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IMMUNE MODULATING FUNCTIONS BY SOYPEPTIDE LUNASIN IN CANCER IMMUNOTHERAPY

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Chun-Yu Tung

In Partial Fulfillment of the

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of

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West Lafayette, Indiana

For my family

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LIST OF ABBREVIATIONS

ACT	Adoptive cell therapy
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cells
CAR	Chimeric antigen receptors
CDC	Complement-dependent cytotoxicity
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CR	Complete responses rates
CRD	Carbohydrate recognition domain
CTL	Cytotoxic T lymphocyte
DBD	DNA binding domain
DC	Dendritic Cell
ERGIC	ER-golgi intermediate compartment
HBV	Hepatitis B virus
HCC	Human hepatocellular carcinoma
HCV	Hepatitis C virus
HPV	Human papilloma virus
HSP	Heat-shock protein
ICAM	Intracellular adhesion molecules
IFN	Interferon
IRF	Interferon regulatory factors
ISG	Interferon stimulated genes
ISRE	Interferon-Stimulated Response Element
IL	Interleukin
LCMV	Lymphocytic choriomeningitis virus

MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
MPL	Monophosphoryl Lipid
NHL	Non-Hodgkin's Lymphoma
NK	Natural killer cell
NSCLC	Non-small cell lung cancer
ORR	Overall response rates
OS	Overall Survival
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBSCT	Peripheral blood stem cell transplantation
PLC	Peptide loading complex
PRR	Pattern recognition receptors
RER	Rough endoplasmic reticulum
ROS	Reactive oxygen species
SNARE	Soluble NSF attachment protein receptor
TAA	Tumor associated antigens
TAP	Transporter associated with antigenic process
TCR	T cell receptor
TIL	Tumor-infiltrated lymphocytes
TLR	Toll-like receptor

ABSTRACT

Tung, Chun-yu. Ph.D., Purdue University, May 2016. Immune Modulating Functions by Soypeptide Lunasin in Cancer Immunotherapy. Major Professor: Hua-Chen Chang, Ph.D.

Chemotherapy is currently the mainstay of treatment for most cancer patients. Despite its efficacy in eliminating cancer cells, a high percentage of chemotherapy patients eventually relapse or suffer progression of the disease. Immunosurveillance is capable of recognizing and eliminating continuously arising transformed mutant cells, and thus cancer immunotherapy is one of the emerging therapeutic strategies that harnesses the power of the immune system to eradicate chemotherapy-resistant cancerous cells. However, the adverse side effects of chemotherapy impede the therapeutic effects of immunotherapy. Our previous studies demonstrate that lymphoma patients are refractory to clinical immunotherapy because of chemotherapy-induced immune dysfunction. In addition, tumors can induce immune suppression, which allows them to escape immunosurveillance. Thus, it is prudent to develop an efficacious immunotherapy that would enhance anti-tumor immunity in cancer patients who are most often immunodeficient.

Lunasin, a 43-amino acid peptide, was originally isolated from soybeans. The current study discovered a novel function of lunasin as a vaccine adjuvant, which enhanced the development of protective immune responses to soluble vaccine antigens. It was found that lunasin-treated conventional DCs (cDCs) not only expressed elevated levels of co-stimulatory molecules (CD86, CD40) but also exhibited up-regulation of cytokines (IL1B, IL6) and chemokines (CCL3, CCL4). Lunasin-treated cDCs induced higher proliferation of allogeneic CD4+ T cells when compared with a medium-only

control in the mixed leukocyte reaction (MLR). In addition, lunasin enhanced cross-presentation of soluble antigens by mouse CD11c+DCs and CD8α+DCs, resulting in effective priming of antigen-specific IFNγ producing CD8+ T cells. Immunization with etoposide-treated B-lymphoma cells and lunasin provided nearly 100% protection against tumor growth. Furthermore, inclusion of lunasin in the cancer vaccine model prevented tumor relapse after chemotherapy.

The immunomodulatory function of lunasin has also been identified in the STAT4 deficiency model. Our previous studies demonstrated that lymphoma patients were refractory to IL-12-based immunotherapy because of chemotherapy-induced immune dysfunctions associated with acquired deficiency of STAT4. To directly determine the requirement for STAT4 in response to lunasin-based cancer vaccination, a syngeneic B-lymphoma in a prophylactic model was utilized to compare the inhibition of tumor growth in wild-type BALB/c (WT) mice versus STAT4 deficient (*Stat4*$^{-/-}$) mice. B-lymphoma cells subcutaneously implanted into *Stat4*$^{-/-}$ mice have similar tumor growth and progression when compared to WT mice. Lunasin-based whole tumor vaccination induces the development of tumor-specific CD4+ and CD8+ T cells in WT and *Stat4*$^{-/-}$ mice. In conclusion, *Stat4*$^{-/-}$ mice do not exhibit accelerated subcutaneous tumor growth over WT mice following lunasin-based vaccination in a syngeneic B-lymphoma model.

Collectively, these studies provide the evidence for lunasin as an immunomodulatory agent that enhances the cross-presentation activity of DCs and promotes antigen-specific antitumor immune responses in cancer immunotherapy.

CHAPTER 1. LITERATURE REVIEW

1.1 Dendritic Cells

Dendritic cells (DCs) are powerful antigen presenting cells (APCs) and play important roles in bridging between the innate and the adaptive immunity. DCs were first discovered in the peripheral lymphoid organs of mice and were named for their stellate morphology (Steinman and Cohn, 1973). It was subsequently shown that DCs are 100-fold more potent than monocytes and macrophages in initiating immune responses (Steinman and Witmer, 1978). DCs detect the presence of pathogens via a set of pattern recognition receptors (PRRs) that are capable of binding to pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). Upon recognition of pathogens, DCs process antigens to produce peptides that are presented to T cell receptors (TCRs) in the context of major histocompatibility complex (MHC) molecules. Subsequently, DCs become activated, resulting in a pro-inflammatory milieu that is crucial for the development of adaptive T cell responses (Reis e Sousa, 2004).

1.1.1 DC subsets and functions

Dendritic cells, which can be broadly categorized by phenotypic markers and anatomical locations, are a heterogeneous population. (Schlitzer and Ginhoux, 2014). In this current literature review, the scope will be concerned with the subsets grouped as conventional DCs (cDCs).

Conventional DCs, which can be divided into at least two subsets characterized by expression of CD8α/CD103 or CD11b in the murine system, can be found in lymphoid tissues as well as non-lymphoid tissues. (Pulendran et al., 1997). CD8α+ DCs found in the lymphoid organs, including lymph nodes and spleens, are considered resident DCs (Crowley et al., 1989, Schlitzer and Ginhoux, 2014). In the non-lymphoid

organs, CD103+DCs do not express CD8α, and are considered as the equivalent population to CD8α+DCs in the lymphoid organs (Bursch et al., 2007). CD103+DCs are migratory DCs, reside in the peripheral tissue under steady state conditions, and travel to lymphoid organs upon activation (Randolph et al., 2005). The development of CD8α+ lymphoid DCs and CD103+ non-lymphoid DCs requires the same transcription factors including basic leucine zipper ATF-like 3 transcription factor (BATF3), interferon regulatory factor 8 (IRF8) and inhibitor of DNA binding 2 (ID2) (Tussiwand et al., 2012). It is known that CD8α+/CD103+ DCs are specialized in cross-presentation activity and express the chemokine receptor XCR1 (Bachem et al., 2010, den Haan et al., 2000). CD8α+/CD103+ DCs express several members of Toll-like Receptors (TLRs), including TLR3, 4, 7, 9, 11 and 13. DNGR1 (CLEC9A), a C-type lectin receptor that binds to dying cells and facilitates cross-presentation of cell-associated antigens, is also expressed by this subset of DCs (Zelenay et al., 2012). CD8α+/CD103+ DCs are the essential subsets that stimulate CD8+T cell immunity through secretion of IL-12 which promotes Th1 differentiation in intracellular pathogen infections. It was found that CD8α+/CD103+ DCs are the main producers of IL-12 (Mashayekhi et al., 2011).

CD11b+DCs, on the other hand, are associated with induction of Th2 and Th17 immunity (Plantinga et al., 2013). CD11b+DCs are heterogeneous and most of the functions still need to be characterized. The development of CD11b+DCs requires V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog B (RelB), neurogenic locus notch homolog protein 2 (Notch2) and IRF4 (Mildner and Jung, 2014). Functionally, CD11b+DCs have higher expression of MHCII compared to CD8α+/CD103+ DCs subsets (Dudziak et al., 2007), and are characterized by their production of cytokines including IL-6 and IL-23 (Persson et al., 2013). CD11b+ DCs are equipped with TLRs 5, 6, 7, 9 and 13 as well as cytosolic sensors, retinoic acid-inducible gene 1 (RIG1) (Schlitzer and Ginhoux, 2014).

Plasmacytoid dendritic cells (pDCs) are different from cDCs and are known to have an exceptional ability in producing type I interferons upon recognition of antigens (Colonna et al., 2004). It requires transcription factor basic helix-loop-helix transcription factor (E protein) (E2-2) for pDC development in both human and mouse systems (Cisse

et al., 2008). Plasmacytoid DCs are characterized by high expression of TLR9 and TLR7, which recognize CpG products and viral products and generate anti-viral activities (Nakano et al., 2001). In addition to the response to viral antigens, it was found that in human pDCs have cross-presentation activity to apoptotic cells, which is type I interferon dependent (Hoeffel et al., 2007). Although pDCs are relatively poor at taking up exogenous antigens compared to cDCs (Villadangos and Young, 2008), they play a vital role in initiating innate and adaptive antiviral responses. (McKenna et al., 2005)

In mice and human immune systems, cDCs can be organized in parallel into two major subsets: mouse CD8α+/CD103+ DCs, which are related to human CD141+ (BDCA3) DCs, with the characteristics in potent cross-presentation and Th1-polarizeation properties; and murine CD11b+DCs which are related to human CD1c+ (BDCA1) DCs, exhibiting Th2 and Th17- polarization capabilities.

Human CD141+ (BDCA3+) DC are found in the lymphoid organs as well as the non-lymphoid tissues including lung, liver and skin. CD141+DCs also express XCR1 and DNGR1 (CLEC9A) on the surface to facilitate cross-presentation activity as in mouse CD8α+/CD103+ DCs. Unlike murine CD8α+/CD103+ DCs, CD141+DCs do not express TLR9 (Poulin et al., 2010, Haniffa et al., 2012). CD141+DCs are able to take up exogenous materials including apoptotic or necrotic cells and present to CD8+ T cells upon stimulation with the TLR3 agonist poly I:C (Poulin et al., 2010).

Human CD1c+ (BDCA1+) DCs are the major subset in human peripheral blood, lymphoid organs and non-lymphoid tissues. CD1c+DCs express TLR1-10 as well as other PRRs such as Dectin 1 and 2 (Harman et al., 2013). It was thought that the functions of CD1c+ DCs are unique since they are equipped with TLR4 and have a higher response to bacterial antigens such as *Staphylococcus* infection compared to CD141+DCs (Jin et al., 2014). On the contrary to CD141+DCs, CD1c+DCs could have an immune-modulatory function by secreting IL-10 and suppressing T cells in *E.coli* infected DC models (Kassianos et al., 2012).

1.1.2 DC maturation

In the steady state, DCs are mostly considered immature, which are highly endocytic with low T cell activation potential since their main goal is to maintain immune tolerance by hindering adaptive immune cells to attack self-tissues (Dudek et al., 2013, Lutz and Schuler, 2002). Immature DCs provide immune check point signals such as cytotoxic T-lymphocyte associated antigen 4 (CTLA4) and programed cell death protein 1 (PD1) to T cells, leading to T cell anergy or differentiation to regulator T cells (Pardoll, 2012)

Upon PRR activation, DCs undergo dramatic sets of functional and morphological changes and this process is called "maturation", which happens rapidly after contact of pathogen stimulation (Trombetta and Mellman, 2005). The maturation process allows DCs to transition from antigen acquisition to antigen presentation to initiate adaptive immune responses (Reis e Sousa, 2006). During the maturation process, DCs undergo several cellular changes including down-regulation of macropinocytosis, upregulation of major histocompatibility complex (MHC) class II (Boes et al., 2002) and upregulation of co-stimulatory molecules such as CD80 (B7.1), CD86 (B7.2) and CD40 (Lenschow et al., 1993). DEC205 (CD205), mainly expressed on DCs, is an endocytosis-mediating receptor and is upregulated during the maturation process, which can enhance antigen presentation to the MHC class II complex (Birkholz et al., 2010, Mahnke et al., 2000). To optimize antigen processing, increased lysosomal acidification leading to enhanced proteolysis has been found in DCs during maturation (Trombetta et al., 2003). Because the maturation process is to facilitate DCs to present antigens to T cells, increased migration of DCs can also be found through upregulation of CCR7, a key chemokine receptor responsible for DC homing to lymph nodes (Bonasio and von Andrian, 2006). In addition, DCs secrete a wide variety of cytokines and chemokine such as IL-12, IL-1, IL-15, IL-18, type I interferons, IFNγ, IL-4 and TNFα. The secretion of these cytokines depends on the nature of stimuli, maturation phase of DCs and other existing cellular microenvironment. Generally, the cytokines secreted from different stages of DCs will ultimately determine the polarization of Th1 or Th2 cells (Moser and Murphy, 2000).

1.1.3 Antigen process and presentation

DCs are specialized in antigen processing and presenting endogenous and exogenous antigens to MHC class I and MHC class II, respectively. In the following sections, the mechanisms of conventional antigen processing and antigen presentation will be discussed. Non-conventional antigen presentation mechanisms will also be discussed.

1.1.3.1 Antigen capture and processing

Dendritic cells acquire self-antigens and foreign antigens through different forms of endocytosis. Endocytosis can be achieved by the following mechanisms: phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-dependent endocytosis and nonclathrin/non-caveolae endocytosis. Cells utilize different mechanisms of endocytosis determined by the size of the molecules and the structure on the plasma membrane. (Marsh and McMahon, 1999).

Phagocytosis is the process of ingestion of large particles using actin cytoskeleton to push a protrusion of the plasma membrane to surround these particles. The size of the particle varies from 0.1 to 10 μm. Phagocytosis often occurs after recognition of an appropriate ligand on the surface of the target. For example, DCs recognize phosphatidyl serine on apoptotic cells by the T cell immunoglobulin mucin (TIM) 1 or TIM4 (Kobayashi et al., 2007). It was found that the phagocytosis activity of DCs was reduced after DC maturation. Freshly isolated immature DCs were highly phagocytic, but lost their ability when cultured under inflammatory stimuli (Reis e Sousa and Germain, 1995, Wilson et al., 2006).

Macropinocytosis is the process in which cells ingest extracellular fluid in a large endocytic structure (Falcone et al., 2006). DCs are constitutively undergoing macropinocytosis (Sallusto et al., 1995). Clathrin-mediated endocytosis or receptor-mediated endocytosis occurs at the coated pits, which are specialized patches coated with clathrin and adaptor molecules. Clathrin-coated vesicles can engulf up to 100-150 nm diameter of molecules (Rappoport, 2008). Caveolae are small (~50 nm) membrane

structures which are rich in cholesterol and glycolipids. Caveolae are stabilized by the major protein caveolin, which inserts as a loop into the inner leaflet of plasma membranes. There are increasing numbers of receptors that have been identified and considered relevant for uptake of foreign antigens by phagocytes such as DCs (Kerrigan and Brown, 2009). C-type lectins, is the largest family of endocytic receptors that have been found in DCs. Members in this family have a conserved structure of carbohydrate recognition domain (CRD) with a calcium binding pocket (Figdor et al., 2002). The dendritic and epithelial cell receptor with a molecular weight of 205 kDa (DEC205), a well-characterized C-type lectin, is expressed by DCs and is known for its role in facilitating antigen presentation (Jiang et al., 1995). Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-intergrin (DC-SIGN), is another C-type lectin that binds to some endogenous ligands and intracellular adhesion molecule ICAMs. Binding of a soluble ligand to DC-SIGN can induce rapid internalization of antigens. It was found that HIV-1 utilized this mechanism to be internalized into DCs and transmitted to T cells (Engering et al., 2002)

Extracellular molecules are engulfed by cells through various endocytic mechanisms and then transported into cytoplasm, where they fuse with each other with other membrane compartments. All of these compartments are termed the endosomal membrane system, comprising the early/recycling endosome, multi- vesicular bodies, late endosomes and lysosomes, which are more acidic and more active in proteolysis (Huotari and Helenius, 2011). Different compartments can be distinguished by markers such as Rab proteins, morphologies and the time it takes for endocytosed substances to reach them. For example, EEA1 (Rab5a), Rab 6 or Rab11 are for early, late or recycling endosome, respectively (Stenmark, 2009). The final localization of antigens in the endosomal compartments may determine the fate of antigen presentation to MHC class I or MHC class II (Burgdorf and Kurts, 2008). Antigens targeted to late endosomes are more efficient in presenting to MHCII, while antigens targeted to early endosome are most efficient to MHCI (Burgdorf and Kurts, 2008).

To initiate the adaptive immune response, T cells recognize peptide fragments after they have been processed and presented on the surface of MHCs. CD8+ T cells

recognize peptides presented by the MHC class I, while CD4+ helper T cells recognize peptides presented by the MHC class II. MHC class I expressed on all nucleated cells and it contains two noncovalently bound transmembrane α chain and β2-microglubulin chain. The peptide binding groove of MHC class I mostly binds 8-10 amino acid in length. MHC class II predominantly expressed by antigen presenting cells including DCs, macrophages and B cells. The structure of MHC class II is composed of two transmembrane noncovalently bound α and β chains, and the α1 and β1 domains form the peptide binding groove which binds to 13-18 amino acid in length (Owen et al., 2009)

Antigens have to be processed and degraded through a proteolysis pathway in order to be loaded onto the peptide-binding groove of MHC molecules that are recognized by T cells (Blum et al., 2013). There are two proteolytic systems: the cytosolic pathway that is mediated by proteasomes and the endosomal pathway that is mediated by lysosomal proteolysis.

For the cytosolic pathway, internal proteins such as endogenous viral proteins and organelle proteins are degraded within the cytoplasm by proteasome. In the APCs, the β1, β2 and β5 subunits of proteasome are replaced to specialized IFNγ-inducible subunits called β1i, β2i and β5i. The proteasome that is equipped with the special subunits is called the immunoproteosome, which has a higher proteolysis activity compared to the constitutive proteasome (Ferrington and Gregerson, 2012). For the endosomal pathway, exogenous proteins are degraded by lysosomal proteolysis. Exogenous antigens are acquired into cells through various endocytosis routes, and are passing through a series of intracellular compartments of increasing acidity (Owen et al., 2009).

1.1.3.2 Classical antigen presentation pathway

The peptides that result from the proteasome degradation of cytosolic antigens are transported across the membrane on the rough endoplasmic reticulum (RER) by transporter associated with antigenic process (TAP). TAP is a heterodimeric protein that is composed of two subunits, TAP1 and TAP2. The transport process of peptides through TAP requires an ATP-dependent process (Suh et al., 1994). Peptides transported into the

ER lumen are ready for loading to MHC class I. To form a peptide loading complex (PLC), initially the MHC class I α chain is stabilized by calnexin, and calnexin is lost when α chain binds to β2-microglobulin. The calreticulin and tapasin then bind to the MHC class I, and tapasin brings the whole MHC into the vicinity of the TAP which facilitates the peptide loading. After peptide loading, MHC class I molecules dissociate from calreticulin and tapasin, and are transported to the cell surface (Owen et al., 2009). Patients who have mutations on TAP proteins do not have MHC class I exposed on the cell surface because the peptides could not be transported into ERs. Lack of peptide binding groove contributes to unstable structures of MHC class I (Gadola et al., 2000).

On the other hand, externally-derived antigens are being processed in the endocytic compartments and are loaded on to the MHC class II molecule on the ER membrane. Initially, MHC class II α chain and β chain bind to the invariant chain (I chain) to form a chaperon, blocking the peptide binding groove. Once MHC class II enters the endosomal compartment, I chain is degraded, leaving a small fragment called class II-associated invariant chain peptide (CLIP) on the peptide binding groove (Wu and Gorski, 1996). CLIP is removed by a MHC-encoded heterodimeric glycoprotein, DM, which loads a peptide on the MHC class II. The peptide-loaded MHC II complex is then transported to the cell surface (Owen et al., 2009).

1.1.3.3 Cross-presentation

The MHC class I antigen presentation pathway allows the immune system to remove cancer cells and virus-infected cells through the presentation of antigen by MHCI on the cell surface. Although all nucleated cells express MHC class I, naïve CD8+ T cells cannot directly recognize the antigens on the transformed cells or virus-infected cells to elicit cytotoxicity. Naïve CD8+ T cells are first needed to be activated by APCs to become effector cytotoxic T cells (Joffre et al., 2012). When APCs are not infected, they need to acquire exogenous antigens from infected cells or tumor cells and present the antigen on the surface of MHC class I. This pathway is called “cross-presentation.”

The detailed mechanism of cross-presentation in APCs is still under investigation. There are two main intracellular pathways that have been reported, the cytosolic pathway and vacuolar pathway (Figure 1.1).

In cytosolic pathway, after phagocytosis, antigens are exported to cytosol, processed by proteasome, and the peptides are loaded onto the MHC class I on the ER membrane (Fehres et al., 2014). The peptides after proteolysis by the proteasome can be transported into phagosome and loaded onto the MHC class I in the phagosome. The connection between ER and phagosome is one possible mechanism of promoting cross-presentation. DC phagosomes have many ER-resident proteins, particularly for those related to the MHC class I loading (Guermonprez et al., 2003). One of the Soluble NSF attachment protein receptor (SNARE) proteins, SEC22B, localized in the ER-golgi intermediate compartment (ERGIC), is required for the recruitment of proteins from the ER compartment to phagosome. It was shown that silencing of SEC22B decreased the cross-presentation activity by the *Toxoplasma gondii* infection model. The defects of cross-presentation related to SEC22B were from the impaired transportation of antigens to phagosomes (Cebrian et al., 2011).

In the vacuolar pathway, phagocytosed antigens are degraded into peptides in the phagosome, and peptides can be loaded on to the recycling MHC class I in the phagosome (Fehres et al., 2014). The proteolytic activity in the DCs is weaker compared to other professional phagocytes including macrophages and neutrophils. It was found that the limited antigen degradation in the DCs is associated with effective cross-presentation due to lower levels of lysosomal proteases and the decreased activity of proteases, caused by relatively high pH (Delamarre et al., 2005). Type I interferons play a role in decreasing antigen degradation in the endosomal compartment, which enhances cross-presentation activity by mouse CD8α+ DCs (Lorenzi et al., 2011).

Among resident DCs, CD8α+ DCs are thought to be the most potent DC subsets in cross-presenting exogenous antigens in mice. However, other DC subsets such as CD8α-CD11b+ DCs can efficiently cross-present antigens to the MHC class I by engaging c-lectin receptor signaling or cytokine stimulations (den Haan and Bevan, 2002). Mouse pDCs are generally considered poor APCs, but it was found that *ex vivo*

stimulation of pDCs by TLR 9 agonist, CpG, could enhance cross-presentation activity *in vitro* (Mouries et al., 2008). Human CD141+/BDCA3+ DCs are thought to be the most potent DCs in inducing cross-presentation, in corresponding to mouse CD8α+DCs. However, it was shown that human CD1c+/BDCA1+ DCs present exogenous antigens as efficient as BDCA3+ DCs when antigens are targeted to the early endosome, using anti-CD40 monoclonal antibody. On the other hand, if targeting antigen to the early endosome using the DEC205 or the mannose receptor endocytosis pathway, it decreases the efficiency of cross-presentation by human CD1c+ DCs (Chatterjee et al., 2012). Altogether, it was suggested that the intracellular location of antigens are important for cross-presentation.

Given the important role of cross-presentation to elicit an effective antitumor immunity, studies on improving cross-presentation in cancer vaccinations have emerged as a promising approach for immunotherapeutic intervention (Fehres et al., 2014). To induce long-lasting T cell immune response that leads to effective tumor elimination, long synthetic peptides are more effective than short peptides (Faure et al., 2009). Stimulation with cytokines or agonists enhances DC maturation, resulting in a more effective cross-presentation in vivo (Joffre et al., 2012). Targeting C-type lectin receptors, such as CD207, DC-SIGN, CLEC9A, or other surface receptors including integrins, heat-shock protein (HSP) receptors and glycolipids, have contributed to an effective induction of cytotoxic T cell responses by enhancing the cross-presentation pathway in the mouse models (Singh-Jasuja et al., 2000, Caminschi et al., 2008). However, the translation from mouse to human is still limited, due to the difference of surface marker expression and the functional divergence of DC subsets. Integration of different strategies of immunotherapy to improve DC-induced CD8+ T responses is essential to maximize clinical benefits for cancer patients.

1.2 Cancer Therapy

1.2.1 Types of current cancer therapy

Cancer is the second most common cause of death in the United States. According to the annual report from the American Cancer Society, cancer accounts for almost 1 of

every 4 deaths. The high morbidity and mortality of this disease represents a significant economic and medical burden. The types of cancer treatments include surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy and stem cell transplant. Clinically, patients received different types of cancer therapies based on their own history, types of cancer and pathology results. Take Non-Hodgkin's Lymphoma (NHL) as an example, CHOP-based chemotherapy (Cyclophosphamide, Hydroxydaunorubicin, Vincristine and Prednisolone) is the standard treatment. Patients with aggressive NHL who relapse after conventional chemotherapy are eligible to receive high-dose chemotherapy (e.g. Etoposide, Carmustine and Cyclophosphamide) and autologous stem cell transplantation (Robertson et al., 2005). However, more than 50% of patients will develop recurrent diseases following autologous stem cell transplantation. Other treatments, such as targeted therapy and immunotherapy, have been alternatively used as to improve the clinical outcomes (Klingemann and Phillips, 1991).

1.2.1.1 Immunodeficient tumor microenvironment in cancer patients

In principle, cancer cells express antigens that can be recognized by CD8+ cytotoxic T cell. However, cancer cells often develop strategies to escape from the host immune system (Swann and Smyth, 2007). Cancer cells evade the immune system through several mechanisms including immune tolerance, defective antigen priming of tumor antigen and immunoregulatory effects (Gajewski et al., 2013).

Early therapeutic approaches of cancer immunotherapy were focusing on identification of tumor antigens to develop tumor-antigen-based therapeutic vaccines. However, most tumor cells express shared antigens that are also expressed by self-tissues, leading to immunologic tolerance by cytotoxic high-affinity CD8+ T cells (Bos et al., 2012). Tumor cells generate neoantigens through point mutations, and these antigens could be good candidates for target antigens, but with the limitation of individual differences. Exome sequencing is one of the powerful methods to identify mutant antigen individually for a personalized cancer therapy (Robbins et al., 2013).

In addition to immunologic tolerance of tumor antigen in cancer patients, the tumor microenvironment is mostly immunosuppressive. Generally, CD8+ T cell infiltration can be a good prognosis for solid tumors, including colorectal cancer, melanoma, breast cancer and renal cell carcinomas (Gajewski et al., 2013). Although a huge amount of T cells infiltrates into a tumor site, most of the time, the T cells have inhibitory phenotypes instead of immunostimulatory phenotypes. It was found in the melanoma model that IFNγ produced by CD8+ T cells increased the expression of Programmed-death ligand 1 (PDL1) and indoleamine 2,3-dioxygenas (IDO), contributing to the infiltration of CD4+FoxP3+ regulatory T cells into the tumor site (Spranger et al., 2013).

Dendritic cells in the tumor microenvironments are mostly immunosuppressive rather than immunostimulatory. It was found in human and mouse models that tumor-associated DCs, especially plasmacytoid dendritic cells, are tolerogenic. Plasmacytoid DCs in the tumor microenvironment express IDO and have an impaired ability of producing type I interferon, leading to progression of cancers (Chen et al., 2008, Demoulin et al., 2013). Other myeloid cells, such as M2-macrophages and myeloid-derived suppressor cells (MDSCs), can also contribute to immunosuppression in the tumor microenvironment. Tumor associated macrophages secrete CCL22 that recruits regulator T cells in human ovarian carcinoma (Curiel et al., 2004). M2-macrophages are also known to produce inhibitory cytokines, including IL-10 and TGFβ, which contribute to the immunosuppressive environment (Quatromoni and Eruslanov, 2012). MDSCs are one of the suppressor cells and are known for the expression of arginase that deprives L-arginine from the tumor microenvironment to inhibit T cell functions (Rodriguez et al., 2009). MDSCs can also induce antigen-specific CD8+ T cell tolerance by increasing nitration of tyrosine in the TCR complex (Nagaraj et al., 2007).

The stromal components can also contribute to the immunosuppressive environment. Fibroblasts, vascular endothelial cells and extracellular matrix could support tumor growth, which support immune cell infiltration. On the other hand, fibroblasts secrete fibroblast activating protein-α (FAP), which is associated with immunosuppression. Ablation of FAP-expressing cells in several cancer models

including Lewis lung carcinoma and pancreatic ductal adenocarcinoma could induce hypoxic necrosis of cancer cells through activation of IFNγ and TNFα. This suggests the role of FAP-expressing stromal cells for tumor support and immunosuppression (Kraman et al., 2010).

