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

Cellular and Molecular Biology

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Contents

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1.0 Introduction

1.1. Rationale

Immunologists continue to seek a greater understanding of the intricate nature of the immune system and more effective methods for autoimmune diagnosis and treatment. Currently, diagnoses are founded primarily on clinical disease manifestation and laboratory tests, but the majority of biomarkers available lack high sensitivity and specificity [1]. Several proteins and signaling pathways have been identified in the onset and development of autoimmune diseases including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [2], and many of these have also been mapped and studied in greater depth [3]. In particular, interferon regulatory factor 4 (IRF4) and interferon regulatory factor 5 (IRF5) are molecules of interest for their role as regulatory transcription factors in several immune functions, along with implications in autoimmune disease [4]. Recent findings have established kinetically distinct roles between the two proteins [5], but there is a need for more specific investigation into where in the course of immune cell development these proteins function. Linking protein kinetics - during processes such as activation and specialization - with progression and impact on disease offers promise to identify potential biomarkers and tools for autoimmune disease diagnosis and therapeutics. This study investigated the kinetics of IRF4 and IRF5 in B-cell receptor (BCR) and T-cell receptor (TCR) induced immune cell activation. Based on current knowledge of these proteins, it was hypothesized that in B-cells, IRF5 would have an earlier role in the activation process and function with IRF4 in later stages, while the opposite would occur in T-cells, with IRF4 functioning very early and IRF5 taking a role in late-stage activation.

1.2. Literature Review

1.2.1. The Immune System and Interferon Regulatory Factors (IRFs)

In response to antigen activation and with the aid of several transcription factors and signaling pathways, including B-cell receptor (BCR), Toll-like receptor (TLR), and T-cell receptor (TCR) signaling, naive B- and T-cells undergo maturation to generate specialized cells critical for innate and adaptive immune responses [6-11]. When B- and T-cells become autoreactive and/or have imbalanced cell numbers, including memory B cells, plasmablasts (PBs) or antibody secreting cells (ASCs), T-helper (Th) cells, cytotoxic T cells, and T-regulatory (Treg) cells, it can lead to the onset and development of autoimmune disease, but at the same time, provide potential new targets for therapeutic intervention [12-14]. 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, along with interferon (IFN)-driven disease [4, 15-16]. Interferon regulatory

factor 4 (IRF4) and interferon regulatory factor 5 (IRF5) have been implicated in various processes in BCR and TCR activation, along with B-cell and T-cell development and function [3, 17-20].

1.2.2. IRF4 and IRF5 in B-Cell Activation and Development

In a recent study, IRF5 was identified to regulate early stages of B cell activation and differentiation in response to BCR and TLR signaling. Specifically, knockdown (KD) of IRF5 in human primary naïve B cells resulted in a significant reduction in the B cell activation marker, CD86, supporting an early regulatory role for IRF5 [3, 5]. Although somewhat distinct from IRF5, IRF4 has demonstrated expression in B cells at most developmental stages and has been identified to control class-switch recombination (CSR), regulation of germinal center (GC) formation, and PB differentiation and development [5, 21-23]. IRF4 expression is typically low in pro-B cells, elevated in pre-B cells, and high in ASCs after differentiation, and 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 [3, 23]. 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 [21, 24-25]. Through upregulation of AID, Bcl6, and Blimp1, IRF4 regulates CSR, GC reaction initiation and termination, and PB differentiation, respectively, and it has an important role for B cell function and homeostasis [26]. IRF4 has also been identified as necessary for pre-B cell development, receptor editing at the immature B cell stage, the primary way B cells revise antigen-receptors and maintain self-tolerance, and mature B-cell generation, and it is a major regulator of antibody-secreting cell (ASC) differentiation, proliferation, and cell cycle control [22-23, 25]. Despite a variety of distinct functions, studies suggest that IRF5 is also involved in CSR, possibly through AID regulation, and the IRF5-Ikaros axis was found to be a key modulator of IgG2a/c class switching [3, 27-28]. Furthermore, IRF5 was found to be a regulator of Blimp1, and it stimulates Blimp1 expression, indicating a role in GC reactions [29].

1.2.3. IRF4 and IRF5 in T-Cell Activation and Development

Both IRF4 and IRF5 have been identified as having roles in the development and function of T-cells. IRF4 was identified as a mediator of TCR signaling, and in murine experiments, expression levels were found to peak very early, at 6-9 hours, before declining by 12-16 hours [17, 19]. IRF4 was shown to be crucial for CD4+ and CD8+ T-cell function, expansion, effector differentiation, and cell fate, along with production of pro-inflammatory cytokines including IL-4, IL-17, and IL-21 [17, 19, 26, 30]. Furthermore, IRF4 is important for the differentiation, development, and responses of a variety of T-cell subtypes, including Tc17, Th2, Th17, and T-follicular helper (Tfh) cells, and IRF4 expression was also found to give Treg

cells the ability to suppress Th2 responses [19, 26, 31]. Interestingly, the role(s) of IRF4 in GC formation and GC B cell differentiation through its regulation of Tfh differentiation and development, highlights its ability to link B- and T-cell interactions [32]. IRF4 has also been identified as a molecular "rheostat" capable of sensing TCR signal strength and directing signaling outcomes [17-18]. 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 [20, 33]. More specifically, in *Irf5*$^{-/-}$ mice, Th cell differentiation was skewed from Th1 cells to Th2 cells [33]. This causes an imbalance in cytokine production and protective responses, as Th1 cells produce cytokines including interferon gamma (IFNγ), IL-21, and tumor necrosis factor beta (TNFβ) for phagocyte-dependent responses, while Th2 cells produce cytokines including IL-4, IL-5, IL-10, and IL-13 for protective responses independent of phagocytes [34].

