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PEPTIDE VACCINE FORMULATION CONTROLS THE DURATION OF ANTIGEN PRESENTATION AND MAGNITUDE OF TUMOR-SPECIFIC CD8+ T CELL RESPONSE

Hiep Khong

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**PEPTIDE VACCINE FORMULATION CONTROLS THE DURATION OF ANTIGEN
PRESENTATION AND MAGNITUDE OF TUMOR-SPECIFIC CD8+ T CELL RESPONSE**

by

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A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

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Graduate School of Biomedical

Sciences in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

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Houston, Texas

May, 2017

DEDICATION

I dedicate this dissertation to my parents, for all sacrifices they have made for me.

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PEPTIDE VACCINE FORMULATION CONTROLS THE DURATION OF ANTIGEN PRESENTATION AND MAGNITUDE OF TUMOR-SPECIFIC CD8+ T CELL RESPONSE

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Despite remarkable progresses in vaccinology, therapeutic cancer vaccines have not achieved their full potential. We previously showed that the duration of antigen presentation critically affected the quantity and quality of T cell response and subsequent anti-tumor efficacy. Here we describe L-tyrosine amino acid-based microparticles as a novel peptide vaccine adjuvant for the induction of tumor-specific T cells. L-tyrosine microparticles did not induce inflammasome activation, but instead extended antigen presentation time. The consequent prolongation in antigen presentation translated into prolonged T cell proliferation and superior numbers and anti-tumor function of vaccination-induced CD8+ T cells. Indeed, prolonging antigen presentation by repeated injection of peptide in saline resulted in an increase in T cell numbers similar to that observed after vaccination with peptide/L-tyrosine microparticles. These results suggest that the duration of antigen presentation is critical for optimal induction of anti-tumor T cells, and can be manipulated through proper vaccine formulation.

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ABBREVIATIONS

ACT: adoptive cell therapy

ADAP: adhesion and degranulation promoting adaptor protein

Ag: antigen

AP1: activator protein 1

APC: antigen presenting cell

BTLA: B and T lymphocyte attenuator

cDC: conventional dendritic cell

CDK: cyclin-dependent kinase

CDN: cyclic dinucleotide

CFA: complete Freund's adjuvant

CFSE: Carboxyfluorescein succinimidyl ester

cGAS: cyclic GMP-AMP synthase

covax: a cocktail of IL-2, imiquimod and agonist anti-CD40 antibody

CTL: cytotoxic T lymphocytes

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

DAG: diacylglycerol

DAMP: damage associated molecule patterns

DC: dendritic cell

GADS: GRB2 related adaptor protein

GM-CSF: granulocyte macrophage colony- stimulating factor

GRB2: growth factor receptor-bound protein 2

HBV: hepatitis C virus

HPV: human papilloma virus

IFA: incomplete Freund's adjuvant

IFN- γ : interferon gamma

IL: interleukin

IRF: interferon response factors

ITAMs: immunoreceptor tyrosine based activation motifs

ITK: interleukin-2 inducible T cell kinase

LAG3: lymphocyte activation gene 3

LAT: linker for activation of T cell

Lin: lineage markers, including CD3, CD19 and NK1.1

MAPK: mitogen activated protein kinase

MFI: median fluorescence intensity

MHC: major histocompatibility complex

MPL: monophospholipid A

NFAT: nuclear factor of activated T cells

NK: natural killer cells

PAMP: pathogen associated molecule patterns

PD-1: programmed cell death protein 1

pDC: plasmacytoid dendritic cell

PK1: 3-phosphoinositide-dependent protein kinase 1

PD-L1/2: programmed cell death ligand 1/2

PI3K: phosphatidylinositol 3-kinase

PIP₂: phosphatidylinositol-(4,5)-bisphosphate

PKC: protein kinase C

PLA: poly(lactic acid)

PLC γ , phospholipase C γ

PLG: poly(lactide-co-glycolide)

PLGA: poly(lactic-co-glycolic acid)

poly IC: polyinosine-polycytidylic acid

PPR: pattern recognition receptors

PTEN: Phosphatase and tensin homolog

RB: retinoblastoma-associated protein

RLR: C-type lectin receptors and retinoic acid inducible gene (RIG)-I-like receptors

RTK: receptor tyrosine kinase

SLP76: SH2 domain containing leukocyte protein of 76 kDa

STAT: Signal transducer and activator of transcription

STING: stimulator of interferon genes

TCR: T cell receptor

T-eff: effector T cell

Th (1,2,17): helper T cells (type 1, 2, 17)

TIM3: T cell immunoglobulin 3

TIL: tumor infiltrating lymphocytes

TLR: toll-like receptors

TNF: tumor necrosis factor

Treg: regulatory T cell

TRIF: TIR-domain-containing adapter-inducing interferon- β

UV: ultraviolet

VdLN: vaccination site-draining lymph node

ZAP70: ζ -chain associated protein kinase of 70 kDa

CHAPTER 1 – INTRODUCTION

I. Melanoma

Melanoma is a rare type of skin cancer originating from malignant melanocytes. Although it only accounts for 2 percent of skin cancer, melanoma is the most dangerous type which is responsible for 60 percent of total death of skin cancer (1). As of 2015, it was estimated by the National Cancer Institute that there would be approximately more than 73,000 new case of melanoma diagnosed. The incident rate was roughly 30 per 100,000 per year in the US.

