High-Throughput siRNA Screen in Dendritic Cells to Discover New Genes Regulating Immunologic Tolerance

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Immunology
University of Toronto

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University of Toronto

2014

ABSTRACT

The outcome of autoantigen recognition by the immune system—self-tolerance or autoimmune disease—is largely determined by the activation state of the dendritic cells (DCs) that uptake, process, and present autoantigens to autoreactive T cells. In particular, activation of dendritic cells to an immunogenic phenotype is necessary for the full activation of naïve autoreactive T cells, and thus necessary for the initiation of autoimmune disease. A better understanding of the cellular and molecular triggers and mechanisms of DC activation could catalyze the development of novel therapies to induce self-tolerance in patients with autoimmune disease. Towards the goal of discovering new genes and pathways that regulate DC activation and immunologic self-tolerance, we (1) developed a high-throughput siRNA screen in bone marrow-derived DCs and (2) show that BMDC expression of CD80, CD86, MHC II, and IL-12/23-p40 can identify candidate genes that regulate DC activation.
ACKNOWLEDGEMENTS

I would like to thank my research supervisor, Dr. Tak Mak, for giving me the great opportunity to work with him on this exciting project. He is a visionary scientific leader and innovator with whom it was an honour and privilege to work. I am grateful for his constant support, encouragement, and guidance in academic, professional, and personal spheres of life.

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<thead>
<tr>
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
</tr>
<tr>
<td>³H-thymidine</td>
<td>Tritiated thymidine</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td>BLIMPI</td>
<td>B lymphocyte-induced maturation protein 1</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCNB1</td>
<td>Cyclin B1</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine (C-C motif) receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDC</td>
<td>Classical or conventional dendritic cell</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Csf-2</td>
<td>Colony-stimulating factor 2</td>
</tr>
<tr>
<td>Csf-2R</td>
<td>Colony-stimulating factor 2 receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DI</td>
<td>Division index</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Term</td>
<td>Explanation</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sulfate sodium</td>
</tr>
<tr>
<td>DX</td>
<td>Dextran</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>Ebi3</td>
<td>Epstein-Barr virus-induced 3</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eYFP</td>
<td>Enhanced yellow fluorescent protein</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment, crystallizable</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Flt3</td>
<td>FMS-like tyrosine kinase 3</td>
</tr>
<tr>
<td>Flt3L</td>
<td>FMS-like tyrosine kinase 3 ligand</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence-minus-one</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ buffered salt solution</td>
</tr>
<tr>
<td>HEV</td>
<td>High-endothelial venule</td>
</tr>
<tr>
<td>I-A</td>
<td>Mouse MHC (H-2) I-A gene</td>
</tr>
<tr>
<td>I-A&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;i&gt;b&lt;/i&gt; allele of mouse MHC (H-2) I-A gene</td>
</tr>
<tr>
<td>I-E</td>
<td>Mouse MHC (H-2) I-E gene</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Interferon-α</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
</tbody>
</table>
Ig  Immunoglobulin
IL  Interleukin
IRES  Internal ribosome entry site
Itgb8  Integrin beta 8
iTr35  Induced T regulatory type 35
KI  Knockin
KO  Knockout
LCMV  Lymphocytic choriomeningitis virus
LDH  Lactate dehydrogenase
LN  Lymph node
LPS  Lipopolysaccharide
Ly6C  Lymphocyte antigen 6C
M  Molar
M. tuberculosis  Mycobacterium tuberculosis
MACS  Magnetic-activated cell sorting
MAD  Median absolute deviation
MFI  Median fluorescence intensity
MHC  Major histocompatibility complex
MHC I  Major histocompatibility complex class I
MHC II  Major histocompatibility complex class II
mL  Millilitre
mM  Millimolar
mm  Millimetre
MR  Mineralocorticoid receptor
mU  Milliunits
NFkB1  Nuclear factor of kappa light chain polypeptide gene enhancer in B cells 1
ng  Nanogram
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain-like receptor</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomoles</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NR0B2</td>
<td>Nuclear receptor subfamily 0, group B, member 2</td>
</tr>
<tr>
<td>NR3C2</td>
<td>Nuclear receptor subfamily 3, group C, member 2</td>
</tr>
<tr>
<td>NT</td>
<td>Non-targeting</td>
</tr>
<tr>
<td>OTE</td>
<td>Off-target effects</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pattern associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>Polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>Prdm1</td>
<td>Positive regulatory domain zinc finger protein 1</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>Ptpn6</td>
<td>Protein tyrosine phosphatase non-receptor type 6</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RBBP8</td>
<td>Retinoblastoma binding protein 8</td>
</tr>
<tr>
<td>RBBP8NL</td>
<td>Retinoblastoma binding protein 8 N-terminal like</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RIP-GP</td>
<td>Rat insulin promoter-lymphocytic choriomeningitis virus glycoprotein</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RLR</td>
<td>Retinoic acid-inducible gene I-like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SGOT</td>
<td>Serum glutamic oxaloacetic transaminase</td>
</tr>
<tr>
<td>SHP</td>
<td>Small heterodimer partner</td>
</tr>
<tr>
<td>$Shp^{-/-}$</td>
<td>Small heterodimer partner gene knockout</td>
</tr>
<tr>
<td>SHP-1</td>
<td>Src homology region 2 domain-containing phosphatase 1</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokine signaling 1</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper cell type 1</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper cell type 17</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper cell type 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TLR-L</td>
<td>Toll-like receptor ligand</td>
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<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
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<td>Tumour necrosis factor-α-induced protein 3</td>
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<td>T regulatory type 1</td>
</tr>
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<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>V</td>
<td>Volts</td>
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<tr>
<td>VCT</td>
<td>Violet CellTrace</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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<td>Symbol</td>
<td>Description</td>
</tr>
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<td>-------------</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
<td>$z^*$ score</td>
<td>Robust z score</td>
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<td>$\mu F$</td>
<td>Microfarad</td>
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<td>$\mu g$</td>
<td>Microgram</td>
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<tr>
<td>$\mu L$</td>
<td>Microlitre</td>
</tr>
<tr>
<td>$\mu M$</td>
<td>Micromolar</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Ohm</td>
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CHAPTER 1: INTRODUCTION

1.1 AUTOIMMUNE DISEASE AND IMMUNOLOGIC TOLERANCE

Autoimmune diseases are a major cause of morbidity and mortality, afflicting approximately 3-10% of the population in Western countries\textsuperscript{1-3}. There are more than 80 distinct autoimmune diseases, most of which are chronic conditions that often manifest debilitating and life-threatening complications\textsuperscript{4}. Current therapies for autoimmune diseases are suboptimal because they often cause generalized immunosuppression, which predisposes to serious infections and cancer\textsuperscript{5}. Moreover, these therapies fail to correct the fundamental biological defect underlying autoimmune disease pathogenesis: loss of immunologic self-tolerance\textsuperscript{6}.

Towards the goal of addressing the clinical problem of autoimmune diseases, the overall objective of this work is to better understand the cellular and molecular mechanisms of immunologic self-tolerance. Greater understanding of these mechanisms will hopefully lead to better therapies for autoimmune diseases in the clinic.

We have chosen to focus our research on T cell tolerance because most autoimmune diseases are T cell-dependent. This is because the humoral (i.e., B cell) immune response in antibody- and immune complex-mediated autoimmune diseases also requires T cell help for immunoglobulin isotype class switching and immunoglobulin variable region somatic hypermutation\textsuperscript{7}.

1.1.1 Central Tolerance

The physiologic function of the immune system is defense against foreign substances, particularly microorganisms\textsuperscript{8}. To that end, the immune system possesses an immense repertoire
of lymphocytes whose diverse specificities can recognize a multitude of foreign invaders\textsuperscript{9}. However, this extreme diversity in antigen recognition is inextricably linked with self-reactivity and therefore predisposition to autoimmune disease. This is because the normal mechanisms of T cell development guarantee that mature T cells will not only be able to recognize foreign antigens, but also self antigens\textsuperscript{9}. During the process of thymic positive selection, developing T cells (thymocytes) are positively selected for survival when their T cell receptors (TCRs) engage self-peptide/MHC complexes with intermediate to high affinity\textsuperscript{10}. Therefore, all mature T cells are inherently self-reactive. Every T cell that can recognize a foreign antigen can also recognize at least one cross-reactive self-peptide, i.e., the one that selected it for survival during its development in the thymus\textsuperscript{11}.

Following thymic positive selection, the process of thymic negative selection purges the developing T cell repertoire of those T cells whose TCRs can interact with high affinity to self-peptide/MHC complexes\textsuperscript{10}. This helps to ensure that the mature T cell repertoire can only interact with self-peptide/MHC complexes with low or intermediate affinity. However, this clonal deletion is incomplete because not every self-peptide in the body is expressed in the thymus\textsuperscript{11}. Therefore, high-affinity self-reactive T cells can escape from the thymus into the mature T cell repertoire in the periphery (i.e., in the secondary lymphoid organs and peripheral tissues)\textsuperscript{11}. The net result is that every mature T cell in the peripheral T cell repertoire is self-reactive with either low, intermediate, or high affinity.

1.1.2 Peripheral Tolerance

If every T cell is inherently self-reactive, then why are we not all afflicted with autoimmune diseases? Indeed, every time a T cell is activated by a foreign antigen, there should be a pathologic cross-reaction to the self antigen that positively selected that T cell in the
thymus\textsuperscript{11}. This response could be especially damaging if the cross-reacting T cell was a high-affinity T cell that had escaped negative selection. In fact, there is no epidemic of autoimmune diseases because the immune system possesses elegant mechanisms to regulate self-reactive T cells in the periphery. These peripheral mechanisms of tolerance, which include anergy, deletion, and suppression, act to prevent mature peripheral T cells from becoming inappropriately activated and/or acquiring tissue-damaging effector functions\textsuperscript{12}.

In summary, two events must occur in order for a self-reactive T cell to become autoaggressive and cause tissue damage and disease: (1) there must be a failure of one or more of the central or peripheral tolerance mechanisms, and (2) the self-reactive T cell must become activated (Figure 1.1). Thus, we can conclude that if we wish to develop better therapies for autoimmune diseases in the clinic, we need to better understand the mechanisms of self-tolerance and the mechanisms of T cell activation.
1.2 THE CENTRALITY OF THE DC IN IMMUNITY AND TOLERANCE

1.2.1 The DC Activation State Determines the Outcome of Antigen Recognition

What determines whether a naïve self-reactive T cell in the periphery remains tolerant to self or becomes autoaggressive? A major part of the answer is the activation state of the dendritic cell (DC). This is because a naive T cell can recognize antigen only when a DC endocytoses and
processes antigen into peptide fragments that are presented to the T cell in the context of a self-MHC molecule on the surface of the DC\textsuperscript{13}.

How does this DC-T cell interaction determine the balance between tolerance versus immunity? The classic paradigm is that activation of a naïve T cell requires three collaborative but distinct signals\textsuperscript{14}: signal 1, signal 2, and signal 3 (Figure 1.2). Signal 1 is delivered to the T cell when the TCR engages a peptide/MHC complex on the surface of a DC. Signal 2 and signal 3 are delivered to the T cell when the DC is activated via its pattern recognition receptors (PRRs, e.g., Toll-like receptors (TLRs)). In the presence of infection or inflammation, pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) ligate PRRs and cause a profound phenotypic transformation in the DC that results in (1) the upregulation of specific combinations of costimulatory molecules (signal 2) on the DC surface and (2) the production of cytokines (signal 3) by the DC. The delivery of these three signals to the T cell by the DC, and their subsequent integration by the T cell, determines the overall phenotype of the immune response, i.e., the phenotype of the resulting effector T cells and the nature of their effector mechanisms\textsuperscript{14}.

In contrast, in the steady state, i.e., in the absence of infection or inflammation, there are no PAMPs or DAMPs present to ligate PRRs. Therefore, the DC presents signal 1 without signal 2 or 3. The result is T cell tolerance by anergy, deletion, and/or regulatory T cell (Treg) differentiation (Figure 1.3)\textsuperscript{14}. 
Figure 1.2. The classic three-signal model of naive T cell activation. In the presence of PAMPs or DAMPs (e.g., infection or inflammation), DCs are activated and therefore present Signal 1, Signal 2, and Signal 3 to naive T cells. The result is immunity. The magnitude and effector phenotype of the immune response depends on the quantity and quality of these three signals and their integration by the T cell. Adapted with permission from Coates, et al. *Expert Reviews in Molecular Medicine* 4, 1-21 (2002), with the following modifications: (1) In the original figure, a single arrow to the right of the T cell pointed to the words “Th1 response”. I substituted the red arrows and the images of effector cells containing the labels “Th1”, “Th2”, “Th17”, and “CTL”. (2) I added the word “foreign” to the label “peptide”. (3) I changed the label “Donor DC” to “Immunogenic DC”. (4) I added the PRR molecule and its label to the DC. (5) I added the images of PAMPs and DAMPs and the red curved arrow, together with the labels “PAMPs/DAMPs” and “Infection/Inflammation”, to the top left side of the figure.
Figure 1.3. The classic one-signal model of naive T cell tolerance. In the absence of PAMPs or DAMPs (e.g., infection or inflammation), DCs are not activated and therefore present Signal 1 without Signal 2 or Signal 3. The result is T cell tolerance. Adapted with permission from Coates, et al. Expert Reviews in Molecular Medicine 4, 1-21 (2002), with the following modifications: (1) In the original figure, a single arrow to the right of the T cell pointed to the words “Th1 response”. I substituted the red arrows and the images of cells containing the labels “Anergy”, “Deletion”, and “Treg”. (2) I added the word “self” to the label “peptide”. (3) I changed the label “Donor DC” to “Tolerogenic DC”. (4) I superimposed black lines over the labels “Signal 2” and “Signal 3”. (5) I superimposed a black “X” over the images of the cytokine molecules and the CD80/86 and CD28 molecules. (6) I added the PRR molecule and its label to the DC. (7) I added the images of PAMPs and DAMPs and the red curved arrow, together with the labels “PAMPs/DAMPs” and “Steady state”, to the top left side of the figure. (8) I superimposed a black “X” over the images of the PAMPs and DAMPs.

In summary, the outcome of T cell antigen recognition, i.e., immunity versus tolerance and the phenotype of the resulting immune response, depends on the activation state of the DC.
Only DCs that have been activated by PAMPs or DAMPs via their PRRs—thereby upregulating costimulatory molecules and producing cytokines—can initiate a primary immune response by activating naïve T cells. Thus, there is no autoimmune disease without T cell activation, and there is no T cell activation without DC activation (Figure 1.4).

**Figure 1.4. The role of dendritic cells in autoimmune disease pathogenesis.** Adapted and modified from *Autoimmune Diseases: Overview*. Washington: Office on Women’s Health, United States Department of Health and Human Services, 2010. Online at womenshealth.gov. Free of copyright restrictions.

1.2.2 The DC is the Bridge Between the Innate and Adaptive Immune Systems

The widespread tissue distribution of DCs is crucial to their central role in
immunoregulation. DCs are present in most nonlymphoid tissues\textsuperscript{15}, where they serve as the sentinels of the immune system, constantly patrolling peripheral tissues for the presence of danger or infection\textsuperscript{13}. They are equipped with a diverse set of PRRs, including Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (CLRs), which recognize a multitude of diverse PAMPs and DAMPs\textsuperscript{16}. In addition, they constantly sample their environment via endocytosis\textsuperscript{17}. When DCs are activated by PRR ligation, they migrate through afferent lymphatic vessels to the T cell zones of the tissue-draining lymph nodes (LNs), where they present their captured antigens to antigen-specific T cells to initiate the adaptive immune response\textsuperscript{17}.

The migratory ability of DCs is strictly dependent on CCR7, a chemokine receptor that is upregulated on the DC surface upon PRR ligation-induced DC activation\textsuperscript{18}. Afferent lymphatic endothelial cells express CCL21 and CCL19, which bind to CCR7 and thereby mediate DC chemotaxis and migration via lymphatics to the CCL19- and CCL21-expressing T cell zones of the draining lymph nodes\textsuperscript{19,20}. In parallel, naive T cells, which also express CCR7, continuously recirculate from the blood into the LNs, and via lymphatics back into the blood, searching for their cognate antigens\textsuperscript{21}. The antigen-bearing DCs that migrate from peripheral tissues penetrate the LN paracortex and preferentially settle around high-endothelial venules (HEVs), which constitutively express CCL21. This positioning is strategic, as it (1) maximizes the probability of encountering newly arriving antigen-specific T cells, and (2) assures that the newly arriving naive T cells will first be exposed to DCs that have recently been in the peripheral tissues, and thus posses the most up-to-date information about the dangerous or infectious battlefield\textsuperscript{22}.

