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The Transcription Factor MEF2C regulates B cell maturation, proliferation, and function

by

Dustin Khiem

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Dedication

I dedicate this work to my parents, Pieter and Elaine, who came to this country with virtually nothing and have given me everything. Thank you for your unending sacrifice and support of my development as a scientist and human being.

Acknowledgements

The contents of this thesis, my success as a graduate student, and my professional and personal development would not have been possible without the attentive and direct mentorship provided to me by my thesis advisor, Brian Black. Under his tutelage, I've learned to be more attentive to detail while also learning to maintain focus on the view from 10,000 feet up. I've learned that he is not a frog and I am not a rabbit, so we must never jump ahead. I thank Brian for his no-nonsense mentoring style, which, while difficult at times to swallow, provided me with the motivation and direction that every lost graduate student needs. Brian, through his enthusiasm for science, has managed to attract and motivate a dynamic group of amazing individuals. The high caliber of labmates that I've gotten to befriend is truly a testament to Brian's achievement as a successful scientist, principal investigator, and mentor.

I would not have been able to graduate without the support and supervision from my thesis committee, Jason Cyster and Matthias Hebrok. I thank Jason Cyster for his intellectual guidance and for his role as a BMS administrator. Working on an immunology based thesis project in a heart development lab would have been virtually impossible without his direction. Jason has contributed greatly to my work and I thank him for all of intellectual contributions. I am grateful to Matthias for providing an objective, "outsider's" critical eye to my project. His constructive criticism has taught me to be detail oriented and to subject my work to a higher standard of accuracy. While towards the end of my thesis he felt that he could not contribute as substantially, I thank

Matthias for his willingness to serve on my committee and to support me as a graduate student.

I owe a large debt of gratitude to my collaborators in the Cyster lab for providing to me their technical expertise. Robin Lesley taught me core immunology techniques which much of the data from this thesis is based on. Larry Shiow and Guy Cinamon provided me with various reagents and protocols, in addition to making me feel welcome in the lab. I thank Ryan Swenerton, Maha Abudalla, and Zachary Mackey from the McKerrow lab for their help with biochemical techniques and T. brucei infections. Without the help of Chris Wilson from the Chuang lab, my reviews for my publication would probably have taken much longer. Thank you for your assistance with lentiviral transductions and for entertaining me while in tissue culture.

I thank the members of the Black lab, present and past, for their friendship, company, laughter, and camaraderie. As any graduate student will tell you, graduate school is often filled with frustrating and depressing days of failure. Without the wonderful individuals that I have gotten to meet through the years I wouldn't have enjoyed my time at UCSF as much as I have. They have supported me both by helping me with experiments and also by creating a lab environment that is fun to work in.

I thank my parents, Pieter and Elaine, who although might not understand the minutiae of this thesis, fully appreciate that this work is the fruit of their labor. I am ever grateful for your trust and confidence in me. Now that I'm ready to go off into the "real world"

I hope the slow repayment can begin.

I could never forget the wonderful friends that I've met in the Biomedical Sciences program at UCSF. Thanks guys for the beers, parties, bbqs, and random days of playing hooky. You have made this trial by fire, known as grad school, a truly memorable experience and I am forever grateful.

Much of the work and its accompanying figures were submitted for publication to the *Proceedings of the National Academy of Sciences* by Dustin Khiem, Jason G. Cyster, John J. Schwarz, and Brian L. Black, entitled: **A p38 MAPK-dependent MEF2C transcriptional pathway regulates B cell proliferation.**

Abstract

B lymphocytes are an integral part of the adaptive immune system. Upon antigen binding to the B cell receptor (BCR), B cells rapidly proliferate and differentiate into antibody-secreting plasma cells. The p38 mitogen activated protein kinase (MAPK) pathway functions downstream of the BCR to control cell proliferation, but the transcriptional effectors of this pathway in B cells have remained elusive. The MADS domain transcription factor MEF2C functions as a signal-dependent transcriptional switch to control a wide array of developmental and physiological processes. In addition to its established role in muscle and neural lineages, MEF2C is also expressed in B cells, but its function in that lineage had not been determined previously. In the present study, we inactivated *Mef2c* exclusively in B cells by conditional gene targeting in mice. Loss of MEF2C function resulted in a reduced immune response to antigen and a severe defect in B cell proliferation, and we show that MEF2C regulates proliferation in response to BCR stimulation via the p38 MAPK pathway. These studies establish MEF2C as an important regulator of B cell proliferation in response to BCR stimulation via its role as a downstream transcriptional effector of the p38 pathway.

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General Introduction

The immune system

The immune system of higher organisms, in particular mice and humans, consists of multiple cell and tissue types that function to protect the organism from infection and associated pathology. It is a highly adaptable system that is capable of recognizing an extremely diverse array of foreign antigens, while also possessing the ability to discriminate self antigens derived from the host organism {DeFranco, 1987 #47}. A properly developed immune system is vital to the survival of the host organism as immunodeficiency can leave the host defenseless against infection; while conversely, an over active immune system can lead to lymphoma {Bende, 2007 #61}. The following studies focus on the development and function of one component of the immune system, the B cell. The signaling pathways, transcriptional networks, and genes of B cells are investigated with the goal of understanding the molecular mechanisms underlying the development, function, and proliferation of this cell type.

The immune system is largely broken down into two main components: innate and adaptive immunity. The innate immune system consists of such cells as macrophages, neutrophils, basophils, and eosinophils that are capable of immediately recognizing and clearing foreign pathogens. However, innate immunity functions in a largely generic, non-specific manner, and does not confer lasting protection. The adaptive immune system complements the innate immune system by reacting to foreign pathogens with much higher specificity and has the ability to develop immunological memory. The main

effectors of the adaptive immune system are B and T cells.

B cells differentiate from a common hematopoietic lineage known as HSCs. These HSCs can be found in the yolk sacs of embryos as early as embryonic day 7.5. B cells expressing key markers have been identified as early as embryonic day 12.5 in the fetal liver of mice {de Andres, 2002 #66}. B-cell differentiation is controlled by a series of transcription factors {Kee, 2001 #67}. The E proteins, E12 and E47, and early B-cell factor (EBF) transcription factors regulate the rearrangement and expression of the IgH and Igκ gene loci, and are essential for B-cell lineage commitment and subsequent development. The Pax5/BSAP protein is expressed in the earliest B-cell precursors, and its expression has been shown to favor B-cell lineage commitment through the repression of key genes which promote the differentiation of other cell lineages, including T cells {Nutt, 1999 #68}. B cells develop in successive stages and undergo multiple rounds of selection and proliferation {Cooper, 2002 #1}. It is these mature B cells that function to survey the body for foreign antigen and combat infection {Fu, 1999 #30}. Follicular (Fo) B cells are the main subset of mature B cells and play a critical role in an effective adaptive immune response by secreting highly specific antibodies {DeFranco, 1987 #47}. These antibodies are capable of neutralizing foreign antigens and recruiting other components of the immune system to aid in the clearing of the pathogen. Much has been learned about the development and function of Fo B cells, however, the genes and transcriptional pathways that are critical to the development and function of B cells have yet to be fully elucidated.

The transcription factor MEF2C

Transcription factors are proteins that control the precise spatial and temporal transcription of genes. MEF2C is one such transcription factor that has been demonstrated to play a critical role in regulating the differentiation and proliferation of many tissue types {Black, 1998 #11; McKinsey, 2005 #55; Czubryt, 2004 #56}. *Mef2c* is a member of the MADS domain family of transcription factors, named for the first four members of the family to be identified MCM-1, *Agamous, Deficiens*, and Serum Response Factor (Shore and Sharrocks, 1995). It is composed of a DNA binding and protein dimerization domain on the N-terminal end with transactivation domains near the C-terminus of the protein. Mice have four MEF2 family members: MEF2A, MEF2B, MEF2C, and MEF2D. MEF2 transcription factors form homo-dimers or hetero-dimers with other MEF2 family members and bind to a consensus DNA-binding element (5'-YTAWWWWTAR-3') {Black, 1998 #11}. *Mef2c* has been well studied for its essential role in skeletal and cardiac muscle development. Underscoring its importance, inactivation of *Mef2c* in mice results in embryonic lethality at E10 due to heart morphogenic and myogenic defects {Lin, 1997 #12}. MEF2C functions as an integrator of specific upstream signals and has the ability to both activate and repress genes. This is accomplished through various post-translational modifications to MEF2C, which can toggle its association with histone acetylases or deacetylases {McKinsey, 2001 #13}. The mitogen activated protein kinase p38 is capable of phosphorylating MEF2C on 3 amino acids which then activates this transcription factor. On the other hand, the activity of MEF2C can also be regulated by the addition of other post-translational modifications such as sumoylation, which serves to repress MEF2 activity {Gregoire, 2005 #69}.

Interestingly, *Mef2c* has been demonstrated to be highly expressed in B cells, but not T cells of the spleen and lymph node {Swanson, 1998 #15}. Since this observation, other studies have described potential roles for MEF2C in B cells. Gene expression profiling studies on B cells have demonstrated *Mef2c* expression levels to be significantly increased in anergic B cells, while slightly downregulated in activated B cells {Glynne, 2000 #16}. MEF2C has also been implicated in the transcriptional regulation of the J-chain gene, required for the proper secretion of IgM pentamers {Rao, 1998 #17}. Despite these studies, the exact function of MEF2C in B cells has not been directly tested.

The studies presented here focus on understanding the function of MEF2C in B cells. In Chapter 1, we outline a strategy to inactivate MEF2C exclusively in B cells by conditional gene targeting in mice. We show that loss of MEF2C function resulted in a profound decrease in Fo B cells of the spleen and lymph node. These results suggest a critical role for MEF2C in proper peripheral B cell development. Chapter 2 focuses on the functional consequences of the loss of MEF2C in B cells. We show that mice lacking MEF2C in B cells exhibit a reduced humoral response upon immune challenge. Further investigation revealed that this dampened immune response was due to a severe proliferative defect in B cells lacking functional MEF2C, uncovering a novel role for this transcription factor in B cells. Finally, in Chapter 3, we investigate the molecular mechanisms by which MEF2C controls proliferation in B cells. We show that MEF2 activity is strongly stimulated by BCR stimulation, and that this activation is dependent upon direct p38 MAPK phosphorylation of MEF2C. Taken together, these studies uncover novel roles for MEF2C in B cell development and function.

Chapter 1: MEF2C is essential for normal peripheral B cell development

Introduction

B cells are critical for protection against a wide range of pathogens including bacteria, viruses, and fungi. In order for the immune system to be capable of responding to such an expansive and diverse array of antigens from potential pathogens, it requires the continuous development and maintenance of a large pool of B cells {Srivastava, 2005 #62}. This process of B cell development occurs first in the bone marrow, and then in the periphery, usually the spleen. Although many studies have described the role of various transcription factors involved in early B cell development, there exists a gap in the literature regarding those transcription factors involved in peripheral B cell development.

The development of Fo B cells in the adult begins in the bone marrow where they undergo selection for the successful rearrangement of the B cell receptor (BCR). B cells that express a BCR then exit the bone marrow, and enter into the spleen to undergo further peripheral development. These B cells are termed transitional B cells, and consist of at least 2, possibly 3 sub-populations based on surface marker expression and function. The least mature of these cells are known as Transitional 1 or T1 B cells and are characterized by a high degree of apoptosis upon BCR stimulation. T1 B cells that survive selection for self reactivity continue to mature into T2 B cells. However, it is unclear whether T2 B cells proliferate like mature B cells or undergo apoptosis upon BCR stimulation. What seems to be clear is that T2 cells are more responsive to T cell proliferation cues compared to T1 cells {Allman, 2001 #4}. From this pool of T2 B cells,

mature, long-lived B cells emerge. There are 3 major subsets of mature B cells: B1, marginal zone (MZ), and follicular (Fo) B cells. The development and origins of the B1 B cell lineage remains to be further elucidated, however this sub-group is not thought to undergo similar pathways as MZ B or Fo B cells. The vast majority of these mature B cells are the classical, recirculating Fo B cells, while a smaller percentage develops into MZ B cells.

The MADS domain transcription factor MEF2C is highly expressed in B cells. In the present study we interrogate B cell development from the bone marrow to the periphery in mice lacking MEF2C in B cells. We show that MEF2C is required for proper B cell development. Inactivation of *mef2c* in B cells results in a severe reduction in mature B cells, specifically within the follicular B cell populations, suggesting a critical role for MEF2C in peripheral B cell development.

Results

Stage-specific Mef2c expression peaks in Follicular B cells during B cell development.