It requires a complex interplay of exogenous (environmental) and endogenous (genetic) factors for melanocytes to transform to melanoma (2). Environmental factors such as ultraviolet (UV) radiation, including UVA and UVB, are major risk factors for melanoma pathogenesis (3, 4). Briefly, UV radiation has been implied to cause mutation of melanoma associated genes such as *N-RAS*, *BRAF* and *CDKN2A*(5, 6). In addition, UV radiation has been shown to suppress the immune system, allowing tumor growth which otherwise would be rejected (7, 8). Genetic factors also contribute: around 8 percent of all patients have a family background of melanoma. Among them, 40 percent carry germline mutations in the cyclin dependent kinase inhibitor 2A (*CDKN2A*) locus (9). *CDKN2A* encodes for 2 tumor suppressors, p16^{INK4A} and p14^{ARF}, responsible for controlling the cell cycle by inhibiting CDK4/6 and MDM2 respectively (Figure 1). Accordingly, loss of function of *CDKN2A* will disrupt cellular homeostasis. Another germline mutations found in familial melanoma patients is *CDK4* (10).

In terms of molecular mechanism of the disease pathogenesis, genetic alterations lead to the change of intracellular signaling pathways including mitogen activated protein kinase

(MAPK) and PI3K/AKT. For example, under normal condition, MAPK gets activated when receptor tyrosine kinases bind to their ligands (i.e. growth factors). In 50 percent of melanoma patients, a substitution of valine to glutamic acid at position 600 (V600E) makes BRAF constitutively active which in turn activates the MAPK pathway. Another example is PTEN loss, caused by point mutation or deletion, which activates the PI3K pathway (Figure 1).

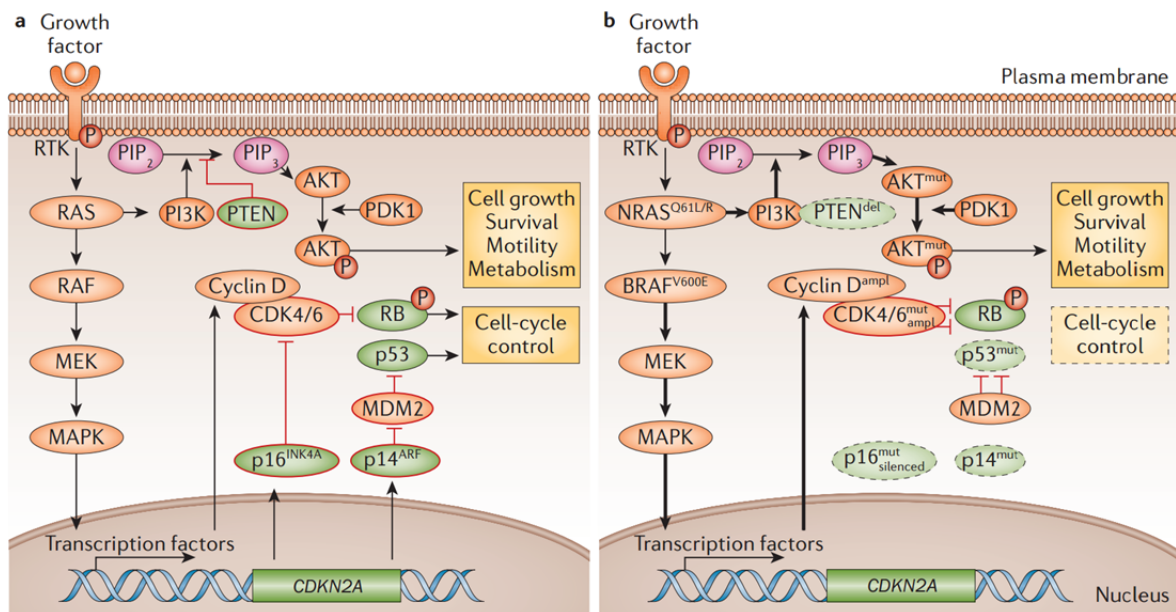


Figure 1. Molecular pathogenesis of melanoma. a) In normal cells, mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-AKT signaling control the balance of cellular function including cell cycle, survival, motility and metabolism. B) In malignant cells, genetic alterations cause the loss of function of p16, p14 and PTEN or constitutive activation of NRAS or BRAF. CDK, cyclin-dependent kinase; MDM2, E3 ubiquitin-protein ligase MDM2; MEK, MAP/ERK kinase; P (in a red circle), phosphate; p14^{ARF}, splice variant encoded by the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene; p16^{INK4A}, splice variant encoded by the CDKN2A gene; PDK1, 3-phosphoinositide-dependent protein kinase 1; PIP₂ phosphatidylinositol-(4,5)-bisphosphate; PIP₃, phosphatidylinositol-(3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog; RB, retinoblastoma-associated protein; RTK, receptor tyrosine kinase. Reused by permission from Macmillan Publishers Ltd: *Nature Review Disease Primer*, "Melanoma", D. Schadendorf *et al.*, ref. (2), copyright © 2015.

