J-LEAPS VACCINES ARE SUFFICIENT TO ACTIVATE AND DIRECT AN IMMUNE RESPONSE THROUGH DENDRITIC CELLS

A dissertation submitted to Kent State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

Patricia R. Taylor

August, 2010
Dissertation written by
Patricia R. Taylor
B.S., The University of Akron, 2005
M.S., The University of Akron, 2007
Ph.D, Kent State University, 2010

Approved by

Ken S. Rosenthal, Advisor

Jennifer L. Marcinkiewicz, Committee Member

Gary K. Koski, Committee Member

Gary. D. Neihaus, Committee Member

Chun-che Tsai, Committee Member

Accepted by

Robert V. Dorman, Director of the School of Biomedical Sciences

Timothy Moerland, Dean of the College of Arts and Sciences
TABLE OF CONTENTS

LIST OF FIGURES..................................................................................vii

LIST OF TABLES....................................................................................ix

INTRODUCTION.........................................................................................1

Vaccines.................................................................................................1

Dendritic cells.......................................................................................9

T cell immune response........................................................................26

J-LEAPS vaccines..................................................................................34

METHODS...............................................................................................43

Mice........................................................................................................43

Peptides...................................................................................................43

J-LEAPS immunizations.......................................................................44

Immunization cytokine profile...............................................................45

Spleen cell preparation...........................................................................46

Bone marrow cell preparation..............................................................47

Immature dendritic cell preparation....................................................48

ELISA......................................................................................................49

Mouse flow cytometry...........................................................................49

Bone marrow cytokine arrays...............................................................50
Co-cultivations ................................................................. 51
Protein extraction .......................................................... 51
Western immunoblotting ............................................... 52
Cell inhibitor preparation .............................................. 53
Human monocyte preparation ........................................ 53
Human lymphocyte preparation .................................... 54
Human cytokine arrays ................................................ 55
Human flow cytometry .................................................. 56
Allotypic T cell assay .................................................... 56
Antigen specific co-cultivations ..................................... 56
JgD-DC vaccine preparation ......................................... 57
JgD-DC vaccine injections ........................................... 57
HSV-1 dermal abrasion infection .................................... 58
HSV-1 infection scoring ............................................... 59
RESULTS ........................................................................... 60
J-LEAPS vaccines’ cytokine response ............................. 60
J-LEAPS mouse target cell .......................................... 74
J-LEAPS human cell response ..................................... 102
JgD antigen specific response ...................................... 117
DISCUSSION ................................................................. 130
J-LEAPS cytokine survey ............................................. 131
Mouse J-LEAPS target cell………………………………………………139
J-LEAPS human cell response…………………………………………150
JgD antigen specific response…………………………………………..155
CONCLUSION………………………………………………………………159
BIBLIOGRAPHY…………………………………………………………160
APPENDICES………………………………………………………………171
Appendix AI…………………………………………………………………171
Appendix All…………………………………………………………………173
Appendix Alll…………………………………………………………………175
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Th1 activation</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>J-LEAPS vaccine constructs</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>JgD cytokine survey</td>
<td>63</td>
</tr>
<tr>
<td>4</td>
<td>Cytokine arrays of sera</td>
<td>67</td>
</tr>
<tr>
<td>5</td>
<td>J &amp; JH cytokine survey</td>
<td>68</td>
</tr>
<tr>
<td>6</td>
<td>J, JgD, and JH cytokine survey</td>
<td>70</td>
</tr>
<tr>
<td>7</td>
<td>IL-12 ELISA</td>
<td>72</td>
</tr>
<tr>
<td>8</td>
<td>Splenocyte cytokine arrays</td>
<td>77</td>
</tr>
<tr>
<td>9</td>
<td>Bone marrow cytokine survey</td>
<td>79</td>
</tr>
<tr>
<td>10</td>
<td>Co-culture cytokine survey</td>
<td>81</td>
</tr>
<tr>
<td>11</td>
<td>Cytokine survey of co-culture washes</td>
<td>83</td>
</tr>
<tr>
<td>12</td>
<td>Flow cytometry of CD86, MHC 11, CD11c</td>
<td>86</td>
</tr>
<tr>
<td>13</td>
<td>Flow cytometry of CD8 and IL-12</td>
<td>88</td>
</tr>
<tr>
<td>14</td>
<td>Lck activation in T cells</td>
<td>90</td>
</tr>
<tr>
<td>15</td>
<td>Lck western blot</td>
<td>92</td>
</tr>
<tr>
<td>16</td>
<td>Flow cytometry of Lck inhibited IL-12</td>
<td>94</td>
</tr>
<tr>
<td>17</td>
<td>Flow cytometry of Cyclosporin A IL-12</td>
<td>96</td>
</tr>
<tr>
<td>18</td>
<td>Purified iDCs results</td>
<td>99</td>
</tr>
<tr>
<td>19</td>
<td>Phenotypic human cell results</td>
<td>105</td>
</tr>
<tr>
<td>Page</td>
<td>Section Title</td>
<td>Page</td>
</tr>
<tr>
<td>------</td>
<td>---------------</td>
<td>------</td>
</tr>
<tr>
<td>20</td>
<td>Cytokine survey of human cells</td>
<td>107</td>
</tr>
<tr>
<td>21</td>
<td>Human lymphocyte cytokine arrays</td>
<td>110</td>
</tr>
<tr>
<td>22</td>
<td>Allotypic T cell assay</td>
<td>115</td>
</tr>
<tr>
<td>23</td>
<td>JgD-DC IP vaccine survival curve</td>
<td>122</td>
</tr>
<tr>
<td>24</td>
<td>JgD-DC IP vaccine disease score</td>
<td>123</td>
</tr>
<tr>
<td>25</td>
<td>JgD-DC IP &amp; ID vaccine survival curve</td>
<td>125</td>
</tr>
<tr>
<td>26</td>
<td>JgD-DC IP &amp; ID vaccine disease score</td>
<td>126</td>
</tr>
<tr>
<td>27</td>
<td>All J-LEAPS IP &amp; ID vaccine scores</td>
<td>128</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th></th>
<th>Table Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Definitions of immune responses</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Development of dendritic cells</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Dendritic cell isolation</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>Dendritic cell cytokines</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>Th1 subsets</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>Sequential Bonferroni of cytokine surveys</td>
<td>69</td>
</tr>
<tr>
<td>7</td>
<td>Cytokine survey of GM/4, GM, and human monocyte</td>
<td>112</td>
</tr>
<tr>
<td>8</td>
<td>Antigen specific co-cultures’ cytokine survey</td>
<td>120</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

Vaccines

Vaccines prevent the spread of infectious diseases by inducing immunity against pathogens and are one of the most efficient ways to prevent epidemics. Successful vaccines promote a protective immune response that often provides lifelong protection without harmful side effects. There are many different ways vaccinologists can manipulate pathogens or antigenic proteins to design a vaccine that initiates immune protection from an infectious disease. Many potentially fatal childhood diseases (such as polio, measles, mumps, rubella, and hepatitis) are seldom seen in the United States due to the effectiveness of vaccines. Immunizations save millions of lives each year. However, there still remains a great need for new vaccines against many other viral diseases, like Herpes Simplex Virus-1 (HSV-1) and Human Immunodeficiency Virus (HIV). The American Social Health Association estimates that nearly 41,000 people per day...
are infected by some type of sexually transmitted disease, like HSV-1. While the World Health Organization (WHO) estimates that approximately 14,000 individuals per day are infected with HIV worldwide. An effective vaccine could have an immense impact on the control of the spread of these infectious diseases.
Table 1. Definitions of components involved in an immune response.

<table>
<thead>
<tr>
<th>Keyword</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease</td>
<td>An incorrectly functioning organ or system of the body resulting from infection.</td>
</tr>
<tr>
<td>Pathogen</td>
<td>A disease causing organism</td>
</tr>
<tr>
<td>Immunity</td>
<td>Host defenses that are mediated by immune cells, following exposure to a pathogen, which elicits an immune response that has specificity, diversity, memory, and self-nonself recognition.</td>
</tr>
<tr>
<td>Antigen</td>
<td>Any substance (usually foreign) that binds specifically to an antibody or a T cell receptor.</td>
</tr>
<tr>
<td>Immunogen</td>
<td>A substance capable of eliciting an immune response.</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>An agent that increases the antigenic response of an immunogen.</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>The capacity of a substance to induce a specific immune response under a given set of conditions.</td>
</tr>
<tr>
<td>Epitope</td>
<td>The minimal portion of an antigen that is recognized and bound by an antibody or a T cell receptor-peptide complex.</td>
</tr>
<tr>
<td>Humoral immunity</td>
<td>Host defenses that are mediated by antibody present in plasma. It protects against extracellular bacteria and foreign macromolecules.</td>
</tr>
<tr>
<td>Cell-mediated immunity</td>
<td>Host defenses that are directed by antigen-specific T cells and mediated by various nonspecific cells of the innate immune system. It protects against intracellular infections, fungi, tumors, etc.</td>
</tr>
<tr>
<td>Cytokines</td>
<td>Small proteins that direct and regulate the nature of an immune response by exerting a variety of effects on lymphocytes and other immune cells.</td>
</tr>
<tr>
<td>CD number</td>
<td>Cluster of Differentiation (CD) is a protein defined by collection of monoclonal antibodies that is found on a particular differentiated cell. The antigen recognized by these antibodies is assigned an identifying number.</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex (MHC) is a complex of genes encoding immune proteins, including proteins that are required for antigen presentation to T cells (HLA in humans and MHC I and MHC II in mice).</td>
</tr>
</tbody>
</table>

*The above definitions of immunological terms that will be used throughout this dissertation to explain immune responses (1).*
Development of Vaccines

The goal of a vaccine is to trick the immune system into thinking that it sees an infection and then elicit the desired immune response in a safe manner. In natural immunity infection is spread throughout the host by an invading pathogen, the pathogen is recognized by an innate immune cell and processed into antigenic peptides. Antigenic peptides are then presented to adaptive immune cells to initiate a protective immune response. In a vaccine, the pathogen or subunits of the pathogen have to initiate these protective immune responses without eliciting disease. Common immunological terms used throughout this dissertation are defined in Table 1.

A vaccine must act as both an adjuvant and an immunogen to initiate protective immunity. An immunogen is an antigen that has the proper “foreignness,” molecular size, chemical composition and complexity to initiate an immune response. Immunogens are usually molecules that are not present in the body and for which there are not tolerance or suppressive mechanisms. The immunogen has to be sufficiently large, usually with a molecular mass of 100,000 daltons. Substances with molecular mass less than 5,000 daltons normally cannot elicit an immune response. Finally if the molecule is not a protein but rather a lipid or carbohydrate, a complete immune response is normally not elicited without attaching the molecule to a carrier protein (1). An antigen may lack the ability to initiate an immune response, but is still recognized by immune
cells. An antigen that is not immunogenic due to small size or lack of chemical complexity is a hapten. The immunogenicity of a hapten can be enhanced by attachment to a carrier protein; such as keyhole limpet hemocyanin (KLH). When a carrier protein is added to a hapten, the hapten becomes accessible to the immune system due to increased size and can function as an immunogen. However, the carrier protein initiates an immune response to itself as well as to the hapten, unrelated to the immune response needed for protective immunity (1).

Another way that immunogenicity can be increased is by adding an adjuvant. Adjuvants help vaccines elicit an early, high and long-lasting immune response with a lower amount of antigen; which can make the vaccine safer and less costly. Adjuvants can be used with immunogens in vaccines to prolong the presence of an immunogen in tissue. This extends the length of time of exposure of the immunogen to the immune cell to allow induction of protective immune responses. Also, with the use of adjuvants, the immune response can be selectively modulated to initiate a specific type of T cell response capable of protection from diseases caused by intracellular pathogens, such as viruses (2, 3). Live vaccines (i.e. measles mumps and rubella) do not use adjuvants. Alum adjuvants are not very potent and are not good activators of immune cells (4). Many subunit and synthetic vaccines (diphtheria-tetanus-pertussis, Hepatitis A & B, Prevnar, and HPV vaccines) that contain poor immunogens require adjuvants
These vaccine components adhere to the alum and are readily phagocytosed by immune cells. Aluminum salts (alum) are the only FDA approved adjuvants in the USA. Stronger adjuvants have been developed that can activate immune cells, but they are not yet FDA approved for human vaccines (5).

Types of vaccines

Multiple types of vaccines can be used to acquire immunity to infectious diseases. There are currently four types of vaccines being used in humans, which includes live attenuated microorganisms, killed microorganisms, toxoids, and subunits or antigenic components of the microorganisms (1, 6, 7). Inoculation with an attenuated microbe or with antigenic components from a pathogen can stimulate an immune response that is very useful in protecting the host from enveloped viruses (8). Attenuated vaccines can induce long term immunity which mimics a naturally occurring response. However, these types of vaccinations are not always safe for all recipients (i.e. immunosuppressed people) and the virus can sometimes revert back to a virulent virus and cause disease (9, 10). Vaccines that contain killed pathogens, subunits of pathogens, or peptides from pathogens are much safer than live virus, but large doses are required, long term protection may not occur, and the vaccines usually just elicit an antibody (humoral) immune response (11).
Peptide vaccines offer the advantage of a well defined immunogen to ensure the generation of a safe and appropriate response. Peptides containing viral epitopes, the smallest portion of an antigen, which can be recognized by B or T cells, can be used in vaccines to initiate protective immune responses. Due to their small size, these peptides are usually insufficient to induce an immune response. Historically, peptides have been attached to large protein carriers to become an immunogen (11). Presentation of the peptide in this manner is not optimal because this usually initiates an immune response against the carrier protein as well as the viral antigen (12, 13). Several technologies have been developed to convert small, epitope bearing peptides into more effective vaccines including the Ligand Epitope Antigen Presenting System (LEAPS) approach (14, 15, 16, 17, 18).

For the LEAPS approach the antigenic peptide is covalently attached by a triglycine linker to an immune cell binding ligand (ICBL), which makes the viral peptide immunogenic (19). An ICBL is a relatively small peptide that binds to a cell surface receptor on an immune cell. If the ICBL is covalently attached to a viral peptide (containing a T cell epitope), immunity can be initiated against that specific virus. Multiple ICBLs have been used to elicit different types of immune responses. The ICBL, termed “J,” ((aa38-50) (DLLKNGERIEKVE)) is from an exposed region of the beta-2-microglobulin component of an MHC I molecule. Attachment of the J-ICBL enhances the immunogenicity of the attached peptide
and promotes a T cell mediated immune response, which is required for
immunity to certain viral infections like HSV-1 and probably HIV (20). The J-
LEAPS vaccines are sufficient to protect mice from lethal HSV-1 infection (14)
and as demonstrated in this study, are capable of activating dendritic cells (a
type of antigen presenting cell) to initiate a cell mediated immune response in
human cells (21).
Dendritic Cells

Dendritic cells (DCs) are antigen presenting cells that bridge the innate and the adaptive immune response (22). Major functions of dendritic cells are to process and present antigen and produce cytokines to direct an immune response. Dendritic cells induce the strongest immune response due to increased surface activation markers, and are the only antigen presenting cells that can initiate an immune response by activating both naïve and memory T cells (23). There are many different types of dendritic cells but all dendritic cells are defined by their morphology with the presence of dendritic extensions (dendrites).

Types of dendritic cells

Different DCs have different origins and functions and include; follicular dendritic cells, plasmacytoid dendritic cells, Langerhans cells, and myeloid dendritic cells.
Follicular dendritic cells (FDCs) are immune cells that have a stromal mesenchymal origin and are localized in the B cell follicles of secondary lymphoid tissues; such as lymph nodes and spleen. FDCs are the only dendritic cell that cannot process antigen (antigen processing will be discussed in more detail later in this dissertation), but rather present intact antigen to B cells. During antigen presentation to B cells, FDCs assist in B cell maturation and induce antibody production and proliferation (24).

Plasmacytoid dendritic cells (pDCs) are a subtype of circulating dendritic cells that have a hematopoietic lymphoid origin. They can be found in the blood and peripheral lymphoid organs. When pDCs are stimulated by viral components and subsequently activated, they produce large amounts of Type I interferons; such as interferon-alpha (IFN-alpha). IFN-alpha is an anti-viral compound that mediates innate and adaptive immune responses to kill viruses (25).

Langerhans cells are a type of myeloid derived dendritic cell that resides in the epidermis. Monocytes differentiate into Langerhans cells when stimulated by colony stimulating factor 1 (CSF-1). Langerhans cells reside in the stratum spinosum layer of the epidermis. When a pathogen invades any area of skin, the residing Langerhans cells will phagocytize and process the microbial antigen to become a fully functional antigen presenting cell (26).
Myeloid dendritic cells (mDCs) can arise either directly from a bone marrow cell (precursor dendritic cell), or a myeloid hematopoietic cell can become a monocyte that is later stimulated to differentiate into an mDC. Myeloid dendritic cells can be found in the bone marrow, blood, or in the spleen in a more mature state. There are different stages of development and function for myeloid dendritic cells. For example, immature myeloid dendritic cells are highly phagocytic and constantly sampling their surrounding environment for pathogens. Mature dendritic cells can produce large amounts of cytokines and can direct immune responses (23, 27). Myeloid dendritic cells are relevant to the J-LEAPS vaccine mechanism and will be a main focus of this dissertation.

Myeloid dendritic cell differentiation

Dendritic cells develop along distinct differentiation pathways in response to internal and external cues. A myeloid progenitor cell differentiates into an immature dendritic cell that can phagocytize and process antigen. After stimulation, a dendritic cell matures into a cell that migrates to the lymph node, activates and presents antigen to the T cell. Mature dendritic cells also produce cytokines that direct the type of T cell mediated immune response (27). Table 2 describes the developmental stages of dendritic cells, cellular morphology of each stage, and the phenotypic changes that occur during cell differentiation.
<table>
<thead>
<tr>
<th>Pictures of cells</th>
<th>Type of Cells</th>
<th>Markers</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>![image](86x573 to 155x632)</td>
<td><strong>Precursor dendritic cell</strong></td>
<td>CD11c&lt;sup&gt;lo&lt;/sup&gt; CD3&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Bone marrow</td>
<td>Myeloid cells found in the bone marrow.</td>
</tr>
<tr>
<td>![image](86x483 to 160x542)</td>
<td><em>Monocytes</em></td>
<td>CD14&lt;sup&gt;high&lt;/sup&gt;</td>
<td>Blood Bone marrow</td>
<td>Pluripotent cells found in the bone marrow or blood. They can differentiate into dendritic cells, macrophages, etc.</td>
</tr>
<tr>
<td>![image](86x385 to 161x449)</td>
<td><em>Immature Dendritic Cells (iDC)</em></td>
<td>CD11c&lt;sup&gt;<em>&lt;/sup&gt; MHC II&lt;sup&gt;lo&lt;/sup&gt;/DR&lt;sup&gt;lo&lt;/sup&gt; OX40L&lt;sup&gt;</em>&lt;/sup&gt; CD80&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Blood Bone marrow</td>
<td>Dendritic cells that differentiated from precursor bone marrow cells or monocytes with high phagocytic activity.</td>
</tr>
<tr>
<td><img src="528x709" alt="image" /></td>
<td><strong>Mature Dendritic Cells (DC)</strong></td>
<td>CD11c&lt;sup&gt;high&lt;/sup&gt; MHC II&lt;sup&gt;<em>&lt;/sup&gt;/DR&lt;sup&gt;</em>&lt;/sup&gt; CD86&lt;sup&gt;high&lt;/sup&gt; CD8&lt;sup&gt;+&lt;/sup&gt; (possible) Can produce IL-6 and other cytokines</td>
<td>Spleen Blood Lymph node</td>
<td>Phenotypic dendritic cells that are antigen presenting cells.</td>
</tr>
<tr>
<td>![image](86x288 to 161x356)</td>
<td><strong>IL-12 producing Dendritic Cells (DC1)</strong></td>
<td>CD11c&lt;sup&gt;high&lt;/sup&gt; MHC II&lt;sup&gt;<em>&lt;/sup&gt;/DR&lt;sup&gt;</em>&lt;/sup&gt; CD86&lt;sup&gt;high&lt;/sup&gt; CD8&lt;sup&gt;+&lt;/sup&gt; (possible) Produces IL-12 and other cytokines</td>
<td>Spleen Blood Lymph node</td>
<td>Mature activated dendritic cells that produce IL-12 cytokine, influencing T cell differentiation.</td>
</tr>
</tbody>
</table>

*Key stages of developing dendritic cells and their cell surface markers and cellular functions.

<sup>*</sup>MHC II is the MHC molecule in mice and HLA-DR is the MHC II molecule in human.

<sup>**</sup>Precursor and mature dendritic cell definition per Geissmann et al (27).

<sup>***</sup>Monocyte and IL-12 producing dendritic cells definition per Zheng et al (28).

<sup>****</sup>Immature dendritic cell definition per Cohen et al (29).
Monocytes are derived from hematopoietic cells that mainly differentiate into macrophages or dendritic cells. Monocytes are released into the bloodstream to patrol for invading pathogens. A monocyte can detect pathogen associated molecular patterns (PAMP) produced by viruses and bacteria through their pattern recognition receptors (PRRs); such as toll like receptors (TLRs). In response to receptor stimulation, a monocyte will migrate quickly to the site of infection and differentiate into a macrophage or dendritic cell dependent on the cytokine environment and receptor stimulation (30). When a monocyte differentiates into a dendritic cell many phenotypic and morphological changes occur, such as formation of dendrites and CD14 expression may decrease. Most monocytes express high levels of CD14, to assist in TLR binding during microbial stimulation. CD14 acts as a co-receptor for the detection of bacterial lipopolysaccharide (LPS) and assists in the binding of LPS to TLR4 on the monocyte (31). Upon TLR activation, the levels of CD14 decrease (CD14\textsuperscript{lo} dendritic cell) while the monocyte differentiates into a dendritic cell (32).