Previous studies have demonstrated that *Mef2c* is highly expressed in B cells. To better resolve the expression profile of *Mef2c* throughout the course of B cell development, we employed Taqman quantitative real-time PCR on B cells sorted from wild type C57/BL6 mice. For comparison of relative transcript expression levels, Taqman QT-PCR also performed on RNA isolated from adult brain, heart, and diaphragm. All samples were

controlled by comparing to the endogenous housekeeping gene *HPRT1*. *Mef2c* expression was relatively low in the B cells of the bone marrow, starting at the Pro-B cell stage, and increased at the Pre-B cell, and immature B cell stages (Fig.1). In the spleen, *Mef2c* expression continued to increase as B cells matured from transitional 1 (T1) and transitional 2 (T2) stages. Follicular B (Fo B) cell populations expressed the highest levels of *Mef2c*. Interestingly, marginal zone B (MZ B) cells did not follow the trend as *Mef2c* expression levels were comparable to that of the Pre-B cell stage. Confirming earlier observations, *Mef2c* expression was practically undetectable in T cells {Swanson, 1998 #15}. The stage-specific increase in *Mef2c* expression levels in B cells, with the notable exception of MZ B cells, suggest that *Mef2c* plays a role in the later stages of B cell development, particularly in the Fo B population.

Conditional inactivation of Mef2c in B cells.

To determine the function of *Mef2c* in B cells *in vivo,* we employed a conditional knockout strategy by crossing $Mef2c^{+/-}$; $CD19^{Cre/+}$ mice to $Mef2c^{flox/flox}$ mice (Fig. 2A). *CD19* is expressed exclusively in B cells from early in development such that this cross resulted in specific inactivation of *Mef2c* in B cells (Fig. 2B) {Rickert, 1997 #28}. *Mef2c* B cell knockout (KO) mice were born alive and in Mendelian ratios (Fig. 2C). In order to circumvent potential inconsistencies due to genetic variation in strain background, $Mef2c^{+/-}$ and $Mefc^{flox/flox}$ animals were backcrossed for 9 generations into a C57J/BL6 congenic background.

MEF2C is not required for early B cell development in the bone marrow

In order to determine the function of MEF2C during the course of B cell development, we isolated bone marrow from femurs of adult 8 week old *Mef2c* B cell KO mice and littermate controls. Bone marrow lavages were then stained for surface markers and sorted by FACS. We observed no significant differences in total cell numbers of Pro-B, Pre-B, and immature B cells between KO and control animals (Fig. 3A, B). However, we did notice a slight increase in mature, recirculating B cells that reside in the bone marrow (Fig 3A, B). These results suggest that MEF2C is not required for proper early development of B cells in the bone marrow. They are also consistent with earlier observations of low expression levels of *Mef2c* in these early B cell developmental stages (Fig. 1).

MEF2C is required for normal peripheral B cell development

Splenocytes from 8 week old adult mice were harvested and analyzed by flow cytometry. Total numbers of B cells isolated from spleens were not different between *Mef2c* B cell KO animals and controls (data not shown). Continuing our analysis of B cell development, we investigated the earliest stages of peripheral B cell development, which are the transitional B cells. Total transitional 1 (T1) B cell numbers were slightly increased in KO as compared to control animals. Conversely, we observed a slight decrease in transitional 2 (T2) B cells in the spleen (Fig. 4). However, these results were not statistically significant.

Since *Mef2c* expression was highest in the Fo B cell population, we hypothesized that the development of this population of mature B cells would be most affected by conditional inactivation of *Mef2c*. We first investigated general B cell maturation and discovered that total mature B cells were significantly reduced in KO animals (Fig. 5A). Upon further investigation, this reduction in mature B cells of *Mef2c* KO animals was mainly due to a significant reduction in Fo B cells. Flow cytometry plots illustrates a loss of a tight focus of Fo B cells in *Mef2c* B cell KO animals (Fig. 5B). Total Fo B cell numbers were reduced by approximately 50% in KO animals as compared to controls (Fig. 5C). Since total splenic B cell numbers were not different between *Mef2c* B cell KO animals and controls, we sought to identify which population of B cells was compensating for the loss of Fo B cells. By separating mature B cells by the surface markers CD21 and CD23, we resolved an unknown population of B cells ($B220^{+}$, $AA4.1^{-}$, $CD21^{-}$, $CD23^{-}$; Fig. 5B). This population had a broader CD23 expression as compared to the B cell population of the control animals and could represent a "dead end" in B cell development, resulting from loss of MEF2C activity. Alternatively, it is possible that MEF2C is required for the proper expression of certain Fo B cell surface markers such as CD23.

In order to determine if the defect in peripheral B cell development extended to the marginal zone B cells, we assayed this population of cells via FACS analysis. We observed a slight, but statistically insignificant increase in total MZ B cell numbers in KO animals (Fig. 5B, C). Since it is possible that the misregulation of the surface marker CD23 could lead to inaccurate cell counts, we further resolved the MZ B population by

CD9 and CD1d, both markers of activation (data not shown). These experiments did not give different results, allowing us to conclude that MZ B cell development was not affected by the inactivation of MEF2C. Lastly, B1-B cells of the peritoneal cavity were assayed by flow cytometry, but did not yield significant differences between control and knockout animals (data not shown).

Discussion

Resolution of an unknown, immature B cell population in MEF2C KO B mice

In this study we identified a critical role for MEF2C in peripheral B cell development. Our data suggest that in B cells, MEF2C controls the stepwise differentiation of transitional B cells to functional Fo B cells. We can find parallels in skeletal muscle development, where MEF2C is critical for the transition of myoblasts to myocytes by inducing expression of a host of downstream muscle genes. In mice lacking *Mef2c* in B cells, we observed a population of functionally immature cells that could represent a previously unseen cell population that have failed to transition into Fo B cells. We suspect that the resolution of this unknown population ($B220^{+}$, $AA4.1^{-}$, $CD21^{-}$, $CD23^{-}$) was only possible due to a proliferative defect in these cells, although others have not reported the observation of such a population. This population could potentially represent 1) a "pre-Fo" B cell population after the T2 stage, 2) an increase in MZ B cells, 3) Fo B cells that have misregulated their CD23 expression or 4) a "dead end" in development. Because these data were also confirmed in lymph nodes, where all B cells should be

mature, we ruled out the possibility that this unknown population of cells could be transitional B cells. To address the possibility of an increase in MZ B cells, we used flow cytometry with antibodies against the activation markers CD9 and CD1d and found no significant increase in this population of B cells. In order to identify this unknown B cell population, functional studies will need to be conducted to circumvent misregulation of key cell surface markers. These experiments are conducted in Chapter 2, when the functional consequences of *Mef2c* inactivation in B cells are interrogated.

MEF2C in peripheral B cell fate decisions

Due to the defect in Fo B cells population in *Mef2c* conditional KO animals, it is possible that MEF2C is playing a role in cell fate decisions between MZ and Fo B cells. Results from studies that have inactivated the zinc finger transcription factor Aiolos and the tyrosine kinase BTK have suggested that the strength of B cell receptor signals plays a critical role during peripheral B cell development . Strong BCR signals have been suggested to promote differentiation of Fo B cells, while weaker signals favored MZ B cell development. Our studies are in agreement with these data and implicate MEF2C as part of a potential sensor for BCR signal strength due to the reduction in Fo B cells. However, the lack of a statistically significant increase in MZ B cells suggest that BCR signal strength alone does not control the cell fate decision of mature B cells into the Fo B or MZ B cell lineage. It is likely that BCR signal strength potentiates a cell fate decision, but that other signals such as Notch signaling are required to fully differentiate B cells.

Figures

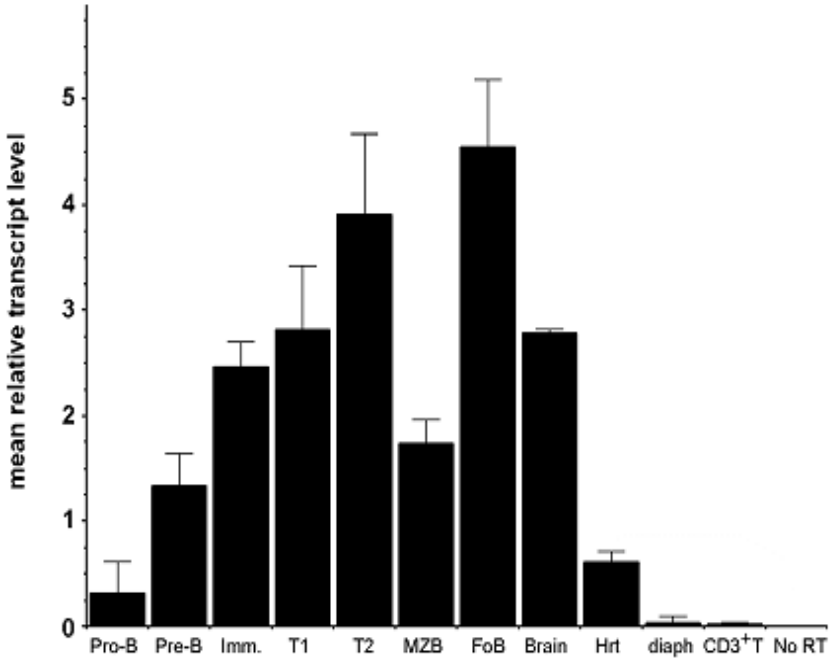
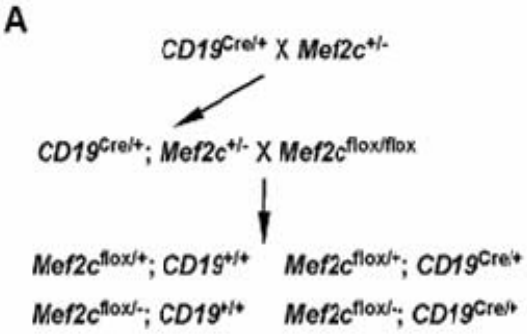
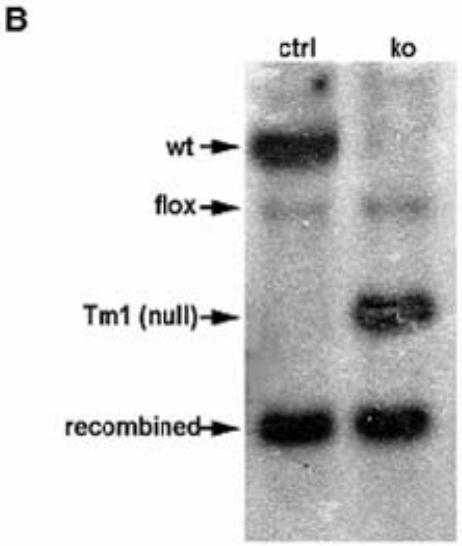


Figure 1. Stage-specific *Mef2c* expression peaks in Follicular B cells during B cell development.

Mef2c expression was relatively low in the B cells of the bone marrow, starting at the Pro B cell stage, slightly increasing at the Pre-B cell, and immature B cell stages. In the spleen, *Mef2c* expression continued to increase as B cells matured from transitional 1 (T1) and transitional 2 (T2) stages. Follicular B (FoB) cell populations expressed the highest levels of *Mef2c*. Marginal zone B cell (MZB) *Mef2c* expression levels were comparable to that of the Pre-B cell stage. Confirming earlier observations, *Mef2c* expression was practically undetectable in T cells. Adult brain, heart (hrt), and diaphragm (diaph) were used as reference for *Mef2c* expression.