Due to the immunosuppressive phenotype of the tumor microenvironment, it is essential to understand the regulatory features used in clinical treatment implications. Blockade of the immunosuppressive functions such as the PDL1-PD1 axis and the usage of IDO inhibitors show promise in the cancer therapy (Muller et al., 2005, Pardoll, 2012). For example, in one Phase I study, NSCLC patients received anti-PD-1 antibody, Nivolumab, intravenously every three weeks with standard platinum doublet chemotherapy regiment. It was found that the 1 year Overall Survival (OS) rate were high at 59-87% (Rajan and Gulley, 2014). Furthermore, studies have found that PD1-PDL1 pathway blockade combined with treatments that activates the immune system such as type I interferons activation could prolong survivals in the mouse melanoma model (Bald et al., 2014).

1.2.1.1.1 Treatment induced immunodeficiency

Chemotherapy is the mainstay of treatment for cancers. The cytotoxic effects of chemotherapy are extensive as chemotherapy also kills other hematopoietic cells and is used as an immunosuppressive agent (Menard et al., 2008). Chemotherpy-induced immunodeficiency is related to T cell depletion, especially on the subset of CD4+ T cells (Mackall, 1999). It was found that alkylating agents such as cyclophosphamide, purine nucleoside analog such as fludarabine monophosphate, and corticosteroids increase the risk of therapy-induced immunosuppression (Mackall, 1999). Take cyclophosphamide as an example, it was found that within one day of the cyclophosphamide treatment, the numbers of CD3+ T cells were substantially reduced in the peripheral blood. The recovery of T cells back to the baseline level is generally within three months (Mackall et al., 1997).

1.2.1.1.2 STAT4 deficiency

Signal Transducer and Activator of Transcription 4 (STAT4) is a critical transcription factor that is responsible for Th1 development and induction of IFNγ in response to IL-12, IL-23, type I interferons and other cytokines (Cho et al., 1996, Jacobson et al., 1995, Watford et al., 2004). STAT4 shares a conserved protein structure with other STAT transcription factor families. It contains 6 distinct domains: N-terminus coiled-coil (CC), DNA binding domain (DBD), linker (LK), SH2, tyrosin activation (Y) and transcriptional +activation domain (TAD) (Lim and Cao, 2006). Upon ligand binding to a transmembrane receptor, the receptor undergoes conformation changes and induces recruitment of a Janus family of receptor associated kinases (JAKs). JAK kinases phosphorylate the receptor, making it a docking site for STAT proteins. The respective STAT binds to the phosphorylated receptor, which in turn gets phosphorylated and activated. Once activated, STAT forms a homodimer or heterodimer with another activated STAT molecule. Dimerized STAT proteins translocate to the nucleus where they bind to their corresponding DNA elements of the target gene and activate gene transcription (Darnell et al., 1994).

IFNγ is known to have pleotropic effects in promoting antimicrobial and antitumor immunities. The production of IFNγ is critical for promoting antitumor immunity through induction of apoptosis in cancer cells, enhancement of MHCI and II expression and induction of cytotoxic T lymphocyte and NK cell cytotoxicity (Dellacasagrande et al., 2002, Steimle et al., 1994).

We have previously found that NHL patients who received autologous peripheral blood stem cell transplantation (PBSCT) are refractory to IL-12 based immunotherapy because of impaired production of IFNγ. CD4+ T cells isolated from these patients fails to differentiate into the Th1 lineage due to deficiency of STAT4 (Chang et al., 2009, Robertson et al., 2005). The underlying mechanism of STAT4 deficiency in the NHL patients is a consequence of cancer chemotherapy. The levels of STAT4 expression were similar between the healthy controls and NHL patients before treatment; however, they were significantly decreased after chemotherapy (Lupov et al., 2011). The decreased STAT4 protein levels after chemotherapy were not caused by inefficient translation but

reduced half-life of STAT4. This is in part due to enhancement of ubiquitination and proteasome degradation following chemotherapy (Lupov et al., 2011). Given the facts that chemotherapy could induce STAT4 deficiency and impaired IFNγ production, it requires optimal strategies that can ameliorate or circumvent this defect to promote optimal antitumor immune responses.

1.2.2 Cancer Immunotherapy

Immunotherapy is one of the emerging therapeutic strategies to harness the power of the immune system to eradicate cancer cells. Both innate and adaptive immune systems act as sentinels in confining malignant cells to a state that can be controlled. For example, natural killer (NK) cells from the innate system can be activated by type I interferons and cytokines (e.g. IL-2 and IL-12) to enhance their cytotoxicity against tumor cells. In addition, activated NK cells produce the inflammatory cytokines such as IFNγ, which is an important cytokine to activate CD8+ cytotoxic T lymphocyte (CTL) cytotoxicity against tumor cells, and to enhance the immunogenicity of tumor cells by increasing the expression of MHC class I and class II allowing them to be recognized by CD8+ and CD4+ T lymphocytes, respectively (Dovedi et al., 2013). Genetically engineered T cells that express the chimeric antigen receptors (CAR) are one of the breakthroughs in cancer immunotherapy. Treatment with the CAR-T cells elicits superior antigen-specific antitumor responses in patients with advanced cancer (Kochenderfer and Rosenberg, 2013).

Cancer patients often have compromised immune responses to immunotherapy. Reduced tumor immunogenicity and increased immunosuppressive factors all contribute to the unsatisfactory outcomes of immunotherapy. For example, tumor cells-derived immunosuppressive cytokines such as IL-10 can recruit regulator T cells, leading to impaired DCs to engage in effective cytotoxic T cell activities (Hanahan and Weinberg, 2011). Also, most myeloid cells in the tumor microenvironment are immature myeloid suppressor cells, which can secrete inhibitory cytokines such as IL-10 and TGF-β, leading to T cell anergy (Huang et al., 2006).

Therefore, to achieve effective antitumor immune responses, there are three major areas that can be improved: (1) enhancing antigen presentation functions by dendritic cells; (2) promoting the protective T cell responses ; and (3) overcoming the immunosuppressive tumor microenvironment (Mellman et al., 2011).

1.2.2.1 Cytokine therapy

Administration of single or a combination of different cytokines to enhance the anti-tumor immune response is one of the approaches for cancer immunotherapy. Interferon alpha (IFNα) is able to recruit and activate NK cells and DCs, eliciting effective antitumor responses. IFNα also has the ability to suppress growth of cancer cells and to induce apoptosis of cancer cells (Krasagakis et al., 2008). IFNα therapy is currently used clinically for the treatment of metastatic melanoma (Minutilli and Feliciani, 2012). Interleukin-2 (IL2) is another FDA approved cytokine for treatment of solid tumors such as melanoma and renal cell carcinoma (Royal et al., 1996). However, IL-2 has a low response rate of about 15% and a high risk of inducing systemic inflammation (Mellman et al., 2011).

Although cytokines play important roles in the regulation of immune responses, cytokine therapy rarely achieve complete cure for cancer patients because of their indirect anti-tumor activity. Nowadays, cytokine therapy is used in combination with other types of cancer treatment.

1.2.2.2 Antibody therapy

Antibody therapy is the use of monoclonal antibodies (mAb) that bind to cancer cells and lead to cancer cell death. Antibodies can be conjugated to radioactive materials or anticancer drugs, resulting in effective elimination of cancer cells (Panowksi et al., 2014).

The expression of CD20 antigen is specific to B cells and is a target for the treatment of hematologic malignancies such as NHL. Binding of CD20 by the anti-CD20 mAb (Rituximab or Rituxan) induces the antibody-dependent cell-mediated cytotoxicity

(ADCC) and complement-dependent cytotoxicity (CDC), which destroy the CD20-expressing B cells (Scott, 1998).

Cytotoxic T lymphocyte associated antigen 4 (CTLA4) is a negative regulator expressed on T cells. It binds to the members of B7 co-stimulatory molecules (CD80 and CD86) on antigen-presenting cells (Lenschow et al., 1993). Interaction of CTLA4 and CD80/86 leads to suppressive T cell responses as well as the induction of regulatory T cells (Chambers et al., 2001). The failure of patients to generate effective antitumor immune responses has been attributed to the overexpression of CTLA4. Inhibiting the suppressive signals mediated by CTLA4 using the blockade antibodies will thereby enhance the effective functions of T cells, leading to a more potent anti-tumor activity. In addition, the anti-CTLA4 antibody can inhibit CTLA4 expression on regulatory T cells. This will limit the ability of Treg in suppressing effector T cells. Blockade of CTLA4 signals using monoclonal antibodies has been proven to be effective in different cancers such as malignant melanoma, lung small cell carcinoma and prostate cancer. In 2011, the monoclonal antibody against CTLA4, iplimumab, was approved by the FDA as the treatment for melanoma (Mellman et al., 2011).

Programmed cell death-1 (PD-1) is another negative regulator for T cell activation. PD-1 is a member of CD28 family, and binds to its ligand PDL1 and PDL2. This binding induces a tolerance by preventing the activation of T cell responses (Parry et al., 2005). PDL1 and PDL2 are highly expressed on some tumor cells. The binding of PD-1 to PDL1/PDL2 inactivates T cell effector responses, which induces immunosuppression in the tumor microenvironment. Blockade of PD1-PDL1 interaction using a monoclonal antibody, nivolumab, has been shown to be safe and effective in non-small cell lung cancer (NSCLC) and melanoma (Topalian et al., 2012).

Antibodies conjugated with radioactive materials have been used in imaging and treatment historically. The radio-antibodies are wildly applied to the radiosensitive tumors such as leukemias and lymphomas (Larson et al., 2015). The fully humanized antibodies that specially target hematological tumors are also available. There are several clinical trials focusing on using radiolabeled CD20 antibodies such as ^{131}I or ^{90}Y-labeled antibodies. It was shown that the overall response rates (ORR) and complete responses

rates (CRs) of radiolabeled anti-CD20 antibodies are high, ranging from 60-80% ORR and 15-40% CRs in relapsed NHL patients compared to unlabeled rituximab (Larson et al., 2015, Witzig et al., 2002).

1.2.2.3 Adoptive cell therapy (ACT)

Adoptive cell therapy (ACT) is a personalized immunotherapy for cancer. Adoptive cell therapy utilizes tumor-specific lymphocytes from patients to generate anti-tumor responses. There are two main strategies for ACT: (1) tumor-infiltrated lymphocytes (TILs) which are natural host immune cells that can target specific cancer cells; and (2) host T cells that have been genetically engineered to express tumor-specific T cell receptors (TCRs) or chimeric antigen receptors (CARs) (Rosenberg and Restifo, 2015).

Tumor-infiltrated lymphocytes are obtained from tumor tissues, and the tissues are digested into single-cell suspensions, growing in IL-2 until tumor tissues are destroyed by the overgrowing lymphocytes. It takes 2-3 weeks to generate a pure culture of lymphocytes and these cells need to be tested for killing activity. These lymphocytes are expended in the presence of feeder T lymphocytes and IL-2, and up to 10^{11} of lymphocytes can be obtained for infusion (Rosenberg et al., 1988). TILs have been applied to clinical trials especially on metastatic melanoma. The number of objective response (OR) rates can range from 30% to 50% which is considered as an effective immunotherapy (Rosenberg and Restifo, 2015). If patients have undergone lymphodepletion before infusion of TILs, the duration of expanded lymphocytes are longer, correlating to a better clinical outcome (Dudley et al., 2002).

To enhance the efficacy of ACT, T cells can be engineered by the integration of genes encoding either conventional αβTCR or chimeric antigen receptors (CARs). CARs can be constructed by linking the variable regions of antibody to intracellular signaling chains such as CD3-zeta and in most cases, including co-stimulatory domains encoding CD28 or CD137 (Maher et al., 2002). The recognition of antigens by CARs is not MHC-restricted, but the antigens must be exposed on the cell surface. CAR-T cells are being

successfully applied to the treatment of hematological malignancies. One of the reasons is because hematological tumors express shared target antigens as normal tissues. The effects of CAR-T cells are limited in solid tumors due to their lack of shared antigens as suitable targets (Rosenberg and Restifo, 2015). In 2010, the first successful CAR T-cell therapy was reported using the CD19-targeted CAR in patients with advanced follicular lymphoma. As a result, the CD19-targeted CAR T-cell therapy destroys all the B lineage cells in the circulation (Kochenderfer et al., 2010).

1.2.2.4 Cancer Vaccines

Cancer vaccines can be divided into two types: prophylactic and therapeutic. The most successfully prophylactic cancer vaccines are used in the prevention of infectious agents such as human papilloma virus (HPV) and hepatitis B virus (Mellman et al., 2011).

It has been suggested that about 20% of cancer incidence is caused by infectious agents and the incidence and mortality rates are higher in developing countries (Vedham et al., 2015). HPV is the causative agent of cervical cancer, which is the third leading cause of cancer death among women. It is known that the early exposure of HPV, mainly through sexual transmitted route, will induce cervical cancer (Satterwhite et al., 2013). Prophylactic vaccines that target HPV types 16 and 18 capsid protein L1 are the most common vaccines available since HPV types 16 and18 are known to cause 70-80% of cervical cancers (Dillner et al., 2011). In the reports from 2007, it was shown that infection with HPV types 16 or 18 have decreased by more than 60% in girls younger than the age of 20 (Lauri E. Markowitz, 2007).

Liver cancer is one of the leading causes of death worldwide and in less developed countries. Hepatocellular carcinoma (HCC) accounts for 70-90% of primary liver cancer and about 80% of HCC are linked to chronic hepatic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) (Torre et al., 2015). HBV and HCV are transmitted through body fluids such as blood and semen. It is known that onset of HBV or HCV infection in early ages is an important factor contributing to the development of

hepatocellular carcinoma. Vaccination is the most effective way to prevent infections with HBV and HCV. Recombinant hepatitis B surface antigen (HBsAg) vaccines are available and recommended to all newborns and infants (Poland and Jacobson, 2004).

Therapeutic cancer vaccines are designed to modulate the endogenous immune response against cancer cells in patients. Therapeutic cancer vaccines are also considered as active immunotherapy because the main purpose is to increase patients' own immune responses to recognize tumor antigens and destroy malignant tumor cells (Melero et al., 2014). To effectively generate antitumor immune responses, three steps are required and summarized by Mellman et al. First, APCs, especially DCs, must capture, process and present tumor associated antigens (TAAs). Second, DCs must be activated to go through a maturation process and migrate to secondary lymphoid organs where they present TAAs to naive T cells. Lastly, antigen-specific T cells must be expanded to reach the numbers that are sufficient to eliminate tumor cells (Mellman et al., 2011).

1.2.2.4.1 DC-based cancer vaccination

To induce potent antitumor immune responses, DCs by far are the most powerful APCs that can induce robust antigen-specific responses. Processed tumor antigens will be cross-presented through the MHC class I for CD8+ T cells, which elicit effective anti-tumor responses. However, cross-presentation of tumor antigens is less efficient, and most often results in tolerance due to the presence of tolerogenic DCs in the tumor microenvironment (Harimoto et al., 2013). It is important to develop strategies that enhance DC functions to induce a long-lasting and robust antitumor immunity.

To generate effective adaptive immune responses, DCs need to be activated through binding with the PRR ligands. Activated DCs loaded with tumor antigens can then be administered into patients, traffic through afferent lymphatics to lymph nodes where they interact with naïve T cells, present antigens, and provide co-stimulatory signals and cytokines to activate tumor-specific T cell responses. Afterwards, the activated T cells migrate out through the efferent lymphatics, enter the blood stream, and

reach the tumor site expressing cognate tumor antigens to induce antigen-specific antitumor immune responses (Topalian et al, 2001).

Modulation of DCs to improve antitumor immunity can be achieved by the following strategies:

Enhance DC maturation signals

Dendritic cells activate naïve T cells through at least three signals: MHC-TCR interaction, co-stimulatory signal and cytokines environment. Therefore, any modulation that enhances these three signals may improve the antitumor immune responses. TLR ligand such as LPS activates DCs that induces DC maturation (Kadowaki et al., 2001). It was shown that a LPS-activated dendritic cell-based immunization was able to induce the Th1 polarized anti-HER2/neu responses in women at an early stage of breast cancer. Monocyte-derived DCs were activated by LPS and these activated, mature DCs were pulsed with the tumor peptide carrying HER-2/neu sequences. Peptide- loaded DCs were then injected directly into lymph nodes at the groin area. The vaccine strategy was focusing on inducing Th1 immune responses that lead to cytotoxic T cell response to eliminate HER-2/neu expressing cells. It was found that patients were able to induce efficient CTL and Th cell responses after immunization (Koski et al, 2012).

Engagement of CD40 on DCs has been shown to enhance the expression of costimulatory molecules CD80 and CD86. Furthermore, enhanced T cells responses against tumor antigen Melan A were observed when CD40 ligands were introduced to DC (Bonehill et al, 2009). Another approach is to activate the toll-like receptor (TLR) pathway, resulting in the increased expression of costimulatory molecules. Incorporation of TLR-agonists has been reported to induce antigen-specific immune responses in the infectious disease and cancer models. *In vivo* mouse studies have shown that delivery of TLR4 agonist and paclitaxel (mitotic inhibitor) into B16-F10-bearing mice have effectively decreased the tumor burden (Roy et al, 2013).

Induction of immune-potentiating molecules

Maturation of DCs can be accomplished by exposure to cytokines. DCs also produce cytokines and chemokines, which recruit more immune cells and direct polarization of T cells. Th1-related cytokines including IL-12, IFN-γ and IFN-α/β are known to induce CD8+ T cell activation through stimulating growth, differentiation and survivals of CD8+ T cells (Sad et al., 1995). Therefore, DCs can be engineered to constitutively produce cytokines that induce antigen specific Th1 responses. DCs modified to constitutively express IL-12 have been proven to increase antigen presentation and costimulatory molecule expression, thereafter inducing increased numbers of activated T cells. Moreover, DCs that have been stimulated to produce IL-12 are able to prime T-cells from melanoma patients to produce IFN-γ. This study suggests that Th1 cytokines are able to induce the development of tumoricidal T cell activity in cancer patients (Carreno et al., 2013).

Natural killer (NK) cells are innate immune cells that are critical in eliminating transformed or cancerous cells by recognizing the altered MHC class I expression on those cells. NK cells are able to induce IFN-γ production which is important in the antitumor immunity. Modification of DCs for Th1 cytokine production, including IL-12 and GM-CSF, has been shown to induce NK activation and recruitment (Martin-Fontecha et al., 2004). It was shown that B16 melanoma-bearing C57BL/6 mice immunized with the MART melanoma antigen-engineered DCs were able to generate antigen-specific cytotoxic T cells and protective responses against the B16 melanoma. The protective immune response was dependent on NK cells (Wargo et al, 2005).

Downregulation of DC negative regulators

The soluble factors such as VEGF and IL-10 can block the activation of DCs, resulting in a loss of effector cell functions. There is a concomitant increase in the numbers of immunosuppressive cells such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), which also contribute to an inhibitory phenotype at the tumor site. Increasing the efficacy of cancer vaccines thus requires the blockade of inhibitory responses against DC functions in the tumor microenvironment (Benencia et

al, 2012). Depletion of Treg by antibody therapy with anti-CD25 has resulted in an increase in the efficacy of cancer vaccine in patients who have metastatic renal cell carcinomas (Benencia et al, 2012). MDSCs are known to interact with antigen-specific CTL through the integrins CD11b, CD18 and CD29. Blocking the integrin interface using the monoclonal antibodies has been shown to abrogate MDSC-mediated suppression of CTL (Lindau et al, 2013).

1.2.3 Conclusion

The use of vaccination for cancer therapy has been scientifically demonstrated using various tumor models in preclinical studies. However, the clinical outcomes are often disappointing. The diversity of tumor antigens among patients has contributed to the unsatisfactory outcome of cancer vaccines. Cancer cells undergo mutations of multiple genes, resulting in the diversity of tumor antigens (Noguchi et al., 2013). This nature of heterogeneity remains a challenge to identify a specific antigenic determinant for a universal cancer vaccine. In addition, maturation signals for APCs are limited in the tumor microenvironment, which lead to the T cell anergy or generation of suppressive cells (Huang et al., 2006). To have a major impact on cancer mortality, cancer vaccines will need to overcome these problems. The next frontier for cancer vaccines is likely to incorporate the experimental targeted therapies. For example, a combination treatment of the checkpoint blockade anti-CTLA4 antibody with the immunostimulatory cytokine GM-CSF has been shown to promote CD8+ effector T cells rather than regulatory T cells (Quezada et al., 2006).

1.3 Lunasin

1.3.1 Structure

Lunasin is a biologically active peptide composed of 43 amino acids. It was originally isolated from soybeans (*Glycine max*), and is encoded as a small subunit peptide from a 2S albumin seed protein (Galvez and de Lumen, 1999). The functional structures of lunasin include a poly-D carboxyl end with eight D-residues and an RGD cell adhesion motif. The negatively charged C-terminus is thought to have a high affinity

for the positively charged hypoacetylated chromatin in the tumor cells (Galvez et al., 2001). The RGD (Arg-Gly-Asp) motif is responsible for internalization of lunasin into cells expressing the surface αVβ3 integrin (de Mejia and Dia, 2009). This RGD motif also allows the peptide to attach to the extracellular matrix in mammalian cells (de Lumen, 2005) (Figure 1.2).

1.3.2 Functions of lunasin

Lunasin has been extensively studied as a chemopreventive peptide (Galvez and de Lumen, 1999, Hernandez-Ledesma et al., 2013). The consumption of soy products is often associated with a lower cancer prevalence in South East Asian cultures, supporting that lunasin may contribute to cancer prevention (de Lumen, 2005). Lunasin has *in vitro* effects on disrupting mitosis on several mammalian cancer cell lines including murine hepatoma, human breast cancer cells and murine fibroblasts C3H 10T1/2 (Galvez and de Lumen, 1999). In a murine model, lunasin has a dose-dependent activity on inhibiting the growth of human breast cancer MDA-MB-231 cells by arresting the cell cycle in S-phase as well as down-regulating the expression of cyclins D1 and D3, CDK4, and CDK6 genes (Hsieh et al., 2010). Lunasin also inhibited the in vivo growth of HT-29 colon cancer cells through decreasing the levels of anti-apoptotic Bcl-2 proteins but increasing the levels of pro-apoptotic Bax protein and caspase-3. All of these molecular alterations lead to an increase in the apoptotic process (Dia and Gonzalez de Mejia, 2011).

The epigenetic effects of lunasin have been well documented. Lunasin acts as a surrogate tumor suppressor by tightly binding to hypoacetylated core histones, resulting in an arrest of the cell cycle (de Lumen, 2005). Since phosphorylation of histone proteins is required for a proper condensation and segregation of chromosome, the binding of lunasin to the hypoacetylated histone tails may block this phosphorylation event and consequently inhibit cell division (Jeong et al., 2007a). It has been shown that lunasin binds to deacetylated histone proteins, resulting in the inhibition of histone H3 acetylation by the histone acetyltransferases (HAT), including yGCN5 and p300/CBP-associated factor (PCAF) (Jeong et al., 2007a, Jeong et al., 2002, Jeong et al., 2007b). Epigenetic regulation by chromatin modification is known to alter gene expression.

Indeed, lunasin has been shown to modulate the expression of genes associated with apoptosis, extracellular matrix and cell adhesion molecules involving in the growth of human cancer cells (Lam et al., 2003). We also found that lunasin is able to regulate the expression of genes involved in the antitumor functions of NK cells and DCs (Chang et al., 2014, Tung et al., 2014).

Lunasin suppressed transformation of mammalian cells stably transfected with the *E1A* or *RAS* oncogenes (Jeong et al., 2002, Lam et al., 2003). Topically applied lunasine can reduce the incidence of skin papilloma in carcinogen-initiated SENCAR mice (Galvez et al., 2001). The chemopreventive effects of lunains have been further demonstrated in the xenograft models. Systemic administration of lunasin decreased the tumor volumes in the xenograft model using the NSCLC H1299 cell line. All these findings support the direct antitumor activities of lunasin by inducing apoptosis of transformed cells or causing cell cycle arrest, which are important in cancer prevenion (McConnell et al., 2015).

In addition to its chemopreventive properties, lunasin has the anti-inflammatory activity against LPS stimulation in a murine macrophage cell line (RAW 264.7). Lunasin inhibits the production of proinflammatory cytokines IL-6 and IL-1β, and other inflammatory mediators such as COX-1, PGE2, and NO through blocking the activity of NF-κB by inhibiting translocation of p50 and p65 subunits. This reduction of NF-κB translocation in the nucleus thus inhibits the transcription of the pro-inflammatory genes (Cam and de Mejia, 2012, de Mejia and Dia, 2009). Lunasin can also suppress allergic airway inflammation in OVA+LPS sensitization asthma model. It was found that lunasin suppressed airway inflammation by significantly reducing total cell and eosinophil infiltration in BAL fluid. The anti-inflammatory effect of lunasin in the asthma model was attributed in part to the induction of antigen-specific Treg accumulation (Yang et al., 2015).

In the pro-inflammatory state following stimulation with LPS, the levels of cell adhesion molecules such as integrins are upregulated in the activated macrophages. Lunasin could inhibit the induction of cell adhesion molecules, thereby decreasing the inflammatory responses mediated by LPS. Lunasin suppressed Akt-mediated NF-κB

pathway through interaction with αVβ3 integrin thereby inhibit inflammation (Cam and de Mejia, 2012). The anti-inflammatory effects of lunasin through interaction of integrin provide one possible intervention in cardiovascular disease (CVD) especially in atherosclerosis because αVβ3 integrin-expressed macrophages are commonly found in atherosclerotic lesions (Gianni et al., 2012). It is also suggested that lunasin may recruit Caveolin-1 to the surface of the plasma membrane through signals mediated by integrins. As lunasin is endocytosed into the cell, its interaction with integrins initiates a signaling cascade that results in an exocytosis of Caveolin-1 to the cell surface membrane. This translocation of Caveolin-1 to the surface may prevent the aggregation of abnormal or aberrant macrophages, thereby leading to an anti-inflammatory response (Cam et al., 2013).

Lunasin exerts an antioxidant activity by decreasing the levels of reactive oxygen species (ROS) in macrophages following LPS stimulation (Hernandez-Ledesma et al., 2009a). Studies on the LPS-treated RAW264.7 macrophages have shown the ability of lunasin in inhibiting the oxidation of linoleic acid (Dia and de Mejia, 2011). Linoleic acid is a polyunsaturated fatty acid, which, upon oxidation, can produce peroxyl radicals that attack host tissues. Lunsin is able to interact with peroxyl radicals, thereby inhibiting lipid peroxidation cycles in LPS-stimulated RAW264.7 macrophages. Various segments of lunasin show the different antioxidant bioactivity due to their specific amino acid sequences that exhibit activity against lipid peroxidation.

Recently, our laboratory has discovered an additional property of lunasin as an immune-modulating agent. We have found that lunasin regulates expression of a number of genes that are important for antitumor immune responses mediated by innate immune cells such as NK and DCs (Figure 1.3). Lunasin exerted robust synergistic effects with several therapeutic cytokines to enhance NK-mediated antitumor functions (Chang et al., 2014) . This synergism leads to a stronger NK activation over stimulation with cytokine alone, resulting in increases in IFNγ production and tumoricidal activity (Chang et al., 2014). Our studies suggest that lunasin represents a different class of immune modulating agent that may augment the therapeutic responses mediated by cytokine-based immunotherapy for cancers.

1.3.3 Conclusion

Given the fact that there is no proven effective therapy that can fully eliminate cancers, many cancer patients seek alternative strategies, often in conjunction with traditional cancer therapies (Kessler et al., 2001). Some natural products, commonly sold as dietary botanical supplements, have been used to prevent different stages of cancer or harness cancer immune surveillance by activating immune effector cells (de Mejia and Dia, 2010, Mueller and Anderer, 1990). Lunasin, a 43-amino acid peptide, was originally isolated from soybeans, which has a potential in cancer prevention (de Lumen, 2005). Not only for the chemoprevention effects, lunasin has anti-inflammatory activity and immune-modulating capacity on different subsets of immune cells (Cam and de Mejia, 2012, Chang et al., 2014). The versatile roles of lunasin make it as an additional type of alternative medicine in cancer therapy; however, there is still much to be investigated about the clinical effects of lunasin.

