IDENTIFYING A POSSIBLE LINK BETWEEN ECTOPIC GERMINAL CENTERS
AND THE EVOLUTION OF TYPE I DIABETES

A Thesis submitted to the University of Arizona College of Medicine -- Phoenix
in partial fulfillment of the requirements for the Degree of Doctor of Medicine

Eduardo C. Alcantar Jr.
Class of 2015

Mentor: Li Wen, MD PhD
Acknowledgements

We would like to offer special thanks to and acknowledge: the National Institute of Diabetes and Digestive and Kidney Diseases, the 2012 Summer Internship Committee, and everyone in the Wen Laboratory, especially Changyun Hu.
Abstract

The multifaceted phenotype of the B-lymphocyte has a remarkably effective role in peptide derived pathogen clearance and the prevention of re-infection. This mechanism of host tolerant defense can be attributed to the actions of particular cellular subsets that arise from B-lymphocytes: memory cells and high-affinity antibody secreting plasma cells. Notably B cell propagation does not commence without the help of follicular helper T cells (\(T_{\text{F}H}\)), a specialized subset of CD4+ cells. \(T_{\text{F}H}\) cells are involved in the maturation and differentiation of B-lymphocytes after antigen stimulation with a thymus-dependent peptide. With this specific stimulus the formation of germinal centers (GCs) within B-cell follicles of secondary lymphoid organs is induced and it is within these centers that \(T_{\text{F}H}\) cells are able to interact with B cells to facilitate immunoglobulin affinity maturation, somatic hypermutation, and isotype class switching. Importantly, these respective processes play a fundamental role in manufacturing high-affinity antibodies for effective pathogen clearance. Conversely, by means not well understood, the occurrence of spontaneous GC formation and the mass production of high affinity autoreactive antibodies have been shown to occur simultaneously with the development of autoimmune diseases. By the same token this incident is of particular interest and could play a role in the destruction of pancreatic insulin secreting \(\beta\) cells consequently driving the pathogenesis of type I diabetes. Our objective is to identify a possible correlation between the evolution of type I diabetes and the proliferatory behavior of B-lymphocytes and \(T_{\text{F}H}\) cells within developing GCs of non-obese diabetic (NOD) mouse models.
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**Introduction**