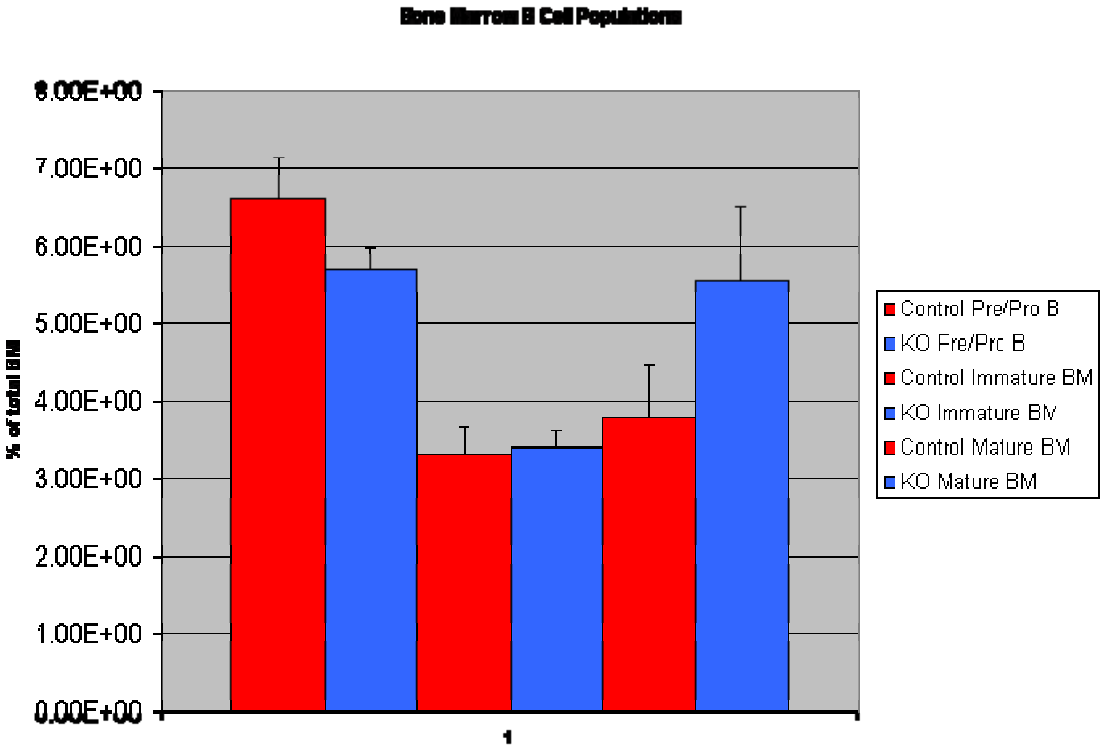
C

	Mef2c$^{flox/+}$	Mef2c$^{flox/-}$
CD19$^{+/+}$	39	40
CD19$^{Cre/+}$	36	41

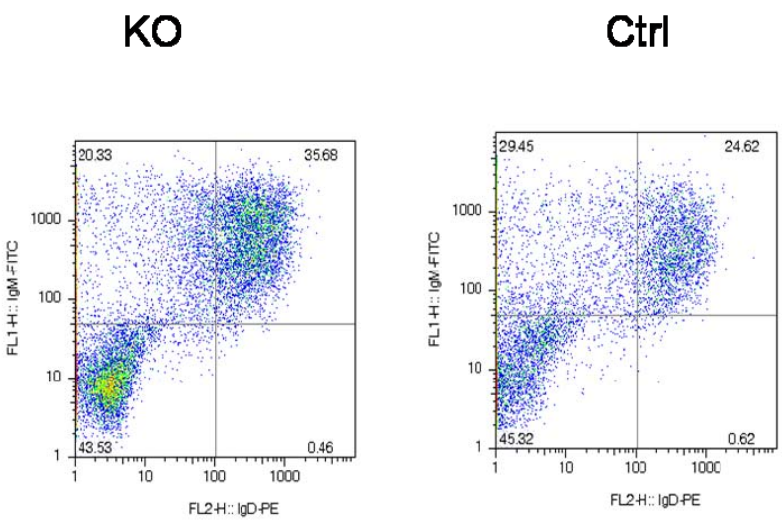
Figure 2. . **Conditional inactivation of *Mef2c* in B cells.**

A) We employed a conditional knockout strategy utilizing Cre recombinase driven by the *CD19* locus to excise *Mef2c* flanked by LoxP sites (*Mef2c*$^{flox/flox}$). *CD19*$^{Cre/+}$ mice were crossed to *Mef2c*$^{+/-}$ mice. The resultant *CD19*$^{Cre/+}$; *Mef2c*$^{+/-}$ mice were then crossed to

Mef2c$^{flox/flox}$ mice to generate knockout animals. All mice were backcrossed into a C57J/BL6 background for 9 generations. For all experiments, *CD19*$^{Cre/+}$; *Mef2c*$^{flox/+}$ littermates were used to control for the effect of *CD19* heterozygosity. B) Deletion of *Mef2c* allele in sorted Fo B cells was confirmed by Southern blot. C) *Mef2c* B cell knockout animals (*CD19*$^{Cre/+}$; *Mef2c*$^{flox/-}$) were born at the expected 1:4 ratio.



A



B

Figure 3. MEF2C is not required for early B cell development in the bone marrow.

A) Total bone marrow cells of control (Red) and *Mef2c* B cell KO (Blue) mice were

analyzed by flow cytometry. No significant differences in B cell numbers were observed in the Pro/Pre B, immature B, and mature/recirculating B cells.

B) Representative flow cyometry graphs comparing control and B cell KO bone marrow B cells. First column is KO, second column is control.

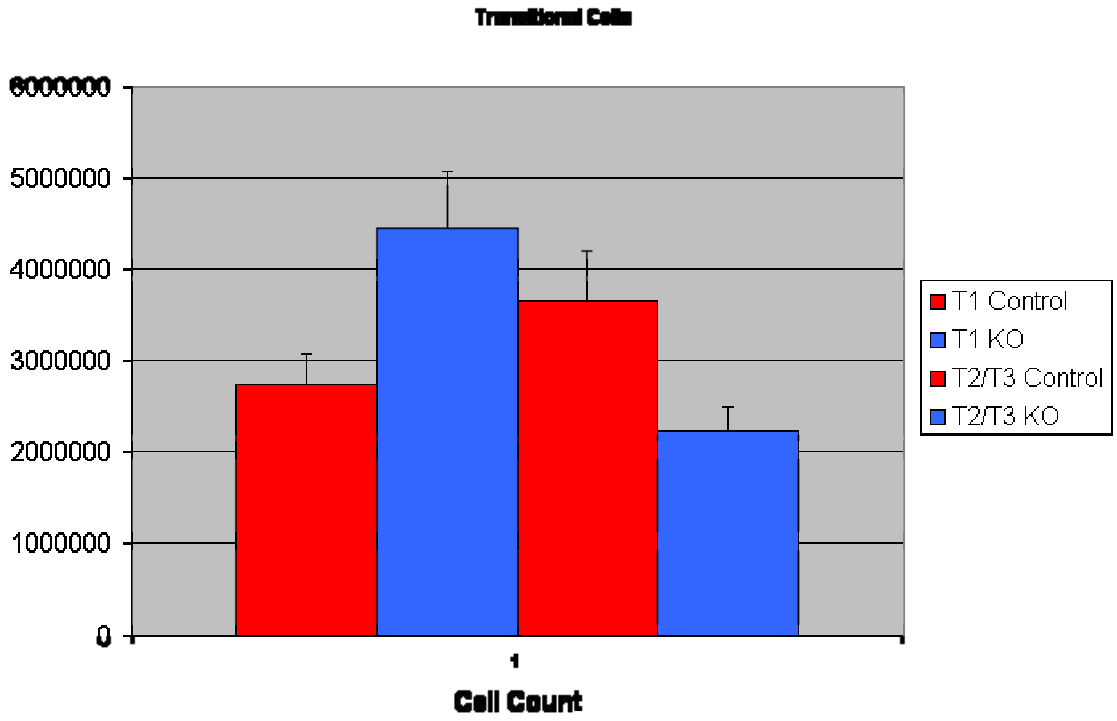
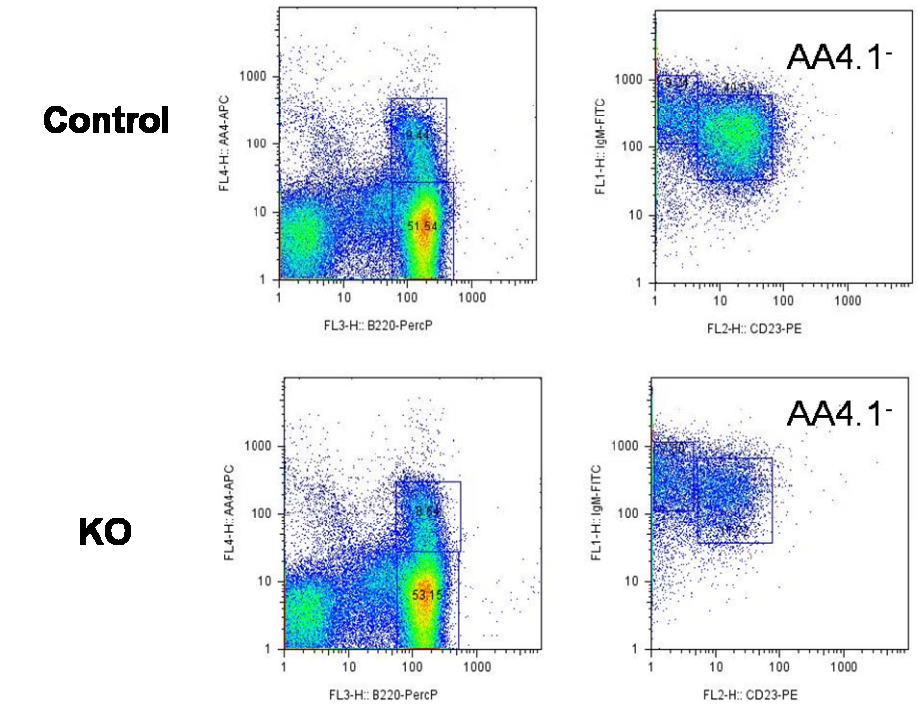
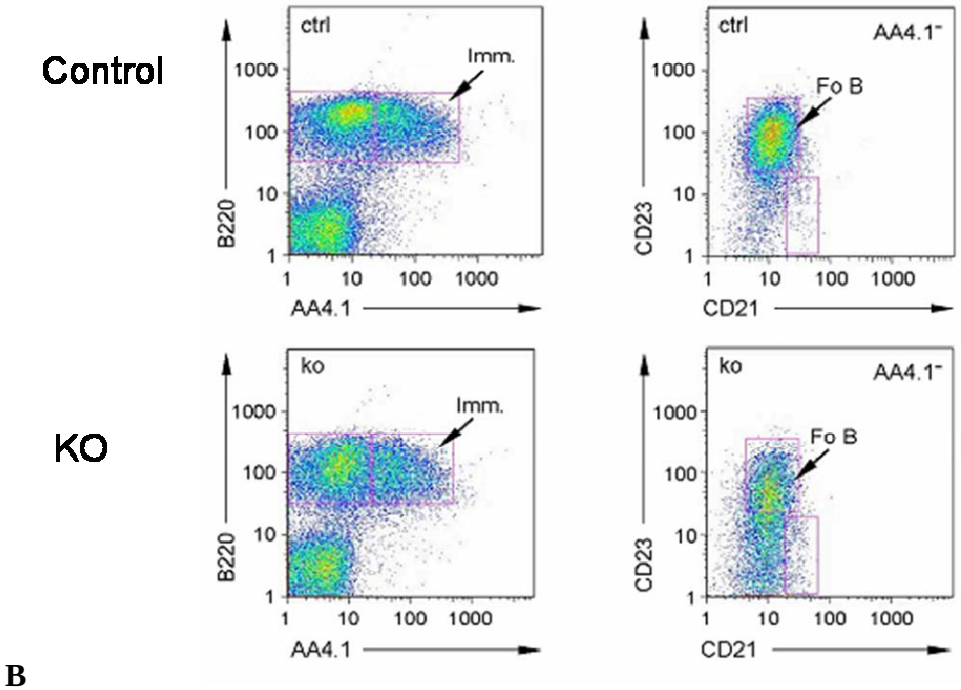


Figure 4. **Development of Transitional B cells are perturbed in *Mef2c* B cell KO animals.**

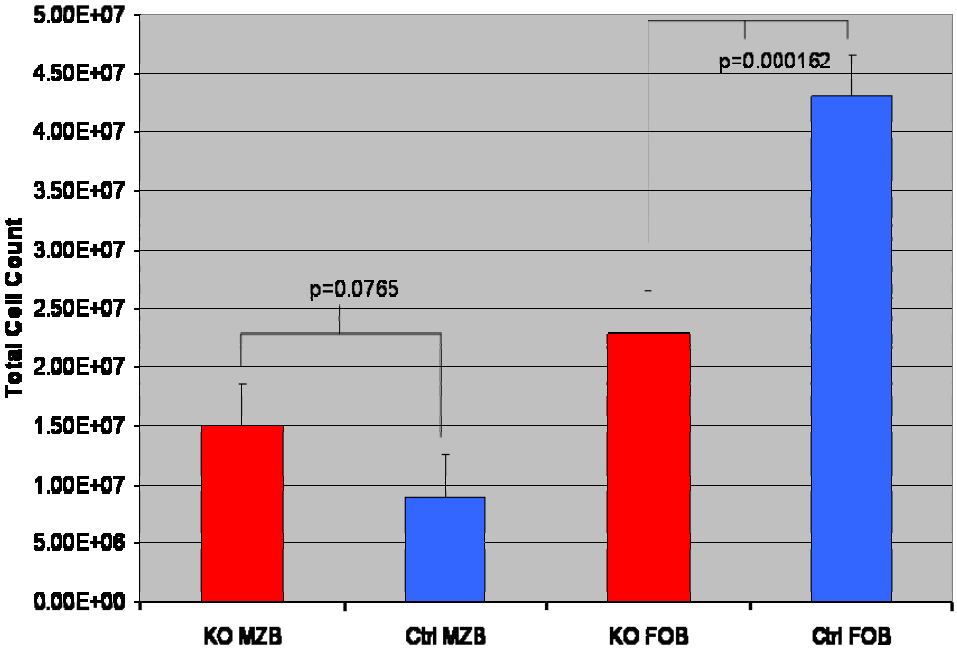
Total transitional cells from control (Red) and *Mef2c* B cell KO (Blue) were compared by flow cytometry. Total T1 cells were increased in KO mice, while T2/T3 cells were decreased. P values are indicated on the graph.


Control
KO
AA4.1-
FL4-H:: AA4-APC
FL3-H:: B220-PercP
FL1-H:: IgM-FITC
FL2-H:: CD23-PE
18.44
51.54
8.69
53.15

A


Control
KO
ctrl
ko
Imm.
Fo B
AA4.1-
B220
AA4.1
CD23
CD21

B



C

Figure 5. MEF2C is required for efficient follicular B cell development.