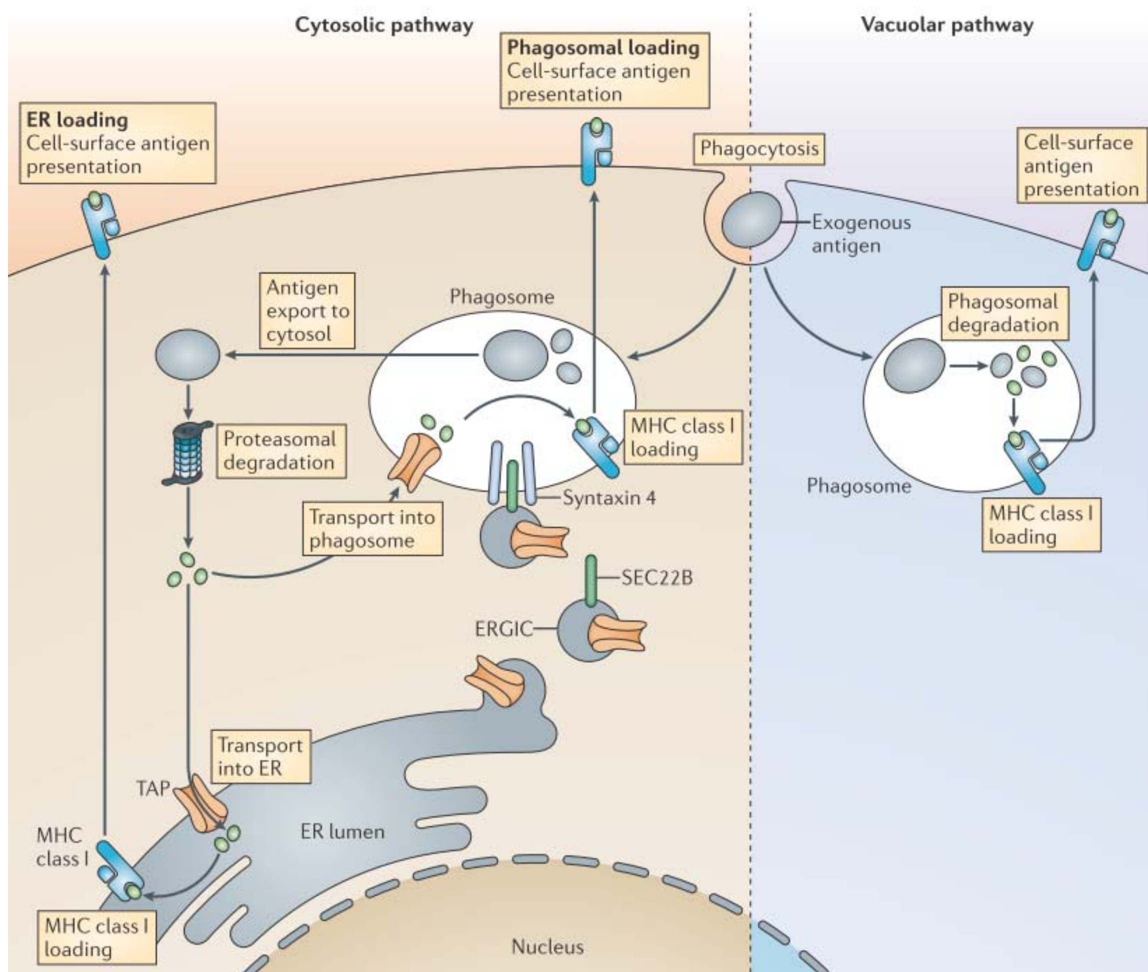


Figure 1.1 Cross-presentation in dendritic cells.

Exogenous antigens could be exported into cytosol after phagocytosis by antigen presenting cells. There are two main intracellular pathways that have been proposed, the cytosolic pathway and vacuolar pathway. In the cytosolic pathway, antigens that are transported into cytosol are processed by proteasome. The peptides after proteolysis by the proteasome can be transported into phagosome and loaded onto the MHC class I in the phagosome directly, or transported through SNARE SEC22B, interacting with syntaxin 4 and TAP phagosome. In the vacuolar pathway, phagocytosed antigens are degraded into peptides in the phagosome, and peptides can be loaded on to the recycling MHC class I in the phagosome (Joffre et al., 2012).

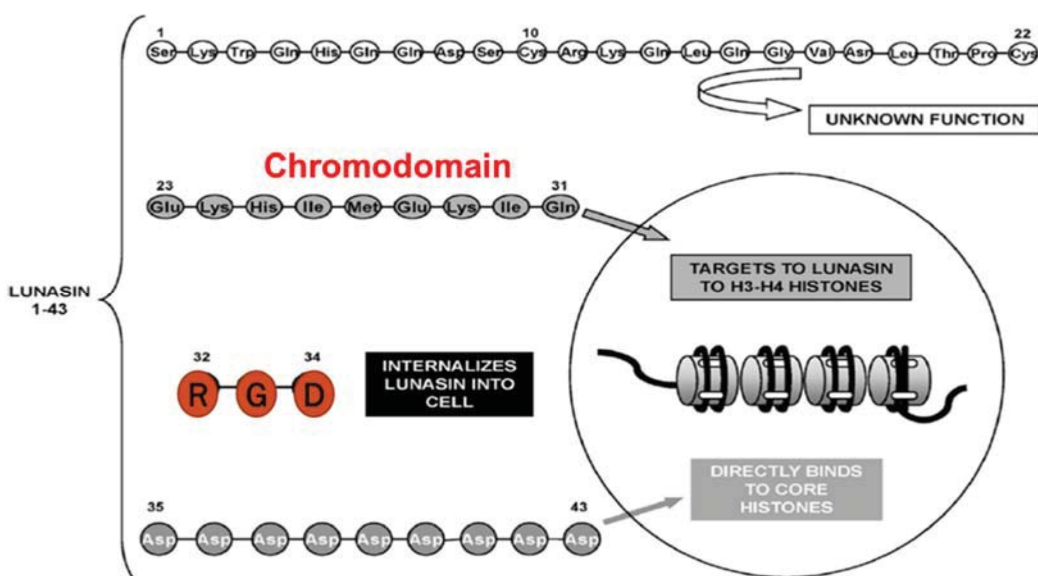


Figure 1.2 Proposed mechanism of lunasin as a chemopreventive agent.
Lunasin is a 43-amino acid peptide with a chromodomain, RGD motif and a negatively charged poly-D carboxyl end. It is believe that the chromodomain is responsible for arresting the cell cycle in transformed cells through binding to deacetylated histone proteins and the RGD domain is responsible for internalization into cells and. The negatively poly-D tail binds to positively charged histone in the nucleus (Hernandez-Ledesma et al., 2009b).

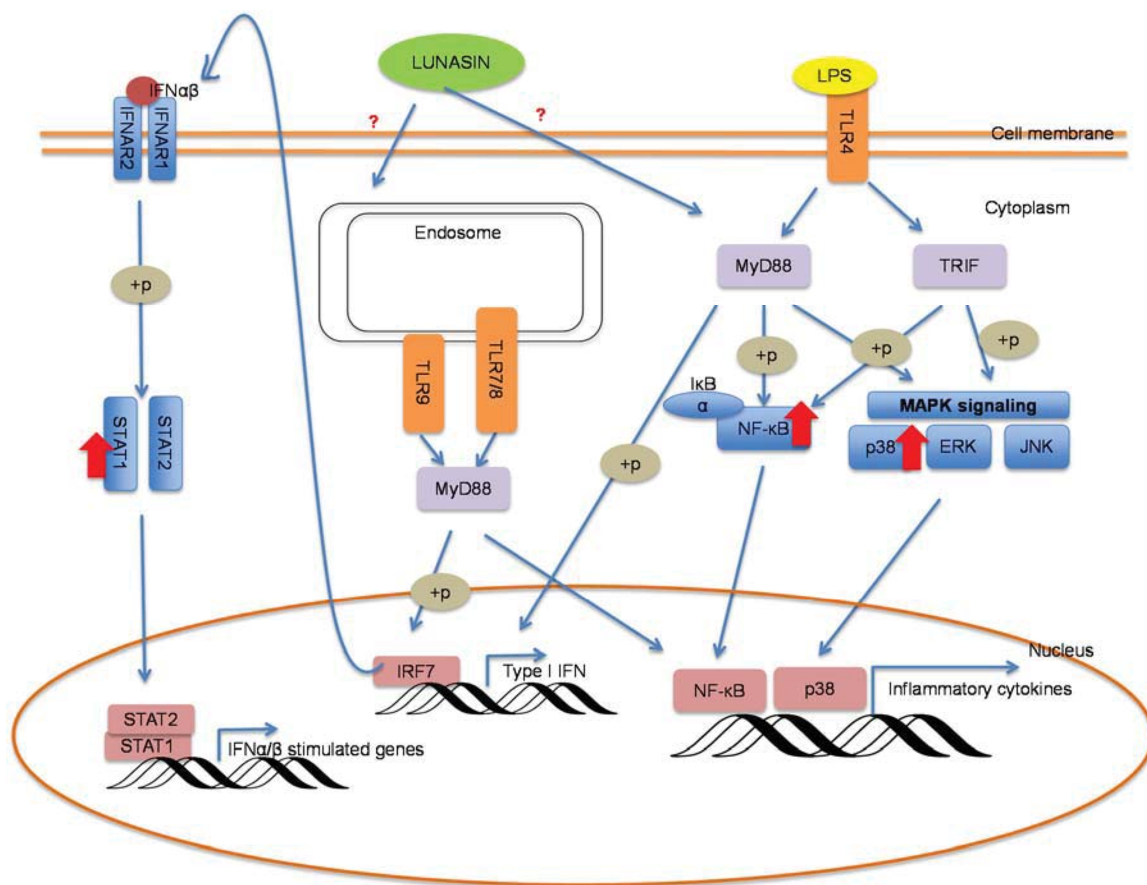


Figure 1.3 Proposed mechanism of lunasin as an immune modulator in dendritic cells. Lunasin stimulation leads to DC activation with increased expression of co-stimulatory molecules, proinflammatory cytokines and interferons. These genes are usually induced upon engagement of PRRs through PAMPs. It remains unknown whether lunasin binds to PRRs; however, increased STAT1 activation was found in lunasin-cultured DCs, suggesting that lunasin induces production of IFNs in DCs and activate STAT1 in an autocrine-loop. Activation of transcription factors, including NF-κB and p38 MAPK (but not ERK1/2) was induced in DCs stimulated with lunasin. These results suggest that lunasin activates these transcription factors, leading to transcription of genes that are important for innate and adaptive immunities.

CHAPTER 2. ACTIVATION OF DENDRITIC CELL FUNCTION BY SOYPEPTIDE LUNASIN AS A NOVEL VACCINE ADJUVANT

Abstract

The addition of an appropriate adjuvant that activates the innate immunity is essential to subsequent development of the adaptive immunity specific to the vaccine antigens. Thus, any innovation capable of improving the immune responses may lead to a more efficacious vaccine. We recently identified a novel immune modulator using a naturally occurring seed peptide called lunasin. Lunasin was originally isolated from soybeans, and it is a small peptide containing 43 amino acids. Our studies revealed stimulatory effects of lunasin on innate immune cells by regulating expression of a number of genes that are important for immune responses. The objective was to define the effectiveness of lunasin as an adjuvant that enhances immune responses. The immune modulating functions of lunasin were characterized in dendritic cells (DCs) from human peripheral blood mononuclear cells (PBMCs). Lunasin-treated conventional DCs (cDCs) not only expressed elevated levels of co-stimulatory molecules (CD86, CD40) but also exhibited up-regulation of cytokines (IL1B, IL6) and chemokines (CCL3, CCL4). Lunasin-treated cDCs induced higher proliferation of allogeneic CD4+ T cells when comparing with medium control treatment in the mixed leukocyte reaction (MLR). Immunization of mice with ovalbumin (OVA) and lunasin inhibited the growth of OVA-expressing A20 B-lymphomas, which was correlated with OVA-specific CD8+ T cells.

In addition, lunasin was an effective adjuvant for immunization with OVA, which together improved animal survival against lethal challenge with influenza virus expressing the MHC class I OVA peptide SIINFEKL (PR8-OTI). These results suggest

that lunasin may function as a vaccine adjuvant by promoting DC maturation, which in turn enhances the development of protective immune responses to the vaccine antigens.

2.1 Introduction

Dendritic cells (DCs) comprise an integral part of the innate immunity that induces the activation of naïve T lymphocytes essential for the adaptive immune responses. DCs process antigens to yield peptides that are presented to T cell receptor (TCR) in the context of major histocompatibility complex (MHC) molecules. Successful priming of naïve T cells also requires strong stimulation upon binding to co-stimulatory molecules expressed by matured DCs. DC maturation can be accomplished by a variety of stimulating agents including microbial products, which bind to various pattern recognition receptors (PRRs) (Iwasaki and Medzhitov, 2004). This binding stimulates the signaling pathways that regulate expression of target genes involved in DC maturation. Thus, an immune stimulating agent or adjuvant that induces DC maturation will ultimately lead to activation of antigen-specific T cells in vaccination.
Conventional DCs (cDCs) express Toll-Like Receptor 4 (TLR4) that responds to lipopolysaccharide (LPS)-based adjuvants (Kadowaki et al., 2001). However, TLR4 is expressed at variable levels among different individuals (Schreibelt et al., 2010). Individuals with low TLR4 expression may not be efficiently primed following vaccination with the adjuvant Monophosphoryl Lipid A (MPL), a derivative of LPS. In addition, patients with immune dysfunctions or in a disease setting may be refractory to stimulation with TLR agonists due to down-regulation of these receptors (Cunningham-Rundles et al., 2006, Monteleone et al., 2008, Yu et al., 2009). Despite the effectiveness of current adjuvant with the TLR4 agonist MPL (Alderson et al., 2006), development of novel immunostimulatory agents that overcome the above limitations may offer alternatives to activate DCs for improved vaccine efficacy.

We recently identified a novel function of soypeptide lunasin as an immune modulating agent that exerts potent synergistic effects with IL-12 or IL-2 on augmenting IFNγ and granzyme B expression by natural killer (NK) cells (Chang et al., 2014). Lunasin was originally isolated from soybeans, and is a naturally occurring peptide

containing 43 amino acids. This peptide exhibits properties that have multiple health benefits, and is a promising chemopreventive agent (Galvez et al., 2001, Galvez and de Lumen, 1999, Jeong et al., 2002, Maldonado-Cervantes et al., 2010). To further define lunasin's potential as an adjuvant, the immune modulating functions of lunasin were established with DCs. Using purified human DCs from peripheral blood mononuclear cells (PBMCs), lunasin's stimulatory effects on the expression of genes important for DC maturation such as cytokines, chemokines, and co-stimulatory molecules were demonstrated. The in vivo effectiveness of lunasin as an adjuvant for a model antigen ovalbumin (OVA) was assessed in the prevention model against the A20-OVA B-lymphoma as well as the PR8-OTI influenza virus. Together, these studies demonstrate the immune modulating effects of lunasin on DC maturation, suggesting its potential as a vaccine adjuvant to enhance the immune responses against the vaccine antigens.

2.2 Materials and methods

Antibodies, lunasin peptide, and other reagents

Fluorochrome-conjugated monoclonal antibodies to human CD1c, CD86, CD40, and to mouse CD11c, B220, CD86, were obtained from BD Biosciences (San Jose, CA). Ficoll-Paque™ PLUS was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). The lunasin peptide with 43-amino acid was chemically synthesized with 97% purity by LifeTein (South Plainfield, NJ) as previously described (Chang et al., 2014). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Guava Technologies (Hayward, CA). Lipopolysaccharide (LPS from *Escherichia coli* 0111:B4) and Concanavalin A (Con A) were from Sigma-Aldrich (St. Louis, MO). Ovalbumin (OVA, chromatographically purified) was from Worthington Biochemical Corp (Lakewood, NJ). Imiquimod (R837) and Alum (Alhydrogel 2%) were from InvivoGen (San Diego, CA).

Human blood samples, primary immune cells, cell line, and virus

Healthy human blood samples were procured from the Indiana Blood Center (Indianapolis, IN). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque™ PLUS, and aliquots of PBMCs were cryopreserved in liquid nitrogen.

Human primary conventional dendritic cells (cDCs) were negative selected from PBMCs using a CD1c (BDCA-1) Dendritic Cell Isolation Kit with ~90% of purity (Miltenyi Biotec Inc., Auburn CA). Human primary natural killer (NK) cells were isolated from PBMCs using a negative selection kit with ~95% of purity (Miltenyi Biotech). Human plasmacytoid DCs (pDCs) were positively selected from PBMCs using BDCA-4 magnetic beads with ~60% of purity (Miltenyi Biotech). Total CD4+ T cells were positively selected from PBMCs using CD4 magnetic beads with ~95% of purity (Miltenyi Biotech). A murine B-lymphoma cell line expressing OVA, A20-OVA ($H-2^d$) was kindly provided by Dr. Gang Zhou (Medical College of Georgia) (Birkholz et al., 2010). Influenza virus expressing the MHC class I OVA peptide SIINFEKL (PR8-OTI) was generated previously (Jenkins et al., 2006).

Characterization of human innate immune cells following stimulation in vitro

Freshly purified human immune cells (cDCs, pDCs, and NK cells) were stimulated for 1 day as indicated. The expression levels of co-stimulatory molecules (CD86 and CD40) on cDCs were evaluated using flow cytometry with staining antibodies. For analysis of gene expression, the cell pellets following 1 day of stimulation were resuspended in Trizol Reagent for total RNA extraction. The first-strand cDNA was synthesized followed by real time qPCR using Taqman Assay with primers for IL-1β (*IL1B*), IL-6 (*IL6*), chemokine (C-C motif) ligand 3 (*CCL3*), CCL4 (*CCL4*), TNFα (*TNFA*), and *ACTB* (β-actin) as endogenous control (Lupov et al., 2011). The supernatants collected from the cultures following 1 day of stimulation were analyzed for the production of CCL3 and TNFα using ELISA (Re and Strominger, 2001).

In vivo administration of lunasin

BALB/c mice were intraperitoneally (IP) injected with PBS (-) or lunasin at 0.4 or 4 mg/kg body weight. Mice were sacrificed and spleens were collected for analysis 18 hours following injection. Splenocytes from these mice were surface stained with B220, CD11c, and CD86 monoclonal antibodies. The expression levels of CD86 were analyzed on DCs gated on CD11c and B220 populations using flow cytometry.

Allogeneic mixed leukocyte reaction (MLR)

Purified cDCs were stimulated as indicated for 1 day and washed prior to co-culture with T cells. Total CD4+ T cells purified from a different donor were stained with CFSE. Treated cDCs were co-cultured with CFSE-labeled allogeneic CD4+ T cells for 5-7 days. Proliferation of alloreactive CD4+ T cells was determined from dilution of CFSE using flow cytometry.

Immunization studies

BALB/c (H-2^d) or C57BL/6 (H-2^b) mice were IP injected twice with PBS or OVA (100 µg) mixed with Alum (Alhydrogel 2%) or lunasin at the dose indicated on days 1 and 7. In the syngeneic B-lymphoma model, immunized BALB/c mice received subcutaneous challenge with 1 x 10^6 A20-OVA (H-2^d) 7 days following the last immunization. Tumor volumes were measured as described (Chang et al., 2014) from day 15 after tumor injection through day 22. Splenocytes processed from these mice were labeled with CFSE, and then cultured in vitro with OVA (100 µg/ml) for 5 days. Proliferation of OVA-specific CD8+ T cells was determined from diluted CFSE using flow cytometry. In the influenza model, immunized C57BL/6 mice were intranasally challenged with a lethal dose of live PR8-OTI at 700 pfu 14 days following the last immunization (Jenkins et al., 2006). Animal survival and body weight was monitored daily for 18 days following challenge.

Statistical analysis

SAS/STAT (SAS Institute Inc, Cary, NC) was used to analyze the data. A mixed model was developed for analyzing the data with within-subject treatments, and the pairwise comparisons among the treatments were performed to determine the *P* values.

2.3 Results

Lunasin stimulation results in elevated levels of co-stimulatory molecules in human cDCs

To determine the responsiveness of DCs to lunasin stimulation, CD1c (BDCA-1) positive conventional DCs (cDCs) purified from PBMCs of healthy donors were stimulated with medium or increasing concentrations of lunasin. Lipopolysaccharide (LPS), a TLR4 ligand, is often used to activate cDCs (Re and Strominger, 2001). Thus, LPS was included in the stimulation as a positive control for comparison. Human peripheral cDCs are known to express CD86 (Mittag et al., 2011), as over 80% of the CD1c+ cells expressing CD86 (Figure 2.1A). LPS stimulation enhanced the surface levels of CD86, resulting in a 5-fold increase in CD86 high populations compared to medium control (Figure 2.1A, 1B). With increasing concentrations of lunasin, greater numbers of CD86 high cDCs were detected (Figure 2.1A, 1B). Unlike CD86, the levels of CD40 expression were low in CD1c+ cDC populations without any stimulation. Following LPS stimulation, 20-25% of these cDC populations expressed CD40 (CD40+). Exposure to 50 μM lunasin resulted in significant increase in CD40+ cDCs compared to medium control (Figure 2.2A, 2B). These results reveal lunasin's innate stimulatory effects as detected by induction of co-stimulatory molecules, including CD86 and CD40 in human cDCs.

Effects of lunasin on the production of cytokines and chemokines by human cDC

As LPS stimulation of cDCs also results in production of cytokines and chemokines (Kadowaki et al., 2001, Penna et al., 2002, Gervassi et al., 2004), we next evaluated whether lunasin had similar effects on human cDCs. We chose lunasin at the concentration of 50 μM that induced comparable surface levels of CD86 as LPS (Figure 2.1B). Lunasin was able to induce gene expression of IL1B, IL6, CCL3, and CCL4, although these genes were induced at higher levels by LPS (Figure 2.3 A). We did not observe any significant changes in TNFA gene expression by human cDCs cultured in medium, LPS or lunasin for 1 day (Figure 2.3 B, upper panel). However, TNFα secretion was observed in LPS-treated supernatants using ELISA (Figure 2.3 B, lower panel). In

contrast, there was no detectable TNFα secreted following lunasin stimulation (Figure 2.3 B, lower panel).

LPS stimulation in innate immune cells also results in secretion of CCL3. Given the essential role of CCL3 in recruiting various immune effectors such as T cells (Trifilo et al., 2003), we next examined CCL3 levels in the supernatants of human cDCs in the Figure 2A. The ELISA results showed no detectable CCL3 in cDCs cultured in medium, while secretion of CCL3 was induced by LPS (Figure 2C). We also observed secretion of CCL3 by lunasin despite at a lower level compared to that induced by LPS (Figure 2.3 C). Collectively, these results demonstrate that treatment of cDCs with lunasin induced the expression of cytokines (IL1B, IL6) and chemokines (CCL3, CCL4). In addition, lunasin did not induce TNFα production, suggesting a distinct stimulatory function from LPS in activating cDCs.

In addition to cDCs, other innate immune cells such as NK cells and plasmacytoid DCs (pDCs) are able to produce CCL3 and TNFα following stimulation (Fehniger et al., 1999, Robertson, 2002, Penna et al., 2002). Although lunasin had little effects on TNFα production by cDCs, it is not known whether lunasin can induce the release of this cytokine by pDCs. Thus, the effects of lunasin on cytokine and chemokine release by NK cells and pDCs were examined. BDCA4+ pDCs and NK cells were purified from human PBMCs for stimulation in vitro. To ensure these purified cells were capable of producing cytokines and chemokines, an appropriate stimulus was included in the treatment as a positive control. For examples, IL-2 was used in NK stimulation, and R837 (TLR7 agonist) was used for pDC. Lunasin alone had no effects on CCL3 secretion by NK cells, while these cells were capable of producing CCL3 when stimulated with IL-2 (Fig 2.3 D). Lunasin stimulation in pDCs resulted in CCL3 secretion, which was also induced by R837 (Figure 2.3 E). In addition, TNFα was induced by R837 stimulation, but was not detectable in the supernatants from lunasin-treated pDC cultures (Figure 2.3 F). These results suggest that lunasin's stimulatory effects are restricted to specific subsets of immune cells, and certain target genes are more susceptible and sensitive to lunasin-mediated regulation.

Effect of lunasin on DCs in vivo

Having found the immune modulatory functions of lunasin in vitro, we next assessed lunasin's in vivo effects on DC populations following administration in mice. BALB/c mice were intraperitoneally (IP) injected with PBS or lunasin (at 0.4 or 4 mg/kg body weight). One day following the treatment, the expression levels of surface co-stimulatory molecule on splenic DCs were analyzed. Lunasin administration at the dose of 4 mg/kg resulted in significantly higher surface levels of CD86 on DC gated on the CD11c+ B220+ populations (Figure 2.4). These results demonstrated the dose-dependent effects of lunasin on increasing the levels of co-stimulatory molecule CD86 on DC populations in mice.

Lunasin-treated human cDCs induce proliferation of alloreactive CD4+ T cells

The stimulatory function of lunasin-treated human cDCs was examined in an allogeneic mixed leukocyte reaction (MLR). Proliferation of alloreactive CD4+ T cells was assessed using flow cytometry to measure the diluted CFSE (Figure 2.5 A). Approximately 25% of alloreactive CD4+ T cells proliferated upon co-culturing with un-stimulated cDCs, while higher numbers of T cells proliferated when co-cultured with cDCs that were pre-treated with LPS or lunasin (Figure 2.5 B). These results demonstrate the ability of lunasin-treated cDCs to effectively stimulate the proliferation of alloreactive T cells.

Immunization with OVA and lunasin confers protection against OVA-expression B-lymphoma

We next evaluated the ability of lunasin to enhance OVA immunization and to confer protection against challenge with OVA-expressing A20 B-lymphomas. Alum adjuvant is non-pyrogenic, and the most often used adjuvant for human vaccines worldwide (Lindblad, 2004b, Lindblad, 2004a). Thus, Alum-adsorbed OVA was included for comparison. Tumor volumes were smaller in mice immunized with Alum-OVA as compared to those receiving PBS (Figure 2.6 A). Between the groups of lunasin-OVA immunized mice, lunasin at the dose of 100 μg/mouse (4 mg/kg) conferred better

protection, resulting in stronger tumor inhibition as evidenced by the smaller tumor volume as compared to lunasin at 10 μg/mouse (0.4 mg/kg) (Figure 2.6 A). Correlating with the tumor growth retardation, we observed higher numbers of OVA-specific CD8+ T cells in mice immunized with Alum-OVA or lunasin-OVA, relative to those immunized with PBS (Figure 2.6 B, 6C). Using this prophylactic model, the adjuvant activity of lunasin was found to be comparable or superior to Alum depending upon the dose, in terms of inducing the antigen-specific CD8+ CTL responses and tumor growth retardation against A20-OVA.

Immunization with OVA and lunasin improves animal survival against influenza virus infection

To further demonstrate the potential of lunasin as an adjuvant in vivo, we tested an immunization and challenge model with influenza virus expressing the MHC class I OVA peptide SIINFEKL (PR8-OTI) (Jenkins et al., 2006). While all of the mice died in the groups immunized with PBS or Alum-OVA, 60% of mice survived in the group immunized with lunasin-OVA at day 13 following challenge (Figure 2.7 A). Although all mice exhibited weight loss, only mice in the lunasin-OVA group showed recovery of weight loss at day 13 following challenge (Figure 2.7 B). These results demonstrate the efficacy of immunization with lunasin and OVA to elicit protective CD8+ CTL responses for improving animal survival against lethal challenge with influenza virus.

2.4 Discussion

Lunasin has been recently identified as an immune modulating agent that exerts synergistic effects with IL-2 or IL-12 on NK activation (Chang et al., 2014). In this study, we found that lunasin by itself was capable of stimulating DC maturation, resulting in elevated surface levels of co-stimulatory molecules (CD86, CD40) as well as induction of cytokines (IL1B, IL6) and chemokines (CCL3, CCL4). Lunasin-stimulated cDCs were functional as evidenced by higher proliferation of allogeneic T cells when comparing with un-stimulated cDCs. Alum and lunasin were compared as adjuvants for a model antigen OVA, and lunasin at 100 μg/mouse conferred better protection than Alum in the

A20-OVA B-lymphoma as well as the PR8-OTI influenza model. Collectively, these results demonstrate the effectiveness of lunasin as a potential vaccine adjuvant that induces DC maturation, which in turn promotes the development of antigen-specific immune responses to confer protection.

The ability of lunasin to induce the expression of several genes, but not TNFA, during DC maturation appears unique, as these genes are often induced upon binding to microbial components such as TLR agonists (Kadowaki et al., 2001, Gervassi et al., 2004). This engagement on receptors such as TLRs initiates a cascade of signaling pathways leading to the activation of transcription factors, such as NF-kB, AP-1, and interferon regulatory factors (IRFs), which contribute to gene transcription. Although we have not identified the transcription factor(s) that are directly activated by lunasin in DCs, lunasin was able to enhance STAT4 activation, contributing to its synergistic effect with cytokines IL-12 or IL-2 on IFNγ expression by NK cells (Chang et al., 2014). The observed changes in gene induction in DCs suggest that lunasin elicits a signaling pathway that leads to the activation of transcription factor(s) bound to the regulatory regions, resulting in initiation of gene transcription. The molecular mechanism for gene regulation by lunasin is of particular interest, and remains to be elucidated. While TNFα is an essential element for DC maturation in subsequent development of the adaptive immunity (Pasparakis et al., 1996, Trevejo et al., 2001), excessive TNFα secretion can cause detrimental toxicity similar to septic shock (Wage et al., 1987). Lunasin did not induce TNFα secretion, suggesting its potential as a safe adjuvant due to the lack of TNFα-associated toxicity.

