

**THE ROLE OF HEAT SHOCK PROTEIN RECEPTOR CD91 IN INITIATION OF
TUMOR-ASSOCIATED IMMUNITY**

by

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THE ROLE OF HEAT SHOCK PROTEIN RECEPTOR CD91 IN INITIATION OF TUMOR-ASSOCIATED IMMUNITY

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Adaptive immune responses against tumors are routinely detected in hosts bearing the tumors. However, the mechanisms of priming T cell responses which are central to the underlying immunosurveillance for nascent tumors remains unclear, given the limited amount of each unique tumor antigens available for cross-priming during the early stages of tumor development and lack of classical PAMPs in general. I provide evidence here that heat shock proteins (HSPs), when released into the tumor microenvironment under these conditions, is the molecular entity necessary for priming specific tumor-associated immunity. These responses require CD91, the endocytic and signaling receptor for the immunogenic HSPs. In this study, I generated CD11c-specific CD91 knockout mice and showed that tumors grew faster in those knockout mice compared to CD91^{+/+} mice, due to a lack of priming efficient T cell responses. In addition, I abrogated the interaction of tumor-derived HSP with CD91 *in vivo* by over-expression of Receptor Associated Protein (RAP), an endogenous inhibitor of CD91. The data showed that: first, tumors expressing RAP grew with significantly faster kinetics than the non-RAP expressing counterparts in wild type mice, although this difference was non-existent in immunocompromised mice; second, RAP-expressing tumors, when used as an immunogen in tumor prophylaxis, were significantly less efficient in priming immune responses; third, inhibition of antigen cross-presentation by RAP reduced T cell proliferation *in vivo*; last, the

competition of RAP and tumor-derived HSP for binding to CD91 was examined *in vivo*. In the presence of RAP, fewer tumor-derived HSPs were taken up by APCs in draining lymph nodes. In summary, this study demonstrates that early in tumor development, the HSP-CD91 pathway is critical for establishment of anti-tumor immunity. Considering that elevated RAP levels have been reported in colon cancer patients, I propose a novel mechanism of immune evasion for tumors expressing competing ligands for immune receptors such as CD91.

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To you all, I dedicate this dissertation.

ABBREVIATION

APCs	Antigen Presenting Cells
ATCC	American Type Culture Collection
cDC	Conventional Dendritic Cell
cDNA	Complementary Deoxyribonucleic Acid
CFSE	Carboxyfluorescein succinimidyl ester
CRT	Calreticulin
CTC	Circulating Tumor Cells
CTLs	Cytotoxic T Lymphocytes
DAMP	Danger-Associated Molecular Pattern
DC	Dendritic Cell
dLN	Draining Lymph Node
DMEM	Dulbecco's Modified Eagle's Medium
GLP	Good Laboratory Practice
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HSPs	Heat Shock Proteins
LDLR	Low-Density Lipoprotein Receptor
LOX-1	Lectin-like oxidized LDL receptor-1

LRP1	Low density lipoprotein receptor-Related Protein 1
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
NK	Natural Killer
ndLN	Non-draining Lymph Node
PAMP	Pathogen-Associated Molecular Pattern
pDC	Plasmacytoid Dendritic Cell
PFA	Paraformaldehyde
RAP	Receptor-Associated Protein
SR-A	Scavenger receptor A
SREC-1	Scavenger receptor expressed by endothelial cell-1
SV40	Simian Virus 40

1.0 INTRODUCTION

1.1 TUMOR-ASSOCIATED IMMUNE RESPONSE

1.1.1 Tumor immunosurveillance

Cancer, the malignant form of a neoplasm, is the leading cause of death in developed countries and the second leading cause of death in developing countries (Jemal et al., 2011). Despite inherent complexities, there are several essential alterations shared by the vast catalog of cancer cells that dictate their malignant growth, including self-sufficiency in proliferative signaling, insensitivity to growth inhibitory signals, evasion of cell death, replicative immortality, sustained angiogenesis, and tissue invasion/metastasis (Hanahan and Weinberg, 2000, 2011). Those genetic and cellular alterations provide the body's immune system with the means to resist or eradicate cancer cells. The long-standing theory of immunosurveillance proposes that normal cells or tissues are constantly monitored by the surrounding immune system and that such surveillance by immune cells leads to detection and elimination of most incipient neoplastic cells (or nascent tumors). This concept was first conceived by Paul Ehrlich (Ehrlich, 1909), and later formally proposed by Sir. Macfarlane Burnet and Lewis Thomas (Burnet, 1957; Burnet, 1970; Thomas, 1959).

With the advent of inbred mice, syngenic tumor cells, and genetic engineering, the hypothesis of immunosurveillance has been tested experimentally. Initially, experiments with the athymic nude mice showed discordant evidence against the immunosurveillance hypothesis, in that the incidence and development of MCA-induced tumor was similar between nude mice and their wild type counterparts (Stutman, 1974). However, certain caveats exist in these experiments. First, nude mice are not completely immunocompromised and still contain a detectable amount of $\alpha\beta$ T cells, B cells, NK cells, etc. Second, the mouse strain used in Stutman's experiments is highly susceptible to MCA-induced tumors, and therefore rate of tumor formation in those mice may overwhelm the protection mechanisms by their immune systems. It was not until other immunocompromised murine models were made available in the 1980s and 1990s, that the concept of immunosurveillance was resurrected. Mice deficient in various components of the immune system were, once again, examined for development of carcinogen-induced tumors. The results showed that tumors arose more frequently and/or grew more rapidly in many immunocompromised mice compared to their immunocompetent controls (Dunn et al., 2002; Schreiber et al., 2011). Specifically, impairment in development (e.g. *rag2*^{-/-}) or function (e.g. *Ifn γ* ^{-/-}, *perforin*^{-/-}) of CD8⁺ cytotoxic T lymphocytes (CTL), CD4⁺ helper T cells, B cells, or Natural Killer (NK) cells each contributed to higher incidence of tumor (Engel et al., 1996; Engel et al., 1997; Girardi et al., 2001; Kaplan et al., 1998; Langowski et al., 2006; Liu et al., 2004; Shankaran et al., 2001; Smyth et al., 2001; Smyth et al., 2000; Swann et al., 2007; Swann et al., 2009; Wakita et al., 2009). Mice with combined immunodeficiencies (e.g. *rag2*^{-/-} *γ c*^{-/-}, *RkSk* mice) were even more susceptible to tumorigenesis (Shankaran et al., 2001). Additionally, a phenomenon called concomitant immunity provides further evidence that supports tumor-associated immune responses. Coined by Paul Ehrlich, this concept describes a hypothesis that

the immune responses primed by a progressive tumor reject the second challenge of the same tumor at a distant site in the host (Ehrlich, 1906). It was later shown in murine models that a significant level of anti-tumor resistance against a later tumor challenge was not expressed until 6 days after inoculation of the primary tumor, suggesting the involvement of adaptive immune responses (North and Kirstein, 1977).

Immunosurveillance also plays a critical role in restraint of cancer in human patients. Several follow-up studies of individuals with primary immunodeficiency or transplant patients who were immunosuppressed showed that they have a significant higher risk of developing cancer (Gatti and Good, 1971; Penn, 1970; Vajdic and van Leeuwen, 2009). However, the vast majority of these cancers were virus-induced, suggesting that reduction of viral load was required to control tumorigenesis in these cases (Penn, 1999). But an increasing body of research has also recently demonstrated that, at least in some forms of non-virally-induced cancer, the immune system serves as a major barrier to tumor formation and development (Hoover, 1977; Penn, 1995, 1996; Pham et al., 1995; Sheil, 1986). In addition, studies suggest a positive correlation between infiltrated lymphocytes in a tumor and increased patient survival (Deligdisch et al., 1982; Epstein and Fatti, 1976; Lipponen et al., 1992; Nacopoulou et al., 1981; Naito et al., 1998; Rilke et al., 1991). In summary, the data from mice and humans highlights the existence and significance of immunosurveillance.

To elicit immune responses that eradicate cancer cells, a succession of events must be initiated and allowed to proceed iteratively, which is termed the 'Cancer-immunity Circle' (Chen and Mellman, 2013)(Figure 1-1). In the first step, neoantigens generated by tumorigenesis must be captured by surrounding antigen presenting cells (APCs). In order to activate APCs and downstream T cell responses, another alarming signal is generally required. These signals

include pathogen-associated molecular patterns (PAMPs) that are expressed by various classes of microbes, and danger-associated molecular patterns (DAMPs) that normally reside in the cells (step 1). APCs then process the neoantigen and present antigenic peptide on MHC I or MHC II to T cells in the context of costimulation (step 2), resulting in the priming of effector T cells (step 3). The final outcome of immune responses is shaped in this stage, by a vital balance between effector T cells and regulatory T cells. Finally, the activated T cells traffic to (step 4) and infiltrate the tumor microenvironment (step 5), where they specifically recognize cancer cells expressing cognate antigen (step 6) and destroy the target cells (step 7). Killing of cancer cells leads to additional release of tumor-associated antigens (step 1 again), which then expand the breadth and depth of anti-tumor immunity.

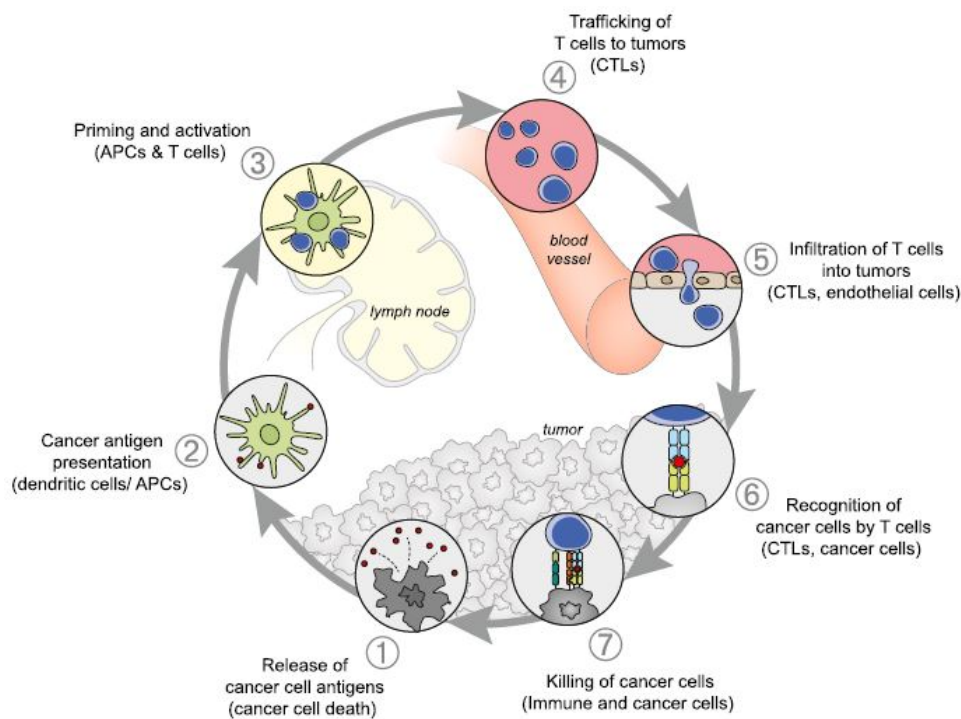


Figure 1-1 Cancer-immunity circle (Chen and Mellman, 2013)

The cancer-immunity circle is a series of self-propagating events that amplify and broaden the antitumor immune responses. Schema of seven major steps in the circle is shown here, with the primary cell types involved and the anatomic location of different activities listed. (Reprinted from *Immunity*, Volume: 39, Chen DS and Mellman I, *Oncology meets immunology: the cancer-immunity cycle*, Pages 1-10, Copyright (2013), with permission from Elsevier)

Various types of APCs including macrophages and dendritic cells (DCs) serve as cellular sentinels in this cancer-immunity circle. While macrophages mainly serve as scavenger cells with greater capacity for antigen uptake, DCs possess superior antigen processing and presentation machinery (Delamarre et al., 2005). DCs represent a heterogeneous population with different morphologies, phenotypes and functions in various tissues (Merad et al., 2013; Steinman, 2012). There are two main subpopulations of DC: plasmacytoid DCs (pDCs) and conventional DCs (cDCs). pDCs morphologically resemble plasma cells but can produce enormous amounts of type-1 interferon (IFN) upon stimulation (Colonna et al., 2004). Recent data have indicated that tumors can induce type I IFN production possibly *via* pDCs, which is required for a spontaneous T cell response *in vivo* (Gajewski et al., 2012; Gajewski et al., 2013). cDCs refer to all DCs other than pDCs. Compared to pDCs, cDCs have an enhanced ability to capture, process, and present antigen to naive T cell responses, which are essential for tumor immunosurveillance (Joffre et al., 2012; Villadangos and Schnorrer, 2007). The surface marker CD11c is generally expressed in various subsets of cDCs, including CD8 α ⁺ DCs, Langerhans cells and CD103⁺ cells. However, CD11c⁺ cells are not limited to cDCs, since spleen macrophages, NK cells, activated T cells, and monocytes also express some level of CD11c (Merad et al., 2013).

1.1.2 Tumor immuno-editing

The repeated cancer-immunity circle is responsible for elimination of most nascent tumors. Immune system also sculpts the tumor cells in return. It has been proposed by Robert Schreiber that, in order to become clinically apparent, tumor cells need to undergo three 'E' phases of immune-editing---Elimination, Equilibrium, and Escape. The elimination phase corresponds to

immunosurveillance (Dunn et al., 2002). In the equilibrium phase, tumor cells keep undergoing the selective pressure of immune destruction, become functionally dormant, and remain clinically unapparent for the life of the host. In the escape phase, cancer cells undergoing stochastic genetic and epigenetic mutation create critical modifications to avoid both innate and adaptive immunological destruction. Meanwhile, the immune system selects more aggressive tumor variants, and the immunologically sculpted tumor cells then grow unhindered, leading to appearance of overt cancer. Various forms of adaptation can be acquired by tumor cells to evade immunosurveillance (Rabinovich et al., 2007). On one hand, tumor cells can undergo cell-autonomous modifications to avoid detection by professional antigen-presenting cells or destruction by effector immune cells; on the other hand, surrounding immune cells and stromal cells (Singh et al., 1992; Zhang et al., 2007) can be modified by tumors to generate a local immunosuppressive microenvironment.

Tumor transplantation experiments provide strong evidence that support immunoediting. Cancer cells established in immunodeficient mice are significantly less efficient for generation of a secondary tumor when transplanted into the syngenic immunocompetent hosts. However, cancer cells that are harvested from immunocompetent mice are equally efficient in initiation of secondary tumor in both immunocompetent and immunodeficient hosts (Smyth et al., 2006). Those phenomena have been interpreted as follows: In the immunocompetent host, highly immunogenic tumor cells have been constantly targeted and eliminated by different mechanisms of immunosurveillance, leaving behind weakly immunogenic tumor variants, which survive this immuno-sculpting and keep growing to eventually generate solid tumors. When transferred, such weakly immunogenic tumors can therefore colonize both immunocompetent and immunodeficient secondary hosts. However, when arising in immunodeficient host,

immunogenic tumor cells are not under pressure of immunoselection, and can therefore prosper along with their weakly immunogenic counterparts. When those nonedited tumors are transplanted into the syngenic recipient, the immunogenic cells will be rejected once they encounter a functional immune system of their secondary hosts for the first time.

1.2 HEAT SHOCK PROTEINS AND THEIR IMMUNOLOGICAL PROPERTIES

1.2.1 HSPs family, and their general functions

Heat shock proteins (HSPs), the most abundant and ubiquitous soluble proteins inside the cell (Lindquist and Craig, 1988), can be classified into ten families, each consisting of one to five closely related proteins (Table 1-1). Under normal condition, HSPs represent up to 5% of the total intracellular proteins. However, their level can rise to 15% or more under stress, including high temperatures, toxins, and glucose deprivation. Since their discovery, an increasing number of various vital functions have been attributed to HSPs, such as molecular chaperones for protein folding and degradation (Gething and Sambrook, 1992; Parsell and Lindquist, 1993), building blocks of multi-subunit complexes (Haas, 1991), buffers of mutations (Queitsch et al., 2002; Rutherford and Lindquist, 1998), and mediator of thermotolerance (Lindquist, 1986). Additional studies also highlight the role of HSPs as a model in transcriptional regulation (Morimoto, 1998), stress responses (Lindquist, 1986), and molecular evolution (Feder and Hofmann, 1999).

Table 1-1. The major families of heat-shock proteins (Srivastava, 2002b)

HSP family	Members	Intracellular location
Small HSPs	HSP10, GROES, HSP16, α -crystallin HSP20, HSP25, HSP26, HSP27	Cytosol
HSP40	HSP40, DNAJ, SIS1	Cytosol
HSP47	HSP47	Endoplasmic reticulum
Calreticulin	Calreticulin, Calnexin	Endoplasmic reticulum
HSP60	HSP60, HSP65, GROEL	Cytosol and Mitochondria
HSP70	HSP72, HSC70 (HSP73), HSP110/SSE, DNAK SSC1, SSQ1, ECM10 GRP78 (BiP), GRP170	Cytosol Mitochondria Endoplasmic reticulum
HSP90	HSC84, HSP86, HTPG gp96 (GRP94, HSP108, endoplasmin)	Cytosol Endoplasmic reticulum
HSP100	HSP104, HSP110, CLP proteins HSP78	Cytosol Mitochondria

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1.2.2 Immunological function of HSPs

The immunological properties of HSPs were first discovered in the 1980s when homogenous fraction of tumor lysates containing HSPs elicited anti-tumor immune responses while same preparation of HSPs from normal tissue did not. The first studies were carried out with the HSP gp96 (Srivastava and Das, 1984; Srivastava et al., 1986; Ullrich et al., 1986). In subsequent years, this phenomenon was extended to hsp70, hsp90, calreticulin, hsp110, and grp170 (Basu and Srivastava, 1999; Tamura et al., 1997; Udono and Srivastava, 1993, 1994; Wang et al., 2001). Interestingly, the HSPs purified from a certain type of cancer can only elicit protective immunity against that particular cancer. Where does this specificity come from? Extensive sequencing studies showed no difference of HSP cDNAs between cancers and normal tissues and from one cancer to another. Instead, the antigenic peptides associated with HSPs, which are generated as a result of normal protein turnover within cells, contribute to the specificity of

HSPs-mediated immunity (Srivastava et al., 1998). HSPs stripped off their chaperoned peptides lose their ability to mount measurable specific immune responses (Peng et al., 1997; Uono and Srivastava, 1993). HSPs chaperone peptides are comprised of the total pool of processed proteins within cells, including antigens derived from mutation (in transformed cells) or pathogen (in bacteria or virus-infected cells). Therefore, those peptides represent the antigenic fingerprint of the cells from which HSPs are purified.

1.2.3 Mechanism of immunogenicity of HSPs

A great amount of research has been conducted to investigate the cellular events that contribute to the immune responses following immunization with purified HSPs. Depletion of T cells or antigen presenting cells (APCs, such as dendritic cells or macrophages) abrogated immunity elicited by HSPs, suggesting that these cells play essential role for HSPs-mediated immune responses (Tamura et al., 1997; Uono et al., 1994). Considering that HSPs are intracellular proteins, their exposure in the extracellular environment was proposed to serve as a danger signal to alert APCs to stresses in the body, such as tumor necrosis (Basu et al., 2000; Somersan et al., 2001). This proposal has been supported by two decades' work of experimental investigations. When purified HSPs are used to interact with APCs, they are capable to elicit both adaptive and innate immune responses (Srivastava, 2002a, b) (Figure 1-2).

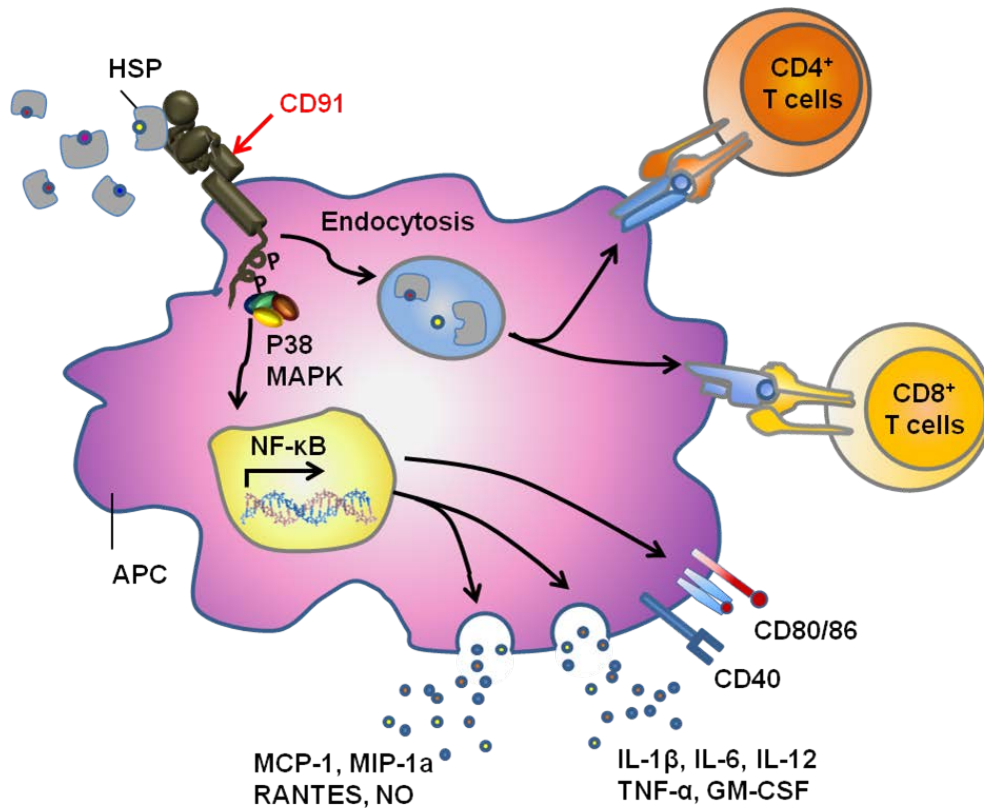


Figure 1-2. The interaction between HSPs and APCs integrate adaptive and innate immune events

HSPs can engage APCs via their receptor CD91 to induce both adaptive and innate immune responses. On one hand, antigenic peptides chaperoned by HSPs are internalized, processed in the APCs, and presented on MHC I or MHC II to CD8+ T cells or CD4+ T cells. On the other hand, signals from HSPs can be relayed to nucleus, leading to activation and maturation of APCs. CD91 is both a scavenger receptor and a signaling receptor for HSPs, mediating downstream adaptive and innate immune responses.