A) Total mature B cells of the spleen were analyzed by flow cytometry. Top row is control group, bottom row is KO group. First column shows similar total numbers of B cells of the spleen, however 2nd column further sorted on AA4.1$^-$ B cells, depicts a significant loss of mature B cells of the spleen (IgM^{mid}, CD23$^+$).

B) Flow cytometry was used to separate mature, splenic B cells into marginal zone (2nd column, CD21$^+$, CD23$^-$) and follicular (CD21$^-$, CD23$^+$) B cells. Note loss of tight Fo B cell grouping in KO group (lower right box). Top row is control group, bottom row is KO group.

C) Total Fo B (FOB) cells in KO (blue) mice are reduced by 50% as compared to control mice (red). Marginal zone B cells (MZB) are not statistically different between the two.

Chapter 2: MEF2C is critical for the proper function and proliferation of follicular B cells

Introduction

In the previous chapter, we identified a novel role for the MADS domain transcription factor MEF2C in peripheral B cell development. We showed that total Fo B cells in animals inactivated for *Mef2c* in B cells were reduced by approximately 50% compared to littermate controls. We now focus on the functional consequences of this severe reduction in those B cells that are critical for the production of antibody during a humoral immune response. We hypothesize that the loss in Fo B cells in *Mef2c* B cell knockout mice will exhibit a reduction in Ig response upon immune challenge.

In the course of a normal immune response, those few B cells that happen to recognize a particular antigen will adopt one of two fates. A small percentage will immediately begin to produce IgM class of antibodies that help to containing the infection. The vast majority of B cells that react to a particular antigen will undergo rapid proliferation in germinal centers located in lymph nodes and spleens. There, B cells undergo competition for growth and survival based on BCR binding strength and specificity to the antigen. B cells will also receive signals from T cells and dendritic cells to undergo both somatic hypermutation to create antibodies of higher specificity and class switching to IgG1, and other immunoglobulin classes. Typically the IgM response to antigen peaks at 7d post immunization, while the more specific IgG1 response peaks between 14d to 21d post immunization. Once the pathogen is cleared, most of the B cells specific for that pathogen undergo apoptosis. However, a small percentage develop into memory B cells, which can be activated rapidly if the immune system encounter the same pathogen,

greatly increasing the efficiency of a secondary response.

In general, there are two classes of antigens: T cell-dependent (TD) or T cell-independent (TI). TD antigens are ancient, multivalent antigens such as bacterial lipopolysaccharide (LPS), and usually elicit a response from marginal zone (MZ) B cells. This type of mature B cell usually responds quickly to antigen stimulation, differentiating to antibody-secreting plasma cells. They are believed to fill the gap between innate immunity, which is rapid, and adaptive immunity which generally peaks in 2 weeks. TI antigens are more complex antigens and mainly elicit a response from Fo B cells, which are the main mature B cell population that comprises germinal centers. The major challenge for the immune system is to select for those few cells that are reactive for a given antigen and expand them considerably to mount a robust response to the foreign pathogen. In this chapter, we show that MEF2C is critical for proper humoral response against a TD antigen. We demonstrate a reduction in IgG1 titers post immunization, which is caused by defects in germinal center formation. We also show that these defects are caused by a defect in proliferation of Fo B cells lacking functional MEF2C.

Results

Mef2c is required for efficient primary humoral immune response

Since mice lacking *Mef2c* in B cells exhibited a severe reduction in Fo B cells, we tested the function of these cells by immunizing 8 week old *Mef2c* B cell KO mice and

littermate controls with a TD antigen, and examining IgM and IgG1 titers at 0, 7, 14, and 21 d post-immunization (Fig. 6A). Peak response for IgG1 at 14 d was two-fold reduced in *Mef2c* B cell KO mice and it remained low at 21 d (Fig. 6A). To determine whether the lower IgG1 titers in *Mef2c* B cell KO mice were due to a defect in germinal center (GC) formation, we examined lymph nodes from B cell KO and littermate control mice histologically at 14 d post-immunization. IgD and peanut agglutinin (PNA) were used to highlight the lymphoid follicle and GC, respectively. IgD expression was similar in *Mef2c* B cell KO and control lymph nodes, suggesting that follicle structure did not depend on MEF2C (Fig 6B). By contrast, *Mef2c* B cell KO mice showed a reduction in the robustness of GC formation in response to antigen (Fig 6B). To quantify the GC response, we compared the total number of GC B cells from lymph nodes isolated from *Mef2c* B cell KO and control mice by flow cytometry. Consistent with the reduced PNA staining by histology, *Mef2c* B cell KO mice had fewer GC B cells (PNA^+, FAS^+, $B220^+$) than control mice (Fig. 6C). These data indicate that *Mef2c* is critical for the primary IgG1 humoral response to immunization and GC formation.

IgM titers were also tested by ELISA, however there were no significant differences in titer levels between control and *Mef2c* KO mice at 3d or 7d post immunization (Fig 7), suggesting that MEF2C is not necessary for efficient production of this isotype.

Secondary IgG1 response was also tested by ELISA after boosting the animals with the same antigen 40 days post initial immunization. Interestingly, no significant difference in secondary immune response in either IgM or IgG1 was detected between *Mef2c* B cell

knockout animals versus littermate controls at 3 or 7 days post boost (Fig 8A, 8). These results suggest that MEF2C is critical for a specific subset of B cells, Fo B cells, while dispensable in other subsets such as the MZ B and memory B cells.

MEF2C regulates B cell proliferation in response to BCR stimulation

We hypothesized that the reduced GC response in *Mef2c* B cell KO mice might be due to a defect in Fo B cell proliferation upon antigen stimulation of the BCR. To test this hypothesis, we measured [^{3}H]-thymidine incorporation into B cells from *Mef2c* B cell KO and control animals (Fig. 9). An equal number of immature, transitional splenocytes (total B220$^+$, AA4.1$^+$) and Fo B cells (B220$^+$, AA4.1$^-$, CD21$^+$, CD23$^+$) with equivalent levels of CD21 and CD23 expression between KO and control animals were isolated and allowed to proliferate *ex vivo* in response to BCR stimulation. B cells lacking MEF2C function exhibited 8-fold less proliferation than control B cells upon BCR stimulation by addition of α-IgM F(ab')$_2$ (Fig. 9, lanes 3, 4). The difference in proliferation between knockout and control animals was slightly reduced upon exposure to higher concentrations of α-IgM F(ab')$_2$, suggesting that strong BCR stimulation could partially overcome the proliferative defect (data not shown).

To determine if the requirement of MEF2C for proliferation was specific to BCR stimulation, we induced proliferation using bacterial lipopolysaccharide (LPS), which stimulates Toll-like receptors and associated signaling pathways, and therefore, initiates B cell proliferation via a non-BCR-dependent pathway {Genestier, 2007 #34}.

Importantly, B cells lacking MEF2C function proliferated as well as control B cells in response to LPS stimulation (Fig. 9, lanes 5, 6), indicating that MEF2C was not required for general B cell proliferation. As expected, immature AA4.1^{+} B cells from both groups failed to proliferate upon BCR stimulation (Fig. 9, lanes 1, 2). Interestingly, the unknown population (B220^{+}, AA4.1^{-}, CD23^{-}, CD21^{-}) cells also failed to proliferate upon BCR stimulation, suggesting that they are functionally immature in their proliferative capacity. These data demonstrate that MEF2C is a critical effector of B cell proliferation in response to antigen stimulation of the BCR.

Discussion

Distinct transcriptional programs control unique aspects of B cell proliferation

B cell proliferation in response to antigen is critical to a robust immune response. During infection, B cell proliferation and differentiation must occur rapidly to limit tissue damage, and in extreme infections, prior to the host being overcome by the infectious agent {Allen, 2007 #2}. Given the importance of B cells in the immune response, it is not surprising that multiple B cell intrinsic receptor pathways (in addition to T cell-derived signaling pathways) regulate B cell proliferation, including Toll-like receptors in response to LPS and B cell receptor upon specific antigen stimulation {Campbell, 1999 #29; Gerondakis, 2007 #51}. LPS/Toll-like receptor-induced B cell proliferation has been suggested to represent a more evolutionarily ancient pathway, characteristic of the innate immune system, whereas BCR directed proliferation functions as part of a more versatile,

adaptive immunity {Lopes-Carvalho, 2004 #10}. There are two distinct classes of mature B cells involved in humoral immunity and they function differentially in immune responses. MZ B cells function in a more innate-like fashion compared to the classical Fo B cells {Lopes-Carvalho, 2004 #10}. Interestingly, we observed that *Mef2c* expression is highest in Fo B cells and lowest in MZ B cells (Fig. 1), supporting a role for MEF2C specifically in a classical, humoral immune response by Fo B cells. In addition, the lack of any significant differences in IgM titers between KO and controls further support the idea that MEF2C functions in the more classic humoral immune response of Fo B cells. By contrast, NFκB is expressed in all classes of B cells, and inactivation of NFκB results in a broad proliferation defect upon stimulation with either LPS or α-IgM {Grumont, 1998 #43; Siebenlist, 2005 #36}. Inactivation of another set of transcription factors, OCT-2 and OCA-B, led to a defect in B cell proliferation only upon LPS stimulation, but not α -IgM stimulation {Schubart, 2001 #42}. Together, these observations suggest that distinct transcriptional programs may function coordinately to control B cell proliferation in response to unique stimuli with some pathways activating both classes of B cells while others promote MZ or Fo B cell proliferation specifically. Our data demonstrate that MEF2C is a critical regulator of the adaptive immune response and B cell proliferation specifically downstream of BCR stimulation.

Selection of Transitional to mature, Fo B cells requires efficient BCR signaling

In Chapter 1 we discussed the resolution of a "pre-Fo" B cell population that was a result of a peripheral B cell developmental defect. Interestingly, our proliferation assay

determined that this population of B cells ($B220^+$, $AA4.1^-$, $CD23^-$, $CD21^-$) was functionally immature, despite having a marker of maturity ($AA4.1^-$). The flow cytometry results appear to resolve a dynamic process of Fo B cell maturation from transitional B cell stages. Selection for Fo B cells could rely on proper B cell receptor signal strength to ensure proper proliferation when triggered by cognate antigen. Supporting this idea, we observed that MEF2C does not play as critical a role in B cells that have already become activated and have differentiated into antibody secreting plasma cells. Indeed, we saw no difference between knockout and control animals in peak IgG titers in the secondary response following a booster injection with NP-CGG (Fig. 8), suggesting that MEF2C is no longer necessary in memory B cells. Other compensatory mechanisms, such as other MEF2 family members or other signaling pathways may play a larger role in these B cells.

Figures

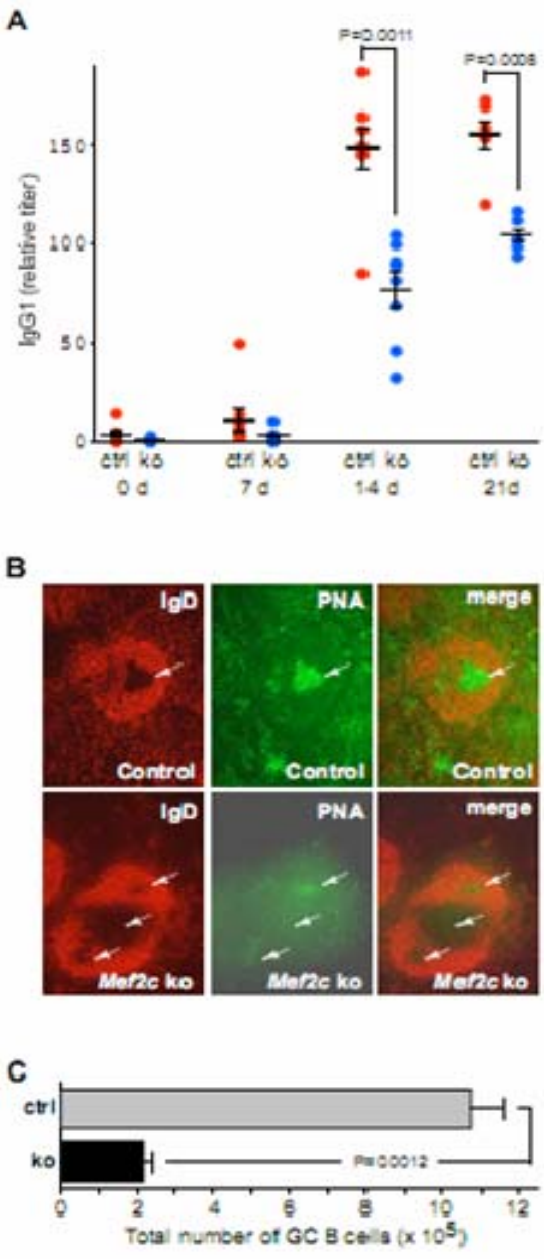


Figure 6. MEF2C is required for efficient IgG1 response to T-dependent antigens.