Despite variable expression of TLRs among DCs (Kadowaki et al., 2001), both cDCs and pDCs responded to lunasin stimulation. We thus speculate the dispensable role of TLRs for lunasin's stimulatory activity in innate immune cells. However, it remains to be determined whether lunasin can bind to TLRs or other PRRs. Nonetheless, lunasin contains a RGD motif that is involved in the binding of integrins that are commonly expressed by various immune cells. Stimulation of DCs with RGD-containing protein osteopontin (OPN) resulted in the secretion of cytokines such as TNFα and IL-12 (Renkl

et al., 2005). To directly verify the requirement of RGD motif for lunasin's stimulatory activity in DCs, we utilized a truncated peptide (32 aa) lacking the last 11 amino acids containing RGD motif as described in our previous study (Chang et al., 2014). This truncated peptide had similar stimulatory activity as the full-length lunasin in DCs (data not shown), suggesting the dispensable role of RGD motif in gene expression regulated by lunasin in DCs. In addition, failure of induction of TNFα and IL-12 (data not shown) by lunasin suggests that its mechanism is different from the integrin signaling pathway upon RGD binding.

In the A20-OVA B-lymphoma model, the protective immune response rely on tumor-specific CTL because high levels of MHC class I on these tumor cells make them more susceptible to the lysis mediated by CD8+ T cells. Depletion of CD8+ T cells resulted in tumor progression and abolished the protective effects induced by soluble OVA formulated vaccination (Hariharan et al., 1995). In addition to CTL, adoptive transfer of antigen-specific Th1 or Th2 caused tumor regression in A20-bearing mice (Nishimura et al., 1999). Alum is known to enhance antibody production and to promote Th2 allergic immune responses (Raz et al., 1996, Chang et al., 2010); however, it is less efficient on eliciting Th1 and CTL responses (Newman et al., 1992, Hariharan et al., 1995). Although Alum-primed antigen specific CD8+ T cells have been reported (Hohn et al., 2002, McKee et al., 2009), these cells exhibit inferior cytotoxicity (MacLeod et al., 2011). In our study Alum-adjuvanted OVA likely induced both CTL and Th2 cellular responses, which together caused tumor regression in mice bearing A20-OVA B-lymphomas. In the PR8-OTI influenza model, however, MHC class I-restricted CD8+ T cells were required to protect mice from lethal challenge. There was no survival advantage for mice receiving Alum-OVA vaccination compared with mice in the negative control group receiving PBS. Thus, CTL and Th2 responses induced by Alum-OVA vaccination were not protective and had little benefits on animal survival following lethal challenge with the PR8-OTI virus. Other studies also showed insufficient protection by Alum alone whereas combination of Alum with MPL resulted in protection against influenza virus (MacLeod et al., 2011). Although lunasin conferred better survival than Alum in vaccination against the PR8-OTI influenza virus, it did not prevent animal

weight loss (Figure 6). Further studies are needed to optimize the formulation or delivery route, which may enhance the adjuvant activity of lunasin in vivo.

In this study, we have characterized the immune modulating functions of lunasin on DC maturation. The novel property of lunasin, a naturally occurring seed peptide, represents a different class of adjuvant for enhancing innate immune cell activation. Using the prophylactic model we have demonstrated the adjuvant activity of lunasin when mixed with a soluble antigen (OVA) to confer protection in mice against subsequent challenge. Collectively, this study suggests the potential application of lunasin as a vaccine adjuvant, which can promote DC maturation for priming of MHC class I-restricted CD8+ CTL protective responses.

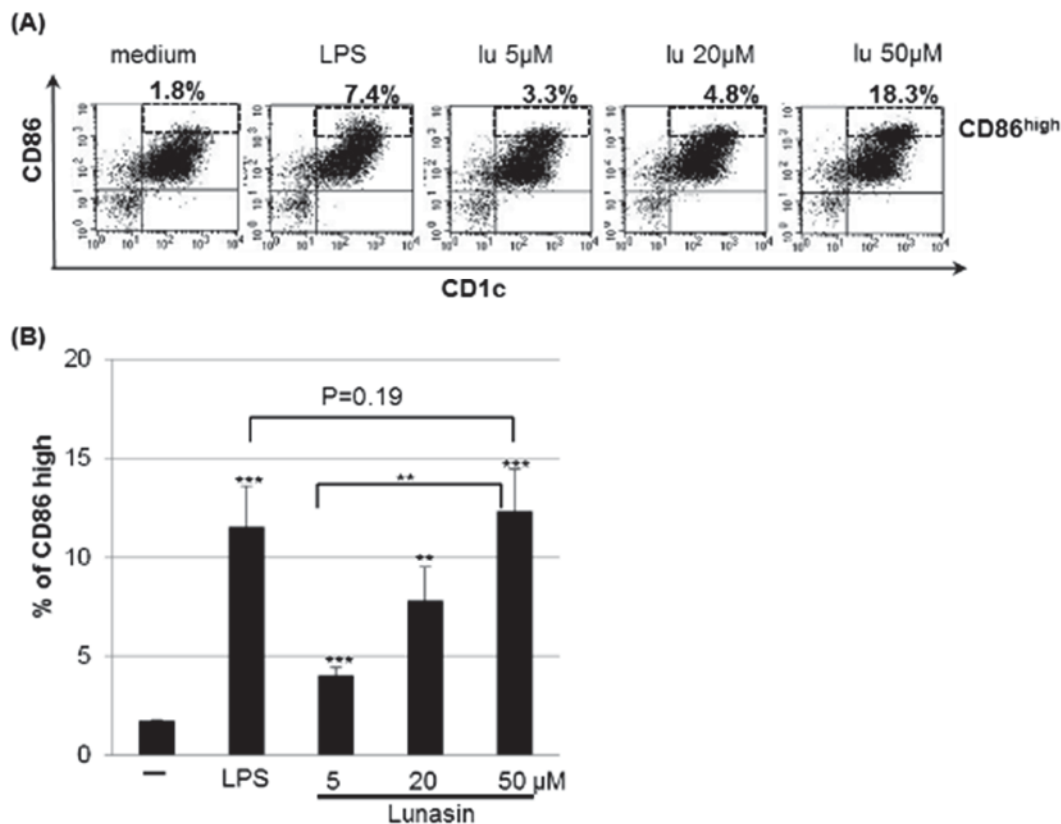


Figure 2.1 Lunasin stimulation results in elevated levels of CD86 in human cDCs. Freshly isolated human cDCs from peripheral blood mononuclear cells (PBMCs) of healthy control subjects using the CD1c (BDCA-1) microbeads (Miltenyi Biotec, Auburn, CA) were stimulated with medium only (-), lipopolysaccharide (LPS) at 1 µg/ml, lunasin peptide (lu) at 5, 20 or 50 µM. Following 24 hours of stimulation, cells were surface stained with APC-conjugated CD1c, PerCP-conjugated CD86, and FITC-conjugated CD40 monoclonal antibodies, washed and fixed. Expression of CD86 and CD40 was evaluated using flow cytometry on 10000 events of gated CD1c positive cell populations. (A) A representative dot plot from one donor shows the percentage of CD1c+ populations that express high CD86 expression (CD86 high) as indicated in the upper right quadrant in the rectangle. (B) The averaged percentage of CD86 high of CD1c+ populations is presented as mean ± SD from 5 different healthy donors. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, relative to the medium only

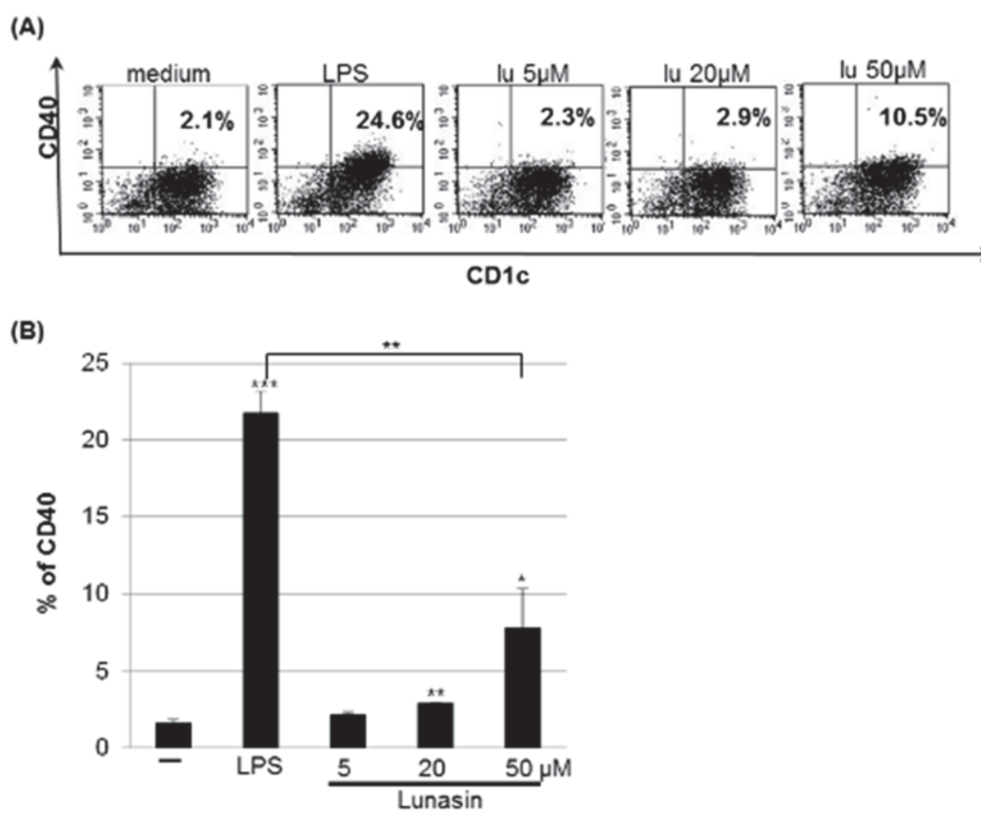


Figure 2.2 Lunasin stimulation results in elevated levels of CD40 in human cDCs. Freshly isolated human cDCs from peripheral blood mononuclear cells (PBMCs) of healthy control subjects using the CD1c (BDCA-1) microbeads were stimulated with medium only (-), lipopolysaccharide (LPS) at 1 μg/ml, lunasin peptide (lu) at 5, 20 or 50 μM. Following 24 hours of stimulation, cells were surface stained with APC-conjugated CD1c, PerCP-conjugated CD86, and FITC-conjugated CD40 monoclonal antibodies, washed and fixed. Expression of CD86 and CD40 was evaluated using flow cytometry on 10000 events of gated CD1c positive cell populations. (A) A representative dot plot from one donor shows the percentage of CD1c+ populations that express CD40 (CD40+) as indicated at the upper right quadrant. (B) The averaged percentage of CD40+ and CD1c+ populations is presented as mean ± SD from 3 different healthy donors. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001, relative to the medium only.

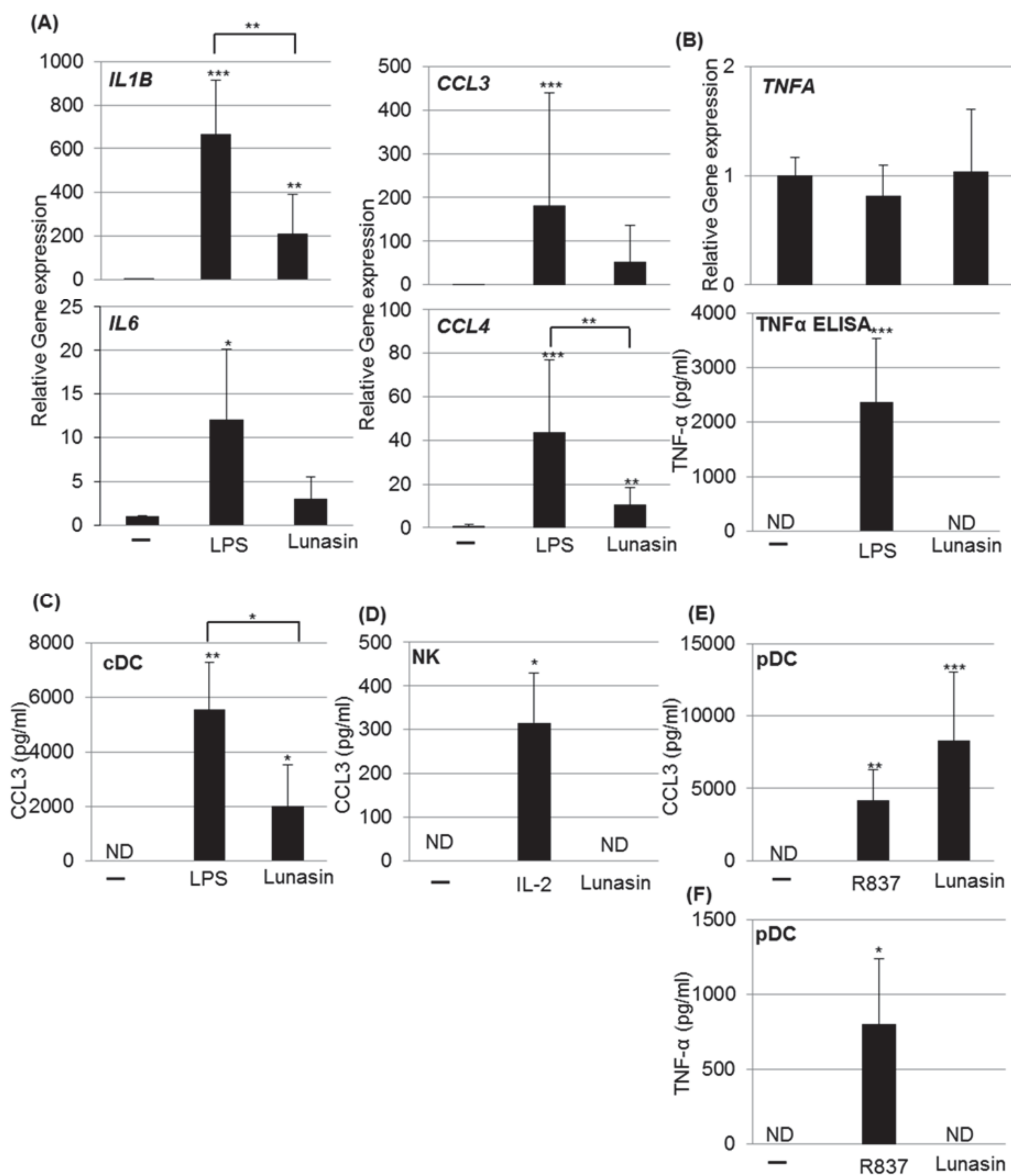


Figure 2.3 Effects of lunasin on the production of cytokines and chemokines by human cDCs, pDCs, and NK cells.

Freshly isolated human cDCs as described in Figure 1 were stimulated with medium only (-), LPS at 1 μg/ml, and lunasin at 50 μM. Following 24 hours of stimulation, the cell pellets were resuspended in Trizol Reagents for total RNA extraction. The first-strand cDNA was synthesized followed by real time qPCR using Taqman Assay with primers for IL-1β (IL1B), IL-6 (IL6), chemokine (C-C motif) ligand 3 (CCL3), CCL4 (CCL4) (A), and TNFα (TNFA) (B, upper panel) in the ABI 7300 (Applied Biosystems by Life Technologies, Carlsbad, CA). The cell-free supernatants collected from the same cultures were evaluated for TNFα (B, lower panel) and CCL3 (C) production using ELISA. Data are presented as mean ± SD averaged from 5 normal controls. (D) Purified human NK cells using negative selection as described [8] were stimulated with medium (-), IL-2 (100 units/ml), and lunasin at 50 μM. Following 24 hours of stimulation, the supernatants were analyzed for CCL3 secretion using ELISA. Data are presented as mean ± SD averaged from 3 normal controls (E) Human pDCs purified from PBMCs using the BDCA-4 magnetic beads were stimulated with medium only (-), R837 at 10 μg/ml, and lunasin at 50 μM. Following 24 hours of stimulation, the supernatants were analyzed for CCL3 (upper panel) and TNFα (lower panel) secretion using ELISA. Data are presented as mean ± SD averaged from 3 normal controls. ND, not detectable; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, relative to the medium only.

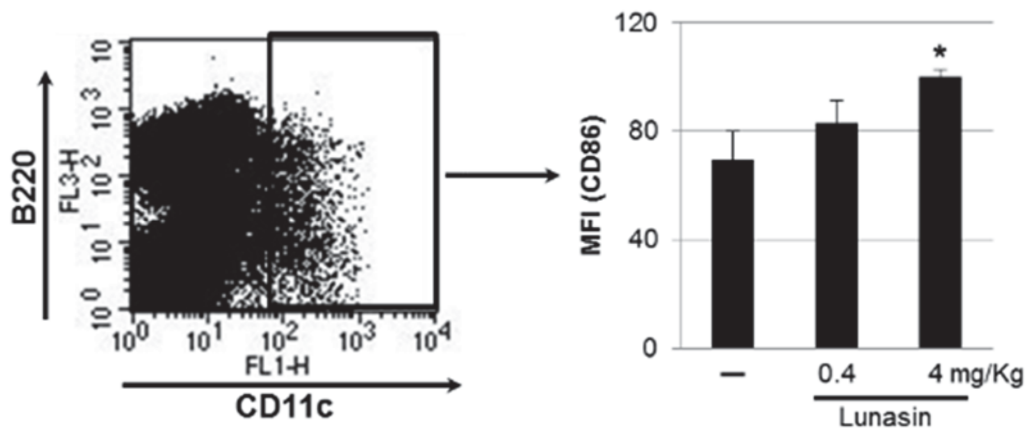


Figure 2.4 Effect of lunasin on DCs in vivo.

BALB/c mice were intraperitoneally (IP) injected with PBS (-) or lunasin at 0.4 or 4 mg/kg body weight. Mice were sacrificed and spleens were collected for analysis 18 hours following injection. DCs gated on 10000 events of CD11c+ and B220+ populations (left panel) were analyzed for surface expression of co-stimulatory molecule CD86 and the geometric mean fluorescent intensity (MFI) was obtained using flow cytometry. Data are presented as Mean ± SD averaged from 3 mice in each group. Statistical significance between groups of mice was determined using an independent sample Student′s t-test. *P ≤ 0.05, relative to the control group receiving PBS only.

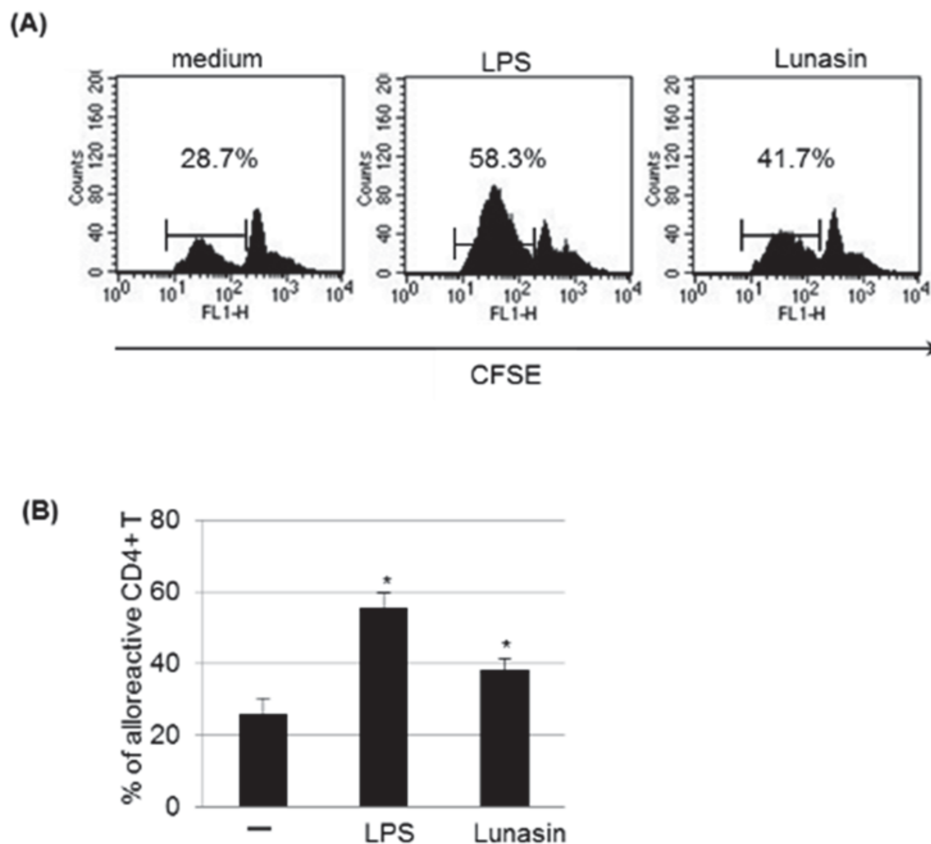


Figure 2.5 Lunasin-treated cDCs induce proliferation of alloreactive CD4+ T cells. Purified human cDCs were stimulated with medium only, LPS at 1μg/ml or lunasin at 50 μM. Following 24 hours of stimulation, these DCs were washed with medium and co-cultured with CFSE-labeled allogeneic CD4+ T cells at a ratio of 1:10 (1 x 10^5 cDCs: 1 x 10^6 CD4+T cells) in a total volume of 500 μl for 5-7 days. CD4+ T cells were purified from different individuals using positive selection with CD4+ magnetic beads (Miltenyi Biotec). Proliferation of alloreactive CD4+ T cells were determined by cells with diluted CFSE using flow cytometry. A representative histogram from one experiment shows the percentage of proliferation as indicated (A). The averaged percentage of proliferation is shown as mean ± SD from 3 independent experiments using different donors (B). *P ≤ 0.05, relative to DCs cultured in medium only.

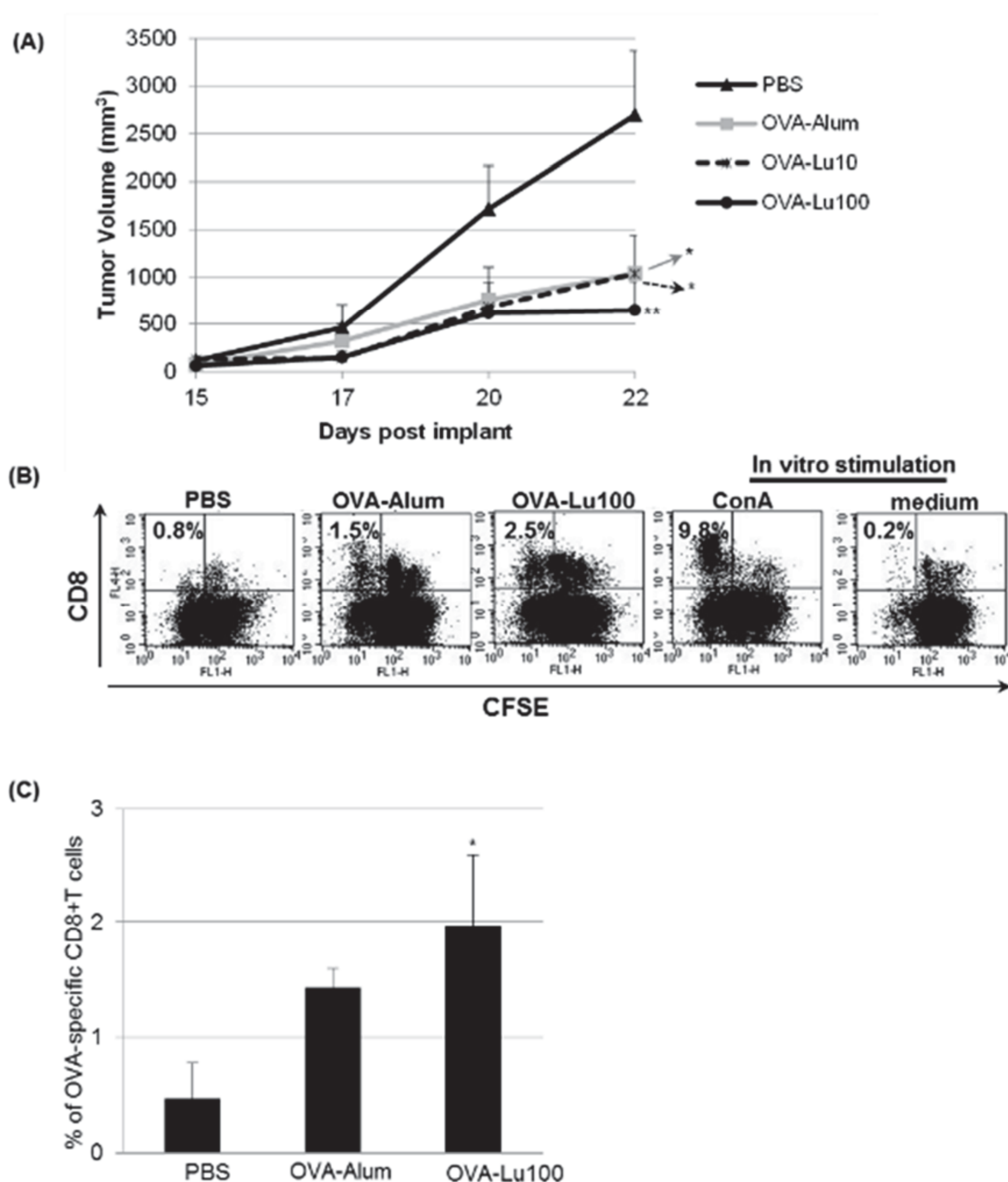


Figure 2.6. Effects of lunasin as an adjuvant in a tumor challenge model with A20-OVA B-lymphoma.

BALB/c mice (female, 6-7 week-old) were intraperitoneally (IP) immunized twice with PBS or OVA (100 µg) mixed with Alum (Alhydrogel 2%, InvivoGen) or lunasin (10 µg or 100 µg per mouse) on days 1 and 7. At day 14, these mice were subcutaneously challenged with 1 x 10^6 cells of OVA-expressing A20 (A20-OVA), a syngeneic B-

lymphoma cell line. (A) Tumor volumes were measured from day 15 after injection through day 22. Data represent the mean ± SEM from 5 mice per group. A mixed model with repeated measure to the data was developed using PROC MIXED in SAS program followed by pairwise comparison test of the mean differences among treatments by different days. *$P \leq 0.05$; **$P \leq 0.01$, relative to PBS-vaccinated group. Results shown are representative from 2 independent experiments with similar profiles. (B) All mice were euthanized 22 days following tumor injection. Spleens collected from these mice were processed into single-cell suspensions followed by labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE-labeled splenocytes were then stimulated with OVA (100 µg/ml) to induce proliferation of OVA-specific T cells. For the in vitro controls, CSFE-labeled splenocytes were cultured in medium only as a negative control, or treated with Concanavalin A (Con A) (2.5 µg/ml) for polyclonal proliferation as a positive control. Proliferation of CD8+T cells was determined from diluted CFSE using flow cytometry. A representative dot plot shows the percentage of diluted CFSE at the upper left quadrant. (C) The averaged percentage of proliferation from OVA-specific CD8+ T cells is shown as mean ± SD from 3 mice per group. *$P \leq 0.05$, relative to PBS-vaccinated group.

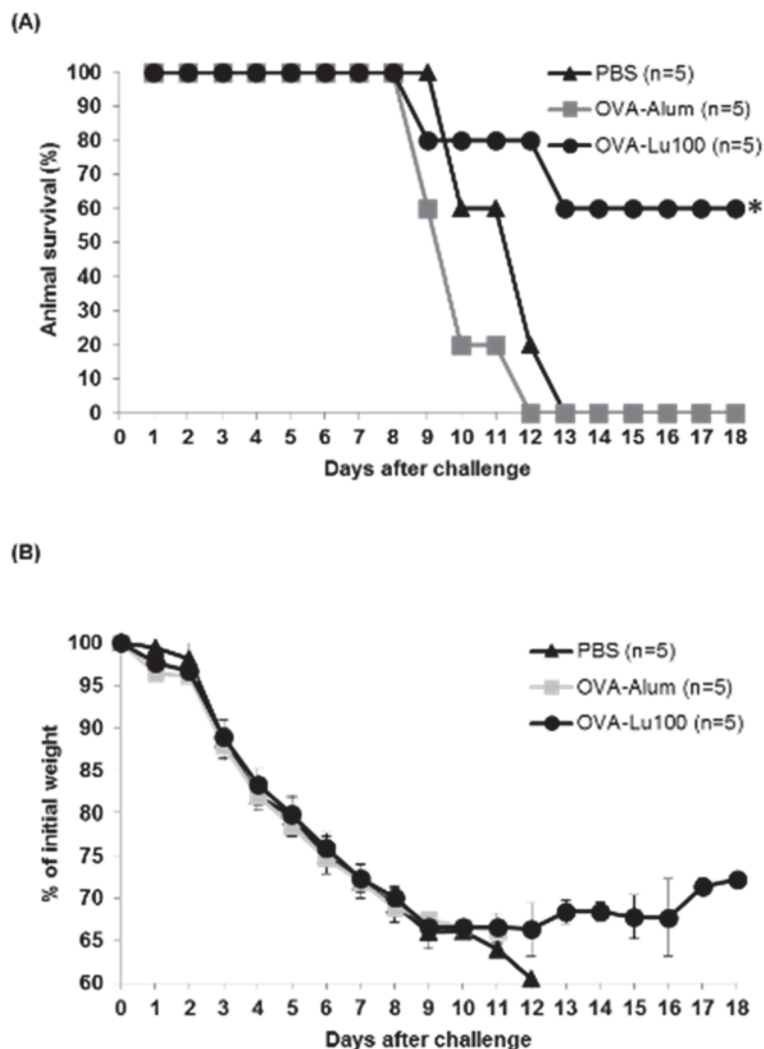


Figure 2.7 Immunization with OVA and lunasin improves animal survival against lethal challenge with influenza virus PR8-OTI.