Bone marrow derived hematopoietic cells can be precursors of dendritic cells (33). Bone marrow derived precursor dendritic cells are defined by their immunological phenotypes. Some of the cell surface markers that define a precursor dendritic cell are CD34\textsuperscript{+}, CD11c\textsuperscript{lo}, and CD3\textsuperscript{–} (27). CD34 is an adhesion molecule that participates in hematopoietic differentiation. CD11c is a type I transmembrane protein present on most human and mouse dendritic cells
and is involved in inducing phagocytosis. CD3 is a T cell co-receptor that is only found on T cells and not myeloid cells. These precursor dendritic cells can be found in the bone marrow of mice and humans where they can be signaled to differentiate into an immature dendritic cell.

Immature dendritic cells can be found circulating in the blood or in the lymphatic system constantly sampling their surrounding environments for pathogens. These cells are committed to becoming a dendritic cell but have not yet been activated to differentiate into an antigen presenting, cytokine producing dendritic cells. The phenotype of immature dendritic cells include CD11c⁺, MHCII⁻, OX40L⁺, and CD80⁺ (29). OX40L is a secondary costimulatory molecule that is involved in the activation of T cells. CD80 is a molecule found on the cell surface of monocytes that provides a necessary costimulatory signal for T cell activation and survival. MHC II (major histocompatibility complex class two) is a heterodimeric molecule found on the cell surface of antigen presenting cells. Processed antigen is presented in the MHC molecule for proper presentation to the T cell receptor (TCR) during T cell activation.

After an immature dendritic cell phagocytizes a pathogen, the dendritic cell presents peptides that generate and present the antigen on MHC I or II. Peptides on MHC II are derived from phagocytized proteins (35). Peptides on MHC I can also be derived from phagocytosed proteins by an antigen presenting cell process called cross presentation, but for most cells the peptides are derived from endogenous proteins that are processed through the proteosome and then
enters the endoplasmic reticulum to bind to MHC I for antigen presentation (36). Peptides on MHC II are usually 11-13 amino acids long and ~8 amino acids long for MHC I (37, 38).

Precursor, monocyte, immature, and mature dendritic cells all have pattern recognition receptors (PRRs) that can be stimulated to activate dendritic cell maturation and cytokine production. After a precursor or immature dendritic cell or monocyte is stimulated through their PRR, the mature dendritic cell forms dendrites (branch-like projections). Also the phenotype changes to a CD11c $^{\text{high}}$, MHC II$^+$, and CD86 $^{\text{high}}$ mature dendritic cell (27). CD86 is a molecule expressed on antigen presenting cells that induces costimulatory signals necessary for T cell activation and survival.

Some mature mouse dendritic cells express CD8. The function of this cell surface marker on dendritic cells is not yet known (27). The CD8$^+$ subset of dendritic cells can be involved with promoting cytotoxic T cell responses (39). T cell proliferation and T cell stimulation can be directed by IL-12 production (40). CD8$^+$ dendritic cells are generated by J-LEAPS immunogens.

After dendritic cells are stimulated, the mature dendritic cells migrate through endothelial venules to the lymph node for antigen presentation to cells. During maturation, the dendritic cells become high cytokine producers to induce specific signals and cellular environments that direct and initiate specific types of adaptive immune responses (27).
Cytokines are signaling proteins that can regulate immunity, inflammation, and hematopoiesis. Transiently produced cytokines activate effector cells by binding to specific cell surface receptors that induce signal transduction pathways and initiate specific immune responses. Specific cytokines have to be produced by dendritic cells to direct T cell differentiation, activation, and function making cytokines an essential component of the immune response. The type of immune response that is initiated by a dendritic cell depends upon the type of cytokines that the DCs produce.

The type of cytokines that an activated dendritic cell produces depends upon the antigen that the DC is processing, the cytokine environment, and the type of ligands that bind to pathogen recognition receptors (PRRs) on the DCs. Dendritic cells recognize foreign pathogens through multiple cell surface receptors, such as PRRs (pattern recognition receptors), TLRs (toll like receptors), and LILRs (leucocyte immunoglobulin- like receptors). When a ligand binds to these cell surface receptors, the dendritic cell is activated to produce specific cytokines. The most defined route of dendritic cell activation and cytokine production is through TLRs. Usually two different TLRs are activated, such as TLR 4 (activated by LPS from bacteria) and TLR 9 (activated by CpG from bacterial DNA) to stimulate cytokine production. This induces an activation cascade that allows NF-kappa-B to migrate to the nucleus to activate the transcription of many genes necessary for production of proinflammatory cytokines (41, 42). Following TLR activation large amounts of proinflammatory...
cytokines such as TNF-alpha (tumor necrosis factor-alpha), IL-1beta (interleukin-1 beta), and IL-6 (interleukin-6) are produced along with a T cell stimulating cytokine known as IL-12 (28, 43, 44). Interleukin-12 (IL-12) is a cytokine that elicits the proper environment for the activation of a T helper 1(Th1) immune response (Th1 immune responses will be discussed later in the dissertation). Dendritic cells that activate Th1 cell mediated immune response are referred to as DC1s.

**In vitro isolation, maturation, and activation of dendritic cells**

Multiple *in vitro* models have been designed to properly study the differentiation, maturation, and activation of dendritic cells. Table 3 describes some of the *in vitro* methods used for DC isolation. Dendritic cells and their precursors can be found in the spleen, blood, and bone marrow in both humans and mice (27). A mature dendritic cell is not usually isolated because dendritic cell differentiation and the induction of various subpopulations cannot be properly studied. In most cases a precursor cell is isolated and treated *ex vivo* to differentiate into a dendritic cell. There are several advantages in establishing a dendritic cell population *ex vivo* from precursors. For example, the identification of phenotypic changes and signals required for maturation of precursor dendritic cells into mature immunostimulatory dendritic cells can be studied, and outcome of the cells can be more controlled.
Most of the dendritic cell populations from spleen are mature dendritic cells rather than precursor and immature DCs. Splenocytes lack precursor dendritic cells and have a high population of T cells that make them a poor choice for \textit{ex vivo} DC studies. Iwasaki et al. found that there was very low expression of CD11c$^+$ cells and there was a decreased ability of these cells to activate allogeneic T cells when compared to other DC populations (45). Precursor or immature dendritic cells are rarely found in mouse blood (27); so most mouse \textit{ex vivo} DC studies are performed on bone marrow derived dendritic cells (33).

Mouse bone marrow cells contain a large number of precursors of dendritic cells. The ability of a biological substance to activate a precursor dendritic cell can be studied on mouse bone marrow cells. Cytokines and stimulating agents can be added to the mouse bone marrow cells to elicit a more specific or primed precursor DC population. For example, when mouse bone marrow cells are cultured in granulocyte macrophage stimulating factor (GMCSF), precursors for steady-state DCs are generated (33). When GMCSF and IL-4 are added to mouse bone marrow cells a primed precursor dendritic cell population is produced that will differentiate into a dendritic cells if the proper stimuli induces DC maturation. Another way that mouse bone marrow cells can be induced to generate a steady-state precursor DC population is by adding Flt3 ligand (46). Flt 3 ligand is a small molecule that binds to a cytokine receptor on the surface of hematopoietic progenitor cells that acts as a growth factor and an
activator of steady state precursor dendritic cells. Flt 3 ligand expands the precursor DC population and signals through STAT3, eliciting normal DC development when the precursor DCs are stimulated (29, 46).

A purified immature mouse dendritic cell population can be obtained by first treating bone marrow cells with Flt3 ligand and then later treating with GMCSF + IL-4. The addition of Flt3 ligand and IL-6 to mouse bone marrow cells generates a precursor dendritic cell population. GMCSF and interleukin-4 (IL-4) is added to enhance and prime the cells for DC differentiation (29).

Human bone marrow cells can also be used for the generation of precursor dendritic cells. Several approaches using stimulating agents can be added to human bone marrow cells to expand the small CD34+ precursor dendritic cell population. For example, one method uses Flt3 ligand or c-kit ligand to expand the CD34+ population and to ensure that the DC progenitor cells stay viable during the differentiation process. GMCSF and IL-3 are then added to enhance DC differentiation and block macrophage maturation, making an immature dendritic cell population (36, 47). Human blood derived monocytes are a good source of precursor dendritic cells and have been used in many dendritic cell studies (34). Large numbers of white blood cells can be isolated from blood through leukapheresis, and the monocyte population separated by elutriation, a centrifuge-like process that separates the cells by size and mass. After the monocyte population has been isolated, GMCSF is added to sustain (keep the cells viable) the cell population. Normally, IL-4 is also added to the monocytes to
produce a precursor dendritic cell that is primed to differentiate into a DC if the proper stimuli induce DC maturation (21, 48, 49).
Table 3. *In vitro* methods for dendritic cell isolation

<table>
<thead>
<tr>
<th>Type of Cells</th>
<th>Stimulators</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow derived mouse precursor dendritic cells</td>
<td>GMCSF</td>
<td>Steady-state, myeloid derived progenitor mouse dendritic cells.</td>
</tr>
<tr>
<td>Bone marrow derived mouse precursor dendritic cells</td>
<td>Flt3 Ligand</td>
<td>CD34⁺ Expanded population of myeloid mouse progenitor dendritic cells.</td>
</tr>
<tr>
<td>Immature mouse dendritic cells</td>
<td>Flt3 + IL-6, GMCSF + IL-4 (added later)</td>
<td>CD34⁺ expanded population of mouse myeloid progenitor dendritic cells that has been primed to enhance DC differentiation.</td>
</tr>
<tr>
<td>Bone marrow derived human precursor dendritic cells</td>
<td>Flt3 or c-Kit GMCSF + IL-3 (added later)</td>
<td>CD34⁺ expanded population of human myeloid progenitor dendritic cells that has been primed to enhance DC differentiation.</td>
</tr>
<tr>
<td>Human monocytes</td>
<td>GMCSF</td>
<td>A human monocyte population that can potentially become precursor dendritic cells.</td>
</tr>
<tr>
<td>Primed human monocytes</td>
<td>GMCSF + IL-4</td>
<td>Potential precursor dendritic cells that have been primed to enhance DC differentiation.</td>
</tr>
</tbody>
</table>

* Methods used to isolate mouse precursor dendritic cells (33).
* Methods used to isolate mouse precursor dendritic cells (46).
* Methods used to isolate an immature dendritic cell population in mouse (29).
* Methods used to isolate human precursor dendritic cells from bone marrow (36).
* Methods used to isolate a human monocyte population (49).
* Methods used to isolate a primed human monocyte derived precursor dendritic cell population (48).
After a precursor dendritic cell population has been isolated, the cells can be manipulated into becoming cytokine producing dendritic cells. There are multiple approaches to promoting maturation of human and mouse dendritic cells into specific cytokine producing DCs. In each case these approaches generate an antigen presenting cell that can activate a naïve T cell to initiate an immune response, demonstrating the key characteristic of a dendritic cell. Table 4 describes some of the methods used to manipulate dendritic cells into producing key cytokines that are involved in initiating specific T cell responses. Although stimulation of two TLRs are the most common method used to manipulate PRRs to promote production of cytokines, there are other cell surface markers on dendritic cells that can induce cytokine production. Some \textit{in vitro} methods used to activate cytokine production in DCs include; activating purinergic receptors, adding cytokines, using calcium ionophores, or using CD40 ligand (50, 51, 52). Dendritic cells can be manipulated \textit{ex vivo} to produce many different cytokines that direct different T cell responses. Specific cytokine production by dendritic cells can initiate at least four different types of immune response. For example, DCs that produce IL-12 can initiate a cell mediated immune response, DCs that produce IL-23 can initiate a T cell inflammatory response, DCs that produce IL-10 can initiate a humoral immune response, and DCs that produce TGF-beta can induce a regulatory response. There are multiple signals, agonists, and ligands that can be used to induce the DCs to produce these types of cytokines. For example, Schnurr et al. used ligands for purinergic receptors to activate IL-12
producing dendritic cells (52). Roses et al used an agonist to only activate one TLR to induce dendritic cells to produce IL-23. When a second TLR agonist was added a typical IL-12 producing dendritic cell was produced (49). Yanagawa et al activated dendritic cells to produce IL-10 through TLR restimulation. When LPS was added to a TLR4/TLR2 activated dendritic cell, TLR4 was restimulated and caused the dendritic cell to produce IL-10 (53). Dumitriu et al. initiated a T regulatory immune response when human dendritic cells were co-cultured with lung carcinoma cells and these DCs produced TGF-beta (54). When human monocytes were cultured in ATP and TNF-alpha, the P2 purinergic receptor was activated, inducing dendritic cell maturation and IL-12 production (52). In this dissertation IL-12 producing dendritic cells is generated by J-LEAPS immunogens.
Table 4. Cytokines produced by mature dendritic cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>In vitro stimulators</th>
<th>Cytokine function</th>
</tr>
</thead>
<tbody>
<tr>
<td>°TNF-alpha</td>
<td>2 TLR agonists</td>
<td>A pro-inflammatory cytokine that is cytotoxic to tumor cells.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It mediates an immune response against bacterial infections.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It plays a role in autoimmune diseases and rheumatoid arthritis.</td>
</tr>
<tr>
<td>°IL-1beta</td>
<td>2 TLR agonists</td>
<td>A proinflammatory cytokine that stimulates T cell proliferation.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It induces B cell maturation.</td>
</tr>
<tr>
<td>°IL-6</td>
<td>2 TLR agonists</td>
<td>It regulates inflammatory responses.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It stimulates B cell differentiation and antibody production.</td>
</tr>
<tr>
<td>°IL-12</td>
<td>2 TLR agonists, 1 TLR agonist &amp; IFN-gamma ATP &amp; TNF-alpha</td>
<td>A regulator of cell mediated immunity.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It induces IFN-gamma production.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It promotes the development of Th1 cells.</td>
</tr>
<tr>
<td>°IL-23</td>
<td>1 TLR</td>
<td>It plays a role in eliciting an inflammatory response.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It promotes the development of Th17 cells.</td>
</tr>
<tr>
<td>°IL-10</td>
<td>TLR4 &amp; TLR2 agonists, then restimulation of TLR4</td>
<td>An immunosuppressive cytokine.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It inhibits the expression of proinflammatory cytokines.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>It enhances humoral immune responses.</td>
</tr>
<tr>
<td>°TGF-beta</td>
<td>Lung carcinoma cells</td>
<td>It regulates cell proliferation and T cell differentiation.</td>
</tr>
</tbody>
</table>

* 2 TLR agonist dendritic cell activation (43).
° 1 TLR agonist and IFN-gamma dendritic cell activation (50)
°° 1 TLR agonist dendritic cell activation (49).
°°° ATP & TNF-alpha dendritic cell activation (52).
°°°° TLR restimulation dendritic cell activation (53).
°°°°° LCC dendritic cell activation (54).
**IL-12 producing dendritic cells**

Interleukin-12p70 (referred to as IL-12) is a heterodimeric cytokine comprised of disulfide-bonded p35 and p40 subunits. The p40 subunit is part of both IL-12 and IL-23. IL-23 initiates a T cell mediated inflammatory response (Th17) that is affiliated with autoimmune diseases, and IL-12 initiates a cell mediated immune response that provides protection from intracellular infection; like viruses. Antigen presenting cells (such as DC1s and macrophages) produce IL-12 to aid in the activation of naïve T cells, stimulation of T cell growth, and to direct T cell function, to initiate a cell mediated immune response. IL-12 plays an intermediary role in cell mediated immunity by promoting a T helper 1 response, while inhibiting the activation of T helper 2 and T helper 17 cells (these responses will be discussed in more detail later in the dissertation). This T cell interaction initiates a cell mediated immune response. To ensure that the proper cytokine environment is maintained, IL-10 (a cytokine that supports a humoral immune response) production is blocked while IL-12 production continues (55). IL-12 producing dendritic cells activates IFN-gamma production in Th1 cells, and IFN-gamma initiates an increase in production of IL-12 in a positive feedback manner. This circular process of IL-12 production ensures that a favorable environment for proper cell mediated immune responses is maintained.
T cell Immune Responses

The T cell receptor (TCR) is restricted to recognizing antigenic peptides that are bound to a MHC I or MHC II molecule (also known as HLA (human leukocyte antigen) in humans) on antigen presenting cells. Antigen presenting cells (such as dendritic cells, macrophages, and B cells) are specially equipped to acquire and present antigen, and prime T cells. These antigen presenting cells also express costimulatory receptors required for proper T cell activation. A proper cytokine environment is also required to induce the proper T cell activation. However, only dendritic cells are capable of activating naïve T cells.

Types of T cell

CD8$^+$ T cells can become cytotoxic T cells after antigen specific activation. The CD8 on the T cell surface acts as a co-receptor to the T cell receptor (TCR) to bind to a non variable portion of the MHC I molecule and bring the TCR complexes closer together during antigen specific T cell activation. The MHC I molecule is found on all nucleated cells including dendritic cells (only dendritic cells can activate a naïve CD8 or CD4 T cell). Cytotoxic T cells express TCRs that recognize antigenic peptides that are ~8
amino acids long bound to MHC I molecules (9). Cytotoxic T cells induce apoptosis in target cells that display epitopes of a foreign antigen bound to MHC I (56). Activated cytotoxic T cells produce large amounts of IFN-gamma (56), which can signal the activation and recruitment of phagocytic macrophages. Macrophages are innate immune cells that can be enabled by IFN-gamma to destroy intracellular pathogens and cells that are infected by these pathogens (57).

CD4+ T helper cells produce large amounts of cytokines that direct the immune response. CD4+ T cells are called “helper” cells because the cytokines that they produce help initiate cell to cell interactions that define the immune response. The cytokines that T helper cells produce can promote lymphocyte growth, activate antibody class switch in a B cell, induce cytotoxic T cell activity, and maximize bactericidal activity of macrophages (58). Following T cell development in the thymus, CD4+ naïve T cells migrate in blood to the lymph nodes where they reside and await activation by a dendritic cell (only dendritic cells can activate a naïve T cell). CD4+ T cells are activated by 11-13 amino acid peptides that are generated by antigen presenting cells and presented on the cell surface of MHC II molecules (59). The MHC II molecule is primarily expressed on antigen presenting cells (such as B cells, macrophages, and dendritic cells). The CD4 on the T cell surface acts as a co-receptor to the TCR to bind the MHC II and bring the TCR complexes closer together during antigen specific T cell activation. Activation of CD4+ T cells is
dependent on two simultaneous interactions between molecules expressed on the surface of the T cell and molecules expressed on the antigen presenting cells. The second signal for T cell activation occurs when CD28 on the T cell binds to CD80 or CD86 on the antigen presenting cell. If the second signal is not activated, the T cell will become anergic (lack of immunity to an antigen). However when CD80 or CD86 bind and activate CD28, costimulation for T cell activation and cytokine production is initiated.

**Subtypes of T helper cells**

There are at least four different types of T helper cells that initiate specific types of immune responses; T helper 1 cells (Th1), T helper 2 cells (Th2), T helper 17 cells (Th17), and T regulatory cells (Treg) (Table 5). The type of T helper cell that is produced depends on the type of cytokines that are produced by the dendritic cell during antigen specific activation. The T helper cell makes different cytokines to promote different immune responses.
Table 5. T helper cell subsets

<table>
<thead>
<tr>
<th>Type of T cell</th>
<th>Cytokine Initiator</th>
<th>Cytokines Produced</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IL-12, IL-15, IL-18</td>
<td>IL-2, IFN-γ, TNF-β</td>
<td>Cell Mediated Immunity, CTL (CD8+ T cell activation), IgG2 Antibody Production, Macrophage activation</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4</td>
<td>IL-10, IL-4, IL-5</td>
<td>Humoral Immune Response, IgG, IgE &amp; IgA Antibody Production</td>
</tr>
<tr>
<td>Th17</td>
<td>TGFβ + IL-6 + IL-21 or IL-23</td>
<td>IL-17, IL-22, IL-21</td>
<td>T-cell Mediated Inflammation, Autoimmunity, Neutrophil Recruitment</td>
</tr>
<tr>
<td>Treg</td>
<td>TGFβ</td>
<td>TGFβ, IL-10</td>
<td>Promote Memory Cell Development, Regulate &amp; Inhibit T cell Responses</td>
</tr>
</tbody>
</table>

*T cell defining cytokine responses (96).
*Information in the above table (9, 60).
Table 5 shows different types of T helper cell functions, with their inducing
and response defining cytokines, and immune responses. Briefly, when a
dendritic cell presents antigen to a naïve T cell in the lymph node and IL-4 is
being produced, a Th2 immune response will be elicited that will initiate a
humoral immune response (61). IL-23 promotes a Th17 immune response that
can initiate T cell mediated inflammation (62). A naïve T cell activated in a TGF-
beta environment by acute phase cytokines initiates a T regulatory immune
response (63). When a dendritic cell produces IL-12, a Th1 immune response
will be elicited that supports cell mediated immunity and antibody production (64,
65). This dissertation will focus on J-LEAPS activating dendritic cells that initiate
a T helper 1 response.