1.2.4. IRF4 and IRF5 in Autoimmune Disease

In addition to their roles in a variety of immune cell processes and functions, IRF4 and IRF5 are molecules of therapeutic interest due to their implications in autoimmune disease since the transcription factors impact a wide variety of downstream genes and pathways. IRF5 hyperactivation and risk polymorphisms are associated with SLE onset and development, and some of these variants in SLE cells are associated with increased IFN production [35-37]. Furthermore, IRF5 was identified as necessary for the production of IgG2a subtype autoantibodies, which are the most prominent in mouse models of autoimmunity, and studies in a murine model of lupus found a cytokine imbalance in *Irf5*$^{-/-}$ mice that favored Th2 polarization, while *Irf5* loss also weakened the in vivo IFN-I signature necessary for SLE pathogenesis [38-39]. IRF4 is also important in autoimmune disease since it is key for Th, Treg, B-cell and DC development, all of which are crucial for the pathogenesis of autoimmune diseases including SLE, MS, inflammatory bowel disease, and type 1 diabetes [27]. IRF4 has been implicated in the development of lupus-like symptoms in a murine model, and another study found that *Irf4*-deficient mice had enhanced cytokines but attenuated lupus nephritis, as disease activity, chronicity, and plasma creatinine levels were significantly lower than in WT mice [40-41]. Furthermore, different IRF4 gene expression signatures were identified in SLE and RA patients, with significantly elevated IRF4 expression in RA, and also increased IRF4 levels in multiple myeloma (MM) [42-43]. Although MM is not an autoimmune disease, some preliminary findings have linked it to autoimmune disease development, and there were several IRF4 target genes in MM oncogenesis and B cell development that were found to overlap [43-47]. Thus, this study aimed to analyze the kinetics of IRF4 and IRF5 in BCR- and TCR-induced immune cell activation, along

with the mechanisms by which these TFs cooperate in regulating these processes, both of which are critical components of the adaptive immune arm.

1.3. Purpose/Hypothesis

The primary objective of this study was to analyze the kinetics of IRF4 and IRF5 expression in response to B- and T-cell stimulation in primary mouse splenocytes. It was hypothesized that IRF5 is an early regulator of B-cell activation in response to BCR and works together with IRF4 to control later stages of differentiation and CSR, while in T-cells, the opposite kinetics occur, with IRF4 functioning earlier in response to TCR and working with IRF5 in later stages of differentiation and T-cell effector function.

2.0 Methodology

**All work was conducted by Author unless otherwise stated*

2.1. Isolation of Murine Total Splenocytes

Age- and gender- matched littermate wild-type (WT) and *Irf5*$^{-/-}$ (KO) mice were humanely euthanized with CO_2 and spleens were removed via dissection, both by authorized laboratory personnel. The tissue was manually minced and homogenized with sterile frosted microscope slides into conical tubes with ammonium chloride (NH_4Cl). Tubes were chilled for 5 minutes, centrifuged with the supernatant discarded, and resuspended in DPBS. 10 μL of suspended cells were taken and a cell count was performed using a hemocytometer. Conical tubes were centrifuged once more, with the supernatant discarded, and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The following experimental setups were utilized:

Type of Mice	Time Points (for Surface + Intracellular Staining)
WT	D1, D4, D7
2 x WT, 2 x KO	D1, D2, D3, D7 (B cells only)
2 x WT, 2 x KO	D0, D1, D2, D3, D7 (B cells only), IRF5 secondary antibody (2° Ab) control
3 x WT	D0, D1, D2, D3, D4 (T cells only) D7 (B cells only), 10x diluted 2° Ab control
2 x WT, 1 x KO	D0, D1, D2, D3, D4, D7, 10x diluted 2° Ab controls, IRF4 isotype controls

2.2. Cell Culture and Stimulation

Suspended cells were diluted with RPMI supplemented with 10% FBS for concentrations of about 2-3 million cells per plated well. Cells for BCR stimulation were plated in 96-well round-bottom plates (200 μL/well) while those for TCR stimulation were plated in pre-treated 24-well flat-bottom plates (500

μL/well). BCR stimulation wells were treated with a C4 cocktail (2.5 μg/mL CpG-B, 10 μg/mL anti-mouse IgM, 100 ng/mL IL-21, and 1μ μg/mL anti-mouse CD40), while TCR stimulation wells were treated with 5 μg/mL CD3 and CD28 in 300 μL DPBS for at least 24 hours. 200 μL cells were plated in each BCR well and 500 μL cells in each TCR well. All plated cells were incubated at 37°C with 5% CO_2 until use for signaling analysis.

2.3. Multicolor Flowcytometry Signaling Analysis

2.3.1. Surface Staining

BCR and TCR stimulated total splenocytes were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) supplemented with 2% fetal bovine serum (FBS) and stained with antibodies against surface markers for 30 minutes (all antibodies were from Biolegend; CD4-PerCP, #100432; CD4-BV510, #100553; CD69-FITC, #104506; CD19-PerCP, #115534; B220-BV510, #103248; CD86-AF700, #105024; CD86-PE). Stimulated B cells were stained for CD19, CD86 and B220 while stimulated T cells were stained for CD4 and CD69. CD19 and B220 were used to differentiate B cells from total splenocytes and CD4 was used to differentiate T cells, as these markers have been shown to be present in various developmental stages and subsets of the respective cell types [48-50]. All cells were also stained with Live/Dead viability discrimination dye (Invitrogen #L34968/#L23101) and fixed with 2% formaldehyde for at least 30 minutes. After fixation, cells were washed and resuspended in DPBS.