A) *Mef2c* B cell conditional knockout (ko, blue circles) exhibit reduced NP-specific IgG1 titers compared to control (ctrl, red circles) mice at 14 and 21 d post-immunization with

NP-CGG, as measured by ELISA. B) Immunohistochemistry on sections from lymph nodes depicts a reduced germinal center response (PNA, green) at 14 d post-immunization in *Mef2c* B cell ko (bottom row) compared to control (top row) mice (white arrows indicate germinal centers). Lymphoid follicle structure (IgD, red) remained intact in both groups. C) Quantification of GC defect by flow cytometry shows an approximate 5-fold reduction in total PNA^+, FAS^+, $B220^+$ GC B cells in lymph nodes of *Mef2c* B cell knockout (ko) compared to control (ctrl) mice.

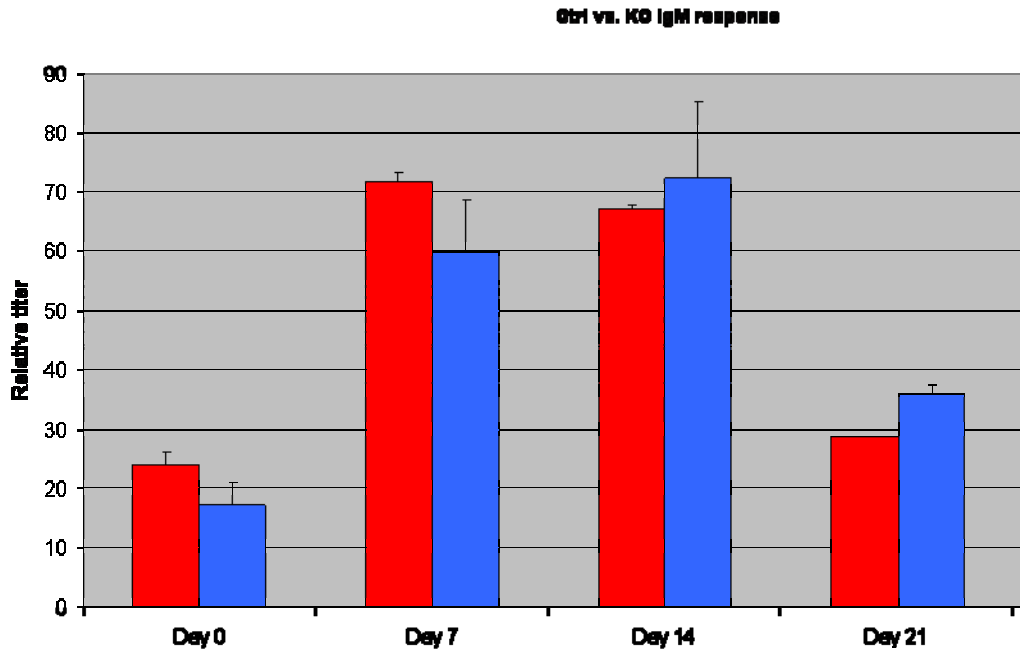
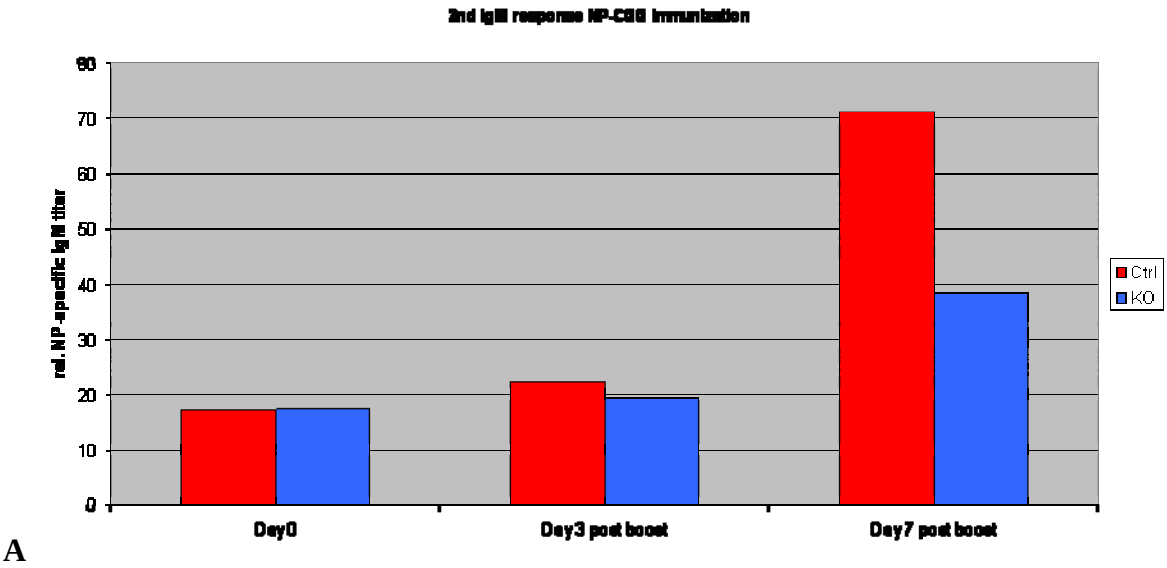
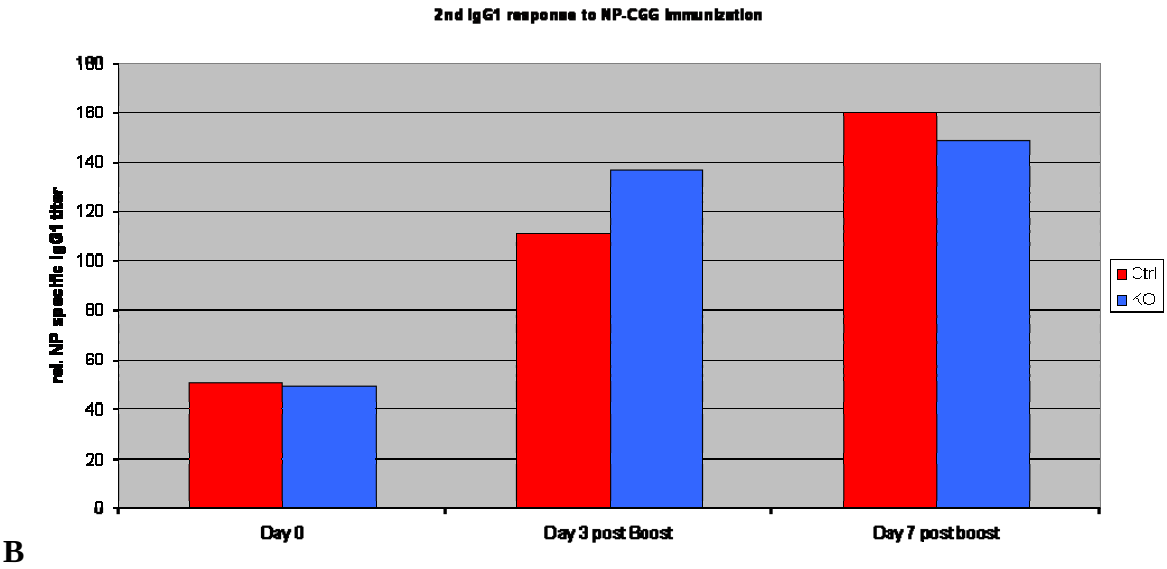


Figure 7. MEF2C is not required for initial IgM response to T-dependent antigen.

Mef2c B cell conditional knockout (blue) exhibited no difference in NP-specific IgM titers compared to control (red) mice at any time point post-immunization with NP-CGG, as measured by ELISA.



A



B

Figure 8. MEF2C is not required for secondary immune response to antigen.

A) ELISA on sera from control (red) and KO (blue) boosted with NP-CGG 45 days post initial immunization showed no difference in IgM titers. B) ELISA on sera from control (red) and KO (blue) boosted with NP-CGG 45 days post initial immunization showed no difference in IgG1 titers.

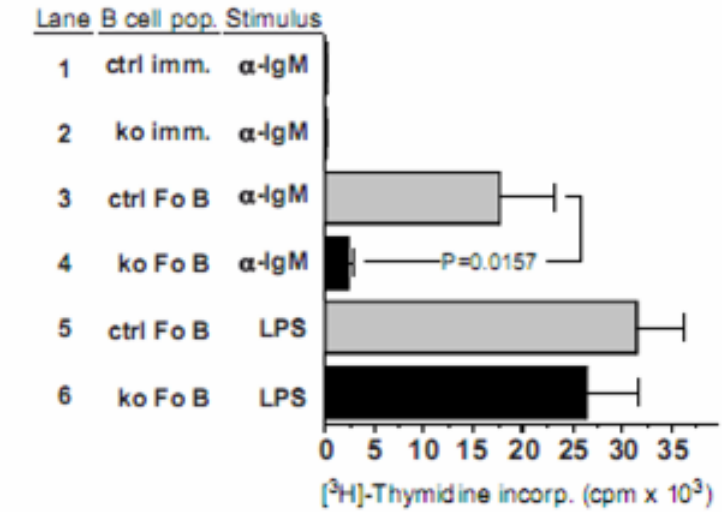


Figure 9. MEF2C regulates B cell proliferation in response to BCR stimulation.

[^{3}H]-thymidine incorporation assay shows an 8-fold reduction in proliferation of sorted Fo B cells from knockout (blue) mice compared to controls (red) upon stimulation of the BCR with α-IgM (lanes 3, 4), but not LPS (lanes 5, 6). Immature B cells (imm.) from either population fail to proliferate upon BCR stimulation (lanes 1, 2). Unknown population of B cells also failed to proliferate

Chapter 3: A p38 MAPK-dependent MEF2 transcriptional pathway regulates follicular B cell proliferation

Introduction

BCR stimulation leads to the initiation of a host of signaling cascades, including the mitogen activated protein kinase (MAPK) pathway, calcineurin cascade, and NFκB pathway. The MAPK pathway consists of 3 subfamilies, which include extracellular-signal regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38. MAP kinases are serine/tyrosine kinases that function in a phosphorylation cascade beginning with MAPKKK to MAPKK to MAPK {Ashwell, 2006 #57}. In the case of the p38 family, MKK6 serves to phosphorylate and activate p38 MAPK, which then can translocate to the nucleus and affect various transcriptional pathways. One important effect of toggling the p38 MAPK pathway is to induce cell proliferation {Raman, 2007 #48}. There are 4 isoforms of p38, α, β, δ, and γ, all of which are expressed in B cells, which suggests that they play an important signaling role in that cell type {Cook, 2007 #70}. However, the transcription factors responsible for controlling cell cycle genes downstream of p38 MAPK in B cells have not been clearly defined.

Recently, MEF2C has been shown to be phosphorylated by p38 MAPK in myocytes and macrophages, which leads to increased expression of known MEF2-dependent targets {Han, 1997 #14; de Angelis, 2005 #54}. MEF2C functions as a signal-dependent transcriptional switch, which allows it to function as an integrator of a variety of upstream signals. We hypothesized that MEF2C controls B cell proliferation downstream of the B cell receptor (BCR) via the p38 MAPK pathway. In Chapter 1, we described the

inactivation of MEF2C exclusively in B cells by conditional gene targeting in mice. We showed that mice inactivated for *Mef2c* in B cells had a severe peripheral B cell developmental defect. In Chapter 2 we showed that the loss of MEF2C function resulted in a profound decrease in peak IgG1 titers upon immunization due to a reduced germinal center response and severely diminished proliferative capacity. In this final chapter, we interrogate the function of MEF2C in BCR induced cell signaling. We utilized gene expression profiling of *Mef2c*-null B cells to demonstrate that many cell cycle genes are downregulated compared to controls, which suggested a defect in the p38 MAPK pathway. We show that MEF2 activity is strongly stimulated by BCR stimulation, and that this activation is dependent upon direct p38 MAPK phosphorylation of MEF2C. These data suggests a pathway in which MEF2C acts downstream of BCR signaling via the p38 MAPK cascade to drive B cell proliferation in response to antigen stimulation.

Results

Activation of MEF2C by BCR stimulation requires p38 MAPK

B cell receptor stimulation results in activation of p38 MAPK signaling and subsequent proliferation {Campbell, 1999 #29}. Previous studies performed in myocytes and macrophages have demonstrated that MEF2C is a direct target of p38 MAPK via phosphorylation of 3 residues in the C terminal transactivation domain {Han, 1997 #14; Yang, 1999 #49; de Angelis, 2005 #54}. Therefore, we reasoned that MEF2C might be an effector of p38 MAPK signaling in B cells in response to BCR stimulation. To test this

notion, we transfected the 2PK3 B cell line with a MEF2-dependent reporter, composed of four MEF2 sites directing luciferase expression, and measured the activity of this reporter upon BCR stimulation. BCR stimulation resulted in an approximately 10-fold increase in MEF2-dependent activation (Fig. 10A, lanes 1, 2). This activation was dependent upon MEF2 binding to the reporter, as transfection with a mutant MEF2 reporter showed no increase in luciferase activity in response to BCR signaling (Fig. 10A, lanes 5, 6). Addition of SB203580, a p38 specific inhibitor {Cuenda, 1995 #50}, blocked the activation of the reporter (Fig. 10A, lanes 3, 4), indicating that p38 activity was critical for BCR induced activation of MEF2.

