

## Research Plan

### Cellular & Molecular Biology

#### Determining the Kinetics of IRF4 and IRF5 Expression in B- and T-Cell Activation

##### A. Rationale

In response to antigen activation and with the aid of several transcription factors and signaling pathways, including B-cell antigen receptor (BCR) and Toll-like receptor (TLR) signaling, naive B-cells undergo developmental and proliferation processes to form antibody-secreting cells (ASCs), also known as plasma cells <sup>[1-2]</sup>. Of the nine members of the interferon regulatory factor (IRF) family of transcription factors, many have been shown to be critical mediators downstream of pattern recognition receptors, and therefore, important for the regulation of both innate and adaptive immunity <sup>[3-4]</sup>. Interferon regulatory factor 4 (IRF4) has demonstrated expression in B cells at most developmental stages, and has been identified to control class-switch recombination (CSR), plasma cell generation, and regulation of germinal center (GC) cell formation, also playing a key role in the interaction of B-cells and T-cells in the GC for B cell differentiation. The function of IRF4 as a transcriptional regulator is critical for the induction of proteins for GC reactions and plasma cell differentiation, and its induction after BCR engagement to cognate antigens is a checkpoint that determines a cell's exit from the GC program into plasma cell differentiation <sup>[5-7]</sup>. IRF4 has also been identified as a major regulator of antibody-secreting cell (ASC) differentiation, proliferation, and cell cycle control <sup>[8]</sup>. It is known that in response to upregulated IRF4, B cells undergo CSR, in which the immunoglobulin (Ig) locus is rearranged, changing IgM to IgA, IgG, or IgE <sup>[9]</sup>. Interestingly, IRF4 was also found to have a role in maintaining the homeostasis and positioning of mature cells in lymphoid environments through NOTCH2 expression and activity <sup>[5]</sup>. IRF4 is induced in response to pathways which also activate nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling, another major transcription factor downstream of IRF4 that is involved in innate and adaptive immunity, and it plays key roles in the generation and function of other immune cells, including T follicular helper cells (Tfh), macrophages, and dendritic cells <sup>[7]</sup>. In a recent study, IRF4 was identified as a downstream target of IRF5, another IRF with several immunity-related and oncogenic implications that include a B-cell intrinsic role in the differentiation of ASCs <sup>[8]</sup>. Targeted IRF5 knockdown in primary naïve B cells resulted in decreased IgG secretion and decreased expression of the enzyme activation-induced deaminase (AID), which suggests a decrease in B-cell CSR, since CSR is dependent on AID <sup>[8]</sup>. However, at the time points examined, *IRF4* transcript expression was not found to be

significantly impacted by IRF5 knockdown, suggesting that while IRF5 bound to the IRF4 promoter in response to BCR signaling, it may not be essential for early IRF4 transcriptional regulation <sup>[8]</sup>. In T cells, IRF4 was identified as a mediator of TCR signaling, along with CD4+ and CD8+ T-cell function, expansion, effector differentiation, cytokine production and cell fate, and in murine experiments, expression levels were found to peak very early, at 6-9 hours, before declining by 12-16 hours <sup>[10-11]</sup>. In contrast, not much is known about IRF5 in T-cells or TCR signaling, but it has been implicated in a pathway that favors chronic infection establishment by suppressing protective CD4+ T cell responses, and studies suggest that IRF5 affects T cell activation and differentiation by impacting cytokine production <sup>[12-13]</sup>. IRF5 polymorphisms and overexpression have been associated with the onset of SLE, and mechanisms of its role in murine disease pathogenesis include regulation of CSR and antigen specificity, along with Ig production <sup>[14-16]</sup>. Increased IRF5 mRNA and protein levels have been observed in the primary blood cells of SLE patients, which correlate with increased risk of disease development <sup>[15]</sup>. Furthermore, the critical roles of IRF5 in IgG2a production, a prominent inducer of autoimmunity, and in direct control of the  $\gamma$ 2a locus in CSR, as observed in murine models, suggest implications for a greater understanding of human SLE <sup>[14]</sup>. Similar to IRF5, dysfunction in IRF4 function can lead to the development of autoimmune diseases, including lupus, multiple sclerosis (MS), and type I diabetes, through hypo- or hyper-production of various cytokines <sup>[17-19]</sup>. TLR9 and BCR activation during the early stages of naive B cells to ASCs revealed distinct kinetic changes in both IRF4 and IRF5, and IRF4 knockdown resulted in IgD reduction and reduced ASC differentiation without impacting early B cell activation (CD86), or regulatory factors of CSR, which suggests key roles for both IRF4 and IRF5 in human ASC differentiation <sup>[20]</sup>. Despite the various roles of IRF4 in B cell function and development, its identification as an IRF5 target gene and the identification of similar functions for IRF4 and IRF5 in the control of plasma cell differentiation suggest distinct and overlapping functions for these two transcription factors in the processes of B cell activation and differentiation, along with T cell activation and further developmental functions. Thus, this study aims to identify the mechanisms by which IRF4 and IRF5 cooperate to regulate the generation of functional B- and T-cells, which are critical components of the adaptive immune arm.

