A STUDY OF THE PROXIMAL CD86-INDUCED SIGNALING MECHANISM THAT REGULATES IgG1 PRODUCTION BY A B CELL

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

The goal of this dissertation was to determine the proximal molecular mechanism by which CD86 engagement signals directly to the B cell to increase the level of IgG1 antibody produced. Previous reports showed that CD86 engagement signals directly to a CD40L/IL-4 primed B cell to increase the level of IgG1 produced in vitro and in vivo, without affecting the level of class switch recombination (CSR). The CD86-induced increase in IgG1 production occurs due to the activation of a unique intracellular signaling network that increases the rate of IgG1 transcription via an Oct-2- and NF-κB-dependent increase in the activity of the 3’-Igh enhancer. The CD86-dependent NF-κB activation is regulated via two proximal signaling networks that induce IκBα phosphorylation, subsequent degradation, and release of NF-κB (p50/p65), and subsequent NF-κB (p65) phosphorylation. Although it was understood how CD86 engagement increases the level of IgG1 produced, the proximal molecular mechanism to one of the most proximal CD86-induced signaling intermediates, PLCγ2, remained unknown. Prior reports showed that PLCγ2 was recruited to phosphorylated tyrosine residues, which are absent within the cytoplasmic domain of CD86. Therefore, the hypothesis tested in this dissertation was that the CD86 cytoplasmic domain associated directly with a protein/protein complex capable of undergoing tyrosine phosphorylation to activate PLCγ2 and was required for the CD86-dependent increase in IgG1 production.
by a B cell. In order to test our hypothesis we designed the following specific aims: 1) To determine if a functional signaling protein(s) associated directly with CD86 to mediate the CD86-induced increase in PLCγ2 activation and IgG1 and 2) To evaluate if the CD86 cytoplasmic domain was required for the CD86-induced increase in IgG1.

Using a proteomics-based identification approach, we show for the very first time that the tyrosine-containing transmembrane adaptor proteins, prohibitin-1 (Phb1) and prohibitin-2 (Phb2), bind to CD86. The expression of Phb1/2 and association with CD86 increased primarily after priming with CD40. The CD86-induced increase in Oct-2 and IgG1 was less when either Phb1/2 expression was reduced by shRNA or the cytoplasmic domain of CD86 was truncated or mutated at serine/threonine PKC-phosphorylation sites, which did not affect Phb1/2 binding to CD86. In addition, we show that an intact CD86 cytoplasmic domain is necessary for an optimal IgG1 response against a T-dependent antigen in vivo. Furthermore, we also show that Phb1/2 and the CD86 cytoplasmic domain are required for the CD86-induced phosphorylation and subsequent degradation of IkBα, which we previously reported leads to NF-κB p50/p65 activation; whereas, only Phb1/2 was required for the CD86-induced phosphorylation of PLCγ2 and PKCa/βII, which we have previously reported leads to NF-κB (p65) phosphorylation and subsequent nuclear translocation. Thus, our findings suggest that Phb1/2 and the CD86 cytoplasmic domain cooperate to mediate CD86 signaling in a B cell through differential phosphorylation of distal signaling intermediates required to increase IgG1. The significance of this dissertation is that it is the first to identify the proximal CD86-induced intracellular signaling mechanism in a B cell that regulates the level of IgG1.
produced. Knowledge gained from this work will provide novel therapeutic targets either to elevate or suppress the level of IgG1 produced.
DEDICATION

I dedicate my Dissertation to my very first scientific collaborators, my mother and father for providing me with inspiration, and to wife for endless hours of unwavering support and scientific discussion.
ACKNOWLEDGEMENTS

I am humbled to have worked with my mentor Dr. Virginia Sanders and am forever grateful for all of her guidance, support, patience, constant enthusiasm, and training me to think like a scientist. She has created an outstanding training environment in her laboratory that fosters scientific excellence and achievement while addressing extremely important immunological mechanisms. I also wish to thank our scientific collaborators outside of The Ohio State University, beginning with Dr. Kerry Campbell at Fox Chase Cancer Center, for his intracellular signaling expertise, constant support, and guidance throughout my graduate years. I also wish to thank our collaborators at the National Institutes of Health, including Dr. Sumeena Bhatia and Dr. Richard Hodes, for providing a critical reagent for this dissertation and for extremely helpful discussions. I would also like to thank Dr. Carlos Castro and Emily Briggs here at The Ohio State University in the College of Engineering, for establishing an exciting live cell imaging model system for our clinical application studies. Also, I wish to thank Dr. John Byrd for his constant support and for providing us with a CLL cell line to begin to translate our findings from our murine model system. I wish to thank my committee members at The Ohio State University, Drs. Jeanette Marketon, Susheela Tridandapani, Michael Freitas, and John Byrd for their guidance and helpful discussions during meetings.
I am very grateful to the Integrated Biomedical Science Graduate Program and to the late Dr. Alan Yates for founding an exceptional program that fosters scientific excellence. I wish to thank all of the past and present members in the Graduate Program office, especially Christine Kerr, Amy Lahmers, and Melissa Stenger for their incredible dedication to graduate students. Also, I wish to thank Dr. James Waldman, for his dedication to graduate students and for our many musical jam sessions. I wish to thank the current Directors, Drs. Jeff Parvin and Joanna Groden, for their support and dedication to the program. In addition, I would like to thank Drs. Ron Glaser and Jan Kiecolt-Glaser of the Institute for Behavioral Medicine Research for creating an outstanding working environment.

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scientific discussions, and our laboratory manager, Matthew Gormley, for his experimental support and his dedication to the laboratory.

I am forever grateful to my parents, Robert and Sandy. It was their enthusiasm and love of science that made me want to be a scientist. Through their guidance, work ethic, values, support, and unconditional love, they taught me the value of educational pursuit through perseverance. I would also like to thank my brothers, Rob and Lee, for their constant support throughout my graduate years. Also, I would like to thank my wife’s parents, Mustapha and Soumya, for their unwavering love and support, as well as the many delicious meals that helped me get through the tough days. I also would like to thank my wife’s sister, Lina, for her unending support.

Finally, I would like to thank my wife, Besma. She shared my successes and failures since the very first day of graduate school where we met. I will be grateful forever for her support, countless scientific discussions, incredible meals, and her love throughout my graduate years.
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Area of Research Emphasis: Immunology.
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>Bap37</td>
<td>B cell receptor-associated protein 37-kDa</td>
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<tr>
<td>B-CLL</td>
<td>B-cell Chronic Lymphocytic Leukemia</td>
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<tr>
<td>BCR</td>
<td>B-cell receptor</td>
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<td>BLNK</td>
<td>B cell linker protein</td>
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<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
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<td>CH</td>
<td>Ig receptor heavy chain</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<td>CLP</td>
<td>common lymphoid progenitor cell</td>
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<td>class switch recombination</td>
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<td>deoxyribonucleic acid</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FDC</td>
<td>follicular dendritic cells</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HSC</td>
<td>hematopoietic stem cell</td>
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<td>I</td>
<td>Intervening</td>
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<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<td>IKK</td>
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<td>IL</td>
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<td>IL-4R</td>
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<td>insulin receptor</td>
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<td>isotype-matched control Ab</td>
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<tr>
<td>Iγ1</td>
<td>interfering γ1</td>
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<td>IκBa</td>
<td>Inhibitor of κBa</td>
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<td>MACS</td>
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<td>Mitogen-activated protein kinase</td>
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<td>mean fluorescence intensity</td>
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<td>MHCI</td>
<td>Major Histocompatibility Complex Class I</td>
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<tr>
<td>MHCII</td>
<td>Major Histocompatibility Complex Class II</td>
</tr>
<tr>
<td>N</td>
<td>Nuclear</td>
</tr>
<tr>
<td>shRNA</td>
<td>short-hairpin ribonucleic acid</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>Oct-2</td>
<td>Octamer binding protein-2</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>p</td>
<td>Phosphorylated</td>
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<tr>
<td>PAMP</td>
<td>pathogen-associated molecular patterns</td>
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<td>PDK-1</td>
<td>phosphoinositolide-dependent protein kinase 1</td>
</tr>
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<td>Prohibitin-1</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositide-3 kinase</td>
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<td>PIP3</td>
<td>phosphatidylinositol 3,4,5-triphosphate</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptors</td>
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<td>protein tyrosine kinase</td>
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<td>pY</td>
<td>Phospho-tyrosine</td>
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<td>Rat sarcoma</td>
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<td>Retinoblastoma</td>
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<td>reactive oxygen species</td>
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<td>SCID</td>
<td>Severely combined immunodeficient</td>
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<td>SDS-PAGE</td>
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<tr>
<td>Shc</td>
<td>Src Homologous and Collagen</td>
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<tr>
<td>SHM</td>
<td>somatic hypermutation</td>
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<tr>
<td>shRNA</td>
<td>short-hairpin ribonucleic acid</td>
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<td>STAT6</td>
<td>signal transducer and activator of transcription-6</td>
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</tr>
<tr>
<td>TBST</td>
<td>tris buffered saline-tween 20</td>
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<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<td>Terbutaline</td>
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Tfh  follicular T helper cell
Th   T helper lymphocyte
TNF  tumor necrosis factor
TNP-KLH  Trinitrophenyl hapten-Keyhole Limpet Hemocyanin
TRAF  TNF-receptor-associated factors
Trunc Truncated
Trunc CD86  CD86 cytoplasmic domain-deficient
VDJ  recombined gene segment variable, diversity, joining
WT  Wildtype
INTRODUCTION AND LITERATURE REVIEW

1.1 The Immune System

1.1.1 The Innate Immune System

Protection afforded by the immune system against foreign pathogens, such as viruses and bacteria, is essential to sustain and support life. The immune system is divided into two separate major components known as the innate system and the adaptive system (1, 2). While the innate immune system provides protection via non-specific mechanisms and without associated immunological memory, the adaptive immune system offers protection in a highly specific manner and is associated with immunological memory (1), (see Figure 1). The innate immune system serves as the first line of defense against foreign invaders with a response that occurs from minutes to hours after initial exposure (1), and consists of physiological barriers and phagocytic immune cells, such as neutrophils, macrophages, and dendritic cells (DCs) (2).

Physiological barriers of the innate immune system include the skin and antimicrobial peptides expressed on the surface of the skin, which non-specifically destroy microbes via cationic amino acid residues (1). If a foreign particle happens to breach the defenses employed by the physiological barriers, the particle may be engulfed
# The Immune System

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**Figure 1.** *Divisions of the Immune System.* The immune system is equipped with two major components for host defense against infectious agents, the innate system and the adaptive system. The innate system consists of physiological barriers and phagocytic immune cells including neutrophils, macrophages, and dendritic cells. Innate immunity employs a rapid response via non-specific recognition mechanisms and is incapable of immunological memory. The adaptive immune system consists mainly of T and B lymphocytes, which can recognize foreign antigens via antigen-specific receptors expressed on the cell surface. Although the response occurs after several days upon antigen exposure, adaptive immunity allows for a high-magnitude response against invading organisms while developing immunological memory.
by one or more phagocytic immune cells, including either the neutrophil, macrophage, or DC, which recognize pathogen-associated molecular patterns (PAMPs) expressed on the microbe surface via pattern recognition receptors (PRRs) expressed on the cell surface (3-5). While phagocytic immune cells function to engulf and destroy foreign particles via lysosomal and free-radical mechanisms, these cells also possess the ability to produce and secrete soluble factors called cytokines (6-9). Cytokines have multiple functions, including cell-cell communication and endothelial cell activation, allowing for adhesion molecules that are expressed on endothelial cells to bind to receptors on the immune cell surface to adhere immune cells from the blood stream to the tissue site of infection and to promote diapedesis (10-12). Phagocytic immune cells may also produce other soluble factors called chemokines, which act as chemo-attractants, promoting immune cell recruitment to the site of infection within the tissue (6-9).

In addition to physiological barriers and phagocytic immune cells, the innate immune system is armed with a series of proteins found in the serum called complement, which are soluble proteins that bind to microbes in a non-specific manner to form a membrane attack complex, which leads to the eventual lysis of the pathogen (13). Furthermore, complement is able to work with an adaptive immune component produced by B lymphocytes, namely antibodies (Abs), which, among serving other functions, can fix and activate complement in order to destroy microbes (13). In summary, the innate immune system consists of physiological barriers, phagocytic immune cells, and complement. Together, these components of the innate immune system serve as initial protection against foreign particles and are necessary for host survival.
1.1.2 The Adaptive Immune System

In addition to the defenses of the innate immune system, the immune system contains a second major component known as the adaptive immune system, which is able to mount a high magnitude response against microbes in a highly specific manner (1). The adaptive immune response occurs during the course of several days to weeks and is able to produce immunological memory against the invading pathogen (1). The adaptive immune system consists of T and B lymphocytes, which are immune cells that express antigen-specific receptors, the T cell receptor (TCR) and the B cell receptor (BCR), on the cell surface. These receptors are able to recognize a highly specific region of a microbe to promote activation of the B cell upon receptor engagement (14). B cells are able to recognize portions of whole antigens via the BCR, which include polysaccharides, lipids, or peptides in an antigen-specific manner (15). In contrast, cells of the innate immune system, including macrophages and DCs, recognize conserved portions of pathogens via PRRs (1). Macrophages, DCs, and B cells are all referred to as antigen presenting cells (APCs) due to their ability to endocytose and process antigens into peptide fragments and display the antigenic-peptide on the cell surface to communicate with CD4$^+$ T helper (Th) lymphocytes (1, 16, 17). Th cells will recognize a highly specific peptide fragment of the antigen which is expressed on the surface of an APC in association with Major Histocompatibility Complex Class II (MHCII) via the TCR (16, 17). An activated Th cell is able to produce cytokines that allow other immune cells to function more effectively, including interferon-gamma (IFN-$\gamma$) to enhance macrophage function, and interleukin-4 (IL-4) to allow B cells to produce high affinity antibodies, which are high molecular weight proteins that bind to, neutralize, and allow for the
destruction of foreign pathogens (18-20). The communication that exists between cells of the innate immune system and cells of the adaptive immune system occurs within secondary organs of the immune system, including either a lymph node or the spleen (21), and is required for antigen-specific T cell activation and the subsequent generation of an adaptive immune response (22-24). A second major type of T cell, called a CD8+ or cytotoxic T cell, recognizes a cognate antigenic peptide in association with Major Histocompatibility Complex Class I (MHCI), and is able to lyse target cells upon their activation via perforin and granzyme B-mediated mechanisms (25, 26).

At the conclusion of an adaptive immune response, a select population of either activated, effector, or memory T or B cells remain alive for months to years following the clearance of an infection, the latter of which are able to mount a robust secondary response upon antigen re-exposure (27, 28) and is referred to as immunological memory, which is a hallmark of the adaptive immune system. In summary, in addition to the innate immune system, the immune system is armed with an adaptive component that is able to promote a robust highly antigen-specific immune response and generate immunological memory.

1.2 The B Lymphocyte

1.2.1 B cell development

The B lymphocyte is a critical cell of the adaptive immune system and is responsible for the production of high affinity antibodies that will neutralize and clear foreign microbial pathogens upon infection (27). Prior to becoming an antibody-producing cell, the B cell undergoes a highly regulated developmental process that occurs in an antigen-independent manner. As reviewed in (29-31), and diagrammed in Figure 2,
the developmental process of the B lymphocyte occurs in the bone marrow where key events include the gene recombination of the antigen receptor, followed by the highly regulated process of selection. The process begins when a hematopoietic stem cell (HSC) that expresses the surface marker CD43 within the bone marrow, receives soluble cues from stromal cells called interleukin-7 (IL-7), which binds to the IL-7 receptor on the HSC surface to promote differentiation to a common lymphoid progenitor cell (CLP). The CLP may either commit to the B or T cell developmental lineage depending on the profile of activated transcription factors within the cell. If the activated transcription factors include EBF, and E2A, followed by activation of Pax-5, the CLP will differentiate into a pro-B cell and express B-lineage specific markers including cluster of differentiation (CD) 19 and CD10 on the surface. During the pro-B cell stage, proteins that are essential for antigen receptor recombination called Rag proteins are expressed and begin to recombine the protein products of the heavy chain locus of the immunoglobulin (Ig) gene, which will ultimately become the heavy chain of the antigen receptor. Once the heavy chain is recombined into a functional protein and expressed on the cell surface, the cell has differentiated from a pro-B cell into a pre-B cell. At the pre-B cell stage, the heavy chain of the antigen receptor binds to a surrogate light chain (v-preB and λ5) and associates with proteins critical for BCR signaling, the Ig-α/Ig-β heterodimer, which allows for clonal expansion. This stage can also be identified by low levels of B220 and the presence of CD43 on the cell surface.
Figure 2. B cell development. The process of B cell development begins in the bone marrow as a hematopoietic stem cell (HSC) receives a signal (IL-7) to differentiate into a common lymphoid progenitor cell (CLP). If the appropriate transcription factors become active (EBF, E2A, and Pax-5), the CLP will differentiate into a pro-B cell and express CD43, CD19, and CD10 on the cell surface. The pro-B cell will begin to recombine the heavy chain of the antigen receptor mediated by the expression of products of the Rag genes and express the antigen-receptor heavy chain (H-chain), the Ig-α/Ig-β heterodimer, and the surrogate light chain forming the pre-BCR on the surface. In addition, the pre-B cell expresses CD43 and low levels of B220 on the cell surface. Once the Rag proteins mediate the formation of the light chain (L-chain) of the antigen receptor, IgM is now expressed on the cell surface. Following a selection process, the cell migrates into the periphery and differentiates into an immature B cell marked by the absence of CD43, and the presence of low levels of IgM on the surface. The final stage of B cell development occurs in the periphery as the cell promotes the expression of IgD on the surface. Once the B cell is IgM⁺/IgD⁺ and expresses CD23 on the surface, the cell is now a mature naïve B cell that is able to respond to antigen stimulation.
As the pre-B cell matures, the light chain of the antigen receptor is recombined and is expressed in place of the surrogate light chain allowing the cell to become an immature B cell which now expresses both heavy and light chain products of the Ig gene called Ig μ or (IgM) that associates with Ig-α/Ig-β to form the BCR complex. Although the BCR is not able to respond to antigen prior to the immature B cell stage, the expression of the BCR complex allows the B cell to respond to antigens present within the bone marrow. If a BCR expressed on the immature B cell recognizes self-antigens within the bone marrow with strong affinity, the receptor may undergo an editing process, or the cell may expire. The process of B cell elimination due to strong recognition of self-antigen is known as negative selection. In contrast, B cells that do not express high-affinity receptors for self-antigens undergo positive selection and migrate out of the bone marrow where they complete the maturation process in the spleen marked by the expression of IgD. Once the B cell is IgM+/IgD+, the developmental process is complete and the cell is referred to as a naïve, mature B cell that has not encountered foreign antigen. The naïve, mature B cell then migrates to lymphoid organs including the lymph nodes in the periphery where the cell can respond to foreign antigens upon BCR engagement and ultimately differentiate into an effector B cell capable of producing high affinity antibodies.

1.2.2 B cell activation: The germinal center reaction

Once the developmental process is complete and naïve, mature B cells populate the secondary lymphoid organs of the immune system including the lymph node and spleen, the cells wait to encounter and respond to foreign antigens upon engagement of the BCR. A B cell may become activated to produce and secrete high-affinity antibodies
in either an antigen-specific T cell-dependent, or –independent manner depending on the type of antigen present (18). If the antigen can be recognized by the BCR on a B cell by multiple recognition sites, the antigen is referred to as multi-valent and consists of many, repeatable recognition determinants (32). In the presence of a multi-valent antigen including polysaccharide or lipid antigens, the B cell can produce high-affinity antibodies in the absence of T cells, and does not require antigen-specific T cell help in the form of surface ligands and cytokines in order to produce antibodies (32). For the purposes of this dissertation, we will focus on the B cell activation events that are dependent on an antigen in which the B cell requires T cell help to produce high-affinity antibodies, a thymus-dependent or T-dependent antigen (22-24).

The coordinated process of B cell activation in response to a T-dependent antigen occurs in the following manner. Upon entering the secondary lymphoid organs of the immune system, either lymph nodes or the spleen, the naïve B cell resides where it waits to encounter foreign antigen (33). A naïve B cell will remain in the marginal zones of the spleen and within primary follicles in the lymph nodes for several hours and will then enter the bloodstream and migrate to another secondary lymphoid organ if the cell does not encounter foreign antigen (21). Upon antigen exposure, the naïve B cell will participate in a germinal center reaction if the antigen is T-dependent (33). The phenomenon known as a germinal center reaction, diagrammed in Figure 3, and reviewed in (34), is divided into three distinct phases that will allow a B cell to become either an antibody-secreting plasma cell or a memory cell. Several days after initial antigen encounter, some of the activated B cells will migrate deep into the follicle and will begin to rapidly expand forming the germinal center (35-37). The first phase of the
germinal center contains a “dark zone” that consists of proliferating cells called centroblasts. It is within the dark zone that centroblasts undergo a process called somatic hypermutation (SHM), which results in the surface Ig receptor altering the affinity for its cognate antigen (38, 39). The reaction continues to the second phase in which centroblast progeny called centrocytes migrate out of the dark zone to a region called the basal light zone where follicular dendritic cells (FDCs) reside (31, 39). Here, a selection process occurs as centrocytes interact with the processes of the FDCs, which express Fc receptors that bind to the Fc portion of soluble antibodies, which will bind to the antigen and present the antigen on the FDC surface to centrocytes (40). The centrocytes that express Ig receptors with high affinity for the antigen will bind and compete for the antigen, as centrocytes that express Ig receptors with lower affinity will enter apoptosis (41-43). The centrocytes with high affinity antigen receptors will survive and migrate out of the basal light zone region and enter the apical light zone of the germinal center. Within the apical light zone, the B cell will express an antigenic peptide in association with MHCII for presentation to a unique subtype of Th cell called a follicular T helper cell (Tfh) (44-49). Surface expression of the chemokine receptor CXCR5 allows the Tfh cell to migrate from T cell zones into the B cell follicle within the context of lymphoid organs (50-52). The Tfh cell has recently emerged as the key Th cell responsible for germinal center formation and B cell differentiation into either an antibody-secreting plasma cell or memory cell, capable of long-term survival and the induction of a robust response upon antigen re-exposure (46-49). Thus, in order for a B cell to participate in a germinal center reaction and ultimately differentiate into either an antibody-secreting plasma cell or a
Figure 3. *The Germinal Center Reaction.* The germinal center reaction occurs in three distinct phases upon cognate antigen recognition within secondary lymphoid organs including the spleen and lymph nodes. Upon engagement of the BCR, the B cell is known as a centroblast and undergoes clonal proliferation and somatic hypermutation in the “dark zone.” Once the cell, now called a centrocyte, migrates into the “basal light zone” where follicular dendritic cells (FDCs) reside, a selection process occurs where only centrocytes expressing a high-affinity B cell receptors (BCRs) will survive. The centrocyte then migrates into the “apical light zone” where follicular T helper cell-centrocyte contact will induce isotype-(class) switch recombination (CSR) allowing the B cell to become a plasma cell and produce/secrete the appropriate antibody to combat an infection. Alternatively, the B cell may become a memory B cell capable of long-term survival, and the ability to induce a high-magnitude immune response upon antigen re-exposure.
memory cell, cell-cell interactions between B cells and FDCs followed by B cells and antigen-specific Tfh cells are required.

