Defining Pathways of T cell Development in Cryptopatch Aggregates in the Small Intestine of the Laboratory Mouse, *Mus musculus*

Introduction

Intestinal intraepithelial lymphocytes (IEL) are composed of mostly T cells that reside in the lining of the intestine. These T cells play important roles in our ability to remain healthy and fight infections in the intestine. Despite their importance, the life cycle of IEL is not well defined. IEL can be divided into two developmentally distinct T cell subsets based on the type of T cell antigen receptor (TCR) they express on their cell surface. The TCR is required for normal T cell development in the thymus in addition the TCR allows T cells in the periphery to recognize foreign antigens (such as those derived from pathogens) during an immune response (Hayday et. al.). Most T cells in the peripheral circulation and resident in non-mucosal sites in the body such as the spleen, express a TCR composed of alpha and beta protein chains (TCR $\alpha\beta$). Populations of IEL however, are composed not only of TCR $\alpha\beta$ cells, but a second T cell population that expresses a TCR composed of gamma and delta protein chains (TCR $\gamma\delta$) (Hayday et. al.).

Previous studies in mice have shown that most TCR $\gamma\delta^+$ cells reside in the intestinal epithelium and the skin. In addition, unlike TCR $\alpha\beta^+$ cells, TCR $\gamma\delta^+$ IEL are present in mice that lack a thymus suggesting that the development of these T cells occurs independently of the thymus. The site outside the thymus where TCR $\gamma\delta^+$ IEL develop however, is not well defined. Recent studies have shown that TCR $\gamma\delta^+$ IEL may derive from progenitor cell clusters located in close proximity to the small intestinal crypts, called cryptopatch aggregates (Suzuki et. al.). The ability of cells purified from cryptopatch aggregates to give rise to TCR $\gamma\delta^+$ IEL in mice that otherwise lack these IEL, strongly supported their role in the development of these cells. Whether cryptopatch aggregates are the primary site where commitment in the $\gamma\delta$ T cell lineage occurs, or whether $\gamma\delta$ T cells accumulate or expand in cryptopatch aggregates after primary development in some other site outside the thymus, is not known (Hayday, et al). While previous studies by Suzuki's lab demonstrated that cryptopatch cell precursors are present in the bone marrow, similar to the thymus, the process whereby bone marrow progenitors develop into mature TCR $\gamma\delta^+$ cells in the cryptopatch aggregate or some other site has not been defined. One approach to addressing this question is to define the cellular and molecular anatomy of lymphoid progenitors in cryptopatch aggregates.

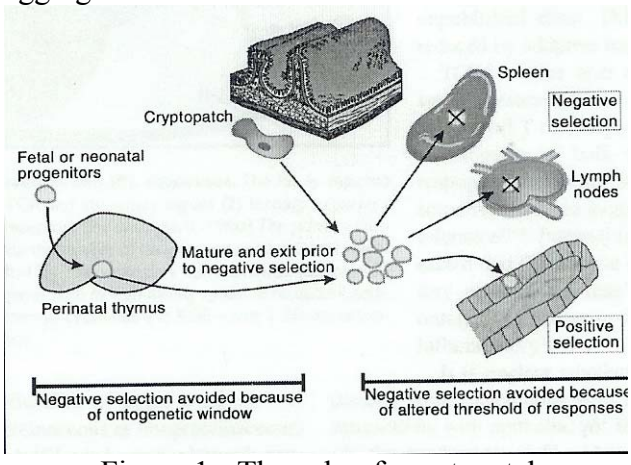

Figure 1. The role of cryptopatch aggregates in the development of IEL. Taken from Hayday et al.

The aim of my project is to define the cellular and molecular composition of cryptopatch aggregates using immuno-fluorescence microscopy coupled with laser-assisted microdissection (LMD). Previous work in

the laboratory of my mentor has used immunohistochemistry to examine the cell surface phenotype of cells in cryptopatch aggregates and DNA PCR to determine the state of TCR variable gamma gene rearrangement in these cells. However, the cell surface phenotype of cryptopatch cells has not been linked to the state of TCR gene rearrangements. For example, do cells that lack surface TCR expression have rearrangement of their TCR genes? My studies will focus on coupling the cell surface phenotype with the molecular state of the TCR genes in defined cryptopatch populations. As TCR gene recombination is the primary characteristic of T cell lineage commitment, I will use this assay to predict IEL lineage commitment in cryptopatch aggregates.