2.3.2. Intracellular Staining

For intracellular IRF4 and IRF5 staining, after fixation, cells were permeabilized overnight in 0.5% Triton X-100 with Trustain [Purified Rat Anti-Mouse CD16/CD32 (BD Biosciences #553142)] and 1% FBS. The next day, cells were washed with 0.1% Triton X-100 and stained for IRF4 and IRF5 with anti-IRF4 antibody (Biolegend #646404/ #646408) and anti-IRF5 primary antibody (Abcam #ab181553) for 1 hour. Cells were then washed and stained with IgG APC secondary antibody (Invitrogen #A-10931) for 30 minutes to analyze IRF5 (secondary antibody was diluted 10x with DPBS for later trials due to high background staining). Cells were washed twice and resuspended in DPBS until samples were run on a BD Fortessa flow cytometer. Isotype controls for IRF4 PE antibody and diluted APC secondary were appropriately stained with PE Rat IgG2a, κ Isotype Ctrl (Biolegend #400507) and 10x diluted IgG APC secondary antibody, respectively, and washed following the same intracellular staining procedure.

2.4. Data Analysis

Representative figures from flow cytometry, activation percentages, and protein mean fluorescent intensities (MFIs) were generated using FlowJo software. Data are reported as mean ± SD and were graphed using GraphPad Prism (version 7.0). Multiple *t* tests assuming unequal SD were conducted to compare corresponding rows for individual expression graphs of IRF5 and IRF4. All statistical analyses were performed using GraphPad Prism (version 7.0). In each figure legend, the number (n) of biological

repeats included in the final statistical analysis is indicated (apart from some D4 and D7 values with fewer repeats). *p*-values ≤ 0.05 were considered significant.

3.0 Results

**All figures were created by Author unless otherwise stated*

3.1. B cell receptor (BCR) stimulation and *Irf5* knockout (KO) impact B cell activation

Among the pathways known to be involved in B cell activation is BCR signaling [51]. IRF5 acts within this pathway to regulate a variety of B cell developmental functions [3]. Analysis of the percentage of B cells expressing CD86, an early marker of B cell activation, showed differences among unstimulated cells treated with IL-21 and BCR stimulated WT and *Irf5*$^{-/-}$cells (Figure 1A). In WT cells, BCR stimulation with C4 cocktail resulted in generally higher activation percentages than unstimulated cells; p≤0.01 D2, D4 (Figure 1B). Furthermore, activation percentage was higher in WT compared to *Irf5*$^{-/-}$ cells, even though both groups were stimulated; p≤0.001 D2, p≤0.05 D3 (Figure 1C). These data further support the role of BCR signaling in B cell activation and suggest a role of IRF5 in the pathway, as activation was defective in knockout mice.

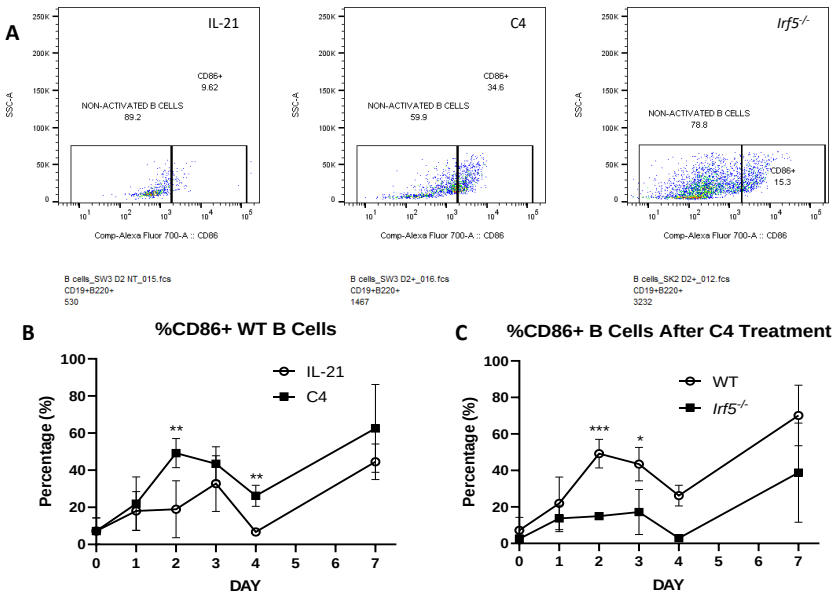

Figure 1 B cell receptor (BCR) stimulation and *Irf5* knockout (KO) impact B cell activation (**A**) Representative images of CD19+B220+ B cells from one wildtype (WT) and one *Irf5*$^{-/-}$ mouse that were stimulated with IL-21 or C4 cocktail for 48h (D2). (**B**) Difference in the percentage of WT B cells expressing CD86, as gated in (A), between cells treated with IL-21 alone or C4 cocktail (n = 5). (**C**) Difference in the percentage of B cells expressing CD86 after treatment with C4 cocktail, as gated in (A), between WT and *Irf5*$^{-/-}$ cells (n = 3). Error bars represent SD. *p≤0.05; **p≤0.01; *** p≤0.001, supporting the role of BCR in B cell activation, and suggesting a role for IRF5 in the pathway.