C57BL/6 mice (female, 4-6 week-old, n=5/group) were intraperitoneally (IP) immunized twice with PBS or OVA (100 µg) mixed with Alum (Alhydrogel 2%, InvivoGen) or lunasin (100 µg) on days 1 and 7. At day 21, these mice were intranasally challenged with a lethal dose of live PR8 virus expressing the MHC class I OVA peptide SIINFEKL (PR8-OTI) at 700 Pfu/mouse. Animal survival **(A)** and body weight **(B)** was monitored daily for 18 days following challenge. *$P<0.05$ relative to PBS-vaccinated group. This experiment was done in collaboration with Dr. Jie Sun's lab in the Department of Microbiology and Immunology, Indiana University School of Medicine.

CHAPTER 3. LUNASIN ENHANCES CROSS-PRIMING OF CD8+ T CELLS BY DENDRITIC CELLS FOR CANCER VACCINATION

Abstract

Despite the efficacy of chemotherapy in eliminating cancer cells, a high percentage of patients eventually relapse or develop progressive diseases. Therapeutic cancer vaccination is designed to stimulate a tumor-specific adaptive immune response that eradicates residual cancerous cells after chemotherapy. However, the adverse side effects of chemotherapy often impede the therapeutic effects of immunotherapy. Many people with cancer pursue natural products that have been empirically used to harness cancer immunosurveillance by activating immune effector cells. However, these products have not been incorporated into conventional practice in oncology because of insufficient scientific evidence on their efficacy and safety. Lunasin, a 43-amino acid peptide, was originally isolated from soybeans. We discovered a novel function of lunasin as a vaccine adjuvant, which enhanced the development of protective immune responses to soluble vaccine antigens. The objectives of this study are to elucidate the immune mechanisms mediated by lunasin, and to determine the possible utility of lunasin in cancer vaccination. We determined the activation phenotypes and functions of DC subsets following lunasin stimulation. Different DC subsets responded to lunasin stimulation as evidenced by increased levels of MHC II and CD86. Lunasin enhanced cross-presentation of soluble antigens by CD11c+DCs and CD8α+DCs, resulting in effective priming of antigen-specific CD8+ T cells. Immunization with a whole-tumor vaccine plus lunasin led to nearly 100% protection against tumor growth in a syngeneic B-lymphma model. In addition, incorporation of lunasin in this B-lymphoma vaccine prevented tumor recurrence after chemotherapy. These results suggest the potential for lunasin as an adjunct to immunotherapy. Lunasin-based vaccination may improve antitumor immune responses in cancer patients after chemotherapy.

3.1 Introduction

Diffuse large B cell lymphoma, the most common subtype of Non-Hodgkin's lymphoma (NHL), is usually treated with CHOP-based chemotherapy. High-dose chemotherapy and autologous stem cell transplantation are the treatments of choice for eligible patients with aggressive NHL who relapse after conventional chemotherapy. Unfortunately, about 40% of NHL patients relapse and exhibit progressive lymphoma between 3 and 12 months post-transplant (Freedman et al., 1993). Currently, there is no proven approach that can increase the complete response rate for NHL patients following high-dose chemotherapy or stem cell transplantation. Thus, it is imperative to develop therapeutic strategies to improve the clinical outcomes in patients with recurrent aggressive NHL.

Cancer immunotherapy is designed to enhance the antitumor immunity and is most effective when the tumor burden is minimal. Thus, it is often given to patients in combination with or after chemotherapy. However, chemotherapy contributes to adverse side effects that impede the efficacy of immunotherapeutic approaches (Lupov et al., 2011). Our previous studies have demonstrated that lymphoma patients are refractory to clinical immunotherapy because of chemotherapy-induced immune dysfunctions that impair immune defense mechanisms (Lupov et al., 2011). Thus, it is prudent to develop an efficacious immunotherapy that overcomes immune dysfunction to enhance antitumor immunity for cancer patients who are most often immune compromised.

Therapeutic cancer vaccination is an alternative strategy that is designed to stimulate tumor-specific adaptive immune responses that eradicate residual tumors from cancer patients. Development of tumor-specific CD8+ Cytotoxic T lymphocytes (CTLs) requires successful cross-priming by potent antigen presenting cells such as dendritic cells (DCs) (Schulz and Reis e Sousa, 2002). Adjuvants are often required in conjunction with tumor antigens to induce potent immunity specific to the tumor antigens. Bacterial products that stimulate the toll-like receptor (TLR) pathway are known to induce DC maturation and promote the cross-presentation of engulfed antigens (Nair-Gupta and Blander, 2013). Enhancing cross-presentation activity of DCs has been shown to induce long-lasting T cell immune response, leading to tumor eradication (Faure et al., 2009).

Nonetheless, it remains a challenge to develop a potent and safe adjuvant capable of inducing effective antitumor immune responses particularly in heavily treated cancer patients who have immune dysfunction.

We recently discovered a novel function of lunasin as an immune modulating agent (Chang et al., 2014, Tung et al., 2014). Lunasin, a naturally occurring seed peptide containing 43 amino acids, was originally isolated from soybeans (Galvez and de Lumen, 1999). We found that lunasin stimulation has resulted in DC maturation and activation, and that lunasin is effective as a vaccine adjuvant against B-cell lymphoma in a mouse model (Tung et al., 2014). The objective of this study is to further determine the possible utility of lunasin in lymphoma immunotherapy and to understand the lunasin's mechanism for promoting antigen-specific CD8+ T cells response. Here, we demonstrated that lunasin is capable of activating different DC subsets, which enhance cross-priming of CD8+ T cells specific to tumor antigens. We also demonstrated the effects of lunasin on a whole-tumor vaccine to prevent B-lymphoma relapse after chemotherapy in a mouse model.

3.2 Materials and Methods

Antibodies, lunasin peptide, and other reagents

Fluorochrome-conjugated monoclonal antibodies to mouse CD4, CD8α, CD11c, CD86, MHC II, granzyme B, IFNγ and IL-4 were obtained from BD Biosciences (San Jose, CA) or Biolegend (San Diego, CA). The lunasin peptide with 43-amino acid was chemically synthesized with 97% purity by LifeTein (South Plainfield, NJ) as previously described (Chang et al., 2014). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Biolegend. Etoposide, Cyclophosphamide, Lipopolysaccharide (LPS from Escherichia coli 0111:B4), and Concanavalin A (Con A) were from Sigma-Aldrich (St. Louis, MO). Ovalbumin (OVA, chromatographically purified) was from Worthington Biochemical Corp (Lakewood, NJ). Imiquimod (R837) was from Invivogen (San Diego, CA)

In vitro cross-priming of naïve CD8+ T cells by DCs

Fresh CD11c+ or CD8α+ DCs were isolated from the spleens of C57BL/6 mice using CD11c+ microbeads or CD8+ Dendritic Cell Isolation Kits, respectively (Mitenyi Biotec, Auburn CA). DCs were incubated with medium only (medium), soluble OVA antigens at 25 μg/ml with or without lunasin or with LPS at 1μg/ml for 18 hours. These DCs were washed, fixed with paraformaldehyde and co-cultured with CFSE-labeled OT-I cells at a ratio of 1:2 for CD11c+ or 2:1 for CD8α+ DCs. Proliferation of OT-I cells and intracellular staining of IFNγ were measured 72 hours later by flow cytometry.

Antigen uptake and process

DQ-Ovalbumin (DQ-OVA) (Life technologies, Grand Island, NY) is a self-quenched fluorescent dye conjugated of OVA, which exhibits bright green fluorescence after proteolytic degradation (de Brito et al., 2011). CD11c+ DCs isolated from C57BL/6 mouse splenocytes were incubated for the indicated times with DQ-OVA at 37°C or at 4°C and were stained with APC-conjugated CD8α. The expression level of green fluorescence was analyzed by flow cytometry (FACS Calibur APC, BD Biosciences).

Western blotting

Purified human cDCs isolated by BDCA1 microbeads were stimulated for 30 mins or 4 hours. Western blot analysis was performed from total protein extracts of cultured cDCs to measure the activation of STAT1 using an anti-phospho-STAT1 (pY701) antibody (BD biosciences), and the same blot was reprobed with an anti-STAT1 monoclonal antibody (Cell Signaling Technology, Danvers, MA) for the total amount of STAT1. Activation of NF-κB was analyzed using anti-phospho-NF-κB p65 (Ser536 or Ser 529) monoclonal antibodies and anti-NF-κB p65 monoconal antibodies for total amount of p65. Activation of MAPK was analyzed using anti-phospho p38 MAPK (T180/Y182), anti-phospho ERK1/2 (T202/Y204), anti-total p38MAPK and anti-total ERK1/2 monoclonal antibodies.

Immunization studies

Whole tumor vaccine model: A20-OVA cells were treated with etoposide for 1 day to induce apoptosis. Lunasin or PBS was incorporated into apoptotic A20-OVA cells as a whole-tumor vaccine. BALB/c mice (6-7 week-old) were subcutaneously immunized twice on the left flank on days 1 and 7 (in some experiments, mice were immunized only once as indicated). These immunized mice were challenged subcutaneously on the right flank with live A20-OVA cells (1 x 10^6) 7 days following the last immunization. Tumor volumes were measured as described (Tung et al., 2014) from day 13 after tumor injection through day 23.

Chemotherapy-cancer vaccine model: BALB/c mice (female and male, 6-7 week-old) were subcutaneously (sc) challenged with $2x10^6$ cells of live A20-OVA on the right flank. Once the tumor sizes reached 5mm in diameter at day 10, all mice received an intraperitoneal (ip) injection with chemotherapeutic drug cyclophosphamide (Cy) in a cytotoxic dose (100mg/kg). After two weeks, Cy-treated mice were randomly allocated into groups that received PBS or etoposide-treated A20-OVA mixed with or without lunasin at days 24 and 31. Tumor volumes were monitored from day 22 after tumor inoculation through day 42.

Statistical analysis

SAS/STAT (SAS Institute Inc, Cary, NC) was used to analyze the data. A mixed model was developed for analyzing the data with within-subject treatments, and the pairwise comparisons among the treatments were performed to determine the P values.

3.3 Results

Effect of lunasin on different subsets of DCs

To investigate the effects of lunasin on different subsets of DCs, CD11c+ DCs were isolated from mice and stimulated with lunasin at different concentrations for 24 hours. TLR4 ligand, lipopolysaccharide (LPS), was used as a positive control to activate CD11c+ DCs (Palha De Sousa et al., 2010). CD11c+ DCs can be separated into $CD11^{med}CD8\alpha^{-}$, $CD11c^{high}CD8\alpha^{-}$ and $CD11^{+}CD8\alpha^{+}$ DCs, and the percentage of these populations among total CD11c+ DCs are 41.6±5.5%, 33.4±2.5% and 16.3±1.3%, respectively (Figure 3.1A). The expression levels of MHCII and CD86 from these subsets of DCs were evaluated using flow cytometry. Each population has different levels of MHCII and CD86 expression (Figures 3.1B and 3.1C, left panel). LPS stimulation enhanced the surface levels of MHCII and CD86 in all the CD11c+ DC subsets (Figures 3.1B and 3.1C, right panel) and $CD11^{+}CD8\alpha^{+}$ DCs had a stronger response to LPS compared to the other subsets. Higher expression levels of MHCII and CD86 were detected in all DC subsets treated with lunasin as compared to medium only (Figures 3.1B and 3.1C, right panel). These results reveal lunasin's stimulatory effects on different subsets of DCs by increasing levels of surface MHCII and CD86.

Lunasin stimulation enhances cross-priming of OT-I cells by CD11c+DCs

Our previous study found that lunasin was effective as a vaccine adjuvant that enhanced the CD8+ T cell responses to immunization with vaccine antigens in mice. Successful priming of CD8+ T effectors specific to soluble antigens such as OVA requires cross-presentation of these antigens by DCs. To demonstrate the effects of lunasin on cross-priming activity, we performed an in vitro assay in which CFSE-labeled CD8+ T cells from OT-I mice were co-cultured with CD11c+ DCs that were previously incubated with OVA in the presence or absence of lunasin. CD8+ T cells from the OT-I transgenic mice express TCRs specific for an MHC class I ($H\text{-}2K^{b}$) restricted epitope of OVA (SIINFEKL peptide, residues 257-264) (Clarke et al., 2000). Activation of OVA-specific CD8+ T cells (or OT-I cells) was then assessed by measuring their proliferation and production of IFNγ after 3 days of culture (Figure. 3.2). Proliferation of OT-I cells

was indicated by the dilution of CFSE as shown in the panel A. In the absence of OVA antigens in DC cultures, there was little proliferation with approximately 3% of CFSE-diluted OT-I cells from gated CD8+ T cells. In the presence of OVA, lunasin stimulation increased the proliferation of OT-I cells showing approximately 50% of CFSE-diluted cells (Figure 3.2A). The production of IFNγ by proliferated OT-I cells was determined using intracellular staining (Figure 3.2A). Approximately 9% of OT-I cells were CFSE-low and positive for IFNγ expression induced by OVA-cultured DCs, and the percentage of OT-I cells that are CFSE-low and IFNγ-positive was increased in the presence of lunasin or LPS. The levels of secreted IFNγ by OT-I cells were determined using ELISA as shown in the Figure 3.2C. The ELISA results also correlated with the intracellular staining, whereby increased levels of IFNγ were found in the OVA-cultured DCs when lunasin or LPS was present.

Among different subsets of DCs, it was known that CD8α+ DCs are more efficient in phagocytosis of dead cells and in presentation of antigens via the MHC class I (Shortman and Heath, 2010). Given the fact that lunasin has the ability in enhancing cross-presentation ability by CD11c+ DCs, we then studied whether lunasin could also enhance this ability by CD8α+DCs using the in vitro assay described in the Figure 2. It was found that lunasin-treated CD8α+ DCs stimulated a greater proliferation of OVA-specific CD8+ T cells and a higher IFNγ production than DCs cultured with OVA alone (Figure 3.3). Overall, these results demonstrate that lunasin can enhance the cross-presentation ability of CD11c+ DCs, including CD8α+ DCs, resulting in promoted cross-priming of antigen-specific CD8+ T cells.

Lunasin stimulation enhances antigen processing by CD8α+ DCs

To define which step of cross-presentation is enhanced by lunasin, we next evaluated the effects of lunasin on processing of internalized antigens by mouse DCs. DQ-OVA exhibits green fluorescence upon internalization and proteolytic degradation inside the cells, and was used in this study (de Brito et al., 2011). After 15 minutes of incubation, the intensity of green fluorescence (DQ-OVA green) appeared to be similar to the CD8α+ DCs among different treatments. However, after 60 minutes of incubation, the

green fluorescent intensity was increased when CD8α+ DCs were incubated with lunasin compared with untreated DCs. The intensity of DQ-OVA green fluorescence was similar in CD8α+ DCs treated with lunasin or LPS (Figure 3.4). These results demonstrate the effects of lunasin in enhancing antigen processing upon internalization by CD8α+DCs.

Lunasin induces Type I IFN expression and STAT1 activation in DCs

Type I interferons play a role in retaining antigens in endocytic compartments, which are essential for cross-presentation by CD8α+DCs (Lorenzi et al., 2011). In addition, signals from type I interferons to CD8α+DCs are required for cross-priming antitumor CD8+ CTLs (Fuertes et al., 2011). To delineate the immune mechanism by which lunasin enhanced the cross-priming, we next analyzed the ability of lunasin in inducing type I IFNs by DCs. We found that lunasin treatment in cDCs indeed induced the expression of *IFNA1* (Figure 3.5A).

In response to type I interferons, signal transducer and activator of transcription 1 (STAT1) becomes phosphorylated, and then it forms a heterodimer with STAT2, which binds to the Interferon-Stimulated Response Element (ISRE) promoter and induces the expression of interferon stimulated genes (ISGs) (Ivashkiv and Donlin, 2014). To determine whether STAT1 was activated in DCs treated with lunasin, we performed Western blot to measure the phosphorylation of STAT1. We observed an increased phosphorylation of STAT1 (P-STAT1) at tyrosine 701 (Y701) following lunasin stimulation for 6 hours, but not 30 minutes (Figure 3.5B). Activation of STAT1 was maintained for 24 hours following lunasin stimulation (data not shown). These results demonstrate the activation of STAT1 in DCs by lunasin.

Lunasin-mediated transcriptional network for gene regulation

To further understand the molecular mechanisms of lunasin on DC activation, we performed Western blot to evaluate the transcription factors that may be involved in the gene regulation by lunasin. Given the fact that lunasin strongly activates the expression of pro-inflammatory cytokines such as IL-6 and IL-1β (Tung et al., 2014), we investigated the activation of transcription factors, including NF-κB and MAPK, which are known to

be involved in expression of these genes. Human cDCs were stimulated with lunasin or LPS, and activation of NF-κB and MAPK (p38 and ERK1/2) was analyzed using Western blot. The levels of phosphorylated NF-κB p65 at Serine 529 and Serine 536 detected in cDCs incubated with LPS or lunasin were increased in comparison to the levels detected in DCs cultured in medium alone (Figure 3.6A). In contrast, the levels of total NF-κB p50 or p65 were not affected in DCs incubated with LPS or lunasin. For MAPK, the levels of phosphorylated p38 MAPK at T180/Y182 were increased in DCs incubated with LPS or lunasin. The phosphorylated ERK1/2 remained unchanged in DCs cultured in medium or lunasin, but it was increased by LPS stimulation (Figure 3.6B). Results from Western blot analysis indicated that activation of NF-κB and p38 MAPK (but not ERK1/2) was induced in DCs stimulated with lunasin.

Effects of lunasin as an adjuvant in a whole-tumor vaccine model

To evaluate the effects of lunasin on enhancing cross-priming of CD8+ T effectors in vivo, we utilized a whole tumor vaccine model in which mice were immunized with apoptotic B-lymphomas using the OVA-expressing A20 cell line (A20-OVA). Etoposide was used to induce apoptosis of A20-OVA cells in vitro. Tumor growth was evaluated in these mice following a challenge with live A20-OVA cells. We found that tumor volumes were smaller in mice immunized with the etoposide-treated A20-OVA cells (A20) compared to those receiving PBS (Figure 3.7). In the presence of lunasin, this whole-tumor vaccine conferred 90% protection and resulted in the strongest inhibition of tumor growth in mice (Figure 3.7).

Effects of lunasin for a whole-tumor vaccine to prevent B-lymphoma relapse after chemotherapy in a mouse model

To further define a practical application of lunasin in lymphoma immunotherapy, we examined the effects of lunasin in a B-lymphoma vaccine to prevent relapse after chemotherapy. Tumor bearing mice were treated with cyclophosphamide at a dose that has been shown to largely reduce the tumor burden (Motoyoshi et al., 2006), but which is followed by tumor progression and eventual relapse (Ding et al., 2014). After 2 weeks,

these Cy-treated mice received therapeutic B-lymphoma vaccination or vehicle control (Figure 3.8A). Incorporation of lunasin into the whole-tumor vaccine (A20+Lu) effectively inhibited tumor growth and resulted in 90% animal survival, while only 20 and 70% survival was observed in mice receiving PBS and A20 vaccination, respectively (Figures 3.8B and 3.8C). The development of effector T cells was evaluated from spleens of these mice at the termination day. In correlation to the higher animal survival, more effector CD8+ T cells that were positive for IFNγ expression were observed in mice receiving A20+Lu compared to the other 2 groups (Figure 3.8D).

3.4 Discussion

In the current study, we defined the potential mechanisms of lunasin on promoting antigen specific CD8+ T cell responses through enhanced cross-presentation activity on different subsets of DCs, including CD8α+ DCs. Increased phosphorylation of STAT1, NF-kB and p38 MAPK was found in DCs treated with lunasin, suggesting that lunasin may mediate a transcriptional network for gene regulation that activates DCs. Immunization with apoptotic B lymphomas and lunasin conferred nearly 100% tumor regression against subsequent challenge. Furthermore, lunasin incorporated in a therapeutic cancer vaccine was effective to prevent relapse after chemotherapy in an immunodeficiency model. Collectively, these results suggest that lunasin works as a potential vaccine adjuvant that enhances cross-presentation activity in DC subsets and promotes antigen-specific immune response in murine models.

Cross-presentation is the ability of antigen presenting cells (APCs) to present exogenous antigens on MHC class I molecules. This ability is critical for the development of antigen-specific CD8+ T cells against tumor antigens that do not infect APCs directly (Mouries et al., 2008). Upon internalization, engulfed antigens must be processed into functional peptides that are subsequently loaded to MHC Class I molecules. Presentation of soluble antigens to MHC class I molecules is likely mediated via the cytosolic or endosomal pathway (Joffre et al., 2012). Although DCs are known to have a superior ability in cross-presentation, other APCs such as plasmacytoid dendritic cells, macrophages and B cells are also capable of cross-priming for CTL induction when

these APCs are activated properly (den Haan and Bevan, 2002, Ke and Kapp, 1996, Kovacsovics-Bankowski et al., 1993). Adjuvants have been shown to activate the machinery for endosomal trafficking and antigen processing, which are involved in the successful process of cross-presentation. Plasmacytoid dendritic cells exhibit the ability of cross-presentation when they are exposed to adjuvants such as TLR-9 agonist CpG (Mouries et al., 2008). The underlying mechanisms of lunasin-mediated cross-presentation by DCs require further investigation. Nonetheless, lunasin was able to increase processing of the model antigens in CD8α+DCs (Figure 3.4), which might contribute to enhancement of cross-priming to CD8+ T cells.

Type I interferons play a critical role in DC-induced cross-priming (Lorenzi et al., 2011, Schiavoni et al., 2013, Spadaro et al., 2012). Signals from type I interferons to CD8α+DCs are required for cross-priming of CD8+ CTLs in the antitumor immune responses (Fuertes et al., 2011). STAT1 activation by host type I IFN signaling is important for DCs in cross-presentation (Helft et al., 2012). In our study, we observed IFNA expression and STAT1 activation in lunasin-cultured DCs (Figure 3.4). Phosphorylation of STAT1 was not detectable until 6 hours and maintained for 24 hours following lunasin stimulation (data not shown). These results suggest that lunasin induces production of IFNs in DCs, which activate STAT1 in an autocrine-loop.

Lunasin has shown its anti-inflammatory and anti-oxidant effects on LPS-activated human and mouse macrophages, inhibiting the release of pro-inflammatory cytokines and reactive oxygen species (ROS) (Hernandez-Ledesma et al., 2009a). It has been shown that the anti-inflammatory function of lunasin is through the inhibition of PI3-kinase/Akt-mediated NF-κB p65 activation via the interaction with αVβ3 integrins (Cam and de Mejia, 2012). Here in our study, we found that lunasin has the ability to activate NF-κB p65 and p38 MAPK pathways. Activation of NF-κB p65 and p38 MAPK leads to transcription of genes that are important for innate and adaptive immunities (Siebenlist et al., 2005). Lunasin is able to induce innate and adaptive immune responses through induction of inflammatory cytokines and chemokines by DCs, and by promoting antigen-specific responses (Tung et al., 2014), indicating that, in contrast to anti-

inflammatory effects on LPS-activated macrophages, lunasin has the ability to activate a pro-inflammatory pathway in DCs.

A variety of stimulations activate different MAPKs (Cargnello and Roux, 2011): ERKs are activated by cell growth and differentiation stimuli which promote cell proliferation; p38 MAPKs, on the other hand, are activated by environmental stress including the pro-inflammatory cytokines IL-1 and TNFα (Nagata et al., 1998). Activation of p38 MAPK but not ERK MAPK in lunasin-treated DCs suggests a distinct activation pathway from LPS-activated DCs.

The same stimulation activates different MAPKs in different cell lines or primary cells (Rao, 2001) because of differences in the expression of various signaling molecules within these cells. For example, studies show that LPS activates p38, ERK and JNK MAPKs in macrophage cell line RAW264.7; however, LPS activates p38 and ERK but not JNK MAPK in human alveolar macrophages (Carter et al., 1999). This may explain the different results of activating transcription factors from our current study to other groups because of the usage of primary cell or cell lines.

Therapeutic cancer vaccine is designed to stimulate a tumor-specific adaptive immune response that eradicates residual tumors from cancer patients (Bodey et al., 2000). Following chemotherapy, DCs undergo homeostatic expansion and largely exhibit immature phenotypes (Salem et al., 2009). Antigen presentation by immature DCs will promote immune tolerance to the tumor antigens, resulting in escape from CTL-mediated surveillance. Adjuvants are required in conjunction with tumor antigens to promote DC maturation and to induce specific anti-tumor immunity. Among the adjuvants for cancer vaccines, GM-CFS has been used in clinical studies. A multicenter Phase III trial conducted in our institution and others, however, did not show a clinical benefit for patients receiving GM-CSF together with a B-lymphoma vaccine (Levy et al., 2014). In this study, we exploited lunasin-based approaches to improve function of DCs and to enhance antigen-specific CD8+ T cell response, leading to a stronger inhibition of tumor growth using murine tumor models. Collectively, these results suggest the therapeutic potential for lunasin, which shows promises to have an impact on clinical outcomes for lymphoma patients.

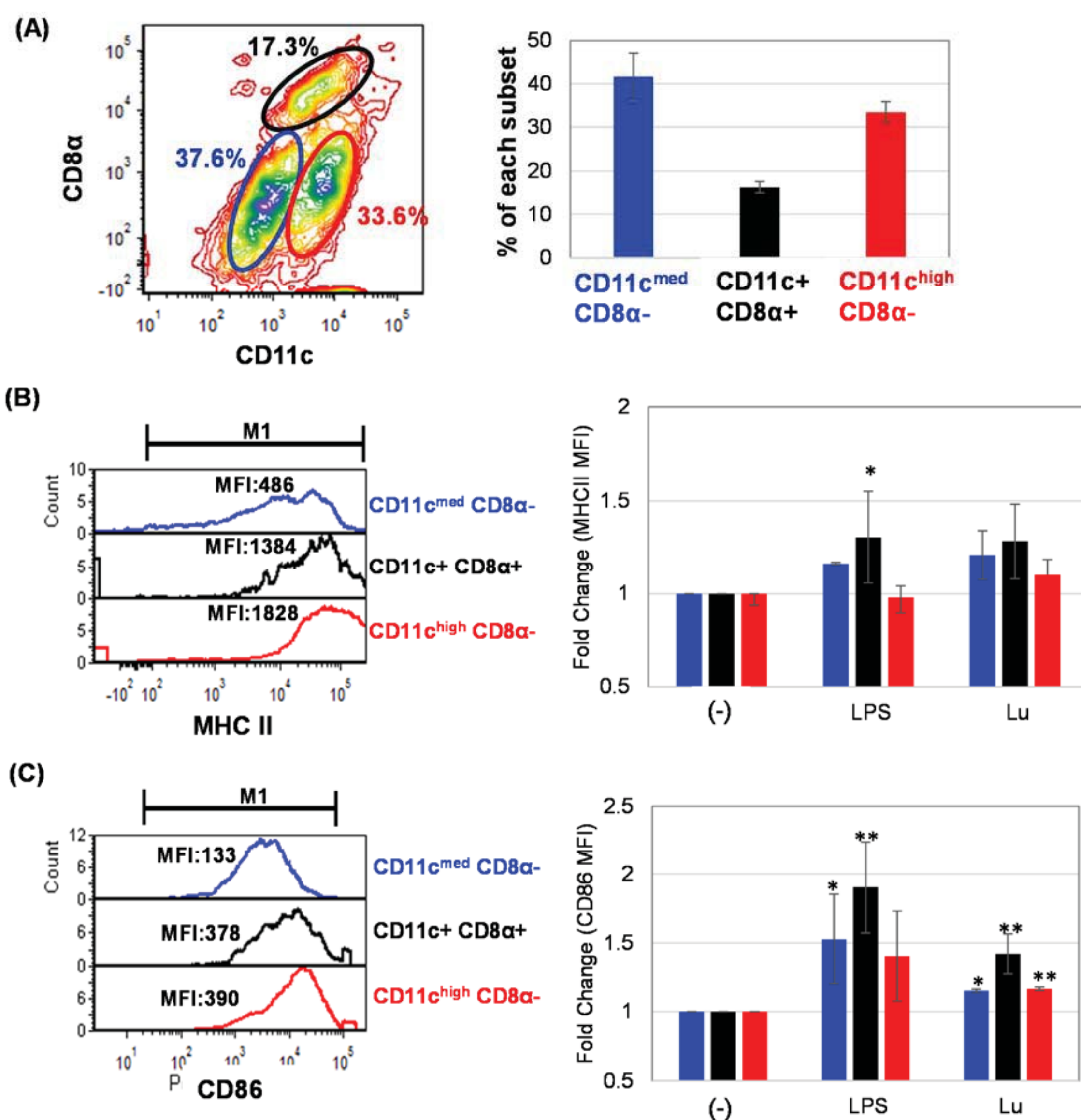


Figure 3.1 Effects of lunasin on different subsets of dendritic cells (DCs).