Thus, the DC is the crucial bridge between the innate and adaptive immune systems\textsuperscript{23}. By
sensing and transmitting information from the peripheral front lines to T cells in the LNs, it regulates the initiation of cellular and humoral immune responses. In recognition of this, the 2011 Nobel Prize in Physiology or Medicine was awarded to Professors Jules Hoffman, Bruce Beutler, and Ralph Steinman for their groundbreaking research in this area. Professors Hoffman and Beutler discovered the PRR molecular sensor system in insects\textsuperscript{24} and mammals\textsuperscript{25}, respectively, while Professor Steinman discovered the dendritic cell and its role in activating adaptive immunity\textsuperscript{26-28}.

1.3 MURINE DC SUBTYPES IN VIVO

In vivo, DCs can be divided into two main categories: conventional or classical DCs (cDCs) and plasmacytoid DCs (pDCs)\textsuperscript{15}. Morphologically resembling plasma cells, pDCs are activated by nucleic acid PAMPs through TLR7 and TLR9 to produce enormous amounts of IFN-\(\alpha\) and to specifically prime viral antigen-specific T cells\textsuperscript{15}. By contrast, cDCs have a dendritic morphology and are dedicated APCs, expressing high levels of MHC II\textsuperscript{23}.

In the mouse, cDCs can be broadly subdivided into two distinct subsets: (1) lymphoid CD8+ cDCs and their nonlymphoid CD103+ counterparts, and (2) CD11b+ cDCs\textsuperscript{15}. Importantly, there is significant functional and genetic heterogeneity within each subset\textsuperscript{23}: some may be superior at antigen presentation, T cell priming, or lymphatic migration, others may preferentially sense pathogens by expressing higher levels of TLRs, and others may be more efficient at secreting immunoregulatory cytokines. In general, CD8+ and CD103+ cDCs are highly efficient at presenting exogenously-derived antigens to CD8+ T cells (cross-presentation), whereas CD11b+ cDCs are thought to preferentially present endogenously-derived antigens to CD4+ T cells\textsuperscript{23}.
To date, no cDC-specific cell surface markers have been found. All cDCs constitutively express CD45, MHC II, and CD11c, and lack markers of the granulocyte, erythrocyte, natural killer (NK) cell, B cell, and T cell lineages\textsuperscript{15}. Notably, CD11c has also been found on lung, intestinal, and splenic macrophages, as well as on monocytes, activated T cells, and NK cells\textsuperscript{15}. High expression of both CD11c and MHC II is also unable to distinguish cDCs; in certain tissues, such as the lung and the intestine, macrophages express levels of MHC II that are as high as those expressed by cDCs\textsuperscript{15}. Moreover, macrophages, like cDCs, upregulate MHC II expression upon activation\textsuperscript{29}.

Intracellularly, few genes are uniquely expressed by cDCs\textsuperscript{15}. However, a comprehensive transcriptional analysis of cDC subsets recently identified a molecular signature that distinguishes cDCs from macrophages in peripheral tissues\textsuperscript{30}. This molecular signature includes the receptor tyrosine kinase FMS-like tyrosine kinase 3 (Flt3), which plays a pivotal role in the regulation of cDC development; CCR7, essential for DC migration; e-Kit, the receptor for stem cell factor; and Zbtb46, a zinc finger transcription factor\textsuperscript{15,30}.

Flt3 ligand (Flt3L) and granulocyte-macrophage colony-stimulating factor (GM-CSF, also known as colony stimulating factor 2 (Csf-2)) are the most important cytokines that regulate cDC development \textit{in vivo}\textsuperscript{31}. Genetic ablation of Flt3 or Flt3L in mice greatly reduces the numbers of DC progenitors, tissue cDCs, and pDCs\textsuperscript{32,33}, whereas systemic administration or conditional overexpression of Flt3L dramatically expands all DC subsets\textsuperscript{34,35}. Underscoring the importance of Flt3L is its ubiquitous expression; it is produced by activated T cells, endothelial cells, and connective tissue cells in multiple organs\textsuperscript{15}. Furthermore, the Flt3L receptor, Flt3, is expressed at all stages of DC development and is also maintained on terminally differentiated cDCs and pDCs, but not on macrophages\textsuperscript{31,36}.
Genetic ablation of GM-CSF or its receptor, Csf-2R, in mice causes differential effects in lymphoid and nonlymphoid tissue. In spleen and LN, GM-CSF or Csf-2R deficiency causes only minor reductions in the DC pool\(^37\). By contrast, in peripheral tissues, GM-CSF deficiency causes substantial reductions in DC numbers\(^38\). These data suggest that GM-CSF is an important regulator of cDC development in nonlymphoid tissues rather than lymphoid ones, which is consistent with the evidence for relatively high production of GM-CSF in peripheral tissues\(^38,39\).

GM-CSF can be produced by many cell types, including epithelial cells, endothelial cells, monocytes, macrophages, mast cells, and activated T cells\(^40\), while Csf-2R is expressed on cDCs and their progenitors.

### 1.4 MURINE IN VITRO-DERIVED cDCs

#### 1.4.1 Flt3L-Cultured BMDCs and GM-CSF-Cultured BMDCs

There are two main approaches to the study DCs in vitro: (1) endogenous (in vivo) DCs can be isolated, or (2) DCs can be differentiated in vitro from isolated hematopoietic progenitors\(^41\). The disadvantage of the former approach is that cell yields are low; approximately 0.1-1 x 10\(^6\) DCs can be isolated from one mouse spleen\(^42\). By contrast, up to approximately 300 x 10\(^6\) DCs can be generated from a single mouse by differentiating them in vitro from bone marrow (BM) cells containing hematopoietic precursors, as will be described below. Two methods are commonly used to generate bone marrow-derived DCs (BMDCs) in vitro: (1) culturing BM cells in the presence of Flt3L, and (2) culturing BM cells in the presence of GM-CSF\(^41\).

In the Flt3L system, BM cells are cultured with Flt3L for nine days to generate three distinct cell types that are phenotypically and functionally analogous to splenic pDCs, CD8+...
cDCs, and CD11b+ cDCs. As such, and coupled with the evidence for the critical in vivo role of Flt3L in DC development presented above, it has been proposed that the Flt3L culture system is a true representation of steady-state DC development from BM progenitors.

Inaba et al. were the first to differentiate BMDCs by culturing BM cells with GM-CSF. This method generates approximately 5 x 10^6 CD11b+ cDCs from one mouse after 6-8 days of culture. By rationally modifying Inaba et al.’s culture conditions, Lutz et al. developed a robust method to generate up to 300 x 10^6 cDCs per mouse after 10-12 days. Instead of culturing BM cells in standard 24-well tissue culture plates, Lutz et al. cultured BM cells in 100 mm bacteriological Petri dishes, which may have enhanced DC development by impairing macrophage development, which can inhibit DC development. Lutz et al. did not deplete any cell types from the starting BM suspension, which may have averted damage to, and/or removal of, critical DC progenitors. Lutz et al. performed three medium changes throughout the culture period without discarding any cells. Additionally and importantly, the DC purity generated by the Lutz et al. method at the end of the standard culture period was 80-90%, compared to 60% purity generated by the Inaba et al. method. Lutz et al. reported generating an average of 9.2 x 10^6 BMDCs per Petri dish. In my hands, the average yield is 10-15 x 10^6 BMDCs per dish (data not shown). As up to 20 Petri dishes can usually be prepared from one mouse BM preparation, the standard maximum yield from one mouse is approximately 300 x 10^6 BMDCs.

Functionally, Lutz et al. used the fluorescein isothiocyanate (FITC)-dextran (DX) endocytosis assay to demonstrate that during the culture period there is a continuous transition of BMDCs from an immature to a mature phenotype. According to the classic paradigm, immature DCs, which express low levels of MHC II, actively uptake antigen via endocytosis; by contrast, mature DCs, which express high levels of MHC II, do not. Thus, DC
maturation may be identified by a reduction in the frequency of MHC II$^{\text{low}}$/DX$^{\text{pos}}$ DC population and a concomitant increase in the frequency of the MHC$^{\text{high}}$/DX$^{\text{neg}}$ or MHC$^{\text{high}}$/DX$^{\text{low}}$ DC population. Lutz et al. found that the starting BM cells were MHC II- and could not endocytose FITC-DX (DX$^{\text{neg}}$). By day 7, the majority of the cells were MHC II$^{\text{low}}$/DX$^{\text{pos}}$, while only a minority were MHC II$^{\text{high}}$/DX$^{\text{neg}}$. Beyond day 10, there was an increasing frequency of MHC II$^{\text{high}}$/DX$^{\text{low}}$ and MHC II$^{\text{high}}$/DX$^{\text{neg}}$ BMDCs.

There is evidence that GM-CSF-cultured BMDCs may be more representative of so-called “inflammatory” Ly6C$^{\text{high}}$ monocyte-derived cDCs that develop in response to infection or inflammation$^{52}$, rather than of steady-state cDCs. First, it was demonstrated that GM-CSF-cultured BMDCs produced more inflammatory cytokines upon TLR ligation than Flt3-cultured BMDCs$^{53}$. Second, it has been shown that GM-CSF-cultured BMDCs arise from a sequential developmental transition: from early myeloid precursors (CD31$^{\text{high}}$Ly6C$^{-}$), to monoblasts (CD31$^{+}$Ly6C$^{+}$), to monocytes (CD31$^{+}$Ly6C$^{\text{high}}$), to BMDCs (CD31$^{+}$Ly6C$^{-}$)$^{54}$. Thus, Ly6C$^{\text{high}}$ monocytes, which are believed to be the direct precursors of endogenous inflammatory cDCs, were also shown to be the direct precursors of GM-CSF-cultured BMDCs.

1.4.2 The Biochemical Profile of Fetal Bovine Serum Influences BMDC Differentiation

In a separate study, Lutz et al. demonstrated that the biochemical profile of the fetal bovine serum (FBS) used to generate BMDCs in vitro has a critical effect on their differentiation and baseline maturation level$^{55}$. Different lots of FBS, even from the same commercial vendor, were shown to induce the differentiation of BMDCs with widely varying levels of surface MHC II expression.
Specifically, different FBS lots produced either predominantly MHC II$^{\text{high}}$ mature BMDCs, balanced levels of all BMDC subsets, predominantly resting immature BMDCs, or completely immature MHC II$^{\text{neg}}$ myeloid precursors. Notably, these different baseline MHC II expression levels did not correlate with the variable concentrations of endotoxin (i.e., a PAMP) in the different FBS lots. Rather, this differential MHC II phenotype was associated with the concentration of two enzymes in the FBS preparation: lactate dehydrogenase (LDH) and serum glutamic oxaloacetic transaminase (SGOT, also known as aspartate aminotransferase (AST)). High concentrations of LDH (greater than 700 mU/mL) and SGOT (greater than 50 mU/mL) correlated with the generation of a high frequency of mature BMDCs (MHC II$^{\text{high}}$/FITC$^{-}$/DX$^{-}$), whereas low concentrations of LDH (less than 300 mU/mL) and SGOT (less than 12 mU/mL) correlated with an inhibition of DC outgrowth and the generation of a high frequency of immature BMDCs (MHC II$^{\text{low}}$/FITC-DX$^{\text{pos}}$).

As LDH and SGOT are found in the liver, heart, and skeletal muscle and are released into the systemic circulation in response to myocardial infarction, stress, or infection, Lutz et al. postulated that these enzymes are surrogate markers for the presence of unidentified DC-activating danger signals that are released into the bovine circulation following slaughter and that are therefore present in commercial FBS.

1.5 DC-DERIVED CYTOKINES (SIGNAL 3) IN ADAPTIVE IMMUNITY

1.5.1 DC Maturation vs. DC Activation

The gold standard approach for determining whether a DC is truly immunogenic is to test whether it can induce an adaptive immune response in vivo, i.e., the clonal activation, expansion, and differentiation of cognate antigen-specific T cells into effector T cells$^{14}$. According to the
classic paradigm of T cell activation discussed above (Chapter 1.2.1), a DC must deliver Signals 1, 2, and 3 in order to induce an adaptive T cell response; if it delivers Signal 1 alone, then the outcome is T cell anergy, T cell deletion, or Treg cell differentiation.

However, in certain circumstances, it has been shown that a DC may deliver Signal 1 and Signal 2 in the absence of Signal 3\textsuperscript{56-60}. What is the outcome of the DC-T cell encounter in these cases? The critical parameter that determines whether the naive T cell differentiates into an effector T cell is the presence or absence of Signal 3, i.e., DC-derived pro-inflammatory cytokines. Although these DCs bear Signal 2, expressing high levels of so-called “maturation markers” such as MHC II, CD80, CD86, CD40, and CCR7, they fail to prime effector T cell responses if they do not produce pro-inflammatory cytokines\textsuperscript{56-60}. These DCs generally do stimulate robust T cell proliferation, but the T cells do not acquire effector functions. Therefore, these DCs are not immunogenic.

Three conclusions can be drawn from these important data. First, it is the production of DC-derived pro-inflammatory cytokines, not costimulation, that enables a DC-T cell cognate interaction to prime T cell immunity. Second, DCs that express high levels of costimulatory molecules or other “maturation markers” may not be immunogenic. Third, the terms “mature DC” and “DC maturation” may be misleading, falsely implying immunogenicity, if their intention was only to indicate the presence of upregulated Signal 2. In light of these conclusions, it has been proposed that the terms “mature DC” and “DC maturation” be used only to indicate the presence of upregulated Signal 2, while the terms “activated DC” and “DC activation” be used to indicate true DC immunogenicity\textsuperscript{14,61}. 
1.5.2 The RIP-GP DC Vaccination Model of Autoimmune Diabetes

The RIP-GP DC vaccination model of autoimmune diabetes\textsuperscript{62} is an example of an \textit{in vivo} system in which autoreactive T cell fate and disease outcome are regulated by the activation state of the DC. Thus, it is a robust \textit{in vivo} system for testing the activation state and immunogenicity of DCs.

RIP-GP mice express the glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV) as a neoself antigen in pancreatic islet beta cells under the control of the rat insulin promoter (RIP)\textsuperscript{63}. In RIP-GP mice, GP-specific T cells are not tolerized in the thymus or in the periphery. Rather, they ignore the neoself antigen. Autoimmune attack of islet beta cell-expressing GP, leading to clinical diabetes, occurs only when GP-specific T cells become activated and therefore differentiate into diabetogenic cytotoxic T cells. This may occur, for example, by infecting the mice with LCMV\textsuperscript{63} or by adoptively transferring immunogenic DCs expressing an immunodominant epitope of GP\textsuperscript{64}.

Building upon this system, Dissanayake et al. developed an experimental DC vaccination protocol in which GP-specific T cell activation and thus clinical diabetes are regulated by the activation state of the DC\textsuperscript{62}. When RIP-GP mice are vaccinated with bone marrow-derived DCs (BMDCs) that have been activated with a TLR ligand and pulsed with LCMV-GP peptides, the RIP-GP mice develop diabetes. In contrast, diabetes does not result when RIP-GP mice are vaccinated with GP peptide-pulsed BMDCs that have not been activated with a TLR ligand (i.e., unactivated BMDCs). Thus, in this system, there is no GP-specific T cell activation, and therefore no clinical diabetes, unless there is prior DC activation.
Importantly, the RIP-GP DC vaccination model corroborates the evidence that Signal 3 is the critical parameter that enables a DC-T cell cognate interaction to prime T cell immunity. Dissanayake et al.\textsuperscript{62} showed that in the absence of TLR-ligand (TLR-L) stimulation, NFkB1-deficient BMDCs induced diabetes upon adoptive transfer into RIP-GP mice. However, compared to wild-type (WT) control BMDCs, these NFkB1-deficient BMDCs did not upregulate MHC I, MHC II, CD80, CD86, or CD40 expression, but did upregulate TNF-α and IL-6 production. Thus, BMDCs delivering Signal 3 (and Signal 1) without Signal 2 were shown to be immunogenic.

1.5.3 The IL-12 Family of Cytokines

The IL-12 family of cytokines is at the heart of immunoregulation, mediating control of major stimulatory and inhibitory effector mechanisms in the immune system\textsuperscript{65}. This family includes IL-12, IL-23, IL-27, which are produced by DCs, and IL-35, which is produced by Treg cells. It is the only family of heterodimeric cytokines, and displays significant chain-pairing promiscuity: IL-12 and IL-23 share the same β-chain (p40), IL-12 and IL-35 share the same α-chain (p35), and IL-27 and IL-35 share the same β-chain (Ebi3).

Interestingly, IL-12 and IL-23 are stimulatory cytokines, while IL-27 and IL-35 are inhibitory cytokines. These interconnected mediators regulate diverse and opposing effects, strategically influencing the phenotype of the immune response. Some key highlights of this immunoregulatory network are discussed below.

By virtue of IL-12 and IL-23, this cytokine family is critically involved in the differentiation of dominant effector T cell subsets. DC-derived IL-12 induces naive T cells to differentiate into IFN-γ-producing CD4+ Th1 cells, which activate macrophages and promote
cell-mediated immunity against intracellular pathogens. IL-12 is also instrumental in differentiating naive T cells into CD8+ cytotoxic T lymphocytes (CTLs), which mediate the killing and clearance of virus-infected and transformed cells. DC-derived IL-23 stabilizes IL-17 production by Th17 cells, which play a key role in defense against extracellular pathogens.

