tissue-tek) of intestines will then be shipped to the University of California, Riverside.
**Intranasal Administration of Allergens**

Silica nano-particles were diluted in 1xPBS and droplets were placed on the noses of naïve, untreated mice. They were then left alone for two weeks until they were sacrificed and their lungs were harvested.

**Environmental Chamber Administration of Allergens**

Silica Nano-particles 100nm in diameter were aerosolized into small particle suspensions using an atomizer. These were then pumped into a Plexiglas environmental chamber built in collaboration with Eric Peng, and Dr. David Cocker, CE-CERT. This chamber regulates air flow (approximately 1 full volume change per hour), humidity (controlled to 30-50%), and light and dark cycles. Mice were kept in this chamber for 4 days, then immediately sacrificed and their lungs were harvested.

**Lung Harvesting Surgeries**

Mice were killed through carbon dioxide inhalation rather than through cervical dislocation to avoid the lungs filling with blood. Next, the animals were sprayed with 75% ethanol, followed by a small incision through the skin on the ventral side, which was subsequently pulled apart, skinning the animal. An incision was then made across the ventral side of the exposed peritoneum so that the diaphragm may be shown. This was then cut to reveal the internal part of the chest cavity. The rib cage was then opened and pulled apart so that the lungs could be visualized. Once this was done, the trachea was identified and a very small incision was made in it to allow for tubing attached to a 22-
gauge needle to be fed through and into the lungs. Next, 1ml of a warmed mixture of 1 part fixative and 2 parts OCT is used to slowly inflate the lungs within the chest cavity (fixative= 4% PFA, 30% sucrose 66% 1x PBS). The trachea was then tied shut with dental floss and the lungs were surgically removed from the chest and frozen whole on dry ice in an OCT mold. This block of tissue was then stored at -20° C

**Peyer’s Patch Harvesting Surgeries (Gut)**

Mice were killed through cervical dislocation, sprayed with 75% ethanol and an incision was made across the ventral side of the animal, through the skin and the peritoneum to expose the intestines. The entire small intestine was then removed and immediately placed into a dish of 1xPBS on the stand of a dissection microscope. Peyer’s Patches were then visualized and cut off of the external wall of the small intestine, briefly rinsed in 1xPBs (to remove feces) and placed into fixative for 20 minutes (fixative= 4% PFA, 30% sucrose and 66% 1xPBS). Finally, the excised Peyer’s Patches are placed into a mold of OCT and frozen on dry ice.

**Cryostat Sectioning, Immunofluorescence Staining, and Confocal Microscopy of Lung and Gut Tissues**

The samples were made into 20µm thick sections on the cryostat and tissues were placed on positively charged slides. For immunofluorescence staining, the O.C.T was removed using 3 washes with 1x PBS. Sections were then permeabilized with 0.5% Tween 20 in 1x PBS for 10 minutes (Fisher scientific). Next, the tissues were washed 3
times in wash buffer (0.1% Tween 20, 1x PBS). Sections were then blocked in avidin/biotin blocker (Vector) for biotinylated antibodies and in casein solution. The following primary antibodies were used: RANKL (Abcam), CD3 (eBiosciences), eBiosciences), Ulex europaeus agglutinin 1 (UEA-1) lectin (Vector), ER-TR7 (Abcam), relB (Abcam), B220/CD45 (eBiosciences), CXCL13 (Abcam). After 1 hour at room temperature, slides were then washed 5 times in wash buffer and incubated with the secondary antibodies for 30 minutes. The secondary’s used were: Alexafluor 674 and Alexafluor 488 (Jackson ImmunoResearch). A final 3 washes were performed before coverslips were mounted with Prolong gold antifade reagent with DAPI (Life technologies). Slides were left in the dark at room temperature to cure overnight. A Zeiss confocal microscope, a BD CARVII Confocal Imager, and Metamorph Imaging Software were used to capture images. Scale bars indicating microns are on each image.

**Hematoxylin and Eosin Staining and Light Microscopy of Gut and Lung**

After sectioning on the cryostat, the tissues are stained for hematoxylin and eosin. First soak in Modified Mayer’s hematoxylin for 6 minutes and then rinse with warm tap water. Next, drop eosin on the slides and only wait 15 seconds before dipping in 75% ethanol 2-3X to rinse. The coverslips were then mounted using Prolong gold antifade reagent and imaged on a compound light microscope.
Literature Review

M cells are well known to be important in pathogen transport across mucosal epithelium and thus play an essential role in immune surveillance. However, other purposes (whether beneficial or harmful to the organism) are less understood. Therefore, much of the literature aims to clarify what M cells do and how they may be induced (Neutra et al., Kraehenbuhl et al.)