On one hand, HSP-peptide complexes are internalized into the APCs. After disassociation with HSPs, the antigenic peptides are then processed via different proposed pathways (Arnold-Schild et al., 1999; Basu et al., 2001; Castellino et al., 2000; Suto and Srivastava, 1995), and presented on the cell surface by MHC I or MHC II to T cells. On the other hand, once HSPs interacted with APCs, they can elicit downstream signal relay, which leads to a series of antigen nonspecific events that enhance immune responses. Those events include: (1) secretion of inflammatory cytokines; (2) induction of inducible nitric oxide synthase (iNOS) and production of nitric oxide; (3) secretion of chemokine; (4) maturation of dendritic cells indicated

by upregulation of MHC II, CD86, and CD40; (5) migration of dendritic cells from site of gp96 injection to draining lymph node (Binder et al., 2000a); (6) nuclear translocation of transcriptional factor NF- κ B (Pawaria and Binder, 2011; Srivastava, 2002a). Therefore, as a single entity, HSP-peptide complex is able to serve as antigen-carrier and adjuvant, and provide the dual signals of antigen and co-stimulation which are required for priming T cell responses.

1.3 CD91: AN HSP RECEPTOR

Typically, mice are immunized with 1-10 μ g of purified HSP-peptide complexes to prime a peptide specific T-cell response. This quantity of HSP-peptide complex contains less than nanogram levels of antigen-specific peptides. Instead, tens to hundreds of micrograms of peptides (with a conventional adjuvant) are usually needed to elicit a similar response. How can the small amounts of peptide chaperoned by HSPs be effective for immunization? Seeking an answer to this conundrum, Srivastava et al. first proposed, entirely on theoretical grounds, that HSP-peptide complexes must be targeted to a highly efficient pathway, possibly via cellular receptors (Srivastava et al., 1994). By utilizing various independent strategies, binding and competition studies showed that HSPs interacted specifically with APCs and could be titrated over a range of protein concentrations, supporting the notion of such a HSP receptor (Arnold-Schild et al., 1999; Binder et al., 2000c; Wassenberg et al., 1999).

1.3.1 CD91 as a common receptor for HSPs

To identify receptor(s) for HSPs, gp96 was immobilized on affinity columns and exposed to membrane protein extracts from APCs. An 85kDa protein that interacted with gp96 was isolated. In a parallel experiment, radiolabeled gp96 was tagged to short photoactivated cross-linkers, which extracted an 80KD molecule on the membrane of macrophage. The 10Å length of the cross-linkers indicated that the 80KDa molecule and gp96 closely interacted as a ligand and its receptor. Protein sequencing by mass spectrometry identified the 85kDa/80KDa molecule as a fraction of CD91 (Binder et al., 2000b). Although structurally unrelated, three other immunogenic HSPs, hsp70, hsp90, and calreticulin also utilize CD91 as a common receptor (Basu et al., 2001).

CD91, also known as the α -2-macroglobulin (α 2M) receptor and low density lipoprotein receptor-related protein 1 (LRP1), is expressed at least at the mRNA level in a variety of cell types, including myeloid cells, hepatocytes, hepatic stellate cells, neuronal cells, fibroblasts, adipocytes, adrenal cortical cells and follicular cells of the ovary. CD91 is translated as a single 600kDa polypeptide and then proteolytically cleaved by a furin-like endoprotease into two subunits of 515kDa and 85kDa (Herz et al., 1990). The significance of this cleavage step is unknown, although it may not be critical for its endocytic functions (Ko et al., 1998). The large extracellular α -subunit of 515kDa contains 4 ligand-binding clusters that can bind to a wide variety of unrelated ligands including ApoE-containing lipoproteins, lipoprotein lipase, complexes of proteinase-proteinase inhibitors, growth factors, and hormone. The α -subunit is non-covalently linked to the 85kDa β -subunit, which contains a small extracellular portion, a single-span transmembrane domain and a short cytoplasmic tail with two tyrosine phosphorylation (NPXY) motifs (Herz and Strickland, 2001). (Figure 1-3)

The role of CD91 in priming immune response is two-fold: First, CD91 is a scavenger receptor. Blocking of CD91 via an anti-CD91 antibody, or its ligands such as α 2M, can abrogate the cross-presentation of peptides chaperoned by the HSPs (Basu et al., 2001). Second, CD91 also serves as a signaling receptor. Once it interacts with HSPs, CD91 is phosphorylated in a unique pattern, which triggers signaling cascades to activate NF- κ B. Each type of HSP induces a unique cytokine profile *via* CD91, which dictates priming of specific T-helper cell subsets (Pawaria and Binder, 2011).

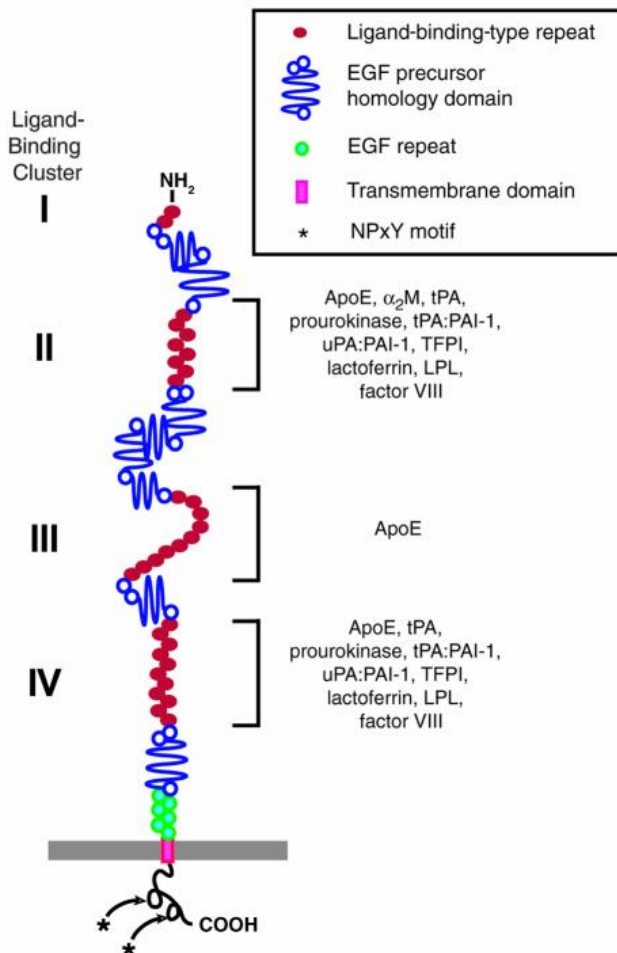


Figure 1-3 Subunits and ligand-binding clusters on CD91 (Herz and Strickland, 2001)

CD91 ligands can bind to the different clusters of ligand-binding repeats on its α -subunit. majority of the CD91 ligands bind to the Cluster II and IV. Cysteine-rich ligand-binding repeats (red ovals) are arranged in four clusters containing 2, 8, 10, and 11 repeats, respectively. Each cluster is followed by 1-4 EGF homology domains (blue), which consist of cysteine-rich EGF repeats (blue circles) and YWTD domains (wavy line). Two NPxY motifs in the cytoplasmic tail of β -subunit are indicated by the asterisks. Copyright (2001) American society for clinical investigation

The aforementioned immunological role of HSP-CD91 interaction was established based on several biochemical (by using anti-CD91 antibody) and genetic evidence (by using siRNA for

CD91)(Banerjee et al., 2002; Binder et al., 2000b; Binder and Srivastava, 2004; Habich et al., 2002; Ling et al., 2007; Robert et al., 2008; Stebbing et al., 2003). In order to validate protein function *in vivo*, knockout mouse models have been a gold standard in recent two decades, although they are time consuming and lab intensive. There are certain caveats associated with these models. For example, the pleiotropic effects caused by various requirements for a particular gene in different locations, or at different stages of embryonic development may complicate the analysis. In the case of CD91, attempts to generate a conventional knockout mouse failed due to an early embryonic lethal phenotype (Herz et al., 1992; Herz et al., 1993). As an alternative, the Cre/LoxP-mediated recombination system is able to overcome these hurdles, by targeting genes in a temporally- or spatially-specific way. Indeed, successful tissue-specific inactivation of CD91 in brain, liver, and vascular smooth muscle cells using this approach have been reported in different functional studies (Boucher et al., 2003; May et al., 2004; Rohlmann et al., 1998; Rohlmann et al., 1996). The structure of the 'floxed' allele is shown in Figure 2-1. Cre-mediated recombination is expected to cause deletion of part of promoter, transcription start site, exon I encoding the signal peptide, and exon II, therefore rendering the allele truncated and untranscribable.

1.3.2 Other molecules proposed to be HSP receptors

The discovery of CD91 as a HSP receptor opened the floodgates in the following years for describing additional HSP receptors in several cases, but with controversial evidence. These include: lectin-like oxidized LDL receptor-1 (LOX-1), Toll-like receptor 2/4 (TLR2/4), CD14, CD40, Scavenger receptor-A (SR-A), and Scavenger receptor expressed by endothelial cell-1 (SREC-1). The experimental evidence for and against each of them is summarized in Table 1-2.

Table 1-2 Evidence for and against possible candidates for HSP receptor [adapted from (Binder, 2009)]

Proposed receptor	Ligands	Supporting data	Discordant data
CD91	Gp96 HSP70 HSP90 Calreticulin	(1) Physical association of HSPs and CD91; (2) Competitive binding of other CD91 ligands or antibodies; (3) Internalization of HSP-peptide complexes; (4) Inhibition of cross-presentation and signal transduction by CD91 siRNA, site-mutation on β -chain of CD91, or anti-CD91 antibodies; (5) <i>In vivo</i> abrogation of HSP-mediated immunity with anti-CD91 antibodies.	(1) CD91-independent binding of HSPs. One caveat is that cells used in this assay lack sufficient fixation; (2) 'Cross-presentation' of HSP-chaperoned peptides is CD91-independent. However, it is shown later that peptides used in this study can be directly presented on MHC I.
LOX-1	HSP70	Binding and competition studies with other CD91 ligands and antibodies.	No data supporting physical interaction
TLR2/4	HSP70 HSP60 Gp96 HspB8 α -Crystallin	HSP-induced cytokine secretion or NF- κ B activation was inhibited in APCs pulsed with anti-TLR2/4 antibodies, or in APCs with dominant negative MyD88 or deficient in TLR2/4	(1) Issues of endotoxin contamination have not been addressed. (2) No data supporting physical interaction
CD14	HSP60 HSP70	(1) Human astrocytoma cells transfected with cDNA for CD14 were able to secrete cytokine in response to HSP; (2) Cytokine released by APCs pulsed with HSPs was inhibited by anti-CD14 antibodies	(1) Issues of endotoxin contamination have not been addressed. (2) No contribution of CD14 in HSP binding assay. (3) No data supporting physical interaction.
CD40	HSP70	(1) Binding and competition studies with other CD91 ligands and antibodies; (2) Internalization of HSP/peptide.	(1) Studies examining physical interaction were conducted with recombinant (non-native) proteins; (2) The consequences of mammalian HSP70-APC interaction were identical in kinetics and magnitude between CD40 ^{+/+} and CD40 ^{-/-} mice.
SR-A	Gp96 Calreticulin	(1) Binding and competition studies with other CD91 ligands and antibodies; (2) Partial cross-presentation of HSP-peptide complexes.	(1) Issues of endotoxin contamination have not been addressed. (SR-A binds to LPS) (2) <i>In vivo</i> data did not demonstrate a role for SR-A in HSP-mediated immunogenicity; (3) No data supporting physical interaction
SREC-1	Gp96 Calreticulin	Binding and competition studies with other CD91 ligands and antibodies.	(1) <i>In vivo</i> data suggested that SREC-I did not function in HSP binding; (2) No data supporting physical interaction

In order to verify the identity of those molecules as a HSP receptor, four major issues need to be addressed. First, purity of HSP preparation is a major caveat in all the experiments where recombinant HSPs purified from bacteria were used, especially with regards to contamination with bacterial endotoxin (e.g. LPS). The LPS receptor TLR2/4 and its co-receptor CD14 have been suggested as HSP receptors in some studies, where deficiency or blockade of these molecules or their downstream adaptors inhibits the activation of APCs (Asea et al., 2002; Vabulas et al., 2002a; Vabulas et al., 2002b). However, it was later shown that HSP-mediated immune responses in the above experiments were indistinguishable from those induced by endotoxin (Bausinger et al., 2002; Gao and Tsan, 2003). Therefore, extra caution needs to be taken when using recombinant HSPs to identify their receptor; second, even if the amount of endotoxin in recombinant HSPs was below detection level, those HSPs may lack proper post-translational modification such as glycosylation or add molecular tags such as glutathione S-transferase (GST) or myc, both of which may interfere with ligand-receptor interaction or introduce artificial binding. Instead, use of proteins as close to their physiological state as possible such as purified endogenous HSPs under the guideline of the Good Laboratory Practice (GLP) * provide a solution to both prevent endotoxin contamination and maintain a much more natural pattern of protein modification and folding; third, most candidates of HSP receptors have been proposed based on the competitive binding or activation assays of APCs *in vitro*. However, few of them have been concluded from direct evidence of physical interaction between ligand and receptors; fourth, because of the caveats mentioned above, verification *in vivo* is required. Unfortunately, with the exception of SR-A (which turned out not to have a role *in vivo*) and CD91, none of the other HSP receptor candidates have been examined via *in vivo* assay.

*: Good Laboratory Practice or GLP refers to a quality control system for research laboratories to ensure the uniformity, reliability and consistency of data generated in non-clinical experiments (W.H.O, 2009).

1.4 RAP, AN ENDOGENOUS ANTAGONIST FOR CD91

Receptor associated protein (RAP) is a three domain 40kDa protein, which functions as an escort protein for receptors of low-density lipoprotein receptor (LDLR) family, including CD91. It helps post-translational processing of CD91, prevents premature intracellular binding of ligands, and assists with delivery of mature CD91 to the cell surface (Bu, 1998). By using immunoelectron microscopy to examine cells that robustly express RAP such as U87 cells and HepG2 cells, RAP was localized most abundantly within the endoplasmic reticulum (ER, 70%) and Golgi compartment (24%) with only 4% in the endosomes and 2% on the cell surface (Bu et al., 1994). Cells expressing RAP include, but are not limited to, CD91-expressing cells [Data from the Immunological Genome Project (www.immgen.org)].

RAP is known to bind with high affinity to CD91 and therefore prevents other ligands from binding to CD91 (Bu et al., 1992; Herz et al., 1991; Warshawsky et al., 1993). Other than CD91, no molecules have been shown to interact with RAP. The mechanisms by which RAP inhibits the binding and uptake of CD91 ligands are not completely understood. However, previous studies suggested that RAP may bind to all ligand-binding sites on CD91, and that each of these RAP binding sites on CD91 may be recognized by various binding determinants on RAP itself. In the case of HSPs, studies *in vitro* demonstrated that their binding to CD91 was also blocked by RAP, which also inhibited cross-presentation of chaperoned peptides (Figure 1-4)(Binder and Srivastava, 2004). Interestingly, no apparent structural similarities were observed between RAP and HSPs. These results led to the idea that the elevated levels of RAP released from necrotic tumor cells *in vivo* could potentially inhibit immunogenicity of HSPs derived from the same tumor.

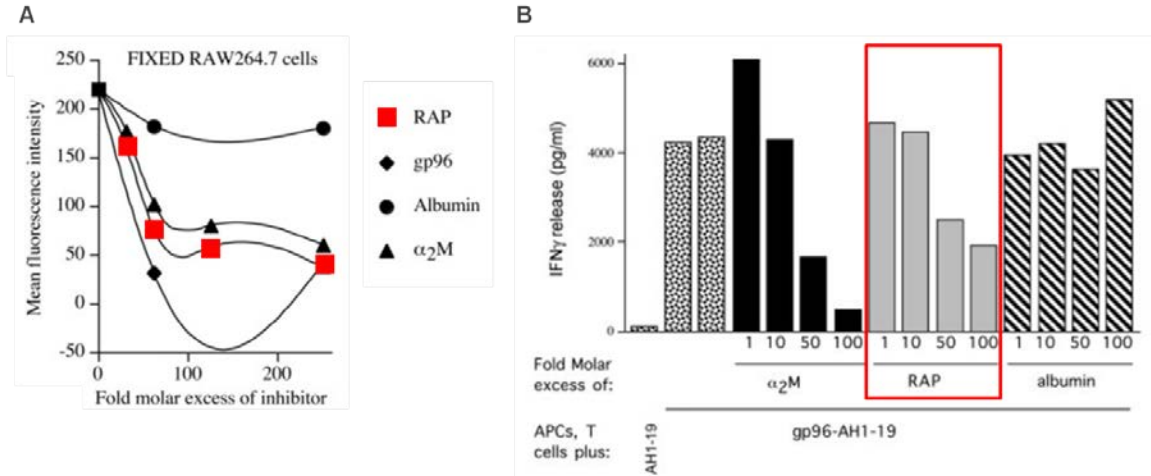


Figure 1-4 RAP inhibited the binding of HSPs to CD91 and blocked cross-presentation of chaperoned peptides *in vitro*. Figure adapted from (Binder and Srivastava, 2004)

(A) RAP competed with gp96 for binding to CD91⁺ macrophage RAW264.7 cells. Paraformaldehyde (PFA)-fixed RAW264.7 cells were incubated with FITC labeled gp96 (gp96-FITC) with or without increasing amount of unlabeled competitors including RAP. Unbound protein was removed by extensive washing. The cells were then analyzed by flow cytometry. The mean fluorescence intensity was measured as an indication of the amount of bound gp96-FITC. Here I highlighted that increasing the amount of RAP (red square) decreased the amount of gp96-FITC bound to cells. (B) The same study examined whether cross-presentation of gp96-chaperoned peptides was inhibited by RAP. Gp96 was complexed to AH1-19, a 19mer peptide containing an L^d-restricted epitope. Gp96-AH1-19 was incubated with the APC RAW264.7 and a CD8⁺ T cell clone that recognized with Ld/AH-1 in a 20 hour re-presentation assay. Stimulation of the CD8⁺ clone was measured by secretion of IFN- γ by the T cells. Again I highlighted here that increasing the amount of RAP inhibited re-presentation of gp96-chaperoned peptides. Copyright (2004) National Academy of Sciences, USA.

1.5 OUTLINE OF THIS THESIS DISSERTATION

The dual roles of HSPs as both an antigen carrier and an adjuvant in one entity make them a good candidate which can initiate tumor-associated immunity efficiently. Therefore, in this thesis dissertation, I examine the requirement of HSP-CD91 pathway in immunosurveillance against tumor. Since HSPs are essential for the survival of cells, depletion of HSPs is not possible. Instead, I target and selectively delete their common receptor CD91 in mice. Chapter 2 of this

thesis describes the generation and characterization of such CD91 conditional knockout mice, and studies whether loss of CD91 expression in APCs affects tumor-associated immunity.

CD91 possess both immunological and non-immunological functions. It is not known whether loss of CD91 expression mainly affects uptake of antigen, compromises the ability of APC to cross-present antigen in general, or both. Chapter 3 of this thesis directly examines the requirement of ligand-binding ability of CD91 for anti-tumor immunity by over-expression of RAP.

It is also important to show that HSPs are the ligands that bind to CD91 or are inhibited by RAP. Therefore, Chapter 4 of this thesis highlights the competition of RAP and tumor-derived HSP for binding to CD91. There, I utilize a strategy to label HSP gp96 with Enhanced Green Fluorescent Protein (EGFP) and co-transfected gp96-EGFP with RAP or a control protein into different tumor cells, so that the localization and trafficking of this fluorescent-labeled gp96 can be monitored *in vivo*.

The results obtained in Chapter 2-4 are further discussed in Chapter 5. Some future directions and the implication of data on the current theory of cancer immunosurveillance and immunoediting are considered.

2.0 LOSS OF CD91 EXPRESSION IN APCs ABROGATES TUMOR-ASSOCIATED IMMUNITY

2.1 RATIONALE

In a majority of patients with cancer and in murine experimental tumor systems, recognition of the tumor by the host's immune system occurs, resulting in priming of concomitant immunity and immunoediting (North and Kirstein, 1977; Schreiber et al., 2011; Shankaran et al., 2001). Conventional mechanisms of priming immune responses to pathogens do not generally apply to tumors because, wherever tested, tumors generally lack sufficient antigen, as a native soluble protein, for cross-priming (Binder and Srivastava, 2005). In addition, being of self origin, tumors in a sterile environment lack the classical PAMPs to activate innate signals and co-stimulation necessary for priming T cells. Given the ability of endogenous, purified HSPs to efficiently prime specific immune responses under conditions of limiting antigen (Binder and Srivastava, 2005; Srivastava et al., 1986), I tested the role of tumor-derived HSPs *in situ* and their receptor CD91 in initiation of immune responses to tumors.

I have explored the HSP-CD91 axis as a mechanism for host priming of anti-tumor immunity for two reasons; i) antigens in the form of peptides are chaperoned by HSPs and are efficiently cross-presented by APCs (Binder et al., 2007; Blachere et al., 1997; Matsutake et al.,

2010; SenGupta et al., 2004; Suto and Srivastava, 1995). The increase in efficiency of cross-presentation of HSP-chaperoned peptides versus peptides or whole antigen protein alone, is several thousand fold and is made possible through the cell surface receptor CD91 on antigen presenting cells (Basu et al., 2001; Binder et al., 2000b; Binder and Srivastava, 2004, 2005; Tischer et al., 2011; Tobian et al., 2004), and ii) our lab has recently shown that HSPs signal through CD91 and activate APCs for co-stimulatory capacity based on secretion of pro-inflammatory cytokines, including IL-1 β , TNF- α , IL-6 and up-regulation of CD40, MHC II and CD86 molecules (Basu et al., 2000; Chen et al., 1999; Pawaria and Binder, 2011; Wang et al., 2002). These observations explain the ability of six intracellular HSPs, gp96, hsp90, hsp70, calreticulin, hsp110 and grp170 to prime immune responses, specific for the peptides they chaperone in cells, once they have been purified from various antigen-bearing cells including tumors and pathogen-infected cells (Basu et al., 2001; Basu and Srivastava, 1999; Binder et al., 2000b; Blachere et al., 1997; Mo et al., 2011; Navaratnam et al., 2001; Srivastava et al., 1986; Tamura et al., 1997; Udono and Srivastava, 1993; Wang et al., 2001; Zugel et al., 2001).

The immunological properties of HSPs make them prime candidates for the initiation of immune responses to tumors. However, HSPs are necessary for survival of cells, so testing their requirement for priming tumor-specific immune responses *in vivo* through simultaneous or sequential deletion is not possible. Instead, I test their requirement by targeting and selectively deleting the HSP receptor, CD91, in mice. This approach is possible because while structurally unrelated, four of the abundant and immunogenic HSPs, gp96, hsp90, hsp70 and calreticulin, utilize the common receptor CD91 to elicit their immune responses (Basu et al., 2001; Binder, 2009; Binder et al., 2000b; Binder and Srivastava, 2004; Pawaria and Binder, 2011). I show that,

unlike wild type mice, mice lacking CD91 expression on dendritic cells fail to elicit tumor-associated immunity.