3.2. IRF5 plays a key role in early- and late-stage B cell activation

Further analysis comparing differences in IRF5 MFI between various groups illustrated a key role for IRF5 in the early and late stages of B cell activation, from about 24-48 h. IRF5 expression levels were significantly higher in activated (CD19+B220+CD86+) compared to total CD19+B220+ cells, $p \leq 0.01$ D1 and $p \leq 0.05$ D2 (Figure 2A), BCR-stimulated compared to non-stimulated cells, $p \leq 0.01$ D1 and $p \leq 0.05$ D2 (Figure 2B), and CD86+ compared to CD86- cells, $p \leq 0.01$ D1/D2 (Figure 2C) from D1-D2. These data suggest that IRF5 is crucial for the entire B cell activation process through a role in BCR signaling, as activated (CD86+) cells drove the increases in expression levels while MFIs for WT cells not expressing CD86 were significant lower during the activation period.

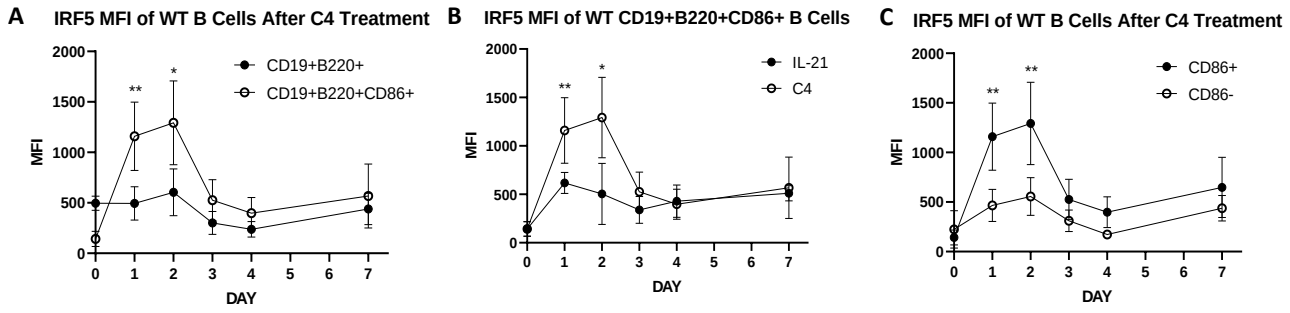

Figure 2 IRF5 plays a key role in early- and late-stage B cell activation. Change in IRF5 mean fluorescent intensity (MFI) from D0-D7 and difference between: (**A**) CD19+B220+ and CD19+B220+CD86+ wildtype (WT) B cells after treatment with C4 cocktail ($n = 4$). (**B**) WT CD19+B220+CD86+ B cells stimulated with IL-21 or C4 cocktail ($n = 4$). (**C**) CD86+ and CD86- WT B cells after treatment with C4 cocktail ($n = 4$). Error bars represent SD. *$p \leq 0.05$; **$p \leq 0.01$ suggesting that IRF5 plays a key role in B cells throughout the activation process (D1-D2) through functions in BCR signaling.

3.3. IRF4 is involved in late-stage B cell activation and subsequent processes

IRF4 has been implicated in several B cell process, particularly in later stages of development [3, 5, 21-25], and it was found that IRF4 expression levels were significantly higher primarily in later activation stages and time points further out. IRF4 MFI was significantly higher in activated compared to total CD19+B220+ cells from D2-D4, $p \leq 0.05$ D2/D4 and $p \leq 0.01$ D3 (Figure 3A), while when comparing BCR stimulated and unstimulated cells, the stimulated cell MFIs were greater at every time point, $p \leq 0.05$ D2/D4/D7 and $p \leq 0.01$ D1/D3 (Figure 3B). When comparing IRF4 expression in WT and *Irf5*$^{-/-}$ cells, WT MFI was significantly higher on D2, $p \leq 0.05$ D2 (Figure 3C), supporting a role for IRF4 in later stages of activation, and a potential interaction between IRF4 and IRF5 or a requirement of IRF5 for IRF4 function at this point, as KO cells did not display any increase in IRF4 expression. Lastly, comparison of CD86+ and CD86- cells illustrated that the increase in IRF4 MFI in WT mice from D2-D3, which is considered late-stage activation, was driven by the activated cells, $p \leq 0.05$ D2 and $p \leq 0.01$ D3 (Figure 3D). Together, these data indicate a role for IRF4 in later activation stages and an interaction/requirement of both IRF4 and IRF5 at this time point, with IRF4 potentially functioning downstream of IRF5.