If detected in early stages (0, I, II and resectable III) melanoma can be cured by surgical removal of lesions and regional lymph nodes (1). The treatment becomes difficult once the disease reaches later stages including non-resectable III, and IV. Current options for metastatic melanoma treatment are chemotherapy, radiation, targeted therapy and immunotherapy (11). Chemotherapy and radiation work by killing actively dividing cells including malignant cells as well as normal stem cells/progenitor cells. Targeted therapy employs small molecules to specifically target certain signaling molecules such as c-KIT, BRAF and MEK (12–14). BRAF (V600E) inhibitors, vemurafenib and dabrafenib were shown to be very effective with high response rates, close to 50 percent both. The responses, however, were not complete and often short-lived (13, 15). Melanoma was shown to acquire resistance to BRAF inhibition by upregulating receptor tyrosine kinase (RTK) or NRAS which makes it predictably sensitive to MEK inhibition (16). Accordingly, combining BRAF and MEK inhibitors remarkably improved the response rate (from 51 percent to 64 percent) and median progression free survival (from 7.3 months to 11.4 months), compared to BRAF inhibitor alone (14). Another form of FDA-approved melanoma therapy is immune-based therapy with cytokines, including interleukin-2 (IL-2) and interferon-alpha (IFN- α). IL-2 is a T cell growth factor, and systemic IL-2 treatment can cure 5-10 percent of patients with metastatic melanoma, albeit with significant, but mostly reversible toxicity (17). IFN- α is modestly effective at reducing the recurrence rate after resection of stage III melanoma, though also with significant toxicity (18). Since the introduction of IL-2 and IFN- α , the advent of novel, more effective immunotherapies has revolutionized the way of treating melanoma because of remarkable effective and durable responses.

II. Immunotherapies for melanoma

1. *T cell checkpoint blockade*

The high load of somatic mutations (more than 10 mutations per megabase of DNA) in the melanoma genome implies expression of a high number of altered proteins (neoantigens), and hence a greater chance of melanoma being recognized by the immune system. In fact, melanoma is very attractive for immunotherapies (figure 2) (19). Unlike other therapies that directly target tumor cells, immunotherapies help the immune system to recognize and kill cancer cells. Major successes of immunotherapies, especially CTLA-4 and PD-1 blockade, have brought immunotherapies back to the mainstream of treatment for metastatic melanoma. Other immunotherapies such as oncolytic virus, adoptive cell therapy, and therapeutic vaccine have been investigated intensively which hopefully to follow the successful path of checkpoint therapy in near future.

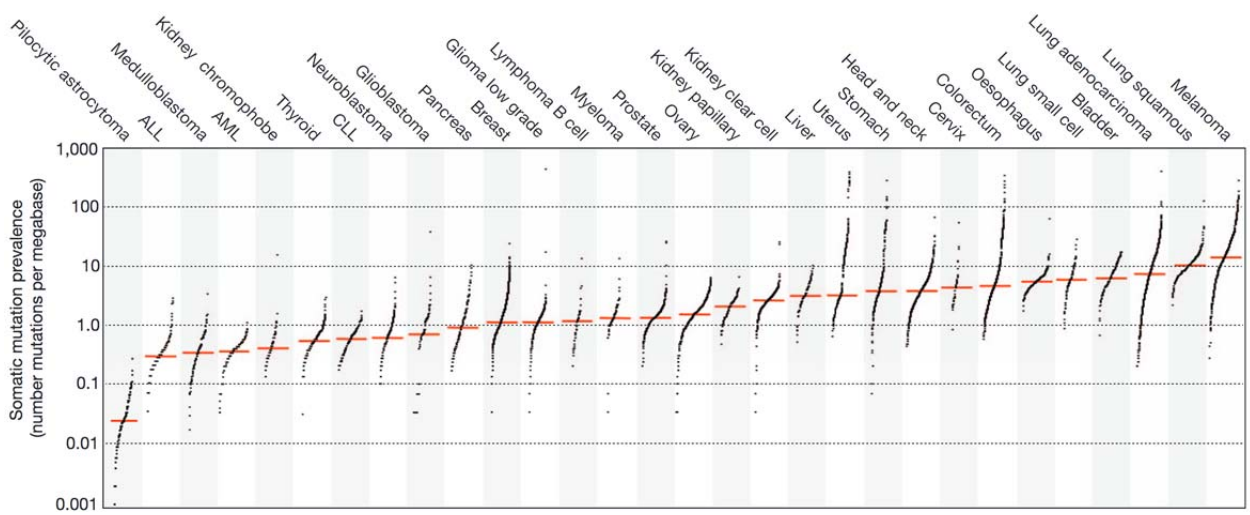


Figure 2. Somatic mutation load in melanoma and other cancers. Melanoma has the highest mutations per megabase. Reused by permission from Macmillan Publishers Ltd: *Nature*, “Signatures of mutational processes in human cancer”, Ludmil B. Alexandrov *et al.* ref. (19), copyright © 2013.

The principle of checkpoint inhibition is to “unleash” the power of the immune system by “releasing the brakes” that serve as a mechanism to avoid tissue damage caused by prolonged T cell reaction. The two most prominent examples of checkpoint inhibition are anti-CTLA-4 and anti PD-1/PD-L1.

When cytotoxic T lymphocyte antigen-4 (CTLA-4) was discovered almost three decades ago (18), it was initially thought to be a co-stimulatory receptor due to its structural homology to CD28 (20–22). Subsequently, the immune attenuating function of CTLA-4 was discovered by independent groups (23–25). Similar to CD28, CTLA-4 is expressed exclusively on T cells. CTLA-4 counteracts antigen-TCR signaling amplification by CD28 by two mechanisms. First, CTLA-4 competes with CD28 for their shared ligands (CD80/CD86) because of its higher affinity for those ligands (26). Second, activated CTLA-4 interacts with protein phosphatase SHP2 which can reverse phosphorylation of TCR and CD28 (27). CTLA-4 also enhances regulator T cell (T-reg) suppressive function by intercepting dendritic cell (DC) and conventional T cells (non T-reg) interaction, thereby limiting T cell priming efficiency (28). Despite the lethal auto-immune and hyper-immune effect of CTLA-4 in knock-out mice, Allison and colleagues demonstrated a therapeutic window for CTLA-4 inhibition was achievable with anti-CTLA-4 antibodies (29). Their pioneering studies paved the way for CTLA-4 blockade to be the first of its class to enter clinical studies, after which it was eventually approved for melanoma treatment (30).