Freshly isolated DCs from naïve C57BL/6 mice using the CD11c+ microbeads (Miltenyi Biotec, Auburn, CA) were stimulated with medium only, LPS at 1µg/ml and lunasin peptide(Lu) at 50 µM. Twenty-four hours after stimulation, cells were surface stained with FITC-conjugated CD11c, APC-conjugated CD8α, PerCP-Cy5.5-conjugated CD86 and PE-conjugated IA/IE monoclonal antibodies. (A) Flow cytometry of DC subsets gated on CD11c and CD8α. The plot represents the gating scheme for identification of 3 major DC

subsets: $CD11c^{medium}CD8\alpha$- (blue, lower left), CD11c+CD8α+ (black, top) and $CD11c^{high}CD8\alpha$- (red, lower right). Frequency of each subset is presented as the percentage of total CD11c+DCs in the bar graph. Expression levels of MHCII (B) and CD86 (C) on different subsets of unstimulated DCs (medium only) are presented as the mean fluorescence intensity (MFI) (left panel). The fold change in MFI indicates the ratios between stimulated and unstimulated DCs (right panel). Data are presented as mean ± SD averaged from 3 mice. $^*P \leq 0.05$; $^{**}P \leq 0.01$; relative to the medium only.

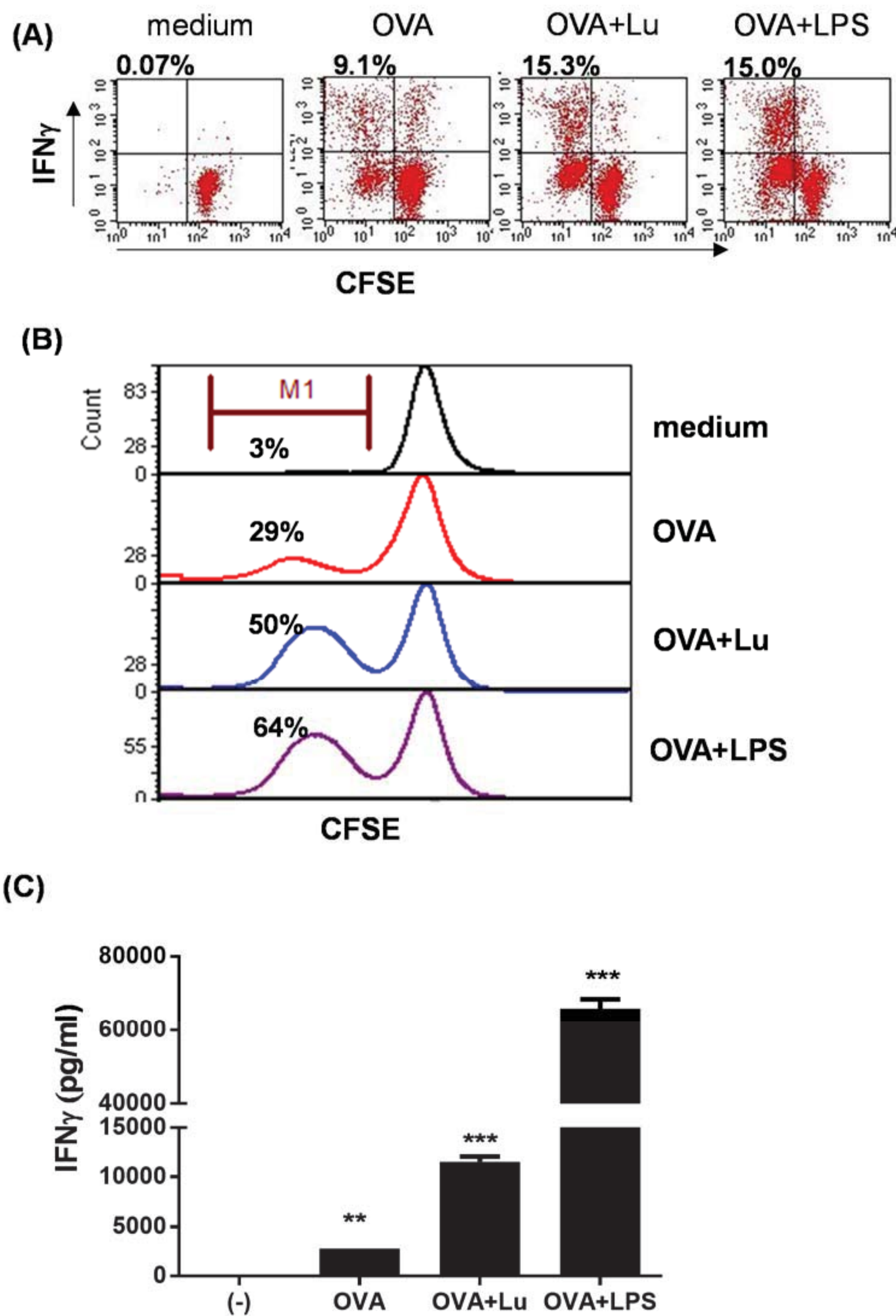


Figure 3.2 In vitro cross-priming of OT-I cells by CD11c+ DCs.

CD11c+ DCs freshly isolated from the spleens of C57BL/6 mice were incubated with medium only (-), soluble OVA antigen alone at 25 μg/ml (OVA), OVA with lunasin (lu)

at 5 μM (OVA+Lu) or OVA with LPS at 1μg/ml (OVA+LPS) for 18 hours. These DCs were washed, fixed with paraformaldehyde, and cultured with CFSE-labeled OT-I cells at a ratio of 1:2 ($1x10^5$ DCs to $2x10^5$ OT-I). Proliferation of OT-I cells and intracellular staining of IFNγ were measured 72 hours later by flow cytometry. Proliferation of OVA-specific OT-I cells is measured by the percentage of diluted-CFSE gated on the M1 region from the total OT-I cells as shown in the histogram (A). Representative dot plots of intracellular IFNγ on diluted-CFSE populations are shown in (B). IFNγ production by OT-I cells in the supernatants collected after 3 days of culture was evaluated using ELISA (C). Data are presented as mean ± SD averaged from 3 mice. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001, relative to the medium only.

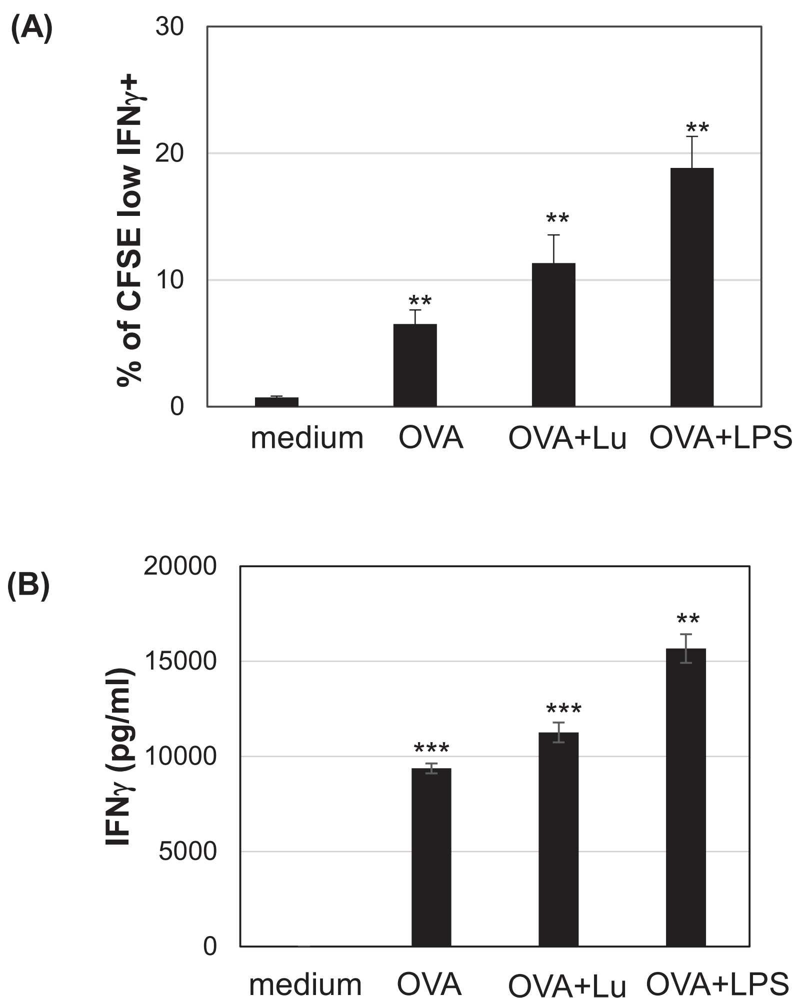


Figure 3.3 In vitro cross-priming of OT-I cells by CD8α+ DCs.

CD8α+ DCs were freshly isolated from the spleens of C57BL/6 mice. CD8α+ DCs were incubated with medium only (medium), soluble OVA antigen alone at 25 μg/ml (OVA), OVA with lunasin (lu) at 5 μM (OVA+Lu) or OVA with LPS at 1μg/ml (OVA+LPS) for 18 hours. These DCs were washed, fixed with paraformaldehyde and co-cultured with CFSE-labeled OTI cells at a ratio of 2:1 ($4x10^5$ DC: $2x10^5$ T cells). Proliferation of OT-I cells and intracellular staining of IFNγ were measured 72 hours later by flow cytometry. The percentage of IFNγ+ and CFSE-low populations is labeled on the upper left quadrate of the dot plots (A). The secretion of IFNγ in the supernatants was measured using ELISA

(B). Data are presented as mean ± SD averaged from 3 mice. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, relative to the medium only.

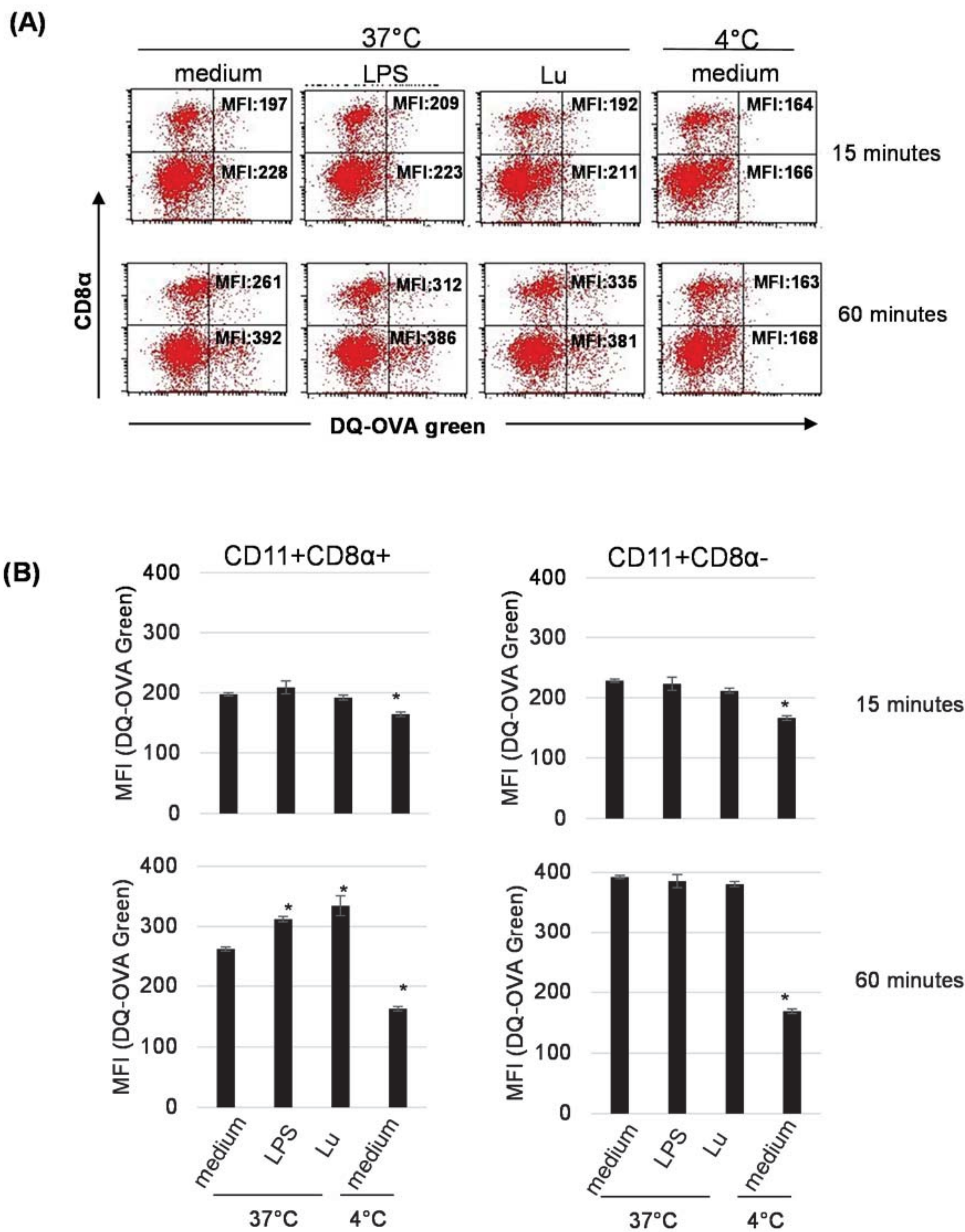


Figure 3.4 Effects of lunasin on antigen processing by mouse CD11c+ DCs.
CD11c+ DCs isolated from the spleens and lymph nodes of C57BL/6 mice were incubated with medium (medium), LPS at 1μg/ml (LPS) or lunasin at 50 μM (Lu) for 15

minutes or 60 minutes with DQ-OVA (Life Technologies, Grand island, NY) at 37°C or 4°C, and were stained with APC-conjugated CD8α. The levels of green fluorescence were analyzed using flow cytometry. A representative dot plot shows the mean fluorescence intensity (MFI) of DQ-OVA green following various treatments in CD11c+CD8α+ and CD11c+CD8α- populations (A). The averaged MFI of DQ-OVA green is presented as mean± SD from 3 different experiments (B). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$, relative to the medium only at 37°C.

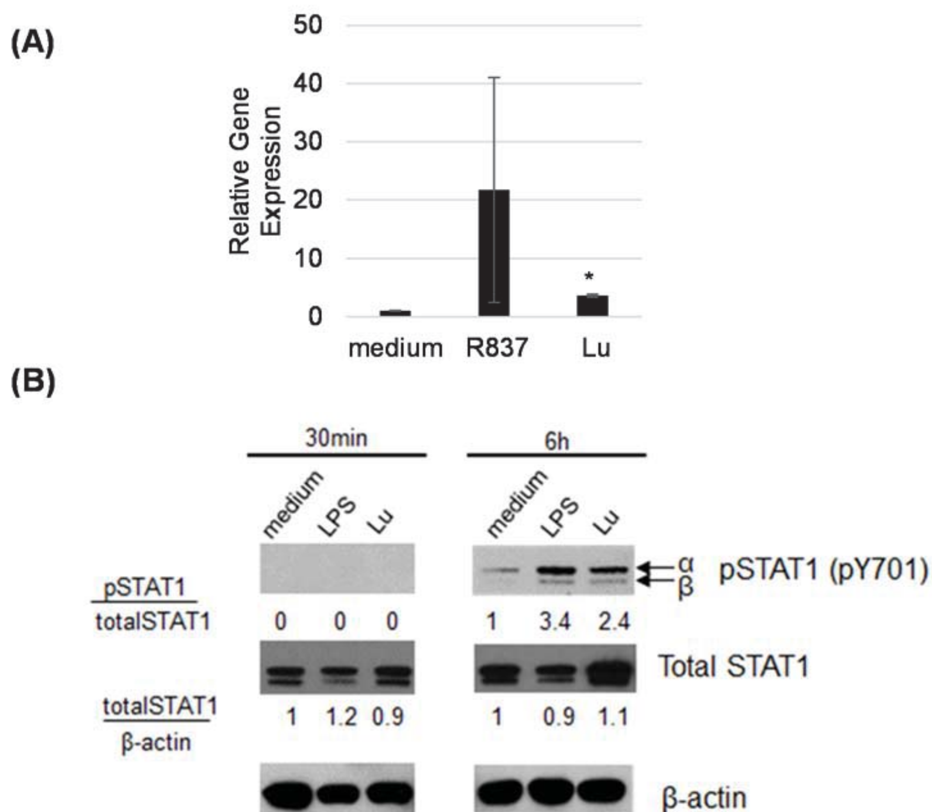


Figure 3.5 Type 1 IFNs expression and STAT1 activation in lunasin-cultured DCs

Freshly isolated human cDCs from peripheral blood mononuclear cells (PBMCs) of normal controls using the CD1c (BDCA-1) microbeads were stimulated with medium only, LPS at 1 µg/ml, or lunasin. Following 24 hours of stimulation, the cell pellets were resuspended in Trizol Reagents for total RNA extraction. The first-strand cDNA was synthesized followed by real time qPCR using Taqman Assay with primers for IFNalpha (*IFNA1*) (A) in the ABI 7300 (Applied Biosystems). Activation of phospho-STAT1 (pSTAT1) and total STAT1 were determined using Western blot of total protein extracts from cultured DCs following 30 minutes or 6 hours of stimulation. An anti-β-actin monoclonal (SC-47778) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for the loading control (B).

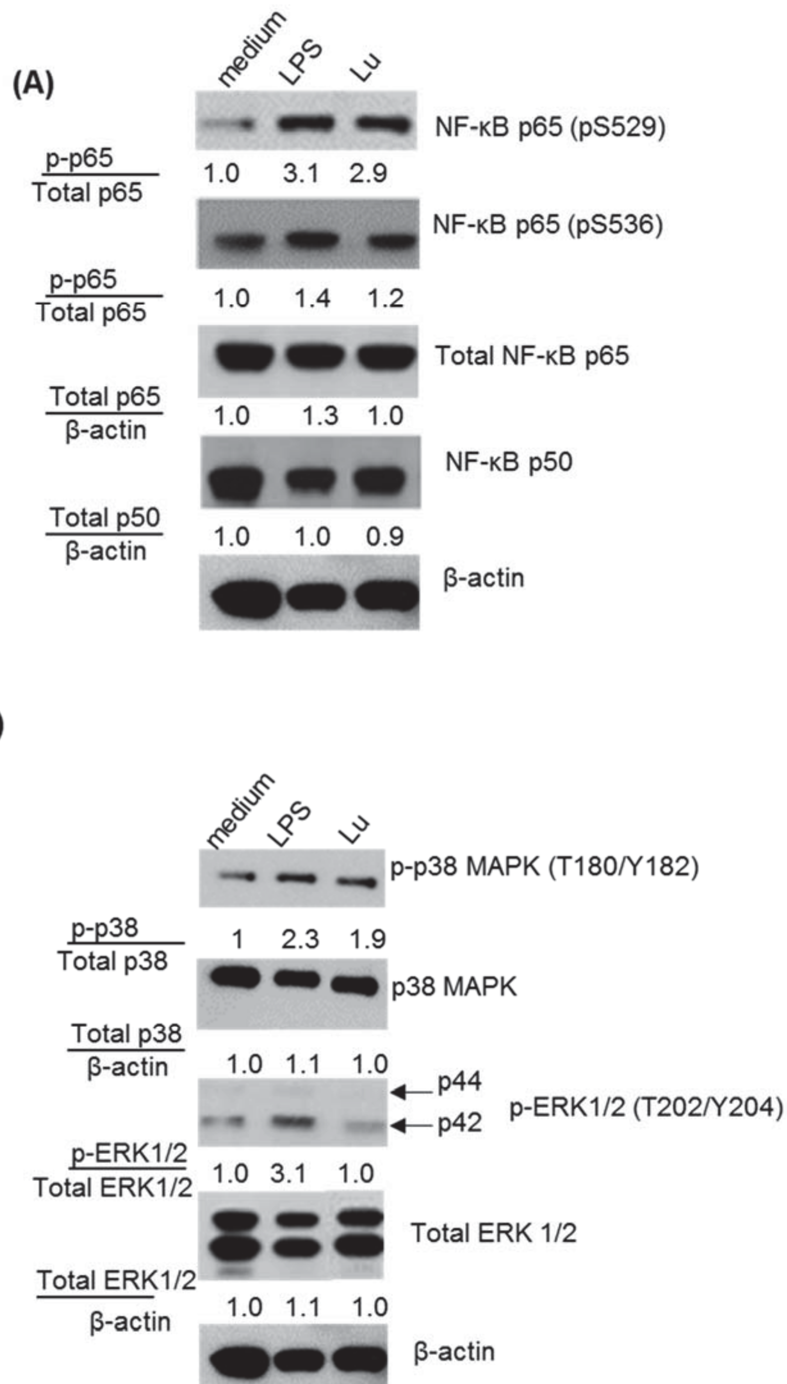


Figure 3.4 NF-κB and MAPK activation in lunasin-cultured DCs.

Freshly isolated human cDCs from peripheral blood mononuclear cells (PBMCs) of normal controls using the CD1c (BDCA-1) microbeads (Miltenyi Biotec, Auburn, CA) were

stimulated with medium only, LPS at 1 μg/ml, or lunasin (Lu) at 50 μM. Activation of NF-κB and p38 MAPK and Erk MAPK was determined using Western blot of total protein extracts from cultured DCs following 30 minutes of stimulation. An anti-β-actin monoclonal (SC-47778) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for the loading control. Results shown are representative from 2 different normal controls with similar profiles.

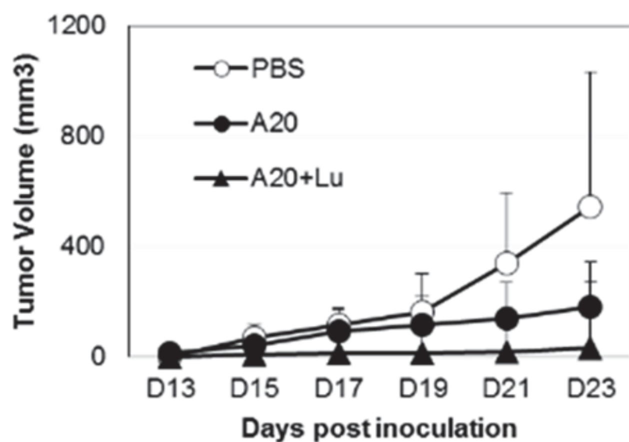
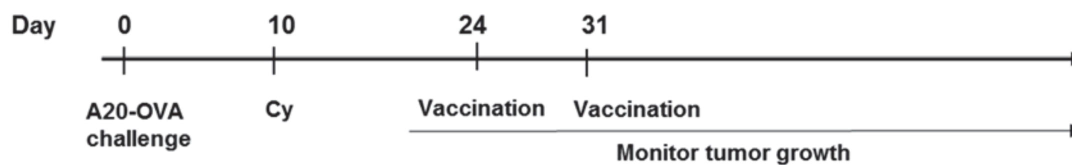
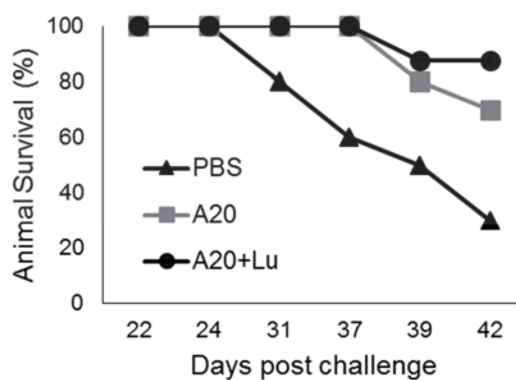
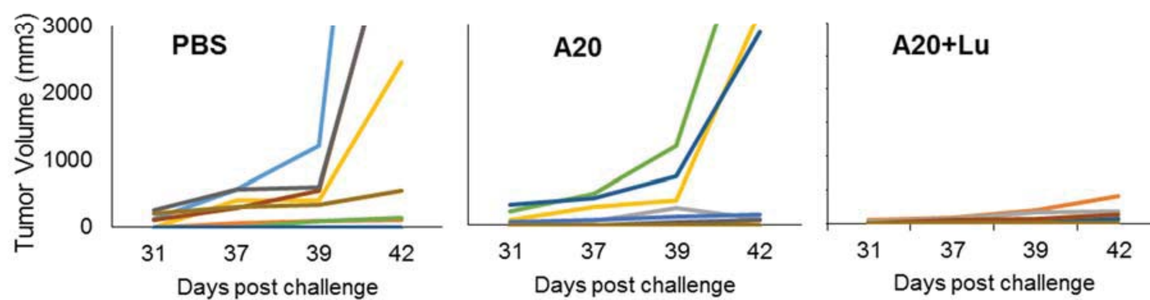


Figure 3.5 Effects of lunasin as an adjuvant in a tumor challenge model with A20-OVA B-lymphoma.

BALB/c mice (female and male, 6-7 week-old) were SC immunized twice on the left flank with PBS or etoposide-treated A20-OVA (10 x 10^6 cells) mixed without or with lunasin (100 µg per mouse) on days 1 and 7. At day 14, these mice were subcutaneously challenged with 1 x 10^6 cells of live A20-OVA. (A) Tumor volumes were measured from day 13 after tumor inoculation through day 23. Data represent the mean ± SEM from 10 mice per group.


(A)
Day
0
10
24
31
A20-OVA challenge
Cy
Vaccination
Vaccination
Monitor tumor growth


(B)
Animal Survival (%)
100
80
60
40
20
0
PBS
A20
A20+Lu
22
24
31
37
39
42
Days post challenge


(C)
Tumor Volume (mm3)
3000
2000
1000
0
PBS
A20
A20+Lu
31
37
39
42
Days post challenge

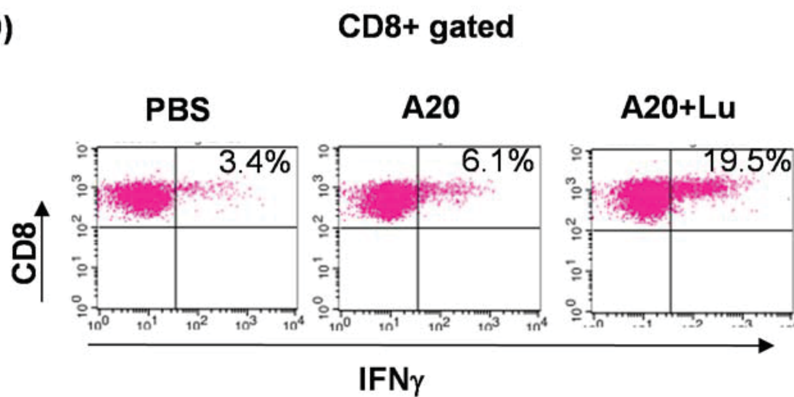

(D)
CD8+ gated
PBS
A20
A20+Lu
3.4%
6.1%
19.5%
CD8
IFNγ

Figure 3.6 Effects of lunasin as an adjuvant in B-lymphoma cancer vaccine to prevent relapse after chemotherapy in a murine model.

BALB/c mice (female and male, 6-7 week-old) were subcutaneously (sc) challenged with $2x10^6$ cells of live A20-OVA on the right flank. All mice were received intraperitoneal (ip) injection with chemotherapeutic drug cyclophosphamide (Cy) in a cytotoxic does (100mg/kg) once the tumor sizes reached 5mm in diameter at day 10. After two weeks, Cy-treated mice were randomly allocated into groups that received PBS or etoposide-treated A20-OVA ($10x10^6$ cells) mixed with (A20+Lu) or without (A20) lunasin (100µg per mouse) at day 24 and 31. Tumor volumes were monitored from day 22 after tumor inoculation through day 42. (A) Schematic diagram of vaccination schedule (A). Animal survivals were monitored from day 22 to day 42 (B). Tumor volumes from individual mouse in different vaccination groups (C). All mice were euthanized days following tumor injection. Spleens collected from these mice were processed into single-cell suspension, following by 4 hours of stimulation with PMA/Ionomycin. The cells were stained with APC-conjugated CD8 and PerCP-Cy5.5 conjugated CD4 monoclonal antibodies, washed, fixed and permeabilized. After washing, cells were incubated with PE-conjugated anti-IFNγ monoclonal antibody. Expression levels of IFNγ were evaluated using flow cytometry on 5,000 events of gated CD8 positive and CD4 negative T cell populations (D).