On the inhibitory side, DC-derived IL-27 has been shown to inhibit Th17 cell development and to induce IL-10-producing anti-inflammatory Tr1 cells. IL-35, the lone member of the family not produced by DCs, is produced by Treg cells and is necessary for their maximal suppressive effects. Treg-derived IL-35 can also induce the differentiation of iTreg cells, which are Treg cells that lack Foxp3, TGF-β, or IL-10 and suppress via IL-35.

In light of the requirement for Signal 3 in the induction of adaptive immunity, and the preeminent role of IL-12 and IL-23 in the differentiation and/or function of Th1 and Th17 cells, one can reasonably conclude that if an IL-12-producing or IL-23-producing DC population is differentiated in vitro or in vivo, it is not only immunogenic, but also likely to exert a significant and dominant immune effect.

### 1.6 DC-INTRINSIC REGULATION OF AUTOIMMUNITY AND INFLAMMATION

In addition to NFkB1 (discussed above), a number of other genes have been found whose deletion in DCs induces spontaneous autoimmune or inflammatory manifestations. When the integrin αVβ8 (Itgb8) is deleted in DCs, latent TGF-β cannot be activated to induce Treg cell differentiation, and the result is polyclonal immune activation and colitis. DC-specific deletion of the transcription factor STAT3 (Stat3) renders DCs refractory to IL-10-induced inhibitory signals, causing excessive Th1 cell differentiation and ileocolitis. DCs deficient in the transcriptional repressor BLIMP1 (Prdm1) spontaneously produce IL-6 and induce T cell help for the differentiation of anti-DNA antibody-producing plasma cells; the result is a disease
resembling systemic lupus erythematosus (SLE)\textsuperscript{75}. DC-specific deletion of the protein tyrosine phosphatase SHP1 (Ptn6) also causes an SLE-like disease due to spontaneous DC maturation, enhanced proinflammatory cytokine release, and enhanced Th1 cell differentiation\textsuperscript{76,77}. Finally, two separate DC-specific A20 (Tnfaip3)-deficient mouse strains exhibit evidence of autoimmune disease. In the first strain, ankylosing spondylitis and colitis result from spontaneous DC maturation, enhanced proinflammatory cytokine release, and Th1 cell differentiation\textsuperscript{76,77}. In the second strain, reduced apoptosis leads to spontaneous DC maturation, increased proinflammatory cytokine release, enhanced presentation of apoptotic cells by DCs, and the development of an SLE-like disease\textsuperscript{78,79}.

The protein products of these genes are either (1) upstream or downstream of key anti-inflammatory signals in DCs, or (2) negative regulators of pro-inflammatory signals in DCs. However, their expression is not unique to DCs. Rather, they are general immune regulators whose genetic ablation in other cell types results in similar manifestations\textsuperscript{23}. Given the centrality of the DC in T cell activation and the identification of a DC-specific transcriptional signature\textsuperscript{30}, it is tantalizing to speculate that DC-specific negative regulators of DC activation exist and play an important role in immunologic tolerance\textsuperscript{23}.

1.7 RNA INTERFERENCE (RNAi)

RNAi is a genetic regulatory mechanism by which small RNAs mediate specific post-transcriptional gene silencing\textsuperscript{80}. Initially discovered in \textit{Caenorhabditis elegans} and later found to exist in plants and mammals, this mechanism is a key component of the innate defense arsenal against viral infection\textsuperscript{81}. 
The explosion in RNAi research over the last decade has not only revolutionized our understanding of RNA and gene regulation, but also our approach to studying loss-of-function phenotypes. Indeed, RNAi has proven to be an effective and efficient alternative to insertional mutagenesis and gene ablation by homologous recombination\textsuperscript{80,82}. Moreover, it has been increasingly harnessed to facilitate high-throughput, genome-scale study of diverse biological processes in a wide variety of cells, tissues, and organisms\textsuperscript{83}.

The small RNA molecules that mediate RNAi include (1) endogenous microRNA and (2) exogenous small interfering RNA (siRNA) and short hairpin RNA (shRNA)\textsuperscript{80}. When these duplex molecules are incorporated into the RNA-induced silencing complex (RISC), the antisense ("guide") strand directs the RISC to the complimentary region of a target mRNA. The result is mRNA degradation or translation blockade and the suppression of gene expression\textsuperscript{80}.

However, this complementarity-based gene specificity is not absolute. Nonspecific, off-target effects (OTEs) are sometimes an unintended consequence of RNAi. In the case of siRNA, a major mechanism of OTEs is complementarity between off-target mRNA 3’ untranslated regions (UTRs) and siRNA “seed regions” (nucleotide positions 2-8 of the guide strand). Importantly, a number of strategies exist for mitigating OTEs. One is siRNA redundancy, which uses multiple effective siRNAs to enable separation of OTEs from target-dependent effects\textsuperscript{84}. Another strategy is chemical modification of the guide strand seed region, which decreases nonspecific interactions with unintended targets\textsuperscript{80}.

Even when RNAi is specific, it can lead to unintended stimulation of the immune system. The recognition of duplex RNA molecules by RNA-binding molecules such as TLR3, TLR7, TLR8, and RIG-I, can lead to the induction of innate responses mediated by type I interferon, IL-6, and TNF-\textalpha\textsuperscript{85}. In the case of siRNA, this innate immune induction may be dependent on a
number of factors, including siRNA sequence and length. GU-rich regions and longer duplexes are more immunostimulatory\textsuperscript{80}.

1.8 RATIONALE, HYPOTHESIS, AND OBJECTIVES

On the basis of (1) the centrality of DCs in the activation of self-reactive T cells and thus the pathogenesis of autoimmune disease, (2) the existence of cell-intrinsic negative regulators of DC activation, and (3) the demonstrated utility of high-throughput RNAi screens to discover new cellular and molecular pathways in biology, we hypothesized that a high-throughput screen of BMDCs using siRNA libraries—silencing individual putative stimulatory or inhibitory DC-intrinsic genes in immature DCs and studying the resulting effects on DC phenotype—would be a highly efficient way to search for new immunoregulatory genes and their associated pathways. Our overall objective is to better understand the cellular and molecular mechanisms of immunologic self-tolerance in order to develop better therapies for autoimmune diseases in the clinic.

To discover new genes and pathways involved in the regulation of BMDC activation and immunologic tolerance, we designed a two-phase experimental approach: (1) an initial \textit{in vitro} screen, in which siRNA-transfected BMDCs are assayed \textit{in vitro} for evidence of maturation (Figure 1.5), and (2) a subsequent \textit{in vivo} screen, in which candidate positive hits from the \textit{in vitro} screen are tested for their ability to induce an adaptive immune response \textit{in vivo} using the RIP-GP BMDC vaccination system (Figure 1.6).
Figure 1.5. Overview of the *in vitro* phase of the siRNA library screen in BMDCs. An siRNA library is electroporated into resting BMDCs. One gene is targeted in each BMDC sample. After 48 hours of putative gene silencing, BMDCs are assayed *in vitro* for evidence of maturation.
Figure 1.6. Overview of the *in vivo* phase of the siRNA library screen in BMDCs. BMDCs are generated *in vitro* from a mouse strain in which a candidate gene hit from the *in vitro* screen is genetically ablated. KO BMDCs are pulsed with LCMV GP peptides with or without TLR stimulation, then adoptively transferred into RIP-GP mice and monitored for the development of autoimmune diabetes. In a different permutation of the *in vivo* screen (not shown), WT BMDCs are generated *in vitro*, then the candidate gene hit is silenced by siRNA. The siRNA-transfected BMDCs are then pulsed with LCMV GP peptides with or without TLR stimulation, then adoptively transferred into RIP-GP mice and monitored for the development of autoimmune diabetes.
CHAPTER 2: MATERIALS AND METHODS

2.1 MICE

Wild-type C57BL/6 mice and gene-targeted IL-12/23-p40-eYFP (B6.129-Il12b<sup>tm1Lky</sup>/J, Stock Number 006412) knock-in mice<sup>86</sup> were purchased from The Jackson Laboratory. Homozygous RIP-GP (“Berlin<sup>+/+</sup>”) mice were previously generated<sup>63</sup> and generously provided by Dr. Pam Ohashi (University of Toronto). Heterozygous RIP-GP (“RIP-GP”) mice were generated by crossing male Berlin<sup>+/+</sup> mice with female wild-type C57BL/6 mice. Gene-targeted SHP-deficient (Shp<sup>-/-</sup>) mice on the C57BL/6 genetic background were previously generated<sup>87</sup> and obtained from Dr. David Moore (Baylor College of Medicine, Houston, Texas). All mice were maintained, and all experiments were performed, at the Ontario Cancer Institute Animal Resource Centre. All procedures were approved by the University Health Network Animal Care Committee.

2.2 IN VITRO GENERATION OF BMDCs

BMDCs were generated in vitro according to the Lutz method<sup>48</sup>. Briefly, bone marrow cells were harvested from the femurs and tibias of mice and cultured in 100 mm bacteriological Petri dishes (BD Falcon) for ten days in RPMI 1640 (Gibco) containing 10% heat-inactivated FBS (Life Technologies), 55 µM of 2-mercaptoethanol (Gibco), and GM-CSF (40 ng/mL for the first 3 days, 20 ng/mL for the remaining 7 days, PeproTech). Medium was changed on days 3, 6, and 8. On day 10, non-adherent BMDCs were collected for further culture or analysis as described below. In the case of further culture without electroporation, the non-adherent BMDCs were washed and re-cultured in 24-well plates at 2x10<sup>6</sup>/mL/well with or without (1) LPS (Sigma).
at 1000, 100, 10, or 1 ng/mL or (2) polyinosinic-polycytidylic acid (poly(I:C), Invivogen) at 100 µg/mL for 16-20 hours. Then, BMDCs were collected for further analysis as described below.

### 2.3 siRNA

The Nuclear Receptors siGENOME siRNA library (Dharmacon) contained 54 siRNA pools (SMARTpools), each consisting of four synthetic siRNA duplexes targeting a single gene, arrayed in a 96-well plate at 0.5 nmol/well. The Cytokine Receptors siGENOME siRNA library contained 158 SMARTpools. A number of siRNA SMARTpools were purchased and used individually, including Non-Targeting Pool #2, siGLO Red Transfection Indicator, CD11c, A20, SOCS1, NR0B2. Lyophilized siRNA library SMARTpools were resuspended in their original library plate in Opti-MEM buffer (Gibco) at 10 mM, then placed on an orbital shaker for 30 minutes at room temperature according to Dharmacon’s instructions. Stock solutions of lyophilized individual siRNA SMARTpools were prepared by resuspension in Opti-MEM buffer at 20 µM or 50 µM, followed by orbital shaking as above.

### 2.4 ELECTROPORATION OF siRNA INTO BMDCs

On day 10 of BMDC culture, non-adherent BMDCs were collected, washed, and resuspended in Opti-MEM buffer at 20x10⁶/mL. Lyophilized siRNA library SMARTpools arrayed in 96-well plates (0.5 nmol/well) were resuspended and shaken in Opti-MEM at 10 mM as described above, then transferred to a 96-well Bio-Rad Gene Pulser MXCell electroporation plate. To each well of the electroporation plate were added 1.1x10⁶ BMDCs in 55 µL of Opti-MEM, producing a final cell density of 10.5x10⁶/mL and a final siRNA concentration of 4762 nM. An exponential waveform pulse of 400 V, 200 µF, and 1000 Ω was delivered to each sample well at room temperature. Immediately following electroporation, BMDCs were
transferred using adjustable multi-channel pipettes to pre-warmed 24-well plates containing 1 or 2 mL of complete 10% RPMI, then incubated at 37°C in 5% CO₂. Forty-eight hours later, BMDCs were collected by gentle pipetting for further experimentation or analysis. For optimization experiments in which pulse voltage and capacitance were varied, the resistance was always held constant at 1000 Ω.

2.5 FLOW CYTOMETRY

**Surface staining:** Cells were collected, centrifuged, transferred to flow cytometry tubes, and washed with cold PBS (without calcium and magnesium) containing 2% FBS and 0.09% sodium azide (“Staining Buffer”). After 10 minutes of Fc receptor blockade with CD16/CD32 monoclonal antibodies (BioLegend) at 4°C, cells were stained for 30 minutes at 4°C in the dark with different combinations of fluorochrome-conjugated monoclonal antibodies, including: MHC II (I-A/I-E or I-Aᵇ), CD80, and CD86 (all from BD BioSciences), and CD11c (eBioscience). Cells were washed with cold Staining Buffer, centrifuged, resuspended in Staining Buffer, and acquired on a FACSCanto flow cytometer (BD).

**Viability staining:** Following surface staining as described above, cells were washed with cold Staining Buffer, centrifuged, and incubated with 50 μL of 7-AAD (BD Biosciences) for 15 minutes at 4°C in the dark. After adding 200 μL of Staining Buffer to each sample, cells were acquired on a FACSCanto flow cytometer.

**Intracellular cytokine staining:** Intracellular cytokine staining was performed using the BD Biosciences Cytofix/Cytoperm Fixation/Permeabilization kit according to the manufacturer’s instructions. Briefly, cells were incubated with GolgiPlug (Brefeldin A) for 5-6 hours. Following surface staining as described above, cells were washed with cold Staining Buffer and
permeabilized by incubation in Cytofix/Cytoperm for 30 minutes at 4°C. Cells were then washed in Perm/Wash buffer and stained with IL-12-p70-specific monoclonal antibody (BD Biosciences) for 30 minutes at 4°C in the dark. Following additional washes in PermWash buffer, cells were acquired on a FACSCanto flow cytometer.

**Data analysis:** Flow cytometry data was analyzed using FlowJo software (Tree Star). When more than one fluorochrome was used, single-stained compensation controls were acquired for compensation analysis, which was always manually performed in FlowJo. Cellular debris was excluded from analysis by setting an appropriate gate in the forward scatter (FSC)/side scatter (SSC) plot. All other gates were set based on fluorescence-minus-one (FMO) control samples. Except for IL-12/23-p40-YFP knock-in BMDCs, all other BMDCs were gated on the CD11c\textsuperscript{high} population before analysis. The CD11c\textsuperscript{high} population frequency was approximately 90% of the FSC/SSC population. IL-12/23-p40-YFP BMDCs were gated on the FSC/SSC population, then analyzed for YFP expression. When viability staining was performed, analyses were conducted on live cells by gating on the 7-AAD-negative population. All MFI values represent median fluorescent intensities. For some histogram overlays, the data were normalized to the peak height at the mode of the distribution (i.e., the number of cells in each bin of a given histogram was divided by the number of cells in the bin containing the largest number of cells). Thus, the y-axis depicts the percentage of the maximum number of cells (i.e., the number of cells at the mode of the distribution).

2.6 FITC-DEXTRAN ENDOCYTOSIS ASSAY

BMDCs were collected, centrifuged, washed, and resuspended in 180 µL of complete RPMI. Twenty microlitres of fluorescein isothiocyanate-dextran (FITC-DX, 10 mg/mL, Sigma) was added to produce a final FITC-DX concentration of 1 mg/mL. Control samples (surface
binding of FITC-DX but no endocytosis) were incubated for 30 minutes at 4°C in the dark while experimental samples (surface binding and endocytosis) were incubated for 30 minutes at 37°C and 5% CO₂ in the dark. After three washes in ice cold Staining Buffer, cells were incubated with Fc block as described above, then surface-stained with MHC II (I-A/I-E)-specific monoclonal antibody (BD Biosciences) for 30 minutes at 4°C in the dark. Cells were washed and then acquired on a FACSCanto flow cytometer.

2.7 CYTOKINE ELISA

BMDC culture supernatants were collected 48 hours after siRNA library transfection and stored at -80°C for future cytokine ELISA analysis. The concentrations of IL-6, IL-12-p70, and TNF-α were determined by sandwich ELISA analysis according to the manufacturer’s guidelines (eBioscience Ready-SET-Go! kits).

2.8 BMDC-T CELL CO-CULTURE

A BMDC-CD3+ T cell co-culture (1:10) with 72 hours of low-dose CD3 monoclonal antibody stimulation (0.1 mg/mL, BioLegend) was prepared in 96-well plates. BMDCs were generated in vitro and electroporated with siRNA as described above. After 48 hours of culture in 24-well plates as described above, supernatants were gently collected and frozen at -80°C for future cytokine analysis. Two mL of complete RPMI was added back to each well for cell resuspension. In order to promote high-throughput workflow, we did not determine the number of cells in each well. Rather, based on our previous data, we assumed that cell recovery per well was 60%, considering viability and plastic adherence. Since each well was originally seeded with 1.1x10⁶ electroporated BMDCs, we assumed that 48 hours later, there were 0.66x10⁶ BMDCs in 2 mL in each well. Thus, 30 µL (0.01x10⁶ cells) of the BMDC suspension in each well was
transferred to separate wells of a 96-well plate. In parallel, CD3+ T cells were purified from wild-type C57BL/6 spleens using a Pan T Isolation Kit II (Miltenyi). Twenty million CD3+ T cells at 10x10^6/mL were stained with Violet CellTrace (VCT, Life Technologies) at 2.5 µM for 20 minutes at 37°C and 5% CO₂. Following quenching with complete RPMI, the CD3+ T cells were incubated for another 5 minutes at 37°C and 5% CO₂. After resuspension at 0.476x10^6/mL in complete RPMI containing 0.1 mg/mL of CD3 monoclonal antibody, 0.1x10^6 (210 µL) of the VCT-stained T cells were added to each well of the BMDC-containing 96-well plate above. After 72 hours of co-culture at 37°C and 5% CO₂, all cells were collected, washed, and acquired on a FACSCanto flow cytometer. Cell division and proliferation analyses were performed using FlowJo software.