The second immune checkpoint pathway which was clinically targeted and approved was programmed death 1 (PD-1). The gene encoding PD-1 was cloned a few years after CTLA-4 was discovered (31). Functional studies of PD-1 using genetically deficient mice revealed its role as a negative regulator in response to antigen (32, 33). Unlike CTLA-4, PD-1 is more

broadly expressed on different cell types including effector T (T-eff), T-reg, B and NK cells (31, 34–36). PD-1 exerts its immunosuppression by enhancing T-reg function and differentiation, inducing IL-10 production, and promoting T cell anergy, exhaustion and apoptosis (34, 37–41). Shortly after PD-1 function was revealed, ligands for PD-1 including PD-L1 and PD-L2 were identified (42, 43). Some interesting observations of PD-L1 expressions should be noticed. First, although PD-L1 is rarely expressed under normal conditions, it can be induced on many nucleated cells in the presence of IFN- γ (44). Second, a variety of freshly isolated human tumor cells express PD-L1 but not their *in vitro* cultured counterparts (39, 45). In vitro cultured cells, however, will express PD-L1 when IFN- γ is added. Consistently, PD-L1 is expressed focally on tumor cells in direct contact with T cells rather than diffusely throughout the entire tumor (46). Finally, PD-1 was found to be highly expressed by T cells in chronic infection or within tumors (41, 47). Since IFN- γ is one important cytokine expressed by T-eff, PD-1 (including PD-1/PD-L1 and PD-1/PD-L2) signaling seems to play as negative feedback mechanism to limit tissue damage in chronic infection and autoimmunity, which can be exploited by the tumor as an escape mechanism. Together, those observations provided a rationale to target the PD-1 pathway in preclinical murine cancers using anti-PD-L1 antibodies (39, 48). Promising results of preclinical studies led to clinical trials with anti-PD-1 antibodies in different human cancers including metastatic melanoma, renal cell carcinoma and non-small cell lung carcinoma (NSCLC) (49, 50). Critical success of PD-1 blockade in melanoma and NSCLC led to their approval for the treatment of these cancers by the US Food and Drug Administration (FDA) (51–53). Recently, anti-PD1 was shown to synergize with anti-CTLA-4 to improve progression free survival remarkably in patients with metastatic melanoma (54). With major successes of

PD-1 and CTLA-4 blockade, the targeting of other checkpoints such as LAG3, TIM3 and BTLA is under investigation (55).

2. *Oncolytic virus*

When used as immunotherapy drugs, viruses can be either native or genetically engineered to selectively kill tumor cells (56). There are two prerequisites of the cancer in order to deploy oncolytic viruses for treatment. First, tumor cells must possess overexpressed or unique surface molecules as anchors for viruses to attach and enter them. For example, herpes virus entry mediator (HVEM) and selected nectins overexpressed by melanoma and different forms of carcinomas can be targeted by HSV-1 (57). Unique cell surface molecules expressed by tumor cells can also be targeted by specifically engineered viruses in the case of ovarian cancer with adenovirus (58). Second, because tumor cells acquire aberrant signaling pathways, viruses can exploit those in malignant cells but not normal ones. For example, many tumor cells have over- or constitutively active RAS signaling which confers them unchecked proliferation and resistance to cell death (59). Protein kinase R (PKR) responsible for protection against viral infection by preventing protein translation is inactivated by RAS (60). Therefore, RAS transformed cells are more susceptible to viral infection and cell lysis to normal cells. Defects in type I IFN signaling, another antiviral mechanism, can also be exploited by vaccinia virus (VV), Newcastle disease virus (NDV), and vesicular stomatitis virus (VSV) and others (56, 61–63). Although the virus-induced lytic effect occurs *in situ*, damage associated molecular pattern (DAMP) signals and “hidden” tumor antigens (due to restricted presentation) released after cell lysis, together with pathogen associated molecular pattern (PAMP) signals of viruses can trigger systemic anti-tumor immunity (56). Therefore, the oncolytic virus approach can be considered as a bridge between traditional chemotherapy/radiation (direct killing of cancer

cells) and immunotherapy (inducing anti-tumor immunity). In 2015, modified herpes simplex virus type 1 (HSV-1) encoding granulocyte-macrophage stimulating factor (GM-CSF), also known as talimogene laherparepvec (T-VEC, Amgen), was approved by the FDA to treat melanoma lesions in the skin and lymph nodes (64).

3. Adoptive cell therapy

Adoptive cell therapies (ACT) are highly personalized approaches which use autologous lymphocytes to treat cancers. ACT includes tumor infiltrating lymphocytes (TIL) therapy, engineered $\alpha\beta$ T cell receptor (TCR) therapy and chimeric antigen receptor (CAR) therapy (65).