With a thymus dependent immune response follicular helper T cells (T\textsubscript{FH}) specialize in promoting protective, long-lived antibody reactions that arise from germinal centers (GCs). Within GCs the diversity and affinity of immunoglobulins for a particular antigen is significantly increased to improve the efficiency of pathogen clearance [1]. Successful cognate interactions between T and B cells induce both cell types to enter the cell cycle and also produces two differentiating pathways for B cells: seeding the GC response or becoming short-lived extrafollicular plasma cells [2]. The events that lead up to GC formation will be discussed, as this differentiation pathway is relevant to our research. Under normal physiologic conditions naïve mature B cells continuously circulate through lymph nodes, spleen, and mucosal associated secondary lymphoid tissues patrolling for invaders. These roaming B cells enter these distinct immunologic areas and rapidly migrate to the primary B cell follicle, which is an area composed of follicular B cells concentrated around follicular dendritic cells (FDCs) [2]. The expression of chemokine receptor type 5 (CXCR5) directs the B cells to the primary B cell follicle via a chemokine gradient of motif chemokine 13 (CXCL13), also known as B lymphocyte chemoattractant (BLC), which is produced by FDCs [3]. The gradient facilitates B cell contact with FDCs, professional antigen presenting cells, to collect antigen from their surface if present; these B cells can also bind soluble antigen if present. If there is no encounter with an antigen the B cells will continue their circulation to another lymphoid tissue. However, if the host has been recently challenged with an infection or immunized with a protein antigen, the naïve B cells will bind the soluble antigen or antigen presented on the surface of the FDC and become active forming a secondary follicle [2]. An activated B cell will internalize the antigen it is confronted with and upregulate Epstein-Barr virus-induced G-protein-coupled receptor 2 (EBI2), which is a migratory receptor that responds to an oxysterol gradient of 7\textalpha; 25–dihydroxycholesterol that facilitates its distribution along the T-B border of a secondary lymphoid organ. At the T-B border B cells can make contact with allied T cells and present fragments of the antigen they recently encountered together with major histocompatibility complex II (MHC II) [2]. The arrival of naïve CD4+ T cells to secondary lymphoid organs relies on the expression of chemokine receptor type 7 (CCR7) and its ligands motif chemokine 19 (CCL19).
and motif chemokine 21 (CCL21). These respective chemokines are expressed on high endothelial venules (in lymph nodes) and on T-cell reticular cells (in the spleen and in lymph nodes) [1, 3]. Activation also requires the help of dendritic cells and is dependent on the effective delivery of two signals: recognition of the MHC complex by the T-cell receptor (TCR) and excitation of the costimulatory signal CD28. CD28 is a major costimulatory receptor expressed on T-cells and is an absolute requirement for T cell priming and subsequent follicular helper T<sub>FH</sub> cell formation. The ligands for CD28 are upregulated by dendritic cells after antigen stimulation via infection or immunization [2], and sometimes, endogenous self-antigens. Upon activation T cells will upregulate inducible T-cell co-stimulator (ICOS) [1, 3]. This protein is essential for T<sub>FH</sub> development as it promotes Bcl-6 expression; the master regulator of T<sub>FH</sub> cell differentiation [1, 2, 4]. Programmed cell death protein 1 (PD-1) is also upregulated by active T cells and is a molecule essential for the eventual function of follicular helper T-cells in the germinal center [1]. Synergistically these stimuli lead to the downregulation of CCR7. Its downregulation aids in the release of these now active T cells from the influence of CCL19 and CCL21, however simultaneous upregulation of CXCR5 and a gradient of chemokine ligand 13 (CXCL13) conveys these cells to the T-B border, so they may interact with B cells, and ultimately allows them to enter their site of action: the B-cell follicle [1, 3]. Particular contact interactions at the T-B border between active B cells and T cells will lead to GC formation provided a reciprocal rapport of positive signals transpires. The symbiotic relationship between these cell types is essential for their respective proliferation and for the GC to flourish. Upon germinal center formation, B cells will migrate between different zones of the GC and compete for a vast number of antigen immune complexes on FDCs. GC B cells begin in an area known as the dark zone where they will proliferate and trigger the machinery for somatic hypermutation. These cells then migrate toward the light zone where B cells with high affinity B cell receptors (BCRs) survive and are able to interact with antigen presented on GC FDCs. GC B cells will internalize the encountered antigen and subsequently present it to a limited number of T<sub>FH</sub> cells in the GC [4]. Only those B cells with high peptide MHC affinity will successfully interact with T<sub>FH</sub> cells and this subset of B cells will then follow one of three potential fates: recycling back to the dark zone for further proliferation/receptor mutation, exit of the GC via the plasma cell fate, or exit
of the GC via the memory cell fate (Figure 1). GC B cells can be identified by their expression of high levels of Fas (CD95) and \(n\)-glycolyneuraminic acid (the ligand of antibody GL-7), binding to peanut agglutinin (PNA), and loss of surface IgD [4]. Throughout many points in this process autoreactive BCRs are negatively selected and eliminated at multiple checkpoints, this process is thought to be largely \(T_{fh}\) cell dependent. In cases of autoimmune disorders inappropriate self-reactive specificities will emerge as a result of somatic hypermutation and if \(T_{fh}\) cells are compromised and intolerant to host antigen, these B-cells will persist forgoing negative selection and ultimately exit the GC to potentially contribute to an autoimmune process [5-7].
**Significance and Rationale for Research Question**

What is currently known about and accepted for the pathogenesis of type I diabetes is a result of aberrant T cell function that leads to autoreactive T cells that are poised to respond to, and eliminate self antigen[6]. Nonetheless a paradigm shift has shed light on of the roles of B-lymphocytes in normal immune responses, in particular their capacity to differentiate, and has focused attention on them as potential therapeutic targets in autoimmune disease. Interestingly perturbations of B cell differentiation and maturation have recently been associated with B-cell hyperactivity and the production of autoreactive antibodies. This has been recently exhibited in models of systemic lupus erythematosus (SLE) [6, 7].
**Goals for the Study**