Materials and Methods

Preparation of intestine: Small intestinal segments will be isolated from appropriate strains of laboratory mice euthanized by CO_2 asphyxiation and cervical dislocation. The Animal Care and Use Committee has approved all procedures. The intestine will be isolated *en bloc*, cleaned in PBS pH 5.5 and blotted to remove excess PBS before placing in OCT medium on dry ice. Frozen small intestinal tissue sections will be stored at –80˚ F until use. We will use athymic and wild-type C57Bl/6 mouse strains for our studies.

Immuno-fluorescence staining and analysis of the small intestine: Small intestine embedded as noted above will be used to generate thin sections that will be used for immuno-fluorescence analysis and harvest of cryptopatch cells. Tissue sections will be generated using a cryostat microtome. Sections will be cut at 7 μm and immediately placed on laser foil-coated Leica slides pre-coated with VECTA-bond membrane to adhere cells and allow immuno-fluorescence staining and laser microdissection. The tissue sections will be fixed in acetone, hydrated in PBS and then incubated with the appropriate concentration of biotin-coupled or directly (PE, FITC, Texas Red or APC) fluorescent-labeled monoclonal antibodies in PBS staining buffer. Sections will be incubated for 30 min in the dark, rinsed in PBS and if necessary, incubated for 30 min with biotin coupled fluorescent labeled (as above) mAb to visualize biotin coupled mAb by incubating tissue sections in 1:500 concentration of fluorescent coupled avidin conjugate. Slides will be rinsed briefly in PBS and viewed under a microscope fitted with 3 fluorescent filters (red, green and blue).

Identification and selective isolation cell populations in cryptopatch aggregates: Serial intestinal sections will be scanned for cryptopatch aggregates. Individual aggregates will be paired across multiple intestinal sections and harvested individually into microfuge caps containing DNA lysis buffer. Within each cluster of related cryptopatch aggregates, zones with TCR $\gamma\delta^+$ or $CD3^+$ cells will be distinguished by cell surface immuno staining from cells that lack these surface markers.

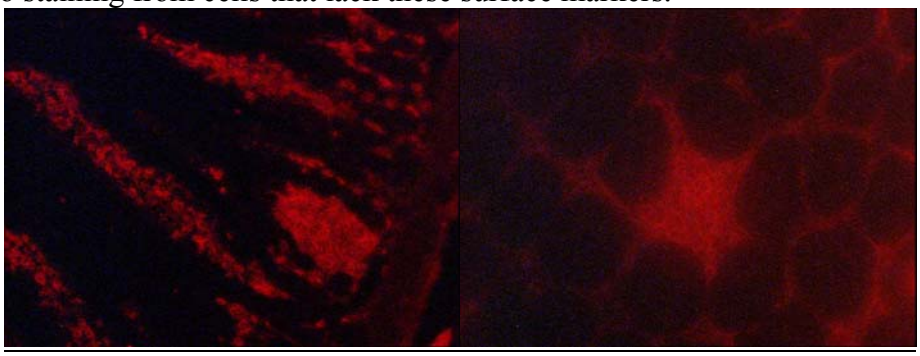

Figure 2. Cryptopatch aggregate stained with CD45 primary antibody.
Pictures from Dr. Camerini's lab.

Preparation of DNA: DNA will be prepared from isolated cryptopatch cells using the PicoPure DNA Extraction Kit (Arcturus Engineering) following instructions provided by the manufacturer. The quantity

of DNA will be determined using a spectrophotometer before real time PCR amplification for TCR variable gamma gene rearrangement. DNA primers that specifically distinguish rearranged from germ-line TCR variable gamma genes will be used to calculate the relative abundance of TCR gene rearrangement among each cryptopatch cell population as compared to mature TCR $\gamma\delta^+$ cells or bone marrow progenitors which lack TCR gene rearrangement. 5' primers will be specific to TCR VG1.2, VG2 and VG5 with a JG1 gene specific 3' primer. If time allows, rearranged TCR VG-J genes will be cloned following preparative DNA PCR amplification and then sequenced and their predicted amino acid sequence determined to compare the frequency of isolated genes that are in-frame and would give rise to a protein if expressed and translated.