TIL therapy selectively expands a patient's tumor-reactive CD8 T cells *ex vivo*, followed by transfusion back to patients (66). The development of TIL therapy relied on several premises. First, lymphocytes from mice immunized against tumor were shown to mediate tumor rejection after being transferred to tumor bearing hosts (67). Second, T lymphocytes can be grown *ex vivo* thanks to the discovery of IL-2. Third, administration of high dose IL-2 can suppress tumor growth in mouse model. Thanks to advances in *in vitro* T cell culture, tumor specific T cells from resected tumor can be expanded using high interleukin (IL)-2 containing medium, up to 10^{11} cells before being transfused back to patients (65). TIL therapy used to induce short-lived tumor rejection but the discovery of lymphodepletion prior to lymphocyte transfusion dramatically enhanced tumor rejection and durability of TIL therapy (68). Clinical trials showed that TIL therapy had objective response rates as high as 50 percent, with some patients remaining cancer-free for 6 years, which is essentially considered a complete cure (65). Although TIL can theoretically be grown from any tumor, melanoma is the only cancer from which tumor reactive lymphocytes can be consistently grown. In addition, the high cost,

labor-intensive and time-consuming processes, together with its highly personalized nature have made TIL therapy difficult to apply widely. As a “living” therapy, TIL does not fit well to “off-the-shelf” practice which further limits its attractiveness to drug companies.

Engineered $\alpha\beta$ T cell receptor (TCR) therapy is an approach that generates lymphocytes having TCR with high affinity against tumor antigens (69). This approach overcomes a disadvantage of TIL therapy that tumor-reactive T cells can neither be found nor expanded in certain patients. Naïve lymphocytes from patients, regardless of their TCR specificity, can be isolated and genetically engineered to express $\alpha\beta$ TCR specific for tumor antigens. Often, antigens of choice are differentiation or overexpressed ones which are shared by normal tissues to some extent. Lack of endogenous T cells specific for these antigens also implies that those TCR have been deleted during negative selection in T cell development. Forcing expression of such TCR may lead to severe autoimmune reaction, also known as on-target, off-tumor effect. For example, genetically modified gp100 and MART-1 specific T cells were ineffective against melanoma in a small clinical trial, but caused severe ear and eye toxicity by targeting gp100 and MART-1 expressing melanocytes in those organs (70).

Similar to engineered $\alpha\beta$ TCR therapy, chimeric antigen receptor (CAR) therapy relies on genetically modified TCR lymphocytes. However, instead of generating conventional $\alpha\beta$ TCR, CAR approach creates receptors with single-chain variable fragment (scFv) derived from immune-globulin (Ig) molecules (found in antibodies or B cell receptors) in fusion with CD3 zeta (ζ) chain (found in TCR complexes) (71). Accordingly, the CAR can bind to its antigen in similar way to that of an antibody but triggers TCR signaling. This first generation CAR T cells had limited survival and expansion because TCR signaling was merely enough for T cell

activation. Indeed, optimal T cell activation and survival requires 3 signals: antigen/TCR, co-stimulatory signaling and cytokines. To overcome those limitations, co-stimulatory domains, such as CD28 and CD137 (4-1BB), were added to second generation CAR (72, 73). Compared to first generation CD3 ζ CAR, second generation CD28/ CD3 ζ and 4-1BB/ CD3 ζ CARs greatly improve T cell proliferation, function and persistence, which directly translated to greater tumor rejection. CAR has certain advantages over engineered traditional $\alpha\beta$ TCR. First, it does not depend on the antigen processing or loading efficiency of antigen to MHC molecules. Second, CAR T cells can mediate killing effect regardless of MHC status on target cell surface. This is very important since some tumors can down-regulate MHC-I to avoid destruction by T cells. Third, CAR therapy therefore does not need to be matched with patient's MHC haplotypes. CAR therapy for B cell malignancies which targets CD19 has been very successful with complete remission rate of 70 to 100 percent (74). Nevertheless, the success of CAR for B cell malignancies is a rare case among cancers because normal B cells which also share CD19, and are killed by the therapy, are dispensable. Solid tumors otherwise have few surface antigens that can be targeted without evoking serious toxicity (71).

4. Therapeutic cancer vaccines

The basics of T cell activation

The fundamental mediator of anti-tumor immunity induced by anti-cancer vaccination is the tumor-specific cytotoxic CD8 T cell (CTL) that kills tumor cells (75). Therefore, understanding how CTL are activated is critical for optimal cancer vaccine design, as depicted in figure 3. In the context of infection by intracellular pathogens where cellular response is chiefly induced, CTL activation occurs in sequential steps. Antigen presenting cells (APC) such

as dendritic cells (DC) are activated *in situ* by sensing pathogen-associated molecular pattern (PAMP) or cell derived damage-associated molecular pattern (DAMP) via pattern recognition receptors (PRR) or DAMP receptors respectively. DC also acquire pathogen-derived antigen (Ag) before migrating to peripheral lymphoid organs. At lymphoid organs, DC presents Ag to CD4 T cells which secrete type 1 helper T cell (Th1) skewing cytokine such as IL-12 and type 1 interferons (IFN). Simultaneously, the DC is licensed by CD40L on Th1 cells to activated cognate CD8 T cells. Licensed DC in turn activate cognate CD8 T cells via 3 signals: antigen, co-stimulatory molecules and cytokines. While signal 1 and 2 are mostly provided by DC (and sometimes B cells), signal 3 – cytokines – can also be provided by many other cell types. Thus, to trigger an optimal CTL response, a vaccine must be carefully designed to provide those 3 signals adequately.