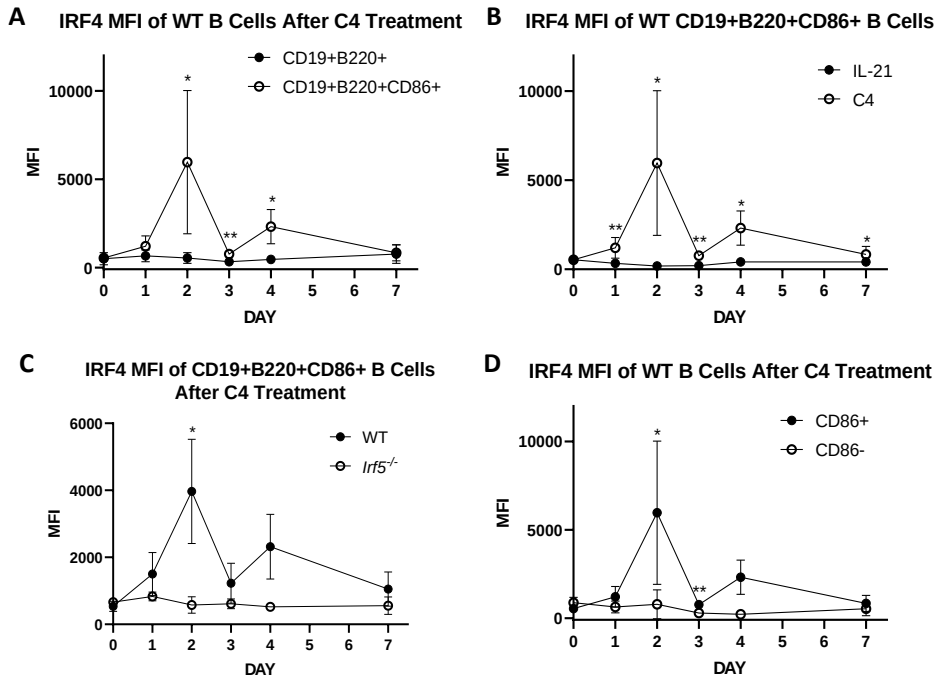

Figure 3 IRF4 is involved in late-stage B cell activation and subsequent processes. Change in IRF4 MFI from D0-D7 and difference between: (**A**) CD19+B220+ and CD19+B220+CD86+ wildtype (WT) B cells after treatment with C4 cocktail (n = 4). (**B**) WT CD19+B220+CD86+ B cells stimulated with IL-21 or C4 cocktail (n = 4). (**C**) WT and *Irf5*$^{-/-}$ CD19+B220+CD86+ B cells after treatment with C4 cocktail (n = 3). (**D**) CD86+ and CD86- WT B cells after treatment with C4 cocktail (n = 4)**.** Error bars represent SD. *p≤0.05; **p≤0.01, suggesting that IRF4 plays a role in later stages of B cell activation and potentially functions downstream of IRF5 starting at this stage.

3.4. T cell receptor (TCR) stimulation and *Irf5* knockout (KO) impact T cell activation

While both IRF4 and IRF5 have been implicated in various T cell activation and functionality processes, much less is known about IRF5 in early stages of T cell development [17-20]. Like CD86 expressing B cells, analysis of the percentage of T cells expressing CD69, one of the earliest markers of activation, showed differences among non-treated, treated WT and treated *Irf5*$^{-/-}$ cells (Figure 4A). TCR stimulation with CD3/CD28 treatment resulted in higher activation percentages in WT cells compared to the untreated cells, p≤0.01 D1 and p≤0.001 D2/D4 (Figure 4B). Comparison between treated WT and *Irf5*$^{-/-}$ cells showed higher activation percentages in WT cells, suggesting an early role for IRF5 in the process, p≤0.05 D2/D3 (Figure 4C). Together, these data suggest the role of TCR signaling in T cell activation along with a role of IRF5 in the pathway, since like in B cells, knockout cell activation was defective, both of which are novel findings to the current understanding of IRF5 in T cells.

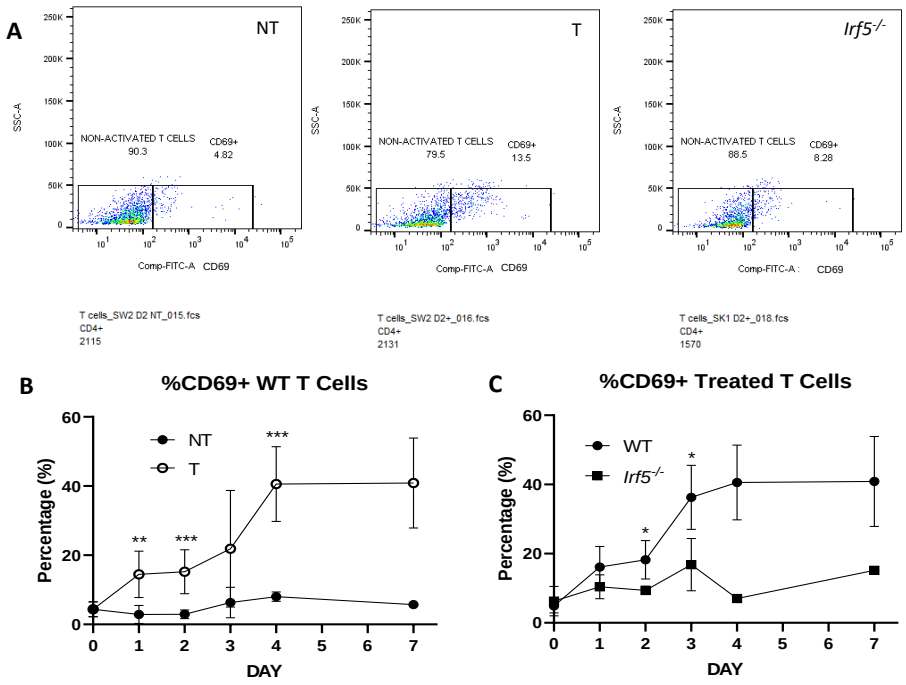

Figure 4 T cell receptor (TCR) stimulation and *Irf5* knockout (KO) impact T cell activation. (**A**) Representative images of CD4+ T cells from one wildtype (WT) and one *Irf5*$^{-/-}$ mouse that were not treated or stimulated with 5 µg/mL CD3 and CD28 for 48 h (D2). (**B**) Difference in the percentage of WT T cells expressing CD69, as gated in (A), between treated and non-treated cells (n = 6). (**C**) Difference in the percentage of treated T cells expressing CD69, as gated in (A), between WT and *Irf5*$^{-/-}$ cells (n = 3). Error bars represent SD. *p≤0.05; **p≤0.01; *** p≤0.001, supporting the role of TCR signaling in T cell activation and suggesting that IRF5 plays a role in the process through functions in the signaling pathway.