When a naïve T cell is activated by an IL-12 producing dendritic cell, a
Th1 cell is initiated to produce specific and four key cytokines; lymphotoxin
(also known as TNF-beta), IL-2, and IFN-gamma. The chemokines include
monocyte chemoattractant protein 1 and 5 (MCP1 and MCP5) and regulated
upon activation normal T cell express sequence (RANTES aka CCL5).
RANTES is a chemoattractant that can also be produced by monocytes, and
dendritic cells. RANTES can signal both CD4 and CD8 T cell activation and
has been suggested to be capable of inhibiting certain strains of HIV (66).
MCP-5 is a chemokine that activates monocytes, immature dendritic cells, and
NK cells; and it chemoattracts T helper 1 cells (67). MCP-1 is produced by
monocytes, fibroblasts, and keratinocytes that can activate dendritic cells and macrophages to induce pathological inflammation (68). Lymphotoxin is a potent mediator of inflammatory and immune responses. TNF-beta can regulate immune cell proliferation and differentiation, and is cytotoxic to many tumor cells (69). IL-2 promotes lymphocyte growth including T cells, B cells, and natural killer cells (70). IFN-gamma is a cytokine produced that is produced by both CD8 and CD4 T cells. IFN-gamma stimulates anti-tumor and anti-microbial responses in macrophages, promotes antibody class switch in B cells, and increases IL-12 production in dendritic cells (71).

IFN-gamma producing T helper 1 cells initiate cell mediated immunity

An antigen presenting cell (like a dendritic cell) producing IL-12 promotes the specific activation of a T helper 1 cell (Figure 1). A T helper 1 cell (Th1) produces IFN-gamma, a cytokine that promotes cell mediated immunity against viral and intracellular bacterial infections. IFN-gamma elicits several immune cell functions including increased production of IL-12 by the DC to induce a positive feedback loop that prolongs the Th1 response, until a regulatory response is initiated to shut down this Th1 response (72). IFN-gamma promotes differentiation of a B cell from IgM antibody production to IgG2a antibody production (aka class switch) (60). The IFN-gamma producing Th1 cells can activate macrophages at the site of infection enabling them to destroy
intracellular pathogens. Also the Th1 cells can stimulate these macrophages to produce other inflammatory cytokines that can clear the host of infectious microbes during cell mediated immunity (73). IFN-gamma inhibits activation of IL-4 to prevent local development of Th2 responses. IFN-gamma also supports CD8 T cell activity at the local site of infection. All of these cell-cell interactions are initiated by IFN-gamma production and are required for cell mediated immunity to protect the host from intracellular infections (74).
Fig. 1. Dendritic cell activation of a T helper 1 cell. Dendritic cell activation of a T helper 1 cell is initiated by the presentation of the antigen peptide on the MHC II molecule on the dendritic cell to the naïve T cell. The second signal for activation is provided by binding of the CD28 molecule on the T cell with the CD80/CD86 molecules on the dendritic cell. This second signal activates a cytokine producing T helper cell. The IL-12 promotes T helper 1 cell differentiation and activation. The activated T helper 1 cell will then produce IFN-gamma; a key signaling cytokine that promotes cell mediated immune responses and reinforces the response.
J-LEAPS Vaccines

Vaccines for Intracellular Pathogens

Many human vaccines initiate humoral immunity; however, humoral immunity is relatively ineffective for protection from intracellular pathogens such as *Mycobacterium tuberculosis* and many viruses (i.e. herpes simplex virus (HSV) or possibly human immunodeficiency virus (HIV)). A humoral response consists of antibody mediated protections. Antibody can block the initial infection of an intracellular pathogen, if there is a sufficient concentration of the pathogen present. But this cannot resolve the infection, because viruses such as HSV can escape antibody control by passing directly from cell to cell, and during latency. Immune protection for HSV and other intracellular infections requires a cell mediated immune response (16).
Fig. 2. J-LEAPS vaccine constructs of JgD, JH, and JM. The J-LEAPS (Ligand Epitope Antigen Presenting System) technology converts a small peptide (containing a T cell epitope) into an immunogen by covalent attachment through a tri-glycine linker to the “J” immune cell binding ligand (J-ICBL). The J-ICBL is a small peptide from beta-2-microglobulin. JgD is an anti-herpes simplex virus-1 vaccine, containing the J-ICBL conjugated to a viral peptide from glycoprotein D of herpes simplex virus. The JH vaccine is a potential vaccine for HIV with a viral peptide (HGP-30) from the gag protein of HIV, and JM is a potential tuberculosis vaccine containing a small peptide from Mycobacterium tuberculosis.
LEAPS J-ICBL

Ligand Epitope Antigen Presenting System (LEAPS) technology was used to develop potential vaccines for intracellular pathogens (such as Mycobacterium tuberculosis, HSV-1, and HIV) that elicit cell mediated immunity (14, 15, 18, 19, 20). J-LEAPS converts a small peptide of 8 amino acids or more containing a T cell epitope into an immunogen by attachment of an immune cell binding ligand (ICBL) (Figure 2). An ICBL is a small peptide that can bind to an immune cell. The LEAPS ICBLs were originally chosen because they were considered to be from portions of immune cell proteins that interact with CD8, CD4, or other T cell surface receptors. The F-ICBL (VQGEESNDK) is derived from IL-1 beta and induces a combined Th1 and Th2 immune response (16). The G-ICBL (NGQEEKAGVVSTGLI) is derived from the MHC II molecule and induces a Th2 immune response (18). The J-ICBL initiates Th1 response, the required immune response for protection against intracellular pathogens and is the focus of this dissertation.

The J-ICBL is a 20 amino acid peptide from the exposed portion of the beta-2 microglobulin of the MHC I molecule. The J-ICBL is derived from the arginine 45 region of beta-2-microglobulin that is accessible for intermolecular interactions since it is found on the outside surface of the MHC I molecular complexes. The J-ICBL is centered on the arginine 45 region of beta-2-microglobulin. In previous studies it was shown that the addition of whole or cleaved potions of beta-2-
microglobulin could enhance cytotoxic T cell activity and promote CD4 T cell proliferative responses. Parham et al showed that a polyclonal antibody to this beta-2-microglobulin region would block allogeneic T cell responses in a mixed lymphocyte population. These results suggested that the beta-2-microglobulin region in the J-ICBL is recognized by T cells and potentially capable of activating a T cell response (14, 15, 18, 19, 75).

**J-LEAPS tuberculosis vaccine**

J-LEAPS vaccines for *Mycobacterium tuberculosis* (JM) has a 21 amino acid peptide from a *Mycobacterium tuberculosis* protein (BrAcDQVHFQPLPPAVVKLSDAL(“M”)) covalently attached to the J-ICBL through a tri-glycine linker (Figure 2) (20). The “M” peptide contains a T cell epitope known as 38.G. Immunization of mice with JM produced no antibody. When the mice received a booster containing the tuberculosis antigen protein, IgG2a, was produced. IgG2a is a specific antibody isotype that is affiliated with Th1 immune responses. This suggests that Th1 cells were generated and induced a secondary-like IgG2a antibody response to the booster. The 38.G epitope portion of the JM vaccine elicited an antigen specific immune response to multiple strains of *Mycobacterium tuberculosis* (20). These results suggested that J-LEAPS heteroconjugates containing an epitope-bearing peptide from an intracellular pathogen elicit a Th1-like immune response.
**J-LEAPS HIV vaccine**

A potential J-LEAPS HIV vaccine was developed by covalently attaching the J-ICBL to a 30 amino acid epitope-bearing viral peptide from the HIV-1 gag protein, HGP-30, (JH) (Figure 2). Cell mediated immunity with a strong CD8+ cytotoxic T lymphocyte (CTL) response has been suggested to be important to control HIV replication (76). HGP-30 peptide contains the proper epitopes to be recognized by T cells that could elicit HIV protective immune responses, but HGP-30 is not immunogenic by itself. HGP-30 was covalently attached to the J-ICBL (JH) or the G-ICBL (GH) to potentially increase its immunogenicity. In the LEAPS HIV vaccine studies, immunogenicity was determined by the antibody isotype, titer, and specificity of antibody produced after a booster with the protein. Sera drawn from JH + adjuvant immunized BALB/c mice contained no antibody; however, GH immunized mice produced large amounts of IgG1 antibodies, an antibody subtype that is affiliated with a Th2 immune response. These preliminary studies showed that the J-ICBL is capable of making this peptide into an immunogen (18).

**J-LEAPS anti-HSV-1 vaccines**

Herpes simplex virus is an intracellular pathogen that causes a cell killing, infection, and then infects the ennervating neuron. In the neuron, the virus can
become latent. CD8 T cells interact with neurons and appear to prevent the replication and reactivation of the virus (77). Virus replication can be activated by stresses that diminish the immune response to cause recurrent disease (77). Immune protection for HSV requires CD8 T cell activation, which is a key component of the cell mediated immune response.

There is still not a licensed vaccine for HSV and the J-LEAPS approach can potentially provide an effective vaccine against HSV. Conjugating the J-ICBL to HSV-1 peptides from the ICP27 (JH1), glycoprotein B (JgB), or glycoprotein D (JgD) generated vaccines that induced protection in mice from lethal infection with herpes simplex virus-1 (HSV-1) (14, 15). The first J-LEAPS anti-HSV-1 vaccine studied was JH1. The J-ICBL is conjugated to a peptide from an immediate early protein from the HSV-1 ICP27 protein (LYRTFAGNPRA)), a T cell epitope recognized by CD8+ T cells in mice (Balb/C) with the MHC type H2d. Balb/C mice immunized twice with JH (emulsified in Seppic ISA51 adjuvant) were protected against a lethal intraperitoneal challenge of HSV-1 with no antibody production (14, 15). However, no protection occurred after immunization of C57BL/6 mice which have a different MHC I type (H2Kb). This suggested that this vaccine is MHC I restricted. These experiments suggest that the J-LEAPS vaccines are capable of initiating protection from intracellular infections. These experiments indicate that J-LEAPS HSV vaccines are capable of eliciting a protective T cell response, because there was protection provided in the BALB/c mice without antibody production. The results suggested that JH1 initiated
protection for HSV through CD8+ T cells because there was MHC I restriction within the HSV protection experiments.

Another J-LEAPS anti-HSV-1 vaccine was JgB. JgB contains a peptide (SSIEFARL) from glycoprotein B of HSV-1(gB). SSIEFARL is the dominant HSV-1 T cell epitope in C57BL/6 mice. The SSIEFARL peptide binds to MHC I and is a cytotoxic T cell target that would elicit a protective CD8+ T cell response (CTL response). This CD8+ epitope is specific for H2Kb which is the MHC I molecule found in C57BL/6 mice. JgB provided no protection against HSV-1 in Balb/C mice. C57BL/6 mice vaccinated with JgB were protected from lethal challenge with HSV-1. No antibodies were produced by the protected mice, suggesting that immunized CD8+ T cells were sufficient for protection (15). SSIEFARL and H1 were known to be peptides received by CD8+ T cells that can potentially initiate a CTL response. For both the JH1 and JgB vaccine studies, a protective immune response was initiated with a vaccine containing these CD8+ epitopes conjugated to the J-ICBL. This indicates that the J-LEAPS HSV vaccines elicit a CTL and cell mediated immune response that is capable of providing protection.

JgD was designed to induce protection in mice or humans by conjugating the J-ICBL to a 16 amino acid peptide (SLKMAFPNRRFRGKDL) from glycoprotein D of HSV-1. Glycoprotein D (gD) contains T and B cell epitopes that are recognized by immune cells that can elicit protective antibody and protective T cell responses to HSV-1(15). The gD cannot initiate an immune response by itself. Subcutaneous vaccination with JgD (emulsified in Seppic ISA-51 adjuvant)
in Balb/C, C57BL/6, and other mice initiated protection from HSV-1 infection upon challenge by intraperitoneal (IP) or skin abrasion routes (14). Disease progression and survival in the skin abrasion-zosteriform spread HSV-1 challenge experiments was determined in multiple strains of mice to be much greater than untreated mice. All mice vaccinated with JgD + Seppic ISA51 vaccine following IP HSV-1 challenge survived (14). Only mice that developed lesions produced antibody. The virus produced in the lesions provided an antigenic boost to allow B cells to make antibody. The antibody isotype favored IgG2a which correlates with a Th1 initiation of cell mediated immune responses (14, 15). These preliminary experiments proved that multiple J-LEAPS vaccines could initiate protective cell mediated immune responses.

Clues to the mechanism of the J-LEAPS vaccines were obtained by using antisera to ablate and neutralize certain components of the immune response elicited by JgD and activated during the subsequent HSV-1 challenge. No protection against HSV-1 infection occurred when CD8+ cells were ablated before vaccination but there was protection when CD8+ cells were ablated after vaccination but before infection. This demonstrates that CD8+ cells are required to initiate the immune response but their role in delivering immune protection cannot be determined. CD4+ cells and IFN-gamma were required to both initiate and deliver the protective immune response. The importance of IFN-gamma is consistent with a protective Th1 initiated cell mediated immune response. These studies proved that the J-LEAPS vaccines induce a Th1-like immune response
that relies on a CD8⁺ initiator cell, CD4⁺ cells (T helper cells), and IFN-gamma, a cytokine that is produced by both CD8⁺ and CD4⁺ T cells during cell mediated immunity (14, 15). Although these findings prove that a Th1 cell is involved in the protective immune response and that a CD8⁺ cell is the initiator cell, these are still just clues towards the identification of the target of the J-LEAPS vaccines. The initiator cell could be a CD8⁺ T cell or a CD8⁺ dendritic cell.

In these studies, it was shown that J-LEAPS anti-herpes vaccines provide protection in mice from lethal challenge with HSV-1 (14, 15). The J-LEAPS vaccines elicited a Th1 immune response by an unknown mechanism, initiated by an unknown CD8 target cell. All the preliminary studies performed on the J-LEAPS vaccines were done on mice.

The work described in this dissertation demonstrates that the CD8 cell that initiates the Th1 immune response in mice is a precursor that differentiates into an IL-12 producing dendritic cell. The J-LEAPS vaccines were also able to initiate a Th1 immune response in human cells by activating monocytes (precursor dendritic cells) into IL-12 producing DCs. Through adoptive transfer of JgD treated bone marrow cells (JgD-DC) it was confirmed that the activation of a precursor dendritic cell is sufficient to initiate protection from HSV-1 infection. These JgD-DCs are also a novel DC vaccine that can initiate protection as a prototype for an immunotherapy.
CHAPTER II

METHODS

Mice

Female A/J or C57BL/6 mice (Charles River, Wilmington, MA) were immunized; serum was obtained and pooled for analysis by cytokine protein array. Female C57BL/6 mice were used (A) to prepare bone marrow cells (Jackson Laboratories, Bar Harbor, ME or Charles River, Wilmington, MA) and (B) for generating pure DC cultures (Biological Testing Branch, Frederick Cancer Research and Development, National Cancer Institute, Frederick, MD). All animals were treated in accordance with Institutional Animal Care and Use Committee (IACUC) policies.

Peptide

The JgD and JH heteroconjugate peptide vaccines consist of an immune cell binding ligand, “J”, ((DLLKNGERIEKVE), amino acid 38-50 from beta-2-microglobulin) conjugated to a peptide from the N-terminus of HSV-1
glycoprotein D (SLKMADPNRFRGKDLP, amino acid 8-23) or the HGP-30 (H) peptide from the p17 HIV gag protein ((YSVHQRIDVKDTEALEKIEEEEQ NKSKKKA (aa 85-115)) through a triglycine linker (16). The vaccine peptides were synthesized by UCB (Atlanta, GA) and supplied by Cel-Sci (Vienna, VA). Lyophilized peptides or peptides frozen in PBS were stable for several years but some degradation was demonstrated by HPLC over the course of days at 30 or 37 C.

**J-LEAPS vaccine immunization**

The peptides were dissolved in Hanks Balanced Salt Solution (HBSS) to produce a stock solution with a concentration of 2mM adjusted to neutral pH. Each of the vaccine solutions was tested by a Limulus Amoebocyte Lysate assay as per manufacturer’s instructions (Cambrex Biosciences Walkersville, MD) for at least 20 EU/100 μl of vaccine solution and shown to be endotoxin free. The vaccine peptide was administered to mice as a 1:1 (vol) emulsion in Seppic ISA-51 (Seppic, Fairfield, NJ) as has been done in previous studies (14). A/J or C57BL/6 female mice were immunized once with the JgD, JH, J, H, or gD-LEAPS peptide subcutaneously with two 50 ul injections of a 2mM solution in the scruff of the neck and in the abdomen. The control mice were injected with HBSS in Seppic ISA-51 adjuvant.
Cytokine Profile following Immunization

Sera collected from three mice was pooled on days 3, 10, and 24 after immunization and 100 μL diluted in 990 μL of blocking buffer was analyzed for 21 different cytokine and chemokine proteins using RayBio R Mouse Cytokine Antibody I array membranes as per manufacturer instructions (Ray Biotech, Inc., Norcross, GA). The following treatment groups were included in the analysis: 1) only adjuvant as a control group; 2) JgD in adjuvant; 3) JH in adjuvant; 4) J in adjuvant; 5) gD in adjuvant; 6) H in adjuvant. Presence of cytokine was detected by chemiluminescence of the membranes and the duplicate spots on film for each cytokine were analyzed by densitometry (Total Lab Array Analysis, Nonlinear Dynamics). Densitometric results were standardized for each membrane by dividing the measured value of each spot by the average values for VEGF (which should not be influenced by the treatments) on that membrane.

Statistical sampling was designed to maximize discovery of trends within the cytokine array results. The serum taken at each bleed was pooled such that it represents the weighted response of three animals. On each membrane, 2 spots (samples) for each cytokine were measured. The replicate spots were treated as a nested source of variance rather than as replicates in the analysis to avoid pseudo-replication. Post-hoc sets for significance were performed using 2 way nested ANOVAs (SAS software system; SAS Institute, Carey, NC) with treatment and day as main factors. Replicate spots were not a significant
source of variation. A third factor in the comparison of JgD values to adjuvant control, strain of mouse, was not a significant source of variation for any of the 21 cytokines. A shared hypothesis (that there would be a response to a treatment) sequential Bonferroni adjustment was performed to allow for multiple comparisons. Critical alpha levels are adjusted to allow for the cumulative probability of type 1 error by this method (78). The data presented in Table 6 includes both the uncorrected P-values and indication of statistical significance after correction (bold box).

Preparation of spleen cells (splenocytes)

Splenocytes were prepared as per Degutt et al. (79). The spleens were obtained from C57BL/6 female mice, and homogenized through a fine mesh in cold Hanks Balanced Salt Solution (HBSS). Red blood cells (RBCs) were lysed using 1M Tris-buffered ammonium chloride and resultant cells were washed 1 time in HBSS. Spleen cells were suspended in serum free tissue culture medium ((SFM) RPMI 1640 containing glutamine and PenStrep) and washed two more times. Decanted cells were resuspended in SFM and 2 X 10^7 splenocytes per well were placed into a 24-well tissue culture plate (Falcon) with 1 mL of SFM and either left untreated or treated with 14.5 micromoles of JgD or JH immunogens. After incubation for 1, 24, and 48 hours at 37°C, cells
were viewed for viability and changes in morphology, tissue 1 mL of culture supernatants were removed and assayed for cytokine production.

Preparation of bone marrow (BM) cells

Bone marrow (BM) cells were prepared as previously described (33, 80). The femurs and tibias were obtained from C57BL/6 female mice, and the ends were removed to expose the hollow bone packed with marrow. BM cells were flushed from the bones with cold Hanks Balanced Salt Solution (HBSS) using a sterile disposable 22g needle and pooled. Red blood cells (RBCs) were lysed using 1M Tris-buffered ammonium chloride and resultant cells were washed 3 times in HBSS. BM cells were suspended in tissue culture medium (TCM) (RPMI 1640 containing Hepes, PenStrep, glutamine, 2-mercaptoethanol, and 5% fetal calf serum) at approximately 5 X 10^6 cells/mL and incubated for 1 hour at 37°C in a 5% CO₂ atmosphere in plastic tissue culture flasks to remove adherent, mature macrophages. Decanted non-adherent cells were resuspended in TCM and 1.5 X 10^6 BM cells in 1 mL were placed into each well of a 24-well tissue culture plate (Falcon) and either left untreated or treated with 14.5 micromoles of J, gD, JgD or JH immunogens. After incubation for 24 or 48 hrs at 37°C, cells were viewed for changes in morphology, tissue culture supernatants were removed and the cells were prepared for flow cytometric analysis.
Generation of immature mouse dendritic cells

Immature DCs were generated from the bone marrow of five normal C57BL/6 female mice as previously described (29). BM cells were harvested as before and cultured at 5 x 10^5/mL in 75 cm^2 flasks at 37°C, 10% CO_2 for 6 days in a complete medium (CM) containing RPMI 1640, 10% fetal bovine serum, 2mM glutamine, 0.1 mM nonessential amino acids, 100 units/mL sodium pyruvate, 100 mg/mL PenStrep, 0.5 mg/mL fungizone, 50ug/mL gentamicin, 50 um 2-mercaptoethanol, supplemented with 10ng/mL of IL-6 (Peprotech, Rocky Hill, NJ) and 10 ng/mL Flt-3 ligand (gift of Amgen, Thousand Oaks, CA). On day 6, the cells were washed twice in PBS, 4 x 10^6 cells/well were transferred to a 24-well cluster plate and cultured in CM supplemented with 10 ng/mL of GM-CSF (gift of Immunex, Seattle, WA), and incubated for 24 hrs. Cells were then analyzed by flow cytometry for expression of CD11c, CD80, CD86, MHC II, CD34, and OX40L, confirming DC purity consistent with previous studies (29).