Upon engagement of TCR to peptide/MHC complex, CD4 and CD8 co-receptors are recruited and bind to MHC class II and class I respectively. Src family kinase LCK associated with CD4/CD8 phosphorylates tyrosine residues on immunoreceptor tyrosine-based activation motif (ITAM) on CD3 and TCR-associated ζ chain. Phosphotyrosine in ITAM recruits ZAP-70, which in turn phosphorylates and activates linker for activation of T cell (LAT) molecule. LAT further recruits multiple signaling molecules to form the LAT signalosome. The most crucial signaling molecule of the signalosome is phospholipase $C\gamma$ (PLC γ). The activation of PLC γ 1 triggers three distinct signaling pathways: Ca^{2+} , NF κ B and MAPK. Activated PLC γ 1 breaks membrane lipid PIP $_2$ into two products: inositol 1,4,5-triphosphate (IP $_3$) and diacylglycerol (DAG). IP $_3$ binds to its receptor on endoplasmic reticulum (ER) membrane to release Ca^{2+} to cytosol from ER. Increased cytosolic Ca^{2+} leading to the activation of nuclear factor of activated

T cells (NFAT). DAG recruits PKC and RasGRP to cell membrane which in turn activates NF κ B and Ras respectively. Activated Ras triggers mitogen activated protein kinase (MAPK) which subsequently activate transcription factor AP-1. Together, transcription factors NFAT, NF κ B and AP-1 induce transcription of genes responsible for T cell proliferation and differentiation (figure 4).

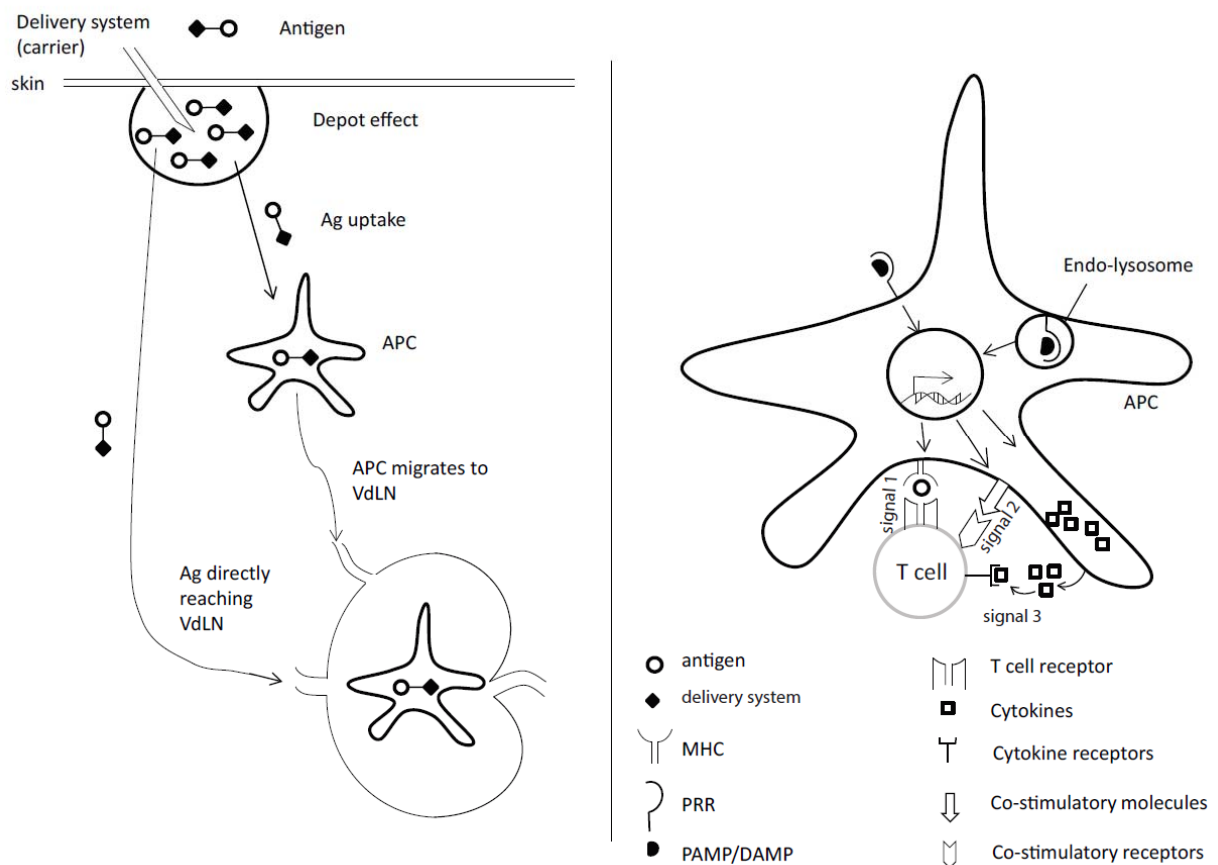


Figure 3. The basics of T cell activation. Left, some adjuvants can function as antigen delivery systems to affect the geographical availability of the antigen (signal 1). Right, adjuvants also commonly stimulate antigen presenting cells (APC) and induce them to upregulate co-stimulatory molecules such as CD80/CD86 (signal 2) and/or produce cytokines such as IL-12 (signal 3). VdLN: vaccination site-draining lymph node. Reused from *Journal of immunotherapy*