3.5. IRF5 plays a role in later stages of T cell activation

More in depth comparisons of IRF5 expression levels with MFIs indicated its importance primarily in later stages of T cell activation (D2). Activated cells (CD69+) had significantly higher MFIs from D1-D3 compared to total CD4+ cells, with the greatest difference on D2, p≤0.05 D1/D3 and p≤0.01 D2 (Figure 5A). Compared to non-treated cells, treated cells had the greatest MFI on D2, but D1 was the only timepoint with a significant difference between the two groups, p≤ 0.05 D1 (Figure 5B). However, a comparison of CD69+ and CD69- cells illustrated that the activated cells drove an increase in IRF5 expression from D1-D3, p≤0.05 D3 and p≤0.01 D1/D2 (Figure 5C). These data suggest a novel role for IRF5 in multiple stages of T cell activation, more importantly in later stages of activation, around 48 h post-stimulation.

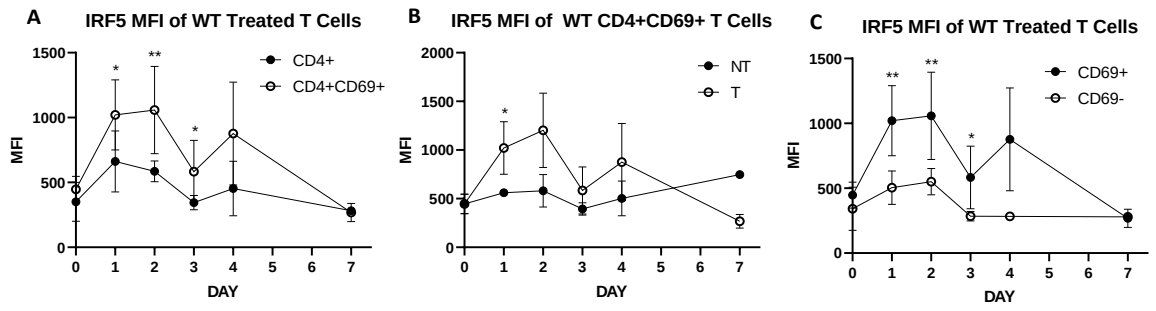

Figure 5 IRF5 plays a role in later stages of T cell activation. Change in IRF5 MFI from D0-D7 and difference between: (**A**) CD4+ and CD4+CD69+ WT T cells after treatment with 5 µg/mL CD3 and CD28 (n = 5). (**B**) Wildtype (WT) non-treated and treated CD4+CD69+ T cells (n = 4). (**C**) CD69+ and CD69- WT treated T cells (n = 5). Error bars represent SD. *p≤0.05; **p≤0.01, suggesting a role for IRF5 in multiple stages of T cell activation, particularly in later stages (around D2)

3.6. IRF4 plays a key role in early- and late-stage T cell activation

Similar to published findings about a very early role for IRF4 in T cell activation [19], analysis of expression levels highlighted a distinct peak 24 h after stimulation (D1) in all four comparisons made (Figure 6A-D). Compared to that of total CD4+ cells, the IRF4 MFI of activated (CD4+CD69+) cells was significantly higher from D1-D3, p≤ 0.01 D3 and p≤ 0.001 D1/D2 (Figure 6A), and these activated cells had significantly higher MFIs on D1-D2 compared to non-treated cells, p≤0.001 D1/D2 (Figure 6B), which demonstrates a key role for IRF4 in TCR signaling. In addition, compared to *Irf5*$^{-/-}$ activated cells, WT activated cells had significantly higher MFIs on D1-D2, which not only suggests a role for IRF4 throughout T cell activation, but also illustrates a role for IRF5 in IRF4-mediated T cell activation, as IRF4 expression levels in KO cells were much lower and did not peak the same, p≤ 0.05 D1/D2 (Figure 6C). Lastly, compared to that of cells not expressing CD69, the IRF4 MFI of CD69+ cells were significantly higher on D1-D3, throughout the T cell activation process, and this correspondence with other peak levels indicates that the activated cells are responsible for the increase in IRF4 expression, signifying the involvement of this protein in the process, p≤0.001 D1-D3 (Figure 6D). Altogether, these data implicate IRF4 in early- and late-stage T cell activation, and they also suggest a novel role for IRF5 in IRF4-mediated T cell activation.