2.9 BMDC VACCINATION OF RIP-GP MICE

BMDCs were prepared in vitro as described above. On day 10, non-adherent BMDCs were collected, washed, and re-cultured in 24-well plates at 2x10^6/mL/well with or without LPS at 10 ng/mL (Sigma) for 16-20 hours. Then, the BMDCs in each well were pulsed with a triple-peptide mix of LCMV peptides (New England Peptide and Washington Biotechnology) for 2-3 hours as follows: 10^6 M gp33-41 (KAVYNFATM), 10^6 M gp276-286 (SGVENPGGYCL), and 1 µg/mL gp61-80 (GLNGPDIYKGVYQFKSVEFD). BMDCs were collected by pipetting up and down, washed with Hanks’ Buffered Saline Solution (HBSS), and resuspended in HBSS at 10x10^6/mL. Two million BMDCs (0.2 mL) were injected intravenously into each RIP-GP mouse via the lateral tail vein. Blood glucose concentrations were measured beginning on day 6 and then every 2-3 days thereafter using an electronic glucometer and chemstrips (Accu-Chek). Diabetes was diagnosed after two consecutive blood glucose readings of 15 mM or higher.
2.10 STATISTICAL ANALYSIS

**Nuclear Receptors siRNA library screen based on CD80 and CD86 (Figure 3.17):** The CD80 MFI value for each experimental siRNA-transfected sample was normalized to the arithmetic mean of the CD80 MFI values of the replicate NT siRNA-transfected control samples. This generated a fold-change CD80 MFI index. The same procedure was performed for the CD86 MFI values to generate a CD86 MFI index. Experimental samples whose MFI index values for both CD80 and CD86 were greater than or equal to 1.1 were defined as hits. This Nuclear Receptors screen was performed six times. Candidate hits were ranked based on the number of times the above hit criteria were met.

**Nuclear Receptors siRNA library screen based on MHC II (Figure 3.19):** As above, the MHC II MFI value for each experimental siRNA-transfected sample was normalized to the arithmetic mean of the MHC II MFI values of the replicate NT siRNA-transfected control samples, generating a fold-change MHC II MFI index. Experimental samples whose MFI index values for MHC II were less than or equal to 0.7 were defined as hits. This Nuclear Receptors screen was performed 3 times. Candidate hits were ranked based on the number of times the above hit criteria were met. The one-sample *t* test (theoretical mean = 1.0) was used to calculate *p* values.

**Cytokine Receptors siRNA library screen based on IL-12/23-p40 (Figure 3.29):** This screen was analyzed using the robust z* score statistic. To generate a robust z* score distribution, we used the following procedure. First, the IL-12/23-p40+ frequency value for each experimental siRNA-transfected sample was normalized to the geometric mean of the replicate NT siRNA-transfected control samples. Second, this normalized raw data was log₂-transformed. Third, the
absolute deviation of each log$_2$-transformed value was calculated. Fourth, the median of all the absolute deviations (MAD) was calculated. Fifth, the robust $z^*$ score was calculated as follows:

$$z^* \text{ score} = \frac{Y_i - \tilde{Y}_N}{\text{MAD}_N \times 1.4826}$$

where $Y_i$ is the $i$th log$_2$-transformed value, $Y_N$-tilde is the median of the log$_2$-transformed values, MAD$_N$ is the median absolute deviation, and 1.4826 is a constant that makes MAD$_N$ equal to standard deviation (SD) when the values are normally distributed.

**Correlation Analysis (Figure 3.31):** Spearman correlation analysis was performed using Prism software (GraphPad).

**T Cell Proliferation Analysis (Figure 3.30):** Robust $z^*$ scores were calculated as above based on the division index (DI) values, calculated by FlowJo software, for each sample.
CHAPTER 3: RESULTS

3.1 OPTIMIZATION OF BMDC CULTURE AND TRANSFECTION CONDITIONS

3.1.1 Minimization of the Baseline BMDC Maturation Level

3.1.1.1 Testing the Effect of FBS on BMDC Maturation Level

To maximize the dynamic range of the screen (i.e., to maximize the probability of detecting a true phenotypic change resulting from BMDC gene silencing), we sought to generate BMDCs with minimal baseline maturation (i.e., as phenotypically immature as possible, while retaining the ability to become fully activated) prior to transfection. Before optimization, the baseline expression of CD80 and CD86 on the surface of our BMDCs was relatively high (Figure 3.1). At first, we assumed that this high level of costimulatory molecule expression was due to inadvertent mechanical stimulation of the cells during their 10 days of in vitro culture and differentiation (e.g., pipetting during cell feedings, etc.). Multiple attempts to reduce this high costimulatory molecule expression by reducing mechanical stimulation of the cells (e.g., gentler pipetting, etc.) were unsuccessful.
Figure 3.1. CD80 and CD86 expression on BMDCs before optimization. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were left unstimulated or stimulated with poly(I:C). After 16-20 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the CD11c<sup>hi</sup> population. Top row histograms: Surface CD86 expression. Bottom row histograms: Surface CD80 expression. Red histograms: Unstimulated BMDCs. Blue histograms: Poly(I:C)-stimulated BMDCs. Numbers indicate (1) the frequency of CD80+ and CD86+ populations, (2) the MFI of CD80 and CD86 expression, and (3) the fold-change in CD80+/CD86+ population frequency and CD80/CD86 MFI upon poly(I:C) stimulation. Data are representative of many independent experiments.

At this point, we discovered the article by Lutz et al.\textsuperscript{55} that reported that the biochemical profile of the fetal bovine serum (FBS) used to generate BMDCs in vitro has a critical effect on their differentiation and baseline maturation level (see Chapter 1.3.3 above). We reviewed the certificate of analysis of the FBS lot that we were using at that time for our BMDC cultures, and
found that the LDH and SGOT concentrations in our FBS were 2136 mU/mL and 148 mU/mL, respectively. These values were greatly in excess of the threshold level for BMDC-stimulating FBS determined by Lutz et al.55. We therefore hypothesized that our FBS lot was at least partially responsible for the excessive BMDC baseline maturation level generated by our cultures.

As such, we ordered from different companies several new FBS lots, all with LDH and SGOT concentrations between 300-700 mU/mL and 12-50 mU/mL, respectively, and tested their effects on BMDC differentiation and maturation. Figure 3.2 shows a representative experiment in which BMDCs were generated using one of the newly ordered FBS lots (Invitrogen, Catalog No. 16000, Lot No. 432023, [LDH] = 332 mU/mL, [SGOT] = 31 mU/mL). As compared to the relatively high frequency of CD86+ BMDCs (54%) that was generated using the old FBS, the CD86+ frequency that was generated with the new FBS was reduced by more than one-third (to 34%). Importantly, the BMDCs generated using the new FBS lot retained the ability to become activated in response to TLR-ligand stimulation, as evidenced by the expected rise in the CD86+ frequency (old FBS: 1.8-fold, new FBS: 2.4-fold) and CD86 MFI (old FBS: 6.8-fold, new FBS: 9.1-fold). In addition, unstimulated BMDCs generated using the new FBS lot did not induce autoimmune diabetes when they were LCMV peptide-pulsed and transferred into RIP-GP mice, whereas poly(I:C)-stimulated, LCMV peptide-pulsed, RIP-GP-transferred BMDCs did induce diabetes (Figure 3.3).
Figure 3.2. FBS with [LDH] and [SGOT] within recommended ranges generated BMDCs with lower baseline maturation level. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days, either with original (pre-optimization) FBS or new FBS containing [LDH] and [SGOT] within the ranges recommended by Lutz et al. On day 10, BMDCs were left unstimulated or stimulated with poly(I:C). After 16-20 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the CD11c<sup>high</sup> population. Top row histograms: Surface CD86 expression on BMDCs cultured in original FBS (same data as those shown in Figure 3.1). Bottom row histograms: Surface CD86 expression on BMDCs cultured in new FBS. Red histograms: Unstimulated BMDCs. Blue histograms: Poly(I:C)-stimulated BMDCs. Numbers indicate (1) the frequency of the CD86<sup>+</sup> population, (2) the MFI of CD86 expression, and (3) the fold-change in CD86<sup>+</sup> population frequency and CD86 MFI upon poly(I:C) stimulation. Data are representative of at least three independent experiments.
Figure 3.3. BMDC vaccination of RIP-GP mice. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days with the new Invitrogen FBS (Catalog No. 16000, Lot No. 432023). On day 10, BMDCs were left unstimulated or stimulated with poly(I:C). After 16-20 hours of culture, BMDCs were pulsed with LCMV triple-peptide mix for 2-3 hours, then adoptively transferred into RIP-GP mice. Blue curve: Blood glucose concentrations in RIP-GP mice (n = 5) injected with poly(I:C)-stimulated BMDCs. Green curve: Blood glucose concentrations in RIP-GP mice (n = 5) injected with unstimulated BMDCs. Data are representative of many independent experiments.

On the basis of this FBS lot-dependent reduction in baseline BMDC maturation level and the preserved ability of the BMDCs to mature, we purchased many bottles of this particular FBS lot so that all of our experiments would be comparable and consistent. When we eventually depleted this lot, we again tested new FBS lots for their effects on BMDC activation and function and selected one (Life Technologies (formerly Invitrogen), Catalog Number 16000, Lot Number
1295595) with a similar biochemical profile ([LDH] = 480 mU/mL, [SGOT] = 40 mU/mL) and comparable and consistent results in standard BMDC assays (data not shown). All of our experiments were performed with one of these two empirically validated lots.

### 3.1.1.2 Removal of GM-CSF from Culture after Day 10 Reduces BMDC Maturation

In parallel, as we were optimizing electroporation experiments for the anticipated start of our siRNA library screening, we made a second important discovery relevant to the minimization of baseline BMDC maturation level. We found that removing GM-CSF from the BMDC culture medium after day 10, at the time when the BMDCs are transferred from bacteriological Petri dishes to tissue culture-treated plastic plates, reduces their level of maturation.

Classically, BMDCs are cultured in the presence of GM-CSF for the first 8-10 days of culture (according to the Lutz method48). When they are subsequently treated with an activating stimulus (e.g., a Toll-like receptor ligand), the BMDCs are re-plated on tissue culture-treated plastic in culture medium without GM-CSF62 or with a reduced GM-CSF concentration48. We had been taught that one reason for the removal or dose reduction of GM-CSF at this juncture is that GM-CSF can inhibit BMDC activation (personal communication). In addition, GM-CSF can contribute to the unwanted differentiation and proliferation of contaminating granulocytes48.

Therefore, up to this point, we had been removing GM-CSF from our TLR-ligand-stimulated BMDC samples in an effort to prevent any GM-CSF-mediated inhibition of BMDC activation. As such, we should have been removing GM-CSF from the matched unstimulated control samples as well. That way, TLR-L stimulation would have been the only difference between the stimulated and unstimulated samples. However, in fact, we had been mistakenly including GM-CSF in the unstimulated control samples, on the false grounds that, since there
was no TLR-L stimulation for GM-CSF to potentially inhibit, it was preferable and acceptable to include it for the health of the BMDCs.

For our anticipated screen, the activating stimulus would be siRNA-mediated gene silencing, which would or would not activate the BMDCs, depending on the identity of the silenced gene. Thus, in order to enable the BMDCs to become activated if the right gene is silenced, we reasoned that GM-CSF should be removed from the post-electroporation culture medium on day 10, just as it is removed when BMDCs are stimulated with a TLR-ligand. However, if GM-CSF is inhibitory and it is removed from the culture, then positive hits (i.e., activated BMDCs) could be the result of either (1) successful siRNA-mediated gene silencing or (2) the absence of the inhibitory effect of GM-CSF on BMDC activation.

Therefore, to distinguish between these two possibilities, we designed an experiment to determine the baseline maturation level of unstimulated BMDCs that were cultured after day 10 without GM-CSF. The results were surprising and striking. We found that the substantial expression of CD86 and CD80 in unstimulated BMDCs was dramatically reduced when GM-CSF was omitted from the culture after day 10 (Figure 3.4). We therefore concluded that (1) GM-CSF does not inhibit BMDC activation, contrary to what we had thought, and (2) any positive hits arising from the screen could be attributed to BMDC-activating effects of siRNA-mediated gene silencing.
Figure 3.4. Removal of GM-CSF from the BMDC culture medium after day 10 reduces BMDC maturation level. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were re-cultured with or without GM-CSF. After 16-20 hours, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the CD11c<sup>high</sup> population. Top row histograms: Surface CD86 and CD80 expression on BMDCs cultured in GM-CSF at 20 ng/mL (CD86 data are the same as those shown in Figure 3.2). Bottom row histograms: Surface CD86 and CD80 expression on BMDCs cultured without GM-CSF. Left column histograms: Surface CD86 expression. Right column histograms: Surface CD80 expression. Numbers indicate the frequency of CD86+ and CD80+ populations. Data are representative of at least three independent experiments.

In summary, by (1) using FBS containing specific concentrations of LDH and SGOT, and (2) removing GM-CSF from the BMDC culture medium after day 10, we succeeded in reducing pre-transfection BMDC maturation markers to a minimal level.
3.1.2 Electroporation Achieves High siRNA Transfection Efficiency with Minimal Effect on BMDC Maturation and Viability

Having minimized the baseline BMDC maturation level, we next attempted to identify electroporation conditions that would simultaneously achieve (1) maximum siRNA transfection efficiency, (2) minimum BMDC maturation, and (3) maximum BMDC viability.

We systematically tested different sets of electroporation conditions, including different pulse voltages and capacitances, siRNA concentrations, and BMDC densities. Our overall strategy was to first exclude electroporation pulse voltage and capacitance parameter sets that resulted in (1) excessive BMDC death, as measured by 7-AAD positivity, and (2) excessive BMDC maturation, as measured by surface BMDC expression of CD80 and CD86. Then, focusing on the subset of electrical parameters that resulted in reasonable BMDC viability and baseline maturation level, we adjusted the sample siRNA concentration and BMDC density in order to achieve maximum transfection efficiency, as measured by BMDC expression of a fluorescently labeled oligonucleotide siRNA duplex (Dharmacon siGLO Red Transfection Indicator).

To assess BMDC viability as a function of different electroporation conditions, unstimulated BMDCs were electroporated on day 10 without siRNA (i.e., mock electroporation), then cultured for an additional 48 hours in order to simulate a period of siRNA-mediated gene silencing. As expected, at any given voltage, increasing the capacitance resulted in increased BMDC death, as measured by flow cytometric analysis of 7-AAD+ cells (Figure 3.5). BMDC death was further increased when voltage and capacitance were concurrently increased. At the same time, these data indicated that over a considerable range of voltages and capacitances, BMDC viability was relatively unaffected (Figure 3.5).
Figure 3.5. BMDC viability as a function of electroporation pulse voltage and capacitance gradients. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were left untreated or electroporated without siRNA (mock electroporation) with different pulse voltages and capacitances. After 48 hours of culture, BMDCs were stained with 7-AAD and analyzed by flow cytometry. Cells were gated on the FSC/SSC population. (A) Zebra plots of the 7-AAD+ and 7-AAD- populations as a function of pulse voltage and capacitance. (B) Zebra plot of the 7-AAD+ and 7-AAD- populations in the untreated (non-electroporated) control. Numbers indicate the frequency of 7-AAD+ and 7-AAD- populations. Data are representative of two independent experiments.

To assess BMDC maturation level as a function of different electroporation conditions, unstimulated BMDCs were electroporated on day 10 without siRNA and cultured for 48 hours in order to simulate a period of siRNA-mediated gene silencing. We found that only at a pulse voltage level of 400 V was there any increase in CD86 expression, which was further augmented as the pulse capacitance was increased (Figure 3.6).
Figure 3.6. CD86 expression on BMDCs as a function of electroporation pulse voltage and capacitance gradients. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were left untreated or electroporated without siRNA (mock electroporation) with different pulse voltages and capacitances. After 48 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD-, CD11c<sub>high</sub> population. Blue histograms: CD86 expression as a function of pulse voltage and capacitance. Red histograms: CD86 expression in the untreated (non-electroporated) control. Numbers indicate the CD86+ frequency. Untreated control: CD86+ frequency = 8.3%. Poly(I:C)-stimulated sample (positive control): CD86+ frequency = 49.2%. Data are representative of at least two independent experiments.
On the basis of these BMDC viability and maturation data, we preliminarily concluded that the delivery of a pulse capacitance of 950 µF was excessive and undesirable, regardless of the pulse voltage level. This conclusion was supported by the data of Jantsch et al., who used a pulse of 400 V/150 µF to achieve excellent gene silencing at the RNA and protein levels. Having narrowed the range of possible pulse voltages and capacitances, we proceeded to optimize siRNA transfection efficiency.