Immature DCs were either untreated or treated with 3.625, 7.25, or 14.5 micromoles of JgD peptide and maintained in CM without GM-CSF. After 48h incubation, spent medium was removed and immediately tested for the presence of IL-12p70 by direct ELISA.
**ELISA**

Sera (100 μL) collected on days 3, 10, and 24 after immunization with J, gD, JgD, or JH LEAPS vaccine were analyzed for IL-12p70 by a direct ELISA (Sigma, St. Louis, MO). The limit of detection for the assay was 250 pg of IL-12p70. Medium (100 μL) was also obtained from cultures of mouse DCs at 48h after treatment with JgD LEAPS heteroconjugate and tested for IL-12p70. The ELISA was repeated and each ELISA sample was run in triplicate.

**Flow Cytometry Analysis of mouse bone marrow cells**

For analysis of CD11c, CD86, and MHC II expression, untreated and peptide treated BM cells, prepared and treated as described above, were labeled with PE-anti-CD11c, FITC-anti-MHC, or PE-anti-CD86 (Beckman Coulter Fullerton, CA). At least 1 x 10^6 cells were analyzed (Altra FACS, Beckman Coulter) using forward and side scatter parameters to limit (gating) the immunofluorescence analysis to cells of the size and granularity of monocytes and dendritic cells.

For CD8 and IL-12p70 expression, CD3+ cells were removed from BM cells using the fluorescence activated cell sorter and then untreated or treated with JgD, J, or JH. Flow cytometric analysis of the sorted population confirmed the removal of CD3 positive cells. The CD3- BM cells were labeled with FITC-
anti-CD8 (Beckman Coulter (clone 53-6.7), fixed with paraformaldehyde, permeabilized with saponin (Intraprep, Immunotech), labeled with PE-anti-IL-12p70 (Beckman Coulter) and then post fixed with paraformaldehyde prior to immunofluorescence analysis.

Cytokine protein arrays of bone marrow supernatants

The 1 mL of spent media from treated spleen cell suspensions were collected at 6, 24, or 48 hours and analyzed for 21 different cytokine and chemokine proteins using RayBio Mouse Cytokine Antibody I array membranes as per manufacturer instructions (Ray Biotech, Inc., Norcross, GA). Presence of cytokine was detected by chemiluminescence of the membranes.

For the bone marrow cell suspension arrays, aliquots of spent medium were obtained after an additional 48 hours and evaluated for cytokine production by cytokine protein array. Presence of cytokine was detected by chemiluminescence of the membranes and the duplicate spots on film for each cytokine were analyzed by densitometry (Total Lab Array Analysis, Nonlinear Dynamics). For these arrays, densitometric results for duplicate cytokine or chemokine spots from two independent trials were normalized to the summation of values for each array. The average of these duplicate results are presented as a fold increase or decrease compared to the untreated control. Error bars represent the standard deviation.
Co-cultivation of immunogen-treated bone marrow cells with spleen cells

Bone marrow cells (2 x 10^6 in one mL) pooled from 3 female C57BL/6 mice were prepared and untreated or treated with JgD or JH (14.5uM) as described above and incubated for 24 hours. At this time, spleen cells (2 x 10^7), prepared and pooled from 3 mice, were either: 1) untreated; 2) treated with JgD (JgD-DC) or JH (JH-DC); 3) added to wells containing the untreated or treated bone marrow cells; or 4) added to wells containing untreated or treated bone marrow cells that had been washed twice (to remove unbound immunogen and extracellular cytokines) and then resuspended in 4 mL of medium. 1 mL aliquots of medium were obtained after an additional 48 hours and evaluated for cytokine production by cytokine protein array. Densitometric results for duplicate cytokine or chemokine spots from two independent trials were obtained and normalized to the summation of values for each array. The average of these duplicate results are presented as a fold increase or decrease compared to the untreated control.

Protein Extraction

CD3⁻ bone marrow cells were prepared as previously mentioned. CD3⁻ bone marrow cells (1,000,000 cells) were plated in 24-well plates and untreated or treated with 14.5 micromoles of J or JgD immunogens and incubated at 37°C in 5 % CO₂ for 24 hours. Cells were collected and washed twice in HBSS. All
supernatants were removed and resuspended in SDS-PAGE sample buffer (0.25M Tris-Cl (pH 6.8), 30% glycerol, 5% SDS, 0.05% bromophenol blue) and boiled at 95 °C for 5 minutes, to prepare cell extract samples for immunoblotting.

**Western immunoblotting**

The cell extract samples were loaded on a continuous 5-20% SDS-polyacrylamide gel. Following electrophoresis, protein was transferred to polyvinylidene fluoride (PVDF) membrane using semi-dry apparatus. The membrane was placed in a block buffer (500 mL Tris-Buffered Saline, 0.5% Tween-20, 5 grams dry milk powder) overnight on the rocker at 8°C. The next day the membrane was washed in wash buffer (500 mL Tris-Buffered Saline, 0.5% Tween-20) three times and placed in 20 mL of an anti- rabbit polyclonal anti-Lck antibody (1: 500 (Biolegend)) and incubated at room temperature for 2 hours. The membrane was washed three times and then placed in 20 mL of anti-rabbit HRP (1:1000 (Biolegend)) and incubated at room temperature for 2 hours. The membrane was washed three times and incubated in ECL for 5 minutes. Presence of Lck protein was detected by chemiluminescence of the membranes. In order to reprobe the membrane, it was placed in a stripping solution per manufacturer instructions (Restore Western Blot Stripping Buffer Pierce Rockford, IL) and placed back in block buffer overnight at 8°C. The membrane was then washed three times in wash buffer and placed in anti-mouse anti-beta-
actin antibody (1:1000 (Biolegend)) and incubated at room temperature for 1 hour. The membrane was washed three times and then placed in 20 mL of anti-mouse HRP (1:5000 (Biolegend)) and incubated at room temperature for 2 hours. The membrane was washed three times and incubated in ECL for 5 minutes. Presence of beta-actin was used to confirm equal protein sampling and detected by chemiluminescence of the membranes.

Cyclosporin A or Lck-inhibition of IL-12 production

Bone marrow cells were prepared as previously described and plated in 24-well plates at 1,000,000 cells per well. One μg/mL of Cyclosporin A (81) or 20 μL/mL of 1 μM of Lck-inhibitor (3-(2-(1H-Benzimidazol-1-yl)-6-(2-morpholinoethoxy)pyrimidin-4-ylamino)-4-methylphenol) per manufacturers instruction (CalBiochem San Diego, CA) was added to the wells and incubated at 37°C in 5 % CO₂ for 1 hour. The cells were then treated with 14.5 micromoles of JgD and incubated for 24 hours and analyzed using flow cytometry for intracellular production of IL-12p70.

Human monocyte preparation and purification

Monocytes (>95% pure) were collected by leukapheresis ((Baxter CS 3000) (Apheresis unit, Cleveland Clinic Foundation), followed by elutriation
(Beckman Elutriator), washed and frozen (51). After thawing, cells were plated at 3 x 10^6 cells/mL in monocyte-macrophage serum free medium (Life Technologies, Gaithersburg, MD) with or without 50 ng/mL recombinant human GMCSF (Immunex, Seattle, WA) (GM-monocytes) or GMCSF+ 500 U/mL IL-4 (Schering-Plough, Bloomfield, NJ) (GM-4 monocytes) for 24 hours at 37º C. After 24 hours, the cells were treated with 14.5 micromoles of JgD, JH, J, gD, or H peptides or HBSS).

**Human lymphocyte preparation and purification**

Human lymphocytes were obtained as a byproduct of leukapheresis (Apheresis unit, Cleveland Clinic Foundation), purified through elutriation (Beckman Elutriator), washed, and frozen. After thawing cells, CD4+ T cells were isolated through high affinity negative selection by T cell enrichment columns (R & D systems St. Paul, Minnesota). CD4+ T cells were plated at 3 x 10^6 cells/mL in RPMI 1640 medium supplemented with 5% human AB serum (Cambrex, East Rutherford, NJ) and treated with 14.5 micromoles of JgD or HBSS (untreated). Culture supernatants were collected and assayed via RayBio® Human Cytokine Antibody Array 3 for cytokine production.
Human cytokine arrays

Medium (1 mL) from peptide treated and untreated cells were obtained after 3 days and assayed for the presence of 42 different cytokine and chemokine proteins using RayBio\textsuperscript{R} Human Cytokine Antibody Array 3 membranes (RayBiotech, Inc., Norcross, GA). Cytokines were detected by chemiluminescence, and the results captured on x-ray film were analyzed by densitometry (Total Lab Array Analysis, Nonlinear Dynamics). In figures 20 and 22 array results were quantitated by densitometry, and normalized to the summation values for each array to allow for comparative analysis of JgD or JH treated to untreated dendritic cell array results. These values were then compared to the values obtained for untreated supernatants and results that were presented as a fold change. Statistical analysis for significant differences for each comparison was performed by equating p-values via ANOVA analysis. In Table 7 densitometric results for each cytokine were divided by the results for EGF (which should not be affected by treatment) to allow comparison of results between array samples.
Human flow cytometry analysis

Untreated and immunogen treated monocytes were labeled with PE-anti-DR or PE-anti-CD86. At least 5 x 10⁵ cells were analyzed by flow cytometry (FACS Calibur; Cell Quest Pro software) (BD Biosciences San Jose, CA)).

Allogeneic mixed leukocyte cultures

Monocytes harvested 24 hours after treatment with JgD or HBSS were co-cultured with CD4 T cells, obtained as a byproduct of elutriation and purified by negative selection (T cell isolation columns; R&D, Minneapolis, MN) (1x10⁶ cells), at a monocyte: T cell ratio of 1:10 for 6 days at 37°C in RPMI 1640 medium supplemented with 5% human AB serum (Cambrex, East Rutherford, NJ). Culture supernatants (1 mL) were collected and assayed via RayBio® Human Cytokine Antibody Array 3 for cytokine production.

Co-cultivation of immunogen-treated bone marrow with spleen cells from JgD immunized mice

C57BL/6 mice were immunized subcutaneously with JgD in Seppic ISA-51 and received a booster one month later. Bone marrow cells from 3 mice were pooled and 2x 10⁶ cells in one mL were treated with JgD or JH (14.5 uM), as described in chapter two, and incubated for 24 h. The bone marrow cells were
then washed twice to remove unbound immunogen and resuspended with JgD immunized spleen cells. Spleen cells \((2 \times 10^7)\), prepared and pooled from 3 JgD immunized mice were either: 1) untreated; 2) added to JH-DC; or 3) added to wells containing JgD-DC. Aliquots of 1 mL of spent medium were obtained after a 48 h incubation period and analyzed for IFN-gamma production, by ELISA, or for cytokine and chemokine production by cytokine protein arrays.

**JgD-DC vaccine preparation**

Bone marrow (BM) cells were prepared as previously described (33, 80). Non-adherent cells were resuspended in TCM and \(1 \times 10^6\) BM cells in 1 mL were placed into each well of a 24-well tissue culture plate (Falcon) and either left untreated or treated with 14.5 micromoles of J, JH, or JgD immunogens. After incubation for 24 hrs at \(37^\circ\)C in 5% of \(\text{CO}_2\), cells were washed three times in HBSS. Cells were re-counted and suspended in HBSS at \(1 \times 10^5\) cells per 150 \(\mu\)L and loaded in syringes for vaccination.

**Injection of JgD-DC vaccines**

In the first trial, six C57BL/6 female mice received equal doses of \(1 \times 10^5\) bone marrow cells treated with JgD (JgD-DC) suspended in 150 \(\mu\)L of HBSS by injecting the cells into the intraperitoneal of the belly (Figure 23 and 24).
second trial, groups of seven C57BL/6 mice received equal doses of $1 \times 10^5$ cells/50 µL by injecting the cells intradermal (near the tail) and $1 \times 10^5$ cells/100 µL intraperitoneally of 1) untreated bone marrow cells (BM), or 2) bone marrow cells treated for 24h with either J-ICBL (J-BM), 3) JgD (JgD-DC), or 4) JH (JH-DC) (Figure 25, 26, and 27). In both trials mice received a booster of an equivalent cellular suspension one week later. After an additional week, mice received a lethal challenge of HSV-1 by dermal abrasion.

**HSV-1 dermal abrasion -zosteriform infection**

Mice were infected with herpes simplex virus strain H129 ($10^6$ PFU) as previously described (14). It was previously shown that HSV-1 strain H129 ($10^6$ plaque forming units (PFU)) is a lethal dose of HSV-1 for multiple strands of mice; including C57BL/6 mice (14). Briefly, mice were shaved and remaining hair on the back right shank to the abdomen was removed with Nair. After 24 hours, skin near the backbone was gently rubbed with sandpaper and 10 µL of herpes simplex virus strain H129 was pipetted onto and spread into the abrasion area.
HSV-1 infection scoring

Disease progression was evaluated daily by the following scale: 1- non-specific evidence of disease (inflammation at the site of the infection); 2-local disease (lesion at the site of the infection); 3- early zosteriform spread (multiple lesions spreading away from the site of infection); 4-late, extensive zosteriform spread (lesions spreading completely down the dermatome to the underbelly); 5-moribund condition (very sick mouse); 6-death. Mice were scored for 14 days after infection (the timeline of the HSV-1 infection).
CHAPTER III

RESULTS

**Question:** What cytokines are produced during a J-LEAPS initiated immune response?

**Approach:** Compare levels of cytokines by protein arrays or ELISA in serum taken from mice immunized with the J-LEAPS vaccines (JgD or JH emulsified in Seppic ISA51), the Seppic ISA51 control, or the vaccine subunits (J-ICBL, gD, H) on days 3 (innate immune response), 10 (intermediate, adaptive immune response), and 24 (more advanced, adaptive immune response).

**Hypothesis:** J-LEAPS vaccines initiate IL-12 production which activates a Th1 cytokine response in mice

In order to evaluate the nature of the immune response elicited by the anti-herpes J-LEAPS vaccine, JgD, the cytokine response and its time course
was determined following immunization with JgD emulsified in the Seppic ISA51 adjuvant. Immunization conditions are similar to previous vaccine studies (14). Production of the appropriate cytokines would confirm that the JgD vaccine elicited a Th1 initiated cell mediated immune response. Also, the nature of the response could lead to the identification of the vaccine’s target cell. To establish a cytokine/chemokine profile, cytokine protein arrays were used to test A/J mouse sera drawn on days 3, 10, and 24 after immunization with JgD emulsified in Seppic ISA51 or HBSS-Seppic ISA51 immunization. Densitometric results for each cytokine or chemokine on the array were standardized to values for VEGF (which did not have any differences in densitometric values amongst treatments, days, or arrays), and presented as a ratio to the values obtained for mice immunized with only the HBSS-Seppic ISA51 adjuvant emulsion to allow comparison between arrays.

The JgD vaccine also elicited protection for C57BL/6 mice. To ensure that the cytokine profile was not strain specific, C57BL6 mice were vaccinated with JgD and their sera were analyzed for cytokine production.

The unconjugated portions of the vaccine, J and gD were also tested as was another J-LEAPS vaccine JH. JH was tested to see if the same cytokine response would be produced if a different viral epitope was added to the J-ICBL.

Each of the vaccine solutions was tested by a Limulus Amoebocyte Lysate assay (LAL assay) to ensure that the vaccines did not contain endotoxin contamination. The vaccine solutions were shown to be endotoxin free (data not
The LAL assay detects minute amounts of endotoxin (20 EU/100 μL). Endotoxin is a component of lipopolysaccharide (LPS) from gram negative bacteria. The presence of an endotoxin could confound the conclusion of the experiment because endotoxins elicit toll like receptor (TLR) activation and this is sufficient to initiate and skew the type of cytokines produced.

**Cytokine profile of JgD vaccine**

The protein array is a sensitive semi-quantitative method for simultaneous evaluation of serum levels of multiple cytokines useful for comparison of responses. The immunization schedule, amounts of vaccine, and adjuvant were similar to that used in experiments that demonstrated that immunization with JgD induced protection from lethal herpes simplex virus challenge (14). The cytokine array results for serum from mice immunized with HBSS in Seppic ISA51 adjuvant were unremarkable. Similarly, no cytokine response was detected at 3, 10 or 24 days following immunization with equimolar amounts of the H (the HIV peptide portion of the JH vaccine) or gD (the HSV-1 peptide portion of the JgD vaccine) peptides in Seppic ISA-51.
Figure 3. Survey of cytokine production following JgD with Seppic ISA51 immunization of C57BL/6 or A/J mice with JgD. C57BL/6 (n=3) or A/J (n=3) female mice were immunized with Seppic ISA51 or JgD in emulsion with the adjuvant. Sera were collected and pooled on days 3, 10, and 24 for each set of mice and evaluated on mouse protein microarrays. Duplicate spots for each cytokine per microarray were quantified by densitometry, means were determined for the four values (two spots per cytokine per both strains of mice), normalized to values for VEGF, and presented as a ratio to the values for the Seppic control. The highest standard deviation per cytokine was 0.04.
Figure 3 shows the ratio of mean values for serum cytokine production for C57BL/6 and A/J female mice following immunization with JgD to values obtained for mice immunized with adjuvant alone. The time course and trends for the values representing serum levels of cytokines generated by immunization of C57BL/6 and A/J mice with JgD were not different and can therefore be presented as an average of the two together.

By the third day after immunization, elevated levels of cytokines and chemokines associated with dendritic cell innate responses were observed. Amounts of IL-12p40 and IL-12p70 (p40 + p35) were 3 to 4 times higher than for mice immunized with adjuvant alone. There was also a two-fold increase in GMCSF, MCP-1 and RANTES. Interestingly, cytokine levels of TNF-alpha and IL-6 were not increased by immunization with JgD. TNF-alpha and IL-6 are acute phase cytokines that are normally produced at the same time as IL-12p70 in TLR activated dendritic cells. There were only small increases in IFN-gamma present on day 3, with increasing levels of IFN-gamma on days 10 and 24. The levels of IL-12p40 and IL-12p70 remained elevated on the 10th and 24th days after immunization with JgD and induced increasing levels of IFN-gamma. Levels of MCP-1 decreased while levels of MCP-5 increased over this time period. IL-17 levels were also elevated on the tenth day but receded by day 24. Early production of IL-12p40 and IL-12p70 with subsequent production of IFN-gamma
in response to immunization with JgD is consistent with activation of a dendritic cell which activates T cells (82).

**Comparison of cytokine profiles of J, JH, or JgD**

The overall response to immunization of A/J mice with JH was not statistically different from the response to JgD (p-value = 0.849) but the values for JgD and JH were significantly different from the adjuvant control treated mice (p<0.001) and from J treated mice (p<0.05) (per a nested ANOVA of three separate samples per treatment group). As for JH, levels of RANTES, MCP-5, IFN-gamma and IL-17 were elevated over the 24 day course of immunization but TNF-alpha and IL-6 levels were not affected by immunization with the JH vaccine (Table 6).

Immunization of A/J mice with the J-ICBL caused different results than JgD or JH (Figure 6 and Table 6). There was a small but not significant increase in IL-12p40 and IL-12p70. Interestingly, IL-10 levels were decreased to only a third as much as the adjuvant control. No other remarkable effect was observed following immunization with the J-ICBL.

The cytokines or chemokines that were produced in significantly different amounts (bold box) by mice immunized with JgD (A/J and C57BL/6 mice), JH or J compared to adjuvant treated A/J mice are noted in Table 6. Significance was determined by a sequential Bonferroni analysis of the uncorrected ANOVA values for multiple contrasts including treatment type and day post treatment.
Cytokine production over the entire time course was analyzed to highlight the significant events that occurred during the 24 day time frame. Per these statistical analyses, sera from JgD or JH immunized mice had significant increases in IL-12p40 and p70, RANTES, MCP-5, IL-2, IFN-gamma, and IL-17 within the 24 day time course. The only difference between the cytokines produced by the JgD and the JH vaccinated mice was that JgD elicited a significant increase in MCP-1 that did not occur in the JH vaccinated mice. The J-ICBL did not elicit any significant differences in cytokine production, other than a significant decrease in IL-10 production that was not seen in the mice that received the JH or JgD vaccines.
Fig. 4. Cytokine protein arrays for sera drawn on day 10 of A/J immunized mice. Sera from A/J mice that were immunized with JgD + Seppic ISA51, JH + Seppic ISA51, J + Seppic ISA51, H + Seppic ISA51, gD + Seppic ISA51, or Seppic ISA51 emulsified in HBSS was collected on day 10 and evaluated on mouse protein arrays. Evaluation by luminescence.
Fig. 5. Cytokine production following immunization of A/J mice with (A) J, (B) JH, or Seppic adjuvant control. Sera was collected on days 3, 10, and 24 and evaluated on RayBiotech® mouse antibody microarrays. “Spots” were quantified by densitometry, means were determined, normalized to values for VEGF, and presented as a ratio to the values for the Seppic control.
Table 6. A sequential Bonferroni analysis of the p-values of JgD, JH, or J vaccinated mice over 24 day time course. A/J mice were immunized with JgD, JH, or J and sera was analyzed as described above. Uncorrected p-values from a two-way nested ANOVA comparing treatment and day post treatment are presented for immunized mice contrasted to adjuvant treated mice. **Dark outlined boxes** indicate cytokines that had a significant p-value <0.0001 over the 24 day time course after the sequential Bonferroni correction for multiple contrasts.
Fig. 6. Selected comparisons of serum cytokine levels following immunization of A/J mice with JgD, JH or J peptides in Seppic adjuvant. Values presented as a ratio to the values for the Seppic control for (i) IL-12p70, (ii) IL-12p40, (iii) IFN-gamma and (iv) IL-10 and plotted with respect to days after immunization.
IL-12 ELISA results for J-LEAPS vaccines

The same sera that were analyzed by cytokine protein arrays (see Figure 3) were also quantitated by a direct ELISA for IL-12p70 (Figure 7). Whereas only trace or undetectable amounts of IL-12p70 were present in sera from mice immunized with J, gD, H, or HBSS-Seppic ISA51, the sera obtained from JgD immunized mice contained 378 pg/ml, 353 pg/ml or 372 pg/ml of IL-12p70 on days 3, 10 and 24, and sera obtained from JH immunized mice contained 334 pg/ml, 376 pg/ml and 386 pg/ml of IL-12p70 on days 3, 10 and 24. These results are consistent with the elevated levels of IL-12p70 detected by the cytokine protein array.
Fig. 7. Quantification of IL-12p70 production in serum from J-LEAPS immunized mice. Female A/J mice were injected with JgD or JH on day 0, sera were drawn on days 3, 10, and 24. A 1:100 dilution was made and an IL-12 ELISA was run on JH, JgD, and Seppic sera. The amount of IL-12 detected was quantitated by plotting the detected amount on a regression scale of a standard curve. Serum levels of IL-12p70 for the Seppic ISA51 treated mice were below detection on days 3, 10, and 24.
Analysis of the cytokine profile produced in response to immunization of mice with the JgD and the JH immunogens is consistent with activation of dendritic cells that promote a Th1 initiated cell mediated immune response. IL-12 cytokine production by dendritic cells can initiate a Th1 immune response in naïve T cells (55). IFN-gamma levels significantly increased by day 10 and continued to increase by day 24. IFN-gamma is a key Th1 cytokine produced mostly by T cells. These results suggest that the initiator cell is a dendritic cell rather than a T cell. Similar responses were observed in two different mouse strains and for two different J-LEAPS vaccines. This cytokine response confirms that the J-LEAPS vaccines elicit a Th1 immune response.
**Question:** Is the CD8⁺ cell important for initiating JgD immune responses in mice a dendritic cell?