### 1.2.3 B Cell Activation: Signal requirements for differentiation

Within a germinal center reaction, two specific signals are required for a naïve IgM⁺/IgD⁺ B cell to differentiate into either an antibody-secreting plasma cell, or a memory cell in the context of a T-dependent antigen including antigen recognition and signals delivered via Th cells (53). First, as diagrammed in Figure 4, within a secondary lymphoid organ, the BCR on the naïve B cell surface recognizes its cognate antigen either in soluble form or in on the context of a DC, since DCs are capable of cradling antigen on the surface in order to present the antigen to a naïve B cell (54). This is the first required signal for a naïve B cell to become active. Upon antigen binding to the BCR and BCR cross-linking mediated by at least two BCRs, a series of intracellular signaling events initiated via the Ig-α/Ig-β hetero-dimer (55) are promoted within the B cell that will ultimately result in the activation of multiple transcription factors. The activated transcription factors will induce a gene expression profile that will induce clonal cellular proliferation, and increase the expression of a critical surface molecule for the subsequent communication that occurs between a B cell and Th cell, B7-2 (CD86) (56). At this point, although the B cell is capable of clonal expansion, the cell cannot produce antibodies without critical signals from an antigen-specific Th cell (22-24).
Figure 4. *B cell and T helper cell activation*. The coordinated process of B/T cell activation occurs in the following manner: (1) The BCR on the naïve B cell surface recognizes its cognate antigen (Ag) and promotes a series of pro-survival intracellular signaling events within the B cell. (2) The antigen-BCR complex becomes internalized and machinery within the B cell processes the antigen into peptide fragments and shuttles the antigenic-peptide to the cell surface in association with MHCII for presentation to T helper cells. (3) Engagement of the BCR promotes an elevation of CD86, a critical costimulatory molecule on the B cell surface. (4) A naïve antigen-specific T helper cell recognizes its cognate antigenic-peptide:MHCII complex on the B cell (or other APC) surface via the TCR. Costimulation afforded via CD86-mediated engagement of CD28 induces intracellular signaling events along with TCR engagement to promote proliferation, cytokine production (IL-4 in the case of a Th2 cell), and the elevation of CD40L on the Th2 cell surface. (5) Th2 cell-dependent IL-4 and CD40L engage the IL-4R and CD40 on the B cell surface, which will induce intracellular signaling networks to induce class-switch recombination within the B cell to produce IgG1.
The B cell activation process continues as the BCR-antigen complex becomes endocytosed directly into the B cell while coordinated intracellular signaling events occur even after the complex is internalized (57). Machinery within the B cell subsequently processes the endocytosed antigen into peptide fragments that become loaded into the cleft of a protein called MHCII, which serves a critical role in the communication that occurs between a T cell and a B cell (16, 17, 58, 59). The MHCII-antigenic peptide complex now migrates to the cell surface in order to present the peptide antigen to an antigen-specific CD4+ Th cell in order to receive the necessary signals to produce high affinity antibodies.

1.3 The role of the T helper cell in antibody-mediated immunity

1.3.1 T helper cell activation

Similar to the required signals for B cell activation, a naïve T helper cell also requires two signals including antigen recognition and costimulation to become active (60). Naïve Th cells are in constant search of cognate antigen as they migrate from the circulation into the lymphoid organs, then back into circulation. Naïve Th cells will recognize their cognate antigen in the spleen if the antigen is blood born, or in draining lymph nodes if the antigen is delivered via DCs through the lymphatic system (61). While the DC is known as the most efficient APC to promote T cell activation, the B cell and macrophage are also capable (61). APCs capture antigen in the tissue at the site of infection, and enter lymphatic vessels bound to draining lymph nodes where naïve T helper cells are actively surveying the lymph node in search of specific antigens (61). While the APC migrates through the lymphatic vessels with captured antigen, the antigen is endocytosed, processed, and presented on the surface of the APC (either a DC,
macrophage, or B cell) in association with MHCII (61). As diagrammed in Figure 4, in the lymph node, the naïve T cell will recognize an antigenic-peptide fragment in association with MHCII via an antigen-specific receptor (61). Engagement of the TCR on the T helper cell surface is known classically as signal 1 in the context of T cell activation.

Although engagement of the TCR has occurred, the T helper cannot become an activated effector cell without a costimulatory signal delivered by an APC (62). This signal, known as signal 2, occurs as either B7-2 (CD86) or B7-1 (CD80) on the APC surface engages CD28 on the T cell surface (62). CD86 is considered the primary ligand for CD28 based on expression studies as maximal levels of CD86 expression levels on the APC surface occur between 12-24 hours after antigen encounter while maximal levels of CD80 occur on the surface 48 hours post antigen exposure (63). In addition, it was reported that CD86 exists as a monomer on the cell surface as does CD28, while CD80 and CTLA-4 exist primarily as a dimer (64). Costimulation afforded via the CD80/CD86 molecules is essential for the production of antibodies directed against a T-dependent antigen, as studies that employed CD80/CD86-deficient animals failed to promote T cell responses including IL-4 production and subsequent antibody production without functional molecules expressed on the APC surface in the presence of a T-dependent antigen (65, 66). Thus, the costimulatory signal delivered via CD80/CD86 is essential for activation of a naïve T cell to become an effector T cell.

When the TCR (CD3) and CD28 are engaged, a series of signaling networks are induced within the cell that will ultimately result in clonal expansion, CD40L upregulation on the T cell surface, the generation of the T cell growth factor IL-2 (62). In
addition, other specific cytokines are produced by the T helper cell depending on the antigen present and will allow the T cell to differentiate into either a Th1 or Th2 cell (67, 68). In summary, two specific signals are required to promote T cell activation in the presence of a T-dependent antigen including antigen recognition via the TCR:antigenic-peptide in association with MHCII and costimulation via the CD80/CD86:CD28 interaction.

1.3.2 T helper cell differentiation

Once the naïve T helper cell becomes an effector cell, the process of differentiation into a specific T helper cell subset, either Th1 or Th2, commences. As diagrammed in Figure 5, and reviewed in (68), the differentiation of a Th cell is governed via the cytokine profile produced by the APC that activated the T cell, as well as the cytokines produced by the T cell. If the cytokine IL-12 is produced either by a DC, a macrophage, or a B cell, the CD4+ Th cell will be driven to produce IFN-γ and differentiate into a Th1 cell and will ultimately either enhance macrophage-dependent antigen clearance, or allow a B cell to undergo CSR to IgG2a (69). However, if the activated T cell produces IL-4, the cell will differentiate into a Th2 cell, and thus, allow a B cell to induce CSR to either produce IgG1 or IgE to combat the antigen (70, 71).

Thus, subsequent to Th cell activation, the cell undergoes a differentiation process that is regulated via specific cytokine profiles secreted via APCs, as well as T cells dependent on the antigen present. Although Th1 cells are critical to host defense to enhance macrophage function or promote B cell production of IgG2a, this dissertation will focus on the signals generated via the IL-4-producing Th2 cell, which allows the B cell to produce IgG1.
Figure 5. *T helper cell differentiation*. A naïve CD4+ T helper cell will become either a Th1 cell or a Th2 cell depending on the cytokines secreted via the antigen presenting cell (APC) present, or secreted from the T cell itself. IL-12 secretion via the APC will allow a naïve T helper cell to differentiate into a Th1 cell capable of IFN-γ production, whereas; IL-4 secretion from a T helper cell will promote Th2 differentiation. While IFN-γ will enhance macrophage function and help B cells produce IgG2a, IL-4 will promote IgG1 and IgE production by a B cell.
1.4 Immune receptor involvement in antibody production by a B cell

1.4.1 Antibody Class-Switch Recombination to IgG1: IL-4 receptor engagement on a B cell

Class-switch recombination (CSR), diagrammed in Figure 6, and reviewed in (72), as mentioned previously, refers to a process in which the constant region of the Ig receptor heavy chain (C\text{H}) is altered via a process regulated via genetic deletion. During Ab CSR, the recombined gene segment variable, diversity, joining (VDJ), which encodes for the N-terminal antigen recognition sites of the Ig receptor on the B cell surface, recombines with a downstream C\text{H} gene segment via nucleotide regions known as switch regions (S). Switch regions are located 5’ to C\text{H} gene segments including C-\(\mu\), C-\(\gamma\), C-\(\epsilon\), and C-\(\alpha\), but not C-\(\delta\) (72). Furthermore, switch region combinatorial events are regulated via the presence of specific Th cell-dependent cytokines depending on the antigen present (72).

In order for a B cell to Ab isotype-(class) switch from IgM to IgG1, two essential signals must be afforded by a Th2 cell, CD40L and IL-4 (73-76). Upon engagement of CD40 and the IL-4 receptor (IL-4R) on the B cell surface, numerous intracellular signaling events are triggered within the B cell that activate transcription factors including nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) and signal transducer and activator of transcription-6 (STAT6). As shown in Figure 7 and Figure 8, while engagement of IL-4R induces STAT6 activation, CD40 engagement allows for the activation of NF-\(\kappa\)B. Several reports in vitro have shown that activation of a resting, naïve B cell with CD40L and IL-4 increases the level of germline \(\gamma1\) produced as well as subsequent CSR and IgG1 (24, 73, 77), suggesting that CD40 and IL-4R signaling are sufficient to promote B cell
Figure 6. **Class-Switch Recombination to IgG₁ by a B cell.** (1) Upon engagement of the IL-4R and CD40 on the B cell surface, intracellular signaling networks within the B cell promote the transcription of germline γ₁, also referred to as interfering γ₁ (Iγ₁), to allow recombination protein machinery access to the switch regions Sµ and Sγ₁ to facilitate their interaction. (2) Once the switch regions fuse, the intervening DNA is removed forming a “switch circle” of DNA. The end product is an IgH gene locus where the constant region has switched from constant (C) Cµ to Cγ₁, which is now transcribed as the heavy chain region. The “switch circle” of DNA now contains portions of Sµ and Sγ₁, and Cµ, Cδ, Sγ₃, Cγ₃, and Iγ₁ is further removed. VDJ represent the variable, diversity, and joining regions of the IgH gene locus, while Eµ represents the intronic enhancer.
differentiation to an antibody-producing cell. Furthermore, NF-κB and STAT6 binding sites are known to exist within the germline γ1, or intervening (I) γ1 promoter providing further support of CD40L/IL-4-induction of CSR to IgG_1. Germline γ1 transcripts been proposed to allow recombination machinery access to the C-γ1 gene segment to promote the interaction between the S-μ and S-γ1 switch regions (78). In support of this hypothesis, studies have shown that the level of germline γ1 positively correlates with the level of CSR (79, 80), suggesting that germline γ1 transcription is a pre-requisite for isotype-(class) switching to IgG_1. Subsequent to switch region binding, the intervening deoxyribonucleic acid (DNA) is deleted resulting in a gene product that will be expressed to ultimately become the mature antibody product immunoglobulin γ1 (IgG_1).

As mentioned previously, during the process of CSR, switch region combinatorial events are governed via specific cytokines secreted by Th cells depending on the antigen present. If the present antigen promotes Th2 cell-dependent secretion of IL-4, engagement of the IL-4R will occur on the B cell surface and trigger the activation of a key transcription factor, STAT-6 (81), as diagrammed in Figure 7. Upon STAT6 activation, the transcription factor can translocate to the nucleus and bind to a consensus site within the germline γ1 promoter region to induce transcription (82). Furthermore, STAT6-deficient B cells failed to produce germline γ1 transcripts upon activation with CD40L and IL-4 (82). Thus, these findings suggested that IL-4R signaling to induce STAT6 activation is required to promote germline γ1 transcription and subsequent CSR to IgG_1.
1.4.2 Antibody Class-Switch Recombination to IgG\(_1\): CD40 engagement on a B cell

In addition to IL-4R-induced activation of STAT6 in a B cell being a prerequisite for germline \(\gamma_1\) transcription, a second critical Th2 cell-dependent signal is required. This second required signal is generated through CD40 engagement on the B cell surface via CD40L expressed on a Th2 cell surface in the presence of a T-dependent antigen. The critical nature of the CD40:CD40L interaction to promote a humoral (antibody)-mediated immune response was documented by previous reports that showed animals that were deficient in either CD40 or CD40L failed to generate IgG\(_1\) antibody responses against a T-dependent antigen \textit{in vivo} (83, 84). Furthermore, antibody-mediated blockade of the CD40:CD40L interaction \textit{in vivo} also prevented IgG\(_1\) antibody responses (85). \textit{In vitro}, either engagement of CD40 with recombinant CD40L or a CD40 Ab in the presence of recombinant IL-4 was shown to promote B cell proliferation and differentiation into an IgG\(_1\) or IgE Ab-secreting cell (23), suggesting that a B cell can undergo CSR in the absence of antigen stimulation. In addition, the CD40:CD40L interaction was shown to be required for germinal center maintenance (86-88), indicating a critical role for the Th2 cell-B cell interaction in the presence of a T-dependent antigen. Together, these findings suggested that in addition to IL-4R engagement on the B cell surface, CD40 engagement on the B cell surface via CD40L expressed by a Th2 cell is required to induce germline \(\gamma_1\) transcription, and subsequent CSR to IgG\(_1\).
Figure 7. *IL-4 receptor signaling networks.* The IL-4 receptor (IL-4R) is comprised of two chains, the IL-4Rα chain, and the common (c)γ chain. Upon IL-4-mediated IL-4R engagement on the B cell surface, Janus kinase (JAK)1 (JAK1) and JAK3 associate with the IL-4R cytoplasmic domain and facilitate phosphorylation events, followed by the dimerization and activation of signal transducer and activator of transcription factor 6 (STAT6). Once active, STAT6 translocates to the nucleus to promote germline γ1 transcription. In addition to the JAK/STAT pathway, IL-4R engagement also induces the activation of the PI3K/Akt pathway, which is dependent on insulin receptor substrate (IRS1/2) activation and the Src Homologous and Collagen (Shc)-mediated activation of the Ras pathway.
Engagement of CD40 on the B cell surface promotes intracellular signaling networks that are believed to play critical roles in CSR induction to IgG1 through the activation of NF-κB, switch recombinase (89), and activation-induced deaminase (90). CD40 is a homo-trimeric transmembrane protein that is constitutively expressed on the B cell surface (91, 92) and a member of the tumor necrosis factor (TNF)-superfamily of cell surface receptors. CD40 possesses a relatively short cytoplasmic domain that is devoid of tyrosine residues, which are well known to induce intracellular signaling networks. However, CD40 associates with several protein adaptor molecules, some via interaction with threonine residues (93) within the cytoplasmic domain of CD40 that are capable of participating in intracellular signaling. Similar to other members of the TNF-superfamily of receptors, CD40 associates with several adaptor molecules called TNF-receptor-associated factors (TRAFs) via cytoplasmic domain interactions that induce signaling events upon CD40 engagement. Of the six known TRAF molecules, four TRAFs (TRAF2, 3, 5 and 6) are capable of direct interactions with CD40 (94). As diagrammed in Figure 8, upon engagement of CD40 on the B cell surface with CD40L expressed by a Th2 cell, TRAF2 and TRAF3 are recruited to the cytoplasmic domain of CD40 (95). Upon TRAF adaptor molecule recruitment to CD40, a series of intracellular signaling networks are induced that are mediated via the activation of the TRAF molecules. Activation of TRAFs promote the subsequent induction of several well defined signaling cascades that induce gene expression to promote a pro-growth and pro-survival phenotype within the B cell. These signaling networks include the phospholipase C (PLC)/protein kinase C (PKC), Rat sarcoma (Ras), c-jun kinase (JNK), mitogen-activated protein kinase (p38 MAPK), and NF-κB (96-100). However, B cell differentiation into
an IgG<sub>1</sub>-secreting cell that occurs via CSR and prior germline γ<sub>1</sub> transcription is regulated via an NF-κB-dependent mechanism (101, 102). Upon CD40 engagement via CD40L, TRAF2 associates with CD40 and becomes phosphorylated, allowing for the downstream activation of NF-κB (94). CD40-induced activation of NF-κB is preceded by the phosphorylation of Inhibitor of κB kinase-α (IKKα), which phosphorylates Inhibitor of κB (IκBα), a molecule that sequesters NF-κB in the cytoplasm rendering NF-κB unable to enter the nucleus. However, upon phosphorylation, polyubiquitination, and subsequent degradation of IκB alpha, IκB alpha releases NF-κB (103). Free NF-κB (p50/RelB) (104) then translocates to the nucleus to induce germline γ<sub>1</sub> transcription via binding to consensus sequences in the germline γ<sub>1</sub> promoter region (101, 102). In summary, engagement of CD40 and the IL-4R on the B cell surface via the Th2 cell signals CD40L and IL-4 activate intracellular signaling networks, which promote germline γ<sub>1</sub> transcription and subsequent CSR to IgG<sub>1</sub> antibody.
**Figure 8.** *CD40 signaling networks.* Upon CD40L-mediated engagement of CD40 on the B cell surface, TRAF2, 3, 5, and 6 are recruited to the cytoplasmic domain of CD40. TRAF2 mediates the activation of nuclear factor κB (NF-κB) through the phosphorylation of inhibitor of κBα (IκBα) through an inhibitor of κB kinase α/β/γ (IKKα/β/γ)-dependent mechanism. IκBα then becomes degraded allowing for the release of NF-κB, subsequent nuclear translocation, and initiation of germline γ1 transcription. In addition, CD40 engagement can also induce the activation of multiple signaling networks including the phospholipase/protein kinase C (PLC/PKC), Ras, Jnk, p38 MAPK.
1.5 Molecular interactions between the B cell and T helper cell

In order for a naïve Th cell to become active, an interaction must occur between CD86 on the APC surface and CD28 on the T cell surface in addition to TCR engagement via MHCII-restricted antigenic-peptide. CD86, also referred to as B7-2, is a 70 kDa transmembrane glycoprotein expressed primarily on APCs including macrophages, DCs, and B cells (56, 105). CD86 is a well-known costimulatory molecule that ligates CD28 and CTLA-4 expressed on a CD4+ T cell, to increase or decrease, respectively, T cell activation signals (65, 106-108), and essential for germinal center formation (66, 109). CD86 expression is low on resting B cells (56), but increases in response to engagement of the BCR (56), CD40 (110), the IL-4R (111), lipopolysaccharide (LPS) receptor (63, 112) or the beta-2 adrenergic receptor (113, 114). An expression kinetic study showed that CD86 is detectable on the B cell surface at 6 hours and occurs at maximal levels 24-48 hours post B cell activation (63). Currently, it is believed that CD86 expression levels are regulated via NF-κB-dependent mechanisms (114-116).

As mentioned previously, prior to a B cell differentiating from a naïve cell into an IgG1 Ab-producing cell via CSR, the B cell must receive signals from an activated Th2 cell in addition to engagement of the BCR via antigen encounter. Furthermore, for a naïve CD4+ to become activated, cognate recognition of antigenic peptide in association with MHCII on the APC surface via the TCR and costimulation afforded via the CD86:CD28 molecular interaction must occur. These two signals will induce T cell clonal proliferation, CD40L expression, and the generation of IL-4 (62). Without the molecular interaction between CD86:CD28, the generation of an optimal antibody-
mediated immune response against a T-dependent antigen cannot occur. This is supported via reports that showed that mice that were either CD28- or CD86-deficient produced significantly less IgG1 in response to a T-dependent antigen relative to wildtype (WT) mice (65, 66). In addition, another report where mice lacked CD80/CD86 only on the B cell produced significantly less IgG in response to an influenza virus relative to WT control mice (117). Furthermore, reports by our laboratory in either severely combined immunodeficient (Scid) mice or Rag2-deficient mice where only the B cell lacked CD86, mice produced significantly less IgG1 in response to either artificial Th2 cell signals, or the T-dependent antigen 2,4,6, Trinitrophenyl hapten-Keyhole Limpet Hemocyanin (TNP-KLH) (118, 119). Together, these findings suggested that the molecular interaction between CD86:CD28 is critical for an optimal IgG1 antibody response in vivo.

1.6 CD86 Direct Signaling in a B cell

1.6.1 CD86-dependent regulation of IgG1 antibody production

Since the interaction between CD86 on the APC surface and CD28 on the T cell surface was shown to be required for optimal T cell activation (65), and the generation of an optimal IgG1 antibody response against a T-dependent antigen in vivo (65, 66, 119), it was presumed that CD86 acted exclusively as a ligand to engage the receptor, CD28. Furthermore, CD86 contains a short cytoplasmic domain that lacks tyrosine phosphorylation sites and was thought not to signal directly. However, the CD86 cytoplasmic domain does contain three putative serine/threonine phosphorylation sites. An observation by Lenschow et. al. (120) suggested that the CD86 cytoplasmic domain becomes phosphorylated due to cellular activation stimuli suggesting that CD86 may signal directly. Studies have reported that CD86 engagement induced a signal directly
within the B cell that increased IgG₄ production in anti-CD40/IL-4 primed human B cells (121), and the murine IgG₄ homolog IgG₁ production in CD40L/IL-4 (113, 118, 119, 122, 123), or LPS (124) primed murine B cells in vitro, as well as in B cells from mice immunized with either Trinitrophenyl hapten (TNP)-keyhole limpet hemocyanin (KLH) (119), or influenza virus (117). It has also been reported that CD86 also signals to regulate other Ig-isotypes including IgE (113, 121), and IgG2a (124) an affect that may be controlled by the priming antigen or stimulus. Collectively, these findings suggested that CD86 on a B cell plays a role in regulating the level of IgG₁ produced.

Studies from our laboratory have shown that CD86 engagement with either an anti-CD86 Ab or CD28/Ig on the surface of a CD40L/IL-4-primed murine B cell signals directly to elevate the level of IgG₁ Ab produced (113, 118, 119, 122, 123), on a per cell basis (113), through an increase in the rate of mature IgG₁ transcription (122). Importantly, CD86 engagement on primed B cells failed to affect class switch recombination (113, 118, 119, 122, 123), indicating that the increase in IgG₁ was due to an effect on the amount of IgG₁ produced per cell and not the number of cells that switched to IgG₁. In addition, the finding suggested that CD86 engagement promoted the activation of a unique signaling network, independent of the signaling networks triggered via CD40 and IL-4R engagement required to induce CSR.

The initial results from these studies led to the search for transcription factors and proximal signaling intermediates required to regulate the CD86-induced increase in IgG₁ production by a primed B cell. Previous studies by our laboratory showed that the CD86-induced increase in the rate of mature IgG₁ transcription occurred through an increase in the level of 3’-IgH enhancer activity (118, 123), a genetic element that is known to
regulate mature antibody transcription (125). These findings suggested that a proximal signaling mechanism activated via CD86 engagement may exist to trigger the activation of a transcription factor(s) that can bind to, and subsequently regulate the activity of the 3’-IgH enhancer to ultimately increase the rate of mature IgG1 antibody transcription.

Previous reports from our laboratory also showed that CD86 engagement promoted the binding of a transcription factor called octamer binding protein-2 (Oct-2) to the hs1,2 and hs4 regions (118), and NF-κB (p50/p65) to the hs4 of the 3’-IgH enhancer (123) to augment its activity to ultimately increase the rate of mature IgG1 transcription. In addition, data from our laboratory showed that CD86 engagement increased the level of Oct-2 mRNA and protein (118, 123), which was shown to be regulated via an NF-κB (p50/p65)-dependent mechanism through activation and (p50/p65) nuclear translocation (118). Importantly, the CD86-induced increases in NF-κB activation, Oct-2 expression, increases in 3’-IgH enhancer activity, and increases in mature IgG1 mRNA and protein production failed to occur in CD86-deficient B cells (113, 118, 122, 123). Taken together, these findings suggested that CD86 direct signaling to a primed B cell promotes the activation of the transcription factors NF-κB and Oct-2 to enhance the activity of the 3’-IgH enhancer to increase the rate of mature IgG1 mRNA and subsequent protein production.

1.6.2 CD86-induced signaling networks that regulate IgG1 production

The findings that Oct-2 and NF-κB regulated the CD86-induced increase in IgG1 production via binding to the 3’-IgH enhancer, and that NF-κB promoted the CD86-induced increase in Oct-2 expression, lead to the identification of proximal signaling intermediates that activated NF-κB upon CD86 engagement. Data from our laboratory
showed that the p50/p65 subunits of NF-κB were activated upon CD86 engagement via a signaling network that allowed for the CD86-induced increase in NF-κB (p65) phosphorylation (S536) that occurred in a PKCα/βII- and PLCγ2-dependent manner (118, 123). Importantly, the CD86-induced increases in PLCγ2 (Y1217) and PKCα/βII (T638/641) failed to occur in CD86-deficient B cells (123). However, data also showed that CD86-induced IκBα phosphorylation and subsequent degradation occurred in the presence or absence of a PKC inhibitor suggesting for the first time that CD86 signaled directly to a primed B cell to regulate NF-κB activation via a PKC-independent mechanism (118). Therefore, we reasoned that CD86 engagement on a primed B cell induced two distinct signaling networks that converged to activate NF-κB with the PKC-independent phosphorylation and subsequent degradation of IκBα occurring proximal to the PKCα/βII-dependent p65 phosphorylation (118).