## **B. Research Questions, Hypotheses**

### **A. Research Questions**

1. Does IRF5 directly regulate IRF4 transcription in B- and T- cell activation and development?
2. Where in the processes of B- and T-cell activation do IRF5 and IRF4 functionally overlap?
3. What proteins, molecules, or signaling pathways are involved in these interactions?

**B. Hypothesis/Goals/Expected Outcomes**

1. **Goal:** To analyze the kinetics of IRF4 and IRF5 expression in response to B- and T-cell stimulation in primary mouse splenocytes.
2. **Hypothesis/Expected Outcomes:** IRF5 will be an early regulator of B-cell activation in response to BCR and will work together with IRF4 to control later stages of differentiation and CSR, while in T-cells, the opposite kinetics will occur, with IRF4 functioning earlier in response to TCR and working with IRF5 in later stages of differentiation and T-cell effector function.

**C. Research Methods**

**A. Procedure**

1. **Isolation of Murine Total Splenocytes**
  - a) **Isolation:** Age- and gender-matched littermate wild-type and *Irf5*<sup>-/-</sup> mice will be humanely euthanized by authorized laboratory personnel. Spleens will be removed via dissection, and the tissue will be manually minced and homogenized with frosted microscope slides into a conical tube with ammonium chloride solution. Animal remains will be appropriately disposed of by qualified laboratory personnel. The cells will be suspended in cold PBS and centrifuged, the supernatant discarded, and the cells resuspended. A cell count will be performed using a hemocytometer.
  - b) **Cell Culture:** Isolated total splenocytes will be suspended in RPMI 1640 medium supplemented with fetal bovine serum (FBS) until use for signaling analysis.
2. **Signaling Analysis:** B cell receptor (BCR) signaling analysis will be performed by treating isolated total splenocytes with anti-IgM and the TLR9 ligand CpG-B, with anti-CD40 and IL-21 added for cell viability, over a time course of 0-7 days. T cell receptor (TCR) signaling analysis will be performed by treating isolated

total splenocytes with CD3 and CD28 over the same time course. Multi-color flow cytometry will be used to analyze both activation and IRF4 and IRF5 protein expression levels. Analyses will be conducted in cells from wild-type and *Irf5*<sup>-/-</sup> mice.

- a) **Flow Cytometry:** Total splenocytes will be surface-stained using antibodies that detect different maturation states of B cells, and then fixed. Intracellular staining will be performed to detect IRF4 and IRF5. All samples will be run on a Fortessa flow cytometer. Cellular protein expression will then be analyzed using the program FlowJo.

## **B. Risk and Safety**

1. This study will involve the use of potentially hazardous chemicals and biological agents such as 2% paraformaldehyde solution, 2% formaldehyde solution, 0.1% and 0.5% diluted solutions of 10% Triton X-100, murine spleens, and total splenocytes. These chemicals and biological agents may cause irritation or have toxic biological effects if handled or disposed of improperly. While the level of risk from all chemicals at their respective concentrations and murine cells is minimal, the use of proper PPE equipment, including goggles, nitrile gloves and a lab coat, will help minimize risk from exposure. All experimentation will be performed in a fume hood under direct lab member supervision to further minimize exposure and increase safety.