A study previously conducted in the Lo lab (Parnell et al), implies that chronically inflamed mucosal epithelia may be more likely to form organized, or unorganized lymphoid structures. This may be due to differential signals in the NFκB pathway. This cascade may be initiated by many different signals and can result in the regulation of a plethora of genes. Thus, NFκB is thought to play a role in both promoting inflammation and in cell differentiation. Two specific signaling molecules, identified in this paper, that are thought to play a role in this are RANKL and TNF-α. Subsequently, these two molecules are also thought to play an important role in inducing M cells at sites of chronic inflammation in the colon. This is proposed due to the findings that inflammation induced M cells in the colon are not inducible in TNFR2KO mice. Therefore, this receptor for TNF-α must somehow partake in this induction. Additionally, it has been stated by others that TNFR2 is involved in the differentiation of oligodendrocytes, adding more evidence that TNF-α may play a vital role in cell differentiation (Patel et al.) On the other hand, it was found that RANKL was expressed in the cells just below the epithelium that possessed these induced M cells. It remains to be determined which NFκB signaling pathways may be involved in the promotion of inflammation and in the
differentiation of inflammation induced M cells (Parnell et al.).

Lastly, the literature shows that CXCR2 is important in neutrophil migration and tracking. Knocking out this gene in mice could significantly alter the inflammatory response in the intestine (Eash et al.).
Figure 1: Peyer’s patches from wildtype mice were harvested and stained for cell markers.

In (A), M cells are detected in the epithelial cell layer (red) and are indicated with a white arrow. Just below, a B cell follicle is identified in purple with T cell infiltrates filling follicular spaces (green). In (B) and (C), the section was stained for RANKL (purple) and ER-TR7 (green) markers. The signal for ER-TR7 is strongly expressed on reticular fibroblasts, thus (C) is included so RANKL expression in cells can be visualized (orange arrow). Lastly, (D) shows nuclear relB in purple. (A), (B), (C) and (D) have DAPI staining of nuclei in blue.

Before any studies could be done in the CXCR2KO model for inflammation, developing a panel of markers for normal Peyer’s Patches is essential. Once a comprehensive, yet also consistent, pattern in staining occurs for all proposed markers, the study may move forward so that the same markers are searched for in the CXCR2KO mice for comparison. Thus, Figure 1 shows staining patterns which have thus far proved
to be consistent and reliable. The first of these is *Ulex europaeus* agglutinin 1 (UEA-1). This is a lectin that specifically binds to fucose molecules present on the surface of M cells. As a result, UEA-1 does not label the entire cell, instead it only marks the surface. However, M cells are not the only intestinal epithelial cells that may be UEA-1 positive. Goblet cells, which are involved in the secretion of mucous (which contains fucose), can also be bound by this lectin. Therefore, it is vital to note that the shape of the fluorescent signal is important in properly identifying M cells. As shown in Figure 2, these cells tend to express UEA-1 in a distinct cap-like, or columnar shape. Goblet cells on the other hand tend to be round, or irregular in shape (Figure 1 does not show any goblet cells). Another consistent marker has been the B220 antibody, which stains B cells by targeting the CD45R receptor on their cell surface. This is shown in Figure 1(A), as well as in Figure 2. In Peyer's patches, these cells often appear as large follicles with a distinct ring-like pattern. Similarly, T cell staining also tends to show this characteristic ring-like pattern (Figure 1(A)), however, they do not form

**Figure 2: M cells versus Goblet cells**

Both M cells and Goblet cells are UEA-1 positive (red) however the distinctions in shape can be used to differentiate them. The round UEA-1 positive cell (orange arrow) is a goblet cell, while the two columnar UEA-1 positive cells with distinct caps are M cells (white arrows). Green staining shows a B cell follicle, blue is DAPI staining of nuclei.
large follicles. Instead, T cells tend to be dispersed throughout the Peyer’s Patch (though they may sometimes form unorganized aggregates). The marker used to identify these cells is CD3, which is a protein present on the surface of all T cells. Another Peyer’s Patch organizational indicator is the presence of reticular fibroblasts. These cells provide the structure and framework of the lymphoid follicle. They have been effectively stained using the ER-TR7 antibody, which detects an antigen that is present within the reticular fibroblasts (see Figure 1(B)). Thus, the signal for this marker is detected throughout the entire patch and can be described as a network. Additionally, relB staining is distributed throughout the Peyer’s Patch, in the cell nuclei, since it is a transcription factor associated with the NFkB pathway. However, this is specifically present in the follicle epithelium (see Figure 1(D)). Lastly, RANKL, a ligand that activates the NFkB pathway, has been stained for as well. This marker can be found in the cells beneath the epithelial cell layer and is suspected to play a role in the differentiation of epithelial cells into M cells.

However, the afore mentioned markers are not the only ones that are important in characterizing Peyer’s Patch structure and morphology. There are many other components that still need to be consistently stained for. These are dendritic cells (CD11c) and CXCL13 (a B cell chemotactic cytokine). Once these final markers can be reliably stained for, a panel of normal organized lymphoid follicle constituents will be completed. The next step would then be to apply these same markers to the intestines of the CXCR2KO mice and note the presence, or absence, of each marker.
Analytic Discussion: Lung

Unlike in the gut, the lung does not possess an example of normal organized lymphoid follicles. Therefore, the panel of markers developed for the gut, will also be used to identify the potential induction of organized lymphoid follicles in the airways in response to chronic inflammation. Currently, experiments are being conducted to determine the ideal exposure time, and method, necessary to allow for the development of lymphoid aggregates and follicles in lungs.