for cancer, “Adjuvants for peptide based cancer vaccine”, Khong H. and W. W. Overwijk, ref.
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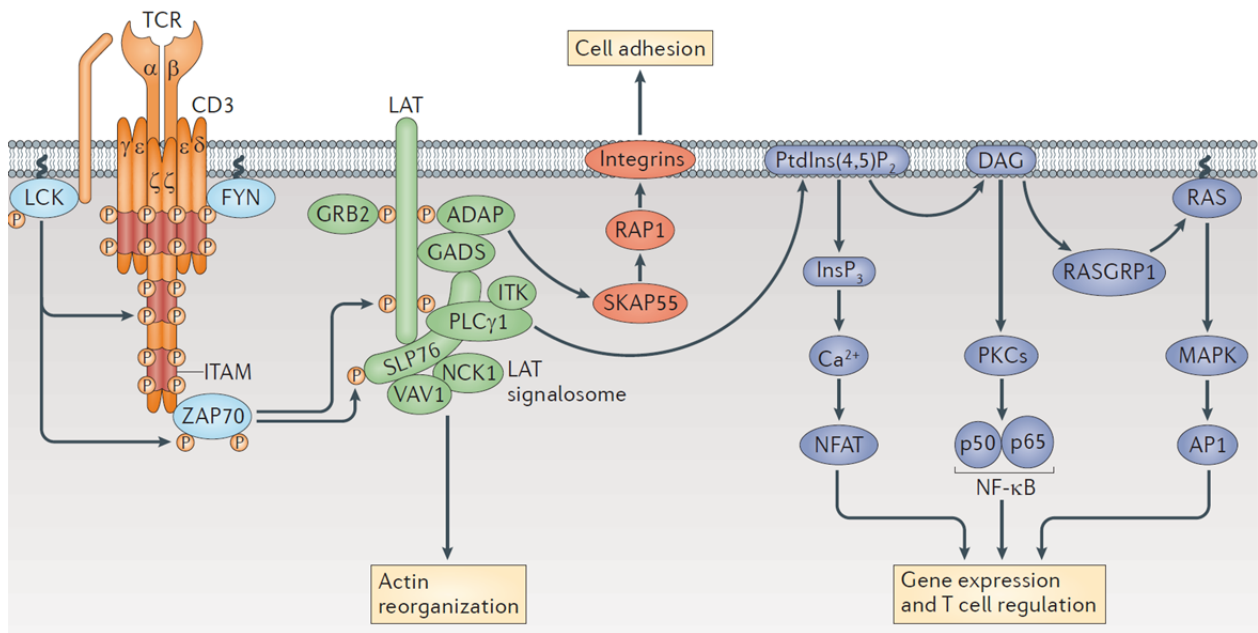


Figure 4. Intracellular signaling of TCR activation. TCR signaling is triggered by the recognition of cognate peptide-MHC molecules. Src family kinase LCK is recruited and phosphorylates ITAMs of CD3 δ -, ϵ - and ζ - chains. Phosphorylated ITAMs enable the recruitment and phosphorylation of ZAP70. Activated ZAP70 phosphorylates and activates LAT. Activated LAT recruits several signaling molecules such as PLC γ , GRB2, GADS, SLP76, ADAP, ITK, NCK1 and VAV1 to form the LAT signalosome. The LAT signalosome initiates 3 major signaling pathways: the Ca^{2+} , NF κ B and MAPK which lead to the activation of transcription factors and genes essential for T cell growth and differentiation. ITAMs, immunoreceptor tyrosine based activation motifs; ZAP70, ζ - chain associated protein kinase of 70 kDa; LAT, linker for activation of T cell; PLC γ , phospholipase C γ ; GRB2, growth factor receptor-bound protein 2; GADS, GRB2 related adaptor protein; SLP76, SH2 domain containing leukocyte protein of 76 kDa; ADAP, adhesion and degranulation promoting adaptor protein; ITK, interleukin-2 inducible T cell kinase; AP1, activator protein 1; DAG, diacylglycerol; InsP₃, inositol-1,4,5-trisphosphate; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PtdIns(4,5)P₂,

phosphatidylinositol-4,5-bisphosphate (PIP₂); RASGRP1, RAS guanyl-releasing protein 1; SKAP55, SRC kinase-associated phosphoprotein of 55 kDa. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, "T cell receptor signaling networks: branched, diversified and bounded", Bronelie J. R. and R. Zamoyska, ref. (77), copyright © 2013.

TCR-Ag/MHC engagement (signal 1) is barely sufficient for T cell activation. A full-fledged T cell activation requires additional stimulations such as co-stimulating signals (signal 2) and cytokines (signal 3) (78). These additional stimulations are provided by APC when their pattern recognition receptors (PRR) and/or damage-associated molecular pattern receptors (DAMP) are activated by PAMP and DAMP, respectively. Details of PAMP/DAMP and their respective receptors will be discussed further in the section of vaccine adjuvants below.

A classic example of signal 2 is the activation of co-stimulatory receptor CD28 upon binding to its ligands CD80/CD86 on professional antigen presenting cells (APC) such as DC (27). This brings the cytoplasmic domain of CD28 close to CD4/CD8, allowing it to subsequently be phosphorylated by CD4/CD8 associated LCK. Phosphorylated tyrosine on CD28 cytoplasmic domain then serves as a docking site for PI3K which in turn recruits AKT to activate PLC γ . In this manner, co-stimulatory signal from CD28 reinforces signaling pathways initiated by TCR activation (figure 5). In fact, all three transcription factors NFAT, NF κ B and AP-1 are required for the transcription of IL-2, an essential cytokine for T cell proliferation and differentiation.