The evidence that CD86 engagement on a primed B cell allowed for the activation of two signaling networks to converge to activate NF-κB, ultimately allowing for an increase in the level of IgG1 Ab produced, led to the search for the proximal molecular signaling mechanism to the CD86-induced phosphorylation of IκBα. Since it was well documented in the literature that IκBα phosphorylation occurred via a distinct IκB kinase (IKK) complex which required its own phosphorylation for activation (126), which has been shown to occur via an Akt-dependent mechanism (127), it was possible that CD86 engagement induced IκBα phosphorylation via an IKK- and Akt-dependent mechanism. Data from our laboratory showed that CD86 engagement promoted a time-dependent increase in the level of IKKα/β and Akt (Thr308) phosphorylation that failed to occur in CD86-deficient B cells (123). Furthermore, chemical inhibition of IKKα/β blocked the
CD86-induced increase in IκBα phosphorylation (123). Together, these findings suggested that the CD86-induced increase in IκBα phosphorylation was IKKα/β-dependent and that CD86 signaled directly to a primed B cell via an Akt-dependent mechanism.

Although our findings suggested that CD86 engagement induced activation of Akt and IKKα/β allowing for the IκBα phosphorylation, the search resumed for the proximal mechanism to CD86-dependent Akt activation. Previous reports showed that activation of Akt was preceded via the activation of phosphoinositide-dependent protein kinase 1 (PDK-1), and phosphoinositide-3 kinase (PI3K) (128, 129), suggesting that CD86 engagement may induce activation of PDK-1 and PI3K prior to the activation of Akt and NF-κB. Data from our laboratory showed that CD86 engagement increased PDK-1 phosphorylation in a time-dependent manner and that chemical inhibition of PDK-1 blocked the CD86-induced increase in Akt phosphorylation (Thr308) (123). In addition, data showed that while CD86 engagement increased PI3K activity, chemical inhibition of PI3K prevented CD86-induced increases in Oct-2 and Akt phosphorylation (Thr308) (123). Together, these findings indicated that in addition to the PLCγ2/PKCα/βII-dependent network that controls p65 (S536) phosphorylation, CD86 signals directly to a primed B cell via the classic PI3K/Akt and subsequent classical NF-κB signaling networks to ultimately promote in increase in the level of IgG1 Ab produced.

Although our findings revealed that CD86 signals directly to a primed B cell to induce the activation of two distinct signaling networks, the search continued for the proximal molecular signaling mechanism to the CD86-induced activation of PI3K. Since previous reports showed that PI3K activation required binding to a phosphorylated
tyrosine residue (130), and no tyrosines existed within the cytoplasmic domain of CD86, it was likely that a signaling intermediate existed capable of undergoing tyrosine phosphorylation upon CD86 engagement. This signaling intermediate would then allow for the CD86-induced activation of PI3K. Since previous reports showed that PI3K bound to the co-receptor CD19 upon BCR engagement to facilitate activation (131), it was possible that CD19 served as a mechanistic link between CD86 engagement and PI3K activation. Data from our laboratory showed that CD86 engagement induced a time-dependent increase in CD19 phosphorylation, an effect that failed to occur in CD86-deficient B cells (119). In addition, CD86 engagement on the surface of CD19-deficient B cells failed to increase CD86-dependent PI3K activity, Oct-2 mRNA, and mature IgG1 mRNA and protein (119). Furthermore, CD86 engagement promoted protein-protein interactions between CD19 and PI3K, and CD19 and the protein tyrosine kinase (PTK) Lyn, as well as an increase in Lyn phosphorylation (119). Taken together, these findings suggested that CD19 is a critical CD86-induced signaling intermediate that is required for PI3K activation and IgG1 production, and the PTK Lyn may be involved with mediating CD86-induced signaling in primed B cells since Lyn-dependent activation of CD19 was reported previously (132).

In summary, as diagrammed in Figure 9, CD86 engagement induces the activation of two distinct signaling networks that ultimately converge to activate NF-κB. One network ultimately allows for CD86-induced phosphorylation of IκBα and consists of activation of the Lyn/CD19 network, followed by the activation of the PI3K/PDK-1/Akt network, and concludes with the IKKα/β-dependent phosphorylation of IκBα. The phosphorylation and subsequent degradation of IκBα allows for the release of
NF-κB (p50/p65). The other signaling network induced via CD86 engagement consists of the activation of the PLCγ2/PKCα/βII network, which allows for the full activation of NF-κB via the PKCα/βII-dependent phosphorylation of NF-κB (p65) (S536). NF-κB then translocates to the nucleus to promote Oct-2 transcription. Oct-2, once translated into protein, along with NF-κB bind to the 3’-IgH enhancer to ultimately increase the rate of mature IgG1 Ab transcription and subsequent protein production.
Figure 9. Proposed CD86 direct signaling model on a CD40L/IL-4-primed B cell. Upon CD86 engagement with either an anti-CD86 Ab or CD28/Ig on the surface of a CD40L/IL-4-primed B cell, two distinct signaling networks are induced. One network ultimately controls the phosphorylation of IκBα through the activation of Lyn, CD19, phosphoinositide-3 kinase (PI3K), phosphoinositide-dependent protein kinase 1 (PDK-1), Akt, and IKKα/β, allowing for NF-κB release (p50/p65). A second signaling pathway is activated upon CD86 engagement and controls the phosphorylation of p65 via a PLCγ2- and PKCα/βII-dependent mechanism. Once fully active, NF-κB (p50/p65) translocates to the nucleus to initiate octamer-binding protein 2 (Oct-2) transcription. Oct-2, along with (p50/p65) bind to the 3’-IgH enhancer to increase the rate of mature IgG1 transcription, independent of CD40L/IL-4-mediated class switch recombination.
1.7 Conclusion and hypothesis

CD86 engagement on the surface of a CD40L/IL-4-primed murine B cell increases the level of IgG1 production through an increase in the rate of transcription via the activation of a unique intracellular signaling network, without an affect on the level of CSR to IgG1 (113, 118, 119, 122, 123). The increased level of signaling intermediate activation and/or Oct-2 that was induced by CD86 engagement on primed B cells resulted in a 2-3 fold increase in IgG1 as compared to primed B cells in the absence of CD86 engagement. Notably, clinical findings have shown that a 2-3 fold increase in serum IgG correlates to a 3-9 fold increase in protection against *Streptococcus pneumoniae* and *pertussis* (133, 134), suggesting that increases of this magnitude in the level of Ab produced are potentially relevant clinically. An understanding of the mechanism by which CD86 signals directly to a B cell to increase not only IgG1, but also IgE production, as shown previously (113, 121), may help direct the rational design of CD86-targeted therapeutic strategies to increase the level of protective antibody in immuno-compromised patients. Conversely, CD86-targeted therapeutic strategies might also be developed to decrease the level of harmful antibodies produced in certain cases of autoimmunity or allergic asthma. Therefore, the present study was designed to identify the membrane-proximal CD86-induced signaling mechanism proximal to the activation of one of the first identified CD86-dependent signaling intermediates, PLCγ2.

Previous studies from our laboratory employed *in vitro* and *in vivo* model systems to test if CD86 engagement on a CD40L/IL-4-primed B cell signaled directly to elevate the level of IgG1 produced. The *in vitro* model system consisted of culturing resting, splenic, murine primary B cells, or transfected A20 or CH12.LX B cells in the presence
of CD40L expressed on the surface of an expired Sf9 cell to engage CD40 and recombinant IL-4 to engage the IL-4R to induce CSR to IgG1 for 16-20 hours. An anti-CD86 Ab or CD28/Ig would then be introduced to the B cell culture system to engage CD86 for various times prior to the execution of a functional assay. The current study utilized a similar model system except for either the transfection of targeted shRNA-plasmids targeted against genes of interest for 24 hours prior to CD40L/IL-4 priming, or transfection of a FLAG-CD86 expression plasmid for two hours followed by CD40L/IL-4 priming (see Figure 10). In the current study, an anti-CD86 Ab was used to engage CD86 to promote intracellular signaling. For our in vivo experiments designed to test if the CD86 cytoplasmic domain were required for an optimal IgG1 response against a T-dependent antigen (see Figure 11), WT CD4+ T cells and either WT or CD86 cytoplasmic-deficient (Trunc CD86) B cells were adoptively transferred into Rag2-deficient mice which lack mature B and T cells. In addition, either WT or Trunc CD86 B cells were adoptively transferred into CD86-deficient B cells (see Figure 11).
Figure 10. *In vitro model system used in the current study.* The following *in vitro* model system was utilized in the current study: (1) Either gene-specific short-hairpin ribonucleic acid (shRNA) plasmids or FLAG-CD86 expression plasmids were transfected into CH12.LX B cells via nucleofection. (2) Either 24 hours (shRNA plasmids) or 2 hours (FLAG-CD86 plasmids) later, the cells were primed with CD40L expressed on an expired Sf9 insect cell and recombinant interleukin-4 (IL-4) for 16-20 hours. (3) Either an anti-CD86 or anti-FLAG Ab was added for various times followed by the execution of a functional assay to measure CD86-induced signaling intermediate activation or the level of IgG₁ produced.
Figure 11. *In vivo model system used in the current study*. The following *in vivo* model system was employed in the current study. In each experiment in the current study, a cellular adoptive transfer approach was used. Wild type (WT) CD4\(^+\) T cells and either WT or CD86 cytoplasmic domain-deficient (Trunc CD86) B cells were adoptively transferred into Rag2\(^{-/-}\) mice via lateral tail vein injection. Likewise, either WT or Trunc CD86 B cells were adoptively transferred into CD86\(^{-/-}\) mice. After 7 and 21 days, mice were immunized with the T-dependent antigen OVA-alum. Serum was collected and analyzed for levels of IgG\(_1\) produced over a 42 day time course. On day 42, total splenocytes were harvested, re-activated *ex vivo* and analyzed for T/B cell levels, CD86 levels, and IL-4 levels via fluorescence activated cell sorting (FACS) analysis.
Although our findings thus far suggested that CD86 engagement on a primed B cell induced the activation of PLCγ2, ultimately allowing for an increase in IgG\textsubscript{1} production by a primed B cell, the membrane-proximal molecular signaling mechanism remained unclear. Another CD86-dependent signaling intermediate was likely to exist since PLCγ2 functions classically by recruitment to proteins containing tyrosine-residues (135, 136), which are lacking in the CD86 cytoplasmic domain. Therefore, it was hypothesized that the CD86 cytoplasmic domain associated directly with a protein/protein complex capable of undergoing tyrosine phosphorylation to activate PLCγ2 and was required for the CD86-dependent increase of IgG\textsubscript{1} production by a B cell. The data presented in the current study are the first to show novel CD86-induced signaling intermediates that cooperatively signal to differentially activate distal signaling intermediates upon CD86 engagement on a primed B cell. Using a proteomics-based identification approach, we show for the very first time that the tyrosine-containing transmembrane adaptor proteins, prohibitin-1 (Phb1) and prohibitin-2 (Phb2), bind to CD86. The basal expression of Phb1/2 and association with CD86 was low in resting B cells, while the level of expression and association increased primarily after priming with CD40. The CD86-induced increase in Oct-2 and IgG\textsubscript{1} was less when either Phb1/2 expression was reduced by shRNA or the cytoplasmic domain of CD86 was truncated or mutated at serine/threonine PKC-phosphorylation sites, which did not affect Phb1/2 binding to CD86. In addition, we show that an intact CD86 cytoplasmic domain is necessary for an optimal IgG\textsubscript{1} response against a T-dependent antigen in vivo. Furthermore, we also show that Phb1/2 and the CD86 cytoplasmic domain are required for the CD86-induced phosphorylation and subsequent degradation of IκBα, which we
previously reported leads to NF-κB p50/p65 activation; whereas, only Phb1/2 was required for the CD86-induced phosphorylation of PLCγ2 and PKCα/βII, which we have previously reported leads to NF-κB (p65) phosphorylation and subsequent nuclear translocation. Together, these findings suggest that Phb1/2 and the CD86 cytoplasmic domain cooperate to mediate CD86 signaling in a B cell through differential phosphorylation of distal signaling intermediates required to increase IgG1.

The significance of this dissertation is that it is the first to identify the membrane-proximal CD86-induced intracellular signaling mechanism in a B cell that regulates the level of IgG1 produced. Knowledge gained from this work will provide a molecular mechanism by which CD86 engagement regulates the IgG1 antibody response by a primed B cell to provide novel therapeutic targets either to elevate or suppress the level of IgG1 produced.
CHAPTER 2

MATERIALS AND METHODS

2.1 Animals - Female pathogen-free BALB/c and CD86<sup>-/-</sup> (BALB/c background) mice were obtained from Taconic (Germantown, NY), female Rag2<sup>-/-</sup> C57BL/6 and CD86<sup>-/-</sup> (C57BL/6) mice were obtained from Jackson Laboratories and were housed in the American Association Accreditation of Laboratory Animal Care (AAALAC)-accredited Animal Research Facility at The Ohio State University (Columbus, OH). All mice were provided autoclaved food and deionized water <i>ad libitum</i> and used at 8-10 weeks of age.

2.2 Cell Lines/Transgenic B cells - CH12.LX is a murine B cell lymphoma line previously described (137) kindly provided by Dr. G. Bishop (University of Iowa, Iowa City, Iowa). WT, transgenic B cells (Line 7 Tg) that overexpress CD86 on a C57BL/6 background were previously described (138). Transgenic B cells that express a CD86 cytoplasmic truncation (Line I2) within a CD80/CD86-double deficient mouse on a C57BL/6 background were previously described (139).

2.3 B cell Isolation/Priming - Naive B cells were isolated using magnetic cell sorting (MACS) anti-mouse CD43 beads following manufacturer’s directions (Miltenyi Biotec,
Auburn, CA). Resting B cells (CD43−) were cultured at 1 x 10^6 cells/ml in either a 24- or 6-well plate (Greiner Bio-One, Monroe, NC) in a final volume of 2.0 or 5.0 ml of cell culture medium consisting of RPMI 1640 medium (CellGro, Herndon, VA), 10% fetal bovine serum (FBS) (Atlas Biologicals, Colorado Springs, CO), 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 50 µM 2-mercaptoethanol, in a humidified atmosphere at 37 °C and 5% CO₂. Cells were primed with CD40L-expressing Sf9 cells or control Sf9 cells lacking CD40L expression at a B cell to Sf9 cell ratio of 10:1, IL-4 [1 ng/ml (eBioscience)], anti-IgM (0.1 µg/ml), terbutaline (Terb) (10^−6 M; Sigma-Aldrich), or LPS (50 µg/ml, Sigma-Aldrich) for 16 hours. Total mRNA and total protein were collected and analyzed via quantitative real-time PCR (qRT-PCR) and immunoblot, respectively. All reagents used were negative for the presence of endotoxin, as determined by Etoxate (Sigma), a Limulus lysate assay with a level of detection < 0.1U/ml.

2.4 In vivo cell transfer and immunization - Seven days before immunization, 7.5 x 10^6 CD4+ T cells and either 15 x 10^6 WT B cells or Truncated-CD86 (Trunc CD86) B cells were adoptively transferred into Rag2-deficient recipient animals, and either 22.5 x 10^6 WT B cells or Trunc-CD86 B cells were adoptively transferred into CD86-deficient animals in a volume of 100 μl PBS in the lateral tail vein. One week and four weeks following the adoptive transfer of cells, mice were administered 100 μg OVA in alum i.p., and serum samples were collected over time. The level of serum IgG1 was determined in various dilutions of serum samples using ELISA. On Day 42, total splenocytes were re-activated as described previously (119). T cell activation was
determined by the number of CD4\(^+\) cells expressing intracellular IL-4 as measured by FACS analysis. CD86 expression levels and percentages of either B or CD4\(^+\) T cells localized to the spleen were determined by FACS analysis.

2.5 Immunoprecipitation - Immunoprecipitations were performed using the Profound Mammalian Co-Immunoprecipitation Kit (Pierce). Briefly, 25 x 10\(^6\) CH12.LX B cells were cultured and primed as described above, lysed with 0.5% Triton-X100 buffer, immunoprecipitated with an anti-CD86 GL1 (eBioscience), or species- and isotype-matched control Ab (Rat IgG2a, eBioscience) following manufacturer’s directions. The precipitates were analyzed via immunoblot for CD86, Phb2, and Phb1. Alternatively, 20 x 10\(^6\) CH12.LX B cells, and 50 x 10\(^6\) WT B cells were activated and lysed as described above, and immunoprecipitated with 50 µg of either anti-FLAG Ab (M2; Sigma) or anti-CD86 Ab (GL1; eBioscience) overnight at 4°C, followed by the addition of 50 µl of protein G agarose beads (Invitrogen).

2.6 Mass Spectrometry - Mass Spectrometry and protein identification were performed by the Mass Spectrometry and Proteomics Core Facility at The Ohio State University (Columbus, OH). The unique band present at ~35 kDa in an anti-CD86 immunoprecipitate of CH12.LX cells from a gel stained with Sypro Ruby (Invitrogen) was excised and digested with sequencing grade trypsin from Promega (Madison, WI) using the Multiscreen Solvinert Filter Plates from Millipore (Bedford, MA). Capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano-LC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray
source operated in positive ion mode. The LC system was an UltiMate™ 3000 system from Dionex (Sunnyvale, CA). Sequence information from the MS/MS data was processed by converting the .raw files into a merged file (.mgf) using an in-house program, RAW2MXML_n_MGF_batch (merge.pl, a Perl script). The resulting mgf files were searched using Mascot Daemon by Matrix Science version 2.3.2 (Boston, MA).

2.7 Immunoblot Analysis - Resting B cells (5-7 x 10^6) were primed as described above. CH12.LX B cells were transfected with scrambled negative control shRNA or Phb1/2-specific shRNA plasmids (1-5 µg/10^6 cells) Phb1 (clones 1, 3, and 4), Phb2 (clones 1 and 3) (SA Biosciences) for 24 hours followed by priming with CD40L/IL-4 for 16 hours or with a FLAG-CD86 expression plasmid (1 µg/10^6 cells) via nucleofection (Amaza, program O-003), followed by priming with CD40L/IL-4 for 16 hours. In some experiments, cells were cultured under serum-free conditions for at least 30 minutes, and engagement of CD86 with anti-CD86 (P03, eBioscience) species- and isotype-matched control Ab (Rat IgG2b, eBioscience), anti-FLAG (M2, Sigma) or species- and isotype-matched control Ab (mouse IgG1, Southern Biotec). Cell lysates were prepared as described previously (118). Nuclear-enriched protein lysates were prepared by washing 5 x 10^6 CH12.LX B cells with 1X PBS following by the addition of 0.5% NP-40 lysis buffer containing 10 mM NaCl, 10 mM Tris-HCl pH (7.4), and 3 mM MgCl2. Nuclei were pelleted and resuspended in 1X lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM Na2VO4, 1 µg/ml leupeptin, 10 mM okadaic acid, and 10 nM tautomycin). Protein
samples, (5-12 µg) were run on a denaturing 10% polyacrylamide gel and transferred to Immobilon-PVDF membranes (Millipore). Membranes were blocked with tris buffered saline-tween 20 (TBST) as described previously (118) and incubated overnight with primary antibodies at 4°C. Membranes were probed with HRP-labeled secondary antibodies, developed using LumiGlo Detection Kit (Cell Signaling) and specific bands were visualized on Kodak Biomax MS film. Antibodies used were anti-CD86 (M-20), anti-CD86 (H-200), anti-GAPDH (FL-335), anti-PKCβII (C-18) (Santa Cruz Biotechnology), anti-Bap37 (Phb2) (Poly6118) (BioLegends), anti-Phb1, anti-phospho-PLCγ2 (Y1217), anti-PLCγ2, anti-phospho PKCα/βII (T638/641), anti-PKCα, anti-phospho-IκBa (S32) (14D4), anti-IκBa (44D4), anti-phospho-p65 (S536) (93H1), anti-p65 (C22B4), anti-Lamin A/C (4C11), anti-GAPDH (14C10), anti-β-Actin (13E5), anti-phospho-tyrosine (pY100) (Cell Signaling), anti-α-tubulin (DM1A) (Sigma).

2.8 Immunoblot Densitometric Analysis - Specific protein bands detected via Immunoblot Analysis were quantified via Densitometry (ImageJ Software, NIH). The scanned image was inverted in order to measure the optical density (OD) of a specific protein band. A measurement box was created around the broadest band on a given gel and used to measure each band on the gel. The numerical value was recorded as an optical density (OD). Background measurements of each band were recorded from the corresponding gel lanes and subtracted from the original ODs. The ODs of Phb1/2 total protein bands were normalized to GAPDH protein loading control band ODs. The ODs of phospho-PLCγ2 (Y1217) protein bands were normalized to ODs of corresponding total PLCγ2 protein bands. ODs from phospho-PKCα/βII (T638/641) protein bands were
normalized to OD values from both PKCα and PKCβII total protein bands. ODs obtained from phospho-IκBα (S32) and total IκBα protein bands were normalized to GAPDH total protein band ODs. The ODs recorded from phospho-p65 (S536) protein bands were normalized to both total p65 protein band ODs and ODs obtained from GAPDH protein bands. The ODs measured from nuclear phospho-p65 (S536) and nuclear p65 were normalized to both Lamin A and Lamin C protein band ODs. The data are expressed as mean Fold Change ± standard deviation (SD)/standard error of the mean (SEM) relative to resting, CD40L/IL-4-priming alone, or species- and isotype-matched control Ab conditions.

2.9 Ribonucleic acid (RNA)-interfering Phb1 and Phb2 Knock-down and quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) analysis - CH12.LX B cells were transfected with scrambled negative control shRNA, or (prohibitin-1) Phb1 (clones 1, 3, and 4) or (prohibitin-2) Phb2 (clones 1 and 3)-specific shRNA (SABiosciences) (1-5 µg/10^6 cells), or a FLAG-CD86 expression vector (1 µg/10^6 cells) via nucleofection (Amaxa, program O-003) according to manufacturer’s instructions, cultured for 24 hours followed by CD40L/IL-4 priming for an additional 16 hours. In some experiments, GFP^+ cells were sorted (ICy-Reflection) and re-cultured in the presence of either anti-CD86 (PO3 eBioscience) or species- and isotype-matched control Ab (RatIgG2b eBioscience), or CD28/Fc chimera (CD28/Ig) (R & D Biosciences) for 24 hours. Total RNA was isolated via Trizol (Invitrogen), complementary DNA (cDNA) was created via reverse transcription with random hexamer primers (Qiagen) and qRT-PCR, also known as Real-time PCR (SYBRGreen, Roche) was conducted as
previously described (122). Briefly, a common master mix [LightCycler-FastStartDNA SYBR Green I (Roche, Mannheim, Germany), 2 mM MgCl₂, 0.5 µM gene-specific primer] was used and the concentration of gene specific complementary (cDNA) was quantified using a standard curve diluted 1:10 for a concentration range of 1 ng/ml to 1 fg/ml. A melt curve was generated after each real-time reaction for sample purity. Real-time PCR was performed using the Rotor-Gene Q (Qiagen). The following primers were used for Phb1 mRNA levels, forward primer: 5’-ATGGCTGCAAGCTGGAGAAGC-3’; Phb2 mRNA levels, forward primer: 5’-GTAGAAGCCAAGCAAGTGTC-3’ reverse primer: 5’-TTCAGCACAAGGTTGTCAGC-3’; CD86 mRNA levels, forward primer: 5’-CGAGCATAATTTGGGCAACAG-3’ reverse primer: 5’-TTTCCAGAAGCACAACAGCGTC-3’; cytoplasmic CD86 mRNA levels, forward primer: 5’- GTCAATGAAGATTTCTCCAAA-3’ reverse primer: 5’-CAGGTGTAGGTCTCTCTGAGC-3’; Oct-2 mRNA levels, forward primer: 5’-ATCAAGGGCTGAAGACCCCAATG-3’ reverse primer: 5’-TGGAGAGGTGCTATGTGCC-3’; germline IgG₁ mRNA levels, forward primer: 5’-CATCCTATCAGGGAGATTGG-3’ reverse primer: 5’-ATCCTCGGGGCTCAAGTTTTC-3’; and mature IgG₁ mRNA levels, forward primer: 5’-TATGGACTACTGGGTCAAGGC-3’ reverse primer: 5’-CCTGGCACAATTTCTTGT-3’ relative to actin mRNA levels, forward primer: 5’-TACACTCCACCACCACAGC-3’ reverse primer: 5’-AAGGAAGGCTGGAAAGAGC-3’or GAPDH mRNA levels, forward primer: 5’-
ACCACAGTCCATGCCATCAC-3’ reverse primer: 5’-

TCCACCACCCTGTGCTGTA-3’.