The first method attempted was an intranasal administration (INA) of Silica Nano-particles (non-biodegradable components of sand) to TNFR2KO X PGRP-S-dsRed and CX3CR1-EGFP X PGRP-S-dsRed mice. The CX3CR1-EGFP X PGRP-S-dsRed animals would thus represent normal mice.

Figure 3: Lungs After Intranasal Administration of Silica Particles in TNFR2KO and CX3CR1 mice

After one INA of Silica particles, the mice were left alone for two weeks. (A) shows lungs stained for UEA-1 (M cells/goblet cells= purple), DAPI (nuclei=blue) and imaged on the Confocal Microscope (these transgenic mice also express GFP labeled macrophages). (B) shows lungs stained with hematoxylin (nuclei= blue) and eosin (proteins=pink). In (A) no significant aggregates or M cells are shown (the purple cells, marked by orange arrows, are goblet cells). In (B) a very small aggregate (white asterisk) can be seen beneath an airway (white arrow). The blue arrow indicates tissue damage due to over inflation of the lung.
while the TNFR2KO X PGRP-S-dsRed might have either a deficiency in pro-inflammatory, or cell differentiation, signaling. After administering the dose of Silica particles, the mice were left alone for two weeks. This was done so that potentially induced lymphoid structures would have time to form. Then the lungs were harvested and prepared for both confocal and light microscopy. The confocal slides were only stained for UEA-1 (purple) and DAPI (blue), though since the mice expressed transgenic reporter genes, macrophages fluoresced green, while neutrophils and M cells would fluoresce red. When analyzing the results in the confocal images, no lymphoid aggregates or follicles were found in either mouse strain. In addition, the only UEA-1 positive cells identified were goblet cells (no M cells were induced). This is shown in Figure 3 (A), where the purple cells shown are the goblet cells that line the airways of the lung. However, when observed through the compound light microscope and a hematoxylin and eosin stain (H&E), some small aggregates were identified near airways in the TNFR2KO X PGRP-S-dsRed (see Figure (B)). The difference could be due to the fact that densely packed nuclei on the confocal microscope are much harder to identify without additional markers than in the H&E. In addition, if they were present, the aggregates that did not express any of the markers that would have potentially characterized it as an organized lymphoid follicle. Therefore, the INA was unsuccessful at inducing them. One of the explanations for this is that the dose reaching the lungs was not high, or frequent enough to trigger sufficient inflammation. In addition, since this was an INA administration, much of the Silica particles could have been coughed up or swallowed, rather than inhaled. This would therefore reduce the dose of particles reaching the lung. Thus, the INA method is
not ideal for inducing chronic inflammation. Instead, the Environmental Chamber method should be more reliable since the particles are directly inhaled and evenly distributed throughout the lungs. Also, something to note is that the lungs are very delicate, thus damaging them during the harvesting and preservation processes is highly probable. In this case, the lungs were over inflated, thus large empty spaces were evident in the images taken (see the blue arrow in Figure 3 (B)). Therefore, future inflations were done much more carefully.

Finally, a chamber study was conducted in which two sets of TNFR1KO X PGRP-S-dsRed, TNFR2KO X PGRP-S-dsRed, and C57BL/6 mice were exposed to the Silica particles for 4 days. One group was then immediately sacrificed and their lungs were harvested, the other was left for a week to allow time for potential lymphoid follicles to form. This week was not completed by the time this was written, therefore the data regarding the second set of mice is not included. However, the results concerning the first set of mice showed that again, no significant lymphoid aggregates or follicles were found in any of the strains used. All of these images show airways. (A) shows is from an animal lacking TNFR1 (B) is from an animal lacking TNFR2 (C) is wildtype. The blue arrows indicate blood vessels.

Figure 4: Lungs After Environmental Chamber Exposure to Silica Particles
developed in any of the mouse strains. This conclusion was reached by analyzing H&E stained slides. However, the tissues were much more well preserved in this collection and blood vessels were visible.
Conclusion

In the gut, specific markers and identifiers can be utilized to characterize organized lymphoid follicle formation. These markers, such as reticular fibroblasts, M cells, B cells, RANKL, and T cells, could thus be used to also identify whether chronic inflammation could result in the induction of organized lymphoid follicles. Yet, future work must be done so that additional markers could also be used for this purpose.

In the lung, a single INA of Silica particles, followed by two weeks of rest, is not enough to induce the formation of lymphoid follicles in the lung. Similarly, a four day exposure in the environmental chamber was not sufficient for induction. Therefore, the mice which were allowed to rest for a week after the 4 day chamber exposure will be pivotal in this study. Since they had time to develop both chronic inflammation (through the four day exposure) and aggregates (from waiting one week), it is likely that this set of mice will show an induction in lymphoid aggregates, and maybe even organized lymphoid follicles and M cells.
Works Cited