P38 MAPK functions via a downstream phosphorylation cascade that includes the MAPK kinase, MKK6 {Zhu, 2004 #20}. Consistent with a role for the p38 pathway in MEF2 activation in B cells, $MKK6_{EE}$, a constitutively active form of MKK6, induced MEF2 reporter activity in 2PK3 B cells by approximately 5-fold (Fig. 10B, lanes 1, 3). Interestingly, BCR stimulation of cells expressing $MKK6_{EE}$ resulted in a strong synergistic activation of the MEF2-dependent reporter, which was blocked by addition of SB203580 (Fig. 10B, lanes 3-5). These results further support the notion that BCR stimulation works through the p38 pathway to stimulate MEF2C activity.

P38 directly phosphorylates MEF2C on 3 residues in the C-terminal transactivation domain {Han, 1997 #14; de Angelis, 2005 #54}. To further test the role of the p38 MAPK pathway in the activation of MEF2C in response to BCR stimulation, we mutated these three p38 phosphorylation sites (T293, T300, and S387) to alanines and tested the

activity of the MEF2-dependent reporter when co-expressed with this phospho-mutant (P-mut) form of MEF2C (Fig. 10C). MEF2C is expressed endogenously in 2PK3 B cells (data not shown), and expression of additional wild type MEF2C did not have an obvious influence on the ability of BCR stimulation to activate the MEF2-dependent reporter (compare ~10-fold activation in Fig. 10C, lane 2 to similar activation in Fig. 10A, lane 2). By contrast, expression of MEF2C(P-mut) in 2PK3 B cells inhibited BCR-dependent activation of the MEF2-dependent reporter (Fig. 10C, lane 4), suggesting that the phospho-mutant form of MEF2C functioned as a dominant negative with regard to endogenous MEF2 activity and indicating that the p38 phosphorylation sites on MEF2C are important for the response to BCR stimulation.

The dominant negative effect of MEF2C(P-mut) observed in Fig. 10C suggested that phosphorylation of MEF2C by p38 is essential for MEF2C to elicit a response downstream of the BCR. If this were the case, then MEF2C(P-mut) might inhibit B cell proliferation in response to BCR stimulation. To test this notion, we utilized lentivirus to transduce MEF2C and MEF2C(P-mut) into primary Fo B cells isolated from wild type C57J/BL6 mice and measured [^{3}H]-thymidine incorporation. Upon stimulation with α-IgM, primary Fo B cells expressing MEF2C(P-mut) showed a greater than 90% reduction in proliferation compared untransfected to cells or cells over-expressing either wild type MEF2C or GFP (Fig. 11, lanes 1-4). By contrast, LPS stimulation of primary Fo B cells resulted in no difference between cells over-expressing wild type MEF2C versus MEF2C(P-mut) (Fig. 11, lanes 7, 8). Interestingly, there was a slight reduction in [^{3}H]-thymidine incorporation when either the wild type or P-mut form of MEF2C was

introduced in primary Fo B cells stimulated with LPS (Fig. 11, lanes 5-8), suggesting that overexpression of unphosphorylated MEF2C might interfere with activation of cell cycle genes in response to Toll-like receptor activation. Taken together with the data presented in figures 9 and 10, these results support a model in which MEF2C regulates B cell proliferation specifically downstream of the BCR and that this function of MEF2C is controlled by direct phosphorylation by p38 MAPK.

To examine whether BCR stimulation results in the direct phosphorylation of MEF2C, we examined MEF2C and MEF2C(P-mut) by two-dimensional (2D) immunoblot in response to BCR stimulation (Fig. 12). Transfection of B cells with MEF2C plasmid resulted in the presence of a single spot on the 2D immunoblot (Fig. 12, compare panels A, B). Stimulation of BCR resulted in phosphorylation of MEF2C, which could be seen by the presence of two additional spots on the 2D immunoblot (Fig. 12, panel C). Detection of the phosphorylated forms of MEF2C was dependent on the p38 phosphorylation sites, since MEF2C(P-mut) did not exhibit a change in isoelectric point in response to BCR stimulation (Fig. 12, panel D). These observations establish for the first time a link between BCR stimulation and the activation of the MEF2C transcription factor via p38 phosphorylation.

Taken together, the results presented in Figs. 11 and 12 suggest that MEF2C is a direct biochemical target of p38-dependent phosphorylation downstream of the BCR. These observations, combined with the role for MEF2C in B cell proliferation, suggested that MEF2C might regulate the expression of cell cycle genes downstream of p38 MAPK

signaling. To test this hypothesis, we examined by Taqman RT-PCR the expression of an array of genes involved in the MAPK pathway, including numerous cell cycle genes, transcription factors, and other genes that are known targets of this signaling cascade (Table 1). Consistent with a MEF2C-dependent cell cycle program downstream of the BCR, we observed that the expression of many cell cycle genes was significantly reduced in unstimulated Fo B cells lacking MEF2C compared to controls, including *Cyclin D3* (fold change = -6.1, p = 0.013), *Cyclin B1* (fold change = -5.7, p = 0.030), *Cdk inhibitor 1b* (fold change = -7.8, p = 0.018), and *Cdk inhibitor 1a* (fold change = -9.6, p = 0.050). These results highlight the requirement for MEF2C for normal expression of cell cycle genes in Fo B cells, and suggest a coordinated p38 MAPK-MEF2C-dependent program for proliferation upon BCR stimulation.

Discussion

A model for BCR induced cell proliferation

In this study, we have provided evidence of a pathway that connects BCR stimulation with the activation of the transcription factor MEF2C and subsequent cell proliferation. We determined the mechanism by which MEF2C functions, and describe a pathway in which BCR stimulation triggers the activation of p38 MAPK via MKK6. This leads to p38 phosphorylation of MEF2C, resulting in its activation. MEF2C then serves to switch on the cell proliferation program by rapidly activating the transcription of key cell cycle genes. The necessity of MEF2C for the initiation of the proliferative program is

highlighted by the fact that many cell cycle genes are downregulated even in resting B cells that have been inactivated for MEF2C. Based on its relatively high level of expression in Fo B cells, we speculate that MEF2C is regulated post-translationally as opposed to transcriptionally in order to effect cell proliferation.

Convergence of signals on MEF2C in B cell proliferation

During the course of our studies, we found that we could not fully activate the MEF2-dependent reporter upon overexpression of a constitutively active form of MKK6. However, we were surprised to see that stimulation of the BCR along with transfection of $MKK6_{EE}$ led to a synergistic increase in reporter activity. The implications of this result are that either full activation of p38 requires other signals, including possibly MKK4, or full activation of MEF2C requires additional signals to de-repress it. Since the designers of the $MKK6_{EE}$ plasmid have seen similar levels of activation of this plasmid compared to endogenously activated MKK6 in their cell system, we will focus our speculation on the second possibility {Zhu, 2004 #20}. However it still remains possible that B cell signaling includes unique circuitry that could explain the less than full activation of the MEF2-dependent reporter by $MKK6_{EE}$. Due to the ability of MEF2 to act as either a repressor or an activator based on its association with other factors, it is likely that full activation of MEF2C requires the convergence of signaling pathways stemming from the BCR. One possibility could be that BCR signaling, through CaMK phosphorylation of class II HDACs, could serve to derepress MEF2C and allow for its full activation {McKinsey, 2000 #60}. Thus, a release of the "brakes" on MEF2C, combined with the

constitutive activation of p38 by MKK6 could result in a large increase in MEF2 reporter activity than by BCR stimulation alone.

Figures

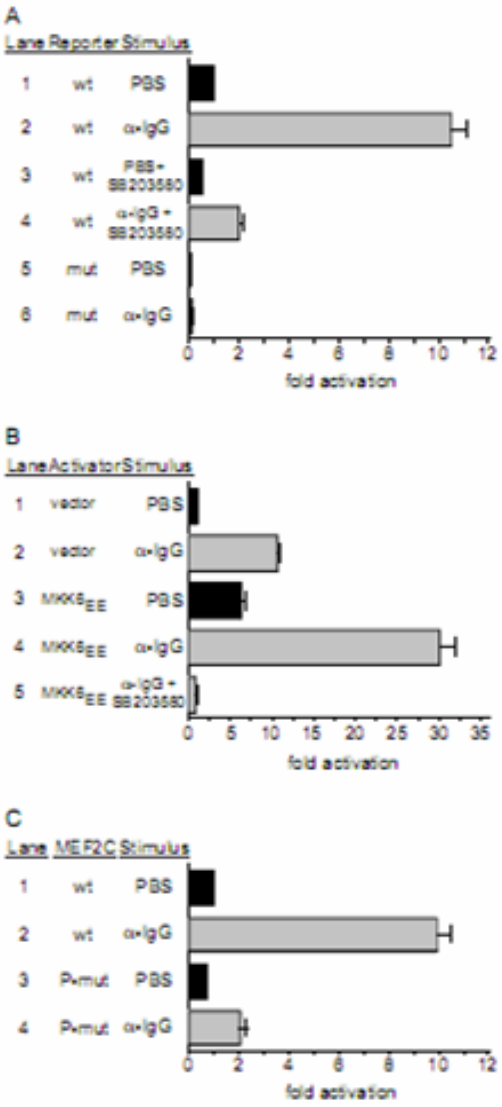


Figure 10. Activation of MEF2C by BCR stimulation requires p38 MAPK.

A) BCR stimulation (α-IgG) of 2PK3 B cells transfected with a MEF2-dependent reporter plasmid (wt) showed a 10-fold increase in reporter activity compared to PBS treated (lanes 1, 2), whereas mutant reporter plasmid (mut) did not (lanes 5, 6). Addition of a p38 specific inhibitor (SB203580) blocked BCR induced reporter activity (lanes 3, 4). B) Co-transfection with $MKK6_{EE}$ and stimulation with α-IgG showed a synergistic

increase in MEF2-dependent reporter activity (lane 4) compared to transfection of $MKK6_{EE}$ without stimulation (lane 3) or reporter alone stimulated with α-IgG (lane 2). Addition of p38 inhibitor (SB203580) blocked this activation (lane 5). C) p38 phosphorylation sites on MEF2C are necessary for its activity. Addition of α-IgG to cells transfected with phospho-mutant MEF2C (P-mut) failed to activate the MEF2-dependent reporter (lanes 2, 4).

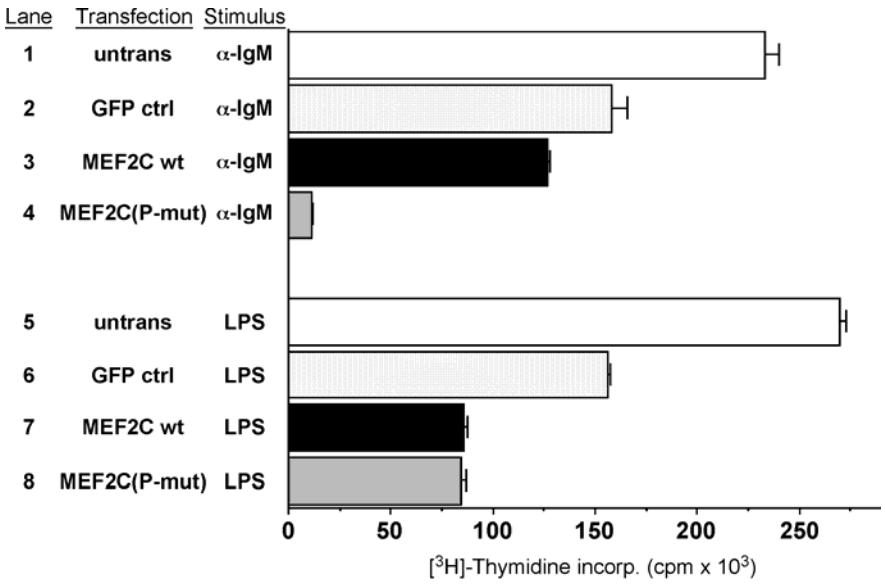


Figure 11.. **Forced expression of MEF2C(P-mut) reduces proliferative capacity of WT Fo B cells.**

Wild type primary Fo B cells forced to express MEF2C(P-mut) proliferate significantly less than Fo B cells forced to express wild type MEF2C (compare lanes 3, 4 $p = 2.5$ E-7). No difference in proliferation was observed when cells were stimulated with LPS (lanes 7, 8).