**Approach:** Bone marrow cells from C57BL6 mice were untreated or treated with J-LEAPS immunogens (J-LEAPS vaccines without Seppic ISA51 adjuvant), and then analyzed for IL-12 production, DC phenotype (morphology & cell surface markers), and biological ability to initiate a Th1 immune response.

**Hypothesis:** Precursors to dendritic cells are the initial target cells of J-LEAPS vaccines in mice

Antibody ablation studies on JgD vaccinated mice demonstrated that CD8⁺ cells are important for initiating immunity (14). The CD8⁺ initiator cell could be a T cell or an antigen presenting cell, such as a dendritic cell. The initial survey of the cytokine response to immunization of A/J or C57BL/6 mice with the JgD or JH immunogens showed that IL-12 was produced soon after immunization with IFN-gamma being produced later. This strongly suggests that a myeloid dendritic cell is the initiator cell that elicits a Th1 immune response.
Since IL-12 is produced by myeloid cells and not T cells, and IFN-gamma by T cells or natural killer cells (if natural killer cells were the initiator cell IFN-gamma would be produced by day 3; ruling out natural killer cells as the J-LEAPS initiator cell).

To identify the CD8⁺ initiator cell, *ex vivo* sources of dendritic cells and naïve T cells had to be utilized. Potential sources of precursor dendritic cells are blood and bone marrow; however, it is very rare that precursor dendritic cells can be found in mouse blood (27). Naïve and immune T cells, and a more mature population of dendritic cells can be found in the spleen. The first attempts to establish an *ex vivo* cell culture system to study responses to the J-LEAPS immunogens were performed on spleen cells however, there was no cytokine response when the J-LEAPS immunogens were added directly to the splenocytes (Figure 8). In Figure 8, three different experiments were performed on splenocytes to determine if the J-LEAPS immunogens were capable of inducing cytokine production when added *ex vivo*. Spent media (1 mL) was collected at different times (6 hours, 24 hours, 48 hours) and analyzed by cytokine protein arrays. There were no increases in any cytokine or chemokine detected when the JgD or JH treated splenocytes were compared to the untreated splenocytes.

Bone marrow is a good source of stem cells for naïve myeloid precursor DCs with few T cells (29, 33, 83, 84). Bone marrow cells were tested and were responsive to the J-LEAPS immunogens. The activation of J-LEAPS
immunogens were tested on bone marrow cells without adherent cells (BM). This population of cells would contain precursor dendritic cells plus other bone marrow cells without mature macrophages. Bone marrow cells deleted of CD3 expressing cells and without adherent cells (CD3⁻ BM) were analyzed by flow cytometry to identify the J-LEAPS initiator cell. This population of cells would contain precursor dendritic cells and some other bone marrow cells without any T cells, NKT cells, or mature macrophages. The purified immature dendritic cells (iDC) were studied to confirm the J-LEAPS initiator cell. This population of cells is bone marrow cells cultured in Flt3 ligand & IL-6 and then later treated with GM-CSF and IL-4 to generate purified immature dendritic cells.
Fig. 8. Cytokines produced by JgD or JH treated splenocytes. JgD or JH were added to spleen cell suspensions and incubated at 37 C in 5% CO\textsubscript{2}. Cells were incubated for: A) 6 hours, C) 24 hours, and D) 48 hours in 3 separate experiments. Spent media from the cell cultures were analyzed on RayBiotech\textsuperscript{R} mouse protein microarrays and there were no specific cytokines produced when compared to the untreated spleen cytokine profile in any of the experiments.
Cytokine profile of BM cells treated with J-LEAPS immunogens

Bone marrow (BM) cell suspensions were untreated or treated with equimolar amounts of JgD or JH immunogens for 48 hours. Media was analyzed for cytokine production by cytokine protein arrays as previously done on mouse sera. The spent media from the JgD or JH treated bone marrow cell suspensions exhibited very similar cytokine responses. Supernatants from the JgD or JH treated bone marrow cells contained 3.5 fold more IL-12p70 and IL-12p40 compared to the spent media from the untreated bone marrow cell suspensions (Figure 9). In addition, the spent media from JgD or JH treated bone marrow cells had approximately a 2-fold increase in some of the Th1 affiliated chemokines, such as RANTES and MCP-5. This suggests that the J-LEAPS immunogens are sufficient to activate bone marrow derived precursor dendritic cells into IL-12 producing DCs.
Fig. 9. Cytokine profile of JgD or JH treated bone marrow cells. Bone marrow cells were treated with JgD or JH immunogens and incubated for 48 hours in two separate repeated experiments (n=4 per treatment group). The spent media was collected and evaluated by RayBiotech® mouse antibody microarrays. “Spots” were quantified by densitometry, means and standard deviation were determined, normalized to total array values to allow comparison, and presented as a ratio of values for treated BM cells to untreated BM cells. Error bars indicate the standard deviation between two separate experiments. The cytokines displayed are the only cytokines that were detected by the array, acute phase cytokines, such as TNF-alpha and IL-6, were below detection.
J-LEAPS treated bone marrow cells co-cultured with naïve splenocytes

To test if the IL-12 producing bone marrow cells were capable of initiating the IFN-gamma production of the Th1 response, BM cells were treated with JgD or JH immunogens as previously described and after 24 hours of incubation, cells that were untreated, JgD treated, or JH treated were incubated with untreated spleen cells, as a source of T cells, and evaluated for IFN-gamma production. This tests whether the J-LEAPS treated bone marrow cells were converted into IL-12 producing dendritic cells capable of activating a Th1 response as defined by IFN-gamma production.
Fig. 10. Cytokines produced by splenocytes co-cultured with J-LEAPS treated bone marrow cells. Bone marrow cells were treated the same way as in Figure 9 (in two separate repeated experiments (n=4 per treatment group)), but they were incubated for only 24 hours and then washed three times in HBSS before co-culturing these cells with splenocytes at a BM : splenocyte ratio of 1:10 for 48 hours. Spent media was collected and analyzed by mouse cytokine microarrays as described in Figure 9. Error bars indicate the standard deviation between the two separate experiments. The cytokines displayed are the only cytokines detected by the array, there were no detectable amounts of acute phase cytokine production (TNF-alpha or IL-6).
As seen in Figure 10, the spent media obtained 48 hours after spleen cells had been treated with JgD-DC or JH-DC (washed free of unbound immunogens) contained elevated levels of IL-12, MCP-1, MCP-5, RANTES, IL-2, and IFN-gamma when compared to untreated spleen cells. Consistent with previous experiments, there was no detectable IL-6 or TNF-alpha in the media following T cell incubation with JgD-DC or JH-DC. Interestingly, media from the spleen cells incubated with untreated bone marrow cells had elevated but low levels of IL-4, IL-5, IL-10, and IL-13 (data not shown), suggestive of a Th2 type of response, while no detectable amounts of these cytokines were present in the samples from spleen cells mixed with the JgD-DC or JH-DC. The presence of IL-2 and IFN-gamma are characteristic of a Th1 response.

There were not any increases in cytokine production and IFN-gamma was not produced by spleen cells following treatment with the cell-free JgD or JH peptides (48 hour results Figure 8). This confirms that the J-LEAPS immunogens are not directly interacting with T cells to initiate a Th1 response and supports the hypothesis that the J-LEAPS vaccines initiate immunity by interacting with a pre-dendritic cell.
Fig. 11. Cytokines produced by splenocytes co-cultured with washed or unwashed J-LEAPS treated bone marrow cells. Mouse C57BL6 bone marrow cells were untreated or treated with JgD or JH immunogens for 24 hours and then either washed 3 times or not washed. The cells were then co-cultured for 48 hours with splenocytes from untreated C57BL6 mice. Spent media was collected and analyzed by mouse cytokine microarrays as described in Figure 9. Error bars indicate the standard deviation between the two separate repeated experiments (n=4 per treatment group). The cytokines displayed are the only cytokines detected by the array, there were no detectable amounts of acute phase cytokines produced (TNF-alpha and IL-6).
Removing unbound immunogen and any previously produced cytokines by washing the treated bone marrow cells prior to addition of spleen cells did not significantly reduce the amount of IFN-gamma production (Figure 11). These results demonstrate that treatment with JH or JgD is sufficient to convert precursor bone marrow cells into an IL-12 producing cell; probably a DC1 cell. Also the IL-12 producing cells are necessary and sufficient for activating naïve splenocytes to produce the Th1 defining cytokines; IFN-gamma and IL-2.

Flow cytometry analysis of treated bone marrow cells

Dendritic cells express specific cell surface markers upon maturation and activation, including CD11c (a type I transmembrane protein found on most human and mouse dendritic cells), CD86 (a cell marker for mature DCs capable of providing co-stimulatory signals to T cells), and MHC II (the molecule that presents antigen to the T cell receptor) (85). To further characterize the IL-12 producing cells, the monocyte population of BM cells was analyzed on the second day after treatment with J, gD, JgD or JH for these DC cell surface markers. Representative flow cytometry results from two independent experiments are presented in Figure 12. The JgD and JH treated bone marrow cells (JgD-DC and JH-DC) expressed higher levels of CD11c (Figure 12A), CD86 (Figure 12B), and MHC II (Figure 12C) than the J-LEAPS peptide treated or the untreated cells. The X-axis value for the mean fluorescence intensity of the cell
population (X-mean) indicated the level of expression of the cell surface markers on the dendritic cells. The untreated monocyte population contained very few CD11c, CD86, or MHC II expressing cells (X-mean = ~ 0.5), whereas the X-mean for CD11c expressing cells was 4.7 for JgD-DC and was 4.0 for JH-DC. X-mean for CD86 expression was 14.3 for the JgD-DC and 12.2 for the JH-DC. X-mean for MHC II expression was 12.1 for JgD-DC and 11.2 for JH-DC. These results confirm that JgD and JH are capable of promoting the differentiation of bone marrow derived precursors into mature DCs.

The response to gD was also tested and there was no increases (X-mean = ~0.5) in CD11c, CD86, or MHC II expression. The J treated bone marrow cells did not have increases in CD11c or CD 86 expression (X-mean =~0.5), but there was a slight increase in MHC II expression (X-mean= 1.7). These results demonstrate that the immune cell binding ligand (J) and the viral peptide (gD) do not promote the maturation or activation of dendritic cells. Induction of maturation only occurred for the heteroconjugates; JgD and JH.
Fig. 12. Flow cytometry of DC maturation markers found on J-LEAPS treated bone marrow cells. JgD, J, gD, or JH were added to the BM cell suspensions and incubated for 48 hrs. A) Cells were then stained with PE-anti-CD11c; B) PE-anti-CD86 and C) PE-anti-MHC II and flow cytometry was performed on cell suspensions with light scatter parameters of monocytes.
Flow cytometry analysis of J-LEAPS treated CD3⁻ bone marrow cells

Ablation studies demonstrated that CD8⁺ cells are involved in the initiation of J-LEAPS vaccine protection against HSV-1 challenge (14). To further identify the CD8⁺ initiator cell, flow cytometry analysis of the JgD or JH treated, IL-12 producing cells were performed. The analysis was performed on a T cell depleted bone marrow cell population. T cells were removed by FACS (Fluorescence Activated Cell Sorting) separation of CD3 expressing cells. All T cells express CD3 (73). The remaining bone marrow cells were incubated with JgD or JH and analyzed for intracellular IL-12p70 expression and surface CD8 (Figure 13). After 24 hours, only 12% of the control cells expressed low levels of CD8 and less than 3% of these cells were positive for IL-12p70. The JgD-DC CD3⁻ cells contained 73% CD8 positive, IL-12p70 producing cells and the JH-DC CD3⁻ cells contained 72% CD8 positive, IL-12p70 producing cells. Treatment with JgD or JH increased the number of CD8 expressing cells or the expression of CD8 and promotes IL-12p70 production in this population of bone marrow cells. Production of IL-12p70 in CD3⁺/CD8⁺ expressing cells confirms that the JgD and JH responsive cells are of myeloid origin and not T cells (82). IL-12 production by J-LEAPS treated CD3 depleted BM confirms that T cells are not needed for the J-LEAPS immunogens to activate the precursor dendritic cells into IL-12 producing DCs.
Fig. 13. Flow cytometry results of CD3- J-LEAPS treated bone marrow cells. BM cells were FAC sorted to remove CD3+ cells, and were untreated or treated with JgD or JH for 48 hours. Intracellular IL-12p70 and extracellular CD8 were evaluated for the entire sorted BM cell population. Immunofluorescence was analyzed and compared to isotype controls. The table represents the percent of positive cells for each quadrant.
Lck involvement with IL-12 production in J-LEAPS treated CD3+ bone marrow cells

There could be a link between CD8 and the activation of IL-12 production since CD8+ cells are producing IL-12p70 (Figure 13). The function of CD8 on dendritic cells is still not known however, the function of CD8 in T cells is well defined. In T cells, CD8 is a transmembrane glycoprotein which initiates a chain of signaling events that leads to cytokine production (86). Binding to the cell surface portion of the CD8 protein activates its cytoplasmic tail which associates with a leukocyte-specific protein tyrosine kinase, Lck, inside of the T cell. Lck then initiates tyrosine phosphorylation cascades that produce a docking site for a signaling enzyme referred to as phospholipase C (PLC). PLC then hydrolyzes phosphatidylinositol (PIP2), generating inositol triphosphate (IP3), which induces the elevation of intracellular free calcium (87). The increased cytoplasmic level of calcium binds to a subunit of a protein phosphatase known as calcineurin. Calcineurin promotes activation of transcription factors such as NFAT, NF kappa-B, and AP-1, which regulate the production of genes for T cell associated cytokines and other proteins (Figure 14) (88). It may be possible that many of the proteins involved in the Lck associated induction of cytokine production in CD8+ T cells are also involved in IL-12 production of CD8+ dendritic cells.
Lck initiated activation of cytokine production in T cells

Fig. 14. Ligand binding to CD8 at the T cell surface promotes association of Lck with the CD8 cytoplasmic tail. Lck activates a cascade of phosphorylation events that involves calcineurin and activates gene transcription for cytokine production.
Lck protein detection of JgD treated CD3^-/CD8^+/IL-12 producing BM cells

To test the hypothesis that Lck is involved in the production of IL-12 in JgD treated CD8^+ dendritic cells, cell extracts of CD3^-/CD8^+/IL-12 producing, JgD treated BM cells were analyzed for the presence of Lck proteins by Western blot. The CD3^+ cells were removed by FACS and the remaining bone marrow cells were untreated or JgD treated for 24 hours. Flow cytometry was performed on a portion of the cells to confirm that the cells were CD8^+ and producing IL-12 with results that resembled that in Figure 13. Western blot was used to detect Lck in protein extracts of untreated or JgD treated CD3^- BM cells (10^6 cells per sample). Lck is present in T cells but the presence of Lck in DCs has not been described (42, 89). Protein extracts of splenocytes (10^6 cells) were used as a positive control for Lck expression, which contain a large amount of T cells. Lck protein was detected in the splenocyte sample by the polyclonal anti-Lck antibody (Figure 15).
Fig. 15. JgD treatment of CD3- bone marrow cells elicits an increase in Lck protein expression. CD3- bone marrow cells were untreated or JgD treated for 24 hours. Protein extracts were analyzed by Western immunoblotting to detect the presence of Lck using polyclonal anti-Lck antibody. The membrane was then stripped and equal protein concentrations were confirmed by using anti-beta-actin antibody.
There was no detectable amount of Lck found in the untreated bone marrow cells. Surprisingly, there was a high amount of Lck protein in the CD3\(^{-}\) bone marrow cells after treatment with the JgD immunogen (Figure 15). Increased synthesis of Lck together with increased expression of CD8 (Figure 13) in the JgD treated, IL-12 producing CD3\(^{-}\) BM cells suggests that JgD is activating a novel pathway in dendritic cells.

**Flow cytometry analysis of intracellular IL-12 production when Lck is inhibited**

To detect if Lck is important in the activation pathway of IL-12 production upon JgD treatment, bone marrow cells were treated with 50 \(\mu\)L of 0.9 micromoles of an Lck inhibitor (3-(2-(1H-Benzo[d]imidazol-1-yl)-6-(2-morpholinoethoxy)pyrimidin-4-ylamino)-4-methylphenol) for 1 hour prior and during JgD treatment of the cells. After 24 hours, the bone marrow cells were analyzed by flow cytometry for IL-12 production. As seen in Figure 16, there was no intracellular IL-12 detected in the untreated bone marrow cells (X-mean=1.9) but there was intracellular IL-12 expressed in the JgD treated bone marrow cells (X-mean=9.5). Like the untreated controls, cells treated with the Lck inhibitor prior to receiving the JgD treatment had no intracellular IL-12 (X-mean=1.8). This provides evidence that Lck is involved in activating IL-12 production by JgD in dendritic cells.
Fig. 16. Lck-inhibitor blocks JγD elicited production of IL-12 in mouse bone marrow cells. Bone marrow cells were cultured as described in Figure 9. Lck-inhibitor was either not added or added to BM cells and after 1 hour the cells were treated or not treated with JγD. Cells were analyzed for intracellular IL-12p70 production by flow cytometry after 24 hours incubation.
Effect of inhibition of calcineurin on intracellular IL-12

In T cells, Lck activation initiates a chain of phosphorylation events and increases cytoplasmic calcium. The calcium binds to calcineurin which promotes translocation of NF-kappa-B or NFAT to the nucleus to activate the production of cytokine proteins (42). If Lck is functioning through the same cytokine activation pathway in dendritic cells as in T cells, then calcineurin might be involved in IL-12 production. To test the hypothesis that calcineurin is involved with activation of IL-12 production by JgD, calcineurin was inhibited by cyclosporin A treatment (100 μg/mL) (90) and IL-12 assayed in BM cells by flow cytometry. Cells were not treated or treated with cyclosporin A for 1 hour and then JgD was added and cyclosporin A treatment was continued (91). Cells were analyzed by flow cytometry for intracellular IL-12 production after 24 hours incubation with no treatment or JgD treatment. There was no intracellular IL-12 production in the untreated bone marrow cells (X-mean=1.4) but there was an increase in intracellular IL-12 production in the JgD treated cells (X-mean=8.9 (Figure 17)). Cells treated with cyclosporin A prior to JgD treatment had no intracellular IL-12 (X-mean=1.9). This indicates that Ca²⁺ and calcineurin are involved in IL-12 production. Also Lck activation is following a similar cytokine activation pathway in dendritic cells as in T cells. It also confirms that Lck and calcineurin activity is required for JgD induction of IL-12 production of dendritic cells.
Fig. 17. Cyclosporin A blocks JgD induction of IL-12 production in mouse bone marrow cells. Bone marrow cells were cultured and either untreated or treated with Cyclosporin A 1 hour prior to JgD treatment. After Cyclosporin A treatment, all cells were either untreated or JgD treated. After 24 hours incubation, cells were analyzed for intracellular IL-12 by flow cytometry.
JgD can activate purified immature dendritic cells into IL-12 producing DCs

The J-LEAPS immunogens are capable and sufficient to activate precursor dendritic cells derived from bone marrow; however, a more purified immature dendritic cell culture was needed to directly prove the hypothesis that precursor dendritic cells are the target cell for the J-LEAPS peptides. The effect of the JgD immunogen was tested on purified immature dendritic cells (iDCs) prepared from BM (29).