To identify a possible correlation between the progression of type I diabetes and the proliferatory behavior of B-lymphocytes and follicular helper $T_{FH}$ cells within developing GCs of NOD mouse models.
**Hypothesis**

We hypothesize an increasing number of GC B and T<sub>FH</sub> cells within the splenocytes of type I diabetic NOD mice versus non-diabetic NOD mice with increasing age. Because this disease has manifested itself, theoretically an increased number of GCs can be attributed to the underlying autoimmune disease process. With this knowledge, we seek to simultaneously examine the presence and function of B-lymphocytes and T<sub>FH</sub> cells in the midst of a GC that was formed via an inappropriate self-antigen stimulus. Exposing the insult and or manner that yields host tolerance could potentially play an essential role in establishing a platform for exclusive autoimmune disease treatments.
Research Materials and Methods

Objective: identify a possible correlation with the progression of type I diabetes and the proliferation of B-lymphocytes and follicular helper T-cells (T\textsubscript{FH}) within developing germinal centers (GC) of NOD mouse models.

Rationale - pre-diabetic and diabetic NOD mice of distinctive ages were examined. Studied mice were not challenged with a peptide vaccine to induce germinal center formation, therefore the spontaneous process was attributed to an autoimmune process - type one diabetes in this case.

Design – Groups of NOD diabetic and non-diabetic mice were selected at different ages and sacrificed. The spleens of these mice were harvested and half of the organ was used for flow cytometry analysis while the other half was utilized for immunofluorescence and immunohistochemistry staining.

Flow Cytometry – Each harvested murine spleen was weighed and subsequently pulverized in phosphate buffered saline (PBS) to isolate splenocytes. Extracted cells were counted and distributed into test tubes according to a specific cell number. Individual tubes were then incubated with a particular fluorescent marker to identify either GC B cells or T\textsubscript{FH} cells. Markers used to identify GC B cells included:

- GL-7\_Alexa488
- PNA\_FITC
- CD-19\_APC

While markers used to identify T\textsubscript{FH} cells were:

- ICOS\_FITC
- CXCR5\_APC
- PD-1\_PE
- CD4\_APC
Immunofluorescence – for each respective mouse, the other half of the harvested spleen was weighed, placed in optimal cutting temperature (OCT), and frozen. Using a cryosection instrument, sections of the frozen organ were mounted onto slides. The slides were then fixed and stained with specific antibodies for either GC B cells or T\textsubscript{FH} and subsequently imaged for analysis. Antibodies used were conjugated with a fluorophore.

Immunohistochemistry – slide preparation was identical to immunofluorescence (see above) however used antibodies for either GC B cells or T\textsubscript{FH} were conjugated with an enzyme that produced a color forming reaction instead of a fluorophore.

Method of Analysis
Flow cytometry – Analysis of splenocytes was completed using the FACSCalibur S to quantify the presence of GC B cells or T\textsubscript{FH} cells based on the expression of their particular fluorescent markers. Acquired data was analyzed using Flowjo software.

Immunofluorescence – slide preparations were imaged with confocal microscopy to determine expression of cell surface markers.

Immunohistochemistry – slide preparations were imaged with light microscopy to determine expression of cell surface markers.

Interpretation of results - there were three potential outcomes for our research.

- A significant increase in the number of GC B and T\textsubscript{FH} cells is distinguished with disease progression in NOD type I diabetic mice. This is a favorable result and would support further analysis of GC B cells and T\textsubscript{FH} within a specific time period to attempt to clarify specific mechanisms of autoimmunity.

- A significant increase in the number of GC B and T\textsubscript{FH} cells is not distinguished with disease progression in type I diabetic NOD mice. This result can be anticipated as GC B
cells and \( T_{FH} \) may not have significant role in the pathogenicity of type I diabetes. Although further analysis would have to be carried out to confirm this.

- A significant increase in the number of GC B-cells and \( T_{FH} \) cells is distinguished with disease progression in non-diabetic NOD mice. This is an unexpected result, however it is possible given the fact that NOD mice have a propensity to develop type I diabetes. Since the disease has not manifested clinically this result would warrant further studies of non-diabetic mice to attempt to identify the course of GC formation.