2.10 cDNA constructs and Site-Directed Mutagenesis - Total RNA was isolated by Trizol (Invitrogen) from CD40L (10 B cells:1 Sf9) and recombinant IL-4 (1 ng/ml) (eBioscience)-primed murine splenocytes for 24 h and cDNA was generated using Accuscript RT (Stratagene). The murine CD86 cDNA was amplified by PCR using the Easy-A High Fidelity PCR Cloning (Stratagene) and the following primers. The forward primer containing EcoRI site: 5’-

GAATTCCGAGACGCAAGCTTATTTCAATGGGACTCATAT-3’ and the reverse primer containing BamHI site 5’-

TTTTGGATCCCTCCTCAGTCTTGGTTTTGCTGAAGC-3’. The CD86 cDNA was then subcloned into the pCR2.1 TOPO TA vector. To generate a FLAG-CD86 expression construct, murine CD86 cDNA was subsequently cloned into the pFLAG-CMV3 vector (Sigma), introducing an N-terminal FLAG epitope tag (DYKDDDK) before the start of the mature polypeptide receptor. Mutant plasmids containing either FLAG-CD86 cytoplasmic truncations at KKPΔ or 282Δ and or single alanine point mutations at positions S285, T291, and S303 were created by using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer’s instructions. The following primers were used to generate specific mutant plasmids:

KKPΔ forward primer: 5’-

ATTGTATGTCAAGGAAGTAGATAGAATCAAGCTAGCAGGCCC-3’ reverse primer: 5’-

GAGGCTGCTAGGCTGATTCTACTTCTTTGTGACATACAAT-3’; 282Δ forward
primer: 5’-AACACAGCCTCTAAGTTATAGCGGGATAGTAACGCTGACAG-3’
reverse primer: 5’-CTGTCAGCGTTACTATAACCCTGTTAGGCTGTGTT-3’;
S285A forward primer: 5’-
GCCTCTAAGTTAGAGCGGGATGCTAAACGCTGACAGAGACTATC-3’ reverse primer: 5’-GGTTGATAGCTCTCTTGTCAGCGTTAGCATCCCGCTCTAAGTT-3’; T291A forward primer: 5’-
ATAGTAACGCTGACAGAGGGCTATCAACCTGAAGAAGAATC-3’ reverse primer: 5’-AGTTTCTCAGGGTTAGCCTCTCTGTCAGCGTTACTAT-3’; S303A forward primer: 5’-AATTTGAACCCCAAATTGCTGCAGCAAAACCAAATGCAGA-3’ reverse primer: 5’-TCTGCATTTGCTTTGCTGCAGCAATTTGGGGTTCAAGTT-3’.

Integrity of all constructs was confirmed by sequencing at the Nucleic Acid Shared Resource facility at The Ohio State University (Columbus, OH). Surface expression of all constructs was confirmed by Flow Cytometry.

2.11 Flow Cytometry - The level of CD86 on GFP⁺ Phb1/2-shRNA⁺ CH12.LX B cells was determined as previously described (113) using allophycocyanin (APC)-conjugated rat-anti-mouse CD86 (GL1). Expression of FLAG-CD86 was measured via fluorescein isothiocyanate (FITC)-conjugated anti-mouse FLAG (M2), (Sigma). The number of B220⁺ and CD4⁺ cells, the percentage/number of CD4⁺ cells expressing intracellular IL-4, and the level of CD86 expression on B cells was also determined. In brief, total splenocytes were collected on day 42 following primary immunization and stained with Phycoerythrin (PE)-conjugated rat anti-mouse B220, PE/Peridinin Chlorophyll Protein (PerCP)-conjugated rat anti-mouse CD4, Allophycocyanin (APC)-conjugated rat anti-
mouse IL-4, rat anti-mouse Allophycocyanin (APC)-conjugated CD86 (BD Biosciences). For intracellular Flow Cytometry, total splenocytes on day 42 were stimulated for 3 hours with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma-Aldrich) and ionomycin (500 ng/mL; Sigma-Aldrich) in the presence of brefeldin A (1 µg/ml; BD Biosciences). Cells were then washed, fixed, and permeabilized using the Cytofix/Cytoperm (BD Biosciences) kit according to the manufacturer’s directions and stained with a rat anti-mouse IL-4 Ab. All samples were analyzed using a FACSCanto II (BD Biosciences). The data were analyzed using FlowJo software (Tree Star).

2.12 IgG1 enzyme-linked immunosorbent assay (ELISA) - For in vivo IgG1 serum samples were collected and frozen immediately at -20°C until analysis as described previously (119). Briefly, Costar 96-well flexi plates (Fisher Scientific) were coated with goat anti-mouse IgG (2 µg/ml) (Southern Biotech), followed by blocking with 20% FBS solution in phosphate buffered saline + 0.02% azide. 20 µl of each sample were incubated on the plate, a standard curve for IgG1 was prepared using known quantities of recombinant IgG1 protein in a range of 1 µg/ml – 1 ng/ml. A secondary Ab, goat anti-mouse IgG1-alkaline phosphatase (AP) (Southern Biotech) was used for detection. p-Nitrophenyl phosphate (PNPP) (Sigma) was added and color development was determined on a Spectramax Plus microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 405 nm.

2.13 In-Cell ELISA - In-Cell ELISAs were performed to detect in-cell levels of phospho-PLCγ2 (Y1217) and total PLCγ2 protein using the In-Cell Colorimetric ELISA
kit (Pierce). Briefly, $1 \times 10^6$ CH12.LX B cells were transfected under Mock conditions or with either scrambled negative control shRNA or Phb1/2-specific shRNA plasmids for 24 hours followed by priming with CD40L/IL-4, or with a WT or cytoplasmic-deficient (KKPΔ) FLAG-CD86 expression plasmid followed by priming with CD40L/IL-4 16 hours. Cells were cultured under serum-free conditions for at least 30 minutes, followed by engagement of either CD86 with anti-CD86, or FLAG-CD86 with anti-FLAG and appropriate controls as described above for 15-30 minutes. The cells were then washed in 1X PBS. $2 \times 10^4$ cells were plated on a 96-well culture plate and fixed with 8% formaldehyde. The remainder of the assay was performed according to manufacturer’s instructions. Antibodies used to detect phospho-PLCγ2 (Y1217) and total PLCγ2 in-cell protein levels were anti-phospho-PLCγ2 (Y1217), and anti-PLCγ2 (Cell Signaling). The horseradish peroxidase (HRP)-conjugate antibody was used at a (1:2000) dilution.

2.14 Statistical Analysis - Data from multiple treatment groups were analyzed using a one-way analysis of variance to determine if an overall statistical change existed. Certain $p$ values were calculated using a Bonferroni post hoc analysis or a two-sided Student $t$ test for comparison of two treatment groups. A $p$ value of $\leq 0.05$ indicated statistically significant results.
CHAPTER 3

RESULTS

3.1 CD86 associates with prohibitin-1 (Phb1) and prohibitin-2 (Phb2) in B Lymphocytes.

CD86 engagement on a CD40L/IL-4-primed B cell is known to activate PLCγ2 (123), a signaling intermediate that requires binding to proteins containing phosphotyrosine residues for recruitment/activation and its ability to implement receptor function. We reasoned that CD86, which does not contain tyrosine residues in the cytoplasmic domain, must associate with a tyrosine-containing adaptor/scaffolding protein to mediate the activation of PLCγ2. Therefore, a proteomics-based approach was used to identify any protein that associated with CD86 in primed B cells. The murine B cell line CH12.LX was cultured overnight in the presence of CD40L/IL-4 and then exposed to either an anti-CD86 or species- and isotype-matched control Ab. CD86 was immunoprecipitated from cell lysates, and proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Sypro Ruby (see Figure 12A). Three unique bands were present at molecular weights of ~50 kDa, ~35 kDa, and ~28 kDa in the anti-CD86 antibody immunoprecipitate, but were absent
Figure 12. Identification of a CD86-associated protein. (A), CH.12LX B cells were primed with CD40L/IL-4 for 16 hours followed by the addition of a species- and isotype-matched control Ab (anti-rat IgG2a ctrl Ab), or rat anti-mouse CD86 (GL1) (anti-CD86) for 15 minutes. Lysates were prepared, followed by immunoprecipitation (Pierce). Immunoprecipitates were separated via SDS-PAGE followed by total protein staining with Sypro Ruby. Unique bands in the CD86 immunoprecipitate are indicated by arrows at ~50, ~35, and ~28 kDa. (B), The band marked with an arrow at ~35 kDa was sequenced via LC/MS Mass Spectrometry. Tryptic peptide fragments were sequenced and matched with known tryptic peptides via a MASCOT search. 14 non-redundant tryptic peptides were identical to murine prohibitin-2 (Phb2).

<table>
<thead>
<tr>
<th>Non-Redundant Phb2 Specific Tryptic Peptides</th>
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<tbody>
<tr>
<td>RAOFKEVA</td>
</tr>
<tr>
<td>KMLEALSKN</td>
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<tr>
<td>RAQVLIR</td>
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<td>RLGLDYERV</td>
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<tr>
<td>KFNQNLTRA</td>
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<tr>
<td>KDQMVNSLVR</td>
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<tr>
<td>RVLSIPNNQIYELPSMYQRL</td>
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<tr>
<td>RYLTAQNLVLNLQDESFRG</td>
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when the control Ab was used. The unknown ~35 kDa protein was excised since it was clearly isolated from other bands and identified by Mass Spectrometry as the adaptor protein prohibitin-2 (Phb2) (see Figure 12B). In all, 14 non-redundant tryptic peptide fragments of murine Phb2 were identified.

Although sequence analysis was not performed on the other two bands, we predict that the CD86-associated ~28 kDa protein band is Phb1 since the literature suggests that Phb2 associates closely with the highly-related Phb1 in a variety of intracellular locales (140). The identity of the CD86-associated protein band at ~50 kDa remains unclear, but may be another adaptor/scaffolding protein similar to that reported for other B cell surface receptors, including CD40 (141-144) and the BCR (55, 145-148) that are also devoid of tyrosine residues within the cytoplasmic domain, but use adaptor/scaffolding complexes to function.

We tested whether Phb1 and/or Phb2 could be specifically co-immunoprecipitated from B cells under primed vs. resting conditions. Parent CH12.LX cells, CH12.LX cells transfected with FLAG-tagged CD86, or primary B cells were cultured overnight in the presence or absence of CD40L/IL-4-priming. Lysates were prepared and immunoprecipitated with control and anti-CD86 Abs, and then analyzed for level of Phb1/2 and CD86 by immunoblot. As shown in (Figure 13A), levels of Phb1 and Phb2 were low or undetectable in CD86 immunoprecipitates from resting CH12.LX B cells, while co-immunoprecipitation of both proteins increased substantially in primed B cells. This increase in Phb1/2 binding to CD86 may be due to a specific CD40L/IL-4-dependent recruitment event, or an increased level of CD86 present in the CD86
Figure 13. *CD86* associates with Phb1 and Phb2 in B Lymphocytes. (A), CH12.LX B cells alone, CH12.LX B cells expressing a FLAG-CD86 recombinant protein, or primary splenic B cells were primed as described above. Immunoprecipitates were immunoblotted for CD86, Phb2, and Phb1. Gels are representative of three independent experiments. (B), FLAG-CD86 transfected CH12.LX B cells with either Full Length FLAG-CD86 or a Truncated FLAG-CD86 (KKPΔ) or non-transfected B cells from WT or Trunc CD86-expressing mice were primed as described above, immunoprecipitated with either anti-FLAG or anti-CD86, and were analyzed for Phb1/2 and CD86 protein levels via immunoblot. The gels shown are representative of three independent experiments (*left panel*) or one experiment (*right panel*).
immunoprecipitates. Similar results were observed when CH12.LX B cells were transfected with a FLAG epitope-tagged CD86 construct, and primary B cells were used (see Figure 13A). To determine if Phb1/2 associated with the cytoplasmic domain of CD86, we tested CH12.LX B cells transfected with a cytoplasmic truncated form of FLAG-CD86 (KKPΔ), as well as primary B cells from transgenic mice that express a truncated form of the CD86 cytoplasmic domain. Phb1/2 were found to associate with CD86 in the absence or presence of the cytoplasmic domain (see Figure 13B), suggesting that a transmembrane-specific association occurred between CD86 and Phb1/2. Taken together, these findings indicated that low levels of Phb1 and Phb2 associated with CD86 in resting B cells, but that the levels increased after CD40L/IL-4-priming, and was independent of most of the CD86 cytoplasmic domain.

3.2 The expression of Phb1 and Phb2 is regulated primarily by CD40 engagement.

A number of studies have reported that Phb1 and Phb2 associate with each other to form a large complex within the inner mitochondrial membrane, and function by stabilizing proteins of the electron transport chain (149). If a similar stabilizer/molecular chaperone function existed for Phb1/2 to facilitate CD86 stability, transport, and insertion into the plasma membrane, then Phb1/2 protein expression would be expected to increase concomitantly with CD86 protein expression when the B cell was primed with CD40L/IL-4, as well as with other B cell stimuli that are known to increase CD86 protein expression. Primary resting B cells were cultured in the absence or presence of IL-4 overnight with either CD40L, anti-IgM to engage the BCR, the beta-2 adrenergic receptor agonist terbutaline (Terb), or LPS, all of which have been reported to increase CD86
Figure 14. CD86 expression in B cells exposed to different stimuli. Naïve splenic B cells were isolated and cultured for 16 hours in the absence or presence of CD40L, anti-IgM, terbutaline (Terb), or LPS in the absence or presence of IL-4. Total mRNA was collected at 16 hours and analyzed via qRT-PCR analysis for CD86 and GAPDH mRNA levels. CD86 mRNA values were normalized to GAPDH and the data are presented as a mean Fold Change in CD86 from primed B cells relative to resting B cells and are expressed as the mean Fold Change ± SEM from quadruplicate samples/condition of either representative (Resting vs. Terb) or from two independent experiments. Statistical analysis was used to determine significant differences between groups. *, *p < 0.05.
expression on a B cell (56, 63, 110-114), and as shown in (see Figure 14). Total mRNA and protein were collected and analyzed for the level of Phb1/2 and CD86 expression by qRT-PCR and immunoblot, respectively. Engagement of CD40 alone promoted a robust increase in the level of expression for both Phb1 and Phb2 mRNA (see Figure 15) and protein (see Figure 16) as compared to resting B cells. IL-4R engagement on a resting B cell had no effect on the level of Phb1/2 expression while concomitant engagement of the IL-4R with CD40 further increased the level of Phb2, but not Phb1, mRNA, and increased the level of Phb1/2 protein as compared to CD40 engagement alone. Treatment with LPS alone also increased levels of Phb1/2 mRNA relative to resting B cells, although the increase was less than that caused by CD40 engagement, and this LPS-induced increase in mRNA was further increased by the addition of IL-4. In contrast, only a weak increase in Phb1/2 protein was detected in LPS exposed B cells in the absence or presence of IL-4. Other stimuli that were able to increase CD86, i.e., IgM and beta-2 adrenergic receptor engagement on a B cell, were unable to upregulate Phb1/2 expression to any significant level in comparison to resting levels. Importantly, control Sf9 cells lacking CD40L expression failed to induce Phb1/2 mRNA or protein levels (see Figure 17). Together, these findings suggested that Phb1/2 protein expression may be regulated primarily by a CD40-dependent mechanism that is independent from the mechanism involved in upregulating expression of CD86.
Figure 15. The expression of Phb1 and Phb2 mRNA is regulated primarily by CD40 engagement. Naïve splenic B cells were isolated and cultured for 16 hours in the absence or presence of CD40L, anti-IgM, terbutaline (Terb), or LPS in the absence or presence of IL-4. Total mRNA was collected and analyzed via qRT-PCR analysis Phb2, Phb1, and GAPDH. Phb1/2 values were normalized to GAPDH and the data are represented as a mean Fold Change in Phb1/2 mRNA from primed B cells relative to resting B cells and are expressed as the mean ± SEM of quadruplicate samples/condition from three independent experiments. Statistical differences are shown relative to resting. *, p < 0.05.
Figure 16. The expression of Phb1 and Phb2 protein is regulated primarily by CD40 engagement. Naïve splenic B cells were isolated and cultured for 16 hours in the absence or presence of CD40L, anti-IgM, terbutaline (Terb), or LPS in the absence or presence of IL-4. Total protein was collected and immunoblot analysis was used to measure total Phb2, Phb1, and GAPDH protein levels. Gels are representative of three independent experiments. Densitometry was performed and measured Phb1/2 band intensity/GAPDH band intensity and data are presented as the mean Fold Change in Phb1/2 protein from primed B cells relative to resting and expressed as the mean Fold Change ± SEM from three independent experiments. Statistical differences are shown relative to resting. *, p < 0.05.
Figure 17. Control Sf9 cells fail to induce Phb1 and Phb2 expression in the absence of CD40L. (A, B) Naïve splenic B cells were isolated and cultured for 16 hours in the presence or absence of control Sf9 cells lacking expression of CD40L (ctrl Sf9 CD40L neg). mRNA (A) and total protein (B) was collected and analyzed for Phb1/2 expression levels relative to actin or GAPDH via qRT-PCR or immunoblot, respectively. Densitometry was performed and measured Phb1/2 band density relative to GAPDH. The data are expressed as the mean Fold Change ± SEM from either three (mRNA) or Fold Change ± SD from 2-3 (protein) independent experiments. Gels are representative of 2-3 independent experiments.
3.3 Phb1 and Phb2 play a role in mediating the effect of CD86 engagement on B cell function.

Although the data thus far showed that Phb1/2 associated with CD86, the functional significance of this association was unclear. Recent studies have reported that Phb1 interacts with various signaling intermediates to modulate cellular processes, and thus, the possibility was supported that by associating with CD86 in primed B cells, Phb1 and Phb2 could be providing CD86 with a cytoplasmic signaling platform to stimulate B cell responses. To test this possibility directly, CH12.LX B cells were transfected with GFP-containing shRNA expression vectors designed to suppress Phb1/2 expression. The effect of transfection on B cell CD86 expression was measured before functional studies were performed. Flow Cytometric analysis showed that CD86 surface expression following CD40L/IL-4 priming was comparable in the absence or presence of Phb1/2 knockdown (see Figure 18). qRT-PCR analysis of FACS-sorted GFP⁺ and GFP⁻ cells showed that both Phb1 and Phb2 expression was reduced by at least 50% in shRNA-expressing GFP⁺ cells (see Figure 19), where reduction of Phb1 resulted in the loss of Phb2 and vice versa (see Figure 20).

We next determined if both Phb1/2 play roles in mediating the CD86-induced increases in Oct-2 and IgG₁ without a change in class switch recombination, as reported previously (113, 118, 119, 122, 123). CD86 engagement on primed GFP⁺ B cells resulted in comparable levels of germline IgG₁, Oct-2, and mature IgG₁ mRNA relative to isotype control-treated B cells, while significant increases in Oct-2 and mature IgG₁ mRNA were observed due to CD86 engagement in GFP⁻ B cells (see Figure 21). To ensure the comparable levels of Oct-2 and mature IgG₁ mRNA were not due to an off target effect
Figure 18. *CD86 expression levels on the surface of GFP⁺-Phb1/2-shRNA transfected CH12.LX B cells.* Phb1 or Phb2 GFP-containing shRNA plasmids were transfected into CH12.LX B cells and primed as described above. Surface expression of CD86 on GFP⁺ and GFP⁻ cell populations were determined by FACS analysis. Isotype ctrl histograms are depicted as (gray lines), and CD86 histograms are depicted as (black lines). Representative histograms are shown from three independent experiments.
Figure 19. Phb1- and Phb2-shRNA effectively suppress Phb1/2 expression levels.

CH12.LX B cells were transfected with either a GFP-containing Phb1- or Phb2- shRNA expression plasmid for 36 hours to silence Phb1 and Phb2 gene expression. At 24 hours post transfection, cells were primed with CD40L/IL-4 for 16 hours. GFP cells containing the Phb1- or Phb2- shRNA plasmids were separated by fluorescence activated cell sorting (FACS). On day 2 or day 6-post cell priming, Phb1/Phb2 mRNA levels were measured via qRT-PCR analysis and normalized to actin. GFP negative cells and scrambled negative control shRNA are depicted as (gray bars) and GFP+ cells containing Phb1/2 shRNA are depicted as (black bars). Data are expressed as mean Fold Change ± SEM relative to either No GFP and represent at least quadruplicate samples/condition from three independent experiments. Statistical differences are shown relative to No GFP. *, p < 0.05.
Figure 20. Phb1 and Phb2 expression in B cells treated with Phb1 and Phb2 shRNA. CH12.LX B cells were transfected with either a GFP-containing Phb1 or Phb2 shRNA plasmids for 36 hours to silence Phb1 and Phb2 gene expression. At 24 hours post transfection, cells were primed with CD40L/IL-4 for 16 hours. GFP cells containing the Phb1 or Phb2 shRNA vector were separated by FACS. Phb1/Phb2 mRNA were measured via qRT-PCR analysis and normalized to actin. Data are expressed as mean ± SEM Fold Change relative to No GFP of quadruplicate samples/condition from 1-3 independent experiments. No GFP cells are depicted as (gray bars) and GFP cells are shown as (black bars). Statistical differences are shown between groups. *, p < 0.05.
Figure 21. Phb1 and Phb2 play a role in mediating the effect of CD86 engagement on B cell function. CH12.LX B cells were transfected with either a GFP-containing Phb1, Phb2, or scrambled, negative control shRNA (neg ctrl shRNA) expression plasmid, for 36 hours, and primed with CD40L/IL-4 after 24 hours for 16 hours. Upon GFP-dependent FACS, the cells were re-cultured with either an anti-CD86 Ab, or a species- and isotype-matched control Ab (iso ctrl). On day 2 or day 6-post cell priming, Oct-2/germline IgG1 and mature IgG1 mRNA, respectively, were measured via qRT-PCR analysis and normalized to actin. GFP- and neg ctrl shRNA transfected cells are depicted as (gray bars) and GFP+ cells containing Phb1/2 shRNA are depicted as (black bars). Data are expressed as mean Fold Change ± SEM relative to iso ctrl of each group and represent at least quadruplicate samples/condition from three independent experiments. Statistical differences are shown relative to iso ctrl of each group. *, p < 0.05.
of the Phb1/2 shRNA plasmids, scrambled negative control shRNA plasmids were transfected into CH12.LX B cells as described. CD86 engagement promoted significant increases in the level of Oct-2 and mature IgG1 mRNA produced (see Figure 21), whereas germline IgG1 mRNA remained unchanged. Together, these findings indicated that Phb1 and Phb2 are both necessary for CD86 engagement on a primed B cell to enhance the level of B cell function.