2.2 MATERIALS AND METHODS

2.2.1 Mice

B6;129S7-*Lrp1*^{*tm2Her*/J} (CD91^{flox/+}) mice (Jackson Laboratory, Bar Harbor, ME) were mated to homozygosity. CD91 conditional knockout mice were generated by crossing B6.Cg-Tg(*Itgax-cre*)1-1Reiz/J (CD11c-Cre) mouse with CD91^{floxP/flox} mouse. Specific depletion of CD91 in CD11c⁺ cells was confirmed by using immunoblot and flow cytometry. All experiments with mice were approved by the Institutional Animal Care and Use Committee (Pittsburgh, PA) and performed in compliance with its guidelines.

2.2.2 Cells and reagents

Tumor cell lines; lung carcinoma D122 and Simian Virus 40 (SV40)-induced SVB6 were obtained from American Type Culture Collection and cultured in complete Dulbecco's modified Eagle's medium (DMEM), which includes 1% sodiumpyruvate, 1% L-glutamine, 1% non-essential amino acids, 1% penicillin and streptomycin, 0.1% 2-mercaptoethanol and 10% fetal bovine serum (GIBCO). All tumor cell lines were free of specific pathogen, tested by IMPACT I PCR Profile (Research Animal Diagnostic Laboratory, RADIL, Columbia, MO). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Ovalbumin 8mer peptide (SIINFEKL) was synthesized by Genemed Synthesis Inc (San Antonio, TX). Complete Freund's adjuvant, incomplete Freund's adjuvant, and LPS were purchased from Sigma (St. Louis, MO). GM-CSF was purchased from Fisher Scientific. Mouse CD8 α ⁺ T cell isolation kit, and mouse CD11c microbeads were purchased from Miltenyi Biotec (Auburn, CA). Mouse IL-1 β ELISA kit and mouse IL-2 ELISA kit were purchased from eBioscience (San Diego, CA). Pacific Blue anti-mouse CD8 α antibody, PerCP-Cy5.5 anti-mouse CD4 antibody, APC anti-mouse CD19 antibody, PE-Cy5 anti-mouse CD3 antibody, PE anti-mouse CD11c antibody, PE-Cy7 anti-mouse CD11b antibody, FITC anti-mouse CD40 antibody, PE anti-mouse CD86 antibody, and PE anti-mouse MHC II (I-A/I-E) antibody were purchased from BD Pharmingen. Anti-CD91 β -chain antibody and anti-CD8 depleting antibody were purified from culture supernatant of 11H4 and TIB210 hybridomas (ATCC), respectively.

2.2.3 Tumor rejection assay after HSP vaccination

In the E.G7 tumor system, gp96 was purified from cultured cells as previously described (Srivastava et al., 1986). Mice were immunized intradermally with 1 μ g gp96 twice one week apart and challenged with 5x10⁵ E.G7 tumor cells in saline one week later. Tumor growth was measured on two axes and expressed as average tumor diameter.

2.2.4 Tumor growth assay

8x10⁵ D122 or 1x10⁶ SVB6 tumor cells were injected intradermally in CD91^{-/-} or control mice, with or without CD8 depletion. Tumor growth was measured on two axes thereafter. CD8 T cells

were depleted with anti-CD8 antibody one day before tumor challenge as previously described (Sayles and Johnson, 1996).

2.2.5 CD91 independent T cell priming

CD91^{-/-} and CD91^{+/+} mice were immunized subcutaneously with 20µg OVA 8 peptide emulsified in Freund's adjuvant at an interval of one week. One week after the last vaccination, 5x10⁶ splenocytes were harvested from OVA 8 peptide immunized CD91^{-/-} or CD91^{+/+} mice, and cultured *ex vivo* for 5 days in the presence of OVA 8 peptide. Specific T cell responses were monitored by IL-2 ELISA.

2.2.6 T cell proliferation assay *in vivo*.

OT-1 cells were harvested from spleens, enriched for CD8+ T cells (Miltenyi Biotec), and labeled with CFSE (Invitrogen). CFSE-labeled OT-1 cells (2.5x10⁶ cells per mouse) were transferred to CD91^{-/-} or CD91^{+/+} mice *via* the retro-orbital route. One day later, recipient mice were immunized intradermally with 20 µg OVA 8 peptide emulsified in Complete Freund's adjuvant. After 3 days, cells from draining lymph nodes were harvested and stained with anti-CD8 antibody. CFSE dilution of the previously transferred OT-1 cells was examined.

2.2.7 Statistical analysis.

An unpaired two-tailed Student's t-test or two-way ANOVA test was used for statistical analyses, and a *P* value of less than 0.05 was considered statistically significant. Error bars were calculated as standard error of the mean (s.e.m).

2.3 RESULTS

2.3.1 Creation and characterization of CD11c-specific CD91^{-/-} mice

Using multiple approaches *in vitro*, our lab and others have demonstrated that CD91 serves as a receptor for immunogenic HSPs including gp96 (Basu et al., 2001; Binder et al., 2000b; Binder and Srivastava, 2004; Pawaria and Binder, 2011; Tischer et al., 2011; Tobian et al., 2004). Conventional CD91 knock-out mice are embryonically lethal (Herz et al., 1992; Herz et al., 1993), thus I created mice lacking CD91 expression in CD11c⁺ cells, to test the role of CD91 for HSP-mediated immunogenicity *in vivo*.

Mice with homozygous floxed CD91/LRP1 (CD91^{flox/flox}) were crossed with mice expressing Cre-recombinase under the CD11c promoter. Expression of Cre in CD11c⁺ cells cause site-specific recombination of lox sequence, which deleted part of promoter, transcription start site, exon I, and exon II of CD91 (Figure 2-1 A). Members of the F1 generation were then back-crossed with the homozygous CD91^{flox/flox} parent and confirmed CD91^{flox/flox} CD11c Cre^{+/-} offspring were mated for all subsequent generations (Figure 2-1 B-D).

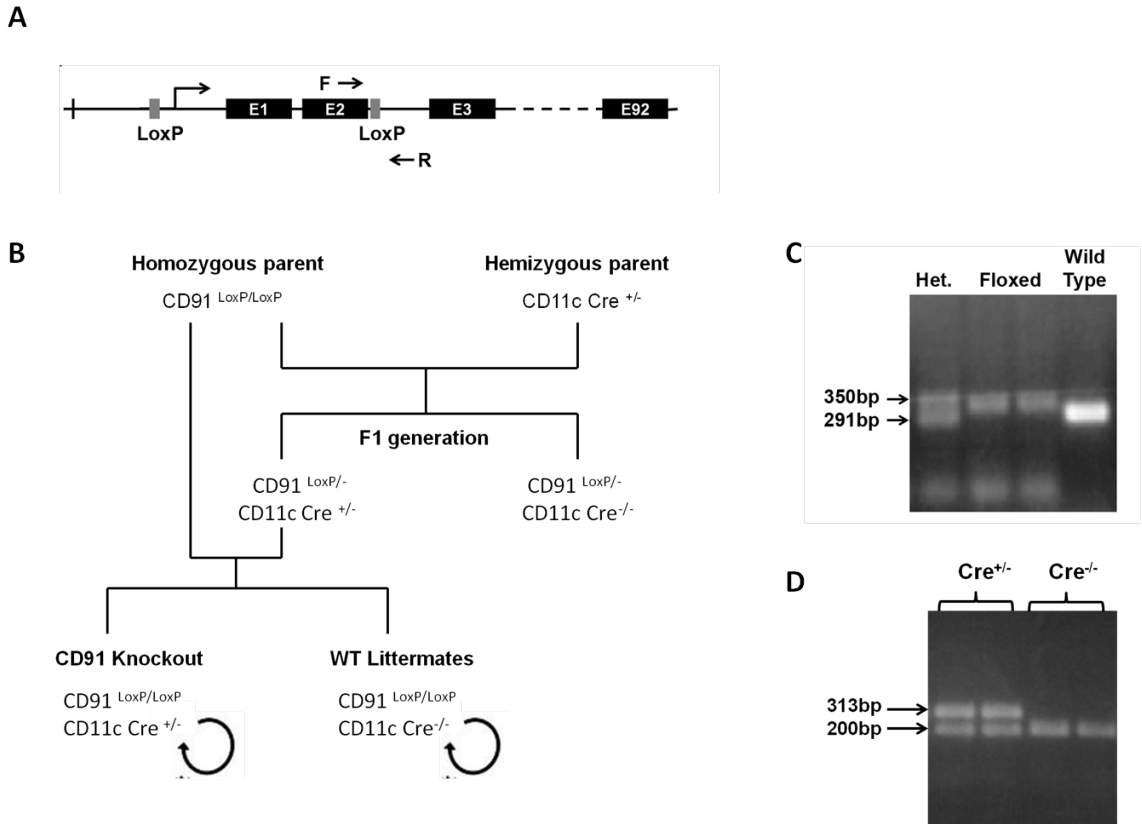


Figure 2-1 Creation of CD11c-specific CD91^{-/-} mice

(A) Schema showing positions of the LoxP sites which occur prior to transcription starting site and at the end of exon 2 of *cd91*. Position of Forward (Abbreviated as 'F') and Reverse (Abbreviated as 'R') primers are also shown, adding a 59bp size to the PCR product. (B). Mating scheme for CD11c specific CD91 KO mice is shown. (C). Representative genotyping of CD91^{flox/flox} is shown and compared to heterozygote CD91^{flox/-} (Het.) and wild type littermate mice. (D) Representative genotyping of CD11c Cre^{+/-} is shown. This assay will not distinguish hemizygous from homozygous transgenic animals. The 313bp band corresponds to the Cre transgene, and the 200bp band corresponds to an internal positive control.

Lack of CD91 expression was confirmed in BMDCs of these mice, generated by culturing bone marrow cells in GM-CSF for 6 days (Inaba et al., 1992). CD11c⁺ cells were purified with magnetic beads to 96% purity. Cells were analyzed by SDS-PAGE and immunoblotting for CD91 protein expression (Figure 2-2 A). In addition, CD11c⁺ cells in the lymph nodes of CD91^{flox/flox} Cre^{+/-} mice were analyzed by flow cytometry. Loss of CD91 expression in CD11c⁺ cells was confirmed (Figure 2-2 B). The CD11c⁺ cells from the lymph node analyzed in Figure 2-2 B included Langerhan's cells, CD103⁺ cells and other subsets of

dendritic cells. Loss of CD91 expression thus occurred on all these cellular subtypes in CD91^{-/-} mice, regardless of their conceivable role in HSP-dependent immunity.

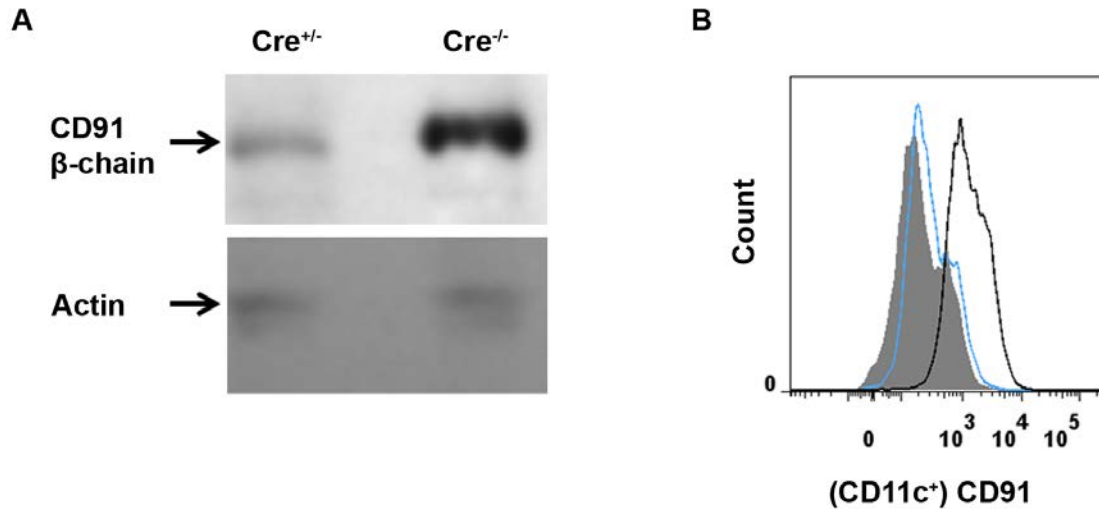


Figure 2-2. CD91 expression is lost in CD11c⁺ cells

CD91^{flox/flox} mice were mated with CD11c⁺-Cre recombinase mice and backcrossed. (A) BMDCs were sorted for CD11c to 96% purity and analyzed by immunoblotting for CD91 β -chain or actin as a loading control. (B) Lymph node cells from CD91^{-/-} (blue) or CD91^{+/+} littermates (black) were stained with anti-CD91 antibody, gated on CD11c⁺ and analyzed by flow cytometry. Filled grey is secondary antibody alone. Experiments were independently performed twice with 2 mice per group.

2.3.2 Selective loss of CD91 in CD11c⁺ cells renders mice unresponsive to gp96

These mice, herein referred to as CD91^{-/-}, had normal percentages of other cellular immune compartments including CD4 and CD8 T cells, B cells, macrophages and DCs when compared to litter mates, CD91^{flox/flox} Cre^{-/-} mice (CD91^{+/+}) (Figure 2-3 A). The ability of BMDCs from CD91^{-/-} mice to mature was tested by pulsing these cells with LPS for 24 hours and monitoring the expression of several maturation markers. CD86, CD40 and MHC II were all up-regulated in response to LPS, and the up-regulation was to similar levels as the BMDCs from CD91^{+/+} littermates (Figure 2-3 B). BMDCs from CD91^{-/-} mice were also able to respond to LPS

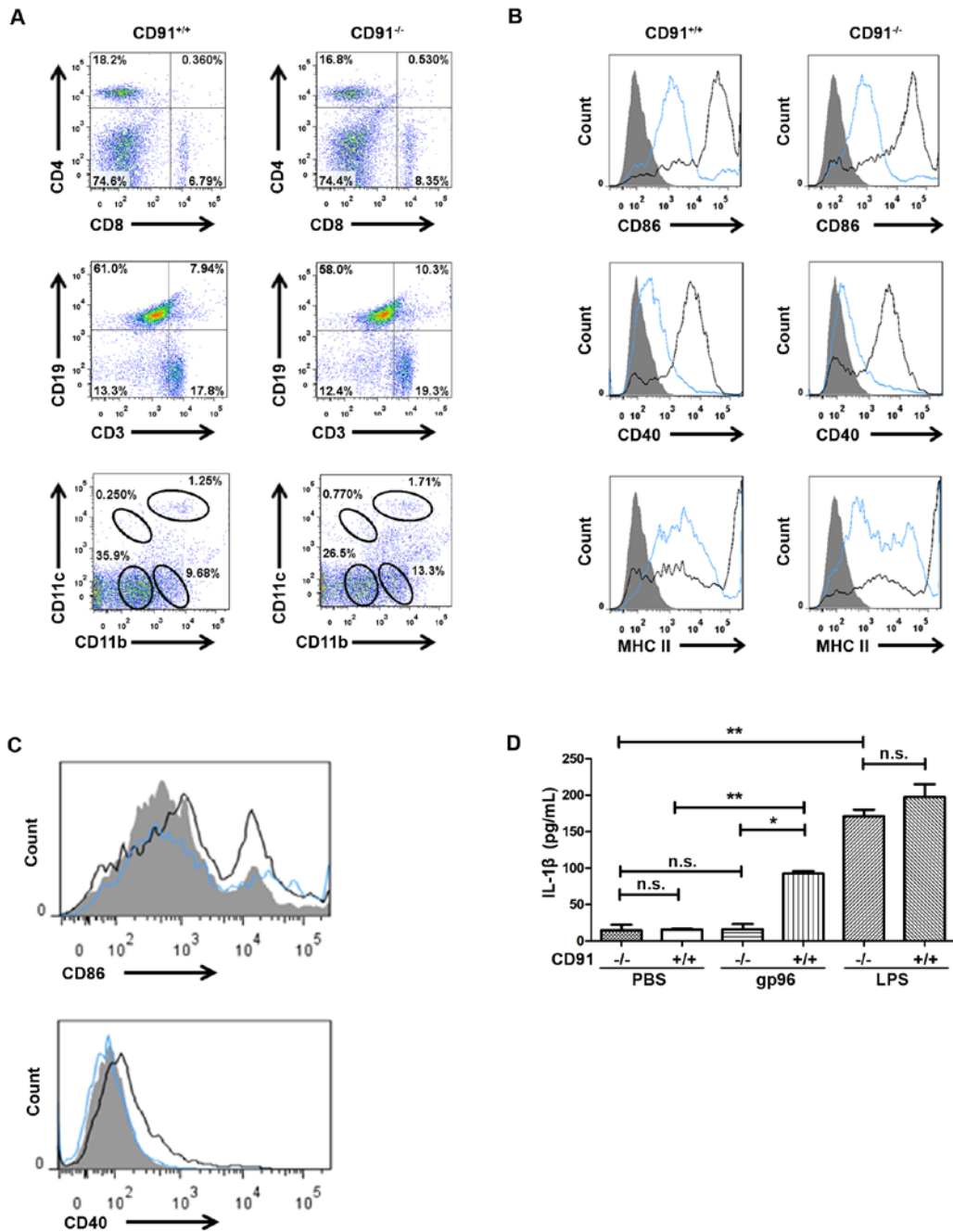


Figure 2-3. Selective loss of CD91 in CD11c⁺ cells renders mice unresponsive to gp96

(A) Lymph node cells from CD91^{-/-} or CD91^{+/+} littermates were phenotyped for markers indicated. (B) BMDCs from CD91^{-/-} or CD91^{+/+} mice were pulsed on day 6 with LPS for 24 hours (black line) or left un-pulsed (blue line). Cells were analyzed for expression of CD86, CD40 and MHC II. Filled grey is secondary antibody alone. (C) BMDCs from CD91^{-/-} (blue line) or CD91^{+/+} (black line) mice were pulsed on day 6 with 100μg gp96 for 24 hours or left un-pulsed (filled grey). Cells were analyzed for expression of CD86 and CD40. (D) BMDCs from CD91^{-/-} or CD91^{+/+} mice were pulsed with 100μg gp96 or 1EU LPS for 24 hours or with PBS. IL-1β was measured by ELISA. * P < 0.05, ** P < 0.01, n.s. not significant. Error bars indicate s.e.m. Experiments were independently performed twice with 2 mice per group. Representative data from 1 mouse was shown in (A-C).

by secreting IL-1 β , measured by ELISA 24 hours later (Figure 2-3 D). Importantly however, CD91^{-/-} BMDCs failed to secrete IL-1 β in response to gp96. Control BMDCs from CD91^{+/+} mice secreted IL-1 β after stimulation with LPS or gp96, consistent with previous observations (Basu et al., 2000; Pawaria and Binder, 2011). Additionally I tested the ability of BMDCs to mature in response to gp96 as previously shown (Messmer et al., 2013). BMDCs from CD91^{-/-} mice failed to upregulate maturation markers CD86 and CD40 in response to incubation with gp96 (Figure 2-3 C). BMDCs from CD91^{+/+} were able to do so.

I next tested the response of CD91^{-/-} mice to gp96 immunization in a prototypical tumor rejection assay (Binder and Srivastava, 2004; Srivastava et al., 1986). CD91^{-/-} or CD91^{+/+} mice were immunized with E.G7-derived gp96. Mice were then challenged with E.G7 and tumor growth was monitored. While tumors were rejected in the gp96-immunized CD91^{+/+} mice (as routinely observed, Binder and Srivastava, 2004; Srivastava et al., 1986), the rejection was absent in the gp96-immunized CD91^{-/-} mice (Figure 2-4).

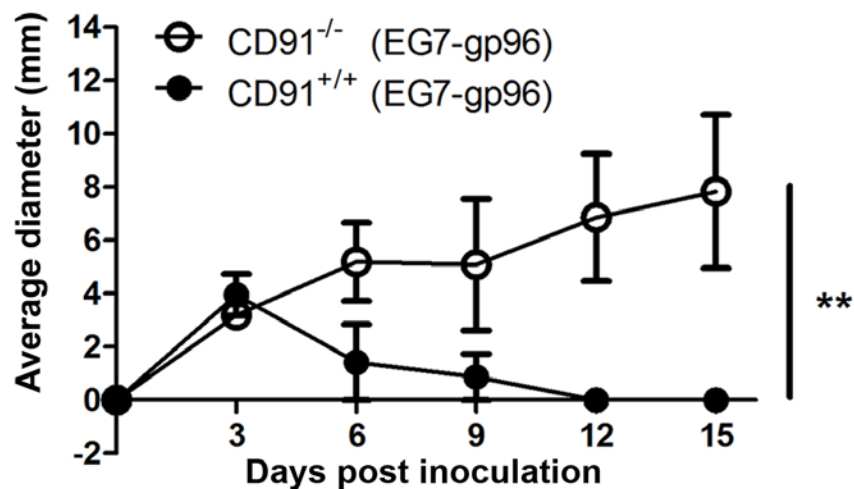


Figure 2-4. CD91^{-/-} mice fail to respond to HSP vaccination

CD91^{-/-} or CD91^{+/+} mice were immunized with 1 μ g E.G7 tumor-derived gp96 twice, one week apart, followed by E.G7 tumor challenge one week later. Tumor growth was monitored. ** P < 0.01. Error bars indicate s.e.m. Experiments were independently performed twice with 3-5 mice per group.

2.3.3 Loss of CD91 expression in antigen presenting cells abrogates tumor-associated immunity

As a measure of tumor-associated immunity, I tested the rate of growth of tumors in CD91^{-/-} mice using the moderately immunogenic tumor D122, a highly metastatic clone of the 3LL (Lewis Lung) carcinoma. When mice were inoculated with 8x10⁵ D122 cells, tumor growth rate was significantly faster in CD91^{-/-} mice versus CD91^{+/+} recipient mice (Figure 2-5 A). The significant difference in tumor growth rate was observed at early time points, up to day 11, with differences then dissipating. To test the role of the adaptive immune response on tumor growth, I depleted CD8⁺ T cells from the CD91^{-/-} or CD91^{+/+} mice. When CD8⁺ cells were depleted, D122 tumors grew with identical kinetics in mice from both groups (Figure 2-5 B). The rate of tumor growth in both groups was identical to CD91^{-/-} mice in Figure 2-5 A. Therefore, specific CD8⁺ T cell priming was impaired in CD91^{-/-} mice. To test the generality of these observations, similar experiments were performed with the highly immunogenic, regressor SV40-transformed tumor SVB6. While SVB6 cells ultimately grew and were rejected in both the CD91^{-/-} and CD91^{+/+} mice, the tumors grew significantly larger and were rejected more slowly in CD91^{-/-} mice (Figure 2-5 C).