Bone marrow cells from C57BL/6 mice (n=5) were cultured for 6 days in Flt-3 Ligand and IL-6 which caused the CD34^+ population to expand (precursor dendritic cells are derived from CD34^+ stem cells). After six days, a portion of the treated bone marrow cells were analyzed by flow cytometry to ensure that a large portion of the bone marrow cells were CD34^+. Cells were washed and plated in fresh tissue culture media and GMCSF and IL-4 (GM-4) for an additional 24 hours. GM-4 primes the cells to differentiate into DC1s if properly stimulated; at this point the cells are immature dendritic cells (29). A portion of the GM-4 treated bone marrow cells were analyzed before JgD treatment by flow cytometry for multiple cell surface markers to ensure that the cells were immature dendritic cells.

Phenotypically, the immature dendritic cells did not display dendrites, and expressed CD11c^+, OX40L^+, and CD80^+, confirming that the cells were immature dendritic cells. The cells (10^6 cells) were incubated in complete media (as
described in materials and methods) and either untreated or treated with 14.5 micromoles of JgD immunogen for an additional 48 hours. After treatment the cells were analyzed for morphological and phenotypic changes.
Fig. 18. Response of purified iDCs to JgD immunogen treatment. Immature DCs from C57BL/6 mice (n=5) were pooled and untreated or treated with 3.25, 7.25, or 14.5 micromoles of JgD. A) After 48 hrs, cells were microscopically examined for morphological changes and B) a direct IL-12p70 ELISA was performed in triplicate on the supernatants from cell suspensions of two independent trials (n=6 per concentration), values were averaged, and error bars indicate standard deviation between ELISA values. Values were significantly different for the different dose amounts (p<0.05) as per ANOVA.
Morphological changes were observed within 48 hrs of addition of JgD to the immature dendritic cell culture (Figure 18A). The cells clustered together and spindling dendrites formed, both of which are characteristics of maturing dendritic cells (94).

Treatment with JgD also promoted a measureable, concentration dependent increase in IL-12p70 production (Figure 18B). Cells (1.5 x 10^6) treated with 3.625 micromoles of JgD produced 8.0 ng of IL-12p70, whereas cells treated with 7.25 micromoles of JgD produced 9.50 ng and treatment with 14.5 micromoles of JgD yielded 12.5 ng of IL-12p70. The increased size, granularity, change of shape and production of IL-12p70 indicate that treatment with the JgD immunogen is sufficient to activate the development of iDCs into DC1s. Also it affirms the hypothesis that the target cell of the J-LEAPS vaccines in mice is a dendritic cell.

In summation, the J-LEAPS immunogens, JgD and JH, are sufficient to promote the differentiation of bone marrow cells into IL-12 producing dendritic cells. The DCs generated by treatment with JgD or JH, ex vivo, were sufficient to steer the response of splenic T cells to produce the prototypic Th1 cytokines, IFN-gamma and IL-2. Interestingly, the lack of response of spleen cells to cell free JgD or JH reiterates that precursor dendritic cells, not mature DCs or T cells, are the initial target cell during immunization with J-LEAPS immunogens.

Both JgD and JH immunogens promoted the development of a DC-like morphology, expression of CD11c and CD86, up regulation of MHC II, as well
as CD8 expression. JgD stimulation of precursor cells to mature into CD8⁺/IL-12 producing DCs without acute phase cytokine production (TNF-alpha and IL-6) suggests that cytokine production is not being initiated by TLR activation (31, 43). Lck and calcineurin play a role in initiating IL-12 production in JgD treated CD8⁺/IL-12 producing dendritic cells and this undescribed way of activating dendritic cells.

The ability of JgD and JH to promote the differentiation of bone marrow cells and specifically, purified dendritic cell precursors, to become IL-12 producing dendritic cells identifies the target cell for the J-LEAPS immunogens as a precursor dendritic cell. These J-LEAPS treated DCs produce IL-12p70 and when co-cultured with naïve splenocytes are capable of initiating a Th1 cytokine response. These results for JH and JgD confirmed that the J-LEAPS vaccines act like an adjuvant to elicit a Th1 immune response.
**Questions:** Can J-LEAPS elicit a response within human cells? What cell is the target cell?

**Approach:** Human blood derived monocytes untreated or treated with J-LEAPS immunogens (JgD or JH without Seppic ISA51 adjuvant), were analyzed for IL-12 production, DC cell surface markers, and the biological ability to initiate a Th1 immune response.

**Hypothesis:** Precursors to dendritic cells are the target cells of J-LEAPS vaccines in human cells

The results of the mouse studies proved that the J-LEAPS immunogens activate and promote the development of an IL-12 producing dendritic cell from precursors in bone marrow. Based on this work, it was hypothesized that JgD and JH will promote the maturation of human dendritic cell precursors into IL12-producing dendritic cells that can elicit the development of Th1 responses; as indicated by relevant cytokine production. The monocyte was chosen as the human dendritic cell precursor (43, 48, 92, 93). Upon treatment with appropriate stimuli monocytes can be converted into DCs. Human monocytes that are cultured in media containing GMCSF sustain the precursor dendritic cell
population. If GMCSF and IL-4 are added to monocytes, the monocytes are primed precursor dendritic cells (46). In this study human GMCSF, GMCSF plus IL-4, or untreated monocytes were treated with J-LEAPS immunogens (JgD or JH without Seppic ISA51) to determine if the J-LEAPS immunogens could promote the maturation of human monocytes into IL-12 producing DCs.

**Morphological and phenotypic changes in J-LEAPS treated human monocytes**

In the first step towards proving the hypothesis, precursors to DCs, obtained by treating purified monocytes with GM-CSF and IL4 (44, 48, 95), were incubated with JgD or JH. The monocytes were isolated from human blood through leukapheresis and a >95% pure monocyte population was obtained by elutriating the white blood cell collection. Experiments were performed on monocytes that were isolated or on cells that were thawed and from previously purified stocks of monocytes. As shown in Figure 19A, monocytes changed from individual and round cells to clumped cells with dendritic extensions after treatment with either JgD or JH. The clustering of the primed monocyte cultures and the formation of dendrites is a morphological property of mature dendritic cells. The immunophenotype of the cells (Figure 19B) also changed with an up-regulation of CD86 and HLA-DR expression within 48 hours of treatment. The morphology, behavior and increased expression of CD86 and HLA-DR of JgD or
JH treated cells is consistent with maturation of the DC precursors to mature DCs (93).
Fig. 19. Treatment with JgD or JH promotes maturation of human monocytes into dendritic cells. Monocytes obtained by leukapheresis of blood and purified by elutriation were cultured in serum free medium + GMCSF and IL-4 for 24 hours. The cells were then treated with 14.5 micromoles of JgD or JH and incubated for 3 days at 37°C. (A) Microscopic photographs of human monocytes show the phenotypic changes after treatment including dendrite formation and clustering of the cells. (B) Cells shown were fixed, stained with PE-anti-CD86 or PE-anti-HLA-DR and analyzed by flow cytometry.
J-LEAPS treatment of human monocytes induces IL-12 production

Different types of DCs are characterized by the cytokines that they produce and the subsequent T cell responses that they mediate (35, 36, 44, 52). A survey of the cytokines produced and released into the medium was performed by protein array to determine the nature of the DC that was produced upon treatment of the DC precursors with JgD or JH. The protein arrays were performed as described before (Figures 8, 9, and 10). The normalized ratio of values from treated or untreated cells provides a semi-quantitative analysis of the cytokine spectrum produced by the cells. Treatment with the unconjugated J, gD, or H caused no significant production of cytokines (data not shown).
Fig. 20. Survey of cytokine production following JgD or JH treatment. Human blood derived monocytes from 3 individuals were treated with JgD or JH in six separate experiments (two separate experiments per donor (3 donors)). Spent media were collected three days post treatment, and evaluated by protein array (RayBio® Human Cytokine Antibody Array 3). Array results were quantitated by densitometry, and normalized to the summation values for each array to allow for comparative analysis of JgD or JH treated or untreated dendritic cell array results. The data shown are the mean scores for the fold increase or decrease to the untreated control for each cytokine detected of the 42 cytokines (IL-12p40 was not on the human arrays) on the replicated arrays. The error bars represent the standard deviation between trials. Inset, human monocytes from different donors produced similar amounts of IL-12p70 after being treated with JgD. Spent media was obtained from monocytes from donor 3, 5, and 8 after incubation with JgD in separate and repeated experiments, and analyzed, as described above. (*) Indicates a significant change in cytokine production from untreated cells.
Figure 20 shows results for a relevant subset of cytokines after a 48h treatment with JgD or JH. The amount of IL-12p70 was significantly increased by >4 fold following treatment with either JgD or JH compared to the untreated control (Per ANOVA, p-values = 2.03E-05 (JgD) and 3.31E-05 (JH) when compared to normalized untreated IL-12p70 values). There was also a visible but not statistically significant change in the levels of the Th1 affiliated chemokines, MCP-2 and RANTES. These results were reproduced for three different individuals and were similar following treatment with either JgD or JH. In each case, IL-12p70 production was enhanced following treatment with either JgD or JH. Production of the acute phase cytokines, IL-1, TNF-alpha and IL-6, was not enhanced and were the same as untreated cells. Production of IL-12p70 without concomitant enhancement of these proinflammatory cytokines is a different outcome than obtained with treatment by two TLR ligands, such as LPS and CpG (28, 43, 44).

**J-LEAPS does not induce cytokine production in human T cells**

The earlier mouse studies demonstrated that J-LEAPS promote differentiation of monocytes to dendritic cells. Purified human T lymphocytes were treated with JgD and analyzed to confirm that the target cell is not a T cell. Spent supernatants were analyzed for the presence of 42 different cytokines and chemokines by cytokine arrays. Per Figure 21, there was no IL-12 or IFN-
gamma produced by T lymphocytes directly treated with JgD and there were no other differences in cytokine production between the JgD treated lymphocytes when compared to the untreated lymphocytes. This confirms that human T cells are not responsive to and are unlikely to be the target cell of J-LEAPS immunogens.
Fig. 21. Cytokine array of human lymphocytes treated with JgD immunogen. Human lymphocytes were not treated or treated with 14.5 micromoles of JgD immunogen. Cells were incubated for 48 hours and spent media was collected and analyzed for cytokine production by protein arrays. There was no quantifiable difference in cytokine production between the untreated and JgD treated lymphocyte media.
J-LEAPS treatment of human monocytes, GMCSF-monocytes, and GMCSF-IL-4 monocytes

Previous studies with mouse bone marrow cells showed that JgD or JH treatment was sufficient to convert precursor dendritic cells into IL-12p70 producing DCs. Similarly, JgD or JH treatment of GMCSF/IL-4 primed human monocytes was sufficient to promote the maturation of these cells into DCs that produce IL-12p70. The addition of GMCSF & IL-4 primes the monocytes for dendritic cell maturation and activation and converts the population into immature dendritic cells. To determine whether the GMCSF or IL-4 was required, the cytokine profile of J-LEAPS immunogen treated human monocytes (untreated), GMCSF (G-monocytes), or GMCSF plus IL-4, (GM4-monocytes) were compared.
Table 7. Cytokine response to JgD of monocytes, GMCSF treated monocytes and GMCSF + IL-4 monocytes.

<table>
<thead>
<tr>
<th>CYTOKINE</th>
<th>MONOCYTE</th>
<th>GMCSF</th>
<th>GMCSF + IL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12p70</td>
<td>3.09*</td>
<td>3.05</td>
<td>3.58</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2.54**</td>
<td>2.45</td>
<td>0.80</td>
</tr>
<tr>
<td>MCP-2</td>
<td>2.45</td>
<td>2.40</td>
<td>1.84</td>
</tr>
<tr>
<td>RANTES</td>
<td>1.61</td>
<td>1.51</td>
<td>1.57</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>1.38</td>
<td>1.44</td>
<td>1.25</td>
</tr>
<tr>
<td>MIP-1 delta</td>
<td>1.15</td>
<td>1.10</td>
<td>1.64</td>
</tr>
<tr>
<td>ENA-78</td>
<td>0.77</td>
<td>0.76</td>
<td>0.74</td>
</tr>
<tr>
<td>MCSF</td>
<td>0.68</td>
<td>0.60</td>
<td>1.49</td>
</tr>
<tr>
<td>MDC</td>
<td>0.68</td>
<td>0.67</td>
<td>1.89</td>
</tr>
<tr>
<td>MIG</td>
<td>0.37</td>
<td>0.37</td>
<td>0.39</td>
</tr>
<tr>
<td>Angiogenin</td>
<td>0.60</td>
<td>0.59</td>
<td>1.00</td>
</tr>
<tr>
<td>Oncostatin</td>
<td>0.52</td>
<td>0.50</td>
<td>1.17</td>
</tr>
<tr>
<td>TARC</td>
<td>0.22</td>
<td>0.24</td>
<td>0.47</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.15</td>
<td>0.21</td>
<td>1.49</td>
</tr>
<tr>
<td>GCSF</td>
<td>0.00</td>
<td>0.00</td>
<td>1.22</td>
</tr>
<tr>
<td>IL-1 alpha</td>
<td>0.00</td>
<td>0.00</td>
<td>0.36</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.00</td>
<td>0.00</td>
<td>0.34</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>0.00</td>
<td>0.00</td>
<td>0.27</td>
</tr>
<tr>
<td>IL-1 beta</td>
<td>0.00</td>
<td>0.00</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Densitometric protein arrays values were normalized to EGF for standardization between cytokine protein arrays and are presented as fold increases found in the spent media of JgD treated cells/untreated cells.

*MCP-1 and 2: monocyte chemoattractant proteins; RANTES: regulated upon activation normal T cell express sequence; PDGF-BB: platelet derived growth factor; MIP-1 delta: macrophage inflammatory protein-1 delta; ENA-78: epithelial neutrophil activating peptide 78; MCSF: macrophage colony stimulating factor; MDC: macrophage derived chemokine; MIG: monokine-induced by interferon gamma; TARC: thymus and activation regulated chemokine; VEGF: vascular endothelial growth factor; GCSF: granulocyte colony stimulating factor; EGF: epidermal growth factor, GMCSF: granulocyte macrophage colony-stimulating factor
Table 7 shows values from the cytokine protein array for monocytes, G-monocytes (GMCSF treated monocytes), or as for the previous experiments, GM-4 monocytes (GMCSF + IL-4 treated monocytes) treated with JgD. The levels of IL-12p70, RANTES, MCP1, and MCP2 produced by monocytes after 48 h treatment with only JgD was most similar to cells pretreated for 48h with GM-CSF. IL-12 was produced by all three of the JgD treated monocyte populations. The GM-4 monocytes also produced elevated levels of IL-12p70 and MCP2 but the trends for some other cytokines and chemokines differed from that of JgD treated monocytes or GM-monocytes. This shows that GMCSF + IL-4 was not necessary for JgD to activate cytokine producing dendritic cells. There was a difference in the chemokine productions of the GM-4 JgD treated monocytes when compared to the JgD treated GM-monocytes and monocytes, suggesting that the GM-4 is a different type of DC precursor than the GM-monocytes and the monocytes. These results suggest that the JgD immunogen was sufficient to activate monocytes without any other cytokines or stimulating agents, or T cell interaction.
JgD treated dendritic cells induce IFN-gamma production in human allotypic T cells

Ultimately, a DC1 cell must be able to activate T cells and promote IFN-gamma and IL-2 production in order to mediate a Th1 cytokine response. The ability of JgD treated monocytes to support allotypic activation of T cells was tested (49). For the experiment depicted in Figure 22, monocytes from two separate donors were treated with JgD or medium for 24 h prior to addition of CD4⁺ T cells. The CD4⁺ T cells were obtained from elutriation following high affinity negative selection from other donors. Spent medium was analyzed after 6 days for cytokine production. Significantly large differences in the Th1 cytokines, IFN-gamma and IL-2 were present in the spent medium from CD4⁺ T cells mixed with JgD treated monocytes compared to those mixed with untreated monocytes. Results were repeated with JgD treated monocytes and allogeneic CD4⁺ T cells from another set of donors. These results demonstrate that the JgD treated monocytes matured into DCs capable of promoting a Th1-like cytokine response by T cells. This demonstrates that J-LEAPS immunogens are capable of activating a human monocyte into an IL-12 producing dendritic cells that can initiate a Th1 immune response. This is similar to the adjuvant effect of JgD and JH demonstrated on mouse cells.
Fig. 22. JgD treated human monocytes activate allogeneic T cells to produce IFN-gamma and IL-2. Separated monocytes and T cells were obtained after elutriation of the human apheresis product. CD4+ T cells were further purified with T cell isolation columns. Monocytes harvested 24 hours after treatment with JgD or HBSS were added to T cell cultures at a DC:T cell (1:10) ratio. Spent media were collected six days after co-culture, and assayed by protein array as described above. (*) indicates significant change in cytokine production from untreated cells.
These results prove that the J-LEAPS immunogens (exemplified by JgD and JH) are sufficient to convert human monocytes (precursor dendritic cells) into an IL-12 producing dendritic cell. This unique dendritic cell produces IL-12 but not acute phase cytokines and is sufficient to interact with T cells and activate Th1 responses. This confirms that the target cell for J-LEAPS vaccines is a precursor of dendritic cells in humans, and that an adjuvant like effect is being demonstrated.
**Question:** Is JgD sufficient to initiate an antigen specific and a protective immune response?

**Approach:** Bone marrow cells from C57BL6 mice were untreated or treated with J-LEAPS immunogens (JgD or JH), and then co-cultured with splenocytes from JgD immunized mice and analyzed for IFN-gamma production. To detect booster response, J-LEAPS immunogen treated bone marrow cells injected into mice that were later lethally challenged with HSV-1 to test for initiation of response and protection from death and disease.

**Hypothesis:** JgD is sufficient to activate the differentiation of mouse bone marrow cells into dendritic cells and is carried by the dendritic cells to elicit an antigen specific and protective immune response in mice.

Previous studies showed that mouse bone marrow (BM) cells treated with J-LEAPS immunogens (JgD or JH) were converted into DCs that produced IL-12p70, and when co-cultured with naïve splenocytes were capable of initiating a Th1 cytokine response. These results for JH and JgD confirmed that the J-LEAPS vaccines act like an adjuvant and can elicit similar Th1 cytokine response. As a vaccine, JgD must be able to initiate an antigen specific and protective immune response. In order to determine whether
J-LEAPS treated dendritic cells can elicit an antigen specific response, an ex vivo and an in vivo approach were taken. JgD treated bone marrow cells (JgD-DC) or JH treated bone marrow cells (JH-DC) were co-cultured ex vivo with splenocytes from JgD immunized mice and analyzed for IFN-gamma production. Positive result for an antigen specific, boost response would be shown if the JgD treated bone marrow cells (JgD-DCs) produce a much higher amount of IFN-gamma production than the JH treated bone marrow cells (JH-DC). An adoptive transfer experiment was performed to test whether JgD-DC can initiates antigen specific protection from lethal challenge with HSV-1. The activity of JgD-DC was compared to untreated bone marrow (BM), J treated bone marrow (J-BM), or JH-DC. JH-DC can act like an adjuvant to promote Th1 cytokine production but would not be able to provide an antigen specific response. JgD-DCs and other J-LEAPS treated or untreated bone marrow cells were injected into mice that were later lethally challenged with HSV-1. If the JgD-DCs initiate a protective response and the JH or J-ICBL treated bone marrow cells do not, then this would prove that the JgD-DCs are capable of eliciting an antigen specific and protective immune response.
J-LEAPS treated bone marrow cells co-cultured with isoantigenic splenocytes from JgD immunized mice

In order to determine whether the JgD-DC cells are capable of antigen presentation to T cells to promote a specific vaccine-like immune response, spleen cells obtained from mice (n=3) immunized with JgD as described for Figure 3, were incubated with JgD-DC or JH-DC (prepared as described for Figure 10). Spent medium was obtained after an additional 48h (72 h after JgD or JH treatment of BM) and cytokine levels were analyzed by protein array and IFN-gamma levels were also analyzed by ELISA (Table 8). Cytokine responses of JgD immunized spleen cells stimulated by the unrelated JH-DC were similar to the previous experiment (see Figure 10) and similar to responses to JgD-DC except for the IFN-gamma response. T cell production of IFN-gamma in response to JH-DC was detected by protein array but was undetectable by ELISA. Importantly, IFN-gamma production was elevated following incubation with the isoantigenic JgD-DC and also detectable by ELISA. This is consistent with the occurrence of an antigen specific booster response of the JgD-splenic T cells mediated by JgD-DC. Also this suggests that the JgD antigen must remain on the cell surface of the matured dendritic cell because the cells were washed free of any unbound antigen and they were still able to initiate an antigen specific response.
Table 8: Cytokine response of co-cultures of JgD immunized splenocytes with JgD-DCs or JH-DCs.

<table>
<thead>
<tr>
<th>CYTOKINES</th>
<th>JH/Untreated</th>
<th>JgD/Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein Array</strong>&lt;sup&gt;1,2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>4.48</td>
<td>7.15</td>
</tr>
<tr>
<td>IL-2</td>
<td>4.33</td>
<td>7.27</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>4.53</td>
<td>7.21</td>
</tr>
<tr>
<td>RANTES</td>
<td>4.43</td>
<td>7.05</td>
</tr>
<tr>
<td>MCP-5</td>
<td>4.27</td>
<td>6.85</td>
</tr>
<tr>
<td>MCP-1</td>
<td>4.17</td>
<td>6.77</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>IL-17</td>
<td>4.06</td>
<td>6.62</td>
</tr>
<tr>
<td>IFN-gamma</td>
<td>4.27</td>
<td>11.83</td>
</tr>
<tr>
<td><strong>ELISA</strong>&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-gamma ELISA</td>
<td>Below detection (&lt;150 pg)</td>
<td>450pg</td>
</tr>
</tbody>
</table>

<sup>1</sup>Average of (2 independent experiments) selected values from protein array of samples obtained at 48 h after culture of splenocytes without or with either JgD-DC cells or JH-DC cells. Cytokine array analysis was performed as described in Figure 10.