### 3.4 The cytoplasmic domain of CD86 also plays a role in mediating the effect of CD86 engagement on B cell function.

The evidence thus far suggested that CD86 associated with Phb1/2 through transmembrane-specific interactions and that Phb1/2 were essential to couple CD86 engagement to a downstream functional increase in IgG1. However, it remained unclear whether or not the cytoplasmic domain of CD86 played any role in mediating the CD86-dependent increase in the B cell functional response. It was proposed previously that the cytoplasmic domain of CD86 had the potential to be phosphorylated at one or more of several putative PKC phosphorylation sites (120), which might provide a mechanism for CD86 to recruit and/or activate downstream signaling intermediates that are known to participate in the CD86-induced increases in Oct-2 and IgG1.

To test this possibility, a series of mutations/truncations were introduced into the FLAG-CD86 cytoplasmic domain that included single-alanine point mutations of the three putative PKC serine/threonine phosphorylation sites, as well as either partial or full truncation of the cytoplasmic domain as diagramed in (Figure 22A). CH12.LX B cells were transfected with the FLAG-CD86 plasmids, and cells were primed with CD40L/IL-
4 in culture for 16 hours, at which time either a species- and isotype-matched control Ab or anti-FLAG Ab (M2) was added. Cells were assessed, via qRT-PCR analysis, for levels of Oct-2/germline IgG1 mRNA on day 2 or mature IgG1 mRNA on day 5 (see Figure 22B). The introduction of alanine-point mutations at all PKC phosphorylation sites had no effect on germline IgG1, but significantly reduced Oct-2 and mature IgG1 mRNA levels, except for Oct-2 induction by the T291A and S285A mutants, as compared to WT controls. Partial and full truncation of the CD86 cytoplasmic domain resulted in significant reductions of Oct-2 and mature IgG1 mRNA levels compared to WT controls. Importantly, levels of either WT or mutant FLAG-CD86 expressed on the B cell surface remained comparable (data not shown). These results emphasize that PKC phosphorylation of the cytoplasmic domain of CD86 is important for signaling function.

In addition to the use of CD86-transfected B cells, B cells were also used that had been isolated from mice transgenic for either WT CD86 (Tg WT) that overexpressed CD86 or a truncated form of CD86 (Trunc CD86) that lacked the cytoplasmic domain. In B cells from the Trunc CD86 mice that were primed, Phb1/2 proteins remained associated with CD86 (see Figure 13), but the level of mRNA expressed for the cytoplasmic domain of CD86, Oct-2, and mature IgG1 was decreased in comparison to WT-expressing B cells, while germline IgG1 was comparable (see Figure 23). Thus, to our knowledge, these data collectively provide evidence that, in addition to Phb1/2, the cytoplasmic domain of CD86 is also required to mediate CD86 signaling to regulate B cell function, likely via phosphorylation of the cytoplasmic domain at serine and/or threonine residues, as the present site-directed mutagenesis data show.
Figure 22. The cytoplasmic domain of CD86 also plays a role in mediating the effect of CD86 engagement on B cell function. (A), A series of single-alanine point mutations (S285A, T291A, S303A) or cytoplasmic truncations (KKPΔ and 282Δ) were introduced into a FLAG-CD86 expression plasmid. (B), Either WT FLAG-CD86 or mutant/truncated plasmids were transfected into CH12.LX B cells before the cells were primed with CD40L/IL-4. Cells were cultured for 16 hours before an anti-FLAG Ab (M2) or species- and isotype-matched control Ab (mouse IgG1 iso ctrl) was added to the culture. 24 hours and 4 days later, respectively, the level of germline IgG1/Oct-2 mRNA and mature IgG1 mRNA was determined by qRT-PCR. Values were normalized to actin and data are expressed as mean Fold Change ± SEM relative to WT mouse IgG1 iso ctrl and represent data of quadruplicate replicates/condition from three independent experiments. Statistical analysis was used to determine statistical significance relative to WT anti-FLAG. *, p < 0.05.
(Figure 22, continued)
Figure 23. Oct-2 and mature IgG₁ expression is regulated via the cytoplasmic domain of CD86. (A) WT-transgenic B cells (WT Tg) or Truncated CD86 (Trunc CD86) B cells were primed with CD40L/IL-4 for 16 hours followed by the addition of CD28/Ig to engage CD86. At 16 hours, levels of cytoplasmic CD86 mRNA were measured by qRT-PCR analysis between WT Tg CD86 and Trunc CD86 groups. Data are expressed as mean Fold Change ± SEM relative to WT Tg resting and are pooled from two independent experiments. (B, C) On day 2 post cell priming, levels of Oct-2 and germline IgG₁ mRNA were quantified by qRT-PCR between WT Tg CD86 and Trunc CD86. Data are expressed as mean Fold Change ± SEM relative to WT Tg CD40L/IL-4 and are pooled from two independent experiments. (D) On day 5 post cell priming, levels of mature IgG₁ mRNA were measured by qRT-PCR analysis between WT Tg CD86 and Trunc CD86. Data are expressed as mean Fold Change ± SEM relative to WT Tg CD40L/IL-4 and represent 1-2 independent experiments. Statistical analysis was performed to determine significant differences among groups (A), or compared to WT Tg CD40L/IL-4 (B to D) *, p < 0.05.
3.5 The cytoplasmic domain of CD86 is required for an optimal IgG₁ response against a T-dependent Ag in vivo.

The need for the cytoplasmic domain of CD86 to mediate a change in IgG₁ production was also tested in vivo. WT CD4⁺ T cells and B cells obtained from WT or Trunc CD86 mice were adoptively transferred into Rag2-deficient or CD86-deficient recipient mice. Both sets of mice were immunized with OVA-alum on day 7, and and re-immunized 28 days post cell transfer. Serum IgG₁ levels were assayed at one-week intervals after primary and secondary immunization. Mice receiving Trunc CD86 B cells were found to produce a consistently lower level of serum IgG₁ at all time points when compared with those mice receiving WT B cells (see Figure 24A and Figure 25A). The phenotype and number of T and B cells at the last time point were determined by Flow Cytometry. Although the total number of splenocytes increased in both groups of mice that received Trunc CD86 B cells compared to mice that received WT B cells, the B/T ratio remained the same, as did the level of CD86 expressed on the B cell surface (see Figure 24B and Figure 25B). However, the number of CD4⁺ T cells and B220⁺ B cells increased in both groups of mice that received the Trunc CD86 B cells when compared to mice that received WT B cells. To test if either the WT or Trunc CD86 molecules differentially affected the level of T cell activation in vivo, total splenocytes were removed from immunized mice and re-activated ex vivo in the presence of PMA and
Figure 24. The cytoplasmic domain of CD86 is required for an optimal IgG1 response against a T-dependent Ag in a Rag2−/− model in vivo. (A, B) WT CD4+ T cells and B cells from either WT or Truncated (Trunc) CD86 mice were adoptively transferred into either Rag-2 deficient mice (n = 5 animals/group). OVA-alum was administered i.p. one and four weeks post cell transfer. (A) Levels of serum IgG1 were analyzed via ELISA. Data are expressed as mean ± SEM. (B) On day 42, total splenocytes were counted by trypan blue exclusion and were analyzed via Flow Cytometry for the number of CD4+ T cells, B220+ B cells, the level of CD86 expressed on the B cell surface, and the % and # of IL-4+ CD4+ T cells. Each data point represents an individual animal and the horizontal bar represents the mean. BLD, below level of detection. Statistical differences are shown between mice that received either WT or Trunc CD86 B cells. *, p < 0.05.
Figure 25. The cytoplasmic domain of CD86 is required for an optimal IgG1 response against a T-dependent Ag in a CD86\(^{-/-}\) model in vivo. (A, B) B cells from either WT or Truncated (Trunc) CD86 mice were adoptively transferred into CD86-deficient mice (n = 4 animals/group). OVA-alum was administered i.p. one and four weeks post cell transfer. (A) Levels of serum IgG1 were analyzed via ELISA. Data are expressed as mean ± SEM. (B) On day 42, total splenocytes were counted by trypan blue exclusion and were analyzed via FACS analysis for the number of CD4\(^{+}\) T cells, B220\(^{+}\) B cells, the level of CD86 expressed on the B cell surface, and the % and # of IL-4\(^{+}\) CD4\(^{+}\) T cells. Each data point represents an individual animal and the horizontal bar represents the mean. Statistical differences are shown between mice that received either WT or Trunc CD86 B cells. *, \(p < 0.05\).
(Figure 25, continued)
ionomycin, followed by FACS analysis for the number of CD4^+ T cells expressing IL-4. The percentages of CD4^+ T cells that expressed IL-4 were comparable in the spleen in both in vivo model systems, (see Figure 24B and Figure 25B), although CD86^-/- mice that received Trunc CD86 B cells had slightly lower levels compared to mice that received WT B cells (see Figure 25B). In contrast, total splenocytes, total numbers of CD4^+ T cells, and total number of B220^+ B cells were found to be greater in mice reconstituted with Trunc CD86 B cells (see Figure 24B and Figure 25B) even though there was an equal percentage of B220^+ B cells and CD4^+ T cells (data not shown). This suggested a potential loss of signal to suppress T and B cell expansion due to the absence of B7-1 (CD80) on the surface of Trunc CD86 B cells, or a potential mechanism to compensate for the loss of signal delivered through CD86 cytoplasmic domain on a B cell. In addition, the number of CD4^+ T cells that expressed IL-4 was significantly greater in T cells from mice that received Trunc CD86 B cells, as opposed to WT B cells, in the Rag2-deficient model (see Figure 24B). In contrast, the number of CD4^+ T cells that expressed IL-4 was comparable in CD86-deficient mice that received either WT or Trunc CD86 B cells (see Figure 25B). This difference may be due to either enhanced function of CD86 expressed on macrophages and DCs in Rag2-deficient Trunc CD86 recipient mice to compensate for the lack of the CD86 cytoplasmic domain, or a defect in circulating immune cells. These findings suggested that CD86 reverse signaling required the intact cytoplasmic domain in order to play a role in increasing the level of IgG1 produced by a primed B cell. We conclude that the cytoplasmic domain of CD86 is critical for CD86 to induce an increase in the level of IgG1 produced by primed B cells.
3.6 Phb1 and Phb2 are necessary for the CD86-induced activation of PLC\(\gamma\)2.

Although findings thus far suggested that both Phb1/2 and the CD86 cytoplasmic domain are required to mediate CD86 signaling to regulate a B cell functional response, the proximal molecular mechanism was unclear. One of the most proximal CD86 signaling intermediates to date, PLC\(\gamma\)2 (123), is classically recruited to phosphorylated tyrosine-containing protein/protein complexes (135, 136). In addition, it was reported that Phb1 binds the protein tyrosine kinase (PTK) spleen tyrosine kinase (Syk) upon BCR engagement in murine B cells (150), indicating that Phb1 may be involved with the activation of PTKs specifically in B cells, and perhaps other membrane-associated signaling intermediates after receptor engagement. Furthermore, since Phb1/2 residues were reported to be phosphorylated, including tyrosines (151-154), it was possible that Phb1/2 became phosphorylated specifically on tyrosine residues and may serve the mechanistic link between CD86 engagement and PLC\(\gamma\)2 activation. To address this, CH12.LX B cells were primed with CD40L/IL-4 followed by CD86 engagement. The level of non-specific tyrosine phosphorylation (pY100) was determined via immunoblot (see Figure 26). CD86 engagement promoted an increase in the level of tyrosine phosphorylation at protein bands possessing molecular weights of 37 and 32 kDa, which corresponded to the molecular weights of Phb1/2, relative to priming alone. These findings suggested that CD86 engagement promotes Phb1/2 tyrosine phosphorylation, which may serve as functional CD86-induced signaling intermediate.

To determine if Phb1/2 were required to mediate the CD86-induce activation of PLC\(\gamma\)2, CH12.LX B cells were transfected with scrambled negative control or Phb1/2-
Figure 26. CD86 engagement induces Phb1 and Phb2 tyrosine phosphorylation.

CH12.LX B cells were primed with CD40L/IL-4 overnight followed by the addition of an anti-CD86 Ab for 5 minutes. The level of non-specific tyrosine phosphorylation of protein bands of molecular weights 37 and 32 kDa (Phb2 and Phb1) was determined via immunoblot analysis relative to Phb2 and Phb1 total protein levels. Densitometry was performed and measured phospho-tyrosine (pY) protein bands at the exact molecular weights of Phb1/2 (films were overlaid prior to scanning) relative to Phb1/2 band intensities. The data are presented as the mean Fold Change in pY from primed B cells where CD86 was engaged relative to priming alone and expressed as the mean Fold Change ± SEM from three independent experiments. Statistical differences are shown relative to priming alone. *, p < 0.05.
specific shRNA plasmids, and either a WT, or cytoplasmic-deficient FLAG-CD86 expression plasmid (KKPΔ) followed by priming with CD40L/IL-4 and CD86 or FLAG-CD86 engagement. Phb1/2 protein knockdown efficiency and the level of PLCγ2 phosphorylation was measured by immunoblot. The presence of Phb1/2-specific shRNA resulted in modest reductions of Phb1/2 protein where reduction of Phb1 resulted in the loss of Phb2 (see Figure 27), and vice versa. Importantly, levels of Phb1/2 among Mock transfected cells vs. cells transfected with scrambled negative control shRNA were comparable (see Figure 27). While Mock and scrambled negative control shRNA transfected CH12.LX B cells promoted a time-dependent CD86-induced phosphorylation of PLCγ2 relative to primed cells alone (see Figure 28A), PLCγ2 phosphorylation upon CD86 engagement in the presence of Phb1/2 shRNA remained comparable to priming alone (see Figures 28B, 28C). Engagement of FLAG-CD86 on B cells transfected with both WT and KKPΔ FLAG-CD86 expression plasmids caused an increase in PLCγ2 phosphorylation relative to a species- and isotype-matched control Ab (see Figure 29). To extend our immunoblot findings, the level of PLCγ2 phosphorylation was measured via In-Cell ELISA. While Mock and scrambled negative control shRNA transfected CH12.LX B cells allowed for a CD86-induced increase in PLCγ2 phosphorylation relative to priming alone, PLCγ2 phosphorylation in the presence of Phb1/2 shRNA remained comparable to priming alone (see Figure 30). Similar to our immunoblot findings, engagement of FLAG-CD86 on B cells transfected with both WT and KKPΔ FLAG-CD86 expression plasmids caused an increase in PLCγ2 phosphorylation relative to a species- and isotype-matched control Ab (see Figure 31). Together, these findings
**Figure 27.** *Phb1 and Phb2 protein expression levels are depleted in the presence of Phb1/2-specific shRNA.* CH12.LX B cells were either Mock transfected, with scrambled negative control, or Phb1/2-specific shRNA plasmids via nucleofection for 24 hours followed by priming with CD40L/IL-4 for 16 hours. Phb1/2 protein levels were measured via immunoblot relative to GAPDH. Densitometry was performed and the data are expressed as the mean % Change ± SEM relative to Mock and are representative of three independent experiments. Statistical differences are shown relative to Mock. *, *p* < 0.05.
Figure 28. Phb1- and Phb2-shRNA depletion prevents the CD86-induced PLCγ2 activation as shown via immunoblot. (A) CH12.LX B cells were either Mock transfected or with scrambled negative control shRNA (neg ctrl shRNA) via nucleofection for 24 hours followed by priming with CD40L/IL-4 for 16 hours. A CD86 Ab (anti-CD86) was administered for 5, 15, 30, and 60 minutes. Levels of phospho-PLCγ2 (pPLCγ2) protein relative to total PLCγ2 were measured via immunoblot. (B and C) Phb1 or Phb2 shRNA plasmids were transfected into CH12.LX B cells followed by CD40L/IL-4 priming, and a CD86 Ab (anti-CD86) was added as described above. Levels of pPLCγ2 protein were measured relative to total PLCγ2 via immunoblot. Representative gels (A to C) are shown from three independent experiments. Densitometry was performed and measured pPLCγ2 relative to PLCγ2 band intensity and the data are presented as the mean Fold Change in pPLCγ2 from primed B cells where CD86 was engaged relative to priming alone (A to C) and expressed as the mean Fold Change ± SEM from three independent experiments. Statistical differences are shown relative to priming alone (A to C). *, p < 0.05.
Figure 29. The CD86 cytoplasmic domain is not involved with the CD86-induced PLCγ2 activation as shown via immunoblot. WT, and CD86 cytoplasmic-deficient (KKPΔ) FLAG-CD86 plasmids were transfected into CH12.LX B cells via nucleofection and primed with CD40L/IL-4 for 16 hours. Either an anti-FLAG Ab, or species- and isotype-matched control Ab (iso ctrl) was added for 15 minutes. Levels of pPLCγ2 protein were measured relative to total PLCγ2 via immunoblot. Representative gels are shown from three independent experiments. Densitometry was performed and measured pPLCγ2 relative to PLCγ2 band intensity and the data are presented as the mean Fold Change in pPLCγ2 from primed B cells where FLAG-CD86 was engaged relative to iso ctrl Ab and expressed as the mean Fold Change ± SEM from three independent experiments. Statistical differences are shown relative to iso ctrl. *, p < 0.05.
Figure 30. Phb1- and Phb2-shRNA depletion prevents the CD86-induced PLCγ2 activation as shown via In-Cell ELISA. CH12.LX B cells were either Mock transfected or with scrambled negative control shRNA (neg ctrl shRNA) via nucleofection for 24 hours followed by priming with CD40L/IL-4 for 16 hours. A CD86 Ab (anti-CD86) was administered for 30 minutes. Levels of phospho-PLCγ2 (pPLCγ2) protein relative to total PLCγ2 were measured via In-Cell ELISA. pPLCγ2 protein OD values were normalized to total pPLCγ2 protein OD values and the data are represented as a mean Fold Change in pPLCγ2 protein from CD86-engaged B cells relative to primed alone B cells of each group and are expressed as the mean ± SD of quintuplicate samples/condition from at least two independent experiments. Statistical differences are shown relative to priming alone of each group. *, p < 0.05.
Figure 31. The CD86 cytoplasmic domain is not involved with the CD86-induced PLCγ2 activation as shown via In-Cell ELISA. WT, and CD86 cytoplasmic-deficient (KKPΔ) FLAG-CD86 plasmids were transfected into CH12.LX B cells via nucleofection and primed with CD40L/IL-4 for 16 hours. Either an anti-FLAG Ab, or species- and isotype-matched control Ab (iso ctrl) was added for 15 minutes. Levels of pPLCγ2 protein were measured relative to total PLCγ2 via In-Cell ELISA. pPLCγ2 protein OD values were normalized to total pPLCγ2 protein OD values and the data are represented as a mean Fold Change in pPLCγ2 protein from FLAG-CD86 engaged B cells relative to primed iso ctrl and are expressed as the mean ± SD of quintuplicate samples/condition from at least two independent experiments. Statistical differences are shown relative to iso ctrl. *, p < 0.05.
suggested that while Phb1/2 are critical for the CD86-induced activation of PLCγ2, the CD86 cytoplasmic domain is not involved, indicating that Phb1/2 and the CD86 cytoplasmic domain must differentially activate signaling intermediates upon CD86 engagement.

3.7 Phb1 and Phb2 are necessary for the CD86-induced activation of PKCα/βII.

Although our evidence suggested that Phb1/2 are necessary for the CD86-induced activation of PLCγ2 independent of the CD86 cytoplasmic domain, whether or not Phb1/2 were essential for the CD86-dependent downstream activity of PLCγ2 remained unknown. Since PLC classically allows for the activation of PKC via cleavage of phosphoinositides into inositol-3 phosphate and diacylglycerol, leading to an increase in Ca²⁺ (155), and our previous findings showed that CD86 engagement on a CD40L/IL-4-primed B cell promoted the activation of PLCγ2 and the downstream activation of PKCα/βII (123), we considered PKCα/βII phosphorylation a viable measurement of PLCγ2 activity. We predicted that Phb1/2 alone would be necessary to induce CD86-dependent PKCα/βII phosphorylation independent of the CD86 cytoplasmic domain since Phb1/2 alone were sufficient to allow for CD86-induced PLCγ2 activation. To address this, CH12.LX B cells were either Mock transfected or transfected with scrambled negative control shRNA, or with Phb1/2-specific shRNA plasmids followed by priming with CD40L/IL-4 and the addition of a CD86 Ab over a 60 minute time course. The levels of PKCα/βII phosphorylation (pPKCα/βII) relative to total PKCα and total PKCβII protein were measured via immunoblot. While CH12.LX B cells that were Mock and scrambled negative control shRNA transfected produced a time-dependent
Figure 32. *Phb1* and *Phb2*-shRNA depletion prevents the CD86-induced PKCα/βII activation. CH12.LX B cells were either Mock transfected, with a scrambled negative control shRNA (neg ctrl shRNA), or with Phb1 or Phb2 shRNA plasmids into CH12.LX B cells via nucleofection for 24 hours followed by priming with CD40L/IL-4 for 16 hours. A CD86 Ab (anti-CD86) was administered for 5, 15, 30, and 60 minutes. Levels of phospho-PKCα/βII (pPKCα/βII) protein relative to total PKCα and PKCβII were measured via immunoblot. Levels of pPKCα/βII protein relative to total PKCα and PKCβII were measured via immunoblot. Representative gels are shown from three independent experiments. Densitometry was performed and measured pPKCα/βII relative to PKCα and PKCβII band intensity and the data are presented as the mean Fold Change in pPKCα/βII from primed B cells where CD86 was engaged relative to priming alone and expressed as the mean Fold Change ± SEM from three independent experiments. Statistical differences are shown relative to Mock priming alone. *, p < 0.05.
Figure 33. The CD86 cytoplasmic domain is not involved with the CD86-induced PKCα/βII activation. WT, and CD86 cytoplasmic-deficient (KKPΔ) FLAG-CD86 plasmids were transfected into CH12.LX B cells via nucleofection and primed with CD40L/IL-4 for 16 hours. Either an anti-FLAG Ab, or species- and isotype-matched control Ab (iso ctrl) was added for 30 minutes. Levels of pPKCα/βII protein relative to total PKCα and PKCβII were measured via immunoblot. Representative gels are shown from three independent experiments. Densitometry was performed and measured pPKCα/βII relative to PKCα and PKCβII band intensity and the data are presented as the mean Fold Change in pPKCα/βII from primed B cells where FLAG-CD86 was engaged relative to iso ctrl and expressed as the mean Fold Change ± SEM from three independent experiments. Statistical differences are shown relative to iso ctrl. *, p < 0.05.
CD86-induced PKCα/βII phosphorylation relative to priming alone, the effect was prevented in the presence of Phb1/2 specific shRNA relative to priming alone (see Figure 32). Engagement of FLAG-CD86 on B cells transfected with both WT and KKPΔ FLAG-CD86 expression plasmids caused an increase in PKCα/βII phosphorylation relative to a species- and isotype-matched control Ab (see Figure 33). Collectively, these findings indicate that while Phb1/2 are necessary for CD86-dependent activation of PKCα/βII, a downstream signaling intermediate promoted through PLCγ2 activity, the CD86 cytoplasmic domain is not involved, providing further support that CD86 engagement allows for differential activation of distal signaling intermediates.