To optimize siRNA transfection efficiency, we studied the uptake of a fluorescently labeled oligonucleotide duplex (siGLO Red Transfection Indicator, Dharmacon) as a function of siRNA concentration, BMDC density, and a refined, narrower gradient of pulse voltages and capacitances. siGLO Red uptake was analyzed by flow cytometry, performed immediately following electroporation.

Figure 3.7 shows representative results from this initial set of experiments. We found that even at the relatively high siRNA concentration of 500 nM, the transfection efficiency was only between 30-45%. However, when the siRNA concentration was increased greatly to 2000 nM, the transfection efficiency rose to 65-80%. When the BMDC density was doubled from $1 \times 10^6$/mL to $2 \times 10^6$/mL (while holding pulse voltages and capacitances constant), there was no significant change in the transfection efficiency at either 500 nM or 2000 nM. However, when the pulse capacitance and siRNA concentration were held constant at 300 µF and 2000 nM, respectively, increasing the pulse voltage from 300 V to 400 V raised the transfection efficiency by more than 10% (from 70% to 80% at either BMDC density). Similarly, when the pulse voltage and siRNA concentrations were held constant at 400 V and 2000 nM, respectively, increasing the pulse capacitance from 200 µF to 300 µF raised the transfection efficiency by more than 10% at both BMDC densities (70 to 80%). At the siGLO concentration of 2000 nM
and the BMDC density of either $1 \times 10^6$/mL or $2 \times 10^6$/mL, there was no significant difference in transfection efficiency when the pulse voltage and capacitance were varied in opposite directions, i.e., to either 300 V/300 µF or 400 V/200 µF.

Figure 3.7. siRNA transfection efficiency as a function of [siRNA], BMDC density, and pulse voltage and capacitance gradients. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with either a fluorescently labeled siRNA (siGLO) or a non-fluorescent, non-targeting (NT) control. Cells were analyzed by flow cytometry following electroporation. Cells were gated on the FSC/SSC population. Blue histograms: NT control. Red histograms: siGLO. Rows show different siGLO concentrations. Columns show different pulse voltages and capacitances. Numbers indicate the transfection frequency, i.e., the frequency of siGLO+ BMDCs. (A) BMDC density = $2 \times 10^6$/mL. Data are representative of at least two independent experiments.
Figure 3.7. (B) BMDC density = $1 \times 10^6$/mL.

On the basis of these results, we concluded that (1) maximum transfection efficiency would require the siRNA concentration to be at least 2000 nM, (2) there was no advantage or disadvantage to doubling the BMDC density from $1 \times 10^6$/mL to $2 \times 10^6$/mL when the siGLO concentration was high at 2000 nM, and (3) applying a pulse of 400 V/300 μF achieved the highest transfection efficiency, but at the cost of (a) slightly more BMDC death (Figure 3.5) and (b) slightly more CD86 expression (Figure 3.6). By comparison, delivering a pulse of 400 V/200 μF resulted in only marginally less transfection efficiency, but also slightly less BMDC death and maturation.
Coupled with the results of Jantsch et al.\textsuperscript{88} indicating that high transfection efficiency and gene silencing required (1) a pulse of 400 V/150 µF, (2) approximately 4800 nM of siRNA\textsuperscript{1}, and a (3) a BMDC density of 20x10\textsuperscript{6}/mL, our results led us to design new optimization experiments focusing on an even narrower range of electroporation conditions. Specifically, we compared (1) a pulse of 400 V/200 µF to a pulse of 400 V/150 µF, (2) an escalating gradient of siRNA concentrations from 2000 nM to 4762 nM, and (3) and escalating gradient of BMDC densities from 2x10\textsuperscript{6}/mL to 20x10\textsuperscript{6}/mL.

Figure 3.8 shows representative results from the first set of these experiments. We found that at the BMDC density of 2x10\textsuperscript{6}/mL, electroporating 4000 nM of siRNA resulted in a higher transfection efficiency (85%) than either 3000 nM (80%) or 1000 nM (50\%) of siRNA. At the siRNA concentration of 4000 nM, there was no advantage or disadvantage in raising the BMDC density to 20x10\textsuperscript{6}/mL from 2x10\textsuperscript{6}/mL (both 80-85\%). Notably, when the pulse voltage was maintained at 400 V but the pulse capacitance was reduced from 200 µF to 150 µF to simulate Jantsch et al.’s conditions, the transfection efficiency dropped substantially from 85% to 70\% (BMDC density held constant at 2x10\textsuperscript{6}/mL). This drop was slightly attenuated when the BMDC density was raised to Jantsch et al.’s level of 20x10\textsuperscript{6}/mL.

\textsuperscript{1} Jantsch et al. used 60 ng/µL/sample of Qiagen siRNA. Although the exact molecular weight of siRNA depends on its sequence, Qiagen reports that 20 µM of their 21-nucleotide duplex siRNA is equal to ~0.25 µg/µL. Thus, 60 ng/µL of siRNA is equal to ~4800 nM.
Figure 3.8. siRNA transfection efficiency as a function of [siRNA], BMDC density, and focused pulse voltage and capacitance gradients. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with either a fluorescently labeled siRNA (siGLO) or a non-fluorescent, non-targeting (NT) control. Cells were analyzed by flow cytometry following electroporation. Cells were gated on the FSC/SSC population. Blue histograms: NT control. Red histograms: siGLO. Rows show different siGLO concentrations. Columns show different pulse voltages, pulse capacitances, and BMDC densities. Numbers indicate the transfection frequency, i.e., the frequency of siGLO+ BMDCs. Data are representative of at least two independent experiments.

From these results, we concluded that (1) maximum transfection efficiency would require at least 4000 nM of siRNA, (2) a pulse of 400 V/200 µF was superior to a pulse of 400 V/150 µF, and (3) high transfection efficiency could be achieved over a 10-fold range of BMDC densities (2-20x10⁶/mL).
To facilitate high-throughput screening, we sought to electroporate sufficient cells to permit multiple functional assays from a single electroporation experiment. We therefore designed an optimization experiment to determine whether high transfection efficiency could be achieved at the BMDC density of 10.5x10^6/mL (Figure 3.9). At an electroporation sample volume of 105 µL, this BMDC density corresponded to an absolute BMDC number of 1.1x10^6/well. This number was strategic because it would permit us to divide the electroporated BMDCs from each well of the electroporation plate into two separate 24-plate wells, each containing approximately 5.5x10^5 BMDCs. After 48 hours of putative gene silencing, and allowing for an expected degree of BMDC death, these divided samples containing 5.5x10^5 BMDCs each would be suitable for at least three functional assays (e.g., flow cytometric analysis of cell surface markers, flow cytometric analysis of FITC-DX uptake, and ELISA analysis of cytokine production).

In this optimization experiment to test transfection efficiency at the BMDC density of 10.5x10^6/mL, we also further increased the siRNA concentration to 4762 nM. We did this in order to (1) promote technical simplicity, because each well of the Dharmacon siRNA libraries contained 0.5 nmol of pre-spotted, lyophilized siRNA (0.5 nmol/105 µL = 4762 nM), and (2) more closely simulate the electroporation conditions of Jantsch et al., who used approximately 4800 nM of siRNA. Figure 3.9A shows that at the BMDC density of 10.5x10^6/mL and the siRNA concentration of 4762 nM, the transfection efficiency was extremely high, approaching 90%. Figure 3.9B shows representative summary data from multiple transfection efficiency experiments performed using 4000 nM or 4762 nM of siRNA. On the basis of these results, we

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2 siRNA is expensive. As we increasingly accumulated evidence that a high siRNA concentration would be required to achieve maximum transfection efficiency, we lowered the sample volume in order to simultaneously raise siRNA concentration and lower siRNA consumption. 100 µL is the lowest sample volume that can be used in the Bio-Rad MXCell electroporator. We chose to use 105 µL so that there would be some extra buffer as a safeguard.
concluded that the ideal electroporation conditions for our anticipated siRNA library screen included the combination of: (1) a pulse of 400 V/200 µF, (2) an siRNA concentration of 4762 nM, and (3) a BMDC density of 10.5x10⁶/mL.

Figure 3.9. Optimized siRNA transfection efficiency approaches 90%. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with either a fluorescently labeled siRNA (siGLO) or a non-fluorescent, non-targeting (NT) control. Cells were analyzed by flow cytometry following electroporation. Cells were gated on the FSC/SSC population. Blue histograms: NT control. Red histograms: siGLO. (A) Histograms showing transfection efficiency (i.e., the frequency of siGLO+ BMDCs). (B) Summary data from multiple independent experiments.

Having successfully optimized the transfection efficiency, we returned to an investigation of BMDC viability and maturation, in order to confirm that our optimized electroporation
conditions would not cause excessive BMDC death or maturation. Figure 3.10 shows that electroporating 10.5x10^6/mL BMDCs with a pulse of 400 V/200 µF (without siRNA) resulted in no change in surface CD80 or CD86 expression.

Figure 3.10. Electroporation with optimized conditions does not cause BMDC maturation. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were left untreated or electroporated without siRNA (mock electroporation) with a pulse of 400 V/200 µF at a cell density of 10.5x10^6/mL. After 48 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD−, CD11c^{high} population. Top row histograms: CD80 expression. Bottom row histograms: CD86 expression. Red histograms: Untreated samples. Blue histograms: Mock-electroporated samples. Numbers indicate CD80+ and CD86+ frequencies. Poly(I:C)-stimulated samples (positive controls): CD80+ frequency = 41.3%, CD86+ frequency = 44.6%. Data are representative of multiple independent experiments.
In summary, we demonstrated in the preceding series of experiments that electroporation could achieve high siRNA transfection efficiency with minimal effect on BMDC viability and maturation.

3.1.3 siRNA Does Not Cause BMDC Maturation or Reduce BMDC Viability

Double-stranded RNA molecules can induce innate immune responses by interacting with TLRs expressed by DCs\(^\text{85}\). Therefore, it was crucial to test whether electroporating our optimized, high dose of siRNA (4762 nM) into our BMDCs would adversely activate them and/or compromise their viability over a 48-hour period of putative gene silencing\(^3\).

We found that high-dose siRNA did not mature the BMDCs, as measured by (1) surface expression of CD80 and CD86 (Figure 3.11) and (2) intracellular expression of IL-12-p70) (Figure 3.12). In addition, high-dose siRNA did not compromise BMDC viability (Figure 3.13). Thus, we concluded that siRNA does not cause BMDC maturation or reduce BMDC viability in our system.

\(^3\) By contrast, in the siGLO experiments described in the previous section, BMDC transfection efficiency was analyzed by flow cytometry immediately following electroporation (i.e., without 48 hours of siRNA exposure).
Figure 3.1. siRNA does not upregulate costimulatory molecule expression on BMDCs. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated without siRNA (mock electroporation) or electroporated with NT siRNA (4762 nM) with a pulse of 400 V/200 µF at a cell density of 10.5x10⁶/mL. After 48 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD-, CD11c<sup>high</sup> population. Top row histograms: CD80 expression. Bottom row histograms: CD86 expression. Red histograms: Mock-electroporated samples (same data as those shown in Figure 3.10). Blue histograms: NT siRNA-transfected samples. Numbers indicate CD80+ and CD86+ frequencies. Untreated controls: CD80+ frequency = 23.7%, CD86+ frequency = 11.1% (same data as those shown in Figure 3.10). Poly(I:C)-stimulated (positive) controls: CD80+ frequency = 41.3%, CD86+ frequency = 44.6% (same data as those for Figure 3.10). Data are representative of multiple independent experiments.
Figure 3.12. siRNA does not upregulate IL-12-p70 production by BMDCs. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated without siRNA (mock electroporation) or electroporated with NT siRNA (4762 nM) with a pulse of 400 V/200 μF at a cell density of 10.5x10^6/mL. After 48 hours of culture, BMDCs were incubated with Brefeldin A for 5-6 hours, then fixed, permeabilized, stained with IL-12-p70-specific monoclonal antibody, and analyzed by flow cytometry. Red histograms: IL-12-p70 expression in mock-electroporated samples. Blue histogram: IL-12-p70 expression in NT siRNA-transfected samples. Numbers indicate IL-12-p70+ frequencies. These data are from a single experiment.
Figure 3.13. siRNA does not reduce BMDC viability. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated without siRNA (mock electroporation) or electroporated with NT siRNA (4762 nM) with a pulse of 400 V/200 µF at a cell density of 10.5x10⁶/mL. After 48 hours of culture, BMDCs were stained with 7-AAD and analyzed by flow cytometry. Cells were gated on the FSC/SSC population. Zebra plots show 7-AAD+ and 7-AAD- frequencies. Untreated control: 7-AAD+ frequency = 16%. Poly(I:C)-stimulated (positive) control: 7-AAD+ frequency = 10.1%. Data are representative of multiple independent experiments.
3.1.4 Transfection of siRNA Targeting a Known Negative Regulator of BMDC Activation Induces BMDC Maturation

Before embarking on an siRNA library screen, it was important to confirm that we could successfully downregulate gene expression in BMDCs using siRNA. We attempted to silence the CD11c gene, whose protein product is highly expressed and easily detectable on the BMDC surface. Figure 3.14 shows that transfection of CD11c-specific siRNA caused a greater than 80% reduction of surface CD11c expression, as measured by flow cytometry.

In a functional prelude to the siRNA library screen, we tested whether we could induce BMDC maturation by transfecting siRNA targeting SOCS1, a known negative regulator of DC activation\(^9\). Transfection of SOCS1-specific siRNA caused a substantial increase in BMDC maturation, as indicated by (1) an increase in BMDC surface expression of CD86 and CD80 (Figure 3.15), (2) an increase in the MHC II\(^{\text{high}}\)/DX\(^{\text{neg}}\) population frequency, and (3) a decrease in the MHC II\(^{\text{low}}\)/DX\(^{\text{pos}}\) population frequency (Figure 3.16).
Figure 3.14. CD11c-specific siRNA downregulates BMDC expression of CD11c. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with CD11c-specific siRNA or NT siRNA (4762 nM) with a pulse of 400 V/200 µF at a cell density of $10^6$/mL. After 48 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD population. Histograms show CD11c expression. Red histograms: NT siRNA-transfected samples. Blue histogram: CD11c siRNA-transfected sample. Data are representative of at least three independent experiments.
Figure 3.15. SOCS1-specific siRNA upregulates the expression of costimulatory molecules on BMDCs. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with SOCS1-specific siRNA or NT siRNA (4762 nM) with a pulse of 400 V/200 µF at a cell density of 10.5x10⁶/mL. After 48 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD-, CD11c<sup>high</sup> population. Top row histograms: CD80 expression. Bottom row histograms: CD86 expression. Red histograms: NT siRNA-transfected samples. Blue histograms: SOCS1 siRNA-transfected samples. Numbers indicate CD80+ and CD86+ frequencies. Untreated control: CD80+ frequency = 18.8%, CD86+ frequency = 6.39%. Mock electroporation control: CD80+ frequency = 26.6%, CD86+ frequency = 12.7%. Poly(I:C)-stimulated (positive) control: CD80+ frequency = 37.2%, CD86+ frequency = 43.6%. Data are representative of at least three independent experiments.
Figure 3.16. SOCS1-specific siRNA upregulates MHC II expression and reduces dextran uptake by BMDCs. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with SOCS1-specific siRNA or NT siRNA (4762 nM) with a pulse of 400 V/200 µF at a cell density of 10.5x10^6/mL. After 48 hours of culture, BMDCs were incubated with FITC-DX, then stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD- population. Density plots show MHC II expression and DX positivity. Red rectangles: mature (MHC II^{high}/DX^{neg}) BMDC frequencies. Blue rectangles: immature (MHC II^{low}/DX^{pos}) BMDC frequencies. Untreated control: mature BMDC frequency = 37.5%, immature BMDC frequency = 34.9%. Mock electroporation control: mature BMDC frequency = 31.6%, immature BMDC frequency = 40%. Poly(I:C)-stimulated (positive) control: mature BMDC frequency = 41.8%, immature BMDC frequency = 15.6%. Quadrant gates were set based on the FITC-DX binding observed on ice in the NT control: mature BMDC frequency = 32%, immature BMDC frequency = 2.0%. Data are representative of at least three independent experiments.
3.2 siRNA LIBRARY SCREEN BASED ON BMDC EXPRESSION OF CD80, CD86, AND MHC II IDENTIFIES CANDIDATE GENES THAT REGULATE BMDC ACTIVATION

Having optimized the BMDC culture and transfection conditions, we proceeded to screen a commercially available siRNA library. We chose to begin our screening with a small library containing 54 siRNA SMARTpools targeting various nuclear receptors (Dharmacon siGENOME siRNA Nuclear Receptors library).