<sup>2</sup>All p values were < 1.8 x 10^-4 per ANOVA compared to untreated cells

<sup>3</sup>ELISA result for JgD immunized spleen cells co-cultured with untreated bone marrow cells was zero.
Lethal HSV-1 challenge of mice receiving adoptive transfer of J-LEAPS treated bone marrow cells

In order to determine whether JgD-DC can activate a sufficient T cell response to provide vaccine-like protection, mice receiving JgD-DC were challenged with a lethal dose of HSV-1 strain H129 by dermal abrasion on the back and evaluated for zosteriform spread of disease and survival as in our previous vaccine study (14). In the first study, immunization with JgD in ISA51 prevented the onset of zosteriform lesions, which would have occurred for unimmunized mice within 3-5 days, and death within 7-10 days (14).
Fig. 23. Kaplan-Meier survival curve of untreated and mice receiving JgD-DC by the intraperitoneal route. Bone marrow cells were treated with JgD immunogens 24 hours prior to injection. Cells were washed to remove all unbound peptide and suspended at 100,000 cells per 150 µL of HBSS (JgD-DC vaccine). Female C57BL/6 mice (n=6) were injected with 150 µL of JgD-DC vaccine into the intraperitoneal cavity. Mice received a booster in the same fashion 1 week later. One week after the booster, JgD-DC mice were challenged with a lethal dose (10^6 PFU) of H129 herpes simplex virus, by the dermal abrasion-zosteriform challenge model. Disease and survival of mice were recorded from day 0 to day 14 and survival is plotted in this Kaplan Meier survival plot.
Fig. 24. HSV-1 disease score of untreated and mice receiving JgD-DC by the intraperitoneal route. Mice treated and infected as for Figure 23. Extent of disease was: 0= no symptoms, 1=non-specific evidence of disease, 2=local disease, 3=early zosteriform spread, 4=late extensive zosteriform spread, 5=moribund condition, 6=death. The results are shown as an average of each treatment group.
Mice were untreated or received $10^5$ JgD treated bone marrow cells (JgD-DC) injected into the peritoneum (IP) on two occasions separated by one week and then challenged one week later with $10^6$ PFU of HSV-1 strain H129. All of the untreated mice died from the HSV-1 infection by day 11. Only 1 mouse died in the JgD-DC vaccine group, while the other 87% survived (Figure 23). Although the majority of the mice in the JgD-DC group survived the lethal infection, many of the mice showed symptoms of the HSV-1 infection (i.e. spread of lesions) and then recuperated from the HSV-1 disease within 14 days (Figure 24). This demonstrated that the JgD-DC vaccine provided protection from death but protection from both death and disease was not obtained by this protocol. The route of administration may be an important influence on the outcome due to its influence on corporal delivery of the DCs to draining lymph nodes. Many of the JgD-DCs could potentially die or be delayed during migration to the lymph node from the peritoneum, which would delay or limit their ability to activate a T cell response. This could result in a weak or delayed immune response.
Fig. 25. Kaplan-Meier survival curve of mice receiving combined intradermal (ID) and intraperitoneal (IP) treatment with DC vaccines. Bone marrow derived vaccines were prepared as described in Figure 23. In this experiment the cell suspension (100,000 cells in 150 μL) was administered as 100 μL IP and 50 μL ID. Mice (n=6 per treatment group) were injected twice with a one week interval and then lethally infected with HSV strain H129 (10^6 PFU) one week after the booster. Kaplan-Meier survival curve was plotted over a 14 day period.
Fig. 26. HSV-1 disease score of mice receiving combined intradermal (ID) and intraperitoneal (IP) treatment with DC vaccines. Disease score was recorded for the vaccine trial described in Figure 25. 0= no symptoms, 1= non-specific evidence of disease, 2= local disease, 3= early zosteriform spread, 4= late extensive zosteriform spread, 5= moribund condition, 6= death. The results are shown as an average of each treatment group.
In a second experiment, mice were treated with $10^5$ JgD-DC split between an intradermal (ID) and intraperitoneal (IP) site on two occasions separated by one week and then challenged one week later with $10^6$ PFU of HSV-1 strain H129 (Figure 25). ID administration of the cells would facilitate their delivery to lymph nodes to promote T cell activation. Mice received untreated BM, J-BM, JH-DC, or JgD-DC.

All mice receiving JgD-DC survived, one of these mice had local disease (score = 2) and one mouse had zosteriform lesions (score=4) that resolved but the other mice had no signs of disease (Figure 25 and 26). In contrast, mice receiving no treatment, or receiving untreated mouse bone marrow cells (BM), mouse bone marrow cells treated with the J-ICBL (J-BM), or mouse bone marrow cells treated with JH (JH-DC) incurred significant disease with zosteriform spread and death of all or a majority of the group within 2 weeks (Figure 27). Although all mice in these groups exhibited zosteriform disease, survival of one mouse (JH-DC group) or two mice (J-BM, untreated groups) is likely to be due to variability in the efficiency, and hence the inoculum, of the infection. The protection elicited by JgD-DC was not due to unbound vaccine since the JgD-DC were washed and resuspended prior to injection. These results indicate a much better outcome than the earlier experiment, in which the JgD-DC was only administered by the IP route and the adoptive transfer prevented death but not disease. This suggests that the route of administration is important for the delivery of the DCs to lymph nodes and to activate a protective T cell response.
Fig. 27. Adoptive transfer of JgD-DC provides antigen-specific protection from HSV disease. All bone marrow vaccines administered ID & IP and performed as previously described (Figures 25 and 26). Disease scores for mice treated with JgD-DC were compared to JH-DC, J-BM, untreated-BM, and no treatment and were recorded for 14 days, where 0 = no symptoms, 1 = non-specific evidence of disease, 2 = local disease, 3 = early zosteriform spread, 4 = late extensive zosteriform spread, 5 = moribund condition, 6 = death. The results are shown as an average of each treatment group.
These results prove that the DCs generated by JgD treatment of bone marrow cells are sufficient to initiate and develop an antigen specific immune response sufficient to provide protection from a lethal HSV infection. The results also indicate that the JgD LEAPS peptide remains on the surface of the DC for sufficiently long periods of time to be able to mediate interaction with T cells both ex vivo and in vivo. The inability of JH-DC to provide protection indicates that antigen specific immunization is required to confer protection. This also confirms that the JgD induces an antigen specific immune response to initiate protection.
The overriding question of this dissertation was, “How do J-LEAPS vaccines and specifically, JgD, elicit a protective immune response?” Ultimately, it was determined that J-LEAPS vaccines are sufficient to promote the differentiation of precursor DCs into IL-12 producing DC1s. The J-LEAPS activated DC1s then initiate a Th1 immune response capable of providing protection against lethal infection by HSV-1. This was determined by: identifying the J-LEAPS initiated cytokine response, identifying the J-LEAPS initiator cell in mice and humans, and by confirming the mechanism by which protection is initiated by a J-LEAPS antigen specific immune response.
Identifying the cytokines that are produced during a J-LEAPS initiated immune response

IFN-gamma production confirms that J-LEAPS vaccines initiate a Th1 immune response

The J-LEAPS vaccines studied were JgD (a potential herpes simplex virus (HSV-1) vaccine) and JH (a potential human immunodeficiency virus (HIV) vaccine). JgD contains a viral peptide from glycoprotein D of HSV-1. JH contains a viral peptide from the gag protein of HIV. A Th1 immune response is required for protection against both of these intracellular viruses. Prior studies determined that J-LEAPS vaccines generated immune activities that are consistent with Th1 responses including immunoglobulin subtype production, and JgD induced antigen specific delayed type hypersensitivity (DTH), and protection from lethal HSV-1 challenge (14, 17, 19). The cytokine profile of sera from JgD or JH immunized mice was analyzed to confirm that the proper cytokines were being produced to initiate a protective Th1 immune response. The cytokines produced on days 3, 10, and 24 in response to JgD and JH provided clues to the immune response elicited by the vaccine at initiation (day 3), delivery (day 10), and acquisition (day 24) of protection. Cytokines are signaling proteins that can regulate immunity, inflammation, and hematopoiesis. Considering the essential functions of cytokines, they are key determinants of the innate and immune
responses (96).

On day 3, both JgD and JH vaccines induced IL-12 production prior to any T cell cytokine production. IL-12 is a cytokine produced by myeloid antigen presenting cells (82), showing that the initial cytokine response was made by a myeloid antigen presenting cell not a T cell. The IL-12 cytokine response progressed to include increased production of T cell cytokines, IL-17 and IFN-gamma, ten days after treatment with either JgD or JH. IL-17 is produced by Th17 cells and IFN-gamma is produced by Th1 cells, which means that two different T cells are being activated during the delivery of the vaccine (96). By day 24, JgD and JH vaccines induced high amounts of IFN-gamma production, continuous production of IL-12, and IL-17 production subsided. The transient IL-17 response may result from increased levels of IFN-gamma inhibiting the production of IL-17 (97). Production of IFN-gamma confirms that a Th1 immune response was initiated by the J-LEAPS vaccines, which correlates with the Th1-like immune activities found in the previous J-gD vaccine studies (17). In the original J-LEAPS vaccine studies it was thought that the J-LEAPS directly activated T cells without interacting with an antigen presenting cell (16). The progress of the cytokine response generated by JgD or JH suggests that the immune response is initiated by an antigen presenting cell that later initiates a Th1 response.
Early IL-12 production and delayed IFN-gamma production provides preliminary evidence that the initiator cell is a type of dendritic cell

Early IL-12 production and delayed IFN-gamma production after JgD or JH immunization of mice indicates that an antigen presenting cell is promoting the development of a Th1 immune response. IL-12 production is required for an antigen presenting cell to promote the development of a Th1 response, while IFN-gamma is the response defining cytokine produced by CD8+ cytotoxic T cells and CD4 Th1 cells during a T cell mediated immune response (64, 65).

The cytokine profile following immunization with JgD or JH also provides hints to the identification of the J-LEAPS initiator cell. The delayed production of IFN-gamma in JgD or JH immunized mice suggests that the initiator cell is not a T cell. IL-12 was produced in response to immunization of immunologically naïve mice with the JgD or JH vaccines, and only dendritic cells can initiate a new immune response (36, 55, 82), suggesting that J-LEAPS interacts with dendritic cells.

The J-LEAPS vaccines were emulsified in Seppic ISA51 adjuvant for immunizations. By itself, the Seppic ISA 51 adjuvant did not activate a relevant cytokine response. Adjuvants can be used in vaccines to enhance immunogenicity by stimulating DCs and by prolonging the presence of an immunogen, extending the time of exposure to the immunogen (5). The longevity of IL-12 production in the JgD or JH immunized mice suggests that
the Seppic ISA51 adjuvant establishes a slow release reservoir for the vaccine. The ability of JgD to activate DCs that can generate booster responses *ex vivo* and protection from lethal HSV infection upon adoptive transfer into mice demonstrate that the adjuvant is not necessary for the activity of the J-LEAPS vaccines but may be necessary to ensure that the peptide vaccine is present long enough to elicit the necessary response.

A Th1 immune response promotes a positive feedback loop for IL-12 and IFN-gamma production. IL-12 producing antigen presenting cells induce T cells to produce IFN-gamma and IFN-gamma producing Th1 cells promote antigen presenting cells to produce IL-12 (65). This cycle of cytokine response could also explain why there was continuous IL-12 production detected over the 24 days. This type of progression would elicit the desired immunity required for protection from HSV-1 and other viral diseases.

*IL-12 production accompanied by negligible amounts of IL-6 & TNF-alpha suggests that a DC1 is activated in a novel way*

In most cases dendritic cells (DCs) are activated to produce IL-12 when toll like receptors (TLRs) bind ligands of microbial origins. The IL-12 production is accompanied by the production of large amounts of proinflammatory cytokines such as TNF-alpha, IL-6, and IL-1 beta (28, 43, 44). Although IL-1 beta was not assayed in the experiments with mice, it was assayed in the
experiments with human cells and was not induced by JgD. The cytokine response to JgD or JH treatments differed from that induced by some TLR activators of DCs since there were only undetectable or negligible amounts of IL-6 and TNF-alpha produced (28, 31, 43, 44). The production of proinflammatory cytokines with IL-12 is initiated by NF-kappa-B and other transcriptional factors upon stimulation of multiple TLRs. The lack of proinflammatory cytokines suggests that the J-LEAPS vaccines activate different receptors and different combinations of pathways than those initiated by TLRs. These differences distinguish the nature of the IL12 response to J-LEAPS immunogens from those activated by TLR ligands (28, 31, 43, 44, 50, 97). This suggests that J-LEAPS vaccines potentially activate dendritic cells in a novel way.

The lack of IL-6 and TNF-alpha production should allow immunomodulation with potentially less immunopathology. Proinflammatory cytokines, such as TNF-alpha and IL-6, can elicit harmful side effects following vaccination. For example, production of TNF-alpha and IL-6 can cause destruction of tissue through chronic inflammation (99). Production of these proinflammatory cytokines is not required to provide protection against intracellular viruses (i.e. HSV-1). Interferons are the important anti-viral cytokines for protection against HSV (100), rather than proinflammatory cytokines like TNF-alpha and IL-6. For example, Gill et al determined that TNF-alpha knockout mice were still protected against genital tract HSV challenge
following treatment with poly (I:C), showing that TNF-alpha is not required for HSV protection (101). J-LEAPS vaccines' induction of IL-12 production in this novel way is likely to generate a safer and appropriate immune response.

**Attachment of the J-ICBL is required to make the gD and H viral peptides immunogenic**

In previous studies of J-LEAPS based vaccines, neither the gD nor the H peptides could elicit an immune response by themselves (16). As expected, neither the gD nor the H peptide could elicit a cytokine response. The gD and H peptides are similar to haptens and are too small to initiate an immune response on their own. The J-ICBL in the JgD and JH vaccines appears to act like a carrier protein making the viral peptide immunogenic. In contrast to J, a carrier protein, like KLH, promotes immunogenicity but initiates an immune response to itself, the carrier protein, as well as to the hapten and this is an extraneous response. The response initiated by a hapten-carrier protein complex is usually a Th2 response that is limited to only antibody protection (1, 102). This is different from the Th1 response that is induced to the gD or H when attached to the J-ICBL.

When the J-ICBL was conjugated to the H or gD peptides, the JH and JgD immunized mice produced large amounts of IL-12 and then IFN-gamma. The J-ICBL by itself was not able to elicit an increase in the production of any T
cell cytokines, and there was only a slight elevation in the amount of IL-12 produced. This shows that the J-ICBL must be covalently attached to the epitope bearing peptide for induction of cell responses.

The J-ICBL (DLLKNGERIEKVE) is derived from beta-2-microglobulin of MHC I (16, 17, 20). The J-ICBL is centered on the arginine 45 region of beta-2-microglobulin. In previous studies it was shown that the addition of whole or cleaved portions of beta-2-microglobulin could enhance cytotoxic T cell activity and promote CD4 T cell proliferative responses. Parham et al showed that a polyclonal antibody to this beta-2-microglobulin region would block allogeneic T cell responses in a mixed lymphocyte population (75). These results suggested that the beta-2-microglobulin region in the J-ICBL is recognized by T cells and potentially capable of activating a T cell response. Considering the background of the J-ICBL, it was originally thought that the J-ICBL was a T cell binding ligand (and was originally referred to as J-TCBL) that would directly bind to and induce responses in T cells without the involvement of antigen presenting cells. The cytokine profiles of JgD or JH treatments show that the J-ICBL requires an antigen presenting cell to initiate an immune response. Thus, the J peptide is not a TCBL and the cytokine profile does not display any evidence that it directly binds to a T cell.

Injection of unconjugated J and an epitope does not induce an immune response (15). Similarly, neither J nor the antigenic peptides, gD or H, could promote cytokine production. The heteroconjugate JgD and JH vaccines are
likely to interact with two different receptors on the same cell. Covalent linkage between the J-LEAPS heteroconjugate immunogen may be necessary to either ensure delivery of both J and antigenic peptide to the same target cell and to crosslink an epitope-binding MHC molecule and a separate J-binding receptor on the cell surface.

Although the J-ICBL was not able to initiate a T cell response by itself, it does elicit a large decrease in IL-10 production in mice. The reduction in IL-10 levels would promote an environment that is more conducive for activation of Th1 immunity, since IL-10 is an antagonistic cytokine of IL-12 (103, 104, 105). The mechanism by which the IL-10 levels are decreased is unknown. The J-ICBL appears to have biological activity but it is not sufficient to induce development of DCs or an immune response by itself.
Identifying the cells that initiate the J-LEAPS induced immune responses in mice

*J-LEAPS immunogens activate precursor bone marrow cells to produce IL-12*

Previous studies demonstrated that antibody ablation of CD8, CD4, and IFN-gamma prevented the development of a protective immune response to lethal challenge of HSV-1 infection. CD8\(^+\) cells were involved in initiating the protective immune response (14). The CD8\(^+\) initiator cell could be a T cell or an antigen presenting cell. The initial survey of the cytokine response to immunization of mice with the JgD or JH immunogens showed that IL-12 was produced soon after immunization with IFN-gamma being produced later. This strongly suggests that a dendritic cell or precursor dendritic cell is the initiator cell that elicits a Th1 immune response. Potential sources of mouse dendritic cells are spleen and bone marrow (27). Spleen cells, which have a rich source of T cells, B cells, and mature dendritic cells, generated no cytokine response detected to the JgD or JH immunogens. The spleen contains T cells and dendritic cells that are more mature than the precursor dendritic cells found in the bone marrow. Bone marrow cells containing precursor and immature dendritic cells were a better source of cells with the potential to be J-LEAPS vaccine target
cells and initiator of the response (27, 33, 83). The JgD or JH treated bone marrow cells clustered, became more granular, and produced IL-12. These findings suggest that the J-LEAPS vaccines are capable of initiating the maturation of precursor dendritic cells, which could be the J-LEAPS initiator cell.

**J-LEAPS immunogens activate precursor bone marrow cells to mature into dendritic cells**

A mature dendritic cell must be capable of activating a T cell response. For a dendritic cell to activate a T cell response three things must occur; antigen recognition, second signal activation, and cytokine production (36). The dendritic cell must present antigen to the T cell in its MHC molecule so that the T cell can recognize the antigen (34). A second signal must occur for T cell activation when CD28 on the T cell binds to CD80 or CD86 on the antigen presenting cell (46). Cytokines must be produced by the dendritic cell to activate and initiate T cell differentiation and direct the subsequent response. IL-12 must be produced to initiate a Th1 immune response and IL-12 was produced by JgD or JH treatment of bone marrow cells and was present in spent medium (44). JgD or JH treatment was sufficient to promote maturation of bone marrow derived precursor cells into mature DCs (JgD-DCs or JH-DCs) that displayed increased expression of MHC II, CD11c, and CD86, which are cell surface markers for a mature DC capable of antigen presentation.
The JgD vaccine protection studies determined that CD8$^+$ cells were required for initiating protective immunity to HSV challenge. When T cell depleted (CD3 expressing cells removed) bone marrow cells were treated with JgD or JH immunogens, a CD8$^+$ precursor dendritic cell population was activated to produce IL-12, confirming that the CD8$^+$ initiator cell is a precursor dendritic cell. T cells were removed from the precursor population to confirm that a T cell is not the initiator cell nor was T cell interaction required for J-LEAPS activation of the dendritic cells.

Interestingly, JgD and JH treatment caused an increase in the number of CD8 expressing cells in the majority of the precursor population. Expansion of the CD8 population could have resulted from either the increased expression of CD8 on all of the cells or the differentiation of a specific DC population that expresses CD8. This CD8$^+$ dendritic cell population is likely to be the initiator cell that is required for immune protection.

**Pathways of DC activation by JgD**

It was originally hypothesized that the J-ICBL would bind to CD8, which could potentially activate Lck, but CD8 might not be the J-receptor. X-ray crystallography does not show an interaction between the J-ICBL portion of beta-2-microglobulin and CD8 (106). Other techniques are being designed to identify the J-receptor. A newly discovered family of antigen presenting cell receptors,
known as leucocyte immunoglobulin-like receptors (LILRs) will be a main focus of future J-receptor studies. These receptors interact with MHC and modulate dendritic cell function. LILRs are capable of initiating DC maturation and directing T cell activation (107).

There is a link between CD8 and the activation of IL-12 production since JgD treatment induced expression of both CD8 and intracellular IL12. Although the function of CD8 on dendritic cells is still not known, the function of CD8 on T cells is well defined. Some of the key proteins involved in the signal transduction of cytokine production in CD8 T cells are Lck and calcineurin. Lck expression on BM cells increased with the increase of CD8 expression after JgD treatment. In T cells, Lck provides a second activating signal through a tyrosine kinase cascade to initiate activation of cytokine gene transcription and T cell responses (87). The link between Lck activation and IL-12 was confirmed when 3-(2-(1H-Benzo[d]imidazol-1-yl)-6-(2-morpholinoethoxy) pyrimidin-4-ylamino)-4-methylphenol, an Lck specific inhibitor blocked IL-12 production by JgD treated precursor dendritic cells. JgD induction of Lck expression and its role in the activation of dendritic cells is a unique finding.