3.8 Phb1/2 and the CD86 cytoplasmic domain are each required for the CD86-dependent activation of NF-κB.

Although our findings suggested that Phb1/2 alone is sufficient to promote CD86-dependent PLCγ2 and PKCα/βII activation, the molecular role of the CD86 cytoplasmic domain remained unknown. Previous reports demonstrated that engagement of CD86 on the surface of a CD40L/IL-4-primed B cell initiates two signaling cascades that converge to activate the p50/p65 subunits of NF-κB (118, 123). While one cascade is essential for CD86-dependent IkBα phosphorylation (pIkBα), the other promotes the phosphorylation of p65 (p-p65) in a PKCα/βII- and PLCγ2-dependent manner (118, 123). To determine which CD86-induced signaling intermediate controlled NF-κB activation, i.e., IkBα and p65 phosphorylation, CH12.LX B cells were either Mock transfected or transfected with scrambled negative control, or Phb1/2-specific shRNA plasmids, and either WT-FLAG CD86 or CD86 cytoplasmic domain-deficient, (KKPΔ) expression plasmids followed by
CD40L/IL-4-priming and engagement of either CD86 or FLAG-CD86 over a 60-90 minute time course. The level of IκBα and p65 phosphorylation, and total levels of IκBα protein was determined via immunoblot. We observed a time dependent increase in the level of phosphorylated IκBα and time-dependent decrease in the level of total IκBα protein upon engagement of CD86 when both Phb1/2 and the CD86 cytoplasmic domain were intact; whereas, Phb1/2 depletion and the lack of the CD86 cytoplasmic domain prevented a CD86-dependent increase in the level of IκBα phosphorylation and decrease in the level of total IκBα protein (see Figures 34 and 35). Furthermore, the CD86-induced increase in p65 phosphorylation was prevented when Phb1/2 was depleted (see Figure 34), and when CD86 lacked the cytoplasmic domain (see Figure 35), relative to either priming alone or isotype control Ab, respectively. However, we speculated that the signaling intermediate Phb1/2, which was essential for PLCγ2 and PKCα/βII activation, may also prevent CD86-induced p65 phosphorylation since previous findings showed that PKCα/βII-dependent p65 phosphorylation occurred distal to IκBα phosphorylation (118). Together, these findings indicated that both Phb1/2 and the CD86 cytoplasmic domain are necessary for the CD86-dependent activation of NF-κB via IκBα phosphorylation/degradation, followed by p65 phosphorylation.
Figure 34. Phb1 and Phb2 are necessary for the CD86-induced activation of NF-κB.

CH12.LX B cells were Mock transfected or transfected with either scrambled negative control shRNA, or with Phb1, or Phb2-specific shRNA plasmids via nucleofection for 24 hours followed by priming with CD40L/IL-4 for 16 hours. The cells were then resuspended in serum-free conditions for at least 30 minutes. A CD86 Ab (anti-CD86) was added to cell cultures for 5, 15, 30, 60, and 90 minutes. Levels of phospho-IκBα
(pIκBα), total IκBα, and phospho-p65 (p-p65) protein were measured relative to GAPDH or total p65 via immunoblot. Representative gels are shown from three independent experiments. Densitometry was performed and measured pIκBα, total IκBα, and p-p65 band intensity relative to GAPDH, and p-p65 band density relative to total p65 and the data are presented as the mean Fold Change in pIκBα, total IκBα, or p-p65 from primed B cells where CD86 was engaged relative to priming alone and expressed as the mean Fold Change ± SEM from at least three independent experiments. Statistical differences are shown relative to priming alone. *, p < 0.05.
Figure 35. The CD86 cytoplasmic domain is necessary for the CD86-induced activation of NF-κB. WT, or CD86-cytoplasmic deficient (KKPΔ) FLAG-CD86 plasmids were transfected into CH12.LX B cells via nucleofection and primed with CD40L/IL-4 for 16 hours. An anti-FLAG Ab was added for 5, 15, 30, and 60 minutes relative to a species- and isotype-matched control Ab (iso ctrl). Levels of pIkBa, total IkBa, and p-p65 protein were measured relative to GAPDH or total p65 via immunoblot. Representative gels are shown from three independent experiments. Densitometry was performed and measured.
pIκBα, total IκBα, and p-p65 band intensity relative to GAPDH, and p-p65 band density relative to total p65 and the data are presented as the mean Fold Change in pIκBα, total IκBα, or p-p65 from primed B cells where FLAG-CD86 was engaged relative to iso ctrl and expressed as the mean Fold Change ± SEM from at least three independent experiments. Statistical differences are shown relative to iso ctrl. *, p < 0.05.
3.9 Phb1/2 and the CD86 cytoplasmic domain are each required for the CD86-dependent nuclear localization of NF-κB (p65).

Although our findings suggested that both Phb1/2 and the CD86 cytoplasmic domain were required for the CD86-induced activation of NF-κB, what remained unclear was whether or not the intermediates were necessary for NF-κB (p65) nuclear entry promoted via CD86 engagement. Since previous findings from our laboratory showed that CD86 engagement promoted an increase in the level of p65 phosphorylation present in the nucleus (118), and findings in the present study indicated that both Phb1/2 and the CD86 cytoplasmic domain were required to promote CD86-dependent NF-κB activation, it was likely that both Phb1/2 and the CD86 cytoplasmic domain were required to promote CD86-dependent p65 nuclear localization. To address this, CH12.LX B cells were either Mock transfected or with scrambled negative control shRNA and Phb1/2-specific shRNA plasmids to deplete Phb1/2, and either WT-FLAG CD86 or CD86 cytoplasmic domain-deficient, (KKPΔ) plasmids followed by CD40L/IL-4-priming and either CD86 or FLAG-CD86 engagement for 90 minutes. Nuclear enriched protein lysates were prepared and levels of p65 phosphorylation and total p65 protein levels relative to nuclear membrane proteins Lamin A/C was measured via immunoblot. While phospho- and total p65 nuclear localization increased upon CD86 engagement when both Phb1/2 and the CD86 cytoplasmic were intact, the effect failed to occur upon CD86 engagement in the absence of the intermediates (see Figures 36 and 37). Furthermore, successful Cytoplasmic and Nuclear-enriched protein lysate fractionation was demonstrated via the presence of α-tubulin, β-Actin, and GAPDH in Cytoplasmic-
Figure 36. *Phb1* and *Phb2* are required for the CD86-dependent nuclear localization of *NFκB* (p65). CH.12LX B cells were transfected via either under Mock conditions, or with scrambled negative control shRNA, or Phb1/2-specific shRNA plasmids via nucleofection for 24 hours followed by CD40L/IL-4-priming for an additional 16 hours. The cells were then resuspended in serum-free conditions for at least 30 minutes. A CD86 Ab (anti-CD86) was added to cell cultures for 90 minutes. Nuclear-enriched protein lysates were prepared and the level of p65 phosphorylation (p-p65) and total levels of p65 protein present in the nucleus were measured via immunoblot relative to Lamin A/C. Gels are representative of three independent experiments. Densitometry was
performed and measured p-p65 and total p65 band intensity present in the nucleus relative to Lamin A/C and the data are presented as the mean Fold Change in p-p65 or total p65 from primed B cells where CD86 was engaged relative to priming alone and expressed as the mean Fold Change ± SEM from three independent experiments. Statistical differences are shown relative to priming alone. *, \( p < 0.05 \).
**Figure 37.** The CD86 cytoplasmic domain is required for the CD86-dependent nuclear localization of NF-κB (p65). WT, or CD86-cytoplasmic deficient (KKPD) FLAG-CD86 plasmids were transfected into CH12.LX B cells via nucleofection and primed with CD40L/IL-4 for 16 hours. An anti-FLAG Ab was added for 90 minutes relative to a species- and isotype-matched control Ab (iso ctrl). The level of p65 phosphorylation and total levels of p65 present in the nucleus were determined via immunoblot. Gels are representative of three independent experiments. Densitometry was performed and measured p-p65 and total p65 band intensity present in the nucleus relative to Lamin A/C...
and the data are presented as the mean Fold Change in p-p65 or total p65 from primed B cells where FLAG-CD86 was engaged relative to iso ctrl and expressed as the mean Fold Change ± SEM from three independent experiments. Statistical differences are shown relative to iso ctrl. *, p < 0.05.
enriched protein lysates, and the presence of Lamin A/C in Nuclear-enriched protein lysates (see Figure 38). Thus, our findings indicated that Phb1/2 and the CD86 cytoplasmic domain were necessary to promote the CD86-dependent nuclear localization of p65, and, therefore, extended the finding that both Phb1/2 and the CD86 cytoplasmic domain are each required for the CD86-dependent NF-κB activation.
**Figure 38.** *Cytoplasmic- and Nuclear-enriched protein lysate fractionation in CH12.LX B cells.* In order to demonstrate sufficient Cytoplasmic- and Nuclear-enriched protein fractionation, CH12.LX B cells were treated with 0.5% NP-40-based lysis buffer, followed by treatment with 1% Triton-X100-based lysis buffer as described in *Materials and Methods*. Either Cytoplasmic (C)- or Nuclear (N)-enriched protein lysates were analyzed for the presence of Cytoplasmic-specific proteins including α-tubulin, β-Actin, and GAPDH, or Nuclear-specific proteins Lamin A and Lamin C. Gels are representative of three independent experiments.
CHAPTER 4

DISCUSSION

4.1 Summary of Results.

Although CD86 was originally thought to act only as a ligand, emerging findings from studies in B cells, DCs, and in macrophages have suggested that, upon engagement, CD86 can act as a receptor and signal directly intracellularly. Previous reports in the B cell literature have shown that CD86 engagement on a primed B cell signals directly in vitro and in vivo, to control the level of antibody produced (113, 117-119, 121-124), to increase the level anti-apoptotic factors (124), and to activate the pro-survival factor, NF-κB (118,123). In addition, CD86 engagement on the surface of a DC enhances the activation of p38 MAPK and NF-κB, leading to an increase in the level of IL-6 and indoleamine 2,3-dioxygenase (IDO) produced (156), as well as the induction of IFN-α and IDO, in a STAT1-dependent manner on CD19+ DCs (157). Furthermore, CD86 ligation on the surface of macrophages increases the level of TNF-α and reactive oxygen species (ROS) produced via an NF-κB-dependent mechanism (158). Collectively, these findings from three different cell types indicates that CD86 possesses the ability to signal.
directly and strongly supports the proposal of CD86 acting as a receptor, in addition to its well-defined role as a ligand.

Previous findings from our laboratory have shown that CD86 engagement on the surface of a CD40L/IL-4-primed B cell increases the level of IgG1 antibody produced, through a mechanism that involves an increase in the rate of transcription (122), without an effect on the level of class-switch recombination (113, 118, 119, 122, 123). In addition, we have shown that the level of IgG1 produced is controlled via the activation of two proximal signaling networks that induce NF-κB activation upon CD86 engagement (118, 119). However, what remained less well understood was the signaling mechanism proximal to one of the earliest CD86-dependent signaling intermediates, PLCγ2. Thus, the goal of the present study was to define a membrane-proximal CD86-induced signaling mechanism proximal to the activation of PLCγ2. Since PLCγ2 was reported to be recruited to phosphorylated tyrosine residues (135, 136), of which none are present within the cytoplasmic domain of CD86, it was hypothesized that the CD86 cytoplasmic domain associated directly with a protein/protein complex capable of undergoing tyrosine phosphorylation to activate PLCγ2 and was required for the CD86-dependent increase of IgG1 production by a B cell.

In the current study, we show for the very first time that expression of the tyrosine-containing adaptor/scaffolding proteins Phb1 and Phb2 increases in a CD40L/IL-4-primed B cell, and associates with CD86. Depletion of either Phb protein proteins renders CD86 unable to mediate an enhancement in the level of Oct-2 and IgG1 produced. We also show for the first time that Phb1/2 and the CD86 cytoplasmic domain is required for the CD86-induced phosphorylation of IκBα, which we previously reported
leads to NF-κB p50/p65 activation (118); whereas, only Phb1/2 is required for the CD86-induced phosphorylation of PLCγ2, which we previously reported leads to PKCα/βII activation and NF-κB (p65) phosphorylation (123). In addition, we show that the CD86 cytoplasmic domain in vivo is required for an optimal IgG1 response against a T-dependent antigen. Thus, Phb1/2 association with CD86 and expression of an intact CD86 cytoplasmic domain are each necessary to mediate CD86 signaling function to enhance the level of the Oct-2/IgG1 response in a primed B cell through the differential activation of distal signaling intermediates.

4.2 Phb1 and Phb2 as CD86-induced signaling intermediates

Phb2, and its closely related homolog prohibitin-1 (Phb1), are evolutionarily-conserved, multi-functional proteins that are expressed in most cell types and typically reside in a variety of cellular compartments, including the inner mitochondrial and plasma membranes (140). Both Phbs have previously been found to be associated with the BCR (159), and were recently found to co-localize with CD3 of the TCR complex (160). Phb2 is also known as either Bap37 (B cell receptor-associated protein 37-kDa) or REA (nuclear repressor of estrogen receptor activity), while Phb1 is also referred to as Bap32 (B cell receptor-associated protein 32-kDa). Phbs are a family of proteins that possess various cellular and molecular functions (140, 161, 162) that have been found to function independently of each other (140, 161), except within the context of the mitochondrial membrane, where they seem to function together (149, 163-165), and when they associate with the BCR and CD3 of the TCR complex, where their function remains less well-understood. Taken together with findings that Phb1/2 residues can be
phosphorylated, including tyrosines (151-154), we considered Phb1/2 as viable candidates to mediate CD86 signaling function.

4.3 Differential activation of CD86-dependent distal signaling intermediates mediated via Phb1/2 and the CD86 cytoplasmic domain

Previous reports demonstrated that NF-κB (p65) phosphorylation is regulated via PKCα/βII, and that PKCα/βII activation is PLCγ2-dependent upon CD86 engagement on a B cell (118, 123). Data in the present study showed that the CD86-induced PLCγ2 phosphorylation failed to occur when Phb1/2 was reduced, but not when the CD86 cytoplasmic tail was deleted. On the other hand, CD86-induced IκBα phosphorylation failed to occur when either Phb1/2 was reduced or the CD86 cytoplasmic domain was deleted, indicating that both were required for NF-κB activation. The present evidence indicated that both Phb1/2 and the cytoplasmic domain of CD86 on a B cell, although not directly associated, appear to each be necessary to elicit CD86-induced back signaling to enhance the level of Oct-2 and IgG1 responses. Previous reports showed in B cells that engagement of cell surface receptors that lack cytoplasmic tyrosine residues, including the BCR (166, 167) and CD40 (168, 169) used adaptor protein complexes to signal intracellularly to activate NF-κB. Since the CD86 cytoplasmic domain is devoid of tyrosine residues, and since our evidence suggested that a novel association exists between CD86 and Phb1/2, the latter of which does express tyrosine residues, we initially thought is was possible that Phb1/2 alone might mediate CD86-dependent IκBα phosphorylation, independently of the CD86 cytoplasmic domain. However, data in the present study show that both an intact CD86 cytoplasmic domain and Phb1/2 are required
to activate IκBα upon CD86 engagement, suggesting that cross talk may exist between CD86-associated Phb1/2 and the cytoplasmic domain for IκBα activation. Data also show that all alanine substitutions of the PKC phosphorylation sites within the CD86 cytoplasmic domain impaired CD86 function, with S303A, causing the most significant reduction of CD86-induced Oct-2 and mature IgG1 mRNA, suggesting that phosphorylation of the CD86 cytoplasmic domain may serve as a potential docking site(s) for an unknown common signaling intermediate which may also interact with Phb1/2 to regulate phosphorylation of IκBα. Although the mechanistic link between Phb1/2 and the CD86 cytoplasmic domain is unclear at present, each appears to be required to promote CD86-dependent phosphorylation of IκBα and the activation of NF-κB p50/p65.

Based on the findings of the current study and our previous reports, we propose the following CD86 signaling model (see Figure 39): Upon CD86 engagement, Phb1/2 and the CD86 cytoplasmic domain promote the phosphorylation of IκBα, allowing for the activation of NF-κB p50/p65. Concurrently, Phb1/2 alone promote CD86-induced PLCγ2 phosphorylation, allowing for subsequent PKCα/βII activation and phosphorylation of p65. NF-κB p50/p65 then initiates Oct-2 transcription, which ultimately regulates 3’IgH-enhancer activity to elevate the rate of IgG1 transcription and the level of IgG1 produced by a B cell.
Figure 39. **CD86 Signaling Model.** (Dotted arrows indicate proposed novel signaling events identified in the current study). CD86 engagement promotes the phosphorylation of IκBα via mechanisms regulated by Phb1/2 and the CD86 cytoplasmic domain allowing for the activation of NF-κB p50/p65. Concurrently, Phb1/2 alone promotes the CD86-induced PLCγ2 phosphorylation, allowing for subsequent PKCα/βII activation and phosphorylation of p65. NF-κB p50/p65 is now poised to initiate Oct-2 transcription, which ultimately regulates 3'IgH-enhancer activity to increase the rate of IgG₁ transcription and the level of IgG₁ produced by a B cell.
4.4 Phb1/2 as a signaling intermediate between CD86 engagement and PLCγ2 activation

Previous reports showed that both Phb1 and Phb2 become tyrosine phosphorylated within their C-terminal domains upon engagement of cell surface receptors such as the TCR and CD28 (151), while Phb1 alone was shown to undergo tyrosine phosphorylation upon insulin receptor engagement (152-154, 170). Recently, it was found that Phb1 and Phb2 are linked to the MAP kinase signaling cascade upon CD3 engagement in T cells (160). Therefore, it was possible that Phb1/2 may serve as critical intermediates for the CD86-induced activation of PLCγ2. We also proposed that the CD86 cytoplasmic domain may not be involved with the CD86-induction of PLCγ2 due to the lack of tyrosine residues within this domain that would be necessary for PLCγ2 binding. Since prior reports revealed that PLCγ2 becomes recruited to tyrosine-containing proteins upon cell surface receptor engagement (135, 136), our findings indicate a potential role for Phb1/2 alone in mediating CD86-induced PLCγ2 phosphorylation, independent of the CD86 cytoplasmic domain, since Phb1/2 contain tyrosine residues, while the cytoplasmic domain does not. Also, it was reported that Phb1 associates with Syk upon BCR engagement (150). Since Syk has been reported to regulate the activation of PLCγ2 via the adaptor protein, B cell linker protein (BLNK), upon BCR engagement (171), it is possible that Phb1/2 serve as a scaffolding complex to link CD86 engagement to PLCγ2 activation. Nonetheless, the modest interdependent reduction in Phb1/2 protein levels in the current study was sufficient to prevent CD86 induction of IgG1 and proximal signaling intermediates, suggesting that Phb1/2 are each required to mediate CD86-induced signaling in B cells.
4.5 The interdependency of Phb1 and Phb2 expression

In the present study, we used RNA-interference to silence Phb1/2 because both Phb1- and Phb2-deficient mice are embryonic lethal. Previous reports revealed that Phb1 and Phb2 protein expression levels were interdependent, with depletion of one causing the loss of the other (172, 173), which is consistent with findings in the current study. Phb1/2 mRNA levels, however, were reported to be comparable in the presence of non-gene-specific Phb1/2 siRNA (173), which is in contrast with findings of the current study. The discrepancy could be due to a mixed population of Phb1/2 shRNA plasmids, as were used in the present study that targeted multiple regions of Phb1/2 transcripts, as Phb1/2 are highly homologous (149), thus promoting a higher likelihood of suppressing mRNA translation.

4.6 The ability of Phb1/2 to mediate cell surface receptor signaling function

To our knowledge, the present findings using RNA-interference are the first to identify a role for Phb1 and Phb2 in mediating the CD86-induced enhancement of the levels of Oct-2 and IgG1 produced by a CD40L/IL-4-primed B cell. Phb1/2 shRNA-mediated depletion in the current study led to modest reductions in Phb1/2 protein levels, suggesting that only a modest amount of Phb1/2 protein in the cell is required to mediate signaling events induced by CD86; whereas, a greater proportion is necessary to regulate mitochondrial function. This is supported by previous reports that showed Phb1/2 depletion resulted in the loss of mitochondrial membrane potential (151, 174), even though other reports have shown Phb1/2 depletion did not affect mitochondrial
membrane potential (173, 175). Also, using gene silencing, Phb1 was shown to have functional roles in cell migration (176), regulation of angiogenic activity and cellular senescence (174), and cellular metabolic activity (177) upon engagement of various cell surface receptors. Likewise, gene-silencing of Phb2 indicated a functional role for Phb2 in muscle cell differentiation (178). Also, Phb1/2 have been reported to associate with the BCR (159) and, recently, CD3 of the TCR complex (160), although the exact functional roles for the associations remain unclear. Furthermore, a direct protein/protein interaction has been shown to occur between Phb1 and Syk upon BCR engagement (150), while another report showed a decrease in MAP kinase signaling upon CD3 engagement when Phb1/2 function was blocked, indicating that Phb1/2 may serve functional roles in BCR/TCR signaling. Thus, the present findings using RNA-interference provide further support that Phb1/2 participate in mediating cell surface receptor-induced regulation of cellular functions.

4.7 The CD86:Phb1/2 molecular interaction

We originally hypothesized that the cytoplasmic domain of CD86 associated directly with Phb1/2 to mediate CD86 signaling function. However, our data showed that Phb1/2 associated with CD86 even in the absence of the CD86 cytoplasmic domain, suggesting a transmembrane-specific interaction. The ability of CD86 to bind to transmembrane proteins is supported by a recent study in DCs where it was shown that CD86 associated with an E3 ubiquitin ligase in the presence or absence of the CD86 cytoplasmic domain (179). It is also supported by previous reports showing that Phb1/2 exist as transmembrane proteins that function to stabilize proteins in the electron
transport chain and to mediate functional changes induced by cell surface receptors (149, 163-165, 174, 176-178). Taken together, these findings support the proposal that CD86 associates with Phb1/2 via transmembrane-specific interactions in B cells.

4.8 The ability of CD40-induced signaling to regulate Phb1/2 expression

4.8.1 CD40-dependent induction of Phb1/2

The present data show that CD40 engagement was able to increase both Phb1 and Phb2 gene expression in comparison to the negligible effect induced by the engagement of other receptors on the B cell that have been reported to increase CD86 expression, except for LPS, which induced an increase in Phb1/2 expression at the mRNA level that was only weakly reflected at the protein level. The reduction of Phb1/2 gene expression by shRNA interference was unable to affect the level of CD86 surface protein expression induced by CD40 engagement, suggesting that expression of CD86 and Phb1/2 are not linked. However, although the mechanism by which CD40 engagement specifically caused the upregulation of both CD86 and Phb1/2 remains unknown, but is likely mediated by the CD40-induced activation of different transcription factors.

4.8.2 CD40 cross-talk communication as a competency signal for CD86 signaling

Although the association of Phb1/2 with the BCR appears to be constitutive and intact on resting primary B cells (159), the present findings indicate that a similar high level of association of CD86 with Phb1/2 on resting B cells prior to CD40 engagement is lacking. The mechanism by which this association increases is unclear, although other
reports allow us to speculate. First, Phb1 has been reported to localize specifically to lipid raft fractions in membranes of B cell lines (180). Second, CD86 appears to be recruited to lipid rafts during a dendritic cell-T cell interaction (181). And third, Lyn, one of the most proximal CD86 signaling intermediates in B cells (119) is constitutively expressed in membrane lipid rafts (182), which is where PLCγ2 has been shown to be recruited (183). Thus, all of the components for CD86 signaling could potentially be recruited to lipid rafts after CD86 priming to promote the association between Phb1/2 and CD86, allowing for the assembly of an effective signaling complex. Also, the BCR is known to localize in a lipid raft upon BCR engagement (184) and has been shown to be in a physical association with Phb1 and Phb2 (159), suggesting that the BCR and Phb1/2 may be localized to a lipid raft to mediate function. An alternative explanation for the CD40-induced increase in Phb1/2 expression to allow for Phb1/2-specific recruitment to CD86 is that CD40 engagement also increases CD86 expression (110), and that simply more CD86 molecules had been purified. Nonetheless, the present findings suggest that CD40 engagement is important for the induction of Phb1/2 expression, which may be required to facilitate CD86 signaling.

Although the mechanism by which CD40 engagement increases Phb1/2 is unclear at present, it is possible that CD40 is involved with potential cross-talk communication with CD86 to deliver a competency signal to allow for CD86-mediated signaling. This is supported by previous reports that showed that the expression of an adaptor molecule to both the TCR and BCR was required to mediate signaling (185), and that prior B cell activation stimuli including LPS (124), the BCR (113, 186), and CD40L/IL-4 (118, 119, 122, 123), were necessary to mediate CD86-induced increases in antibody production.
Also, B cell activation stimuli increase the level of CD86 on the B cell surface from low levels to reach a potential threshold level that is required for CD86 signaling competency. Although engagement of the IL-4R (111) and the β-2 adrenergic receptor (113, 114) allowed for increases in CD86 levels on the B cell surface, CD86 failed to induce signaling events when engaged on the B cell surface in the presence of those stimuli (unpublished findings). Therefore, it is plausible to propose that a CD86 surface expression threshold is not necessary to induce signaling, but the delivery of a competency signal. Even though the present findings suggest that engagement of CD40, and to a lesser extent the LPS receptor, are able to induce Phb1/2 expression levels and not engagement of the BCR, this induction may be the competency signal required to allow for the CD86-dependent regulation of IgG1 production on a primed B cell.