3.2.1 siRNA Transfection Reveals NR0B2 (SHP) as a Negative Regulator of BMDC Maturation

In the first iteration of our Nuclear Receptors siRNA library screen, we asked whether the transfection of siRNAs targeting nuclear receptor genes would cause (1) the upregulation of CD80, CD86, and MHC II on the BMDC surface, (2) the downregulation of DX uptake by the BMDCs, indicating that the putatively silenced genes were negative regulators of BMDC maturation, and (3) the upregulation of inflammatory cytokine production by the BMDCs.

Figure 3.17 shows the top candidate hits from this first iteration of the screen. Transfection of siRNA targeting the top hit, NR0B2, also known as SHP (small heterodimer partner), resulted in an increase in CD80 and CD86 expression compared to the NT siRNA-transfected controls (Figure 3.18). Corroborating these results, in FITC-DX assays, SHP siRNA-transfected BMDCs displayed a concomitant increase in the mature MHC II$^{\text{high}/\text{DX}^{\text{neg}}}$ population frequency and decrease in the immature MHC II$^{\text{low}/\text{DX}^{\text{pos}}}$ population frequency, as compared to NT siRNA-transfected BMDCs (Figure 3.18). On the basis of these data, we concluded that
transfection of SHP-targeting siRNA causes BMDC maturation, and we hypothesized that SHP is a negative regulator of BMDC activation.

Figure 3.17. Top hits from the Nuclear Receptors siRNA library screen (stimulatory siRNAs). Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with the Nuclear Receptors library as described in Chapter 2.4. After 48 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD-, CD11c\textsuperscript{high} population. (A) Histograms show mean + SEM of MFI index for CD86 (green) and CD80 (red) expression. Asterisks indicate p < 0.05 (one-sample t test with theoretical mean = 1.0). (B) Vertical scatter plots of same data as in (A). Horizontal bars represent mean MFI index for CD86 (green) and CD80 (red) expression. Data are from six independent experiments.
Figure 3.18. SHP-specific siRNA induces BMDC maturation. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with SHP-specific siRNA or NT siRNA (4762 nM) with a pulse of 400 V/200 µF at a cell density of 10.5x10^6/mL. (A) After 48 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD-, CD11c^high population. Red histograms: NT siRNA-transfected samples. Blue histograms: SHP siRNA-transfected samples. Data are representative of at least three independent experiments. Untreated control: CD86+ frequency = 5.8%, CD80+ frequency = 5.2%. Mock electroporation control: CD86+ frequency = 12.7%, CD80+ frequency = 11.3%. NT control: CD86+ frequency = 13.7%, CD80+ frequency = 12.9%. Poly(I:C)-stimulated (positive) control: CD86+ frequency = 64.4%, CD80+ frequency = 30.8%. (B) After 48 hours of culture, BMDCs were incubated with FITC-DX, then stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD- population. Density plots show MHC II expression and DX positivity. Red rectangles: mature (MHC II^{high}/DX^{neg}) BMDC frequencies. Blue rectangles: immature (MHC II^{low}/DX^{pos}) BMDC frequencies. Untreated control: mature BMDC frequency = 43.9%, immature BMDC frequency = 26.9%. Mock electroporation control: mature BMDC frequency = 41.6%, immature BMDC frequency = 30.6%. Gates were set based on FITC-DX binding observed on ice in NT control: mature BMDC frequency = 43.7%, immature BMDC frequency = 1.7%. Data are representative of at least two independent experiments.
3.2.2 siRNA Transfection Reveals NR3C2 (MR) as a Positive Regulator of BMDC Maturation

In a second analysis of the same data, we asked whether the transfection of siRNAs targeting nuclear receptor genes would downregulate MHC II on the BMDC surface, indicating that the silenced genes were positive regulators of BMDC maturation. Figure 3.19 shows the candidate hits from this analysis. Transfection of siRNA targeting the top hit, NR3C2, also known as MR (mineralocorticoid receptor), resulted in a greater than 50% mean reduction in the MHC II MFI index, as compared to the NT siRNA-transfected control. In MR siRNA-transfected BMDCs, there was an increase in the immature MHC II\textsuperscript{low}/DX\textsuperscript{pos} population frequency (60%) and a decrease in the mature MHC II\textsuperscript{high}/DX\textsuperscript{neg} population frequency (10%), as compared to NT siRNA-transfected BMDCs (Figure 3.20). On the basis of these data, we concluded that transfection of MR-targeting siRNA inhibits BMDC maturation, and we hypothesized that MR is a positive regulator of BMDC activation.
Figure 3.19. Top hits from the Nuclear Receptors siRNA library screen (inhibitory siRNAs). Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with the Nuclear Receptors library as described in Chapter 2.4. After 48 hours of culture, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD-, CD11c\textsuperscript{high} population. (A) Histograms show mean + SEM of MFI index for MHC II expression. Asterisks indicate p < 0.05 (one-sample t test with theoretical mean = 1.0). (B) Vertical scatter plots of same data as in (A). Horizontal bars represent mean MFI index for MHC II expression. Data are from three independent experiments.
Figure 3.20. MR-specific siRNA inhibits BMDC maturation. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with MR-specific siRNA or NT siRNA (4762 nM) with a pulse of 400 V/200 µF at a cell density of 10.5x10^6/mL. After 48 hours of culture, BMDCs were incubated with FITC-DX, then stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD- population. Density plots show MHC II expression and DX positivity. Untreated control: mature BMDC frequency = 42.3%, immature BMDC frequency = 28.8%. Mock electroporation control: mature BMDC frequency = 40.9%, immature BMDC frequency = 32.5%. Poly(I:C)-stimulated (positive) control: mature BMDC frequency = 32.5%, immature BMDC frequency = 22.9%. Data are representative of at least three independent experiments.

3.2.3 Cytokine Analysis

In an attempt to validate the above data, we assayed the BMDC culture supernatants from the screen for the presence of IL-6, IL-12-p70, and TNF-α by cytokine ELISA analysis. These
supernatants were collected forty-eight hours after siRNA library transfection and stored at -80°C for future use. Surprisingly, despite excellent standard curves throughout four independent experiments, each testing twenty-four independent samples in triplicate for the presence of IL-6, IL-12-p70, and TNF-α, we were unable to detect any significant cytokine production (Figure 3.21-3.23).

**Figure 3.21. IL-6 ELISA analysis.** Sandwich ELISA was used to determine the IL-6 concentrations in BMDC culture supernatants from siRNA library-transfected BMDC samples. (A) IL-6 standard curve. (B) Histograms show mean ± SD of IL-6 concentration in twenty-four samples from the screen in triplicate. Data are representative of four independent experiments. Samples 1 and 2: NT controls. Samples 3 and 4: Mock electroporation (without siRNA) controls. Samples 13 and 14: SOCS1 siRNA (putative positive controls). Poly(I:C)-stimulated (positive) controls: 584.2 ± 18.7 pg/mL and 599.5 ± 16.4 pg/mL.
Figure 3.2. IL-12-p70 ELISA analysis. Sandwich ELISA was used to determine the IL-12-p70 concentrations in BMDC culture supernatants from siRNA library-transfected BMDC samples. (A) IL-12 standard curve. (B) Histograms show mean ± SD of IL-12-p70 concentration in twenty-four samples from the screen in triplicate. Data are representative of four independent experiments. Samples 1 and 2: NT controls. Samples 3 and 4: Mock electroporation (without siRNA) controls. Samples 13 and 14: SOCS1 siRNA (putative positive controls). Samples 23 and 24: Poly(I:C)-stimulated (positive) controls.
**Figure 3.23. TNF-α ELISA analysis.** Sandwich ELISA was used to determine the TNF-α concentrations in BMDC culture supernatants from siRNA library-transfected BMDC samples. (A) TNF-α standard curve. (B) Histograms show mean ± SD of TNF-α concentration in twenty-four samples from the screen in triplicate. Data are representative of four independent experiments. Samples 1 and 2: NT controls. Samples 3 and 4: Mock electroporation (without siRNA) controls. Samples 13 and 14: SOCS1 siRNA (putative positive controls). Poly(I:C)-stimulated (positive) controls: 662.9 ± 30.6 pg/mL and 689.7 ± 28.8 pg/mL.

### 3.2.4 Gene Knockout Reveals SHP as a Positive Regulator of BMDC Maturation

To confirm our hypothesis that SHP is a negative regulator of BMDC maturation, we performed a series of experiments using a gene-targeted SHP-deficient (Shp−/−) mouse strain\(^7\). In contrast to our results with transfection of SHP-targeting siRNA, Shp−/− BMDCs displayed evidence of immaturity compared to WT BMDCs (Figures 3.24-3.26).
The mature MHC I\textsuperscript{high} population frequency was significantly reduced in unstimulated $Shp^{/-}$ BMDCs (10\%) than in unstimulated WT BMDCs (20\%) (Figure 3.24). In FITC-DX assays, the frequency of the immature MHC I\textsuperscript{low}/DX\textsuperscript{pos} population was greater in unstimulated $Shp^{/-}$ BMDCs (75\%) than in unstimulated WT BMDCs (60\%) (Figure 3.25). Strikingly, when stimulated with LPS, $Shp^{/-}$ BMDCs displayed a reduced mature MHC I\textsuperscript{high} population frequency (40\%) as compared to WT BMDCs (50\%) (Figure 3.26). Similarly, LPS-stimulated $Shp^{/-}$ BMDCs displayed a reduced frequency of the mature MHC I\textsuperscript{high}/DX\textsuperscript{neg} population (5\%) and an increased frequency of the immature MHC I\textsuperscript{low}/DX\textsuperscript{pos} population (60\%), as compared to WT BMDCs (15\% and 40\%, respectively) (Figure 3.25). Taken together, these results indicated that genetic ablation of $Shp$ during ontogeny inhibits BMDC maturation, and suggested that $Shp$ is a positive regulator of BMDC activation.
Figure 3.24. Reduced mature (MHC II$^{\text{high}}$) BMDC population in unstimulated $Shp^{-/-}$ BMDCs. Resting BMDCs were generated by culturing WT C57BL/6 or $Shp^{-/-}$ bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD- population. Top row: WT. Bottom row: $Shp^{-/-}$. Numbers indicate MHC II+ and CD11c+ frequencies. (A) Red and blue histograms: MHC II and CD11c staining. Shaded histograms: FMO control staining. (B) Dot plots: MHC II and CD11c staining. Data are representative of two independent experiments.
Figure 3.25. Increased dextran endocytosis in $Shp^-$ BMDCs. Resting BMDCs were generated by culturing WT C57BL/6 bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were re-cultured with or without LPS for 16-20 hours. Afterwards, they were incubated with FITC-DX, then stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD- population. Density plots show MHC II expression and DX positivity. Quadrant gates were set based on FITC-DX binding observed on ice in WT LPS-stimulated control: mature BMDC frequency = 23.2%, immature BMDC frequency = 6.2%. Data are representative of two independent experiments.
Figure 3.26. Reduced mature (MHC II\textsuperscript{high}) BMDC population in LPS-stimulated Shp\textsuperscript{-/-} BMDCs. Resting BMDCs were generated by culturing WT C57BL/6 or Shp\textsuperscript{-/-} bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were re-cultured with or without LPS for 16-20 hours, then stained with fluorochrome-conjugated monoclonal antibodies and analyzed by flow cytometry. Cells were gated on the 7-AAD- population. Top row: WT. Bottom row: Shp\textsuperscript{-/-}. Numbers indicate MHC II+ and CD11c+ frequencies. (A) Red and blue histograms: MHC II and CD11c staining. Shaded histograms: FMO control staining (same data as those shown in Figure 3.24). (B) Dot plots: MHC II and CD11c staining. Data are representative of two independent experiments.

In an effort to resolve the observed discrepancy between our results with Shp\textsuperscript{-/-} BMDCs and SHP siRNA-transfected BMDCs, we tested whether autoimmune diabetes could be induced in RIP-GP mice by vaccinating them with unstimulated or LPS-stimulated Shp\textsuperscript{-/-} BMDCs. If Shp is a negative regulator of BMDC activation, then vaccination of RIP-GP mice with unstimulated
Shp−/− BMDCs should induce diabetes (whereas unstimulated WT BMDCs would not induce diabetes, as usual). In contrast, if Shp is a positive regulator of BMDC activation, then vaccination of RIP-GP mice with LPS-stimulated Shp−/− BMDCs should result in attenuated diabetes (whereas LPS-stimulated WT BMDCs would induce florid diabetes, as usual). We found that (1) neither unstimulated WT nor unstimulated Shp−/− BMDCs induced diabetes, and (2) both LPS-stimulated WT and LPS-stimulated Shp−/− BMDCs induced diabetes (Figure 3.27). Therefore, these BMDC vaccination experiments were inconclusive, providing no support to either of the two hypothesis concerning the role of SHP in BMDC activation.
Figure 3.27. *Shp<sup>−/−</sup>* BMDC vaccination of RIP-GP mice. Resting BMDCs were generated by culturing WT C57BL/6 or *Shp<sup>−/−</sup>* bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were left unstimulated or stimulated with LPS (10 ng/mL). After 16-20 hours of culture, BMDCs were pulsed with LCMV triple-peptide mix for 2-3 hours, then adoptively transferred into RIP-GP mice. (A) Unstimulated WT BMDCs transferred into RIP-GP mouse. (B) Unstimulated *Shp<sup>−/−</sup>* BMDCs transferred into RIP-GP mouse. (C) LPS-stimulated WT BMDCs transferred into RIP-GP mouse. (D) LPS-stimulated *Shp<sup>−/−</sup>* BMDCs transferred into RIP-GP mouse. Blood glucose concentrations after BMDC transfer are shown. Each curve represents an individual mouse. Data are representative of two independent experiments.
3.3 siRNA LIBRARY SCREEN BASED ON BMDC EXPRESSION OF IL-12/23-p40 IDENTIFIES CANDIDATE GENES THAT REGULATE BMDC ACTIVATION

In a revised screening approach, we asked whether the transfection of siRNAs targeting various cytokine receptor genes (Dharmacon siGENOME siRNA Cytokine Receptors library, 158 SMARTpools) would cause the upregulation of IL-12/23-p40 production by BMDCs. Upregulation of BMDC-derived IL-12/23-p40 would indicate that the putatively silenced genes were negative regulators of BMDC activation.

Our first step was to characterize the estimated dynamic range of this new screening approach. To that end, we measured the fluorescence intensity of BMDCs under several key conditions (Figure 3.28). As compared to NT siRNA-transfected WT (i.e., eYFPneg) BMDCs, we detected a mild increase in eYFP expression in unstimulated (1) non-electroporated p40-eYFP KI BMDCs and (2) NT siRNA-transfected p40-eYFP KI BMDCs. Importantly, there was no significant difference in eYFP expression between these two groups. Moreover, when we transfected p40-eYFP KI BMDCs with siRNA targeting A20 (TNFAIP3, a known negative regulator of DC activation)78,79, we detected an upregulation of eYFP expression significantly above the basal level expressed by control NT siRNA-transfected p40-eYFP KI BMDCs.
Figure 3.28. Estimating the dynamic range of IL-12/23-p40-YFP expression. Resting BMDCs were generated by culturing WT C57BL/6 or IL-12/23-p40-YFP knock-in (p40-YFP KI) bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were left untreated or electroporated with A20-specific siRNA or NT siRNA (4762 nM) with a pulse of 400 V/200 µF at a cell density of 10.5x10⁶/mL. After 48 hours of culture, BMDCs were analyzed by flow cytometry. Cells were gated on the FSC/SSC population. Beige histogram: WT BMDCs transfected with NT siRNA. Red histogram: Untreated p40-YFP KI BMDCs. Blue histogram: p40-YFP KI BMDCs transfected with NT siRNA. Green histogram: p40-YFP KI BMDCs transfected with A20 siRNA. Data are representative of at least three independent experiments.

On the basis of (1) the reasonably low level of eYFP expression in NT siRNA-transfected p40-eYFP KI BMDCs (the control baseline), and (2) the significant eYFP upregulation in A20 siRNA-transfected p40-eYFP KI BMDCs, we hypothesized that BMDC-derived IL-12/23-p40 production, as detected by eYFP expression in p40-eYFP KI BMDCs, could be used as a robust indicator of BMDC maturation in an in vitro siRNA screen to identify genes that regulate BMDC
activation. Here, I will describe the results from the screen of the Cytokine Receptors library Plate #2, containing 78 siRNA SMARTpools.