Since CD91^{-/-} mice were deficient in their ability to mount anti-tumor immune responses, I tested their general immuno-competence by priming with a regimen that bypasses a requirement for antigen uptake and intracellular processing. CD91^{-/-} or CD91^{+/+} mice were immunized with the OVA 8-mer (SIINFEKL) peptide plus Freund's adjuvant and spleen cells were examined for T cells specific for the peptide. The peptide used, OVA 8, required no uptake or further processing by APCs prior to binding MHC I. Following peptide stimulation of spleen cells *ex vivo*, IL-2 was measured in cultures by ELISA as an indication of OVA 8-specific T cells

(Figure 2-6 A). Equitable levels of functional responsive OVA 8-specific T cell responses were obtained in either type of mouse ($P = 0.5428$, Figure 2-6 B). I used a second read out to confirm the results in Fig. 2D. CFSE-labeled OT-1 cells were adoptively transferred into CD91^{-/-} or CD91^{+/+} mice one day prior to immunization with OVA 8 peptide plus adjuvant (Figure 2-6 C). Three days later, lymph nodes were harvested and percent dividing OT-1 cells was quantified. No difference in the percent of dividing OT-1 cells was observed between immunization of CD91^{-/-} and CD91^{+/+} mice (Figure 2-6 D). These result are consistent with normal immune phenotype observed in Figure 2-3 and shows that CD91^{-/-} mice are capable of priming T cell responses when immunized with peptide + adjuvant, a regimen that does not require CD91 for uptake.

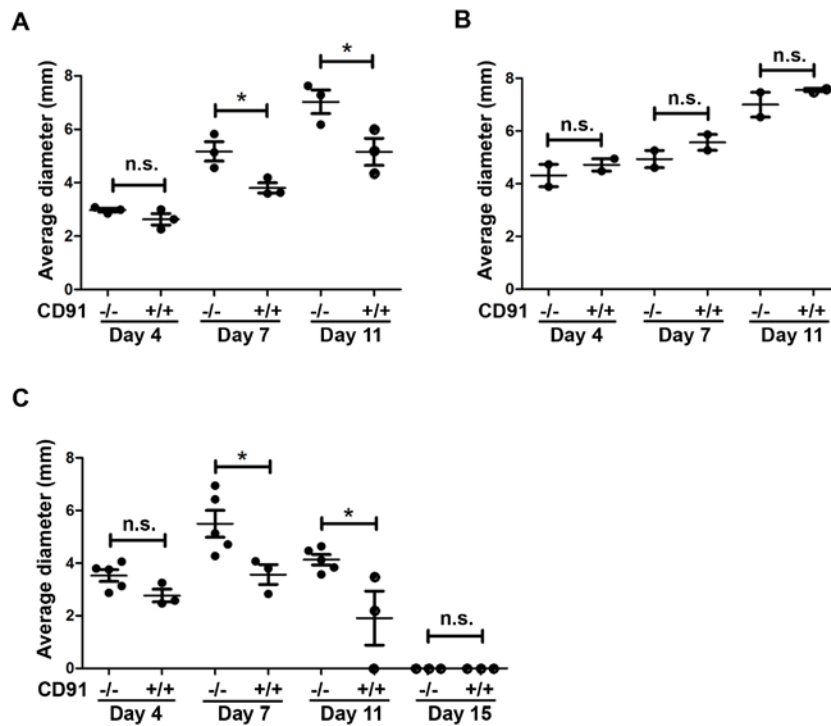


Figure 2-5. CD91 expression in CD11c⁺ cells is required for tumor-associated immunity.

(A) CD91^{-/-} or CD91^{+/+} littermates were challenged intradermally with D122 tumor cells. Tumor growth was measured on two axes. (B) CD8⁺ cells were depleted from CD91^{-/-} or CD91^{+/+} mice 24 hours prior to implantation of intradermal D122. CD8 depletion was continuous for >5 days. Tumor growth was monitored as in (A). (C) SVB6 tumor was implanted intradermally into CD91^{-/-} or CD91^{+/+} littermates. Tumor growth was monitored as in (A). n.s., not significant; * $P < 0.05$. Experiments were independently performed twice with 3-5 (A,C) or 2 (B) mice per group.

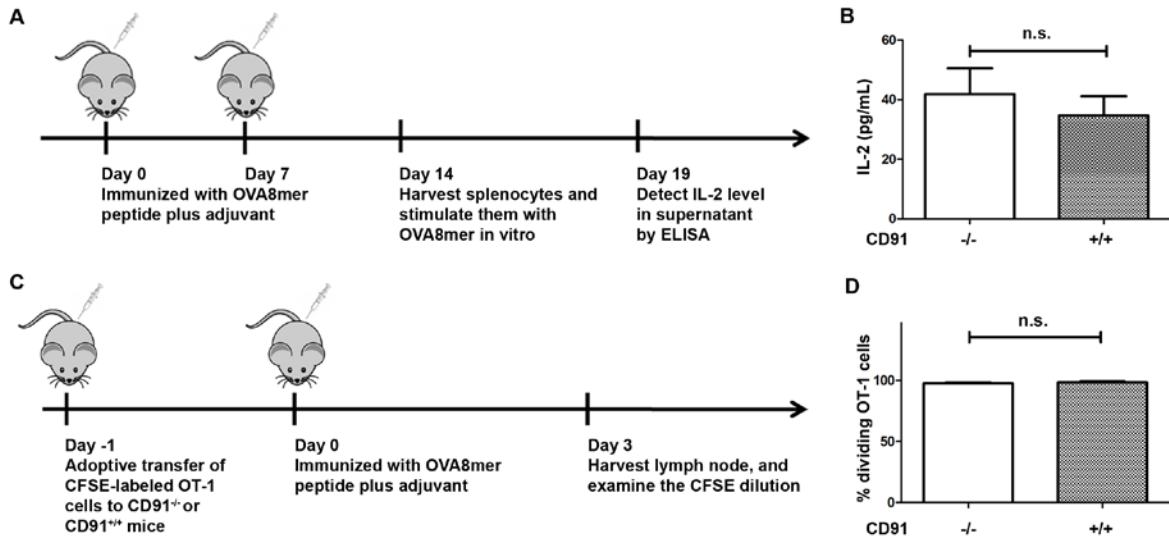


Figure 2-6. CD91^{-/-} mice are not grossly immunocompromised.

(A) Schema for CD91 independent priming. CD91^{-/-} or CD91^{+/+} mice were immunized with OVA8 peptide (SIINFEKL) emulsified in CFA (week 1) or IFA (week2). Supernatants from spleen cells cultured *ex vivo* with OVA8 peptide for 5 days were analyzed for IL-2. (B) IL-2 ELISA was performed on supernatant of cultures. (C) CFSE-labeled OT-1 cells were transferred to mice followed by immunization with OVA8 peptide in CFA. Lymph nodes were harvested on day 3. (D) Percent dividing OT-1 cells in lymph nodes were measured by flow cytometry. n.s., not significant. Experiments were independently performed twice with 3-5 mice per group.

2.4 DISCUSSION

In this chapter, I generated CD11c-specific CD91 conditional knockout mice, and showed that mice deficient in the receptor CD91 on CD11c⁺ antigen presenting cells became unresponsive to gp96, and were unable to mount substantial immune resistance to tumors, in the form of a CD8⁺ T cell response. To my knowledge, these findings are the first reports of a direct role for CD91 in priming tumor-associate immunity *in vivo*. I have taken advantage of my development of a novel system where the tumor and elicitation of tumor-associated immunity can be left unperturbed by experimental manipulation *in vitro*.

By using immunoblotting and flow cytometry, loss of CD91 expression was confirmed in CD11c⁺ cells derived from CD91^{-/-} mice, although residual level of CD91 can still be observed in those cells (Figure 2-2). This residual signal may come from contaminating cells such as macrophages, because it was technically unfeasible to get a higher purity of CD11c⁺ cells than 96% by using MACs isolation in my experiment. Additionally, the expression of Cre is dependent on the promoter of the *cd11c* gene. Therefore, cells that weakly express CD11c may also have a low level of Cre recombinase. In those CD11c^{low}Cre^{low} cells, CD91 expression may not be completely abrogated. However, the loss of CD91 expression was significant, since anti-tumor immune response was inhibited in these CD91^{-/-} mice when compared to CD91^{+/+} mice.

I conducted several studies to examine the phenotype of these newly-generated CD91^{-/-} mice, including percentages of cellular immune compartments, and maturation of CD91^{-/-} BMDC. BMDCs were used because significantly more CD11c⁺ cells were required for this experiment than could be obtained from lymph nodes. BMDCs express a similar phenotype as DC *in vivo*, including the CD11c⁺ marker (Lutz et al., 1999). The characterization continues unabated, in the immunological and non-immunological realm, however all the experiments that are relevant for my hypothesis in this project have been performed. Importantly, unlike WT BMDCs, BMDCs from CD91^{-/-} mice failed to upregulate maturation marker and secrete cytokines in response to HSP gp96. However, CD91^{-/-} BMDCs were still able to respond to an alternative maturation stimulus LPS. This result supported previous findings from our lab and others that CD91 serves as a *bona fide* receptor for four major immunogenic HSPs including gp96.

Besides CD91, several other receptors have also been suggested to be involved in HSP-mediated signaling and activation of APCs, such as TLR2/4. The involvement of TLR2/4 as HSP

receptors was based on a series of studies in which cells transfected with TLR2 and/or TLR4 became responsive to recombinant HSPs, as measured by NF- κ B activation or release of cytokines/chemokines. These immune effects were reduced in cells from knockout mice that lack TLR2/4 or their downstream adaptors MyD88 or TRAF-6, or cells incubated with anti-TLR2/4 antibodies (Asea et al., 2002; Vabulas et al., 2002a; Vabulas et al., 2002b). However, as mentioned in the Introduction (refer to chapter 1.3.2), the recombinant HSPs used in these studies were contaminated with bacteria-derived endotoxins such as LPS, which also use TLR2/4 as a receptor. Therefore, loss of TLR2/4 or their downstream adaptors actually abrogated immune response induced by contaminating LPS, but not HSPs *per se*. Although removal of LPS was attempted in some studies by using polymyxin B beads, residual amount of LPS may still remain in the bacteria-derived recombinant HSPs. In the presence of gp96, TLR ligands such as LPS have been shown to activate DC even at a low concentration (<1 ng/ml). However, TLR ligands alone were unable to activate DC at this concentration (Warger et al., 2006).

I also compared kinetics of tumor growth between CD91^{-/-} mice and their wild type littermates. My data demonstrated that tumors proliferated faster in these knockout mice, possibly due to impaired priming of CD8⁺ T cells. Interestingly, the significant difference in tumor growth rate was observed only at early time points, up to day 11. The disparity dissipated thereafter. It suggested that CD91-independent mechanisms may come into play to restrict tumor growth when tumor volume increases significantly. Therefore, the relatively large amount of inoculated tumor cells (8×10^5 D122 used in this case) should be factored in to explain the short-lived impact of CD91 deficiency on tumor growth. Fewer tumor cells will be inoculated in future experiments to examine the importance of CD91 for extended duration before tumors become firmly established.

When CFSE-labeled OT-1 cells were used to examine the capacity of CD91^{-/-} and CD91^{+/+} mice to prime immune responses after peptide plus adjuvant treatment, I observed that, although the percentage of dividing OT-1 cells was similar, OT-1 cells divided more completely in CD91^{+/+} mice compared to CD91^{-/-} mice (data not shown). It suggested that, besides antigen uptake, CD91 may play a role in enhancement of T cell proliferation.

3.0 LIGAND-BINDING ABILITY OF CD91 IS REQUIRED FOR ANTI-TUMOR IMMUNITY

3.1 RATIONALE

CD91 exerts both immunological and non-immunological functions. Its α -chain can bind to an array of ligands, which participates in several physiological functions, such as lipoprotein metabolism and transport, cell migration, clearance of protease and neurotransmission. As shown in the previous chapter, CD91 is required to control tumor growth, possibly *via* priming of CD8⁺ T cell responses. However, the precise role of CD91 in this process is still unknown. CD91 may be required for ligand endocytosis, signaling, antigen presentation, T cell priming, etc. Both *ex vivo* assay on cytokine secretion and *in vivo* assay on T cell proliferation in previous chapter showed no difference between CD91^{-/-} and CD91^{+/+} mice, suggesting that antigen presentation and downstream T cell priming remain intact in the knockout mice. In this chapter, I then directly examined ligand-receptor interaction for CD91 and its influence on antigen presentation and anti-tumor immune responses.

RAP is a 40-KDa molecular chaperone of CD91, which assists delivery of CD91 from ER to cell surface and prevents premature binding of other ligands during membrane trafficking. The reason why it blocks access of other structurally and functionally distinct ligands to CD91 is that RAP can bind to all four ligand-binding domains on CD91 with high affinity. Indeed,

previous studies *in vitro* showed that RAP competed away HSPs from binding to CD91 and inhibited downstream cross-presentation of HSP-chaperoned antigenic peptides (Figure 1-3). It is therefore a powerful tool to introduce RAP *in vivo* to scrutinize the importance of ligand-binding ability of CD91 on antigen-transfer and tumor-associated immune responses. To this end, I transfected different tumor cell lines with RAP-expressing vector. In the event that tumor cells lose their membrane integrity, I hypothesized that RAP would be released concurrently with HSPs and therefore prevent HSPs from binding to CD91 on surrounding APCs.

In this chapter, I transfected different tumor cell lines with RAP plasmid, and demonstrated that tumors expressing RAP grew with significantly faster kinetics than the non-RAP expressing counterparts in wild type mice, although this difference was non-existent in immunocompromised mice. Inhibition of antigen transfer and downstream cross-presentation by RAP abrogates T cell proliferation *in vivo*, and therefore decrease the immunogenicity of tumors.

3.2 MATERIALS AND METHODS

3.2.1 Mice.

Female BALB/c, C57BL/6, C.129S7(B6)-Rag1^{tm1Mom}/J (*rag1*^{-/-} BALB/c), and B6(Cg)-Rag2^{tm1.1Cgn}/J (*rag2*^{-/-} C57BL/6), C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-1), B6.SJL-Ptprc^aPepc^b/BoyJ (CD45.1) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the animal facility at the University of Pittsburgh. All experimental mice were 6–8-weeks old. All experiments with mice were approved by the Institutional Animal Care and Use Committee (Pittsburgh, PA) and performed in compliance with its guidelines.

3.2.2 Cells and Reagents.

Tumor cell lines; fibrosarcoma CMS5 and Simian Virus 40 (SV40)-induced SVB6 were obtained from American Type Culture Collection (ATCC) and cultured in complete Dulbecco's modified Eagle's medium (DMEM), which includes 1% sodium pyruvate, 1% L-glutamine, 1% non-essential amino acids, 1% penicillin and streptomycin, 0.1% 2-mercaptoethanol and 10% fetal bovine serum (GIBCO). RAP or control vector-transfected tumor cells were cultured in complete DMEM media plus blasticidin (3 μ g/mL, Invitrogen). All tumor cell lines were free of specific pathogen, tested by IMPACT I PCR Profile (Research Animal Diagnostic Laboratory, RADIL, Columbia, MO). All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Ovalbumin, complete Freund's adjuvant, incomplete Freund's adjuvant, LPS, and mitomycin-C were purchased from Sigma (St. Louis, MO). GM-CSF was purchased from Fisher Scientific. Ovalbumin was rendered free of endotoxin by detoxi-Gel columns (Thermo Scientific). Mouse CD8 α ⁺ T cell isolation kit, and mouse CD11c microbeads were purchased from Miltenyi Biotec (Auburn, CA). APC anti-mouse CD45.2 was purchased from BD Pharmingen. Anti-V5-HRP antibody, 53kDa V5-containing protein (positope) was purchased from Invitrogen (Carlsbad, CA). Anti-hsp90 antibody were purchased from Enzo Life Sciences (Farmingdale, NY).

3.2.3 Plasmid construction and transfection.

Total RNA was prepared from mouse liver by using the RNeasy Mini Kit (Qiagen, ON, Canada) and treated with RNase-free DNase to eliminate any potential genomic DNA contamination. Aliquots of 1 μ g of total RNA were used to synthesize first-strand cDNA by using the Omniscript

RT kit (Qiagen, ON, Canada). The cDNA was amplified to generate full-length RAP and short-length RAP which lacks the N-terminal signal sequence, by the following primer pairs respectively: (1) GAGATGGCGCCTCGAAGAGAGA (RAP-NT1) and GAGTTCATTGTGCCGAGCCCTTGA (RAP-CT); (2) GAGATGGGTTACTCGCGAGAGAAGAA(RAP-NT2) and GAGTTCATTGTGCCGAGCCCTTGA (RAP-CT). PCR amplification was performed by using 2X Mango Mix (Bioline USA Inc, MA) for initial 5 cycles (94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min) and additional 25 cycles (94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min). After amplification, the PCR products were inserted into pEF6/V5-His-TOPO vectors (Invitrogen), which were then transformed into One Shot[®] TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA). CMS5 and SVB6 tumor cells were stably transfected with each of the plasmids by using Fugene[®] 6 transfection reagent (Roche Molecular Biochemicals). DNA sequencing was performed at Genewiz or at the Genomics and Proteomics Core Laboratories at the University of Pittsburgh.

3.2.4 Tumor growth assay

To test the effect of RAP on immunogenicity of tumors, BALB/c (or C57BL/6) mice were challenged with 1×10^6 RAP- or control vector-transfected CMS5 (or SVB6) cells. In a separate experimental setting, immunocompromised *rag1*^{-/-} BALB/c or *rag2*^{-/-} C57BL/6 mice were challenged with 1×10^6 corresponding RAP- or control vector-transfected tumor cells. Tumor growth was measured on two axes for 2-3 weeks after challenge.

3.2.5 *In vitro* proliferation assay

The *in vitro* proliferative rates of those tumors were determined by the Click-iT EdU Assay Kit (Invitrogen, Carlsbad, CA).

3.2.6 Tumor rejection assay

To test antigen transfer from RAP-expressing tumor cells to APCs, BALB/c mice were immunized intradermally with titrated dose of mitomycin C-treated, RAP- or control vector-transfected CMS5 cells. Two weeks later, mice were challenged with 1×10^6 untransfected CMS5 cells. Tumor growth was measured on two axes thereafter.

3.2.7 T cell proliferation assay *in vivo*.

Endotoxin-free OVA was introduced to CMS5 cells expressing RAP or control protein control by electroporation at 200 V for 30 ms (Bio-rad). The OVA-loaded cells were then rendered replication incompetent by treatment with mitomycin-C. CD45.2⁺ OT-1 cells were harvested from spleens, enriched for CD8⁺ T cells (Miltenyi Biotec), and labeled with CFSE (Invitrogen). CFSE-labeled OT-1 cells were transferred to CD45.1⁺ C57BL/6 or CD91^{-/-} mice via the retro-orbital route (1.5×10^6 cells per mouse; total OVA in these cells is <1 ng). One day later, recipient mice were immunized intradermally with 1×10^5 replication-incompetent OVA-transfected CMS5 with or without RAP expression. After 3.5 days, cells from draining lymph nodes were harvested and stained with anti-45.2 and anti-CD8 (for the experiment in CD45.1⁺ C57BL/6 mice, Fig. 5 D)

and anti-CD8 antibody only (Fig. 5 E) to enrich for the previously transferred OT-1 cells. CFSE dilution of those OT-1 cells was examined.

3.2.8 Microscopy

Transfected tumor cells were cultured overnight in 35 mm glass bottom culture dishes (MatTek Corp. Ashland, MA) at 37°C, washed with PBS, fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked with 2% BSA. For RAP transfected cells, mouse anti-V5 antibody and Cy3-conjugated goat anti-mouse IgG antibody were used to stain RAP; rat anti-HSP90 antibody and DyLight488-conjugated goat anti-rat IgM antibody were used to stain HSP90; DAPI was used to stain nucleus. For gp96-EGFP transfected cells, rhodamine conjugated Phalloidin (Invitrogen/Life Technologies, Grand Island, NY) was used to stain cytoskeleton; DAPI was used to stain nucleus, according to a protocol developed by the Center for Biologic Imaging (CBI, University of Pittsburgh). Tumor tissues were incubated with 2% paraformaldehyde for one hour, then with 30% sucrose at 4°C overnight. After freezing, tumor tissues were cut into 8µm thick sections using a cryo-microtome (HM505E Microm). The sections were permeabilized with 0.1% Triton X-100 and blocked with 2% BSA. Mouse anti-V5 antibody and Cy3-conjugated goat anti-mouse IgG antibody were used to stain RAP. Rat anti-HSP90 antibody and DyLight488-conjugated goat anti-rat IgM antibody were used to stain HSP90. DAPI was used to stain nucleus. All images were captured using an Olympus FV1000 inverted confocal microscope with 100x objective (Fig. 3 C and D, and 6 B) or 60x objective (Fig. 3 K) and Fluoview v. 2.1 acquisition software (Melville, NY). Imaris v. 7.2.1 (Bitplane, Zürich, Switzerland) and Photoshop v. 7.0 (Adobe, San Jose, CA) were used for analysis and to prepare the images for publication.

3.2.9 Statistical analysis.

An unpaired two-tailed Student's t-test or two-way ANOVA test was used for statistical analyses, and a *P* value of less than 0.05 was considered statistically significant. Error bars were calculated as standard error of the mean (s.e.m).

3.3 RESULTS

3.3.1 CD91-HSP interaction is required for priming anti-tumor immunity

To determine if abrogation of ligand binding to CD91 was responsible for the decreased immune responses to tumors (seen in Fig 2), I constructed two types of plasmids: (1) plasmid expressing wild type RAP, and (2) plasmid expressing modified short-length RAP, which lacks its N-terminal signal sequence necessary for ER translocation (Figure 3-1 A). Unlike wild type RAP which is expressed in the endoplasmic reticulum, modified RAP will be expressed in the cytosol. The rationale behind expressing a cytosolic version of RAP is that I can still study the effect of elevated RAP levels on tumor immunity even if only plasma membrane, but not ER membrane, loses its integrity in some physiological setting. Indeed, the result from experiments in this chapter showed a greater inhibitory effect of the cytosolic RAP compared to its wild type counterparts (data not shown). Therefore, I present data based on cytosolic RAP thereafter.

The cDNA of RAP was amplified by PCR and inserted into pEF6/V5-His-TOPO vectors, in which RAP and a V5 tag were expressed under a strong promoter $P_{EF-1\alpha}$ (Figure 3-1 B). I then expressed and quantified RAP in the moderately immunogenic fibrosarcoma CMS5, or SVB6

(Figure 3-2). Tumors were determined to express ~10 fg of RAP per cell for both tumors. Tumors transfected with RAP was then stained with anti-V5 antibody representing RAP, and anti-HSP90 antibody as a marker for cytosol. Fluorescent microscopy observed co-localization of the stains for RAP and HSP90, indicating the cytosolic expression of RAP (Figure 3-3).