4.8.3 Physiological relevance of CD40-dependent regulation of Phb1/2

The physiological role for an upregulation of Phb1/2 after CD40 engagement on a B cell is unknown. However, we know that CD40 engagement on a B cell functions physiologically in the context of an antigen-specific T-B cell interaction (187). Therefore, it is possible that CD40 engagement promotes the upregulation of both Phb1/2 and CD86 expression to allow for the formation of a CD86-Phb1/2 signaling complex to ultimately enhance the level of IgG1 produced. Thus, the level of CD40 engagement on a B cell, which is dependent on the level of CD40L expression after T cell activation, may be a mechanism by which the level of IgG1 is regulated in a B cell. For example, the level of an infection may dictate the level of T cell expression of CD40L and, subsequently, the level of CD86 and Phb1/2 expression on a B cell, resulting in different
levels of CD86/Phb1/2 complexes that would form to mediate a change in the amount of 
IgG1 produced to mediate protection. Therefore, a low level infection would not require 
as high a level of antibody to be produced for protection as would be required for a high 
level infection. The specificity of CD40 for induction of the highest level of Phb1/2 is 
unknown. One report showed that engagement of CD86 via an anti-CD86 Ab on an LPS-
primed B cell also increased the level of IgG1 produced (124). Our data might explain 
this finding since LPS induced Phb1/2 expression on a B cell, albeit at a low level 
suggesting a potential link between LPS-induced Phb1/2 expression and CD86 signaling 
function. Nonetheless, given these caveats when using LPS, the present results suggest 
that CD40 engagement upregulates a high level of Phb1/2 expression that can be linked 
to an increase in the association of Phb1/2 with CD86 to promote CD86 function.

4.9 In vivo relevance for the CD86 cytoplasmic domain

The present findings suggest that the decrease in serum IgG1 production by 
immunodeficient mice in which B cells that lacked the CD86 cytoplasmic domain (Trunc 
CD86) B cells were adoptively transferred is due to a lack of back-signaling through the 
cytoplasmic domain of CD86, and not due to a decrease in the number of splenic CD4+ T 
cells found after antigen priming, although the percentage of CD4+ T cells expressing IL-
4 was similar, but with a slight decrease found in the CD86-/- recipient mice. The finding 
on T cell number was unexpected since previous reports showed that CD28 engagement 
on a naïve T cell is critically important for the activation and expansion of CD4+ T cells 
in vivo (188). However, our finding might be explained by the fact that the adoptively 
transferred Trunc CD86 B cells also lacked expression of B7-1 (CD80) (139), which is
the structural and biochemical molecule that preferentially binds to CTLA-4 (64, 189). CTLA-4 is a molecule that is expressed by T cells and, upon engagement with CD80, reduces the level of T cell cycle progression and IL-2 production that is caused by T cell activation (190). In addition, an in vivo study showed that blockade of CD86 with a monoclonal antibody (mAb), as compared to blockade of CD80, resulted in more efficient blocking of Ag-specific T cell expansion (191). Yet the number, as opposed to percentage, of CD4\(^+\) T cells expressing IL-4 was significantly greater in Rag2\(^{-/-}\) mice that received Trunc CD86 B cells, while the number did not increase in CD86-deficient mice that received Trunc CD86 B cells compared to WT B cells. This difference between number and percentage in the CD86-deficient recipients vs. the Rag2-deficient recipients might be explained by a compensatory mechanism that was mediated by the presence of CD86/CD80-expressing macrophages and DCs in the Rag2\(^{-/-}\) mice, allowing for more T cells to become activated to express IL-4. Taken together, we conclude that the difference in the amount of serum IgG\(_1\) produced in mice receiving the truncated CD86 B cell is due to the loss of CD86 back-signaling within truncated CD86 B cells and not due to a lack of adequate CD4\(^+\) T cells to provide help for B cell differentiation. The latter conclusion is supported further by present and previously published (119) findings that class switch recombination occurred to a normal level in immunodeficient mice that received CD86-deficient B cells.

4.10 Clinical relevance

The clinical relevance of CD86 back-signaling into a B cell and the small changes induced in IgG\(_1\) remains unknown, but has been suggested previously (117-119), and is
confirmed and extended in the present study. Two-to-three fold changes in human serum IgG have been associated with 3-9 fold increases in protection against agents, such as *Streptococcus pneumoniae* (133), suggesting that small changes in IgG exert a large protective effect that may be relevant for chronically ill individuals. For example, chronic lymphocytic leukemia (CLL) express a high number of transformed non-functional B cells and a very low number of normal B cells, and these individuals also suffer from multiple secondary infections, such as pneumonia, that result in mortality (192). No effective therapeutic strategies have been designed to elevate the level of antibody produced by the small pool of normal B cells in CLL patients, but this would be needed if one wanted to enhance the efficacy of pneumococcal vaccination in mediating protection (192) by the limited number of normal B cells that would expand in number after vaccination. In support of this possibility, one study reported an increase in IgG₄ production when CD86 was engaged on primed human B cells (121). Thus, an understanding of the molecular mechanism by which CD86 and Phb1/2 function together to increase IgG₁/ IgG₄ production per B cell, without affecting class switch recombination, may allow for the development of CD86-targeted therapeutic strategies that would increase the level of Ab-mediated protection in immuno-compromised patients. In addition, findings from the current study will help us to understand the mechanism by which CD86 signals directly to a B cell to increase not only IgG₁, but also IgE production, as shown previously (113, 121). Conversely, CD86-targeted therapeutic strategies might also be developed to decrease the level of harmful antibodies produced in certain cases of autoimmunity or allergic asthma.
4.11 Future directions

4.11.1 The CD86:Phb1/2 signaling complex in multiple cell types

Findings presented in the current study suggested that Phb1/2 are novel CD86-associated functional signaling proteins that are required along with the CD86 cytoplasmic domain, to differentially activate distal signaling intermediates required to increase the level of IgG1 produced. In addition, the evidence suggested that both Phb1/2 and the CD86 cytoplasmic are required to induce CD86-dependent activation of NF-κB. Since CD86 engagement on the surface of B cells, DCs, and macrophages has been reported to activate NF-κB to ultimately promote a functional change at the cellular level (118, 123, 156, 158), it is possible that Phb1/2 along with the CD86 cytoplasmic domain mediates the activation of NF-κB in multiple cell types. The activation of NF-κB upon CD86 engagement has been reported to be important for the production of IL-6 and IDO in DCs (156), as well as TNF-α and ROS in macrophages (158), suggesting that an understanding of a CD86 signaling complex within all types of APCs would allow for the rational design of therapeutics to inhibit the induction of CD86-induced signaling events that regulate a variety of cellular phenotypes that could promote a disease phenotype.

Although it is quite possible that Phb1/2 and the CD86 cytoplasmic domain cooperate to mediate the activation of NF-κB in multiple cell types, the molecular signaling mechanism remains less well understood. Findings in the current study suggest that Phb1/2 and the CD86 cytoplasmic domain are required to allow for IκBα phosphorylation and subsequent degradation, although the proximal mechanism remains unclear. However, it was reported that Phb1 and Phb2 physically associate with Akt to implement receptor functions (170, 178). Since Akt is a CD86-dependent signaling
intermediate known to be activated proximal to IκBα phosphorylation/degradation, and Akt is known to allow for the activation of IKKα/β to phosphorylate IκBα, it is possible that Phb1/2 bind Akt upon CD86 engagement to allow for NF-κB activation. Although no evidence in the literature exists that suggests a link between the CD86 cytoplasmic domain and Akt activation, it is possible that Akt may bind to the CD86 cytoplasmic domain upon CD86 engagement. This is supported by previous studies that showed Akt interacting with the phosphorylated form of PDK-1 (Ser241) (193) and phosphatidylinositol 3, 4-bisphosphate (PIP3) (194). Thus, it is possible that Phb1/2 and the CD86 cytoplasmic domain serve as a docking complex to allow for the activation of Akt upon CD86 engagement to allow for NF-κB activation. In summary, the CD86:Phb1/2 complex could potentially exist in multiple cell types in addition to B cells, including DCs and macrophages, to allow for NF-κB activation which may occur via an Akt-dependent mechanism. Understanding this unique signaling complex, in the context of multiple cell types, would provide a strong rationale for therapeutic intervention.

4.11.2 A role for additional protein tyrosine kinases (PTKs) in CD86 signaling

The current study was designed to define a membrane-proximal CD86-induced signaling mechanism in B cells proximal to the activation of PLCγ2. However, CD86 engagement also promotes the activation of another early signaling intermediate, the PTK Lyn (119). Lyn activation is regulated via a CD45-dependent mechanism through the removal of a C-terminal inhibitory phosphate group to allow for autophosphorylation (195). Therefore, the possibility exists that CD86 engagement allows for CD45-
dependent activation of Lyn. Although a constitutive physical interaction between CD45 and CD86 may not exist due to the absence of a unique protein band in our CD86-associated protein identification experiment (see Figure 12), a weak association may occur between the two molecules upon CD86 engagement. In addition, Lyn did not co-immunoprecipitate with CD86 suggesting that CD86-induced Lyn activation may not occur in a physical manner (data not shown). Alternatively, it is possible that CD86 may enter a lipid raft upon engagement as documented previously (181) where CD45 and Lyn are known to be enriched (196). Thus, an understanding of the relationship between CD86 and CD45 would allow for a greater appreciation for the CD86-induced activation of Lyn.

Although findings in the current study suggest that CD86 engagement on the surface of a primed B cell allows for tyrosine phosphorylation of Phb1/2, the molecular mechanism responsible and functional significance remains ill defined. It is possible that the PTK Lyn is responsible for the tyrosine phosphorylation of Phb1/2 upon CD86 engagement since Phb1/2 is predicted to bind −SH2 domain-containing proteins on tyrosine residues within their C-terminal coiled-coiled domain (197). To address this, co-immunoprecipitation experiments of either Phb1/2 or Lyn would address if a physical association of the molecules occurred upon CD86 engagement. Alternatively, another unidentified PTK perhaps Syk or Bruton’s tyrosine kinase (Btk) may be responsible for the CD86-induced phosphorylation of Phb1/2.

In order to test the functional significance of Phb1/2 tyrosine phosphorylation in the context of CD86-dependent regulation of IgG1, recombinant Phb1/2 plasmids could be introduced into B cells containing either phenylalanine mutants to block potential
tyrosine phosphorylation, or glutamic acid mutants to mimic constitutive tyrosine phosphorylation of Phb1/2 upon CD86 engagement. The CD86-induced Phb1/2 tyrosine phosphorylation suggests that Phb1/2 act as functional signaling intermediates upon CD86 engagement that may mediate the recruitment of additional signaling intermediates including PLC\(\gamma\)2, although preliminary co-immunoprecipitation results were inconclusive. Therefore, conducting a CD86 engagement time course followed by the immunoprecipitation of either Phb1/2 or PLC\(\gamma\)2 could address whether or not a physical association exists between the molecules, suggesting a signaling intermediate recruitment role for Phb1/2 in the context of CD86 signaling.

Our findings in the current study suggest that Phb1/2 alone are required for the CD86-induced activation of PLC\(\gamma\)2. However, the exact molecular mechanism is unknown. It is possible that PLC\(\gamma\)2 activation may be a direct downstream target of Lyn since it was reported that Lyn physically interacts with, and facilitates the activation of PLC\(\gamma\)2 (198). Alternatively, additional PTKs including Syk and Btk along with the adaptor proteins B cell linker protein (BLNK) and SH2 domain containing leukocyte protein of 76kDa SH2 domain containing leukocyte protein of 76kDa (SLP-76) may be involved with the CD86-induced activation of PLC\(\gamma\)2, since these molecules have all been reported to be important for PLC\(\gamma\)2 activation (171, 199-202).

4.11.3 Signaling mechanisms of the CD86 cytoplasmic domain

Findings in the current study suggested that the CD86 cytoplasmic domain, in addition to the novel CD86-associated molecules, Phb1/2, is required for the CD86-
induced increase in IgG₁ production \textit{in vitro} and \textit{in vivo}. In addition, our evidence implicates phosphorylation of the CD86 cytoplasmic domain on the PKC-dependent serine/threonine phosphorylation sites is a plausible mechanism. However, the exact potential signaling intermediate recruitment event(s) or signaling intermediates triggered upon CD86 engagement mediated via the cytoplasmic domain remains undefined. To identify additional signaling intermediate(s) that may be recruited to the CD86 cytoplasmic domain, a CD86 engagement time course followed by a proteomics based identification approach would begin to address if a signaling intermediate(s) is recruited directly to CD86 upon engagement. This experiment could also be executed in the presence or absence of the FLAG-CD86 cytoplasmic domain, or phospho-preventative alanine, or phospho-mimetic aspartic acid point mutations of the PKC serine/threonine phosphorylation sites within the CD86 cytoplasmic domain. Furthermore, the activation status of the known CD86-induced signaling intermediates could be evaluated in the presence or absence of the CD86 cytoplasmic domain. An understanding of the exact signaling mechanism employed via the CD86 cytoplasmic domain, including the exact amino acid reside(s), as well as if other signaling intermediates are recruited to the cytoplasmic domain upon CD86 engagement, will allow for the rational design of therapeutic targets for the CD86 cytoplasmic domain.

\textbf{4.12 Concluding remarks}

When the execution of the experiments presented in the present study began, it was known that CD86 engagement on the surface of a CD40L/IL-4-primed B cell signaled directly within the B cell \textit{in vitro} and \textit{in vivo} to elevate the level of IgG₁.
produced through an increase in the rate of transcription, without affecting the level of
CSR to IgG\textsubscript{1} (113, 118, 119, 122, 123). In addition, it was known that CD86 engagement
activated two proximal molecular intracellular signaling networks that converged to
activate NF-κB to allow for Oct-2 transcription, and a subsequent increase in the rate of
IgG\textsubscript{1} transcription, controlled via an increase in the activity of the 3′-IgH enhancer, (118,
119, 122, 123). The signaling networks activated upon CD86 engagement on a primed B
cell proximal to NF-κB activation included the Lyn/CD19 pathway and the PI3K/PDK-
1/Akt pathway, which controlled IκBα phosphorylation, and the PLC\textgamma{2}/PKCα/β\textsubscript{II}
pathway, which allowed for NF-κB (p65) phosphorylation (118, 119, 123). However, the
molecular mechanism proximal to the activation of PLC\textgamma{2} remained unknown. The
present data show that the tyrosine-containing transmembrane adaptor proteins,
prohibitin-1 (Phb1) and prohibitin-2 (Phb2), bind to CD86. The basal expression of
Phb1/2 and association with CD86 was low in resting B cells, while the level of
expression and association increased primarily after priming with CD40. The CD86-
induced increase in Oct-2 and IgG\textsubscript{1} was less when either Phb1/2 expression was reduced
by shRNA or the cytoplasmic domain of CD86 was truncated or mutated at
serine/threonine PKC-phosphorylation sites, which did not affect Phb1/2 binding to
CD86. In addition, we show that an intact CD86 cytoplasmic domain is necessary for an
optimal IgG\textsubscript{1} response against a T-dependent antigen \textit{in vivo}. Furthermore, we also
show that Phb1/2 and the CD86 cytoplasmic domain are required for the CD86-induced
phosphorylation and subsequent degradation of IκBα, which we previously reported leads
to NF-κB p50/p65 activation; whereas, only Phb1/2 was required for the CD86-induced
phosphorylation of PLC\textgamma{2} and PKCα/β\textsubscript{II}, which we have previously reported leads to NF-
κB (p65) phosphorylation and subsequent nuclear translocation. Together, these findings suggest that Phb1/2 and the CD86 cytoplasmic domain cooperate to mediate CD86 signaling in a B cell through differential phosphorylation of distal signaling intermediates required to increase IgG1.

The contribution of the findings of the current study to the understanding of an IgG1 antibody response against T-dependent antigen in vivo is that not only is the extracellular domain of CD86 required to induce CD4+ T cell activation in vivo to produce IL-4, but also that the presence of an intact CD86 cytoplasmic domain is necessary for optimal levels of IgG1 produced, suggesting for the first time that CD86 signals directly to the B cell via its cytoplasmic domain in vivo to regulate the level of IgG1 produced.

In conclusion, the major contribution of the present findings to the field of Immunology is that the results of this dissertation research show for the very first time that CD86, a B cell surface molecule originally thought to be merely a ligand, associates directly with prohibitin-1 and prohibitin-2, two tyrosine-containing scaffolding proteins that are known to be directly involved with intracellular signal transduction. Furthermore, our results show for the first time that Phb1/2 are functional CD86-induced signaling proteins and necessary for the CD86-dependent increase of IgG1. In addition, the results show that an intact CD86 cytoplasmic domain is required to produce optimal levels of IgG1 in vitro and in vivo. The findings presented are the first to identify a membrane-proximal signaling mechanism induced via CD86 engagement on a primed B cell in which two novel CD86-associated molecules cooperate with the CD86 cytoplasmic domain to differentially activate distal signaling intermediates required to
regulate the level of IgG\textsubscript{1} produced. It is our sincere hope that knowledge gained from this dissertation research will be used to intervene in a unique CD86-induced signaling network activated within a B cell either to elevate or suppress the level of IgG\textsubscript{1} produced.
CHAPTER 5

CLINICAL APPLICATION OF CD86 SIGNALING

5.1 Introduction

Engagement of CD86 on the surface of a CD40L/IL-4-primed murine B cell signals directly within the B cell to induce signaling intermediates that ultimately increase the level of IgG1 produced, without affecting the level of class-switch recombination (113, 118, 119, 122, 123). In addition, CD86 was reported to signal directly to increase the level anti-apoptotic factors (124), the level of IgE produced (113), and to activate the pro-survival factor, NF-κB (118, 123). To date, merely one report showed that CD86 could signal directly to a human B cell to enhance the level of IgG4/IgE produced on B cell primed with anti-CD40 and IL-4 (121). Therefore, the role of CD86 direct signaling in the context of human B cell biology is better understood, but the role played in the context of B cell clinical immunology remains unknown.

Previous reports from our laboratory identified a unique signaling cascade that is activated upon CD86 engagement on a primed murine B cell that includes the activation of numerous distal signaling intermediates that are considered to allow a cell to promote a pro-growth/pro-survival phenotype including the activation of PI3K, Akt, and NF-κB (118, 119, 123). Therefore, the possibility existed that CD86 engagement on the surface of a human B cell would induce a pro-growth/pro-survival phenotype, which is a strongly
undesired effect within a cancerous B cell. Thus, we began to translate our findings from our murine model system into a clinically relevant scenario, namely the B cell malignancy disease of Chronic Lymphocytic Leukemia (CLL).

CLL is a disease of mature B cells (B-CLL) and is the most common adult leukemia in the United States (203, 204). The disease is characterized by cells that expand in number because they evade apoptosis and invade major organ systems, causing mortality (203, 204). For many years, CLL was thought to be merely a disease of apoptotic evasion and not a proliferative leukemia. However, previous reports have documented that B-CLL proliferation occurs in the context of anatomical sites, including the lymph nodes, spleen, and bone marrow where B-CLL cells interact with either costimulatory ligands and/or soluble factors from immune cells or the microenvironment (205-210). Within the confines of the lymph nodes and spleen, actively proliferating B-CLL cells often associate with T cells that produce ligands and/or soluble factors to promote cellular survival and proliferation upon interaction with B-CLL cells (205, 211, 212). Furthermore, studies in CLL patients have shown that B-CLL cells display a wide range of phenotypic proliferation, which is attributed to the disease state, as well as the mutational status of the Ig variable heavy chain (IgVH) (213, 214). Taken together, these findings suggested that B-CLL cells are able to undergo proliferation, in addition to evading apoptosis.

Currently, the most commonly employed treatments for CLL include chemotherapeutic agents, including alkylating agents (such as cyclophosphamide, chlorambucil, and bendamustine), purine analogs (such as pentostatin, cladribine, and fludarabine), monoclonal antibodies (such as rituximab and alemtuzumab), as well as
numerous other chemical compounds currently in clinical trials (215-225). Current CLL treatment regimens are reviewed extensively in (215). Alkylating agents act by non-specifically alkylating DNA and thereby disrupt DNA replication causing programmed cell death (apoptosis) (215, 226). The recently described alkylating agent bendamustine can also induce pro-apoptotic gene expression (227). Purine analogs inhibit ribonucleotide reductase and DNA polymerase and therefore initiate apoptosis (204, 215, 228). Lastly, the monoclonal antibodies act via recognition of B-CLL cell surface antigens and ultimately lead to B-CLL cell depletion (224, 229-232). However, drug resistance develops in CLL patients promoting eventual relapse (203, 215, 225) and, with the rare exception of some cases of allogeneic stem cell transplantation (228), the disease is not curative.

An attractive non-drug therapeutic approach is to elevate CD8$^+$ cytolytic T cell-targeted destruction of B-CLL cells by enhancing the level of expression of co-stimulatory immune molecules, such as CD86 (formerly known as B7-2), on the surface of B-CLL cells to augment the cytolytic response of CD8$^+$ T cells (233, 234). However, previous reports showed that incomplete B-CLL target cell lysis was evident in the presence of activated cytolytic T cells where CD86 would likely be elevated on the B-CLL cell surface (235, 236). In addition, it was reported that engagement of CD86 on the B cell surface is capable of activating intracellular signaling networks, including the activation of Akt and NF-$\kappa$B (118, 123), which have been linked to a pro-growth and survival phenotype in many human cancers (237). Also, it was reported that engagement of CD86 induced the expression of anti-apoptotic molecules, including Bcl-w and Bcl-x(L), suppressed levels of the pro-apoptotic molecule, caspase-8, in a murine B cell
lymphoma cell line, and promoted proliferation (124). Furthermore, CD86 engagement on the surface of human tonsillar B cells was shown to potentiate proliferation induced by CD40/IL-4 priming (121). Together, these findings suggested that CD86 signaling could have detrimental effects within B-CLL cells while administering cytolytic T cell therapy.

Recently, it was found that CD86 associates with, and signals directly to, murine primary B cells through prohibitin-1 (PHB1), a highly conserved, ubiquitously expressed protein found primarily in the mitochondrial and plasma membranes (Lucas, et. al, The Journal of Immunology. In Press. 2012); (170), and over-expressed in a variety of human malignancies (238). A variety of roles have been defined for PHB1, including mitochondrial localization for chaperone function (239), as well as transcriptional (240), cell cycle (241), and cell signaling regulation (170, 176). PHB1 has been shown to be required for signaling networks that promote growth and survival, including Ras-mediated Raf activation (176), and direct interaction with the insulin receptor (INSR), phosphatidylinositol 3,4,5-triphosphate (PIP3), and Akt to play a modulatory role in the PI3K/Akt pathway (153, 154). Constitutive activation of Ras-dependent Erk and Mek occurs in a subset of CLL patients (242) and, thus, it is possible that PHB1 functions to regulate constitutive activation of these signaling networks. Since CD86 has been reported to signal directly within human B cells (121), and PHB1 is known to regulate intracellular signaling pathways that promote growth and survival (153, 154, 176), it is possible that PHB1 regulates CD86 signaling that promotes growth and survival within B-CLL cells.