Anticipated Outcomes and Significance

I anticipate that immuno-staining of cryptopatch aggregates will reveal two dominant cell populations in cryptopatch aggregates characterized by their expression of TCR (or CD3) and CD117. CD117 identifies expression of the stem cell factor receptor, a protein expressed by lymphoid progenitors and characteristic of many cells in cryptopatch aggregates. By contrast, the expression of TCR $\gamma\delta$ or CD3ε, an invariant chain of the TCR complex, identifies mature T cells that my derive from $CD117^+$ cells. In addition, I expect to find a variable $CD117^{+/low}$, $CD3^{+/low}$ population that may be intermediate between $CD117^+/CD3^-$ and $CD117^-/CD3^+$.

With regard to TCR variable gamma gene rearrangements, I expect first, that the frequency of TCR VG5 gene rearrangement will be higher in $CD117^-/CD3^+$ cryptopatch cells isolated by LMD than either TCR VG1.2 or TCR VG2 genes. Second, I expect that cells that lack surface TCR expression (CD3) and co-express CD117 ($CD117^+/TCR^-/CD3^-$) will either lack rearrangement of TCR VG genes or have a lower frequency of rearrangement of TCR VG5. However, as rearrangement of TCR VG genes occurs prior to surface expression of the TCR in the thymus, it is possible that these cells may have similar levels of rearrangement in other TCR VG genes (i.e. equally abundant TCR VG1.2, TCR VG2 and TCR VG5). If this were the case, I would expect that these cells would be $CD3^-/TCR^-$ $CD117^+$ or perhaps have a lower level of expression of CD117. This would suggest that the level of CD117 corresponds with developmental progression in cryptopatch aggregates. Determining the fraction of TCR VG genes that are productively rearranged (in-frame) across the antigen binding grove of the TCR VG gene relative to genes that are not-productively rearranged (out-of-frame) or contain a stop codon, would provide further evidence of whether expression of a surface TCR directs the development of cells in cryptopatch aggregates in a way similar to the thymus.

The project will help further address the mechanisms whereby TCR $\gamma\delta^+$ IEL arise via pathways outside the thymus, of which, little is presently known. The mechanisms of development in the cryptopatch may challenge generally accepted views on the development and role of the thymus in its selection of T cells. Moreover, it has been shown that defects in the cryptopatch aggregate development are associated with defects in TCR$\gamma\delta^+$ IEL development and in the development of inflammation in the intestine. A better understanding of T cell development independent of the thymus can give insight to the pathogenesis of tissue specific responses to injury and inflammation in the intestine (Makita, S. et. al.).

Project Timeline

Currently, I am already familiar with preparation of small intestine on to foil-coated Leica slides with a VECTA-bond membrane for immunohistochemical staining. In addition, I know the procedures for immunohistochemistry, laser assisted micro dissection, and I am somewhat familiar with PCR amplification analyzed with gel electrophoresis. I will need to fine-tune these procedures specifically for CD3 and TCR $\gamma\delta$ staining the cryptopatch aggregate. Dr. Camerini and a research specialist in the laboratory are well versed in these procedures.

Week 1-3: Develop a good procedure for acquiring consistent results of CD117+/CD3$^-$ versus CD3 (or TCR γδ) staining of cryptopatch aggregates in thin sections of the small intestine mounted on Leica slides. Familiarize myself with finding the cryptopatch aggregates under fluorescent filters and the typical pattern of staining using the markers stated above. I anticipate that I will examine 5-10 individual mice during the course of these studies.

Week 4-5: Determine the number of cryptopatch cells (aggregates) required to give sufficient DNA to test for gene recombination accurately and reproducibly. Find a method to precipitate or isolate small amounts of DNA without RNA co-precipitation so that the quantity DNA can be obtained in a spectrophotometer. Familiarize myself with real time PCR procedure and analysis.

Week 6-7: Gather PCR data for TCR variable gamma gene usage in different cryptopatch cell populations.

Week 8: Compare data for gene recombination in cryptopatch aggregates to TCR γδ$^+$ IEL.

Week 9-10: Assess the outcome of the project and plan future tests to confirm results or trouble shoot unexpected problems and time constraints.

References

Hayday, Adrian, Efstathios Theodoridis, Elizabeth Ramsburg, and John Shires. 2001. Intraepithelial lymphocytes: exploring the Third Way in immunology. *Nature Immunology*. 2(11): 997-1003.

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