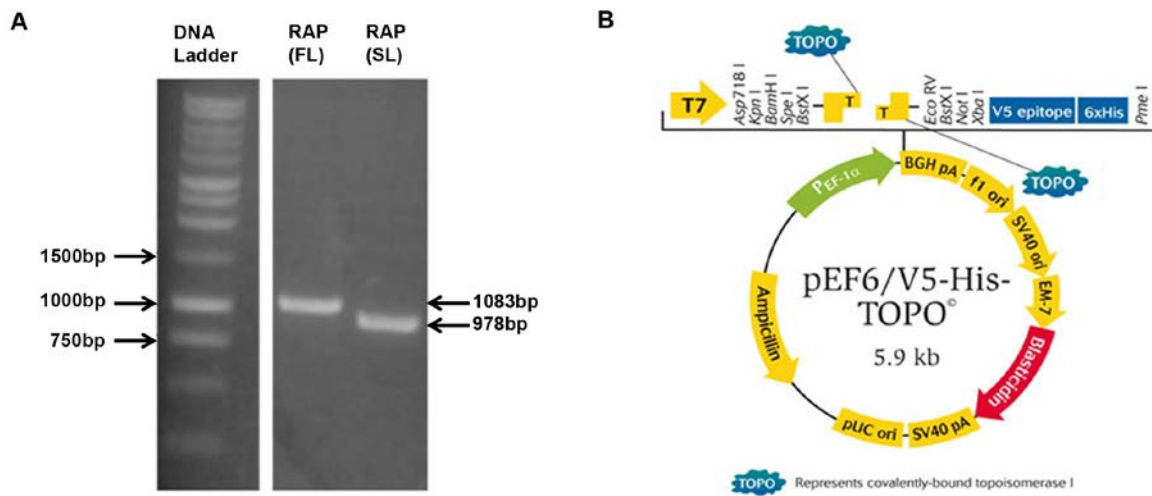


Figure 3-1. RAP is expressed under a strong promoter P_{EF-1a}

(A) Full-length RAP cDNA (1083bp) and short-length RAP cDNA (978bp) were cloned from murine hepatocytes. (B) Full-length or short-length RAP, in tandem with a V5 tag, was expressed by the pEF6/V5-His-TOPO vector, under a strong promoter P_{EF-1a}.

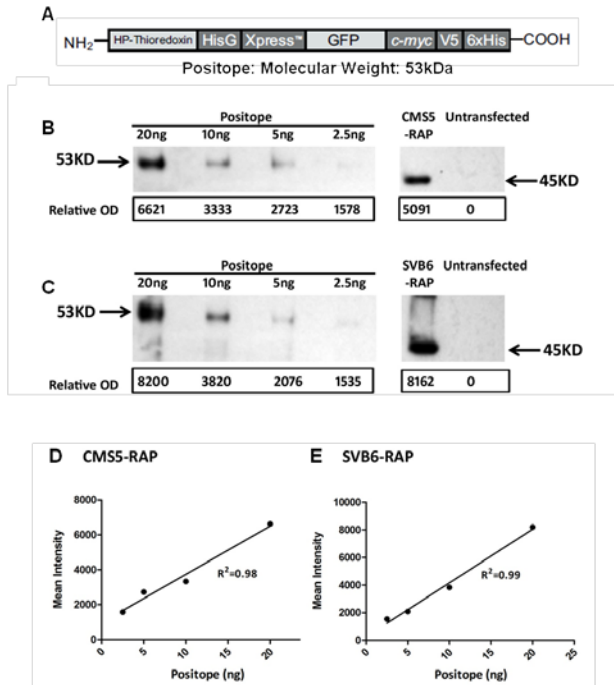


Figure 3-2. Quantification of stable RAP expression in tumor cells.

The CD91 inhibitor, RAP, was transfected and stably expressed in tumor cells, as confirmed by immunoblot in CMS5 cells (A), or SVB6 cells (B). Titrated amount of a 53 kDa V5-expressing protein was blotted and scanned to obtain relative optical density (OD). (D and E) a linear relationship was established between the titrated amounts of the V5-expressing protein and their relative OD. (B) and (D) were used to quantify RAP expression in CMS5 cells. (C) and (E) were used to quantify RAP expression in SVB6 cells. The relative OD values of CMS5 and SVB6 bands (2×10^6 cells equivalent) were 5091 and 8162, respectively. Experiments were independently performed twice.

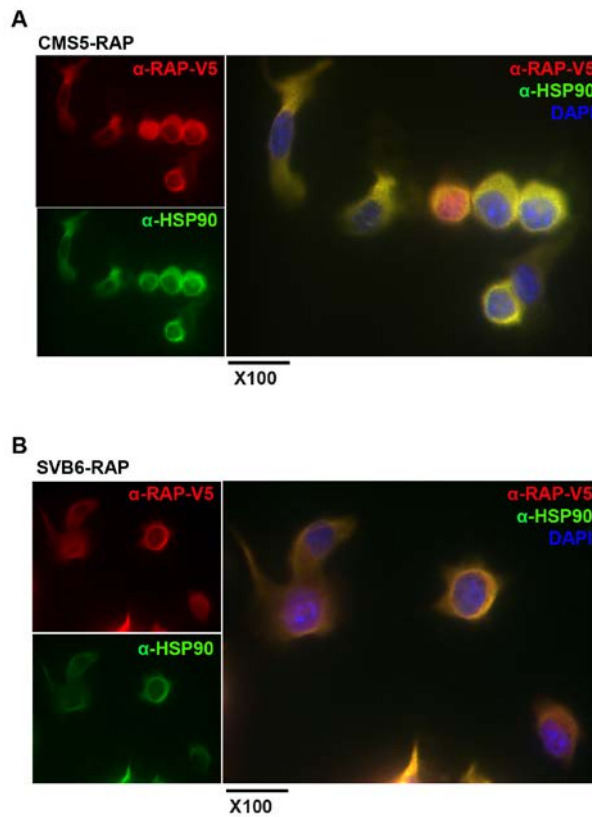


Figure 3-3. RAP is expressed in the cytosol of tumor cells.

Cytosolic localization of RAP in CMS5 (A) cells, or SVB6 (B) cells was examined by confocal microscopy comparing the signal of RAP with the cytosolic protein HSP90. The two signals co-localized to give the yellow color. Experiments were independently performed twice.

Tumors expressing RAP or a control (non-CD91 binding) protein were then implanted into BALB/c mice and tumor growth was monitored. In the tumor growth assay, RAP-expressing CMS5 grew with significantly faster kinetics when compared to control CMS5 tumor cells (Figure 3-4 A). However, when the same tumor cells were implanted into immunocompromised *rag1*^{-/-} mice, there was no difference in growth rate between the RAP and non-RAP expressing tumor cells (Figure 3-4 B). Since tumor growth rate is determined largely by the balance between tumor cell proliferation and lysis (mediated in part by immune effectors), I tested if RAP transfection had any effect on cellular proliferation. There was no difference in growth rate between RAP and non-RAP expressing cells when tested in a proliferation assay *in vitro* that measured incorporation of EdU dye into DNA (Figure 3-5 A-B). These results were confirmed in the SVB6 tumor system in C57BL/6 mice. RAP-expressing SVB6 tumors grew to a significantly bigger size and were rejected more slowly than control SVB6 tumor cells (Figure 3-4 C). In immunocompromised *rag2*^{-/-} mice, however, the growth of tumors was comparable irrespective of RAP expression (Figure 3-4 D). Similar to the CMS5 model, SVB6 tumors proliferated comparably *in vitro* whether they expressed RAP or not (Figure 3-5 C). The reduction of immunogenicity of RAP-transfected cells depends on the continual expression of RAP. Therefore, RAP expression in tumors was monitored throughout the duration of the experiment and was found to be preserved 15 days after implantation (Figure 3-6). These results highlight the role of CD91 as a receptor for ligand binding, in mounting tumor-associated immunity.

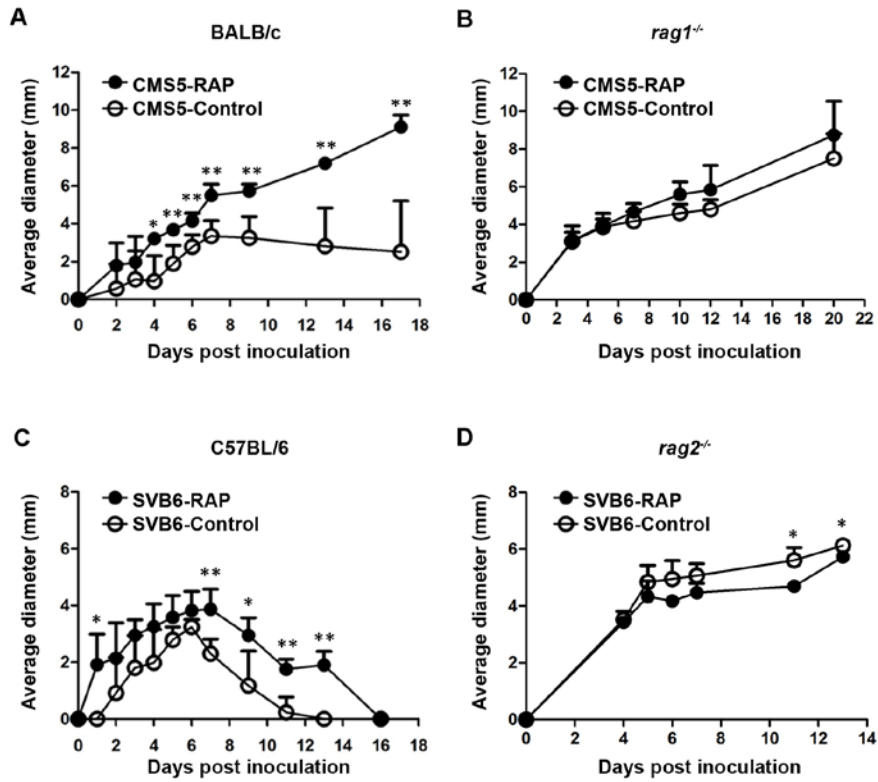


Figure 3-4. Inhibition of ligand binding to CD91 abrogates priming of anti-tumor immunity

(A and B) CMS5 cells expressing RAP or a control protein were implanted intradermally in BALB/c (A) or *rag1*^{-/-} (B) mice and tumor growth was monitored. Average tumor diameter was calculated. (C and D) SVB6 cells expressing RAP or control protein were implanted into C57BL/6 (C) or *rag2*^{-/-} (D) mice and tumor growth was monitored. * P < 0.05, ** P < 0.01. Experiments were independently performed thrice with 3-5 mice per group. Error bars indicated s.e.m.

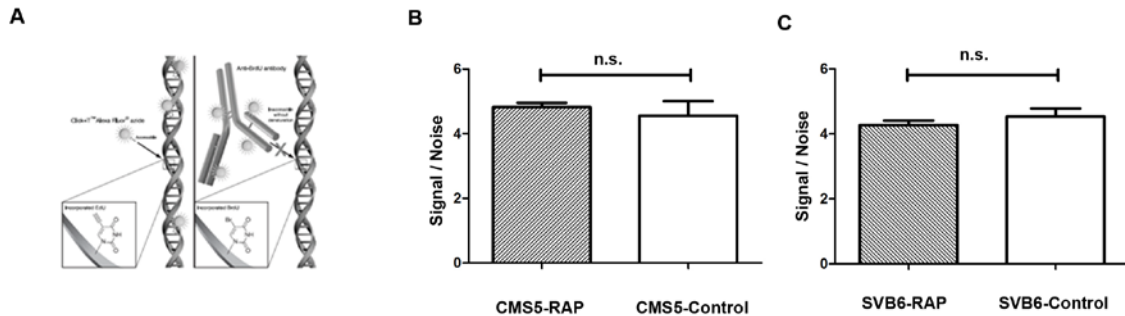


Figure 3-5. The inherent proliferative rates of tumors with or without RAP are identical.

(A). The Click-iT EdU Flow Cytometry Assay Kit from Invitrogen was used for the *in vitro* proliferation assay. It directly measures DNA synthesis in S phase, similar to the BrdU assay. (B-C) Proliferation of CMS5 cells (B) or SVB6 (C), with RAP or control protein expression, was determined by EdU incorporation into DNA. n.s., not significant. Experiments were independently performed twice. Error bars indicated s.e.m.

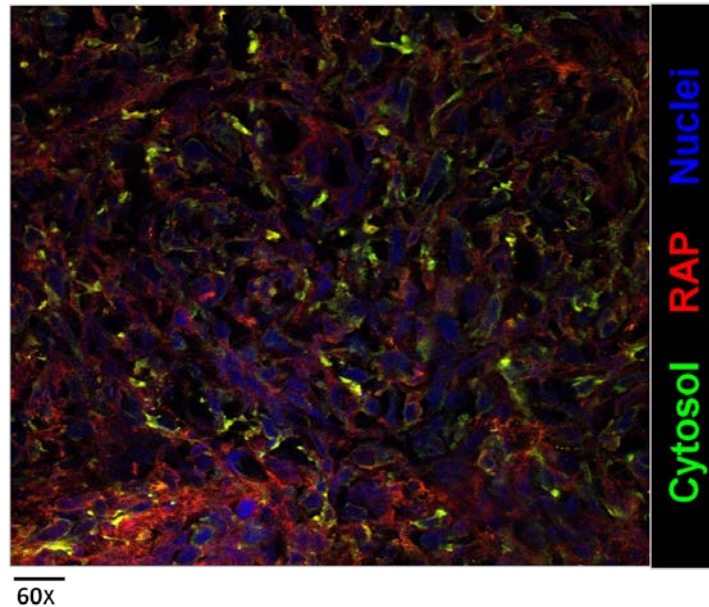


Figure 3-6. RAP expression is maintained throughout the tumor growth assay.

CMS5 tumor expressing RAP were established intradermally in BALB/c mice for 15 days. Tumor was harvested, sectioned and stained with antibodies to V5-tagged RAP and cytosolic HSP90. Experiments were independently performed twice.

3.3.2 RAP abrogates antigen transfer *in vivo* and inhibits T cell priming

To examine the transfer of antigen from tumor cells to APCs in the presence or absence of RAP *in vivo*, I rendered the transfected tumor cells replication incompetent and used them as a source of antigen. Mice were immunized with titrated doses of replication-incompetent CMS5 tumor cells expressing RAP or control protein (Figure 3-7 A). Two weeks later, the mice were challenged with 1×10^6 wild type CMS5 cells and tumor growth was monitored. No protection was seen at the 1,000 immunizing dose in either group (Figure 3-7 B). However, when mice were immunized with 10,000 cells, RAP-expressing cells failed to protect mice from a subsequent tumor challenge while the control tumor cells were able to elicit protection (Figure

3-7 C). These results are consistent with RAP-expressing cells failing to transfer their antigen to APCs leading to inefficient cross-priming of T cells. At $>1 \times 10^5$ immunizing cells, tumor cells expressing either protein were able to protect mice suggesting that at higher antigen doses, CD91-independent mechanisms for antigen transfer to cross-prime APCs may come into play (Figure 3-7 D and E).

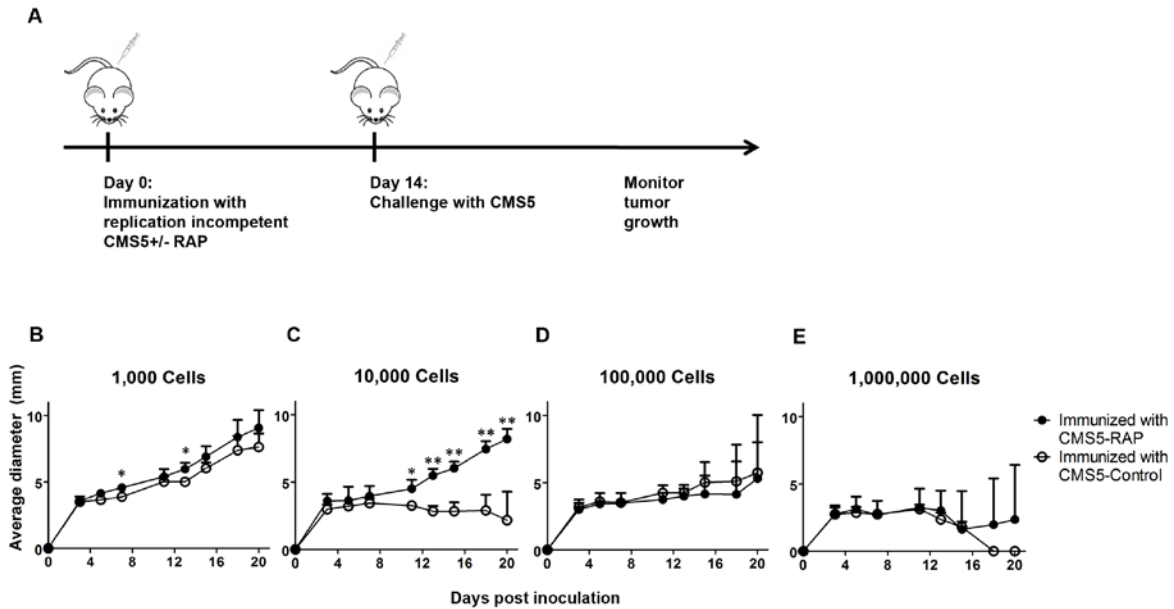


Figure 3-7. RAP abrogates antigen transfer *in vivo* and inhibits T cell priming.

The ability of RAP-expressing CMS5 to transfer antigen and prime immune responses was tested in a tumor prophylaxis assay. (A) Schema showing the experimental design, in which BALB/c mice were immunized with titrated doses of replication incompetent, RAP-expressing cells and later challenged with parental (wild type) CMS5 cells. (B-E) Mice were immunized with 1000 (B), 10,000 (C), 100,000 (D) or 1,000,000 (E), RAP or control protein expressing CMS5 tumor cells rendered replication incompetent. Mice were challenged with 1×10^6 parental CMS5 cells in all groups and tumor growth was measured. * $P < 0.05$, ** $P < 0.01$. Experiments were independently performed twice with 3-5 mice per group. Error bars indicated s.e.m.

3.3.3 Inhibition of antigen cross-presentation by RAP reduces T cell proliferation *in vivo*.

Upstream of anti-tumor responses, I tested the ability of RAP to prevent antigen transfer by measuring levels of antigen presented to T cells in the draining lymph nodes. CFSE-labeled CD45.2⁺ OT-1 cells were adoptively transferred into naïve mice (CD45.1⁺) which were immunized a day later with 1x10⁵ ovalbumin-loaded CMS5 tumor cells (Figure 3-9 A). This number of cells was estimated to be loaded with a total of approximately 1 ng of ovalbumin (Figure 3-8). The ovalbumin-loaded CMS5 tumor cells either expressed RAP or a control protein. The CMS5 tumor is of the H-2^d haplotype and cannot directly present the OVA8 peptide to OT-1 cells. Therefore stimulation of the OT-1 cells will be an indication of cross-presentation by resident APCs of the (H-2^b, CD45.1⁺) C57BL/6 mouse. Lymph node cells were harvested after 3 days and the percent of dividing CD8⁺CD45.2⁺ cells was determined by flow cytometry. I show CFSE dilution in OT-1 cells from representative mice in Figure 3-9 B and C. In multiple mice (Figure 3-9 D) I show that the presence of RAP in the tumor cells accounted for a decrease in proliferation of OT-1 cells when compared to cells expressing a control protein. This is despite the fact that both cell types were loaded with exactly the same amount of OVA. In parallel experiments, CFSE-labeled OT-1 cells were transferred into CD91^{-/-} mice one day prior to immunization with replication incompetent, ovalbumin loaded CMS5 cells (Figure 3-10). CMS5 cells were expressing RAP or an irrelevant protein. Lymph node cells were harvested after 3 days and the percent of all dividing CD8⁺ cells was determined by flow cytometry. Regardless of RAP expression, no OT-1 proliferation was observed in these CD91^{-/-} mice. When mice were immunized with 10 times more OVA loaded CMS5 cells (1x10⁶, 10 ng), no difference was

observed in dividing OT-1 cells when cells expressed RAP or a control protein (Figure 3-11). This latter result was consistent with results in Figure 3-7 D,E when antigen doses are high.

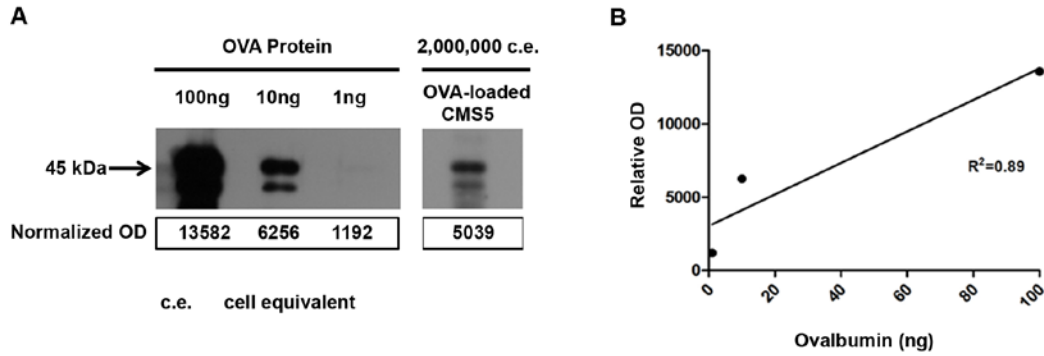


Figure 3-8 The amount of ovalbumin protein loaded in CMS5 cells

(A) Titrated amount of ovalbumin protein and 2×10^6 cell equivalent (c.e.) of lysate from OVA-loaded CMS5 were analyzed by SDS-PAGE and immunoblotted with anti-OVA antibody. The immunoblots were scanned and quantified, and the relative optical density (OD) units are indicated below the blots. (B) A semi-quantitative relationship was established between the titrated amounts of ovalbumin protein and their relative OD. It was established that 1×10^5 cell immunizing dose contained approximately 1 ng of OVA.