This study sought to determine if engagement of CD86 on the surface of MEC1 B-CLL cells delivered a pro-growth signal, capable of causing proliferation. We show
for the first time that CD86 engagement promoted an increase in the number of viable
MEC1 B-CLL cells. CD86 engagement induced the activation of the pro-growth/-
survival factor NF-κB as shown by immunofluorescence time-lapsed imaging and Flow
Cytometry. Furthermore, CD86 associated with PHB1, as revealed by
immunoprecipitation followed by immunoblot. Moreover, PHB1 depletion via shRNA
inhibited MEC1 B-CLL proliferation. Thus, a novel CD86-PHB1 signaling complex
may be involved in mediating a signal in B-CLL cells directly to promote a proliferative
response, and therefore, counteract the intended reason to increase CD86 to promote
cytotoxic T cell-mediated B-CLL cell death.

5.2 Materials and Methods

5.2.1 Cell lines

MEC1 B-CLL cells are a stable, human B cell Chronic Lymphocytic Leukemic
cell line described previously (243).

5.2.2 Culture Conditions

5 x 10^4 MEC1 B-CLL cells were cultured in the presence of either an anti-CD86
Ab (IT2.2) or a species- and isotype-matched control Ab (mouse IgG2b) (BD
Biosciences) at 20 µg/ml over a four day time course. In some experiments, an Fc
blocking reagent was used to negate undesired effects mediated via the mouse IgG2b
isotype control Ab (Human Fc blocking reagent, Miltenyi). The number of viable MEC1
B-CLL cells was determined by hemacytomic cell counting (trypan blue exclusion) and
Flow Cytometric analysis.
5.2.3 RNA-interfering shRNA

RNA-interference was used to deplete PHB1 expression levels. Either scrambled, negative control shRNA, or PHB1- (clones 1-4) specific shRNA plasmids (4-5 µg/10^6 cells) (SA Biosciences) were transfected into MEC1 B-CLL cells via nucleofection (Amaxa, program O-003 and U-013) and allowed to proliferate over a four day time course. The level of MEC1 B-CLL cell proliferation was measured by Flow Cytometric analysis. Knockdown effectiveness was determined at the mRNA level by quantitative real-time PCR analysis, and at the protein level by immunoblot analysis.

5.2.4 Hemacytomic Cell Counting

The number of MEC1 B-CLL cells was determined by trypan blue exclusion (1:1 dilution). An average viable cell count of four 16-square grid was reported.

5.2.5 Flow Cytometry

Flow cytometric analysis was conducted as described previously (113). 7-amino actinomycin D (7-AAD) and PE-conjugated Annexin V (BD Biosciences) was used to determine the number of viable MEC1 B-CLL cells. Cellular proliferation was assessed via the Violet V450 nm proliferation dye (BD Biosciences). The GFP-based Cignal Reporter construct (Qiagen) was used to measure the level of NF-κB activity. The data were analyzed using FlowJO Software (Tree Star).
5.2.6 Immunoprecipitation

Immunoprecipitations were performed using the Profound Mammalian Co-Immunoprecipitation Kit (Pierce). Briefly, MEC1 B-CLL cells, \((25-30 \times 10^6)\) were cultured, lysed with 0.5% Triton-X100 buffer, immunoprecipitated with an anti-CD86 Ab [EP1158Y] (abCAM) following manufacturer’s directions. The precipitates were analyzed using immunoblot for CD86 and PHB1.

5.2.7 Immunoblot analysis

Protein samples, (10 µg), or samples from CD86-immunoprecipitates from either resting or primed (anti-CD40 (MAB89) (AbCam) 0.2 µg/ml and recombinant IL-4 200 units/ml (eBioscience), were run on a denaturing 10% polyacrylamide gel and transferred to Immobilon-PVDF membranes (Millipore). Membranes were blocked with tris buffered saline-tween 20 (TBST) as described previously (118) and incubated overnight with primary antibodies at 4°C. Membranes were probed with HRP-labeled secondary antibodies using LumiGlo Detection Kit (Cell Signaling) and specific bands were visualized on Kodak Biomax MS film. Antibodies used were anti-CD86 (H-200) (Santa Cruz), and anti-PHB1 (Cell Signaling).

5.2.8 Quantitative reverse transcriptase real-time PCR analysis

Total RNA was isolated via Trizol (Invitrogen), cDNA was created via reverse transcription (Qiagen) and qt-RTPCR (SYBR Green, Roche) was conducted as previously described (122) for PHB1 mRNA levels, forward primer: 5’- ATGGCTGCCAAAGTGTTTGAGTC-3’; reverse primer: 5’-
TCAGTGGGAAGCTGGAGAAGC-3’; and actin mRNA levels, forward primer: 5’-
ACCAACTGGGACGACATGGAGAAA-3’ reverse primer: 5’-
TAGCAGCCTGGATAGCAACGTA-3’.

5.2.9 NF-κB Cignal Reporter Assay and immunofluorescence

1-2 x 10^6 MEC1 B-CLL cells were transfected with 1-2 µg of GFP-NF-κB Cignal
Reporter construct (Qiagen) via nucleofection (Lonza, solution V, program U-013)
according to manufacturer’s instructions for 24 hours. The cells were washed with 1X
PBS and resuspended in clear medium or clear Hanks buffered saline solution (HBSS)
(Life Technologies) followed the addition of either anti-CD86 (clone IT2.2) or species-
and matched- isotype control antibody (mouse IgG2b, κ) (20-40 µg/ml) for 24 hours.
Levels of GFP indicative of NF-κB activity was assessed via Flow Cytometry.
Alternatively, images were taken in real time post-addition of anti-CD86 every 5 minutes
for 12 hours at room temperature via an immunofluorescence microscope (Nikon) for
DIC, GFP (488 nm), or 7-AAD (560 nm) to assess viability.

5.2.10 Statistical analysis

Data from multiple treatment groups were analyzed using a one-way analysis of
variance to determine if an overall statistical change existed. Certain p values were
calculated using a Bonferroni post hoc analysis or a two-sided Student t test for
comparison of two treatment groups. A p value of ≤ 0.05 indicated statistically
significant results.
5.3 Results

5.3.1 CD86 signals directly to increase the number of MEC1 B-CLL cells

An attractive, novel, non-drug therapeutic approach to CLL is to elevate CD8\(^+\) cytolytic T cell-targeted destruction of B-CLL cells by enhancing the level of expression of co-stimulatory immune molecules, such as CD86, on the surface of B-CLL cells to augment the cytolytic response of CD8\(^+\) T cells (233). However, previous reports showed that CD86 signals directly to the B cell to promote activation of pro-growth/survival factors including the activation of Akt and NF-κB (118, 123), which have been linked to a pro-growth and survival phenotype in many human cancers (237). Since CD86 engagement on WEHI 279 murine B cells and on human tonsillar B cells was reported to induce proliferation (121, 124), it was possible that CD86 could signal directly to a B-CLL cell to promote cellular proliferation. To determine if CD86 engagement on the B-CLL cell surface alone could induce cellular proliferation in B-CLL cells, MEC1 B-CLL cells were cultured in the presence or absence of an anti-CD86 Ab or species- and isotype-matched control Ab over a four day time course. The presence of the anti-CD86 antibody caused a significant increase in the number of viable MEC1 B-CLL cells as measured by hemacytometric cell counting and (7-AAD)/PE-Annexin V staining via Flow Cytometry (see Figure 40). Together, these findings suggested that CD86 could signal directly to MEC1 B-CLL cells to increase the number of MEC1 B-CLL cells and thus induce cellular proliferation.
Figure 40. CD86 signals directly to increase the number of MEC1 B-CLL cells. (A and B) MEC1 B-CLL cells were cultured in the presence of a CD86 Ab (anti-CD86) or a species- and matched- isotype control Ab (iso ctrl) over a four-day time course. The number of viable cells was determined via a hemacytometer (A) or via 7-AAD/PE-Annexin V staining by Flow Cytometry (B). The data are expressed as the mean number of viable cells ± SEM and represent three independent experiments. Statistical differences are shown between groups. *, p < 0.05.
5.3.2 CD86 signals directly to promote NF-κB activation in MEC1 B-CLL cells

Although our evidence revealed that CD86 engagement alone on the MEC1 B-CLL cell surface was capable of direct signaling to promote cellular proliferation, what remained unclear was the molecular mechanism responsible for the effect. NF-κB is a critical transcription factor known to be active and important for tumorigenesis in CLL (203, 206). Since previous reports from our laboratory showed that CD86 engagement on a CD40L/IL-4-primed murine B cell signals to activate NF-κB (153, 154); (Lucas et al. The Journal of Immunology. In Press. 2012), we hypothesized that CD86 engagement would induce NF-κB activation in MEC1 B-CLL cells. To address this, MEC1 B-CLL cells were transfected with GFP-NF-κB Cignal Reporter construct (Qiagen) via nucleofection followed by the addition of an anti-CD86 Ab or species- and matched-isotype control Ab (iso ctrl). The level of NF-κB activity was assessed in real time via time-lapsed imaging. The level of GFP-NF-κB increased in a time-dependent manner upon CD86 engagement relative to MEC1 B-CLL cells treated with the iso ctrl Ab (see Figure 41). In addition, NF-κB activity was also measured via Flow Cytometry and found to increase upon CD86 engagement relative to untreated MEC1 B-CLL cells 24 hours post engagement (see Figure 42). Together, these findings suggested that CD86 signaled directly to the MEC1 B-CLL to induce NF-κB activation.
Figure 41. CD86 engagement induces NF-κB activation in MEC1 B-CLL cells as shown via immunofluorescence time-lapsed imaging. MEC1 B-CLL cells were transfected with the NF-κB GFP-based Cignal reporter construct (Qiagen) for 24 hours via nucleofection. The cells were resuspended in clear HBSS medium followed by the addition of either an anti-CD86 Ab (anti-CD86) or species- and matched-isotype control Ab (iso ctrl) over a 12-hour time course. The level of GFP-NF-κB activity was measured via an immunofluorescent microscope (Nikon). Bright field or fluorescent (488 nm excitation) images were taken at 5-minute intervals during the time course. Individual cells were tracked (blue circles) during the course where the mean fluorescence intensity (MFI) (488 nm) is graphed as a function of time. The image represents ~ 8.5 hours post engagement. The y-axis maximum value is shown. Representative images are shown from at least three independent experiments.
Figure 42. CD86 engagement induces NF-κB activation in MEC1 B-CLL cells as shown via Flow Cytometry. MEC1 B-CLL cells were transfected with the NF-κB GFP-based Cignal reporter construct (Qiagen) for 24 hours via nucleofection followed by the addition of an anti-CD86 Ab (anti-CD86) for an additional 24 hours. The level of GFP-NF-κB activity was measured via Flow Cytometry. The data are expressed an individual measurements of Median Fluorescence Intensity ± SEM and represent three independent experiments.
5.3.3 PHB1 associates with CD86 and is important for cellular proliferation in MEC1 B-CLL cells

Although our findings thus far indicated that CD86 could signal directly to a MEC1 B-CLL to promote an increase in the number of B-CLL cells, and mediate NF-κB activation, the proximal molecular mechanism remained unclear. Since previous findings in our laboratory showed that CD86 associated directly, and signaled via a scaffolding molecule called PHB1 in murine B cells (Lucas, et. al. The Journal of Immunology. In Press. 2012), and PHB1 is over-expressed in a variety of human malignancies (238), and capable of signaling to promote growth and survival (153, 154, 176), it was possible that PHB1 associated directly with CD86 in MEC1 B-CLL cells potentially acting as a novel oncoprotein in CLL. To address whether or not PHB1 expression levels were altered in CLL, data was analyzed from a microarray experiment that compared normal vs. CLL gene expression levels (kindly provided by Dr. John Byrd). PHB1 expression levels were ~ 2.3 Fold higher in CLL B cells relative to normal B cells (see Figure 43A). The protein level of PHB1 from MEC1 B-CLL cells was ~ 2 Fold greater compared to PHB1 protein from normal B cells (see Figure 43B). To determine if PHB1 associated directly with CD86 in MEC1 B-CLL cells, CD86 was immunoprecipitated from MEC1 B-CLL cell lysates, followed by immunoblot (see Figure 44). The presence of PHB1 in the CD86 immunoprecipitates under constitutive and primed conditions suggested that PHB1 associates directly with CD86 in MEC1 B-CLL cells. To determine if PHB1 were important for cellular proliferation in MEC1 B-CLL cells, PHB1 expression levels were depleted via RNA-interference. Either negative, scrambled shRNA, or PHB1-shRNA plasmids were transfected into MEC1 B-CLL cells via nucleofection and cultured over a
four day time course where the level of proliferation was determined via Flow Cytometry.

PHB1-specific shRNA decreased levels of PHB mRNA by ~50% (see Figure 45A) and PHB1 protein by ~25% two days post transfection (see Figure 45B). Furthermore, the presence of PHB1 shRNA inhibited MEC1 B-CLL cell proliferation in a time-dependent manner (see Figure 46). Together, these findings suggested that PHB1 associates directly with CD86 and may act as a novel oncoprotein in MEC1 B-CLL cells.
Figure 43. PHB1 is overexpressed in B-CLL cells. (A) To determine if PHB1 is overexpressed constitutively in B-CLL cells, a microarray experiment was conducted in collaboration with Dr. John Byrd that measured total genome profiles from CLL patients, or normal healthy volunteers. (B) To determine if PHB1 is overexpressed constitutively at the protein level, either normal, or lysate from MEC1 B-CLL cells were obtained and analyzed for PHB1 total protein levels relative to GAPDH. Densitometric analysis was performed and quantified the level of PHB1 relative to GAPDH where the data are expressed as the mean OD ± SEM from three independent experiments.
Figure 44. *CD86 associates with PHB1 in MEC1 B-CLL cells.* MEC1 B-CLL cells were cultured in the presence or absence of priming stimuli (anti-CD40 and IL-4) for 16 hrs followed by total protein isolation and immunoprecipitation with a CD86 antibody. PHB1 and CD86 protein levels in CD86 immunoprecipitates were analyzed via immunoblot.
Figure 45. *PHB1 expression levels are reduced in MEC1 B-CLL cells.* Either scrambled (*neg ctrl shRNA*) or PHB1-specific shRNA plasmids were transfected via nucleofection into MEC1 B-CLL cells to deplete PHB1 expression levels for 24 hours. Total mRNA (A) or protein (B) was collected after 48 hrs post transfection and was analyzed via real-time PCR or immunoblot analysis, respectively for levels of PHB1 mRNA relative to actin or protein relative to GAPDH. The data in (A) expressed as the PHB1 mRNA mean Fold Change ± SEM and represent three independent experiments. The data in (B) are expressed as the PHB1 protein mean % Change and a representative is shown from two independent experiments.
Figure 46. *PHB1 is involved with proliferation in MEC1 B-CLL cells.* Either scrambled (*neg ctrl shRNA*) or PHB1-specific shRNA plasmids containing a GFP reporter were transfected via nucleofection into MEC1 B-CLL cells to deplete PHB1 expression levels for 24 hours. The cells were then cultured over a four day time course. Proliferation was assessed by Flow Cytometry on the GFP$^+$ cell population. Representative histograms of the negative control shRNA (*red*) or PHB1 shRNA (*blue*) are shown from three independent experiments.
5.4 Discussion

A novel therapeutic approach for CLL patients is to promote a cytolytic T cell response against B-CLL cells through elevating the level of CD86, a protein expressed at very low levels on the B-CLL cell surface. Since previous reports showed that CD86 direct signaling to B cells to WEHI 279 murine B cells and human tonsillar B cells promoted proliferation (121, 124), it was possible that CD86 engagement on a B-CLL cell might cause a growth signal upon cell:cell contact with a cytolytic T cell. Thus, the purpose of the current study was to determine if CD86 engagement on MEC1 B-CLL cells signaled directly to induce B-CLL cellular proliferation. Here, we show for the first time that CD86 engagement increased the number of viable MEC1 B-CLL cells and NF-κB activation, as compared to cells where CD86 was not engaged. Also, we show that PHB1 expression levels are elevated in B-CLL cells. In addition, PHB1 associated directly with CD86 and is involved with cellular proliferation. Together, our findings suggest that a unique CD86 signaling complex exists within MEC1 B-CLL cells that mediates a direct signal upon engagement to promote a proliferative response, and that PHB1 may act as a novel oncoprotein in MEC1 B-CLL cells when it attaches to CD86.

Findings in the present study suggest that engagement of CD86 transmits a direct signal to MEC1 B-CLL cells to promote cellular proliferation. This is supported by a number of reports that provided strong evidence that CD86 possessed the capacity to signal directly to the B cell to cause an increase in the level of antibody produced in vitro (113, 118, 119, 122, 123) and in vivo (118, 119), providing a rationale to test the ability of CD86 to signal to B-CLL cells to promote a cellular response. The ability of CD86 to
promote cellular proliferation is linked with previous findings that showed that engagement of CD86 on WEHI 279 cells, a murine B cell lymphoma, promoted the activation of anti-apoptotic factors and induced proliferation (124). In addition, another report showed that CD86 engagement on anti-CD40/IL-4-primed human tonsillar B cells potentiated proliferation (121), suggesting that CD86 could signal directly to affect proliferation in both murine and human B cells. Of interest, while WEHI 279 murine B cells were primed with lipopolysaccharide (LPS), and human tonsillar B cells were primed with anti-CD40/IL-4 prior to CD86 engagement, findings in the current study suggest that CD86 engagement alone on a human MEC1 B-CLL cell line was sufficient to induce CD86-dependent signaling. This discrepancy may be due to either the differential levels of CD86 expressed on the cell surface below a level required to induce signaling, or the differential composition of the cytoplasmic domain of CD86 between murine and human CD86. While the murine cytoplasmic domain of CD86 is composed of three putative protein kinase C (PKC) phosphorylation sites, and one protein kinase A (PKA)/PKC phosphorylation site, the cytoplasmic domain of human CD86 contains nine potential serine/threonine phosphorylation sites. Since human CD86 contains many more potential phosphorylation sites, it is possible that multiple intracellular signaling proteins bind to the CD86 cytoplasmic domain upon engagement compared to murine CD86. Furthermore, phosphorylation of the cytoplasmic domain of CD86 is supported in the murine literature (120); (Lucas et. al. The Journal of Immunology. In Press. 2012), providing support for the potential for CD86-dependent signaling, as well as a potential mechanism in MEC1 B-CLL cells subsequent to CD86 engagement. Yet another possibility is that while priming with LPS and anti-CD40/IL-4 my have induced a
competency signal to allow for CD86 signaling in WEHI 279 and human tonsillar B cells, respectively, CD86 on the MEC1 B-CLL surface may possess a constitutive ability to signal directly upon engagement. Together, these findings suggest that CD86 engagement alone on the MEC1 B-CLL surface is able to mediate a direct signal to the human B-CLL to promote a cellular response, and defines a novel role for CD86 in the context of CLL.

Previous findings have suggested that CD86 associates with, and signals via, PHB1, a protein found to be over-expressed in many human cancers (238), and prohibitin-2 (PHB2), a highly related homolog that is not readily linked to human cancer, but is expressed on murine B cells to influence a cellular effect in the form of elevating the level of antibody produced (Lucas, et. al. *The Journal of Immunology. In Press.* 2012). This is consistent with findings in the current study that indicate an association between CD86 and PHB1. Although we have yet to establish a link between CD86 signaling and PHB1 in MEC1 B-CLL cells, findings in the current study suggest that PHB1 in addition to CD86 direct signaling is linked to proliferation. In addition to PHB1, it is possible that PHB2 also plays a role in mediating proliferation in MEC1 B-CLL cells. However, the focus of the current study was to specifically study PHB1, since PHB1 has been linked to the progression of many human malignancies (238) and this protein, as opposed to PHB2, increased expression in the MEC1 B-CLL cells after CD40L-induced activation. Future studies will determine whether or not PHB2 plays a role in both CD86 direct signaling and proliferation in CLL. Although the role of PHB1 in CLL is not well understood, one report showed an elevation of PHB1 in B-CLL cells treated with phorbol-ester (244) suggesting a potential role for PHB1 in activated B-CLL.
cells. The finding that PHB1 is involved with cellular proliferation is consistent with a previous report where PHB1 expression levels were stably depleted with shRNA in a variety of human cancer cell lines (173). PHB1 was also reported to be critical for Ras-mediated Raf activation (176), indicating a potential mechanism employed by PHB1 to control signaling networks that promote growth in cancer cells. Since PHB1 was found to associate with, and participate in, cell signaling induced by two receptors expressed on the B cell, i.e., the B cell receptor (BCR) (150, 159) and CD86 (Lucas, et. al. The Journal of Immunology. In Press. 2012), which also signals to activate pro-growth/-survival factors (118, 123, 206, 245), it is likely that PHB1 regulates pro-growth signaling networks within B-CLL cells. This is also supported by previous studies that provide evidence for PHB1 being critical for the activation of proximal signaling networks known to promote cellular growth including phosphatidylinositol 3,4,5-triphosphate (PIP3), Akt, and NF-κB (153, 154); (Lucas et. al. The Journal of Immunology. In Press. 2012). Although PHB1 was proposed to play a role as an oncoprotein in a variety of cancers, other reports have proposed a tumor suppressor role since PHB1 was reported to interact with retinoblastoma (Rb) in the nucleus to suppress E2F-mediated transcription (240), as well as bind to p53 to mediate p53-dependent transcriptional activity (246). Although the role of PHB1 in the context of CLL is not yet clear, our report is the first to provide evidence for PHB1 acting as a potential oncoprotein in CLL.

While findings in the current study suggest that CD86 and PHB1 are linked to proliferation in MEC1 B-CLL cells, what remains unclear is whether or not CD86 and PHB1 affect survival in B-CLL cells. Since previous reports and findings in the current
study suggest that CD86 signals directly to activate the pro-survival factor NF-κB (118, 123); (Lucas, et. al. The Journal of Immunology. In Press. 2012), and additional proximal signaling intermediates including Akt and PI3K (119, 123), it is possible that CD86 direct signaling promotes a pro-survival phenotype in B-CLL cells. Since a previous report showed elevated levels of NF-κB activation among B-CLL cells present in lymph nodes (206), it is likely that signals from the microenvironment including T cell:B-CLL cell contact where an interaction would occur between CD86 and its natural ligand CD28 could increase NF-κB activation. Likewise, previous reports have shown that PHB1 is directly involved with cell signaling pathways that affect survival including Ras-mediated Raf activation (176), and the PI3K/Akt pathway (153, 154) suggesting that PHB1 in addition to CD86 signaling may mediate a pro-survival phenotype in B-CLL cells. Future studies will address whether CD86 and PHB1 affect survival in B-CLL cells.

Although progress has been made to treat CLL, the disease remains incurable. Therefore, it is critical to explore novel therapeutic approaches. An attractive approach is to promote a cytolytic T cell response against B-CLL cells through enhancing the level of co-stimulation afforded to cytolytic T cells through the elevation of CD86, since levels of CD86 are underexpressed by B-CLL cells (233). However, as shown in the present study, if engagement of CD86 signals directly to induce proliferation (see Figure 47), this competitive effect may either predominate and/or compete with a cytolytic T cell response. Therefore, specifically targeting CD86 direct signaling to B-CLL cells potentially through inhibition of PHB1, could block the proliferative effect induced through CD86 engagement if a direct link exists between CD86 signaling and PHB1 in
B-CLL cells. Thus, a novel therapeutic approach to CLL could be to elevate the level of CD86 on the B-CLL to provide co-stimulation to induce a cytolytic T cell response, while inhibiting CD86 direct signaling, blocking B-CLL proliferation.
Figure 47. Working CD86 signaling model in MEC1 B-CLL cells. A novel therapeutic approach in B-CLL cells is to promote a cytolytic T cell response by increasing the level of co-stimulation afforded to the T cells via increasing the expression of CD86 on the B-CLL cell surface. However, our findings suggest that engagement of CD86 on the MEC1 B-CLL surface signals directly to promote cellular proliferation that may occur via an NF-κB-dependent mechanism. In addition, we show that PHB1 associates directly with CD86 and appears to be involved with proliferation. Therefore, understanding this novel signaling mechanism in B-CLL cells could lead to targeted therapies within the B-CLL to prevent cellular proliferation in the presence of cytolytic T cell therapy.
LITERATURE CITED


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