An increased level of cytoplasmic calcium is induced by the Lck initiated cascade of phosphorylation events in T cells (87). Calcium binds to a subunit of a protein phosphatase known as calcineurin. Calcineurin promotes activation of transcription factors including NFAT, NF kappa-B, and AP-1, which regulate the production of genes for cytokines. Also, Ca\(^{2+}\) influx with a calcium ionophore can
activate the differentiation of precursors into dendritic cells (51). When calcineurin was inhibited by cyclosporin A, JgD treated precursor dendritic cells did not produce IL-12. The inhibition studies of Lck and calcineurin indicate that both of these proteins are important and that calcium ion and NFAT are probably involved in DC activation.

The J-ICBL binds to a receptor on a precursor dendritic cell (CD8, LILR, or other J-receptor) which causes the increase in CD8 expression or the expansion of CD8\(^+\) cells. Considering that there are no other stimulating agents added to the cells, such as Flt-3 or GMCSF, to expand the population, the J-LEAPS immunogens appear to be sufficient to promote the increase in CD8 expression. A potential model for how J-LEAPS activate CD8\(^+\) dendritic cells to produce IL-12 can be developed. Heteroconjugate J-LEAPS immunogen (such as JgD) binds to and cross links two different receptors on the same cell to activate an unknown mechanism that increases the concurrent expression of CD8 and Lck. After CD8 is expressed, the cytoplasmic tail of CD8 activates Lck to initiate the tyrosine kinase cascade, which increases cytoplasmic levels of calcium. Calcium binds to the calcineurin that generates active transcriptional factors which promote the expression of IL-12 cytokine genes. This would initiate a CD8\(^+\) dendritic cell to produce IL-12 and require Lck and calcineurin activation to initiate cytokine production.
**JgD immunogen is capable of initiating the activation of DC1 from purified immature dendritic cells**

The *ex vivo* J-LEAPS bone marrow studies provided evidence that the J-LEAPS initiator cell is a precursor dendritic cell however, the bone marrow cell suspensions contained precursor dendritic cells and other cells and dendritic cells and macrophages differentiate from the same precursor cell (27). A mature dendritic cell and a mature macrophage can express the same cell surface markers and both can produce IL-12. This makes it difficult to distinguish whether the J-LEAPS treated cells differentiate into a macrophage or a dendritic cell.

To focus on the maturation of DCs, the J-LEAPS immunogens were tested on a purified culture of immature dendritic cells. Immature mouse dendritic cells expressed CD11c, CD80, CD86, MHC II, CD34, and OX40L, as shown by flow cytometry, confirming the type of cell and purity of the immature dendritic cells (29). The purified immature dendritic cells treated with the JgD immunogen formed dendrites. Dendritic extensions (dendrites) are one of the characteristics that distinguish dendritic cells from macrophages. As discussed later, only DCs can initiate an immune response and this was demonstrated in the adoptive transfer studies. These results confirmed that the J-LEAPS vaccine induces the differentiation of precursor cells into dendritic cells not macrophages. The JgD treated immature dendritic cells were also activated to produce IL-12.
This demonstrates that the J-LEAPS immunogens are sufficient to directly activate a precursor cell into a DC1 without any other cell involvement.

In most protocols for *ex vivo* dendritic cell generation, multiple agonists and stimulating agents, such as LPS and IFN-gamma, are required to initiate maturation and IL12 production by dendritic cells (29, 34). JgD did not require any other stimulating agents to induce this dendritic cell activation. Unlike DCs generated by other protocols, there were no inflammatory cytokines accompanying IL-12 production. This is different from the response initiated by other types of vaccines.

*J-LEAPS activated bone marrow cells are capable of initiating a Th1-like cytokine response in splenocytes*

Dendritic cells are the bridge between an innate and an adaptive immune response. DCs bridge these two immune responses by activating and directing T cell immunity (22). A functional dendritic cell should be able to activate immune-naive and memory T cells (23). The *ex vivo* series of experiments demonstrated that JgD acts as an adjuvant, to promote IL12 production and maturation of precursors into DCs. The DCs that were generated were capable of antigen presentation and induction of a Th1 response to splenic memory T cells, *ex vivo*, and could initiate an immune response in immunonaive mice. Interestingly, JgD-DC and JH-DC could also modulate the immune environment by promoting
antigen-nonspecific production of interferon gamma from immunonaive or JgD stimulated splenocytes.

The JgD treated BM cells and JH treated BM cells were sufficient to induce interferon gamma production in splenic T cells from naïve or JgD immunized mice. Induction of the response in splenic T cells was performed with J-LEAPS treated DCs washed free of unbound vaccine and unbound media components. Treatment of the BM cells to generate DCs and treatment of the T cells with the JgD-DC or JH-DC could not be done with the Seppic ISA51 adjuvant since this oil in water adjuvant would be toxic to cells in tissue culture. Clearly, the Seppic ISA 51 adjuvant is not required for JgD or JH activity on isolated cells. The Seppic ISA51 is likely to be required for immunization of mice with JgD to prolong the presence of the vaccine in the mouse. The depot effect of the vaccine may be important to extend the presence of the vaccine to allow the vaccine to promote the maturation of DC precursors in the periphery so that they can migrate and home to lymph nodes to activate T cells.

It is very unusual for a peptide vaccine to be able to activate a specific T cell response without the use of any adjuvant. Most subunit and synthetic vaccines (such as diphtheria-tetanus-pertussis, Hepatitis A & B, Prevnar, and HPV vaccines) that contain poor or non-immunogenic forms of toxins, bacteria, or viruses, similar to the small and non-immunogenic gD and H peptides, require an adjuvant to initiate immunity (2, 3, 4). However, the J-LEAPS immunogens probably only require a delivery method to initiate an immune response rather
than requiring an adjuvant to promote their immunogenicity. JgD and JH are acting like an adjuvant by initiating a cytokine response in DC precursors and naïve splenocytes. Adjuvants can initiate a specific type of innate or T cell response but are not antigen specific (2, 3). The JgD-DCs and JH-DCs initiated very similar cytokine responses with naïve splenic T cells in ex vivo studies suggesting that the cells interact with the T cells and the IL12 that they produce is sufficient to activate and direct the nature of the cytokine response to a Th1-like interferon gamma production. This is similar to what was obtained after immunization of mice (14).

**J-LEAPS vaccines are capable of initiating an antigen specific immune response**

The previously discussed ex-vivo studies with BM cells demonstrate that J-LEAPS immunogens act as an adjuvant that can stimulate differentiation of precursors into DC1s that can initiate a Th1 immune response. A vaccine must also act like an antigen to activate an antigen specific immune response which can provide protection from disease. The JgD vaccine does elicit protection from lethal HSV-1 infection and promotes an antigen specific immune response (14). Similarly, JgD treated bone marrow cells (JgD-DCs) induced an antigen specific booster response when mixed with splenic T cells from JgD immunized mice. No booster response was obtained with JH treated bone marrow cells (JH-DCs). The JH-DCs could only elicit the lower level adjuvant response. The antigen
specific booster response could be distinguished from the adjuvant-like response by the elevated levels of interferon gamma and interleukin 2, the response defining cytokines of a Th1 response. This type of IFN-gamma booster response suggests that JgD is capable of becoming a functioning antigen presenting cell that can present the gD peptide to a JgD specific T cell to initiate an antigen specific Th1 immune response. This confirms that the J-LEAPS immunogens and the J-LEAPS treated bone marrow cells are capable of delivering an antigen specific immune response.

It is not known if the viral peptide of the J-LEAPS vaccines (i.e. gD or H) directly binds to the MHC complex or if it is first processed and then presented in the MHC complex. It is known though that the antigen stays on the cell surface in the MHC complex for a sufficient amount of time to activate T cells since the JgD treated bone marrow cells were washed before being added to the splenic T cells and were still capable of enhancing an antigen specific immune response. This means that the JgD or the gD peptide remained bound to the cell surface and the MHC molecule to activate antigen specific T cells and initiate an antigen specific Th1 immune response.

The aforementioned studies demonstrate that JgD and JH act as adjuvants to activate and steer the DC response to promote an interferon gamma response and that JgD-DC act as antigen presenting cells that can reactivate memory T cells in an antigen specific manner. The adoptive transfer studies with JgD-DC, discussed later, demonstrated that the JgD promotes the maturation of
bone marrow cells into mature DCs that can initiate a new immune response with naïve T cells.
The J-LEAPS vaccines induce responses in human cells

*JgD or JH immunogens are capable of activating human monocytes into IL-12 producing dendritic cells*

All studies of J-LEAPS vaccines have been performed on mice, but ultimately a vaccine must work in humans. Human blood derived monocytes were used to study J-LEAPS vaccine responses in human cells. Similar experiments were performed on human monocytes as performed in mice on myeloid derived precursor dendritic cells. Human monocytes provide a defined and available source of human precursor dendritic cells for study.

The conventional method used to isolate human precursor dendritic cells *ex vivo* is to purify human monocytes from blood derived leukocytes collected through leukapheresis and separated by elutriation. These cells can be sustained by GMCSF and primed to become DCs by treatment with IL-4 (21, 48, 49). Unlike other DC activation protocols, the JgD immunogen was able to initiate DC differentiation and activation of human monocytes in tissue culture media only (monocytes), GMCSF treated monocytes (GM monocytes), and GMCSF + IL-4 primed monocytes (GM-4 monocytes) (43, 44) and to form dendrites and produce IL-12. *Ex vivo* activation of dendritic cells through TLR agonists not
only requires more steps but also more stimulating agents than the JgD immunogen. Growth factors and cytokines are required with TLR agonists to initiate cytokine production (43, 44). The JgD immunogen was sufficient to initiate IL-12 production in dendritic cells, when added directly to human monocytes with no other stimulating agents.

A functional dendritic cell is capable of bridging the innate and adaptive immune responses through T cell activation. T cell activation cannot be initiated without the proper cytokine environment. The JgD immunogen was sufficient to induce similar amounts of IL-12 in all three monocyte cultures (monocytes, GM-monocytes, and GM-4 monocytes). The cytokine spectrum generated by the JgD treated monocytes and GM-monocytes showed the same trends. This was unlike the JgD treated GM-4 monocytes which produced different chemokines. This suggests that the GM-4 monocytes are different than the monocytes and GM-monocytes and generate a different DC after JgD treatment. In Roses et al., it was determined that the GM-4 monocytes were different than the GM-monocytes and that IL-4 inhibits production of some cytokines, such as IL-23 (not assayed in this dissertation) (49). Most importantly, the JgD immunogen treated monocyte studies demonstrated that J-LEAPS immunogens are sufficient (by themselves) to induce IL-12 production in monocytes without the need for GMCSF or IL-4 to be added to the cell suspensions.

For mice, it was found that the J-LEAPS vaccines were capable of inducing IL-12 production without the production of proinflammatory cytokines.
JgD or JH treated human monocytes were also activated to produce IL-12 without the accompaniment of proinflammatory cytokine production. Although this cytokine profile is similar to that induced in mouse cells, it is different from the profiles generated by other protocols for DC1 activation. The type of cytokines that an activated dendritic cell produces depends upon the antigen that the DC is processing, the cytokine environment, and the type of ligands that bind to cell surface receptors on the DCs. The most defined route of dendritic cell activation to induce IL-12 production is through multiple TLR activation, which initiates high levels of proinflammatory cytokines to be produced along with IL-12 (28, 43, 44). The lack of proinflammatory cytokines suggests that the J-LEAPS vaccines activate different receptors and different combinations of pathways than those initiated by TLRs. This suggests that J-LEAPS vaccines promote the differentiation of precursors into novel forms of IL-12 producing human and mouse dendritic cells and in a novel way.

The JgD or JH treated GM-4-monocytes were then analyzed for morphological and phenotypic changes. These dendritic cells expressed the dendritic extensions (dendrites) (36). This demonstrated that within 48 hours of JgD or JH immunogen treatment, JgD or JH immunogens have activity on human cells and their activity on human cells parallels that on mouse cells.

A mature dendritic cell must be capable of activating a T cell response. Phenotypically, at least two cell surface DC maturation markers are expressed that are required for a dendritic cell to activate a CD4 T cell response, HLA-DR
(MHC II) and CD86. Processed antigen is presented in the HLA-DR complex to
the T cell receptor (TCR) as the first signal and CD86 binds to CD28 on the T cell
to initiate the second signal, required for proper T cell activation. Both JgD and
JH induced increased expression of HLA-DR and CD86 in human GM-4
monocytes. This suggests that JgD or JH are sufficient to generate a mature
antigen presenting cell that can activate a T cell response through antigen
presentation and second signal activation (27).

**JgD immunogens are sufficient and capable of initiating a Th1 immune response in human cells**

To demonstrate that the DCs generated by J-LEAPS vaccines are capable
of initiating an immune response in human cells, *ex vivo* allotypic T cell assays
were used as a correlate of DC activation of T cell immune responses.
Production of IFN-gamma in the reaction indicates a Th1 response (49).
Initiation of a Th1 cytokine response in the allotypic assay by the JgD treated
DCs demonstrated that sufficient amounts of MHC, co-activating receptors, and
IL-12 are expressed by the JgD treated DCs to activate T cells and steer their
response to an IFN-gamma producing Th1 response. Since antigen specificity
cannot be determined in this assay, only the adjuvant activity of the J-LEAPS
vaccines can be demonstrated but not their immunogen activity. In summary
these studies demonstrated that J-LEAPS immunogens can act on human cells
and dendritic cells and initiate a specific type of T cell response. This
demonstration strongly supports the possibility that J-LEAPS vaccines can act in
humans in a manner similar to that in mice.
**JgD is sufficient to activate mouse bone marrow cells into dendritic cells and is carried by the dendritic cells to elicit an antigen specific and protective immune response in mice.**

*Adoptive transfer of JgD treated DCs initiate an antigen specific and protective immune response*

Heretofore, we showed that JgD and JH can promote the maturation of precursor dendritic cells in mouse and human. These J-LEAPS activated dendritic cells can produce IL-12 and activate T cells to produce IFN-gamma, reminiscent of a DC1 response initiating a Th1 immune response. JgD can also initiate a booster like response *ex vivo*, characteristic of an antigen specific immune response. Ultimately, JgD must be able to initiate a protective immune response through DCs in an immunonaive mouse as demonstrated by protection of mice from lethal challenge of HSV-1 after adoptive transfer of JgD treated bone marrow cells (JgD-DCs). There was no protection provided by mice that were inoculated with untreated bone marrow cells (BMs) or other J-LEAPS treated bone marrow cells (J-ICBL treated bone marrow cells (J-BMs) or JH treated bone marrow cells (JH-DCs)). Protection with JgD-DC and not JH-DC demonstrated that the adjuvant effect induced by either was not sufficient for protection. Development of Th1 IFN-gamma producing cells may be a property of the JH-DC but an antigen specific T cell response is necessary for protection. It is known if the JgD-DCs initiated CD8\(^+\) T cells, CD4\(^+\) T cells, or both to confer
protective immunity. Previous antibody ablation studies determined that JgD vaccinated mice required CD4⁺ cells to deliver a protective immune response against a lethal HSV-1 challenge (14, 15). It can be inferred that CD4⁺ T cells play a role in the JgD-DC protective immune response. Like JgD immunization of mice, adoptive transfer of JgD-DC is unlikely to induce antibody production unless there is lesion formation. Ultimately, the ability of JgD-DCs, but not JH-DCs, to provide protection from lethal HSV-1 challenge demonstrates that JgD-DCs are sufficient to initiate, direct, and deliver a complete and protective immune response.

*The antigenic component of JgD-DC vaccines are bound to the dendritic cell*

JgD-DCs were washed before immunization of mice and were still capable of initiating a protective immune response. This means that the JgD or the gD peptide remained bound to the cell surface and the MHC molecule to activate antigen specific T cells to initiate a protective immune response. Considering that the JgD-DC vaccine is delivered bound to a cell, there is no free antigen released in the host during treatment. This decreases the chances of improper responses being activated by excess antigen and enhances the safety and efficiency of this type of vaccine delivery. In addition, only a minimal amount of the immunogen is required to activate and generate the protective dendritic cell.
This decreases the cost and increases the safety of potential J-LEAPS DC vaccines.

The JgD-DCs were administered in HBSS and did not require any other enhancer, adjuvant, or excipient to initiate protection. Adjuvants and excipients may induce harmful side effects. For example, immunization with the JgD vaccine peptide requires Seppic ISA51 adjuvant to initiate protection, and Seppic ISA51 is not a FDA approved adjuvant for human use. The JgD-DC vaccine method eliminates the need for such an adjuvant.

**J-LEAPS DC vaccines can be utilized for multiple immunotherapies**

The development of a J-LEAPS herpes simplex virus vaccine (i.e. JgD) provided testable models for other vaccines. The mouse and human ex vivo results for JH parallels those for the JgD peptide and suggests that a JH-DC vaccine could potentially be established for immunotherapy of HIV.

J-LEAPS immunogen treatment of precursor cells (i.e. bone marrow cells) is sufficient to produce antigen bearing DCs for adoptive transfer immunizations and for mice they generated a safe and direct immune response. This type of vaccine could potentially be used for immunotherapies or immunomodulators. This suggests that the J-LEAPS vaccines could be utilized to prevent or treat chronic infections, chronic inflammatory diseases, and cancer (Rosenthal KS, Zimmerman DH, Taylor PR. Compositions and methods for modulating
CHAPTER V

CONCLUSION

In conclusion, the J-LEAPS vaccines initiate IL-12 production which activates a Th1 cytokine response in mice. Precursor dendritic cells are the target cells of J-LEAPS vaccines in mice. Precursor dendritic cells are the target cells of J-LEAPS vaccines in human cells. JgD is sufficient to activate mouse bone marrow cells into dendritic cells and is carried by the dendritic cells to elicit an antigen specific and protective immune response in mice. The success of the JgD adoptive transfer experiment opens the door to other J-LEAPS activated DCs for preventing or treating infectious or chronic diseases.
BIBLIOGRAPHY


[73] Swanson MA, Lee WT, Sanders VM. IFN-gamma production by Th1 cells generated from naïve CD4 T cells exposed to NE. Journal of Immunology 166 (1) (2001) 232-40.


Appendix A I.

Fig. A1. Human monocytes obtained by leukapheresis of blood and purified by elutriation were cultured in serum free medium + GMCSF and IL-4 for 24 hours. The cells were then treated with 14.5 micromoles of JgD or JH and incubated for 2 days at 37°C. Cells shown were fixed, stained with FITC-anti-CD14 and PE-anti-CD83 and analyzed by flow cytometry.
CD14 expression is decreased and CD83 expression is increased upon TLR induced maturation into DC1 cells. CD14 acts as a co-receptor for the detection of bacterial lipopolysaccharide (LPS) and assists in the binding of LPS to TLR4 on the monocyte. CD83 is a DC maturation marker that normally is upregulated with CD86 during DC activation. Neither CD14 nor CD83 expression changed following JgD or JH treatment of human monocytes despite other changes in these cells to make them resemble DCs. This suggests that a novel IL-12 producing DC is developed by the J-LEAPS vaccines.
Appendix A II.

Fig. AII. Flow cytometry of DC maturation markers found on J-LEAPS treated bone marrow cells. The J-ICBL was added to the BM cell suspensions and incubated for 48 hrs. Cells were then stained with PE-anti-CD11c or PE-anti-CD86 and flow cytometry was performed on the entire population of the cell suspension.
When the J-ICBL is added to mouse bone marrow cells and the flow cytometry analysis is focused on the monocyte population there is not an increase in cell expression of CD11c or CD86. CD86 and CD11c are myeloid markers that can be found on antigen presenting cells (such as macrophages, dendritic cells, and B cells) that have matured to become functional antigen presenting cells. Upon restriction of analysis to monocytes, dendritic cells, and macrophages within the bone marrow cells, based on their light scatter parameters (gating), no increase in CD11c or CD86 expression was observed. In contrast when the entire bone marrow population was analyzed, J treatment induced a measurable increase in expression of CD11c and CD86. This suggests that the J-ICBL interacts with and activates a subset of bone marrow cells other than monocytes, dendritic cells, and macrophages. The J-ICBL could potentially be activating a precursor B cell or other cells in this bone marrow population to mature into a CD11c$^+$, CD86$^+$, mature antigen presenting cell.
Appendix A III.

Fig. AIII. Survey of cytokine production following J-collagen treatment. Human blood derived monocytes from 2 individuals were treated with J-collagen in two separate experiments. Spent media were collected three days post treatment, and evaluated by protein array (RayBio® Human Cytokine Antibody Array 3). Array results were quantitated by densitometry, and normalized to the summation values for each array to allow for comparative analysis of treated or untreated dendritic cell array results. The data shown are the mean scores for the fold increase to the untreated control for each cytokine detected of the 42 cytokines.
J-collagen (CEL-2000) is a potential immunomodulator of rheumatoid arthritis that contains the J-ICBL conjugated to an epitope bearing peptide from human collagen 2. CEL-2000 stopped the progression of disease in the mouse model of rheumatoid arthritis induced by collagen and adjuvant treatment. CEL-2000 treatment of human monocytes induced an increase in IL-12 production, as did JgD and JH, but it also caused an increase in IL-10 production. Increased IL-10 production was not observed following JgD or JH treatment. IL-10 is an immunosuppressive cytokine that could be beneficial in treating a chronic inflammatory disease such as rheumatoid arthritis. This suggests that either there are two different cell subsets being produced, one that initiates an IL-12 producing DC that would probably activate a Th1 immune response and another IL-10 producing DC that would probably initiate a Th2 immune response or much less likely, a unique dendritic cell is producing both IL-12 and IL-10.