Pitfalls and modifications - the results of this experiment relied heavily on precision and accuracy, which constituted, in part, the technique of the student. User error was accounted for since the assays performed were novel to the student. In addition, the project relied heavily on the availability and health of the experimental mice. A delay in experimentation occurred, as it was necessary to allow mice to age or exhibit clinical symptoms of disease. Adjustments in the protocol were made for time particular time periods as mice of different age became available and were ready for use.

Compliance - trainings completed through Yale University:

- Yale Animal Resource Center: Facility Training
- Yale Animal Resource Center: Animal Care & Use
- Biosafety
- HIPAA Privacy Training
- Laboratory Chemical Safety
- Medical Surveillance for Animal Handlers
- Fire Extinguisher Training
Results

Our data comparing the quantity of GC-B and T<sub>FH</sub> cells demonstrate that NOD non-diabetic mice had a higher number of GC B (P=0.0147) and T<sub>FH</sub> (P=0.0221) cells when compared to NOD diabetic mice that were age and sex matched.

Diabetic versus non-diabetic

Flow cytometry analysis exhibiting the quantitative comparison of splenocytes expressing T<sub>FH</sub> cell markers: CXCR5, CD4, PD-1, and ICOS in age and sex matched NOD mice. P=0.0221 (paired student t-test) (Figure 2). Flow cytometry analysis exhibiting the quantitative comparison of splenocytes expressing GC-B cell markers: CD19 and PNA-R with the loss of IgD in age and sex matched NOD mice. P=0.0147 (paired student t-test) (Figure 3).

Age Comparison

Flow cytometry analysis exhibiting the quantitative difference of splenocytes expressing T<sub>FH</sub> cell markers: CXCR5, CD4, PD-1, and ICOS in age and sex matched NOD mice at various time points (Figure 4). Flow cytometry analysis exhibiting the quantitative difference of splenocytes expressing GC-B cell markers: CD19 and PNA-R with the loss of IgD in age and sex matched NOD mice at various time points (Figure 5).
Figure 2

TFH

% of PD-1+CXCR5+ in CD4+ICOS+

Non-Diabetic  Diabetic

0.0  0.5  1.0  1.5  2.0  2.5

T FH

% of PD-1+CXCR5+ in CD4+ICOS+
Figure 4

The diagram shows the percentage of PD-1+CXCR5+ cells in CD4+ICOS+ Tfh cells across different ages. The x-axis represents age (2m, 4m, 6m, 8m) and the y-axis represents the percentage (%). The data suggests a trend of increase in the percentage with age.
Figure 5

[Graph showing the percentage of IgD-PNA+ in CD19+ cells across different ages (2m, 4m, 6m, 8m) with error bars indicating variability.]
**Discussion**

The higher number of GC-B and T\textsubscript{FH} cells before diabetes onset suggests an active interaction between these two cell types during the pre-diabetic period. This interaction could support the production of high affinity antibodies and the generation of memory B cells consequently contributing to the pathogenesis of diabetes and perhaps the progression of its clinical manifestation. By the same token, the lower number of GC-B and T\textsubscript{FH} cells after diabetes onset indicates that the interaction of GC-B and T\textsubscript{FH} cells is likely to remain at a steady state.

The age comparison outcomes are diffuse as there is no significant correlation appreciated in the number of GC B and T\textsubscript{FH} cells with increasing age. These results suggest that diabetes progression stage, rather than age, of the NOD mice is more important in the pathogenesis of type 1 diabetes development.
Future Directions

Investigate GC-B cells and T<sub>FH</sub> cells in the affected tissue – pancreas and its draining lymph nodes as the results from a distal organ (spleen) might be different from the affected site. Also, study GC-B cells and T<sub>FH</sub> cells in an islet autoantibody specific model system, such as 125 Tg NOD mouse that carries anti-insulin specific B cells.
Conclusions

Our preliminary data might be useful for prediction of diabetes progression. Therefore the number of GC-B and \( T_{FH} \) cells could be used as a biomarker to predict diabetes development. Our study also provides a possibility for preventing diabetes development by targeting GC-B cells and \( T_{FH} \) cells.
References