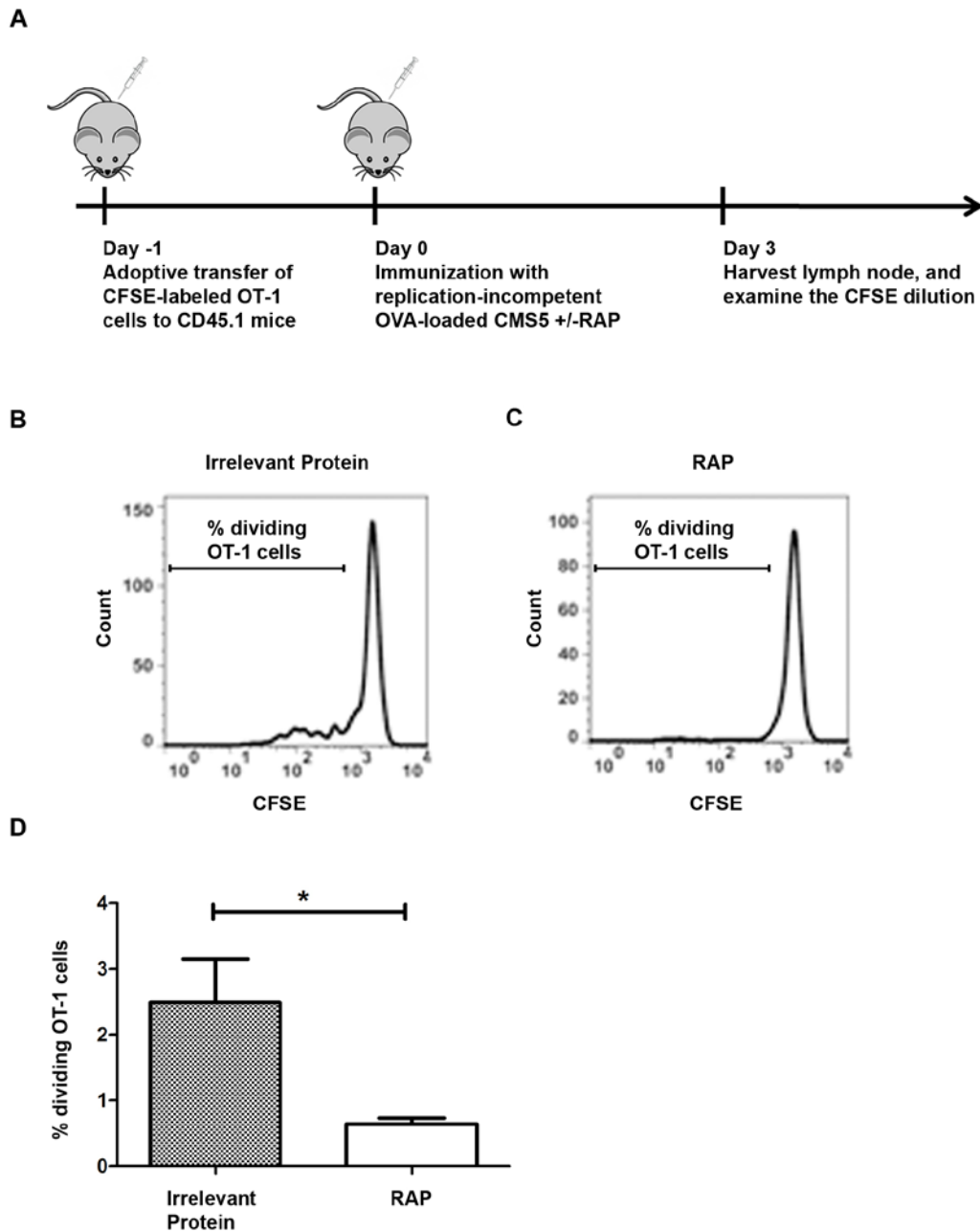


Figure 3-9. Inhibition of antigen cross-presentation by RAP reduces T cell proliferation *in vivo*.

The effect of RAP on T cell proliferation *in vivo* was examined. (A) CD45.1⁺ C57BL/6 mice were adoptively transferred with CFSE-labeled CD45.2⁺ OT-1 cells (1.5×10^6 per mouse), one day before immunization with mitomycin-C treated OVA-loaded CMS5 with or without RAP expression. After 3 days, the draining inguinal and axillary lymph nodes were harvested and stained for CD45.2 and CD8 expression to differentiate adoptively transferred OT-1 cells from endogenous responses. (B and C) Histograms from the flow cytometry analysis of gated CD45.2⁺CD8⁺ OT-1 cells from representative mice of each group. (D) The percentages of dividing OT-1 cells were compared in mice immunized with OVA-loaded CMS5 cells, expressing RAP or control protein. * $P < 0.05$. Experiments were independently performed twice with 3-5 mice per group. Error bars indicated s.e.m.

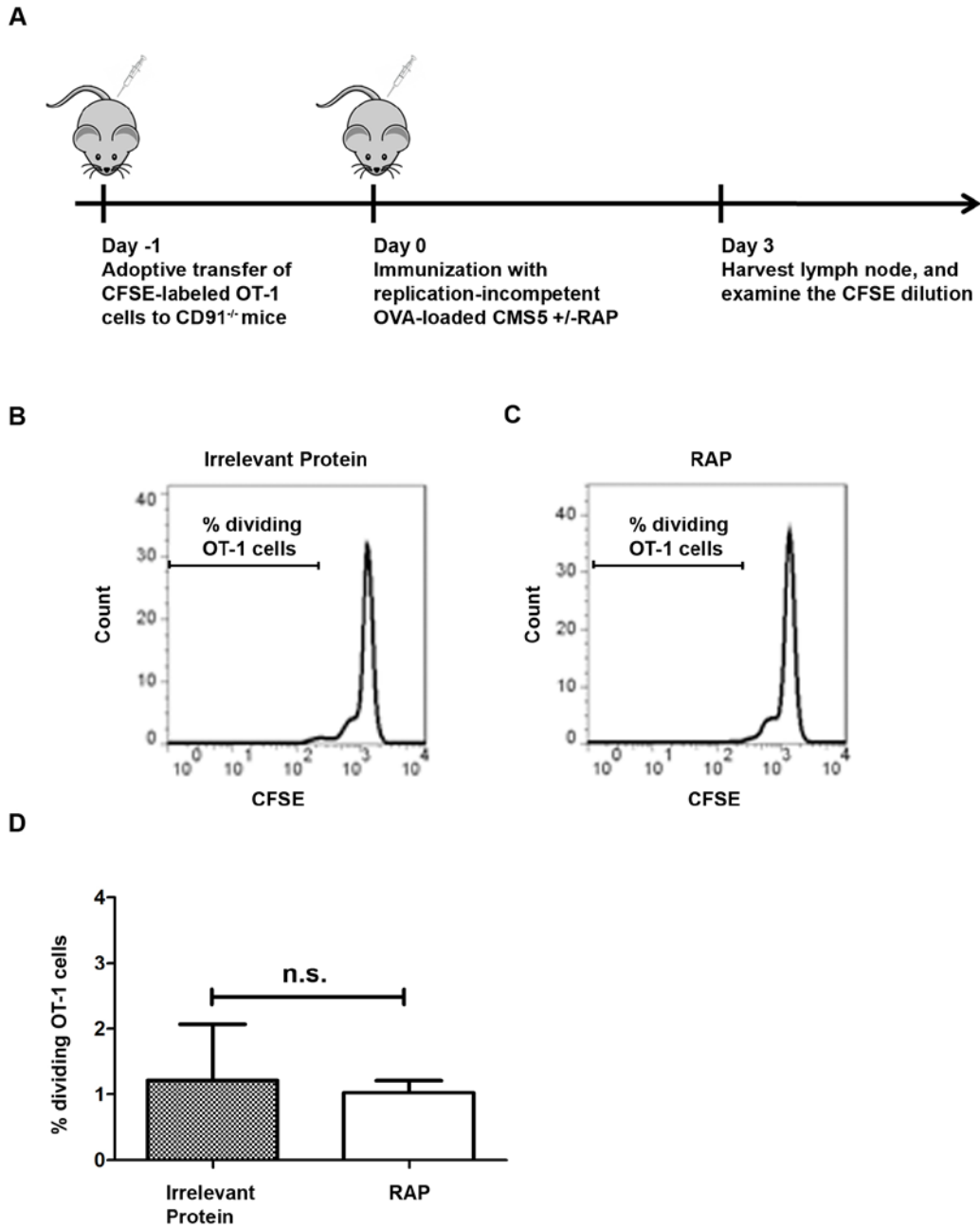


Figure 3-10 Cross-presentation is abrogated in CD91^{-/-} mice

(A) CFSE-labeled OT-1 cells were adoptively transferred into CD91^{-/-} mice followed by immunization with replication incompetent, ovalbumin loaded CMS5 cells one day later. The CMS5 cells expressed RAP or an irrelevant protein. On day 3, lymph nodes were harvested and stained for CD8. (B-C) Representative histograms for CFSE on CD8⁺ cells is shown. (D) the percentage of OT-1 cells that are dividing is shown. Error bars indicate s.e.m.

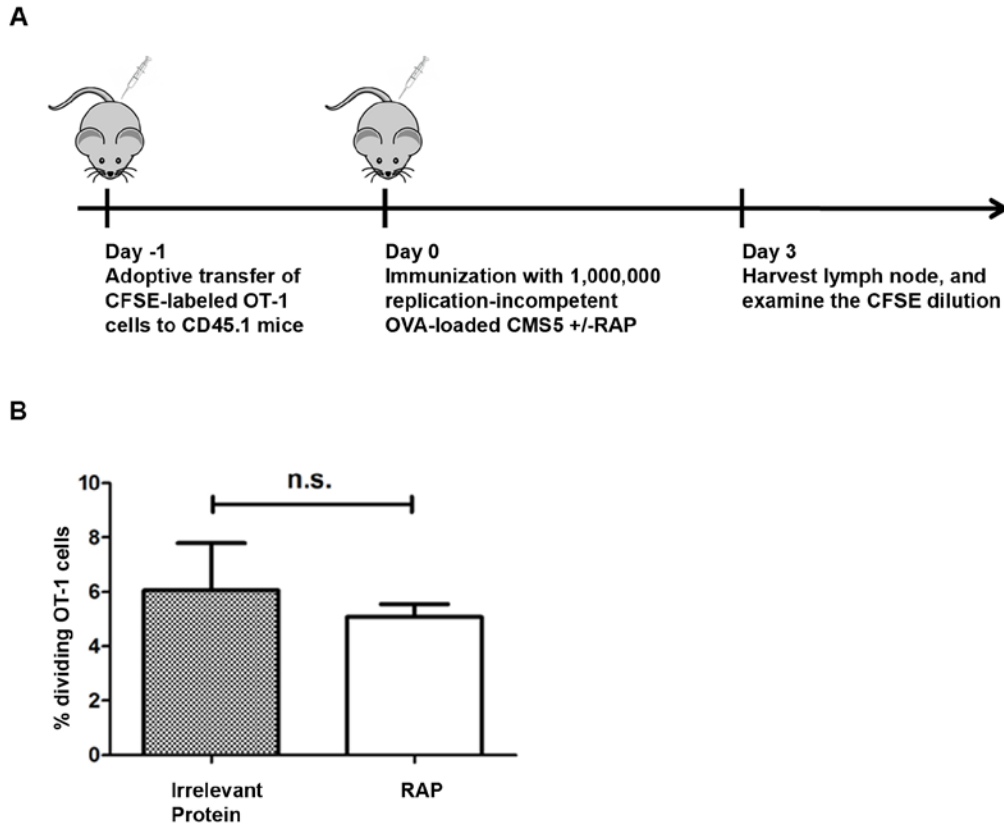


Figure 3-11 The effect of RAP on T cell proliferation is abrogated when mice are immunized with high dose OVA-loaded tumor cells

(A) CFSE-labeled CD45.2⁺ OT-1 cells were adoptively transferred into CD45.1⁺ C57BL/6 mice, one day before immunization with high dose (1x10⁶ cells) of mitomycin-C treated OVA-loaded CMS5 with or without RAP expression. Three days post immunization, the draining inguinal and axillary lymph nodes were harvested and stained for CD45.2 and CD8 expression to differentiate adoptively transferred OT-1 cells from endogenous responses. (B) The percentages of dividing OT-1 cells were compared in mice immunized with OVA-loaded CMS5 cells, expressing RAP or control protein. n.s. not significant. Experiments were performed with 3-4 mice per group. Error bars indicated s.e.m.

3.4 DISCUSSION

In this chapter, I directly examine the requirement of ligand-binding ability of CD91 in mounting substantial immune resistance to tumors by utilizing molecules known to inhibit binding of ligands to CD91, such as RAP. The result demonstrated that tumors expressing RAP exhibited the same phenotype of decreased anti-tumor immunity as shown in CD91^{-/-} mice.

Instead of wild type RAP that resides in the ER as an escort protein for CD91, a cytosolic version of RAP that lacks N-terminal signal sequence was transfected to tumors. Thus it allowed us to study the effect of elevated RAP levels on tumor immunogenicity even if only plasma membrane, but not ER membrane, was compromised in some physiological setting. However as a caveat, several post-translational modifications that normally occur in the lumen of ER/Golgi complex, such as forming of disulfide bonds and glycosylation, may be impaired in the cytosolic version of RAP. It may ultimately affect conformation of RAP and its binding to CD91. Although no disulfide bonds are possible for the sequence of RAP, it does have a sole glycosylation site (McCormick et al., 2005). However, previous study in our lab demonstrated that recombinant RAP lacking glycosylation can still inhibit binding of HSPs to CD91 *in vitro* (Binder and Srivastava, 2004). Additionally, the cytosolic RAP molecules used in this project were shown to decrease tumor immunogenicity, further negating the concern that lack of glycosylation in the RAP may inhibit its interaction with CD91.

Tumors expressing RAP grew with significantly faster kinetics than the control protein-expressing counterparts in wild type mice. The control protein used was *E.coli* LacZ, as described in the methods section. LacZ did not contribute to immunogenicity because tumor cells expressing this protein grew with identical kinetics as untransfected cells (data not shown). Interestingly, the difference observed in wild type mice was non-existent in *rag1/2^{-/-}* immunocompromised mice, corroborating the effect of RAP transfection on tumor immunogenicity, instead of the inherent proliferation rate of tumors. Most likely, RAP inhibited priming of the adaptive immunity, characterized by T cell responses which are the primary cell type responsible for tumor rejection (Boon et al., 1994; North, 1984; Udono et al., 1994). However, innate immune response may be dampened by RAP as well, since *Rag1/2^{-/-}* mice not

only lack T/B cells, but also NKT cells. It is thus conceivable that NKT cells may be involved in HSP-mediated immune response. By using the NKT knockout mice (J(alpha)281(-/-)mice), we can examine the involvement of NKT cells in HSP-mediated immunity in the future. In addition, RAP-expressing tumors grew faster than non-RAP expressing counterparts, at early time points post tumor inoculation (e.g. day1 for SVB6). However, the tumor growth in *rag1/2^{-/-}* mice was not measured between day 0 to day 4. These early time points may contain important information about the role of RAP on HSP-mediated innate immune responses, which may curb tumor growth before establishment of adaptive immunity.

To validate the generality of the effect of RAP on tumor immunogenicity, a second line of tumor cells was used in this project---SVB6, which is a highly immunogenic regressor tumor. The T-antigen (Tag), the antigenic epitope of SVB6, cause the regression of SVB6 regardless of the dose of inoculation, which is similar to other T Ag expressing tumor studied previously (Chapes and Gooding, 1985). The immune response against T-antigen is presumably T cell-mediated. Previous studies showed that substitutions in four T cell epitopes would compromise T cell receptor recognition (Lill et al., 1992). Also, induction of CTLs directed against these Tag epitopes might control Tag-induced tumors (Schell et al., 1999). Despite the high immunogenicity of parental tumor cells, RAP-expressing SVB6 tumors grew to a significantly bigger size and were rejected more slowly than control SVB6 tumor cells in immunocompetent mice. In immunocompromised *rag2^{-/-}* mice, however, the growth of tumors was comparable irrespective of RAP expression until day 11, with the SVB6-Control cells growing faster than the SVB6-RAP thereafter. It further enhanced the validity of my conclusion.

RAP-expressing cells, when used as immunogen in a prophylaxis assay (especially at low dose), were significantly less efficient in priming immune responses. It suggests that HSP-CD91

pathway is important very early in tumorigenesis. This phenomenon is echoed in T cell proliferation assay (Figure 3-11). It has been shown that depletion of CD8⁺, but not CD4⁺ T cells in the priming phase abrogates immunity elicited by gp96 (Udono et al., 1994). But it is unclear, in my system, which subset of T cells was affected by RAP during the priming phase. Therefore future studies will examine the involvement of CD4 and/or CD8 T cells in the same prophylaxis assay, by using anti-CD4 and/or anti-CD8 antibody to deplete CD4/8 T cells.

When antigen is abundant, acquisition of antigens by APCs for cross-presentation and cross-priming can occur through several mechanisms which are prominent in infectious disease settings (Yewdell et al., 1999). These mechanisms are less reliant on the source of antigen or the type of APC (Norbury et al., 2004). Majority of tumor rejection antigens however derive from mutations of self proteins and so are estimated to be limited in quantity at the time of initiation of tumor-associated immunity (Srivastava and Srivastava, 2009). As previously demonstrated for transplantable tumors, concomitant immunity is established by day 3 when intradermal tumors are barely visible (North and Kirstein, 1977). A careful quantification and titration of the amount of antigen in tumors available for cross-presentation at this early stage is ~6 orders of magnitude less than is required if the antigen is transferred as a whole protein (Binder and Srivastava, 2005). This necessitates a special mechanism to efficiently transfer antigens from the bearing (tumor) cell to the cross-presenting cell. Here I show that the HSP-peptide complex with its receptor CD91 provides such a mechanism. This study is supported by a large body of work *in vitro* demonstrating the efficient cross-presentation HSP-chaperoned peptides by a variety of CD91 expressing APCs (Binder, 2009; Binder et al., 2007; Blachere et al., 1997; Matsutake et al., 2010; SenGupta et al., 2004; Suto and Srivastava, 1995). Future studies will quantify the

amount of its immunodominant epitope (a mutated form of ERK2 protein in this case) (Ikeda et al., 1997) from titrated dose of CMS5 cells by western blotting or Mass spectrometry.

Under physiological conditions, majority of RAP resides within the ER. Soon after the biosynthesis of LRP, RAP associates with the receptor and initiates the formation of multi-molecular aggregates. Within these aggregates LRP is inactive in ligand binding. Following their trafficking together to the medial-Golgi compartment, RAP dissociates from LRP as a result of the low pH (-6.4-6.6) within the Golgi compartment (Bu et al., 1995; Chanat and Huttner, 1991). Intriguingly, because of increased glucose metabolism and poor perfusion, H⁺ production and excretion are generally increased in cancers (Schornack and Gillies, 2003), resulting in an acidic extracellular pH in solid tumors (pH 6.5–6.9) compared with normal tissue (pH 7.2–7.4)(Estrella et al., 2013; Gillies et al., 1994; Stubbs et al., 2000; van Sluis et al., 1999). Therefore, extracellular RAP may gradually dissociate from CD91 as solidly established tumors grow larger and generate a lower pH environment.

I also tested ability of RAP to prevent antigen transfer and confirmed the importance of CD91 pathway when the amount of antigen is limited; on the nanogram level in the experiment shown in Figure 3-9 (The loading efficiency has been shown in Figure 3-8). It explains why the percentage of OT1 proliferation in response to OVA loaded cells was relatively small. I can easily incorporate supraphysiological amounts of antigen (microgram to milligram levels into the system and obtain stronger OT1 proliferation presumably as has been done by many others) (Quah et al., 2007). Obtaining ‘strong’ OT1 proliferation, typically achieved with supraphysiological amounts of antigen, is not a goal of this thesis project. This issue is also negated by the fact that T cell responses are sufficient to slow down growth of tumors as shown in Figure 2-5, Figure 3-4 and Figure 3-7.

4.0 CD91 IS REQUIRED FOR UPTAKE OF IMMUNOGENIC HSPS AND TRANSFER OF ANTIGEN

4.1 RATIONALE

The form of antigens that are transferred from tumor cells or infected cells to APCs to prime downstream immune responses has been a longstanding debate. Several possible forms of antigen include intact proteins, free peptides derived from protein, peptides presented by MHC class I molecules (as a part of the process of cross-dressing), or chaperoned by HSPs. Among them, peptides chaperoned by HSPs has been shown to be necessary and sufficient for cross-prime of CD8+ T cells. Indeed, tumor cell lysates in which four major immunogenic HSPs were depleted can no longer elicit downstream T cell responses. Addition of either one of these HSPs restored the activity.

However, depletion of intact antigenic proteins did not abrogate the immunogenic activity of tumor cell lysates. In addition, the efficiency of intact protein to prime T cell responses is much lower than peptides chaperoned by HSPs. Six orders of magnitude higher amount of intact protein is required to elicit immune response as robustly as that mounted by HSP-peptide complexes. High efficiency of antigen transfer is particularly relevant during the early development of tumor when antigen dose is limited.

With regard to peptides derived from protein, several pieces of evidence showed that they cannot exist in their free form: (1) free peptides are too small to assume secondary and tertiary structures necessary to shield hydrophobic residues; (2) A robust search for free peptides in cells found none (Menoret et al., 1999); (3) The high efficiency of antigen processing and presentation indicates that random peptide diffusion is not an option. Indeed, degradation of only 30-5000 proteins are needed for each peptide presented on MHC I (Finelli et al., 1999; Yewdell, 2001).

Peptides presented by MHC class I molecules may serve as another form of antigen transferred for cross-dressing, a process in which intact cell-surface proteins (MHC-peptide complexes in this case) are rapidly exchanged between cells that are in contact with each other. However, cross-dressing may have a significant role only in activating previously primed CD8⁺ T cells, but not naive T cells (Wakim and Bevan, 2011). Therefore, this process may have minimal, if any, role in transferring antigen from nascent tumors to APCs.

As shown in the previous chapter, the ligand-binding ability of CD91 is required for anti-tumor immune responses. However, the identity of immunogenic ligand(s) that are impacted by the deficiency or blockade of CD91 is unknown. Since HSPs-chaperoned peptides are both the necessary and sufficient form of antigen transferred from tumor cells to APCs, I designed experiments in this chapter to dissect the role of CD91 on uptake of HSPs. I utilized a strategy to label HSP gp96 with Enhanced Green Fluorescent Protein (EGFP) and co-transfected gp96-EGFP with RAP or a control protein into different tumor cells (Figure 4-1). The localization and trafficking of this fluorescent-labeled gp96 was then monitored *in vivo*. My general hypothesis is that RAP would compete away gp96-EGFP from binding to CD91, and hence reduce EGFP-derived green signal detected on immune cells in the draining lymph nodes.

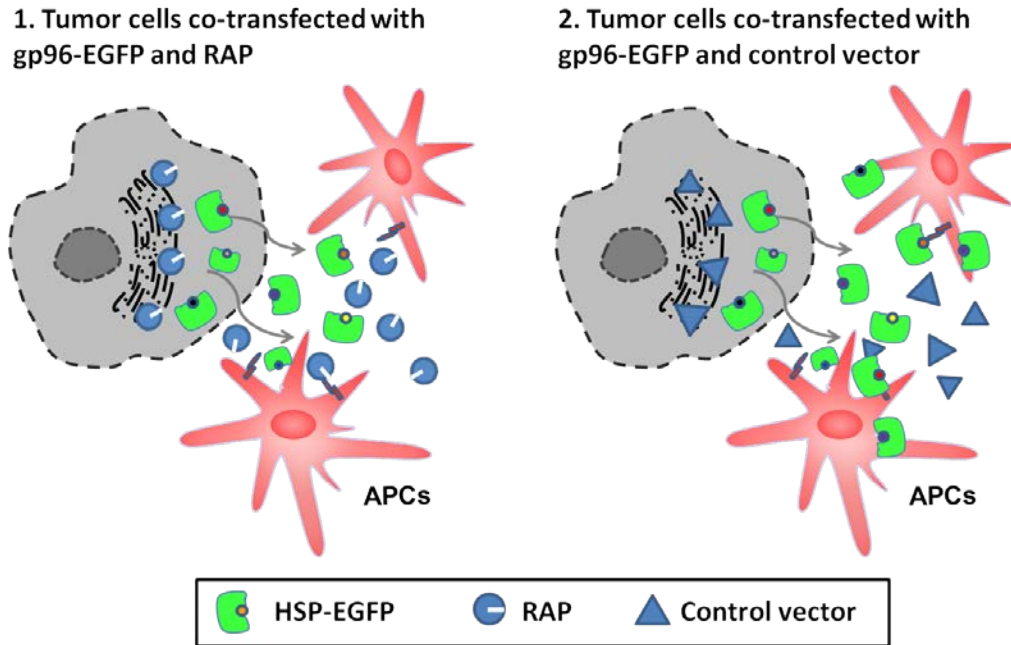


Figure 4-1. Strategy to examine uptake of HSPs *in vivo*

CMS5 cells were constructed to express gp96-EGFP, simultaneously with either RAP or control protein, in order to examine the role of RAP on HSP transfer to APCs. I hypothesized that RAP inhibits APCs from taking up tumor-derived EGFP-labeled HSPs in draining lymph nodes.