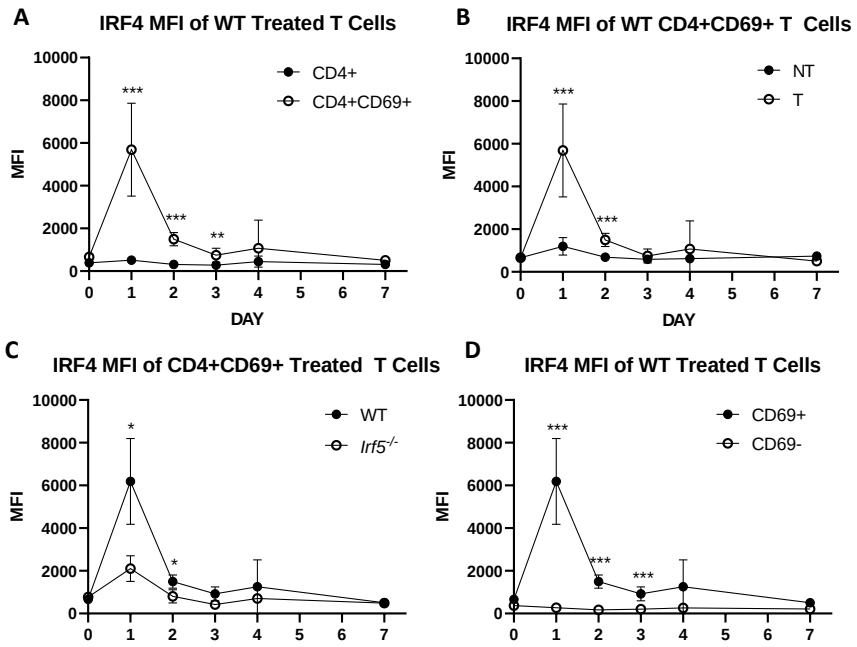

Figure 6 IRF4 plays a key role in early- and late-stage T cell activation. Change in IRF4 MFI from D0-D7 and difference between: (**A**) CD4+ and CD4+CD69+ WT T cells after treatment with 5 µg/mL CD3 and CD28 (n = 7). (**B**) Wildtype (WT) non-treated and treated CD4+CD69+ T cells (n = 6). (**C**) WT and *Irf5*$^{-/-}$ CD4+CD69+ treated T cells (n = 3). (**D**) CD69+ and CD69- WT treated T cells (n = 5). Error bars represent SD. *p≤0.05; **p≤0.01; *** p≤0.001, suggesting a key role for IRF4 throughout T cell activation along with a novel role of IRF5 in IRF4-mediated activation.

3.7. IRF4 and IRF5 have distinct kinetics of expression during B- and T-cell activation

In accordance with the main objectives of this study, the cumulative kinetics of IRF4 and IRF5 in both B and T cells were analyzed. In BCR-stimulated activated B cells, IRF5 expression is highest on D1-D2, while IRF4 expression peaks at D2 and is also elevated at D4 (Figure 7A). The distinct D1 peak of IRF4 expression, along with an elevated MFI through D2 is demonstrated in TCR-activated T cells, while IRF5 expression was relatively lower but has peaks at D2 and D4 (Figure 7B). Furthermore, while IRF4 and IRF5 appear to overlap at certain timepoints, particularly D1-D2, both proteins have kinetically distinct roles in B and T cells over the seven-day period (Figure 7C-D). Taken together, these data show the importance of both IRF4 and IRF5 in various stages of B and T cell activation, along with some of the distinctions between their most prominent expression levels and likely, their most important functions.

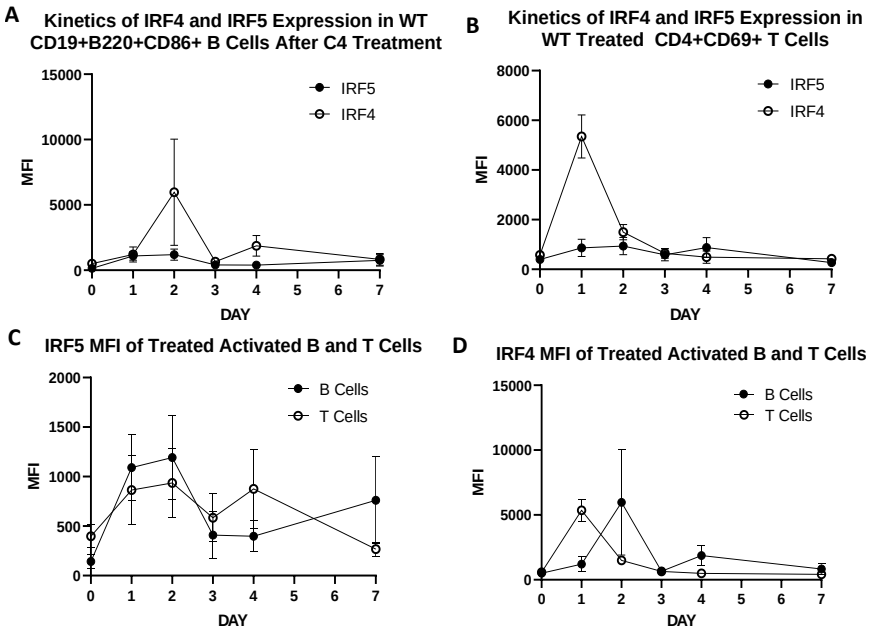

Figure 7 IRF4 and IRF5 have distinct kinetics in B- and T-cell activation and development. From D0-D7, changes in: (**A**) IRF4 and IRF5 MFI of WT CD19+B220+CD86+ B cells after C4 treatment (n = 5). (**B**) IRF4 and IRF5 MFI of WT CD4+CD69+ T cells after treatment with 5 µg/mL CD3 and CD28 (n = 7). (**C**) IRF5 MFI of treated activated B and T cells (n = 5). (**D**) IRF4 MFI of treated activated B and T cells (n = 5). Error bars represent SD.

4.0 Discussion and Limitations

4.1. IRF4 and IRF5 in B- and T-cell activation

To protect the body against foreign antigens, several signaling mediators (transcription factors) and their respective pathways (BCR, TLR and TCR) enable naïve B- and T-cells to specialize into a variety of cell subsets for innate and adaptive immune responses [6-11]. In particular, the IRF family of transcription factors has been identified as key regulators of the immune system and various B- and T-cell processes [15]. Two of these proteins, IRF4 and IRF5, have been implicated in several B- and T-cell developmental stages and functional processes [3-5, 17-20], but a more comprehensive kinetics analysis of both proteins in the early specialization of both cell types has not yet been done. It was shown that both IRF4 and IRF5 expression increased in B- and T-cells following BCR and TCR stimulation, respectively, $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ (Figures 2B, 3B, 5B and 6B), suggesting that both proteins are involved in the pathways and are involved with some of the earliest stages of maturation. To characterize activation, CD86 and CD69 were used as markers for B- and T-cells respectively, as both are expressed during early activation, immediately after BCR and TCR engagement [52-53]. Comparison of IRF4 and IRF5 expression levels between activated cells (CD86+/CD69+) and total B and T cells showed both proteins were more highly expressed in the activated cells, notably during the later stages of the activation process, $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ (Figures 2A, 3A, 5A and 6A), which suggests that both proteins are necessary for driving the

activation of both cell types. In addition, to determine if increases in IRF4 and IRF5 expression levels were driven by activated cells, the four subsets (both proteins in both cell types) were compared to cells that did not display the activation markers (CD86-/CD69-), and indeed, expression levels were significantly higher in the activated cells, $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ (Figures 2C, 3D, 5C and 6D), suggesting that both proteins are involved with activation and have roles in later developmental stages, as expression levels increased.