## **C. Data Analysis**

1. Representative dot plots from flow cytometry analysis will be generated and both cell activation percentages and protein expression levels will be quantified using FlowJo software. Multiple *t*-tests, with one per row, will be conducted to compare corresponding samples for individual expression graphs of IRF5 and IRF4 in order to determine statistical significance.
2. Data will be graphed and analyzed using GraphPad Prism 7.

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**Item #1: Human Participants Research:** N/A

**Item #2: Vertebrate Animal Research:** N/A

**Item #3: Potentially Hazardous Biological Agents:**

Potentially hazardous biological agents will include murine total splenocytes from male and female BALB/c and CS57BL/6 wildtype (WT) and *Ir5<sup>-/-</sup>* mice. Since these cells pose little to no threat of infection in healthy adults, they can be considered Biosafety Level 1 agents. Proper training will be undertaken in order to learn techniques for appropriate handling and disposal of agents, and all work will be done under the supervision of qualified laboratory personnel. Proper equipment will be worn to minimize exposure.

### **Murine Total Splenocytes**

- Spleen cells from wild-type and *Irf5*<sup>-/-</sup> mice
  - Murine total splenocytes pose a risk of irritation or infection from contact due to potential of cells to harbor an infectious agent. Thus, various safety measures and precautions will be taken to prevent injury and/or illness. All cells will be suspended in RPMI 1640 medium supplemented with fetal bovine serum (FBS) and stored at 4°C until use for analysis. Personal Protection Equipment (PPE), which include nitrile gloves and a lab coat, will be worn for all procedures. Special care will be taken to avoid contact with skin or eyes, and all procedures will be performed using proper aseptic technique under a fume hood. After completion of work, all waste media and equipment will be appropriately disinfected in accordance with institutional guidelines, and any remaining cells will be disposed of in an appropriate biohazard waste container.

### **Item #4: Hazardous Chemicals, Activities & Devices:**

There are no hazardous activities or devices involved, but the handling of all hazardous chemicals will be done under the supervision of qualified lab personnel. Hazardous chemicals will include paraformaldehyde, formaldehyde and 10% Triton X-100. PFA and formaldehyde will be diluted with DPBS to concentrations of 2% for use in experimentation. Proper training will be undertaken in order to learn techniques for safe experimental use of chemicals. Proper equipment will be worn to minimize exposure.

### **Paraformaldehyde (PFA) – Solid**

- Product T353-500, Fisher Scientific
  - PFA is a caustic, flammable substance capable of causing serious skin/eye irritation or damage, and has acute oral and inhalation toxicity. PFA will be kept locked in a tightly closed corrosive-resistant container that is in a well-ventilated place. PPE will include nitrile gloves and a lab coat, and all work will be conducted under a fume hood for adequate ventilation. Skin or eye contact will be avoided and PFA will be kept away from heat, sparks, open flames, and hot surfaces. It will be disposed of to an appropriate waste disposal plant.

### **Formaldehyde – 37% by weight**

- Product BP531-500, Fisher Scientific



- Formaldehyde is a caustic, flammable substance capable of causing serious skin/eye irritation or damage, and has acute oral and inhalation toxicity. Formaldehyde will be kept locked in a tightly closed corrosive-resistant container that is in a well-ventilated place. PPE will include nitrile gloves and a lab coat, and all work will be conducted under a fume hood for adequate ventilation. Skin or eye contact will be avoided and PFA will be kept away from heat, sparks, open flames, and hot surfaces. It will be disposed of to an appropriate waste disposal plant.

**Triton X-100 – 10%**

- Product BP151-500, Fisher Scientific
  - Triton X-100 has acute oral toxicity, and can cause skin irritation or serious eye irritation/damage. It will be stored in a tightly closed container kept in a cool, dry, and well-ventilated place. PPE will include nitrile gloves and a lab coat, and all work will be conducted under a fume hood to minimize exposure. Skin or eye contact will be avoided and Triton X-100 will be disposed of to an appropriate waste disposal plant.

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**NO ADDEMDUMS EXIST**