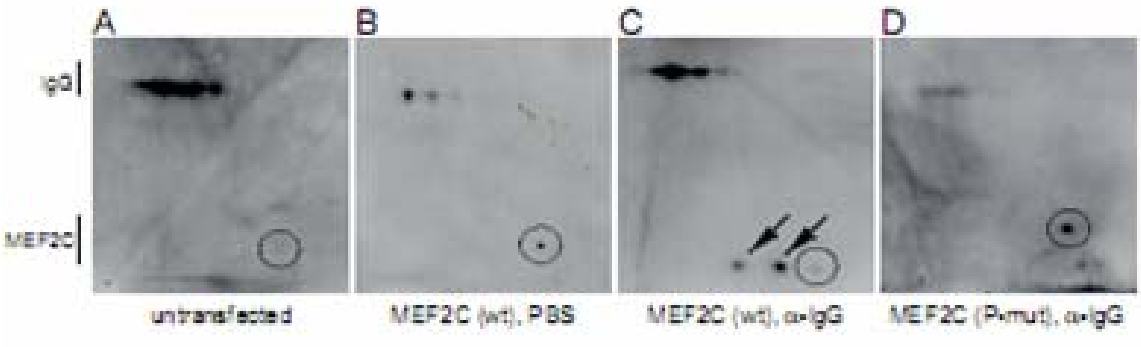


Figure 12. BCR stimulation results in phosphorylation of MEF2C.

2D immunoblot demonstrates that stimulation of BCR by α-IgG resulted in a shift in isoelectric point of MEF2C (B, C), whereas phospho-mutant MEF2C (P-mut) did not (D). Encircled is MEF2C, arrows point to shifts in isoelectric point of MEF2C. Spots were identified by molecular weight (MEF2C-FLAG = ~53 kD, IgG = ~150 kD) as compared to a standard protein ladder run in non-denaturing conditions.

Gene Name	Fold Change	p value	Gene Name	Fold Change	p value	Gene Name	Fold Change	p value
Araf	**-3.61**	0.001136	Ets1	-1.62	0.401633	Mapk10	-1.51	0.8337
Atf2	**-4.17**	0.026319	Ets2	**-2.17**	0.485416	Mapk11	-1.41	0.560768
Ccna1	**-3.71**	0.01426	Fos	**-2.01**	0.59343	Mapk12	1.57	0.473684
Ccna2	**-10.41**	0.05887	Grb2	-1.61	0.900978	Mapk13	**3.56**	0.045239
Ccnb1	**-5.66**	0.029677	Hras1	-1.37	0.180299	Mapk14	-1.06	0.56543
Ccnb2	**-3.94**	0.011774	Hspa5	**-2.56**	0.682536	Mapk3	**-3.31**	0.463476
Ccnd1	**-3.21**	0.009506	Hspb1	**-3.22**	0.289003	Mapk6	**-3.39**	0.045957
Ccnd2	**-2.87**	0.06755	Jun	**-3.26**	0.043844	Mapk7	**-2.73**	0.584072
Ccnd3	**-6.08**	0.013067	Kcnn1	**-3.97**	0.191843	Mapk8	**-2.37**	0.945837
Ccne1	-1.88	0.017151	Kras	**-6.74**	0.364158	Mapk8ip1	-1.17	0.980257
Cdc42	**-3.86**	0.054512	Ksr1	**-2.39**	0.01257	Mapk8ip2	1.06	0.008416
Cdk2	**-3.85**	0.025312	Map2k1	**-3.13**	0.925675	Mapk8ip3	-1.32	0.870214
Cdk4	-1.74	0.014033	Hsp90ab1	-1.05	0.04039	Mapk9	1.23	0.062914
Cdk6	**-2.75**	0.011096	Gapdh	-1.08	0.240559	Mapkapk2	-1.91	0.888216
Cdkn1a	**-9.59**	0.050013	Actb	1.72	0.050558	Mapkapk5	1.29	0.607717
Cdkn1b	**-7.79**	0.017502	Gusb	**-3.15**	0.046355	Max	-1.30	0.496133
Cdkn1c	**-4.62**	0.013574	Hprt1	-1.52	0.026495	Mef2c	**-3.32**	0.031053
Cdkn2a	**-3.70**	0.034863	Map2k1ip1	-1.17	0.692906	Mknk1	**-3.03**	0.767517
Cdkn2b	**-2.52**	0.049855	Map2k2	-1.34	0.995945	Mos	**-2.14**	0.302586
Cdkn2c	**-2.70**	0.013206	Map2k3	1.01	0.939548	Myc	**-3.06**	0.864793
Cdkn2d	**-2.26**	0.144003	Map2k4	-1.11	0.017166	Nfatc4	-1.49	0.412855
Chuk	-1.12	0.776621	Map2k5	**2.84**	0.042799	Nras	-1.35	0.831375
Col1a1	**-2.37**	0.234316	Map2k6	-1.87	0.493096	Pak1	-1.67	0.638628
Creb1	**-6.29**	0.196178	Map2k7	**-4.54**	0.116385	Rac1	1.23	0.847943
Crebbp	**-7.54**	0.050784	Map3k1	**-2.70**	0.324276	Raf1	-1.15	0.807132
Dlk1	**-4.84**	0.587851	Map3k2	**-3.72**	0.555791	Rb1	-1.28	0.045106
E2f1	**-4.76**	0.717268	Map3k3	**-2.14**	0.351709	Sfn	-1.57	0.895166
Egfr	**-2.68**	0.605971	Map3k4	**-6.09**	0.286642	Smad4	-1.25	0.937582
Egr1	**-3.14**	0.661391	Map4k1	**-2.07**	0.629011	Trp53	-1.08	0.046798
Elk1	**-3.51**	0.06049	Mapk1	1.36	0.882588			

Table 1. Table S1. MEF2C is required for the activation of many cell cycle genes.

Select genes involved in the MAPK pathway were analyzed by Taqman real-time PCR for expression in sorted, splenic Fo B cells isolated from *Mef2c* B cell knockout mice compared to littermate controls. Data are expressed as fold change compared to controls. Blue numbers indicate fold decrease in gene expression; pink numbers indicate fold increase in gene expression. P values were determined using a two-tailed t test; n = 6 independent replicates

Final Conclusions

The expression of MEF2C in B cells was documented over a decade ago {Swanson, 1998 #15}, and yet the function of this transcription factor in that cell type had not been tested directly. The studies presented in this thesis for the first time investigate the function of MEF2C in B cells. We demonstrate that MEF2C is critical for the proper development, function, and proliferation of B cells. We also describe the mechanism by which MEF2C functions to translate BCR stimulation into the initiation of the cell proliferation program. Although at first glance, B cell development appears to have little in common with the development of those tissues that MEF2C is best known for, i.e. cardiac and skeletal muscle, there does exist parallels to the role MEF2C plays in each tissue type. MEF2C is crucial for the differentiation of myoblasts to fully mature myocytes. During this transition, MEF2C initiates a developmental program that includes the activation of key muscle markers. Similarly, MEF2C governs the critical step from transitional B cell to a mature, functional Fo B cell. Indeed, our studies that B cells lacking MEF2C fail to efficiently undergo this transition, and instead, we see a population of cells that could represent Fo B cells, but are functionally immature.

As mentioned previously, MEF2C is known to play a role in the proliferation of many tissue types and it is not surprising that it plays an important role in B cell proliferation as well. However, the study of B cell development includes a complexity that other tissue types do not have, and that is the role of selection. Selection and proliferation go hand-in-

hand during the course of B cell development as the immune system first screens for those B cells that are capable of recognizing MHC in the early stages, and then screens for self-reactive clones in the periphery. It would be interesting to determine the role of MEF2C in the intersection of proliferation and selection. Since MEF2C is capable of integrating a host of upstream signals, it would not be surprising to discover that MEF2C plays an important role in selection. However, we speculate that MEF2C is downstream of selection and can utilize its ability to both positively and negatively control gene expression to either drive the proliferative program or pause it.

Implications for MEF2C in B cell-related immune diseases

Dysregulation of B cell proliferation can cause inadequate immune response, immunodeficiency, or leukemia {Ehrlich, 2001 #24; Danilov, 2006 #52}. Our results highlight an important role for MEF2C in regulating B cell proliferation. In other tissues, MEF2C is known to function as a phosphorylation-dependent switch, and as such can serve as either an activator or repressor of transcription {McKinsey, 2001 #13; Miska, 1999 #40; Ma, 2005 #39}. In this regard, it is attractive to speculate that MEF2C may regulate B cell proliferation both negatively and positively depending on its phosphorylation state. Consistent with this notion, we observed that a mutant form of MEF2C, which cannot be phosphorylated by p38 {Han, 1997 #14; Cox, 2003 #53}, blocked BCR-dependent activation of a MEF2-dependent reporter in B cells (Fig. 4). Interestingly, deregulated *Mef2c* expression has been shown recently to accelerate Sox4-induced myeloid leukemia in a population of cells that share a common progenitor with B

cells {Du, 2005 #25}. Our studies suggest that modulation of the p38 MAPK-MEF2C pathway may be an important target for controlling B cell proliferation in leukemia and other diseases involving aberrant B cell growth and function. However, it would be interesting to test the function of MEF2C in B cell diseases by overexpressing a constitutively active form of MEF2C under the control of a B cell specific enhancer. We can only speculate that in the event that the proliferative drive is capable of overcoming negative selection of B cells, these animals could eventually develop B cell leukemia.

Methods

Transgenic Mice and genotyping

CD19$^{Cre/+}$, *Mef2c*$^{+/-}$ and *Mef2c*$^{flox/flox}$ mice have been described previously {Rickert, 1997 #28; Vong, 2005 #26}. For our studies, we backcrossed each of these strains for 9 generations into a pure C57J/BL6 background. Genotyping was performed as published previously {Rickert, 1997 #28; Verzi, 2007 #27}.

To genotype for the TM1 allele, the TM1 5' probe was created by PCR using tail DNA from MEF2C null (TM1) animal and the following primers:

Neo2: 5'-GGCATGCTGGGGATGCGGTGGGCTC-3
MEF2C 5' PST for2: 5'- GAGATTATCTGGGTTAATGTGGGC – 3'

The resulting ~1.2kB fragment was then cloned into pCR2.1 via TOPO. This plasmid, TM15' pCR2.1 was then cut sequentially with BglII and XmaI resulting in 3 bands: 3.5 kB, 1 kB, and 600 bp. The 600 bp piece was excised and purified and used as the TM1 5' southern probe. The sequence of TM1 5' southern probe is as follows:

5'TCTAGATTTAAGAGTTACAATTAAGTGGCAGTAAGAAGTTAACTACCCTGT
GTAAGATCCATTGTGTTTAAAGAAAATAAATGCTAAATAAACCAGTCAGACT
TCCAAAGTATGAGCCTCAAAAATGTATATATTTCTTAAAATAAATAAATAAA
TAATAAATAATTAAGCACATGATACTGTCAGCCATTAGTTGGGGAAGCCGGT

TGTAATTTAAAAGGTTTCTGAGCCTTTGATACAAGAGTCTGCCTTACTACTAA
ATCTGTGAGACGCTAGGACAGGTCTTGGAAGTCTTTTCTTGGAATGATACAGT
ACATAATGCTTGCTTGCAAACACCAGACCATATATGGGTTTGTAGTTAATGTT
GTTCATGCTGTTGGGTGGATTGACCAGGACAAGCTAACATTTAAAGAACACT
GAAATTGCAAGTGTGGAATATGTTCATTTACTGCGACTGGCGAGTGAAGGGC
ATGCAGAACTGGAAACAGGAGCTTAAGGGGGCAGTTCCTGTTGGAAAAGCGT
GTGCACAGAATAAGATGAATAGAAGACAATGATTCCAGGCAGGGAGAGCAT
CCAGGCCAGGAAGCTGCTGGGCTGCCC - 3’

Tail preps were digested overnight with PstI. Following standard Southern blot protocols, expected band sizes are as follows: WT = 3.3 Kb, TM1 = 1.8 Kb, flox = 2.7 Kb, recombined = 1.3 Kb.

If desired, a PCR protocol was developed by Michael Verzi. The following primers are used:

MADS rev: 5’ – CGGCTCTCGTGCGGCTCGTTGTACT – 3’
Neo: 5’-GGCATGCTGGGGATGCGGTGGGCTC-3
Schwarz MADS fwd: 5’ – AGCACAACGAGCCGCACGAGAGCCGG – 3’

Briefly, 2ul of 100ng/ul MADS rev primer, 1ul of 100ng/ul of Neo, 1ul of 100ng/ul Schwarz MADS fwd were used in a standard PCR reaction. The PCR program is as follows:

1) 94 C – 3 minute

2) 94 C – 45 seconds

3) 43.5 C – 45 seconds

4) 72 C – 1 minute

Repeat steps 2-4 for 38 cycles.

5) 72 C – 10 minute to polish.

The amplified WT MEF2C band is approximately 700bp, while the TM1 band is 600 bp.

To genotype for CD19 Cre, digest tail preps with BamHI and use standard Cre probe created by Analeah Heidt. Upon Southern blotting, expected band size is 2.3 Kb for presence of Cre, no band is visible if Cre is absent.