CHAPTER 4. ROLE OF STAT4 IN ANTITUMOR IMMUNE RESPONSES BY LUNASIN-BASED CANCER VACCINATION

Abstract

Our previous studies demonstrated that lymphoma patients were resistant to IL-12-based immunotherapy because of chemotherapy-induced immune dysfunctions associated with acquired deficiency of STAT4. STAT4 is a critical transcription factor required for the development of Th1 and IFNγ production. Given the importance of STAT4 in antitumor immune responses, STAT4 deficiency in the immune system is likely to impair not only cytokine-based immunotherapy, but any therapeutic approach that requires effective antitumor immunity. However, the role of STAT4 in a productive antitumor immunity in cancer immunotherapy remains unclear. In addition, it is not known whether or not a productive antitumor response is compromised in STAT4 deficient mice lacking Th1 immunity. To directly determine the requirement for STAT4 in response to lunasin-based cancer vaccination, a syngeneic B-lymphoma in a prophylactic model was utilized to compare the tumor growth inhibition in wild-type BALB/c (WT) mice versus STAT4 deficient (*Stat4*$^{-/-}$) mice. The development of tumor-specific cellular and humoral immune responses was analyzed. When B-lymphoma was subcutaneously implanted into STAT4 deficient mice, these mice had similar tumor growth and progression compared to that of WT mice. Immunization with etoposide-treated OVA-expressing B-lymphoma and lunasin conferred protection against challenge in WT and *Stat4*$^{-/-}$ mice. Lunasin-based whole tumor vaccination induces the development of tumor specific CD4+ and CD8+ T cells in WT and *Stat4*$^{-/-}$ mice. In conclusion, *Stat4*$^{-/-}$ mice do not exhibit accelerated subcutaneous tumor growth over WT mice following lunasin-based vaccination in a syngeneic B-lymphoma model.

4.1 Introduction

Cancer is the second most common cause of death in the United States, accounting for almost 1 in every 4 deaths. The high morbidity and mortality of this disease presents a significant economic and medical burden. Currently, chemotherapy is the mainstay of treatment for most cancer patients. Despite the effectiveness of chemotherapy in eliminating cancer cells, a high percentage of patients eventually relapse or develop further diseases (Palucka and Banchereau, 2012). Novel strategies are urgently needed to eliminate chemotherapy-resistant cancerous cells in patients who relapse after conventional chemotherapy. Immunotherapy is one of the emerging cancer therapeutic strategies that utilizes the host immune system to remove residual cancer cells after regular chemotherapy (Mellman et al., 2011). Therapeutic cancer vaccines are considered to be an active immunotherapy because the main purpose is to increase patients' own immune responses to recognize tumor antigens and destroy malignant tumor cells (Melero et al., 2014). Dendritic cells (DCs) are powerful antigen presenting cells (APCs), which constantly engulf antigens and induce antigen-specific immune responses mediated by T cells (Guermonprez et al., 2002). To generate effective antitumor immune responses, cross-presentation of tumor antigens by APCs to CD8+ T cells is a critical process (Blachere et al., 2005). Among the dendritic cell subsets, CD8α+ DCs are by far the most efficient DCs for cross-presenting soluble antigens to CD8+ T Cells (Schulz and Reis e Sousa, 2002). However, cross-presentation of tumor antigens is less efficient, and most often it results in immune tolerance in cancer patients (Gilboa, 2007). Given the important roles of DCs in antigen presentation to induce adaptive immunity, manipulating the function of DCs is one of the major targets in cancer vaccinations.

The rationale of targeting DCs in cancer vaccination is appealing; unfortunately, the adverse side effects of chemotherapy hamper the therapeutic effects of immunotherapy. In our lab, we previously found that lymphoma patients were refractory to clinical IL-12 based immunotherapy as a consequence of chemotherapy-induced immune dysfunctions associated with acquired deficiency of Signal Transducer and Activator of Transcription 4 (STAT4) (Lupov et al., 2011, Robertson et al., 2005).

STAT4 is a critical transcription factor required for Th1 and IFNγ production. Given the fact that Th1 and IFNγ are critical for eliciting cytotoxic T cell responses (Knutson and Disis, 2005), lack of STAT4 may contribute to insufficient antitumor immune responses and therefore promote residual tumor cell growth after chemotherapy. The critical role of STAT4 in Th1 cell development has been well studied; however, the role of STAT4 for a protective antitumor immunity in cancer immunotherapy remains unclear. Furthermore, it is not known whether or not a productive antitumor response is compromised in STAT4 deficient mice (*Stat4*$^{-/-}$) lacking Th1 immunity.

Lunasin, a 43-amino acid peptide found in soybeans, is known for its chemopreventive activity (Hernandez-Ledesma et al., 2013). Recently we have discovered a novel function of lunasin as an immune modulating agent that affects innate immune cells, including natural killer (NK) cells (Chang et al., 2014) and DCs (Tung et al., 2014). Lunasin activates DCs including, conventional DCs (cDCs) and plasmacytoid DCs (pDCs), and acts as a vaccine adjuvant for the model antigen OVA in the prophylactic model against OVA-expressing A20-B lymphomas (Tung et al., 2014). Collectively, these results suggest the effect of lunasin as an immune modulating agent that may circumvent existing immune dysfunctions in patients after chemotherapy and augment the antitumor immune responses in cancer immunotherapy.

In this study, we determined the requirement of STAT4 in response to lunasin-based cancer vaccination, and a syngeneic B-lymphoma in a prophylactic model was utilized to compare the tumor growth inhibition in wild-type BALB/c (WT) mice versus *Stat4*$^{-/-}$ mice. The functions of innate and adaptive immune cells from *Stat4*$^{-/-}$ mice were evaluated. The development of tumor-specific cellular and humoral immune responses was also analyzed.

4.2 Materials and Methods

Antibodies, lunasin peptide, and other reagents

Fluorochrome-conjugated monoclonal antibodies to mouse CD4, CD8α, CD11c, CD11b, PDCA-1, CD86, MHC I, MHC II granzyme B, IFNγ, and IL-4 were obtained from BD Biosciences (San Jose, CA) or Biolegend (San Diego, CA). The 43-amino acid lunasin peptide was chemically synthesized with 97% purity by LifeTein (South Plainfield, NJ) as previously described (Chang et al., 2014). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from Guava Technologies (Hayward, CA). Etoposide, Lipopolysaccharide (LPS from *Escherichia coli* 0111:B4), and Concanavalin A (Con A) were from Sigma-Aldrich (St. Louis, MO). Ovalbumin (OVA, chromatographically purified) was from Worthington Biochemical Corporation (Lakewood, NJ).

Mice, primary immune cells and cell line

Wild-type BALB/c ($H\text{-}2^d$) and STAT4-deficient ($Stat4^{-/-}$) mice on a BALB/c background were obtained from The Jackson Laboratory (Bar Harbor, ME). Mouse primary dendritic cells (DCs) were positively selected from mouse splenocytes using CD11c magnetic beads with ~90% purity (Miltenyi Biotec Inc., Auburn CA). A murine B-lymphoma cell line expressing OVA, A20-OVA ($H\text{-}2^d$), was kindly provided by Dr. Gang Zhou (Medical College of Georgia) (Ding et al., 2010).

Whole-tumor vaccination in a syngeneic B-lymphoma model

A20-OVA cells were treated with etoposide for 1 day to induce apoptosis. Apoptotic A20-OVA cells were used as a whole-tumor vaccine. Lunasin or PBS was incorporated into apoptotic A20-OVA cells as a whole-tumor vaccine. BALB/c and $Stat4^{-/-}$ mice (6-7 week-old) were subcutaneously immunized twice on the left flank on days 1 and 7. In some experiments, mice were immunized only once as indicated. These immunized mice were challenged subcutaneously on the right flank with live A20-OVA cells (1×10^6) 7 days after the last immunization. Tumor volumes were measured as described (Chang et al., 2014) from day 15 after tumor injection through day 22.

Evaluation of OVA-specific IgG production

Serum samples were collected from euthanized mice for analysis of anti-OVA IgG levels (Brimnes et al., 2003). Ovalbumin (20 μg/ml) was absorbed onto 96-well plates at 4 °C overnight. Plates were blocked with FACS buffer for 1 hour at room temperature. Serially diluted sera were added to the wells and incubated for 2 hours at room temperature. Thereafter, plates were washed and incubated with horseradish peroxidase (HRP) conjugated anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA) and washed with PBST. Subsequently, TMB (3,3',5,5'-tetramethylbenzidine) substrate (Thermo Fisher, Waltham, MA) was added and the reaction was stopped by 2M sulfuric acid. The OD values were read at 450 nm.

Recall immune responses following vaccination and challenge

Spleens were harvested to evaluate the memory T cell responses to OVA. Splenocytes were labeled with CFSE, and then cultured in vitro with OVA (100 μg/ml) for 6 days. The supernatants were collected for analysis of cytokine secretion using ELISA. The remaining cells were used for intracellular IFNγ and IL-4 staining following 4 hours of stimulation with PMA/ionomycin. Proliferation of OVA-specific CD4+ T and CD8+ T cells was determined from diluted CFSE using flow cytometry.

Phenotypic analysis of DCs from naïve mice

Spleens and lymph nodes (inguinal and axillary) were obtained from naïve BALB/c and *Stat4*$^{-/-}$ mice, and the single-cell suspensions were analyzed for the surface levels of maturation markers (MHC I, II, and CD86) followed by flow cytometry analysis.

Characterization of DCs following stimulation in vitro

CD11c+ dendritic cells freshly purified from naïve BALB/c and *Stat4*$^{-/-}$ mice were stimulated for 1 day as indicated. The expression levels of surface markers (MHCI, MHCII and CD86) on different subsets of DCs were evaluated using flow cytometry with

staining antibodies. The supernatants collected from the cultures following 1 day of stimulation were analyzed for the production of CCL3 and IFNβ using ELISA.

Responses of NK and CD8+T cells to cytokine stimulation

Splenocytes from naïve BALB/c and *Stat4*$^{-/-}$ mice were either cultured in medium alone, with hIL-2 (1000 units/ml), or with mIL-12 (5 ng/ml). Following 1 day of stimulation, the expression levels of granzyme B on NK cells and CD8+ T cells were evaluated by intracellular staining using flow cytometry (Stevenson et al., 2010).

Statistical analysis

SAS/STAT (SAS Institute Inc, Cary, NC) was used to analyze the data. A mixed model was developed for analyzing the data with within-subject treatments, and the pairwise comparisons among the treatments were performed to determine the *P* values.

4.3 Results

Impaired antitumor effects in STAT4 deficient mice in response to a single immunization with a whole-tumor vaccine against B-lymphomas

To evaluate the antitumor immune responses in *Stat4*$^{-/-}$ mice, we performed a single immunization model in which mice received a one-time etoposide-treated A20-OVA vaccination. Etoposide is a topoisomerase inhibitor that has generally been used in the regular chemotherapy regiment since 1980s. It is known to inhibit DNA synthesis and promote apoptosis in cancer cells (Baldwin and Osheroff, 2005). Phagocytosis of apoptotic cells by dendritic cells induces cross-presentation of cell-associated antigens, which generates effective cytotoxic T cell responses (Blachere et al., 2005). One of the potent cancer vaccinations uses apoptotic cancer cells as tumor antigens to elicit effective anti-tumor immunity (Henry et al., 1999). Wild type BALB/c or *Stat4*$^{-/-}$ mice received etoposide-treated A20-OVA cells subcutaneously as a cancer vaccine. One week after vaccination, live A20-OVA cells were subcutaneously injected into the opposite flank, and tumor volume was monitored daily until 30 days after challenge.

Wild type mice that received one time vaccination conferred better protection, resulting in stronger tumor inhibition as evidenced by smaller tumor volumes compared to the PBS group. On the other hand, tumor volumes were similar in *Stat4*$^{-/-}$ mice immunized with PBS or etoposide treated A20-OVA (Figure 4.1A). In correlation with the tumor growth inhibition, higher numbers of OVA-specific CD8+ T and CD4+ T cells were observed in WT mice compared to those of *Stat4*$^{-/-}$ mice (Figure 4.1B). Furthermore, higher numbers of IFNγ producing OVA-specific CD8+ T cells or CD4+ T cells were detected in WT mice than in *Stat4*$^{-/-}$ mice (Figure 4.1B). In this single vaccination model, it was found that *Stat4*$^{-/-}$ mice have impaired antitumor immune responses in tumor inhibition and have defects in the development of antigen-specific CD4+ or CD8+ T cells.

Cellular immune responses to cytokines in *Stat4*$^{-/-}$ lymphocytes

It is known that deficiency of STAT4 results in impaired IFNγ production in response to IL-12 stimulation (Morinobu et al., 2002, Kaplan, 2005). STAT4 regulates

the expression for target genes, including perforin and granzyme B in natural killer cells (Salcedo et al., 1993). Since we observed that *Stat4*$^{-/-}$ mice had impaired anti-tumor immune responses following a single immunization, we next evaluated whether the cellular immune responses to cytokines are impaired in *Stat4*$^{-/-}$ lymphocytes. Freshly isolated splenocytes were obtained from WT or *Stat4*$^{-/-}$ mice and stimulated with human IL-2 or mouse IL-12. After 1 day of stimulation, the expression of granzyme B in natural killer cells or CD8+ T cells was analyzed using flow cytometry. We found that the expression levels of granzyme B in NK cells and CD8+ T cells were comparable in *Stat4*$^{-/-}$ and WT mice in response to IL-2 stimulation (Figure 4.2A and 4.2B). As expected, NK cells and CD8+ T cells from *Stat4*$^{-/-}$ mice failed to respond to IL-12 stimulation in granzyme B production (Figure 4.2).

Functions of CD11c+ DCs in *Stat4*$^{-/-}$ mice

Effective priming of antigens to cytotoxic T cells by antigen presenting cells is an important process in generating protective anti-tumor immune responses (Palucka and Banchereau, 2012). Therefore, we evaluated the functions of DCs in *Stat4*$^{-/-}$ mice to investigate whether or not the impaired antitumor immune responses were the result of defective DC functions. Mouse DCs are heterogeneous and are sub-classified based on the surface marker expression, including CD8α+ DC and CD11b+ DCs (Shortman, 2000). Among the heterogeneous population, it is known that CD8α+ DCs are more efficient in phagocytosing dead cells and cross-presenting to naïve cytotoxic T cells (Schulz and Reis e Sousa, 2002). To evaluate the function of different subsets of dendritic cells in *Stat4*$^{-/-}$ mice, total CD11c+ cells were isolated using microbeads and incubated with DQ-OVA. DQ-OVA has a self-quenching ability upon proteolytic degradation. Upon degradation, DQ-OVA exhibits green and red fluorescence when enough fragments accumulate in the organelle (de Brito et al., 2011). It was found that the antigen process abilities in CD11c+DCs from *Stat4*$^{-/-}$ or WT mice were similar based on the expression of green fluorescence after DQ-OVA incubation (Figure 4.3A). In addition to antigen process activity, we also evaluated the expression levels of surface markers from different subsets of DCs in *Stat4*$^{-/-}$ or WT mice. The levels of co-stimulatory molecule

CD86 or surface MHCI and MHCII expression in CD8α+DCs, CD11b+DCs or PDCA1+ plasmacytoid dendritic cells were similar in both strains of mice (data not shown). The functions of CD11c+DCs in response to TLR stimulation were also evaluated by cytokine secretion. Freshly isolated mouse CD11c+ DCs from *Stat4*$^{-/-}$ or WT mice were incubated with TLR4 agonist LPS for 24 hours. LPS stimulation results in cytokine and chemokine secretion of CCL3, which is known to recruit effector cells, including T cells (Trifilo et al., 2003). It was found that CD11c+DCs from both strains of mice had similar abilities in secreting chemokines such as CCL3 upon stimulation (Figure 4.3B). Overall, the functions of *Stat4*$^{-/-}$ DCs, including antigen processing, cytokine and chemokine secretion, and surface marker expression, are similar to those of WT mice.

A prime-boost immunization with apoptotic A20-OVA cells confers protection against B-lymphomas in STAT4 deficient mice

Next, we evaluated the abilities of *Stat4*$^{-/-}$ mice in response to a prime-boost vaccination. Mice received etoposide-treated A20-OVA twice and were challenged with live A20-OVA cells one week after their last vaccination. Tumor volumes were significantly decreased in both *Stat4*$^{-/-}$ and WT mice immunized twice with etoposide-treated A20-OVA (Figure 4.4A). While the tumor volumes were similar in *Stat4*$^{-/-}$ and WT mice, the proliferation and IFNγ secretion activity in OVA-specific CD8+ T cells from WT mice were higher compared to *Stat4*$^{-/-}$ mice (Figure 4.4B and 4.4C). We also observed lower humoral immune response against OVA antigen in *Stat4*$^{-/-}$ mice compared to WT mice after vaccination (Figure 4.4D). These results demonstrated that prime-boost vaccination of tumor antigen is able to induce comparable levels of protection against B lymphoma challenge despite lower humoral and antigen-specific CD8+ immune response in *Stat4*$^{-/-}$ mice.

4.4 Discussion

In the current study, we have determined the requirement for STAT4 in cancer vaccination by using a prophylactic model with a whole-tumor vaccine. Following vaccination by a single-dose antigen exposure, *Stat4*$^{-/-}$ mice had impaired antitumor

immune responses in eliminating the subcutaneous challenge of A20-OVA B lymphoma with lower antigen-specific CD8+ and CD4+ T cell responses compared to WT mice. While a single immunization failed to confer protection against B-lymphomas in STAT4 deficient mice, tumor growth was attenuated following a prime-boost vaccination. STAT4 is critical for Th1 cell development and IFNγ production, and IFNγ is known for improving the efficacy of cancer vaccination to induce cytotoxic T cell responses (Dezfouli et al., 2003). These results demonstrate the importance of STAT4 in protection against B-lymphomas following a single immunization in a cancer vaccine murine model.

A whole-tumor cancer vaccine requires active presentation of cancer antigens to CD8+ cytotoxic T cells to elicit effective antitumor immune responses (Cicchelero et al., 2014). Upon uptake of apoptotic cancer cells, cross-present to CD8+ T cells by DCs is the critical process to induce effective antitumor immunity (McDonnell et al., 2010). Antigen presenting cells that can cross-present antigen to CD8+ T cells and CD8α+ DCs are thought to be the most efficient DCs in cross-presenting antigen to cytotoxic T cells (den Haan et al., 2000). In a rapamycin model, *Stat4*$^{-/-}$ DCs have defective alloreactivity in stimulating allogenic T cell proliferation and in inducing antigen specific CTL responses (Chiang et al., 2004), indicating the importance of STAT4 in DCs to induce antigen-specific T cell responses. In the current study, the antigen uptake and processing of soluble antigen DQ-OVA by CD8α+ and CD8α- DCs in WT and *Stat4*$^{-/-}$ mice were similar. Also, the frequencies and numbers of CD8α+ DCs were similar in *Stat4*$^{-/-}$ mice (Figure 4.3A), suggesting the DCs from *Stat4*$^{-/-}$ mice were capable of processing antigens. We have not only observed the antigen processing activity of DCs in *Stat4*$^{-/-}$ mice, but have also the functions of STAT4-defeicient DCs in response to pathogen-associated molecular pattern (PAMP) stimulation (Figure 4.3C). Overall, it was suggested that STAT4-deficient mice do not have defects in antigen processing, co-stimulatory molecule expression and chemokine expression compared to WT mice.

The prime-boost strategy is generally considered to be the most effective approach to inducing high levels of memory T cells to achieve better vaccine efficacy (Woodland, 2004). With the help of adjuvant, the immunogenicity of vaccine antigens is improved by activating DCs or enhancing local inflammatory immune responses

(Schreibelt et al., 2010). Lunasin was used as an adjuvant in combination with etoposide-treated apoptotic A20-OVA cancer vaccines. Although *Stat4*$^{-/-}$ mice had deficient antitumor immunity in the suboptimal single-dose cancer vaccination, *Stat4*$^{-/-}$ mice had similar antitumor effects in eliminating subcutaneous A20-OVA B lymphoma to WT mice in the prime-boost vaccination model (Figure 4.4A). It was found that the rejections of syngeneic mammary carcinoma were similar in WT and *Stat4*$^{-/-}$ mice (Ostrand-Rosenberg et al., 2000), suggesting that there may be compensation mechanisms in antitumor immunity in *Stat4*$^{-/-}$ mice. In addition, in terms of antigen-specific T cells, there are no differences in numbers or frequencies of lymphocytic choriomeningitis virus (LCMV) specific CD4+ and CD8+ T cells from WT and *Stat4*$^{-/-}$ mice (Mollo et al., 2013, Suarez-Ramirez et al., 2014), and *Stat4*$^{-/-}$ mice immunized with the model antigen OVA and complete Freund's adjuvant did not have a dramatic decrease in proliferative responses to OVA restimulation compared to WT mice (Kishimoto et al., 2000), indicating that STAT4 is not crucial for antigen-specific T cell maintenance and expansion.

STAT4 is required for IFNγ expression in CD8+ T cells during LCMV infection (Suarez-Ramirez et al., 2014). Similar results can be found in the current cancer vaccine model, showing that IFNγ production by antigen-specific CD8+ T cells was decreased in *Stat4*$^{-/-}$ mice compared to WT mice. It was shown that immunization of STAT4-deficient mice has a typical Th2 immune response (Kaplan, 2005); however, in this study, prime-boost of a whole cancer cell in *Stat4*$^{-/-}$ mice did not have better humoral immunity regarding anti-OVA IgG production in the serum. The detailed mechanism of this effect needs to be elucidated in the future. Despite the lower levels of anti-OVA IgG and IFNγ-producing CD4+ and CD8+ T cells in *Stat4*$^{-/-}$ mice, these mice did not exhibit accelerated subcutaneous tumor growth over WT mice following whole cell cancer vaccination in a syngeneic B-lymphoma model. This suggests that there may be other STAT4-independent pathways that promote Th1 differentiation in a cancer vaccination model.

Clinically, chemotherapy-induced acquired STAT4 deficiency has been found in lymphoma patients, and these patients failed to respond to IL-12 cytokine immunotherapy (Lupov et al., 2011). In the current study, *Stat4*$^{-/-}$ mice did not have

defects in antitumor immunity after a prime-boost vaccination; even their immune cells failed to respond to IL-12 stimulations like humans do. Researchers normally use mice as the main *in vivo* immunological experiments, since mice mirror human immune systems; however, due to the distinct evolutional divergence and different genetic backgrounds, it is unsurprising that there are significant differences between mice and humans (Mestas and Hughes, 2002). Take STAT4 activation as an example: type I IFNs can activate human STAT4 but not in mice. This is due to the minisatellite insertion of mouse *Stat2*, which changes the carboxyl terminus and then disrupts its capacity to activate STAT4 (Farrar et al., 2000). Differences in IgG subtypes can also be found in mouse and human immune systems (Snapper et al., 1999). Therefore, it is possible that a STAT4 deficient mouse could have different antitumor immunity from what we would expect in humans.

In this study, we have characterized the role of STAT4 in lunasin-based whole-tumor cancer vaccination, showing that the role of STAT4 may not be critical for antitumor immunity in the prime-boost cancer vaccination. The antitumor activity in NK cells and CD8+ T cells and functions of DCs from *Stat4*$^{-/-}$ mice are comparable to WT mice. Despite the decreased IFNγ production from antigen-specific CD8+ and CD4+ T cells, *Stat4*$^{-/-}$ mice did not have accelerated subcutaneous tumor growth over WT mice after lunasin-based cancer vaccination.

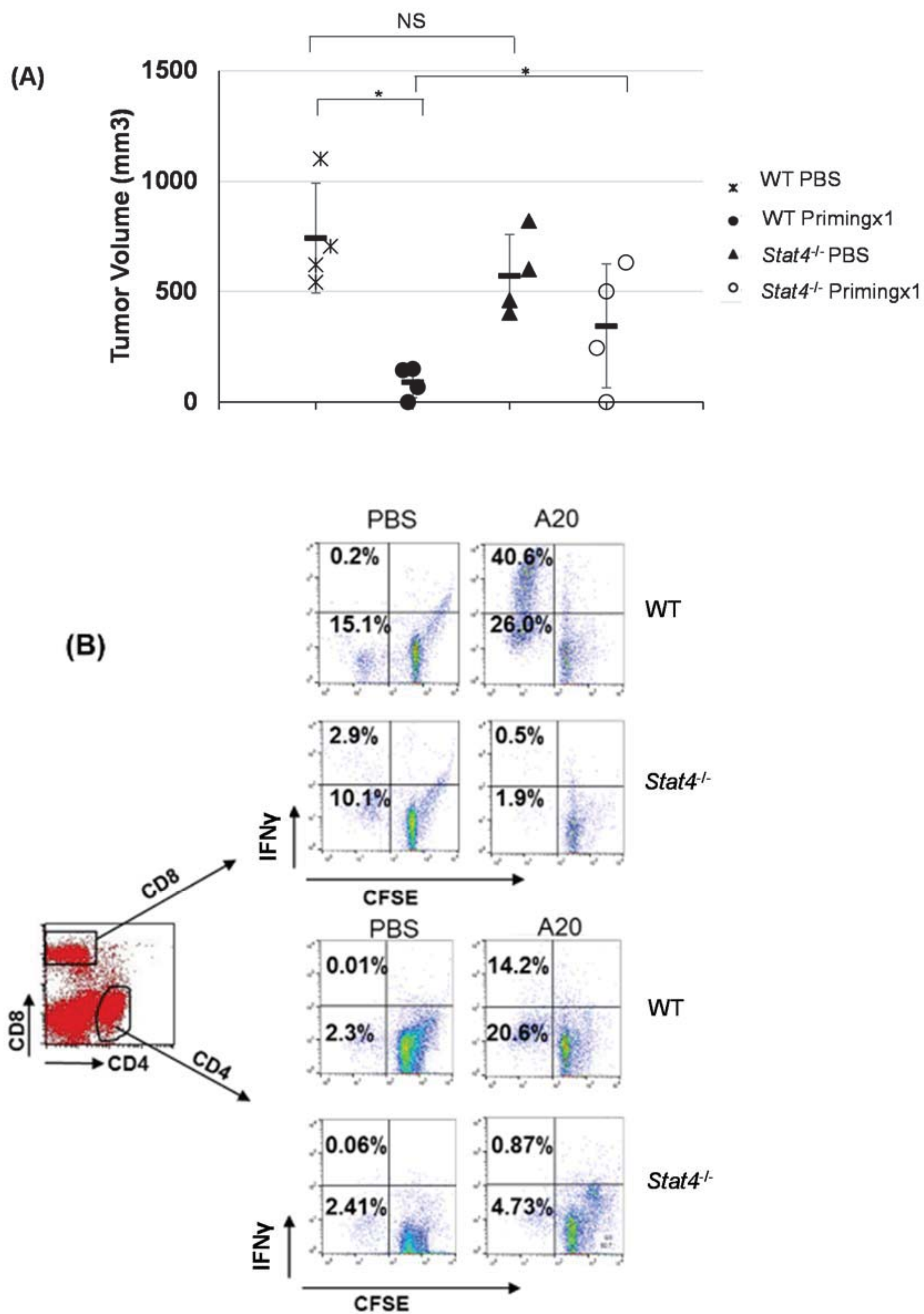


Figure 4.1 Antitumor Effects of STAT4 deficiency in response to a single immunization with a whole-tumor vaccine against B-lymphomas.

BALB/c and *Stat4*$^{-/-}$ mice (female and male, 6-7 week-old) were SC immunized one time on the left flank with PBS or etoposide-treated A20-OVA (10 x 10^6cells) on day 1. At day 7, these mice were subcutaneously challenged with 1 x 10^6 cells of live A20-OVA. Tumor volumes were measured from day 7 after tumor inoculation through day 23. Each symbol represents tumor volume from individual mouse, and the averaged tumor volumes from 5 mice per group are presented (A). All mice were euthanized 23 days following tumor inoculation. Spleens collected from these mice were processed into single-cell suspensions followed by labeling with CFSE. CFSE-labeled splenocytes were then stimulated with OVA (100 μg/ml) to induce proliferation of OVA-specific T cells. Expression of IFNγ was analyzed on gated CD8+ or CD4+ T cells as in (B). A representative histogram and dot plot show the percentages of IFNγ production from CFSE-labeled cells (B). Data are presented as mean ± SD averaged from 4-5 mice. *$P \leq 0.05$, relative to the PBS only or WT mice.