### 3.3.1 siRNA Transfection Reveals IL-17C and RBBP8NL as Positive Regulators of BMDC Maturation

Figure 3.29 shows the composite summary of the results from our Cytokine Receptors siRNA library Plate #2 screen. Notably, there was no significant difference between (1) the top hits based on IL-12/23-p40 upregulation (towards the left-hand side of the graph) and (2) the NT controls (shown in blue). On the other hand, we found a striking difference between (1) the top hits based on IL-12/23-p40 downregulation (towards the right-hand side of the graph) and (2) the NT controls. These latter hits represented genes whose putative silencing resulted in dramatic IL-12/23-p40 downregulation (robust $z^*$ scores between 3 and 4 median absolute deviations below the median). Importantly, these data exhibited minimal variability over two independent experiments (Figure 3.29).
Figure 3.29. Cytokine Receptors siRNA library screen based on BMDC expression of IL-12/23-p40. Resting BMDCs were generated by culturing IL-12/23-p40-YFP knock-in bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with the Cytokine Receptors library as described in Chapter 2.4. After 48 hours of culture, BMDCs were analyzed by flow cytometry. Cells were gated on the FSC/SSC population. Histograms show median robust z* score + interquartile range of IL-12/23-p40-YFP+ population frequency. Red histograms: A20 siRNA (positive control). Blue histograms: NT siRNA (negative control). Purple histograms: Left, IL-17B siRNA; right, IL-17F siRNA. Orange histogram: CCNB1 siRNA. Green histogram: IL-17C siRNA. Yellow histogram: RBBP8NL siRNA. Data are from two independent experiments.
As a correlate to these data, we analyzed CD3+ T cell proliferation in response to coculture with siRNA-transfected BMDCs in the presence of low-dose CD3 monoclonal antibody stimulation (Figure 3.30). Reassuringly, the T cell proliferation data recapitulated the IL-12/23-p40 production data. The top three hits based on inhibition of T cell proliferation (towards the right-hand side of the graph) were identical to the top three hits based on downregulation of IL-12/23-p40 production.
Figure 3.30. Cytokine Receptors siRNA library screen based on T cell proliferation in coculture with siRNA-transfected BMDCs. Forty-eight hours after electroporation, the siRNA-transfected BMDCs from the experiment depicted in Figure 3.29 were co-cultured with Violet CellTrace-stained CD3+ T cells in the presence of CD3 monoclonal antibody. After 72 hours of culture, T cell proliferation was analyzed by flow cytometry. Cells were gated on the FSC/SSC population. Histograms show the robust z* score of the division index (DI, calculated by FlowJo software). Red histograms: A20 siRNA (positive control). Blue histograms: NT siRNA (negative control). Purple histograms: Left, IL-17B siRNA; right, IL-17F siRNA. Orange histogram: CCNB1 siRNA. Green histogram: IL-17C siRNA. Yellow histogram: RBBP8NL siRNA. Data are from a single experiment.
To exclude the possibility that the robust z* score differences that we observed were due to differences in BMDC viability rather than due to true differences in IL-12/23-p40 production, we analyzed the correlation between BMDC viability and the robust z* scores for IL-12/23-p40 production (Figure 3.31A). For this correlation analysis, we used the frequency of BMDCs in the initial flow cytometry analysis FSC/SSC gate (Figure 3.29) as a surrogate indicator of BMDC viability. We found that there was low correlation between BMDC viability and the robust z* scores for IL-12/23-p40 production (Spearman r = 0.3, 95% confidence interval: 0.1 to 0.5, p = 0.001). Therefore, we concluded that differences in BMDC viability were not responsible for the observed differences in the robust z* scores for IL-12/23-p40 production.

To exclude the possibility that the robust z* score differences that we observed were due to differences in post-electroporation sample well harvest order (i.e., the order in which we transferred samples from the 96-well electroporation plate into the 24-well plates for 48-hour culture), rather than due to true differences in IL-12/23-p40 production, we analyzed the correlation between the sample well harvest order and the rank order of the observed robust z* scores for IL-12/23-p40 production (Figure 3.31B). We found that there was low correlation (Spearman r = 0.4, 95% confidence interval: 0.2 to 0.6, p = 0.0003) between sample well harvest order and the rank order of the robust z* scores for IL-12/23-p40 production. We therefore concluded that differences in sample well harvest order were not responsible for the observed differences in the robust z* scores for IL-12/23-p40 production.
Figure 3.31. siRNA-transfected BMDC production of IL-12/23-p40 is not confounded by differences in BMDC sample viability or well harvest order. (A) For the experiment depicted in Figure 3.29, the correlation between BMDC viability (the frequency of cells in initial FSC/SSC gate) and the z* scores for IL-12/23-p40 production was analyzed using Prism software. (B) For the experiment depicted in Figure 3.29, the correlation between the rank order of the z* scores for IL-12/23-p40+ frequency and the sample well harvest order was analyzed using Prism software.

Figure 3.32 and 3.33 show representative raw data from the top hits of the Cytokine Receptors siRNA library screen, highlighting the results of siRNA transfection targeting IL-17C and RBBP8NL. Transfection of siRNA targeting IL-17C and RBBP8NL resulted in (1) greater than 80% reduction in BMDC production of IL-12/23-p40 and (2) almost 60% reduction in T cell proliferation, as compared to NT siRNA-transfected controls. On the basis of these data, we concluded that transfection of IL-17C and RBBP8NL-targeting siRNA inhibits BMDC
maturation, and we hypothesized that IL-17C and RBBP8NL are positive regulators of BMDC activation (Figure 3.32 and 3.33).

Figure 3.32. BMDC transfection of IL-17C-specific and RBBP8NL-specific siRNA downregulates BMDC production of IL-12/23-p40. Resting BMDCs were generated by culturing IL-12/23-p40-YFP knock-in bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with the Cytokine Receptors library as described in Chapter 2.4. After 48 hours of culture, BMDCs were analyzed by flow cytometry. Cells were gated on the FSC/SSC population. (A) Representative raw data from the experiment depicted in Figure 3.29. Histograms show IL-12/23-p40-YFP expression. Blue histograms: NT siRNA-transfected BMDCs. Red histograms: Experimental siRNA-transfected BMDCs, as indicated. Numbers indicate IL-12/23-p40+ frequencies. Untreated control: IL-12/23-p40+ frequency = 11.4%. Mock electroporation control: IL-12/23-p40+ frequency = 15%. A20 (positive) control: IL-12/23-p40+ frequency = 30.1%. (B) Histograms show IL-12/23-p40-YFP+ frequency index (i.e., the normalization of the indicated experimental siRNA data to NT siRNA data).
Figure 3.3. BMDC transfection of IL-17C-specific and RBBP8NL-specific siRNA downregulates T cell proliferation. Resting BMDCs were generated by culturing IL-12/23-p40-YFP knock-in bone marrow cells in the presence of GM-CSF for 10 days. On day 10, BMDCs were electroporated with the Cytokine Receptors library as described in Chapter 2.4. Forty-eight hours after electroporation, the siRNA-transfected BMDCs from the experiment depicted in Figure 3.29 were co-cultured with Violet CellTrace-stained CD3+ T cells in the presence of CD3 monoclonal antibody. After 72 hours of culture, T cell proliferation was analyzed by flow cytometry. Cells were gated on the FSC/SSC population. (A) Representative raw data from the experiment depicted in Figure 3.30. Histograms show Violet CellTrace dye dilution. Grey histograms: untreated BMDCs. Blue histograms: NT siRNA-transfected BMDCs. Red histograms: Experimental siRNA-transfected BMDCs, as indicated. (B) Histograms show T cell division index (DI) index (i.e., the normalization of the indicated experimental siRNA data to NT siRNA data).
CHAPTER 4: DISCUSSION

The overall goal of our work is to discover new genes and pathways that regulate BMDC activation and immunologic tolerance. In this study we (1) developed a high-throughput siRNA screen in BMDCs, and (2) showed that BMDC expression of CD80, CD86, MHC II, and IL-12/23-p40 can identify candidate genes that regulate BMDC maturation.

4.1 TWO-PHASE SCREENING APPROACH

Although the gold standard approach for determining whether a DC is immunogenic is to test whether it can induce an adaptive immune response in vivo, we designed and implemented a two-phase screening approach: (1) an initial in vitro screen, in which siRNA-transfected BMDCs are assayed in vitro for evidence of maturation (Figure 1.5), and (2) a subsequent in vivo screen, in which candidate positive hits from the in vitro screen are tested for their ability to induce an adaptive immune response in vivo using the RIP-GP BMDC vaccination system (Figure 1.6). We reasoned that a preliminary in vitro screen would achieve a strategic balance between (1) the desire for a high-throughput screen, and (2) the need for the screen to be highly sensitive, specific, and cost-efficient.

Although this study only reports the results of the screening of 132 targets, the methods of our in vitro screen design and workflow make clear that high efficiency, high-throughput siRNA library screening could be readily performed. The commercial availability of siRNA libraries, our use of a 96-well electroporator, and our implementation of adjustable multi-channel pipetting techniques all promote high efficiency workflow. The use of adjustable multi-channel pipettes is especially strategic because it enables the rapid transfer of samples between the 96-well electroporation plates and the pre-warmed, culture medium-containing 24-well plates.
Importantly, this rapid transfer also promotes cell viability by (1) minimizing the electroporated BMDCs’ exposure time to potentially cell-damaging pH extremes near the electroporator electrodes, and (2) bathing the BMDCs in warm serum-containing medium as soon as possible after electroporation.

The revision of our screening protocol to include the use of BMDCs derived from the IL-12/23-p40-eYFP knock-in mouse also promotes high-efficiency workflow by obviating the need for any further experimental manipulation following the period of putative gene silencing. The BMDCs can simply be collected, washed, and directly analyzed by flow cytometry, significantly streamlining the screening approach.

On this basis, we are currently planning to screen a large transmembrane receptor siRNA library in BMDCs. At optimum workflow, 320 targets could be screened per week, whereas four weeks and at least 16 to 20 mice would be required to test a single target with full readout of clinical diabetes in the RIP-GP model, with no guarantee that true validation or rejection of candidate targets would be revealed. In contrast, equipped with evidence from relatively robust *in vitro* assays like BMDC production of IL-12/23-p40, the validation of a subset of promising hits could be more confidently pursued *in vivo*, using gene silencing or gene knockout in a variety of animal models of immunologic disease.

**4.2 TYPE OF RNA INTERFERENCE**

We decided to employ siRNA rather than shRNA to silence genes in BMDCs based on a number of considerations. First, we reasoned that since siRNA does not require viral transfection of BMDCs, there should be less risk of PRR-mediated BMDC maturation that would mask the detection of true gene silencing-mediated BMDC activation. Second, since siRNA is not
plasmid-based, it should not interfere with the endogenous transcriptional machinery of the host cell. Third, when chemical or electrical transfection methods are employed, the transfection efficiency of short, 21-mer duplex RNA molecules (i.e., siRNA) is much higher than that of the comparatively longer plasmid DNA molecules containing shRNA inserts. Fourth, we found an article in the literature by Jantsch et al. that reported high-efficiency transfection of siRNA into BMDCs that were generated using the Lutz method. Importantly, the transfection efficiency and cell viability were high, no adverse effects on BMDC functionality were apparent, and excellent gene silencing at the RNA and protein levels was observed.

Although siRNA transfection does not lead to stable, long-term gene silencing, we hypothesized that this would not compromise the integrity of our screen. For the in vitro phase of the screen, we hypothesized that phenotypic changes in transfected DCs would be detectable within 48 hours following siRNA-based silencing, as has been demonstrated in a wide variety of other cell types. For the in vivo phase of the screen, we reasoned that once naïve recirculating T cells are activated in the secondary lymphoid organs by the adoptively transferred siRNA-transfected DCs, further gene silencing in the transfected DCs should no longer be necessary, because the transferred DCs will have already initiated the clonal activation, expansion, and differentiation of of the naive T cells into diabetogenic effector CTLs.

4.3 TYPE OF TRANSFECTION

We decided to employ electroporation to transfect siRNA into BMDCs because of the Jantsch et al. article mentioned above that demonstrated high efficiency transfection of siRNA into BMDCs without BMDC maturation or reduction in BMDC viability. Notably, this report showed that, in contrast to electroporation, lipofection of BMDCs (using Lipofectamine 2000) resulted in a severe reduction in BMDC viability. Specifically, Jantsch et al. found that, after 48
hours, 30-40% of the transfected BMDCs displayed propidium iodide-positive staining. In addition, lipofection impaired the ability of BMDCs to mature in response to TLR stimulation.

The electroporation in this report was performed using the commercially available Bio-Rad Gene Pulser XCell electroporator, a single-cuvette device that enables rational adjustment of critical electroporation parameters such as voltage, capacitance, and resistance. To enable our screen to be high-throughput, we designed our protocol using a related Bio-Rad electroporator, the Gene Pulser MXCell, which is biophysically identical to the Gene Pulser XCell but can electroporate samples in 96-well electroporation plates.

4.4 CRITICAL PARAMETERS OF THE IN VITRO SCREEN

To endow the in vitro screen with maximum sensitivity and specificity for detecting true positive hits, i.e., genes that truly regulate BMDC activation, it was necessary to optimize the BMDC culture and transfection conditions. We first sought to achieve the following goals: (1) minimization of baseline (pre-transfection) BMDC maturation, in order to maximize the dynamic range of the screen; (2) maximization of siRNA transfection efficiency, in order to enable maximum gene silencing and thereby maximize the magnitude of any resulting phenotypic change; and (3) maximization of BMDC viability, in order to (a) maximize the detectability of any resulting phenotypic change and (b) minimize the risk of BMDC maturation that could result from exposure of live BMDCs to necrotic BMDCs or their extruded contents.

The baseline maturation level of our day 10 pre-transfection BMDCs was initially quite high and therefore unsuitable for a high-dynamic range screen. We succeeded in greatly reducing this baseline BMDC maturation level when we made two key experimental changes. First, we found and followed Lutz et al.’s guidelines to empirically select FBS lots containing LDH and SGOT concentrations within specified ranges.
Second, we removed GM-CSF from the BMDC culture medium on day 10, at the time when the BMDCs are transferred to tissue culture-treated plates for a further period of culture. This eliminated the observed and known\textsuperscript{91} potential stimulatory effect of GM-CSF.

As our objective was to perform a high-throughput screen, it would have been highly inefficient to optimize the magnitude of silencing of every individual gene in a given library prior to screening. Since every gene has individual expression dynamics, every gene could theoretically require different electroporation conditions to maximize its silencing. Thus, instead, accepting the risk of generating false-positive and false-negative hits, we attempted to optimize and maximize the siRNA transfection efficiency as the surrogate for successful transfection. We reasoned that uniform delivery of siRNA across all BMDC samples would be the best high-throughput approach to promote maximum library-wide gene silencing, and thereby maximize the magnitude of any resulting phenotypic change. By rationally and systematically modulating various electroporation parameters, we were able to achieve an siRNA transfection efficiency approaching 90% without maturing or killing the BMDCs. In other words, using our optimized conditions, we demonstrated that electroporation does not cause BMDC maturation and does not cause a reduction in BMDC viability. Along the same lines, we demonstrated that siRNA does not cause BMDC maturation and does not cause a reduction in BMDC viability.

To determine whether our optimized conditions could be used for a large-scale siRNA library screen, we conducted two proof-of-principle experiments. First, we demonstrated that we could successfully downregulate BMDC surface expression of CD11c, a molecule highly expressed on the BMDC surface, by transfecting BMDCs with CD11c-specific siRNA. Second, we demonstrated that we could induce BMDC maturation by transfecting BMDCs with siRNA targeting a known negative regulator of BMDC activation, SOCS1\textsuperscript{89}.
To endow the *in vitro* screen with high sensitivity and specificity for detecting true positive hits, it was also critical that we incorporate robust *in vitro* readouts of BMDC activation into our screening protocol. The BMDC maturation markers that we initially chose to assay for the *in vitro* phase of our screen were the classic costimulatory molecules, CD80 and CD86. We chose these markers for several reasons. First, although it is true that non-immunogenic and tolerogenic DCs may express substantial levels of these molecules, it is also true that full activation of naive T cells requires the interaction of CD80 and CD86 on the BMDC surface with CD28 on the T cell surface. Second, CD80 and CD86 are reliably and significantly upregulated on BMDCs that have been stimulated via TLRs, the prototypic PRRs. Third, at the outset of our screen's development, we preferred to generate more false positive candidate hits than false negative ones, and CD80 and CD86 are more sensitive than they are specific. Fourth, towards the goal of developing a high-throughput workflow, we favoured the technical simplicity of flow cytometric detection of cell-surface molecules stained with fluorochrome-conjugated antibodies, which we could easily employ to analyze BMDC surface expression of CD80 and CD86.

At the outset, we recognized the limitations of CD80 and CD86 as markers for BMDC activation. We therefore designed our initial protocol to include the collection of siRNA-transfected BMDC culture supernatants for ELISA-based analysis of BMDC-derived cytokines, for follow-up validation of candidate hits arising from the interrogation of surface CD80, CD86, and MHC II. As described above, inflammatory cytokine production is the key DC-derived signal necessary for effector T cell differentiation and adaptive immunity.
4.5 SCREEN VALIDATION

4.5.1 First Screening Approach

We began our screen with the Dharamacon siGENOME siRNA Nuclear Receptors library. As nuclear receptors play fundamental roles in cell biology as transcriptional regulators of gene expression, we hypothesized that the silencing of individual nuclear receptors in BMDCs would reveal new mechanistic insights into the regulation of BMDC activation.