Immunization and ELISA

8 week old animals were immunized with 50 μg of sterile 4-hydroxy-3-nitrophenylacetyl conjugated to chicken gamma globulin (NP-CGG; Biosearch Technologies #N-5055-5) precipitated in 9% $AlK(SO_4)_2$, pH = 7.25 by intra-peritoneal injection. To precipitate NP-CGG, dilute NP-CGG to 1 mg/mL in dH20. Add 10N NaOH to bring pH to 7-7.5. Solution will turn yellow near correct pH range. Allow the NP-CGG mixture to precipitate for 30 minutes at room temperature. Spin 2000 rpm for 5 min at 4C. Aspirate supernatant and wash with sterile, cold PBS. Resuspend to 0.25 mg/mL. Inject 100 ug/mouse intraperitoneally using 23-26 AWG needle. For booster injections inject $1/10^{th}$ or 10 ug NP-CGG. Tail bleeds were performed at 0, 7, 14, and 21 d post-immunization.

For endpoint ELISA assays, plates were coated with 200 μg of NP-BSA overnight at 4ºC. Sera were added in 5-fold serial dilutions beginning at 1:200 to 1:25,000. For detection of NP-specific IgG1 titers, a 1:2000 dilution of goat α-mouse IgM-AP (Southern biotech #1140-04) or of goat α-mouse IgG1-AP (Southern Biotech #1070-04) was applied after multiple washes with 0.05% Tween-20 in phosphate buffered saline (PBS). Para-nitrophenylphosphate (pNPP, Southern Biotech #0201-01) was used as a substrate for the AP conjugated antibodies and plates were read at 405nm on a Molecular Devices Spectramax 190 luminometer. Relative titer concentrations were calculated by comparing to a standard curve from serial dilutions of sera from hyper-immunized wild type C57J/BL6 mice.

FACS

Spleens, bone marrow, and lymph nodes were harvested and dissociated using a plunger from a 5 mL syringe to mash through a 70 μM cell strainer into 2 mL/spleen Hank's buffered salt solution (HBSS++) with 4% fetal bovine serum (FBS) to create a single cell suspension. For spleen harvests, add 13 mL cold RBC Lysis solution (2.06g Tris, 7.47g NH_4Cl in 100 mL dH20) and let lyse for 5 min on ice. Spin down 1200 RPM at 4C for 5 min. Resuspend cells in 1 mL/spleen HBSS++. Cells were stained for 30 min with the following antibodies at a 1:40 dilution: B220-PerCP (BD Biosciences # 553093), CD21-FITC (BD Biosciences #553818), CD23-PE (BD Biosciences # 553139), and AA4.1-APC (eBiosciences #17-5892-82). 5 ul of cells were taken out for single cell controls.

Cells were washed twice in HBSS++ and filtered through a 40 μM cell strainer to a final concentration of ~2.7E7 cells/mL. Cell viability was measured by addition of propidium iodide (5μg/ml). Sorting was performed on a BD Bioscience FacAria cell sorter. Cells were collected in FACS tubes containing 1 mL RMPI 1640 and 1 mL FBS.

[^{3}H]-thymidine Incorporation Assay

Immature ($B220^+$, $AA4.1^+$) and Fo ($B220^+$, $AA4.1^-$, $CD21^+$, $CD23^+$) B cells were isolated by FACS as described above. $2X10^5$ cells were added in duplicate to a 96 well plate in RPMI-1640 + 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U/mL Penicillin/Streptomycin and 50 μM β-mercaptoethanol (growth medium). Cells were stimulated by addition of α-IgM $F(ab')_2$ (7.5 μg/ml, Jackson Immunoresearch #115-006-006) or LPS (5 μg/ml) to each well. B cells were incubated for 44 hr at 37ºC, prior to addition of 1 μCi [^{3}H]-thymidine to each well. Cells were then allowed to proliferate for another 4 h, when they were harvested, washed, and counted on a scintillation counter.

Plasmids and tissue culture

The MEF2 reporter plasmid, pMEF2x4-E1b-luc was constructed by cloning 4 MEF2 consensus binding sites (GGGTTATTTTTAGAGCGATCC) into a modified pGL2-Basic vector (Promega) that contains the adenovirus minimal promoter E1b. pMEF2x4-E1b-luc (mut) contains mutations in each of the consensus binding sites (CTACCGGTAG). This mutagenesis was done in four steps using the following primers all in 5' to 3' direction:

1) Not*BamE1bXhoF – GGC CGG GGA TCC ACT AGA GGG TAT ATA ATG GAT CGG GC 3'

2) Not*BamE1bXhoR – TCG AGC CCG ATC CAT TAT ATA CCC TCT AGT GGA TCC CC

3) Bam4thMef2B*F – GAT CCG ACA GGG TTA CCG GTA GAG C

4) Bam4thMef2B*R – GAT CGC TCT ACC GGT AAC CCT GTC G

5) Bam3rdMef2B*F – GAT CCC ACT GGG TTA CCG GTA GAG C

6) Bam3rdMef2B*R – GAT CGC TCT ACC GGT AAC CCA GTG G

7) B*1st2ndMef2B*F – GAT CGC TCT ACC GGT AAC CCT GTC GAC AGG GTT ACC GGT AGA C

8) B*1st2ndMef2B*R – GAT CGT CTA CCG GTA ACC CTG TCG ACA GGG TTA CCG GTA GAG C

Each oligo pair was annealed. Bluescript plasmid was double digested with NotI and XhoI first two primer pair was ligated in, destroying the NotI site. The resulting plasmid was then cut with BamHI and the 3rd and 4th primer pair was then ligated in, destroying the BamHI site that it went into, but introducing another BamHI site. This plasmid now containing E1b and the 4th mutant MEF2 site was then cut with BamHI and the 5th and 6th oligos containing the 3rd mutant MEF2 site was ligated in. The resulting plasmid was once again linearized with BamHI and the final 7th and 8th oligo pair was ligated in creating 4 mutant MEF2 sites followed by E1b in pBS. The entire construct was then excised from pBS by SacI and XhoI into 4XMEF2E1bluc.

PRK5-MEF2C-VP16 has been described previously {Verzi, 2007 #27}. MEF2C(P-mut)

was created by mutating the *Mef2c* cDNA at the regions encoding amino acid residues T293, T300, and S387 in the mouse protein (accession #AAH26841) to alanines via PCR SOE mutagenesis. T293A and T300A were mutated simultaneously using the following primers:

Mut phos mef2c arm1a rev: 5' – CTACGGAAACCACCGGGGCAGCCAATG – 3'
Mut phos mef2c arm1b for: 5' – CGGTGGTTTCCGTAGCAGCTCCTAC – 3'

S387A was mutated using the following SOE arm primers:

Phos mut mef2c arm2a rev: 5' – TCAACAGGAGCCCTCCCCGCCTCGTGG – 3'
Phos mut mef2c arm2b for: 5' – CGAGGCGGGGAGGGCTCCTGTTGACAG – 3'

The $MKK6_{EE}$ expression plasmid has been described previously and was kindly provided by T. Gulick {Zhu, 2004 #20}. Briefly, amino acid 207 was mutated from serine (nucleotides: TCT) to glutamic acid (nucleotide: GAA) and amino acid 211 was mutated from theronine (ACA) to glutamic acid (GAA).

2PK3 B cells were maintained in growth media. For Amaxa transfections, cell line kit V (VCA-1003) was used as follows: $1X10^6$ cells were resuspended in 100 µl of Amaxa solution V. 1 µg of reporter plasmid and 1 µg of activator plasmid were added to the cells, which were then subjected to electroporation using program X-001 on an Amaxa Nucleofector II machine. Cells were then plated in 1 ml of growth media in a 12-well

plate, harvested 24 h post transfection, and lysed by either rapid freeze-thaw in 100 µl of 0.1M Na_2HPO_4, pH=7.4 or by passive lysis with B cell lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X 100, 10 µg/ml aprotinin, 2 µg/ml leupeptin, 100 µM PMSF, 5 mM DTT, 1 mg/ml lysozyme, 5 mM EDTA, 100 µM Na_3VO_4). 25 µl of the supernatant post cell lysis was added to 75 µl of luciferase substrate (Promega #E1500), and assayed on an Applied Biosystems Tropix luminometer. 15 µg of α-IgG $F(ab')_2$ (Jackson Immunoresearch) was added to cells for BCR stimulation studies. The p38 inhibitor SB203580 or SB202190 (Sigma) was used at a final concentration of 10µM in DMSO.

RT-PCR and Taqman

Applied Biosystems Assays on Demand primer + probe for *Mef2c* (assay # Mm01344729_m1) and *HPRT* (assay # Mm00446968_m1) were used in studies to detect *Mef2c* transcript expression levels throughout B cell development. RNA was isolated from sorted B cells from 10 wild type C57J/BL6 mice using Qiagen RNeasy mini or micro kits. B cell stages were defined using surface markers as follows: pro-B cell ($B220^+$, $CD43^+$, IgM^-), pre-B cell ($B220^+$, $CD43^-$, IgM^-), immature B cell ($B220^+$, $CD43^+$, IgM^+, IgD^-), transitional 1 ($B220^+$, $AA4.1^+$, IgM^+, $CD23^-$), transitional 2 ($B220^+$, $AA4.1^+$, IgM^+, $CD23^+$), follicular B cell ($B220^+$, $AA4.1^-$, $CD21^+$, $CD23^+$) and marginal zone B cells ($B220^+$, $AA4.1^-$, $CD21^+$, $CD23^-$). For cDNA synthesis, 1 µg of RNA was used in Invitrogen Superscript kit. Taqman-PCR was performed on an ABI 7500 Real-Time PCR machine.

For RT-PCR array experiments, Fo B cells ($B220^+$, $AA4.1^-$, $CD21^+$, $CD23^+$) were harvested and sorted from KO and control animals as described above. RNA was purified from sorted Fo B cells using Qiagen RNeasy mini kit 1 μg of RNA was then prepared into cDNA using SuperArray RT^2 First Strand Kit. cDNA template and SuperArray 2x PCR master mix were then applied to SuperArray Mouse MAP kinase signaling RT-PCR array and run on a Stratagene Mx3005p RT-PCR machine.

Two-dimensional immunoblot

B cell lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X 100, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μM PMSF, 5 mM DTT, 1 mg/ml lysozyme, 5 mM EDTA, 100 μM Na_3VO_4) was used to lyse transfected 2PK3 B cells for 30 min on ice. Lysates were pelleted and resulting supernatants were subjected to BioRad Ready-Prep 2-D cleanup kit. Protein concentration was measured using a nanodrop spectrophotometer (available at CAT on 2^{nd} floor GH). BioRad Ready Prep 2-D Starter Kit (163-2105) was used for the 2-D electrophoresis. Briefly, lysates with equivalent amount of protein were allowed to absorb overnight in BioRad IPG pH 3-10 11 cm strip (#163-2014). Mineral oil was used to cover the strips so as to prevent evaporation of the lysate. Strips were equilibrated in rehydration buffer I and II for 5 minutes each. Isoelectric focusing was performed for 20,000 V-Hr (takes ~8hrs) and IPG strips were then run in 10% BioRad Criterion XT native gel (#345-0115) for 2 hrs at 150V. Gels were then transferred to Immobilon PVDF membranes for 1.5 hrs at 150V. MEF2C-FLAG protein was detected

using standard western blotting technique using α-FLAG M2 primary (Sigma F3165) and goat α-mouse IgG peroxidase conjugate (Sigma A4416) secondary antibodies. Membranes were then developed using Amersham ECL kit on Amersham Hyper ECL film.

Lentiviral transduction

MEF2C and MEF2C(P-mut) was amplified from MEF2C pCDNA1.1 and MEF2C(P-mut) pCDNA1.1 using the following primers that added a PacI site at the 5' end and an AscI site at the 3' end.

Mef2PacIF: 5' CGG CTT AAT TAA ATG GGG AGA AAA AGA TTC 3'

Mef2AscIR: 5' GGC GCG CCC TAT TAA GTA ATA ATG TGA TCA 3'

The PCR product was then ligated into FuPw expression vector at the unique PacI and AscI sites in the multiple cloning site. EcoRI was used to test digest the mini products which are predicted to yield a ~2.5 kB product. 293T cells were used to package the lentivirus and transfected with Lipofectamine 2000 in the following manner:

1) 4.44 μg of FUPW, 3.33 μg pCMVΔ8.9, 2.22 μg of pVSV-G was added to 750 μl of serum free OPTI-MEM.
2) 30 μl of Lipofectamine 2000 was diluted in 750 μl of serum free OPTI-MEM, let incubate benchtop for 5 min.
3) Add DNA mixture to Lipofectamine mixture and let incubate for 20 minutes on benchtop.
4) Add transfection mix to 293T cells and change media after 6 hours.

5) Harvest virus 48 hours post transfection.

6×10^5 sorted primary Fo B cells were then added to a 12 well plate. 2 mL of syringe filtered supernatant from 293T cell transfection with 5 μg/mL polybrene. "Spin-fect" B cells by spinning for 1 hour at 2000 rpm in swinging bucket bench-top centrifuge at room temperature. After spin is complete, carefully aspirate viral supernatant and transfer B cells into RPMI growth medium in a 96 well plate. Add appropriate stimuli and follow protocol for thymidine incorporation assay.

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