4.2. Role of IRF5

Cells from *Irf5*$^{-/-}$ mice were also studied to further analyze the roles of IRF5 in activation, and interestingly, IRF4 expression was significantly lower in *Irf5*$^{-/-}$ cells compared to WT cells during the activation stage, $p \leq 0.05$ (Figures 3C and 6C), which suggests an interaction between the two proteins in the process of activation that is novel in T cells and corroborates published findings in B cells. Previous studies in human naïve B cells have identified IRF4 as a downstream target of IRF5 and have found non-redundant roles for both proteins in B cell activation, proliferation, and differentiation [3, 5]. Even in a murine model, this study provides further evidence for an interaction between the two proteins in B and T cells along with insight into another link between two members of the IRF family in immune responses. In addition, comparing changes in activation percentage to changes in IRF4 and IRF5 expression demonstrated that generally, greater activation occurred at timepoints that also had greater protein expression, $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ (Figures 1 and 4), further corroborating the link between both proteins and cell activation. Similar to lower protein expression in *Irf5*$^{-/-}$ cells, activation percentages were also lower during some activation stages, $p \leq 0.05$, $p \leq 0.001$ (Figures 1C and 4C), indicating that IRF5 plays a role in both BCR and TCR signaling that impacts the processes.

4.3. Kinetics Analysis

Lastly, analysis of the full kinetics of IRF4 an IRF5 in B and T cells highlighted distinct roles for both proteins and a preliminary understanding of how expression differs in the seven-day period after BCR or TCR stimulation. In B cells, IRF5 expression levels were high in both early and late activation, while IRF4 was elevated in later activation stages and further in the development process (Figure 7A), which suggests that the proteins first interact in late-stage activation and may continue to do so through the maturation and differentiation process. In T cells, a characteristic IRF4 expression peak in early activation [19], followed by an immediate decrease, was observed, while IRF5 expression levels increased in later activation and development (Figure 7B), suggesting a very early role for IRF4 and later novel roles for IRF5 which may be related to the decrease of IRF4. Protein expression levels throughout the time course do have several points of overlap in both activation and later stages of development (Figure 7C-D), which

indicate related roles of IRF4 and IRF5. More importantly, an intricate relationship is shown between the two in B and T cells, which serve to further our understanding of these two key proteins in successful immune cell development and responses.

4.4. Limitations

Despite significant findings in activation percentage, protein expression, and the impact of *Irf5* KO, certain limitations arose throughout experimentation and were addressed in analysis. Samples were run and analyzed through flow cytometry, and background staining impacted earlier results until proper dilutions and isotype controls were used, which limited the number of biological repeats for graphing and statistical analysis. Alternate experimental procedures for protein expression analysis, namely Western Blots, would provide further evidence for any changes in expression levels. Furthermore, a limited number of $Irf5^{-/-}$ mice and limited data for later timepoints, particularly D4 and D7, resulted in increased variation as not many replicates were available for comparison or to identify outliers, so additional replicates may provide more significant expression level changes at these time points and may highlight distinct roles for IRF4 and IRF5 in later development processes. In addition, this study used murine total splenocytes and differentiated between cell types by gating for surface markers specific to B and T cells, but repeating the same procedure with isolated naïve B and T cells, along with performing similar experiments in human primary immune cells, may provide more specific results with closer relations to human immune dysfunction and autoimmune disease. Last, analysis of intermediate time points may help to target where both proteins have the greatest expression and functionality.

5.0 Conclusions and Future Studies

Taken together, results identify distinct and related roles for IRF4 (Figures 3, 6) and IRF5 (Figures 2, 5) in BCR- and TCR-induced activation (Figures 1, 4, 7), along with a more targeted effect of IRF5 on IRF4 expression (Figures 3C, 6C). As originally hypothesized, in B cells, IRF5 expression was high at early time points, and in T cells, IRF4 expression also peaked, indicating early regulation in both instances. However, IRF4 in B cells and IRF5 in T cells demonstrated increased expression earlier than expected, starting in later activation stages, and therefore likely interact with the initially expressed protein much sooner than previously thought. Furthermore, from study of *Irf5* KO splenocytes, a link between IRF4 and IRF5 in B and T cell activation was observed, implicating IRF4 as a downstream target of IRF5 and finding a novel role for IRF5 in early T cell development, along with a novel interaction between the two proteins in T cells. For significant findings in all data sets, p values ranged between $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

This study suggests roles of both IRF4 and IRF5 in B and T cell activation and serves as a basis for further investigation of both proteins in more specific stages of activation and later stages of maturation. Their role so early in the specialization of immune cells makes IRF4 and IRF5 molecules of interest for study in autoimmune disease, as protein dysfunction could have more severe consequences, especially if the proteins have key roles in early processes. Since IRF4 and IRF5 both play roles in B and T cell activation, any dysfunction of these proteins could result in negative repercussions in immune cell development and the generation of successful immune responses. From both an academic and therapeutic standpoint, these results help further our understanding of the interaction(s) and cross-talk between proteins and signaling pathways on the immune system and how we could potentially utilize this information for the development of new therapeutic targets to treat patients with autoimmune disease.

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