We were heartened to find that the mean of the CD80 and CD86 MFI indices for most of the top hits was significantly increased compared to the NT control (Figure 3.17A). However, the overall magnitude of the increase in the CD86 MFI index was relatively low. None of the top hits exhibited at least a two-fold increase in mean CD86 MFI index, with only SHP (the top hit) reaching a mean of only 1.7. In the case of CD80, only SHP reached a two-fold increase in mean MFI index to 2.4, while the others reached between 1.7 and 1.8. Moreover, the scatter plot of the data (Figure 3.17B) highlighted the presence of considerable variability in the results, with some data points falling close to the NT control baseline level.

In our second analysis of the same data, we were impressed to find that the MHC II MFI index for most of the top hits was significantly decreased compared to the NT control (Figure 3.19). Moreover, the decrease in the mean MHC II MFI index for NR3C2, the top hit, was greater than 50%, with relatively low variability.

In an effort to clarify and validate these data, we analyzed the BMDC culture supernatants from the screen for the presence of IL-6, IL-12-p70, and TNF-α. These culture supernatants were collected and stored at -80°C forty-eight hours after siRNA library transfection, at the time of screening BMDC harvest. To our surprise, we were unable to detect
any cytokines in any of the supernatants (Figures 3.21-3.23). There are at least two possible explanations for this unexpected finding. First, perhaps there truly was no production of IL-6, IL-12-p70, or TNF-α by our nuclear receptor-siRNA-transfected BMDCs. Second, although the BMDC culture supernatants were stored at -80°C, perhaps there was degradation of the cytokines, for unknown reasons.

Nonetheless, we attempted to validate SHP, the top hit, in vivo using an existing Shp-deficient mouse strain. Since this mouse strain had already been generated on the C57BL/6 genetic background, and some of our data suggested that SHP-siRNA transfection could greatly increase costimulatory molecule expression as well as increase the MHC II\textsuperscript{high}/DX\textsuperscript{neg} population frequency and decrease the MHC II\textsuperscript{low}/DX\textsuperscript{pos} population frequency (Figure 3.18), we reasoned that it was worthwhile to import this strain for the purpose of testing our hypothesis that SHP is a negative regulator of BMDC activation.

The Shp\textsuperscript{−/−} strain produced results in vitro that were opposite to those that we had obtained in vitro using SHP-siRNA-transfected BMDCs. Whereas the latter had provided evidence that SHP was a negative regulator of BMDC activation (Figure 3.18), the former suggested that SHP was a positive regulator of BMDC activation (Figures 3.24-3.26). One possible explanation for these conflicting data is that the SHP-siRNA transfection phenotype was due to an off-target siRNA effect. Alternatively, in the Shp\textsuperscript{−/−} strain, there may have been developmental upregulation of one or more genes to compensate for the constitutive germline SHP deficiency. If so, then inducible or conditional Shp deletion might reveal that SHP truly is a negative regulator of BMDC activation. In support of this hypothesis, a recent study used the Shp\textsuperscript{−/−} strain to show that SHP is a negative regulator of TLR signaling in macrophages\textsuperscript{93}. 

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To reconcile our conflicting data, we tested the ability of \( \text{Shp}^{-/-} \) BMDCs to induce autoimmune diabetes in the RIP-GP model (Figure 3.27). Unfortunately, there was no difference between the \( \text{Shp}^{-/-} \) and WT BMDCs, whether unstimulated or stimulated with low-dose LPS. There are three possible explanations for these negative findings. First, as above, developmental gene compensation may have masked the true phenotype. Second, SHP may not play a biological role in RIP-GP autoimmune diabetes. Third, even if SHP does play a role in RIP-GP diabetes, we may not have used the right experimental conditions to reveal it. For example, perhaps a phenotype would have been detectable had we stimulated the transferred BMDCs with different doses of LPS or with a different TLR.

Taken together, we can draw three conclusions from the data discussed so far. First, our \textit{in vitro} screen based on CD80, CD86, and MHC II did successfully identify candidate genes that regulate BMDC maturation. In other words, our screen did detect \textit{in vitro} differences in the phenotype and functionality of BMDCs that were transfected with siRNAs targeting nuclear receptors as compared to NT controls.

Second, since \textit{in vitro} data may not always reflect \textit{in vivo} data, rigorous functional validation is crucial both \textit{in vitro} and \textit{in vivo}. \textit{In vitro}, it is essential to determine whether target-specific siRNA has truly downregulated target gene expression at the RNA and protein levels, using methods such as real-time quantitative polymerase chain reaction (qPCR) and Western blotting. If specific gene silencing is confirmed, then it is essential to validate further by confirming the consistency of the phenotype in response to a variety of different siRNAs against the same target.

Third, even if off-target effects are ruled out and it is clear that the observed \textit{in vitro} phenotype is due to specific gene silencing, functional \textit{in vivo} validation may require the testing
of multiple conditions, possibly in more than one animal model, to reveal whether the hit is true or false and demonstrate biological and clinical relevance.

4.5.2 Second Screening Approach

At this juncture, we re-evaluated our screening approach. Specifically, we questioned whether the in vitro indicators that we had been employing to screen for BMDC activation (i.e., CD80, CD86, MHC II, DX uptake) were sufficiently robust to warrant further expenditure of time, effort, and money pursuing the validation of the candidate hits that were generated on their basis. In an attempt to enhance the reliability and robustness of the in vitro phase of our screen, we adopted a new experimental approach with three key improvements.

First, BMDC-derived IL-12/23-p40 production was chosen as the primary in vitro indicator of BMDC activation (Figure 4.1). This readout was chosen for two main reasons. First, as discussed above, IL-12 and IL-23 are crucial regulators of cell-mediated immunity. Thus, if transfected DCs upregulate the production of IL-12/23-p40, it would be reasonable to conclude that they are immunogenic and that they will mediate significant immune effects. Second, we discovered that an IL-12/23-p40-eYFP knock-in mouse strain (p40-YFP KI) was commercially available and therefore readily accessible.

In this approach, BMDCs were generated in vitro using bone marrow cells harvested from the p40-YFP KI mouse. This transgenic strain has an IRES-eYFP sequence inserted downstream of the endogenous IL-12/23-p40 stop codon, thereby allowing normal expression of IL-12/23-p40 and simultaneous eYFP expression. Thus, BMDC expression of this preeminent DC-derived immunoregulatory cytokine could be easily measured by flow cytometric detection of eYFP.
Second, we used a T cell proliferation assay as a functional correlate to BMDC-derived IL-12/23-p40 production (Figure 4.1). Purified CD3+ T cell cells were co-cultured with siRNA-transfected BMDCs in the presence of low-dose CD3 monoclonal antibody stimulation, and T cell proliferation was measured by flow cytometric analysis of Violet CellTrace dye dilution.

Figure 4.1. Overview of the revised in vitro phase of the experimental screening approach. BMDCs generated in vitro from an IL-12/23-p40-YFP knock-in mouse are transfected with an siRNA library. After 48 hours of putative gene silencing, one portion of the siRNA-transfected BMDCs are analyzed for IL-12/23-p40-YFP expression by flow cytometry. In parallel, splenic CD3+ T cells are purified and stained with Violet CellTrace, then co-cultured with a second portion of the siRNA-transfected BMDCs in the presence of low-dose CD3 monoclonal antibody stimulation. After 72 hours of co-culture, T cell proliferation is analyzed by flow cytometry. Co-culture supernatants are harvested for future cytokine ELISA analysis.
Third, we refined our statistical approach to data analysis. Instead of defining hits based on the rank order of the index of fold change in the frequency of a given BMDC population (e.g., CD80+CD86+), we defined them based on the rank order of the robust z score ($z^*$ score) of the frequency of a given BMDC population (e.g., p40-eYFP+). Due to their nonparametric nature, $z^*$ scores are more robust (i.e., insensitive) to outliers. This is relevant to a high-throughput RNAi screen, in which true hits should behave like outliers. As such, robust statistics like the $z^*$ score are especially appropriate and recommended for analysis of data from RNAi screens\textsuperscript{94,95}.

After generating evidence that the dynamic range of a screen based on IL-12/23-p40-eYFP expression would be acceptable (Figure 3.28), we proceeded to screen the Dharmacon siGENOME Cytokine Receptors siRNA library. In view of (1) the fundamental role of cytokines and their receptors in all aspects of immune regulation, and (2) the potential for transmembrane molecules to be used as targets for antibody- and/or fusion protein-mediated clinical therapy, we hypothesized that the silencing of individual cytokine receptors in BMDCs would uncover new biological insights into the regulation of BMDC activation.

Interestingly, none of the transfected siRNA pools upregulated BMDC production of IL-12/23-p40 relative to the NT controls (Figure 3.29). However, we were delighted to observe that several of the transfected siRNA pools dramatically downregulated IL-12/23-p40 production, with mean robust $z^*$ scores from two replicates exceeding three median absolute deviations below the median (Figure 3.29). T cell proliferation data from an siRNA-transfected BMDC-T cell co-culture experiment with CD3 monoclonal antibody stimulation corroborated these findings (Figure 3.30), and correlation analyses ruled out meaningful associations between the robust $z^*$ scores and cell viability and sample well harvest order (Figure 3.31).
One of the top hits from the Cytokine Receptors screen, IL-17C, is of special interest to us. Whereas IL-17A and IL-17F have been previously studied and revealed to play important roles in clearing extracellular pathogens and mediating autoimmune inflammation\(^69\), relatively little was known about IL-17C until several high profile studies recently demonstrated a biological role for this cytokine in mucosal immunity and in T cells\(^96-98\). Notably, none of these studies investigated a possible role for IL-17C in DC biology.

Key findings from these studies include the demonstrations that epithelial cells produce IL-17C in response to stimulation through TLR2, TLR4, and TLR5 and by TNF-\(\alpha\) and IL-1\(\beta\)\(^98\), and that IL-17C enhances IL-17A and IL-17F production by Th17 cells\(^97\). Additionally, gene-targeted \(Il17c^{-/-}\) mice exhibited reduced incidence and severity of EAE, with reduced infiltration of Th1 and Th17 cells into the central nervous system (CNS)\(^97\). \(Il17c^{-/-}\) mice were also found to be resistant to dextran sodium sulfate (DSS) colitis. Finally, \(Il17re^{-/-}\) mice, in which the IL-17C receptor (IL-17RE) is genetically ablated, were found to be more susceptible to bacterial gastrointestinal infections\(^96\).

Taken together, these data suggest a possible model\(^99\) in which PAMP- and/or cytokine-mediated stimulation triggers IL-17C production by mucosal epithelial cells. IL-17C then acts (1) in an autocrine manner to induce the mucosal epithelial production of infection-fighting defensins, and (2) in a paracrine manner to stimulate the release of IL-17A and IL-17F from Th17 cells. In turn, IL-17A and IL-17F promote neutrophil recruitment and B cell production of immunoglobulin A (IgA). Importantly, this model could account for the observed gastrointestinal and CNS phenotypes in the \(Il17c^{-/-}\) and \(Il17re^{-/-}\) knockout mice. First, in the absence of IL-17C, the mucosal epithelium produces less defensins in response to infection, thereby increasing susceptibility to infection. Second, in the absence of IL-17C, there is less mucosal inflammation
in response to DSS-induced damage, thereby conferring resistance to DSS colitis. Third, in the absence of IL-17C, Th17 cells produce less IL-17A and IL-17F, thereby reducing EAE severity.

There is only one study in the literature that contains any possible examination of the role of IL-17C in DC biology. Yamaguchi et al.\textsuperscript{100} induced collagen-induced arthritis (CIA) in mice, isolated cells from the arthritic paws, sorted various cell populations using fluorescence activated cell sorting (FACS), and used qPCR to analyze the expression of IL-17C in the FACS-sorted cell populations. Their data suggest that IL-17C is upregulated in arthritic paw-derived CD11c+MHC II+ cells. However, no further characterization of these cells was performed. Therefore, the identity of these CD11c+MHC II+ cells is unclear.

Importantly, two other IL-17 gene family members, IL-17B and IL-17F, were also contained in the Cytokine Receptors library that we screened. However, there was only a marked phenotype when IL-17C-specific siRNA was transfected, suggesting that the putative role for IL-17C in DC biology may be unique to IL-17C and not a general IL-17 family-member phenomenon.

Another one of the top hits that is of particular interest to us is RBBP8NL (retinoblastoma binding protein 8 N-terminal like), for two reasons. First, there are no publications in the scientific literature concerning this gene. Second, RBBP8, to which RBBP8NL is related, has to been shown to have important roles in transcriptional repression, DNA repair, DNA replication, and tumour suppression\textsuperscript{101}. Thus, based on our data, it is tantalizing to speculate that it also has an important role in DC biology.
4.6 CRITICAL REVIEW OF THE SCIENTIFIC LITERATURE

The key study by Jantsch et al.\textsuperscript{88} was the first to show that electroporation could be used to transfect siRNA into BMDCs with high efficiency and without adverse effects on viability or maturation level. Similar to Jantsch et al., we found that an exponential pulse waveform of 400 V/200 $\mu$F was ideal (Jantsch et al. used 400 V/150 $\mu$F). Like Jantsch et al., we also found that high siRNA transfection efficiency required almost 5000 nM of siRNA (we used 4762 nM, Jantsch used approximately 4800 nM).

One notable difference between our respective methods was the electroporation time point. Although Jantsch et al. used the Lutz method\textsuperscript{48} for in vitro BMDC generation as we did, they electroporated their BMDCs on day 7-8, whereas we electroporated our BMDCs on day 10. We strategically chose this later time point for two main reasons. First, the BMDC yield per dish on day 10 is typically more than double that on day 8 (data not shown), facilitating the performance of multiple experiments from one bone marrow culture. Second, the frequency of CD11c$^{\mathrm{high}}$ BMDCs is typically around 90% on day 10, whereas it is more than 75% on day 8 (data not shown). Thus, the BMDC culture on day 10 is more homogeneous and enriched for true BMDCs.

When we began this project, there were no reports of RNAi screens in DCs in the scientific literature. Since then, two such reports have been published. In the first report\textsuperscript{102}, Singhal et al. used two Dharmacon siGENOME siRNA libraries (Calcium/Calmodulin Protein Kinases and Cysteine Proteases) to identify genes in BMDCs that regulate regulate \textit{M. tuberculosis} survival. This report contains significant methodological differences compared to our screening approach.
First, using the original Inaba et al. method\textsuperscript{47} to generate BMDCs \textit{in vitro}, Singhal et al. transfected not BMDCs, but bone marrow precursors, sometime at the beginning of that culture process, then added GM-CSF at five hours post-transfection. Thus, it would seem that their putative gene silencing began extremely early in BMDC development, in cells that were not yet DCs. Second, they transfected the cells for seventy-two hours using lipofection. There was no information provided on whether this had any adverse effects on cell viability or activation level. Third, they reported that their BMDC culture protocol yields a homogeneous population of DCs that was 99\% pure. However, the article that they referenced in support of this claim seems to suggest otherwise\textsuperscript{103}. Although they do not show frequency gates on the flow cytometry plots, there appears to be considerable overlap between the control isotype antibody staining profile and both CD11c and I-A (MHC II) antibody staining profiles. Moreover, other sources report that the standard Inaba et al. method yields a DC purity of 60\% by day 8\textsuperscript{48}. Fourth, following the seventy-two hour transfection period, they stimulated their cells either with TLR ligands, \textit{M. tuberculosis} antigens, or \textit{M. tuberculosis} infection. Thus, although they transfected unactivated DCs, their primary readout, survival of \textit{M. tuberculosis} in DCs, was assayed in stimulated DCs.

In the second report\textsuperscript{104}, Moita et al. used an shRNA library to screen for kinases and phosphatases that regulate antigen presentation by BMDCs. This study also contains important methodological differences compared to our own. First and foremost, the authors used lentiviral infection of BMDCs to to transfect shRNAs for gene silencing. There was no information provided on whether this had any adverse effects on cell viability or activation level. However, they did exclude from their analysis any samples containing less than 20,000 viable cells because reproducibility was reported to be low at these low cell numbers. Second, the primary readout in this screen was T cell proliferation (measured by \textsuperscript{3}H-thymidine incorporation) rather than a DC-intrinsic effector function. Third, the authors used a z score analysis to define hits. As discussed
above, this statistic is sensitive to outliers and therefore the robust z* score is preferable for RNAi screens³⁴,³⁵.

**4.7 CONCLUSION AND FUTURE DIRECTIONS**

In conclusion, we (1) developed a high-throughput siRNA screen in BMDCs and (2) demonstrated that BMDC expression of CD80, CD86, MHC II, and IL-12/23-p40 can identify candidate genes that regulate DC activation. We are currently conducting validation studies on these candidate genes. Moreover, our revised screening approach is unique in that: (1) it employs a *critical DC-intrinsic effector function necessary for effector T cell differentiation* (i.e., production of IL-12/23-p40) as the primary readout, (2) it employs the *electroporation* of *siRNA* into *immature* BMDCs, and (3) it is highly streamlined because the BMDCs are derived from a fluorochrome knock-in mouse strain, greatly simplifying and streamlining the experimental protocol. Our revised screening approach facilitates large scale, high-throughput studies, which we are currently planning for the near future.
REFERENCES