4.2 MATERIALS AND METHODS

4.2.1 Cells and reagents

Double transfected tumor cells (gp96EGFP+RAP, or gp96EGFP+control vector) were cultured in complete DMEM media plus blasticidin (3 μ g/mL, Invitrogen) and geneticin (0.7g/L, GIBCO). APC anti-CD45 antibody was purchased from Invitrogen (Carlsbad, CA). Anti-grp94 antibody, anti-PDI antibody, anti-Hsc70 antibody were purchased from Enzo Life Sciences (Farmingdale, NY). Anti-EGFP antibody was purchased from Clontech (Living Colors Full-length A.V. polyclonal antibody) (Mountain View, CA)

4.2.2 Plasmid construction and transfection.

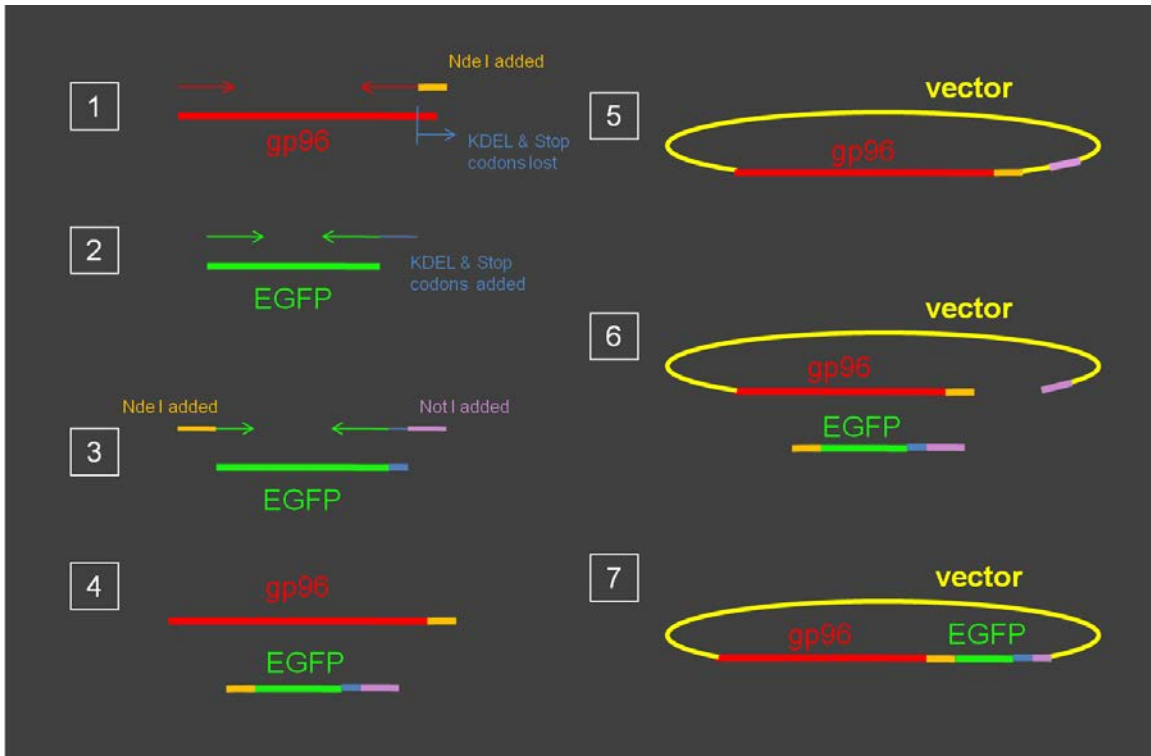


Figure 4-2. Gp96-EGFP Construct Strategy

The diagram illustrates the procedure to generate gp96-EGFP vector (Courtesy of Dr. Michelle Messmer)

The gp96EGFP (C-terminal fusion protein) was generated by sequential RT-PCR and subcloning. The gp96 cDNA was amplified from murine RNA using an N-terminal primer upstream of the gp96 start codon and a C-terminal primer with complementarity starting at nucleotide 2483, and the codons for KDEL, an ER retention signal sequence, and the stop codon were replaced with an NdeI restriction cut site. This construct was inserted into the pEF6/V5-His-TOPO vector and orientation was confirmed by restriction enzyme digestion and sequencing analysis. EGFP was prepared via 2-step PCR. First, EGFP cDNA was amplified from a commercial vector (Invitrogen), using a complementary N-terminal primer and a complementary

C-terminal primer with an overhang for the KDEL and stop codons. EGFP was then reamplified using an N-terminal primer with an NdeI restriction site overhang and a C-terminal primer complementary to the C-terminus with KDEL and stop codons and with a NotI restriction site overhang. The final EGFP construct was ligated into the gp96 containing vector and the final product was fully sequenced to confirm the sequences were in frame and that no mutations occurred during this construct assembly process. CMS5 tumor cells were stably co-transfected with gp96-EGFP vector (with blasticidin resistant gene) and RAP/Control vector (with geneticin resistant gene), by using Fugene® 6 transfection reagent (Roche Molecular Biochemicals).

4.2.3 HSP transfer experiments *in vivo*.

5x10⁶ CMS5 cells co-transfected with RAP and gp96-EGFP, or CMS5 co-transfected with control vector and gp96-EGFP, were injected in one footpad of wild type BALB/c or CD91^{-/-} mice. Two days later, the draining (dLN) and contra lateral (non-draining, ndLN) popliteal lymph nodes were harvested. EGFP signal in CD45⁺ cells in the lymph nodes were compared. Due to minor variations in auto-fluorescence observed in each mouse and in fluorescence of the gp96-EGFP expressing cultured cells over time, the signal was normalized to these two parameters by accounting for background signal in contralateral (ndLN) popliteal lymph nodes and fluorescence of gp96-EGFP expressing cells on the day of the experiment respectively (Figure 4-5 C-D; Table 4-1; Table 4-2). The following formula was used:

$$\text{Normalized green signal intensity} = \frac{\text{MFI (dLN for CMS5-EGFP)} - \text{MFI (ndLN for CMS5-EGFP)}}{\text{MFI (CMS5-EGFP before injection)} - \text{MFI (Untransfected CMS5 before injection)}} \times 100\%$$

4.2.4 Statistical analysis.

An unpaired two-tailed Student's t-test or two-way ANOVA test was used for statistical analyses, and a *P* value of less than 0.05 was considered statistically significant. Error bars were calculated as standard error of the mean (s.e.m).

4.3 RESULTS

CD91 is required for uptake of immunogenic HSPs

To determine whether CD91 was essential for HSP-peptide cross-presentation *in vivo*, an assay was developed to monitor HSP transfer to APCs and the localization of the HSP in the lymph node. CMS5 cells were constructed to express gp96-EGFP, simultaneously with either RAP or control protein (Figure 4-1). Examined by Coomassie stain (Figure 4-3 A), immunoblot (Figure 4-3 B), and confocal microscopy (Figure 4-3 E), gp96-EGFP was expressed robustly at roughly the same level as endogenous gp96. It was expressed in the ER (as indicated by co-localization with PDI), but not in the cytosol (as indicated by absence of co-localization with Hsc70) (Figure 4-3 C-D).

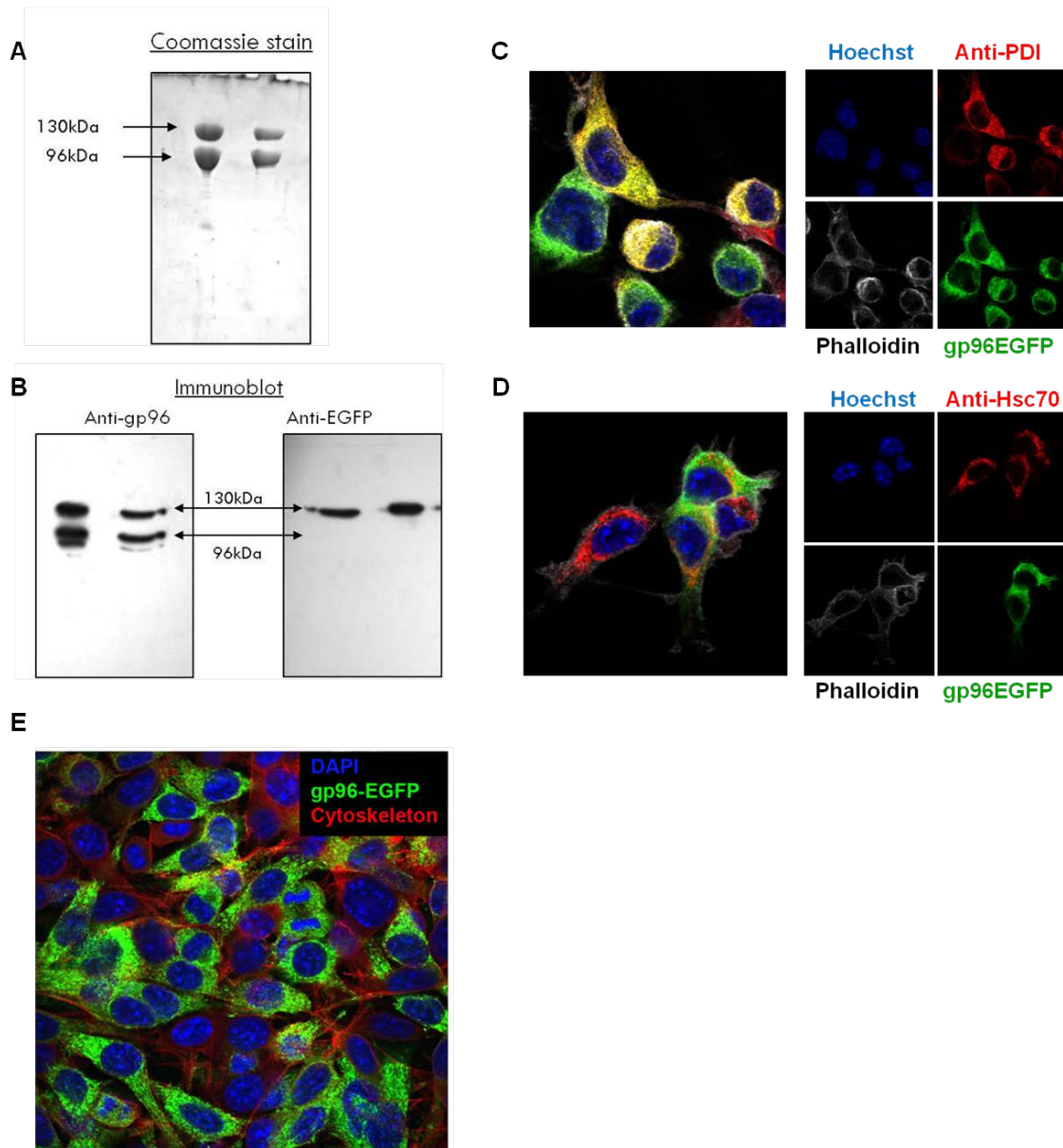


Figure 4-3. Robust expression of gp96-EGFP in transfected CMS5

(A-B) Gp96 was purified from transfected CMS5 cells by using previously described method. Two fractions of elution buffer from the DEAE column were loaded in a 10% SDS-PAGE gel, separated by electrophoresis, and stained with Coomassie Blue. Proteins on the gel were then transferred to an Immobilon-P membrane, blotted with anti-gp96 or anti-EGFP antibodies, and developed in a X-ray film. The gel (A) and film (B) was imaged with the Kodak Image Station. (C-E). Robust fluorescence of gp96-EGFP was analyzed by confocal microscopy. Gp96-EGFP transfected CMS5 cells were labeled with: (C) Hoechst staining for nuclei, Phalloidin staining for cytoskeleton, and anti-PDI antibody staining for ER; or (D) Hoechst staining for nuclei, Phalloidin staining for cytoskeleton, and anti-hsc70 antibody staining for cytosol; or (E) DAPI staining for nuclei and Phalloidin staining for cytoskeleton. (Data from Dr. Michelle Messmer)

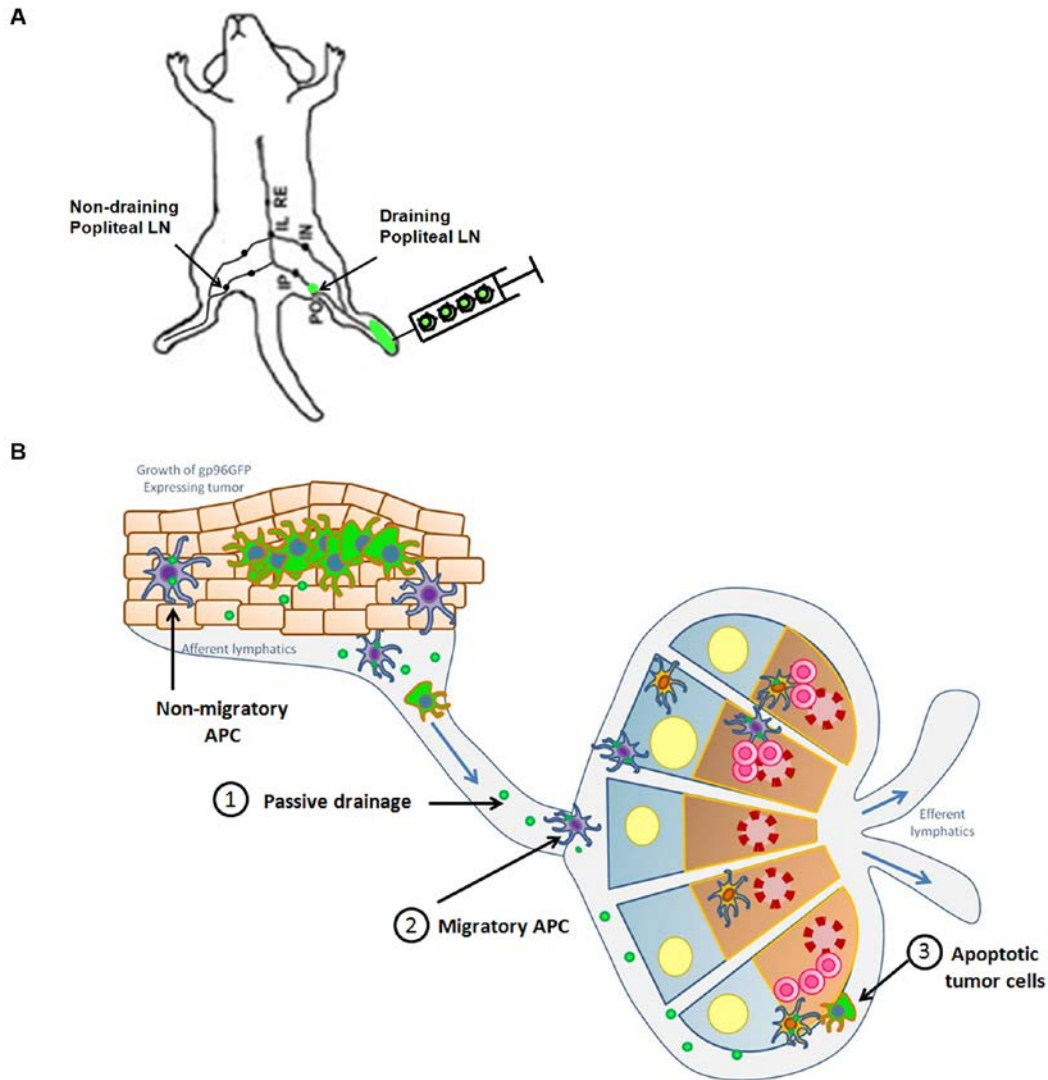


Figure 4-4. Experiment design and multiple scenarios for HSPs trafficking

(A) CMS5 tumor cells co-transfected with gp96-EGFP/RAP or gp96-EGFP/Control protein were inoculated into one footpad of mouse. Two days later, both draining and non-draining popliteal lymph nodes were collected for analysis; (B) Schema for possible routes of HSPs trafficking from tumor cells in peripheral tissue to draining lymph node. (1) HSPs released from tumor microenvironment can be passively drained to lymph node and taken up there by the LN-resident APCs; (2) HSPs, once released from tumor cells, can be immediately taken up by peripheral APCs. Those peripheral APCs are then migrated to draining lymph node; (3) apoptotic tumor cells can be directly transferred to draining lymph node. (Courtesy of Dr. Michelle Messmer)

The two tumor types were implanted into individual foot pads of BALB/c mice and draining lymph nodes were harvested 2 days later (Figure 4-4 A). The popliteal lymph node cells were stained and gated on CD45 to identify hematopoietic cells and exclude tumor cells that

potentially drained directly, from the analysis (Figure 4-4 B; Figure 4-5 B). The amount of EGFP fluorescence was measured by flow cytometry (Figure 4-5 C-D). Due to minor variations in auto-fluorescence observed in each mouse and in fluorescence of the gp96-EGFP expressing cultured cells (Figure 4-5 A), the signal was normalized to these two parameters by accounting for background signal in contralateral (non-draining) popliteal lymph nodes and fluorescence of gp96-EGFP expressing cells on the day of the experiment respectively (Figure 4-5 C-D; Table 4-1; Table 4-2). Significantly less EGFP was detected in lymph nodes when tumors expressed RAP compared to control protein ($P = 0.0002$, Figure 4-6 A).

I tested the transfer of HSP to APCs in CD91^{-/-} mice using the same system. CMS5 cells expressing gp96-EGFP with or without RAP were implanted in the footpads of CD91^{-/-} mice. Draining lymph node cells were harvested after 2 days and analyzed as in Figure 4-6 A. As shown in Figure 4-6 B, I did not detect gp96-EGFP signal in the lymph node regardless of whether cells expressed RAP or not. The importance of CD91 for HSP transfer and trafficking is thus highlighted in cases of deficient expression in APCs and antagonism via RAP.

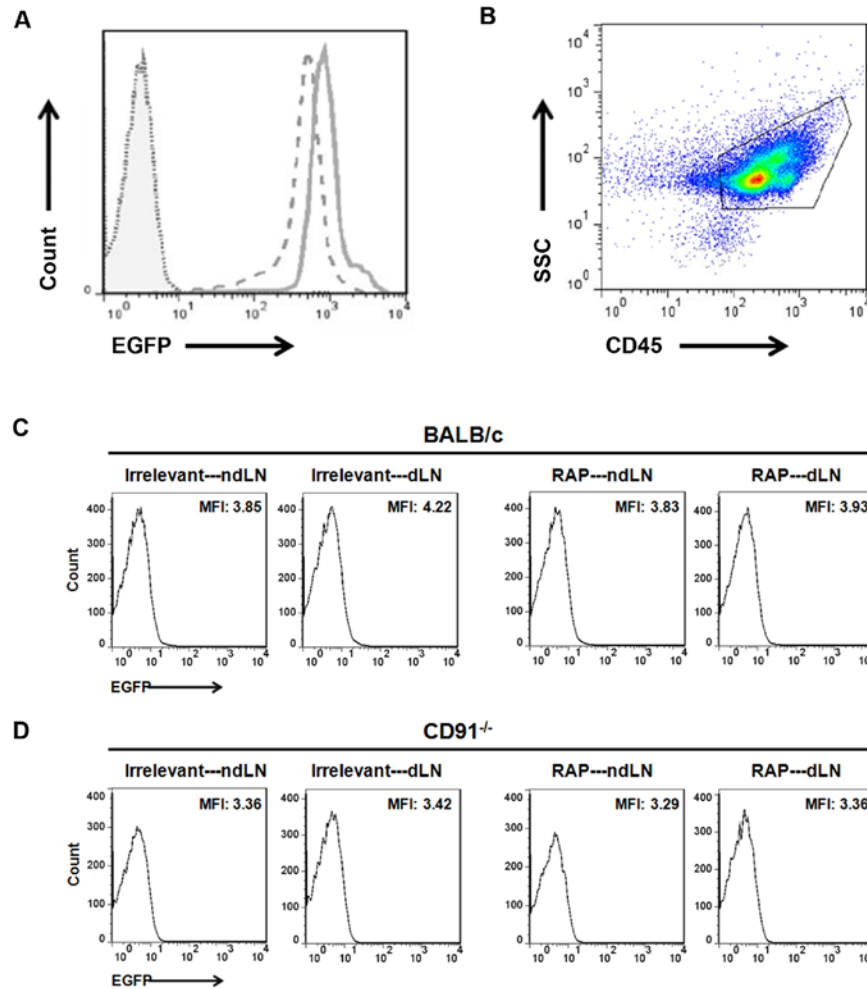


Figure 4-5. Transfer of tumor-derived gp96 to lymph node cells *in vivo*.

CMS5 cells expressing RAP or control protein were transfected with gp96-EGFP. (A) Robust fluorescence of gp96-EGFP was analyzed by flow cytometry. (B) Following implantation of cells into the foot pad of BALB/c mice, draining lymph nodes were isolated and stained for lymphocytic marker CD45 and analyzed by flow cytometry for EGFP as shown in Figure 6. A representative plot shows the CD45 gated population. (C and D) Representative histogram shows the EGFP-containing CD45⁺ cells in the non-draining lymph node (ndLN) or draining lymph node (dLN) following implantation of CMS5 cells expressing gp96-EGFP, and RAP or an irrelevant protein, into BALB/c (C) or CD91^{-/-} (D) mice.

Table 4-1. MFI of all BALB/c mice from HSP transfer experiment *in vivo*

MFI of all BALB/c mice from one experiment are shown. Denominator is the MFI (CMS5-EGFP before implantation) - MFI (untransfected CMS5 before implantation). These numbers are imported into the formula described in the Materials and Methods to obtain the normalized signal.