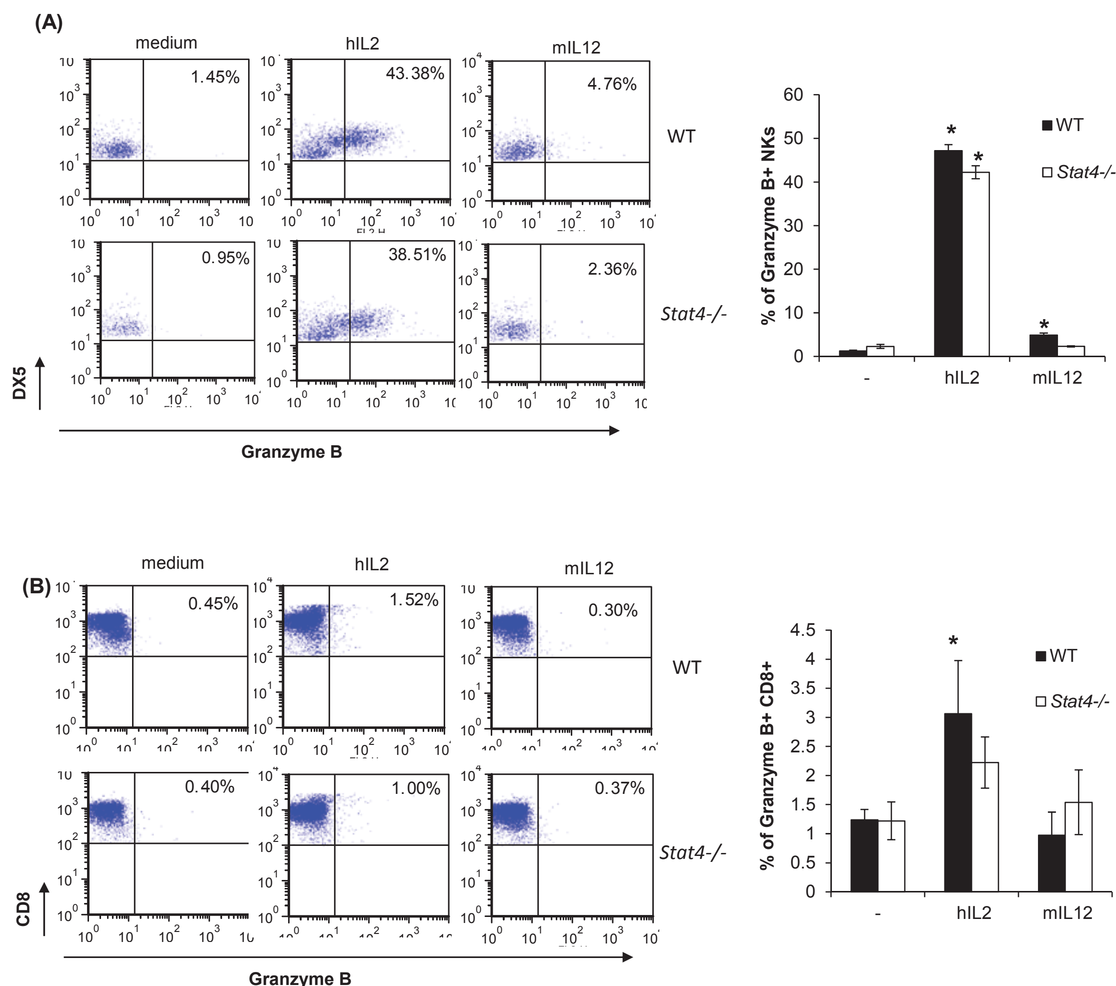


Figure 4.2 Expression of granzyme B by natural killer cells and CD8+ T cells.

Splenocytes isolated from naive BALB/c and *Stat4*$^{-/-}$ mice were stimulated with medium, human IL-2 at 1000U/ml or mouse IL-12 at 5ng/ml for 1 day. Cells were surface stained with PerCP-conjugated CD3, APC-conjugated CD8 and FITC-conjugated DX5 monoclonal antibody. The cells were washed, fixed and permeablized. After washing, cells were incubated with PE-conjugated granzyme B monoclonal antibody. Single-cell expression levels of granzyme B were evaluated using flow cytometry on 5,000 events of gated CD3 negative and DX5 positive NK cell populations (A) or CD3 positive and CD8 positive T cell populations (B). The average percentage of granzyme B producing NK cells or CD8+T cells are presented as mean ±SD from 3 different mice. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$, relative to medium only.

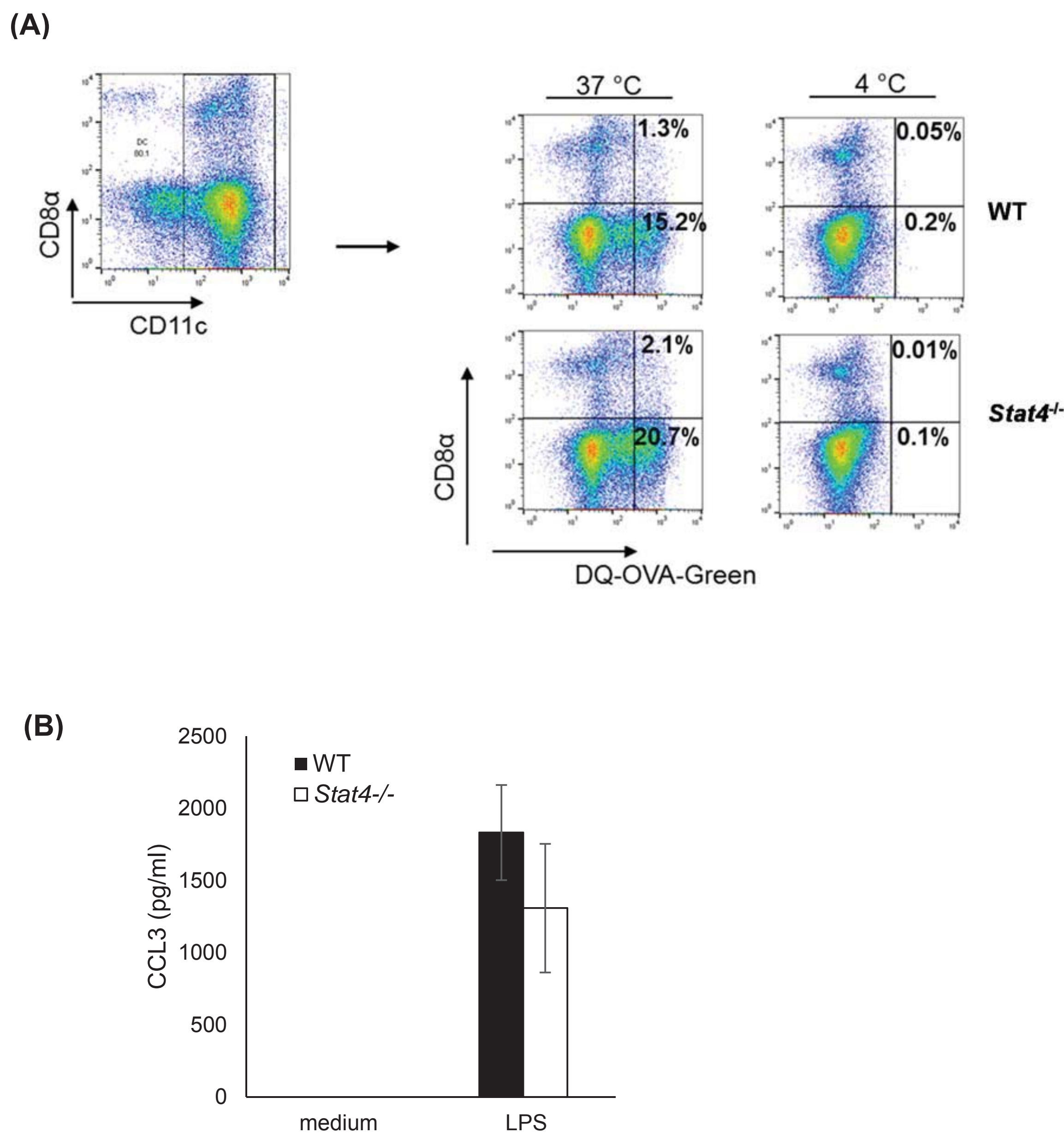
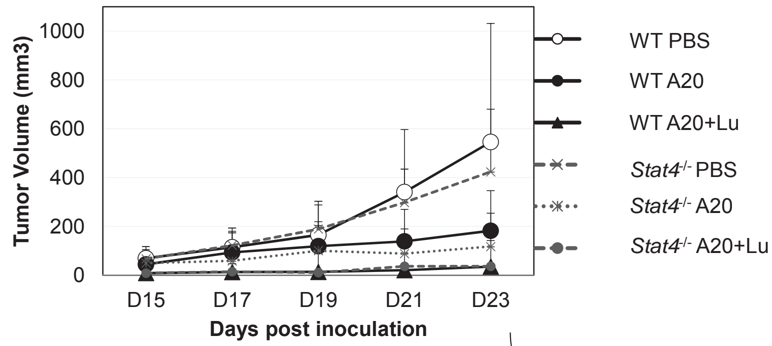


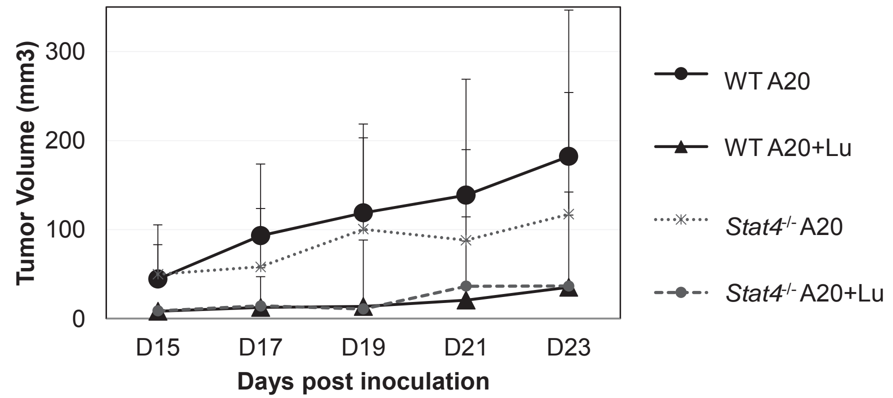
Figure 4.3 Functions of mouse CD11c+ DCs in WT and *Stat4*$^{-/-}$ mice.

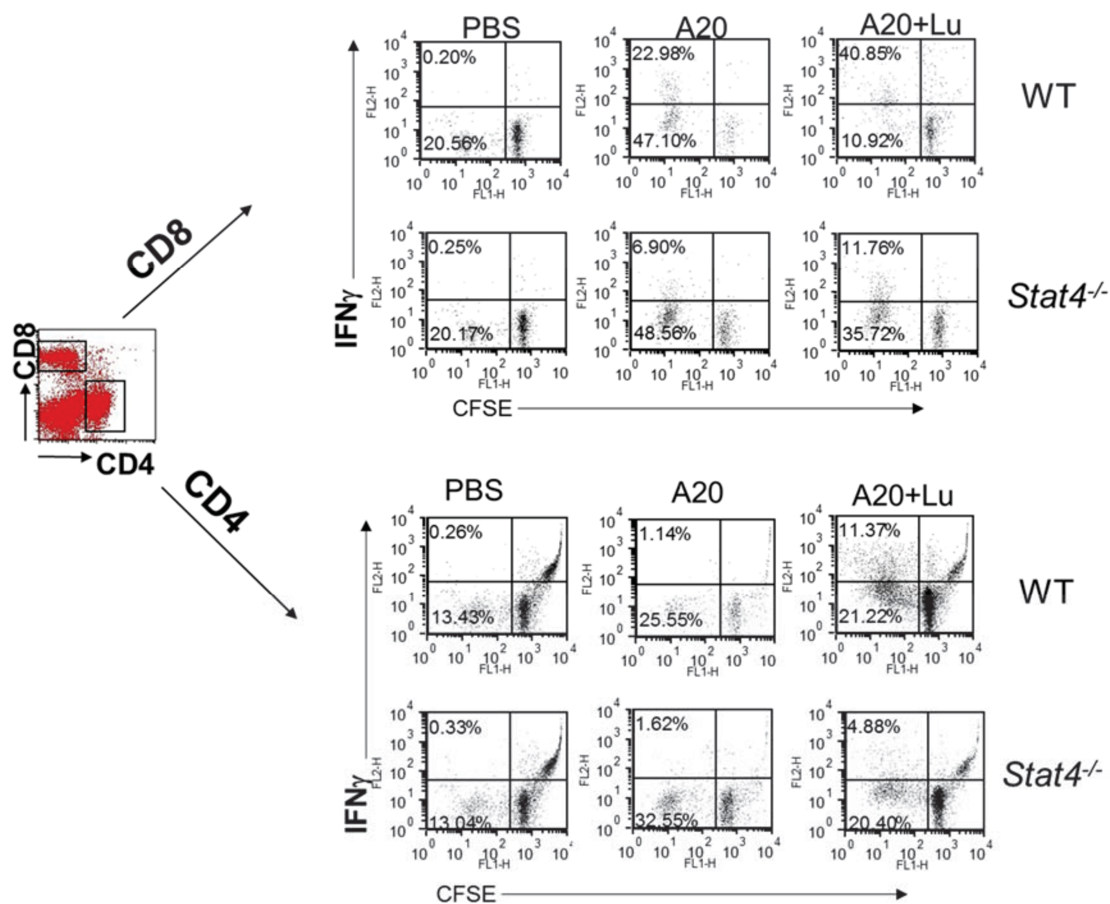
CD11c+ DCs isolated from BALB/c or *Stat4*$^{-/-}$ mouse splenocytes and single cells from lymph nodes were incubated with medium for 60 minutes with DQ-OVA (Life Technologies, Grand island, NY) at 37°C or 4°C and were stained with FITC-conjugated CD11c and APC-conjugated CD8α. The expression level of green or red fluorescence was analyzed by flow cytometry. A representative dot shows the percentage of DQ-OVA green

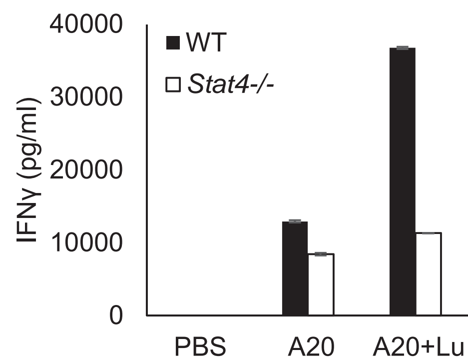
expression in CD11c+CD8α+ and CD11c+CD8α- populations (A). CD11c+DCs were stimulated with medium or LPS at 1μg/ml. Following 24 hours of stimulation, CCL3 production was measured using ELISA (B). Data are presented as mean±SD averaged from 3 different mice.

(A)






(B)
PBS
A20
A20+Lu
WT
Stat4-/-
CD8
CD4
IFNγ
CFSE
0.20%
20.56%
22.98%
47.10%
40.85%
10.92%
0.25%
20.17%
6.90%
48.56%
11.76%
35.72%
0.26%
13.43%
1.14%
25.55%
11.37%
21.22%
0.33%
13.04%
1.62%
32.55%
4.88%
20.40%


(C)
IFNγ (pg/ml)
40000
30000
20000
10000
0
WT
Stat4-/-
PBS
A20
A20+Lu

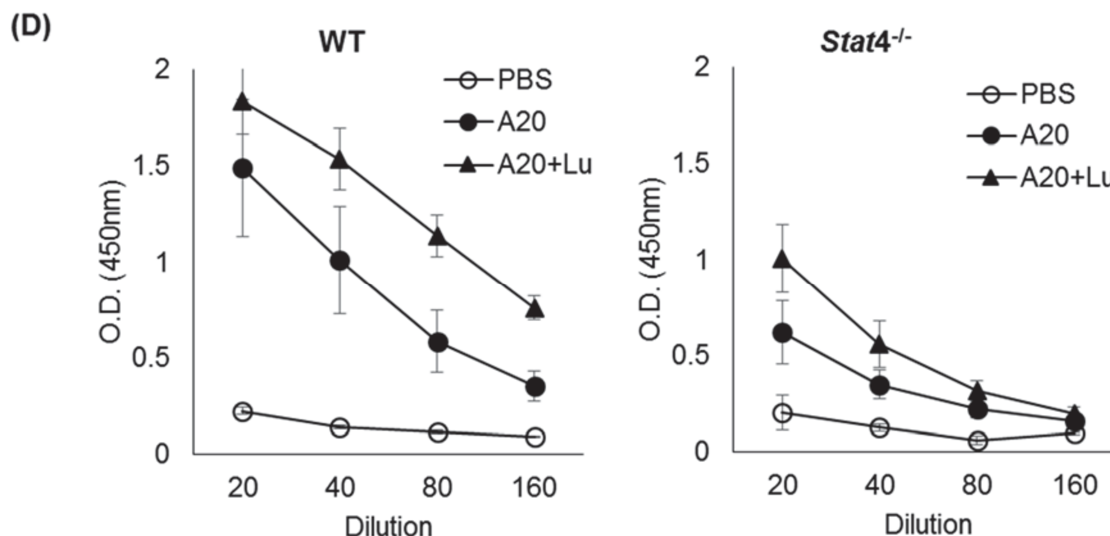


Figure 4.4 Effects of STAT4 in prime-boost cancer vaccination model challenged with A20-OVA B-lymphoma.

BALB/c and *Stat4*$^{-/-}$ mice (female and male, 6-7 week-old) were SC immunized one time on the left flank with PBS or etoposide-treated A20-OVA (10 x 10^6 cells) mixed with or without lunasin at 100µg/ml on days 1 and day 7. At day 14, these mice were subcutaneously challenged with 1 x 10^6 cells of live A20-OVA. Tumor volumes were measured from day 13 after tumor inoculation through day 30 (A). All mice were euthanized 23 days following tumor inoculation. Spleens collected from these mice were processed into single-cell suspensions followed by labeling with CFSE. CFSE-labeled splenocytes were then stimulated with OVA (100 µg/ml) to induce proliferation of OVA-specific T cells. Expression of IFNγ was analyzed on gated CD8+ or CD4+ T cells. A representative dot plot shows the IFNγ production from CFSE-labeled cells (B). The secretion of IFNγ in the supernatants was measured using ELISA (C). OVA-specific serum antibody titers for total IgG were determined by ELISA (D)

CHAPTER 5. FUTURE DIRECTIONS

The most significant finding of the present study is that lunasin acts as an immune modulating agent in activating human and mouse DCs. The in vivo functions of lunasin as a vaccine adjuvant in model antigen soluble ovalbumin (OVA) were assessed in the prophylactic model against OVA-expressing A20-B lymphomas. The underlying mechanisms of lunasin on promoting antigen-specific immune responses were through enhancement of cross-presentation exogenous antigens by DCs as well as activating the NF-κB pathway and MAPK pathways. In addition, lunasin also acted as a vaccine adjuvant in a therapeutic cancer vaccine model and prevented the relapse of OVA-expressing lymphomas after chemotherapy. Furthermore, lunasin worked as an immune modulating agent in the STAT4 deficiency model and promoted antigen specific IFNγ-production in CD4+ and CD8+ T cell immune responses.

Lunasin works as a vaccine adjuvant against OVA-expressing B-lymphoma challenge, and it induces better memory CD8+ T cell immune responses than Alum (Figure 2.6). Many diseases such as tuberculosis and malaria still have no effective vaccines (Appay et al., 2008); therefore, an effective adjuvant that can promote long-lasting humoral and cellular immunity is still needed. Currently, Alum and monophosphoryl lipid A (MPL) are the adjuvants that are licensed in the United States (Macleod et al., 2011). Alum has been used in human vaccines for many years, inducing effective humoral immune responses but poor cellular responses (Hariharan et al., 1995). MPL, a TLR4 agonist, activates effective CD8+ CTL immune responses; however, people with immune dysfunctions who have down-regulation of TLRs may be refractory to MPL stimulation (Monyeleone et al., 2008). Given the fact that lunasin promotes antigen specific CD8+ T cell in a prophylactic OVA vaccination model, application of

lunasin as a vaccine adjuvant in other diseases, including intracellular bacterial diseases and emerging viral diseases, could be another future approach.

A suppressive tumor microenvironment is one of the hallmarks of cancer (Hanahan and Weinberg, 2011). It is generally accepted that the DCs (especially plasmacytoid DCs) in the tumor microenvironment are tolerogenic, or in other words, refractory to TLR stimulation, resulting in low production of type I IFNs (Hartmann et al., 2003). Host type I IFN signals are required by cDCs for the development of tumor specific Th1 cells (Fuertes et al., 2013). Furthermore, cDCs in the tumor bearing host are mostly lacking co-stimulatory signals and have impaired pro-inflammatory cytokine producing activities (Hurwitz and Watkins, 2012). Therefore, the tumor microenvironment is considered to be immunosuppressive. Given the role of lunasin in immuno-activating effects on different subsets of immune cells, including NK cells and DCs, and the phosphorylation of STAT1 that was observed in a autocrine-loop by human cDCs (Figure 3.5), these results demonstrated that lunasin may have an ability to stimulate tolerogenic DCs, which could improve antitumor immune responses. Suppressor immune cells such as regulatory T cells and myeloid-derived suppressor cells (MDSC) are increased in the tumor microenvironment (Hurwitz and Watkins, 2012); the immune-stimulatory effects of lunasin could be another intervention to target suppressor immune cells, which could therefore improve antitumor immunity.

The molecular mechanism of lunasin in promoting cross-presentation activities is another field that needs to be verified. Although the detailed mechanism of cross-presentation is still under investigation, it is thought that targeting antigens to different endosomoal compartments leads to different results of cross-presentation efficiency (Chatterjee et al., 2012). The internalization of lunasin is associated with clathrin-coated structures, and the internalization process can be inhibited by protein trafficking inhibitor, brefeldin A; caveolae-dependent endocytosis inhibitor, nystatin; or clathrin-mediated endocytosis inhibitor, amiloride (Cam et al., 2013). Using lunasin conjugated with fluorescent dyes and co-localizing lunasin with cellular compartments in the DCs may further explain the outcome of enhancing cross-presentation by DCs.

Studies on the functional structure of lunasin suggest that the RGD motif is responsible for lunasin internalization into cancer cell lines, and the poly-D tail is responsible for its direct binding to chromatin once inside the cancer cell lines, which together confers lunasin's ability to induce apoptosis in transformed tumor cells (Galvez and de Lumen, 1999). However, it is not known whether the same structural elements are required for the immunomodulatory properties of lunasin. To directly verify the requirement of these motifs for stimulatory activity in innate immune cells, a truncated peptide Mt.1 (32 aa) lacking the RGD motif and the poly-D tail was chemically synthesized. This truncated peptide induced similar levels of IFNγ in IL-12-cultured NK cells compared to those using the full-length peptide (43 aa) (Chang et al., 2014). However, it was not known whether the RGD motif or poly-D tail was required for lunasin's immunostimulatory function in DCs. The active truncated peptide still contains the region EKHIMEK which has high similarity to the Chromatin Organization Modifier (Chromo) domain (Bottomley, 2004). The chromodomain-containing protein is a central component in the epigenetic regulation of heterochromatic as well as euchromatic gene expression (Bottomley, 2004). The effects of lunasin on epigenetic regulation in chromatin remodeling were examined in NK cells. It was found that lunasin stimulation increased the levels of acetylated histone H3 (AcH3) at the *IFNG* locus in IL-12-cultured NK cells, which was positively associated with upregulation of *IFNG* (Chang et al., 2014). However, it is not known whether lunasin can modulate epigenetic regulation in DCs. Acetylation or methylation of histones is involved in the regulation of gene transcription. It was found that lunasin upregulated of *IFNA1* in human cDCs (Figure 3.5). This may also involve in the epigenetic regulation of chromatin structure, which leads to maintaining a nucleosome structure favorable for promoting *IFNA1* gene transcription.

To investigate the role of lunasin in epigenetic regulation of gene expression in innate immune cells, ChIP assay could be performed using antibodies against histone markers that are known to be positively associated with *IFNA1* transcription, including acetyl-H3 (Chambers et al., 2001), acetylated histone H3 at lysine 9 (Ac-H3K9), and non-immune rabbit serum as control. The chromodomain-containing proteins have been

shown to interact with modified histones such as methylated H3K9 (Bottomley, 2004). Since the active truncated lunasin still contains the region EKHIMEK which has high similarity to the chromodomain, the chromodomain may play a role in lunasin's immunomodulation activity by interacting with histones. Various mutations on the chromodomain of lunasin could be generated and tested.

Several studies have revealed the anti-inflammatory effects of lunasin. It was thought that the anti-inflammatory effects of lunasin are caused by the inhibition of NF-κB translocation in the nucleus and inhibition of the transcriptions of proinflammatory cytokines (Cam and de Majia, 2012). In addition, lunasin could alleviate the OVA+LPS sensitized airway inflammation by inducing antigen-specific regulatory T cells (Yang et al., 2015). We also observed immunosuppressive effects of lunasin in the long-term treatment study. Mice that were subcutaneously injected with B16-F10 or B16-F0 melanoma and received daily lunasin treatment had more aggressive tumor growth compared to those mice that received PBS treatment (Figure A1 and A2). It was found that mice receiving lunasin treatment in a concentration of 10 μg daily had more aggressive tumor growth (Figure A1). These findings are different from the observations in Chapters 2-4, as lunasin activates DCs and works as a vaccine adjuvant in promoting antigen-specific immune responses. The differences between these studies are due to difference in the exposure time. Studies have found that short-term (3 day) and long-term (7 day) exposure to restraint stress on mouse airways had opposite effects; short-term exposure led to fewer inflammatory cells compared to unstressed mice. However, long-term exposure significantly increased inflammatory cell numbers (Forsythe et al., 2004). Therefore, it is possible that short-term versus long-term exposure of lunasin in vivo has opposite effects, suggesting that different mechanisms may be involved. The dose effect of lunasin could be evaluated by analyzing cytokines that are involved in activation or inhibition of immune functions or by analyzing the kinetics of immunosuppressive cells after long-term or short-term lunasin treatment.

The present study reveals the effects of lunasin as an immune modulating agent that can be applied to cancer immunotherapy. In addition, lunasin itself has chemopreventive properties that can arrest the cell cycle and induce apoptosis in

transformed cells including the human breast cell line MDA-MB-231 cells (Hsieh et al., 2010). Given the special characteristics of lunasin in both inducing immune functions and inhibiting tumor growth, it could be used for combined cancer immunotherapy. Currently, the combination of blockade antibody, such as anti-CTLA4 monoclonal antibody (Ipilimumab), to traditional anticancer therapy is being tested in several clinical trials (Callahan and Wolchok, 2013). Lunasin could be used as an immunomodulator with blockade antibodies and other traditional cytotoxic chemotherapies to eliminate cancer cells in different approaches.

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APPENDIX

Appendix A

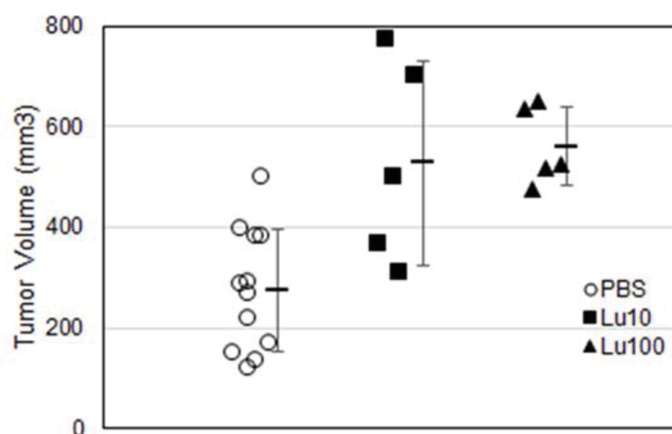

Figure A.1 Long-term treatment with lunasin does not inhibit growth of melanoma following subcutaneous implantation of B16-F10 cells.

C57BL/6 mice were subcutaneously (SC) injected on the right flank with 1 x 10^5 live B16-F10 on day 1. These mice were treated with PBS, lunasin at 10 µg/mouse (Lu10) or lunasin at 100 µg/mouse (Lu100) daily via intraperitoneal (IP) injection starting on the same day of tumor inoculation. Tumor volumes were measured from day 13 after inoculation through day 22.

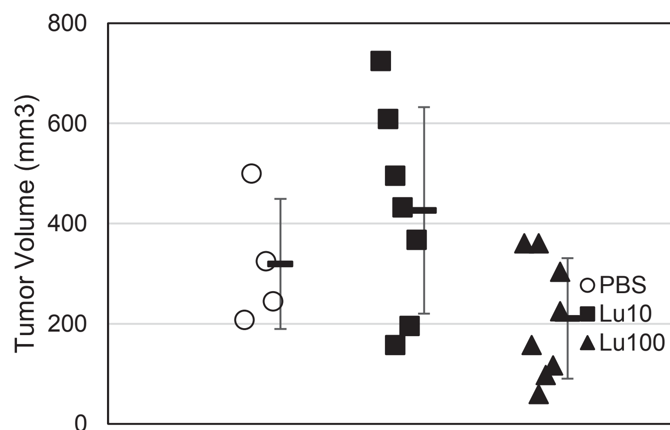

Figure A.2. Long-term treatment with lunasin does not inhibit growth of melanoma following subcutaneous implantation of B16-F0 cells.

C57BL/6 mice were subcutaneously (SC) injected on the right flank with 1 x 10^5 live B16-F0 on day 1. These mice were treated with PBS, lunasin at 10 µg/mouse (Lu10) or lunasin at 100 µg/mouse (Lu100) daily via intraperitoneal (IP) injection starting on the same day of tumor inoculation. Tumor volumes were measured from day 13 after inoculation through day 22.

VITA

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