BALB/c					BALB/c				
Irrelevant Protein					RAP				
	MFI--ndLN	MFI--dLN	Denominator	Normalized Signal		MFI--ndLN	MFI--dLN	Denominator	Normalized Signal
Mouse 1	4.2	4.28	488.19	0.02%	Mouse 1	3.83	3.93	973.33	0.01%
Mouse 2	3.61	3.94	488.19	0.07%	Mouse 2	3.61	3.99	973.33	0.04%
Mouse 3	3.9	4.21	488.19	0.06%	Mouse 3	4.02	4.21	973.33	0.02%
Mouse 4	3.85	4.22	488.19	0.08%	Mouse 4	4	4.27	973.33	0.03%
Mouse 5	3.77	4.14	488.19	0.08%	Mouse 5	3.84	3.99	973.33	0.02%
Mouse 6	1.83	1.97	316.46	0.04%	Mouse 6	2.02	1.86	656.66	-0.02%
Mouse 7	1.76	1.89	316.46	0.04%	Mouse 7	1.86	1.86	656.66	0.00%
Mouse 8	1.71	1.92	316.46	0.07%	Mouse 8	1.81	1.75	656.66	-0.01%
Average				0.06%	Average				0.01%

Table 4-2. MFI of all CD91^{-/-} mice from HSP transfer experiment *in vivo*

MFI of all CD91^{-/-} mice from one experiment are shown. Denominator is the MFI (CMS5-EGFP before implantation) - MFI (untransfected CMS5 before implantation). These numbers are imported into the formula described in the Materials and Methods to obtain the normalized signal.

CD91 ^{-/-}					CD91 ^{-/-}				
Irrelevant Protein					RAP				
	MFI--ndLN	MFI--dLN	Denominator	Normalized Signal		MFI--ndLN	MFI--dLN	Denominator	Normalized Signal
Mouse 1	3.36	3.42	483.49	0.01%	Mouse 1	3.29	3.36	600.13	0.01%
Mouse 2	3.65	3.4	483.49	-0.05%	Mouse 2	3.25	3.2	600.13	-0.01%
Mouse 3	3.47	3.44	483.49	-0.01%					
Average				-0.02%	Average				0.00%

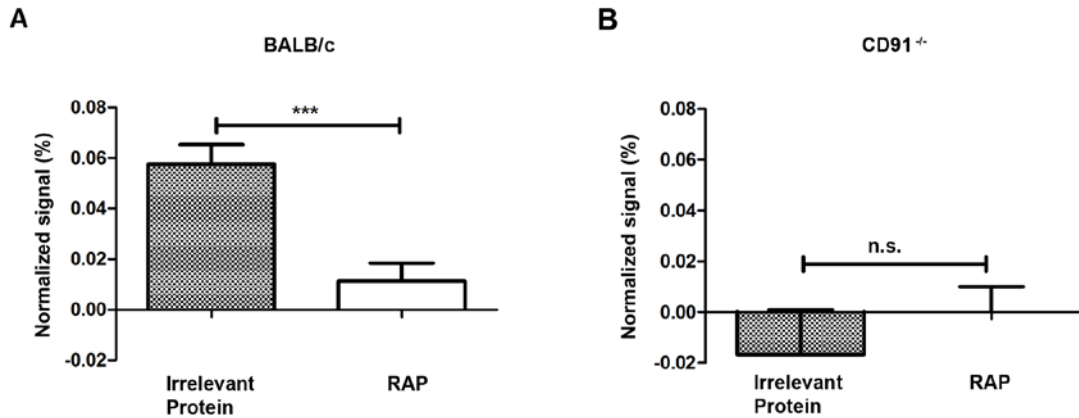


Figure 4-6. Transfer of HSP-peptide complexes to APCs requires engagement of CD91

CMS5 cells expressing RAP or control protein were transfected with gp96-EGFP. The foot pad of BALB/c (A), or CD91^{-/-} (B), mice were implanted with 5x10⁶ of either cell type and the draining or contralateral non-draining lymph nodes were harvested two days later. Cells were stained for the hematopoietic marker CD45 and analyzed by flow cytometry for EGFP. The EGFP signal was normalized against the starting fluorescence in each cell type and the background fluorescence of non-draining lymph nodes. *** *P* < 0.001, n.s. not significant. Experiments were independently performed twice with 8 mice per group (A), or 3 mice per group (B). Error bars indicated s.e.m.

4.4 DISCUSSION

In this chapter, I studied the requirement of CD91 for uptake of immunogenic HSPs, by tracking localization of fluorescent-labeled gp96 in the draining lymph nodes. In the presence of RAP, less fluorescence was detected on CD45⁺ immune cells in draining lymph nodes. When CD91^{-/-} mice were used, however, HSPs were not taken up by any CD45⁺ lymph nodes cells regardless of RAP expression by tumor cells. The implication for this data is two-fold: (1) it suggested that HSP is the molecule that RAP inhibits from binding to CD91 as shown in previous chapter; (2) it supported the importance of CD91 (in CD11c⁺ cells) on HSP trafficking and antigen transfer *in vivo*.

GFP-gp96 is expressed at similar level as endogenous gp96, as shown in the Figure 4-3 A-B. As with the previous chapter, my goal is not to simply present responses with supraphysiological conditions, but to create a system to mimic as close as technically possible to what is physiologically relevant. Therefore, the expression level of gp96-EGFP in cell clones that I chose is close to endogenous levels, and even decreases with passage (data not shown). Similar to endogenous gp96, the transfected gp96-EGFP protein has an ER retention signal, KDEL, on its C-terminal end. Retention of those proteins in the lumen of ER is achieved by their continual retrieval from the cis-Golgi or a pre-Golgi compartment. This process is mediated by a receptor (e.g. KDELRL1) that recognizes and binds the KDEL-containing protein and then returns it to the ER (Pelham, 1990). A possible reason why expression level of gp96-EGFP decreased with passage is that robustly expressed gp96-EGFP exceeds the capacity of those KDEL receptors, so that any excess proteins can escape the ER retention and be secreted into the extracellular environment. To examine this possibility, the amount of extracellular gp96-EGFP in the supernatant of cell culture can be measured by western blotting.

Harvesting lymph nodes 48 hours after tumor injection was a decision made based on my preliminary data. When lymph nodes were harvested 72 hrs after injection, RAP had no effect on HSP uptake (data not shown). At those time points, many other factors such as antigen destruction, aggregation and phagocytosis come into play as previously described (Delamarre et al., 2005). Considering the fact that (i) majority of antigen is destroyed by the expressing cells, (ii) destroyed in the extracellular environment, (iii) APCs that can take up antigen account for ~0.1% of the total lymph node population (Itano et al., 2003; Messmer et al., 2013), the amount of antigen that I detect is certainly meaningful, if not excellent.

Future studies will examine and identify APC subsets that take up gp96-EGFP *in vivo*. In addition, studying sorted APC with gp96-EGFP *in vitro* may provide a hint. However, one caveat is that some APC subsets that are capable to bind to gp96-EGFP *in vitro* may not be able to access and interact with the same antigen *in vivo*, due to anatomical limitation.

5.0 CONCLUSIONS, SIGNIFICANCE AND FUTURE DIRECTIONS

Adaptive immune responses against tumors are routinely detected in hosts bearing the tumor due to immunosurveillance mechanisms. How the immune response to tumors is initially primed remains unclear, given the limited amount of tumor antigen available for cross-priming and lack of classical pathogen associated molecular patterns (PAMPs). I demonstrated in this dissertation that specific ablation of CD91 in antigen presenting cells prevented the establishment of anti-tumor immunity, especially early in tumor development. Anti-tumor immunity was also inhibited when transfer of tumor-derived HSPs to APCs was prevented by using RAP, an endogenous inhibitor of CD91. Inhibition was manifested in a reduction of cross-presentation of tumor-derived antigenic peptides in lymph nodes.

Following priming of anti-tumor immunity, a period called immune-editing occurs where tumors may develop several mechanisms allowing for co-existence with the immune response (DuPage et al., 2012; Matsushita et al., 2012; Schreiber et al., 2011). These mechanisms include induction of immunosuppressive factors, activation of regulatory cells, loss of antigen expression, or tumor-induced impairment of antigen presentation. As a mechanism of evasion of the immune response by tumors, *preceding* or at the early stage of immuno-editing, I hypothesize that tumors may over-express RAP, or other as yet undiscovered endogenous CD91 inhibitors, to disrupt HSP-CD91 pathway and impair antigen transfer. Interestingly, a previous study identified a positive correlation between elevated RAP levels and progressive disease in colon cancer

patients (Sakamoto et al., 2011). I expect this mechanism to occur during initiation of tumors to allow the tumors to be firmly established, because antigen burden is very low, and HSP-mediated antigen uptake prevails at this stage.

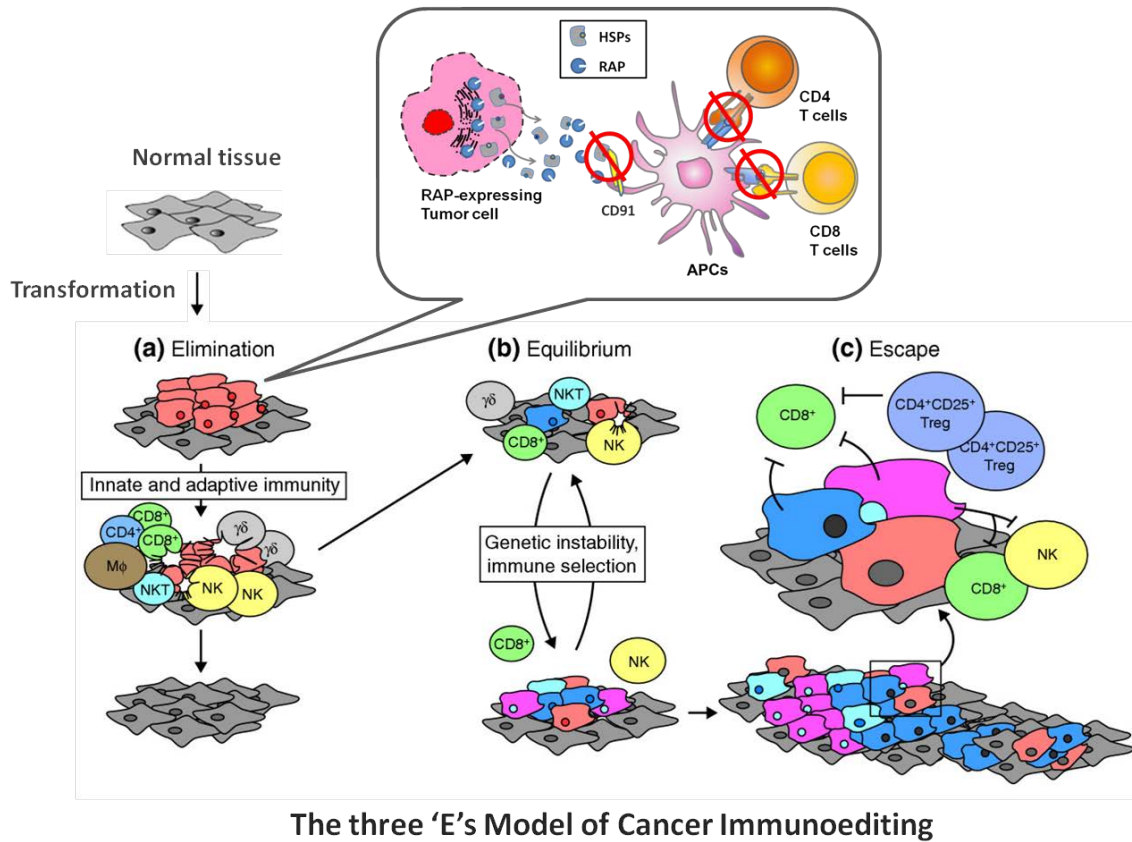


Figure 5-1. Model: Evasion from Immunosurveillance/ Elimination

Schema for the three 'E's model of cancer immunoeediting, which consists of three stages of interactions between cancer cells and immune system: (1) Elimination; (2) Equilibrium; and (3) Escape. It shows how the conclusions from this thesis fits into the model. [Figure adapted from (Dunn et al., 2004)]

I note that as tumor grows and tumor antigen becomes more abundant, other mechanisms of antigen transfer and T cell priming may be evoked, so that the inhibitory effect of RAP on antigen transfer is abrogated. This is hinted at in Figure 3-7, when mice are immunized with larger cell numbers (>100,000). These other mechanisms of antigen transfer may become

dominant in cases of over-expressed self or oncoviral tumor antigens, which may [supported by our own work, (Binder and Srivastava, 2005)] or may not be via HSP-mediated processes [supported by work from other labs, (Norbury et al., 2004; Yewdell et al., 1999)]. Besides HSPs released in the extracellular environment after necrotic death of tumor cells, there are several other means that are possible to transfer HSPs to the surrounding APCs. For example, some cancer chemotherapeutic treatments, such as anthracyclins, induced apoptotic death of cancer cells. While apoptosis imposes a silence signal on immune system in traditional view, the one generated by these chemotherapy agents however can foster a favorable immunogenic environment. Interestingly, the reason behind the phenomenon was that anthracyclins or similar agents induced rapid translocation of an intracellular HSP calreticulin (CRT) to the cell surface of tumors prior to apoptosis, which facilitated phagocytosis of dying tumors (Obeid et al., 2007a; Obeid et al., 2007b). The ability of cell surface CRT to enhance phagocytosis may be correlated with the intrinsic feature of CRT to serve as a binding ligand for receptors on APCs, such as CD91 (Gardai et al., 2005). The exposed HSPs may also be taken up by other receptors than CD91, some of which were mentioned in the chapter 1.3.2. Besides the apoptosis-induced exposure on cell surface, HSPs may also get access to extracellular environment in the form of exosomes. A study *in vitro* demonstrated that exosomes produced by B-lymphoblastoid cell lines under stress condition contained hsp27, hsc70, hsp70, and hsp90. Although this study showed that the exosomes cannot induce direct activation of dendritic cells, they may serve as a vehicle to transport HSP-chaperoned antigens (Clayton et al., 2005). In another study, exosomes derived from resistant anticancer drugs-treated HepG2 cells conferred superior immunogenicity in eliciting HSPs-specific NK cell responses *in vitro* (Lv et al., 2012). However, the role of exosomes to deliver HSP *in vivo* remains unclear. Antigen may even be transfer without the aid

of HSP molecules, in the form of whole proteins, although whole protein is cross-presented extremely inefficiently, and even absent unless used at supraphysiological amounts (Basta et al., 2005; Blachere et al., 2005; Li et al., 2001). In addition, nucleic acid can be another form of tumor antigen. Studies showed that murine DCs transfected with mRNA amplified from the B16 melanoma cells elicited potent CD8⁺ T cell responses, which led to the regression of metastasis in tumor-bearing mice (Boczkowski et al., 2000; Gilboa and Vieweg, 2004). However, the relevance of mRNA *in vivo* as an effective form of antigen transferred from tumor cells to APCs remains to be examined. As a follow-up, we plan to explore these possible mechanisms that obviate the requirement of CD91 pathway when antigen is abundant.

The loss of effect of RAP at high dose of immunization could also be attributed to the generation of acidic extracellular environment as tumor grows larger (Estrella et al., 2013; Gillies et al., 1994; Stubbs et al., 2000; van Sluis et al., 1999). Indeed, previous studies showed that RAP binds to CD91 with high affinity to serve as an escort protein along the secretion pathway of CD91 until it reaches the medial-Golgi compartment, where it then dissociates from LRP due to the low pH (-6.4-6.6) within the Golgi compartment (Bu et al., 1995; Chanut and Huttner, 1991). Similarly, RAP may dissociate from CD91 in an acidic environment within established tumors, and normal uptake of HSP-peptide complexes *via* CD91 can therefore be resumed. However, tumor cells can also take advantage of the acidic environment thereafter, since acidity may be toxic to normal cells and inhibit the immune response to tumor antigens (Gatenby et al., 2006; Lardner, 2001). In contrast, cancer cells develop mechanisms that allow them to survive in acidic environments (Wojtkowiak et al., 2012).

The mechanism of immune evasion by disrupting CD91 pathway may also impact cancer metastasis. In the conventional model of cancer progression, metastasis is viewed as a sequential

multistep process (Chambers et al., 2002; Chiang and Massague, 2008). By gradually acquiring the ability to invade, tumor cells at the primary sites are disseminated into the systemic circulation. Shedding of tumor cells into circulation may occur in large numbers even at early stages of cancer, which contributes to the spread of cancer to distant organs (Husemann et al., 2008; Stoecklein et al., 2008). But only a few circulating cells form overt metastasis. Some examples of impediments to the formation of metastasis includes tight vascular barriers, rare acquisition of organ colonization function, unfavorable microenvironment for survival and immunosurveillance at distant organs (Nguyen et al., 2009). However, cancer cells may develop a variety of mechanisms to adapt to these difficult scenarios. As in the primary sites, limited amount of CTCs expressing RAP or other competing ligands for immune receptors may also gain huge advantage to allow them firmly established at distant organs.

I have used transplantable tumors in these studies because by doing so, I can carefully titrate the input of tumors into mice, measure antigen-specific immune responses and stably introduce CD91 inhibitors such as RAP into the tumors. However, the role of CD91 in *de novo* induction of tumors remains to be tested, as was performed for the dependence of anti-tumor immunity on IFN- γ (Shankaran et al., 2001). I am currently investigating carcinogen- and UV-induction of tumors in CD91^{-/-} mice. Cohorts of age-matched CD91^{-/-} mice and CD91^{+/+} littermates will be injected with methylnanthrene (MCA, dissolved in peanut oil, SIGMA) and will be monitored every week for development of palpable tumors. IFN α R^{-/-} mice will be used as controls. In addition, the KP murine model (developed by Tyler Jacks and available in Jackson Labs) will be used to measure immunosurveillance to spontaneous tumors. Tumor suppressor gene p53 can be deleted and oncogene K-rasG12D can be expressed in these mice under the inducible Cre/LoxP system, by intramuscular injection of viral vectors expressing Cre

recombinase. This system has a unique advantage over the chemically induced system (Aim 1.1), because a specific tumor antigen can be introduced by incorporating the antigen into the vectors alongside Cre recombinase. We plan to make bone marrow chimeras such that the KP mice lack expression of CD91 in CD11c⁺ cells. These studies will allow us to study the role of CD91 in the very early phase of de-novo tumorigenesis. The immunogenicity of these *de-novo* tumors can also be sculpted by the immune system. Tumors that are established in CD91^{-/-} mice, and harvested at <6-8 days, are expected to be more immunogenic and rejected faster when transplanted into wild type mice, when compared to tumors established in wild type mice and transplanted into wild type mice. I expect this because those early tumors established in CD91^{-/-} mice are presumed to be less edited, if edited at all, due to a failure in the establishment of anti-tumor immunity in these mice.

The impact of the loss of expression of CD91 in CD11c⁺ cells also raises several other issues; first, CD91 is known to bind to other ligands (Herz and Strickland, 2001). However, of these ligands, only α_2 M has been shown to have any immunological function (Binder et al., 2001; Chu and Pizzo, 1993). Thus, although not tested here, α_2 M bound to extracellular tumor antigens may be another mechanism for cross-priming. Second, it is important to clarify that the endocytosis of HSPs may not be the only critical feature of HSP-mediated immunity that is disrupted in our CD91^{-/-} mice. Deficiency of HSP signaling through CD91 on CD11c⁺ cells can abrogate maturation of those APCs, which ultimately reduce effective priming as well. To investigate the adjuvant effect of HSP-CD91 pathway, peptide-binding site of HSPs or the tyrosine phosphorylation (NPXY) motifs on the cytoplasmic tail of CD91 will be mutated, and its effect on NF-kB translocation, cytokine secretion, and rate of tumor growth will be assessed. In addition, infiltration of innate immune cells in tumor microenvironment will be examined,

especially in the early time points after inoculation of tumors. Third, in this study, I only examined a prototypic HSP, gp96. However, as our lab has already shown, other types of purified HSPs give rise to different immune outcomes following CD91 engagement. It is therefore important to investigate the relative contribution of each HSP known to bind CD91 to the global immune response. Fourth, given the recent observation that CD169⁺ macrophages are *bona fide* professional antigen presenting cells in the setting of apoptotic-associated antigen in lymph nodes (Asano et al., 2011), I expect a more robust phenotype when CD91 expression is eliminated in both macrophages and dendritic cells. Additionally, the expression of CD91 in different APC subsets has been examined and published recently by our lab (Messmer et al., 2013). Those studies examined APCs subsets from the lymph node *in vivo*. Among different APC subsets, CD91 is most highly expressed on CD11c⁺CD11b⁺ cells, which correlates with their superior capacity to uptake. Other CD11c populations express lower CD91 levels. Therefore, studies are currently underway to create mice with loss of CD91 in both CD11c⁺ and CD11b⁺ cells. Fifth, it is recently shown that a spontaneous CD8⁺ T cell response against transplantable tumors depends on host type I IFN signaling (Gajewski et al., 2012; Gajewski et al., 2013). Gene expression profiling in these studies revealed that tumors having high expression of T-cell markers also exhibit a type I IFN transcriptional signature (Harlin et al., 2009). However, the link between type-I IFN pathway and HSP-CD91 pathway is still unknown, as these two pathways may complement, inhibit or supersede each other.

The findings in this study may also have a bearing on prognosis of immunotherapy against cancer. If the absence of T cells is due to the expression of endogenous CD91 inhibitors by cancer cells, a prevalent approach for adoptive T cell transfer could then be deployed, which

involves isolation of tumor specific T cells from blood of patients, expanding them *in vitro* and re-infusing these autologous, *in vitro* expanded T cells back into the patient (Ho et al., 2002).

In summary, the data from this dissertation describes a novel molecular mechanism for antigen transfer during tumor immunosurveillance. I show that disrupting HSP-CD91 pathway abrogates tumor-associated immunity. This pathway is important very early in tumorigenesis, when antigen load is low. Based on these findings, I propose a novel mechanism of immune evasion for tumors expressing competing ligands for immune receptors such as CD91. A better understanding of these mechanisms will also guide us in designing immunotherapeutic approaches against malignant tumors.

APPENDIX A

RELEVANT PUBLICATIONS

Yu Jerry Zhou, Michelle Nicole Messmer, and Robert Julian Binder.

Establishment of tumor-associated immunity requires interaction of Heat Shock Proteins with CD91.

Cancer Immunology Research. Published OnlineFirst December 31, 2013

doi:10.1158/2326-6066.CIR-13-0132.

Yu Jerry Zhou and Robert J Binder. The Heat Shock Protein-CD91 pathway; a molecular description of tumor immunosurveillance. *Oncoimmunology* 2014 (invited commentary, in press)

Sudesh Pawaria, Michelle N. Messmer, **Yu Jerry Zhou**, Robert J. Binder.

A role for the Heat Shock Protein-CD91 axis in initiation of immune responses to tumors.

Immunologic Research. 2011 Aug;50(2-3):255-60.

Robert J. Binder, **Yu Jerry Zhou**, Michelle N. Messmer, Sudesh Pawaria.

CD91-dependent modulation of immune responses by heat shock proteins; a role in

autoimmunity. *Autoimmune Diseases*. 2012 Nov;2012:863041. doi: 10.1155/2012/863041

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