The types of cytokine (signal 3) induced depend on the nature of the pathogen and the responding cell type. Intracellular and viral pathogens induce the production of inflammatory cytokines such as IL-12 and type I IFNs (α/β). IL-12 is mainly produced by activate inflammatory cells such as DC, macrophages and B cells (79). The cytokine is in fact a heterodimer composed of p35 and p40 subunits which bind to IL-12R β 2 and IL-12R β 1 respectively. The activation of innate immune receptors is not sufficient for the optimal induction of IL-12 heterodimer. Cytokines such as IFN- γ and IL-4 contribute to strong induction of IL-12. Beside cytokines,

molecules involved in direct cell-cell interaction, especially TNF family can also enhance IL-12 production, such as CD40L on activated T cells ligating CD40 on DC and macrophages. Particularly, CD40 signal promotes the formation of IL-12 heterodimer (p35-p40) by preferentially inducing the transcription of p35 gene (80). The engagement of IL-12 to its receptors, mainly expressed by activated T and NK cells, phosphorylates and activates STAT4 signaling which in turn promotes Th1 and cytotoxic T cell differentiation in response to antigen. Type I IFNs can be produced by virtually every nucleated cell in the body in response to innate receptor activation by microbial components. Type I IFNs signal through heterodimeric transmembrane receptor composed of IFNR1 and IFNR2. The engagement of type I IFNs and their receptors leads to the activation of PI3K, MAPK and STAT signaling pathways. Unlike IL-12 which signals through STAT4 homodimers, type 1 IFNs signals through STAT1-STAT2 heterodimers and STAT1 homodimers. STAT1-STAT2 heterodimers, considered as canonical pathway, activate the transcription of several hundreds of interferon stimulated genes (ISG) while STAT-1 homodimers activate IFN- γ associated genes. Together, IL-12 and type IFN promote clonal expansion and enhanced effector function and long-lived memory of CD8 T cells (81). Therapeutically, IFN- α effectively enhances antitumor immunity after peptide vaccination in terms of tumor specific CD8 T cell number, effector function and long-term persistence (82).

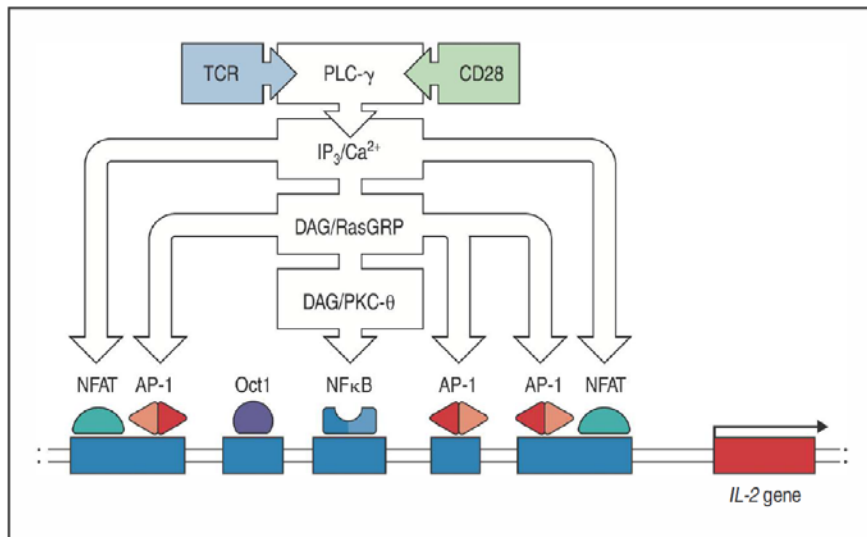


Figure 5. Co-stimulatory CD28 reinforces TCR signaling through PLCγ. Reused by permission from Garland Science Publisher, Janeway's Immunobiology 8th Edition, ref. (83), copyright © 2012.

These insights into T cell activation allow immunologists to tailor vaccine components, especially the adjuvants, to achieve the highest therapeutic effect of anti-cancer vaccination. The goal of a therapeutic cancer vaccine is to induce the activation and proliferation of T cells, in particular CTL, which specifically recognize and kill cancer cells leading to improved therapeutic outcome for the patient. To maximize CTL responses, an ideal vaccine adjuvant must fulfill two major roles. First, it must provide optimal availability of the antigen (Ag, signal 1) by regulating its persistence, location, concentration and presentation by antigen presenting cells (APC). Second, it must enhance the immune response by inducing the expression of co-stimulatory molecules (signal 2) and cytokines (signal 3) by APC (78). Suboptimal delivery of any of these signals can result in poor T cell numbers and/or function.

Antigen delivery systems

Antigen delivery systems facilitate signal 1 by different mechanisms. First, they extend Ag presentation time by protecting Ag from degradation by proteases and peptidases (84). Second, they enhance the uptake of tiny antigenic peptides by APC by forming those peptides into particles of a size similar to that of pathogens (micrometer or sub-micrometer size) (85). Third, some delivery systems can promote the localization of Ag to peripheral draining lymph nodes which increases the chance of encountering draining lymph node-resident APC, resulting in increased Ag-presentation to T cells (86). Collectively, these mechanisms enhance T cells responses by extending Ag presentation time to be optimal for T cell clonal expansion, effector function and/or memory formation (87, 88). Of note, vaccination can also allow for the delivery of immunodominant or neoantigen epitopes, resulting in enhanced anti-tumor efficacy.

Besides signal 1, antigen delivery systems can also deliver signal 2 and 3 by activating innate immune cells. Aluminum, PLG and polystyrene particles were shown to activate the inflammasome complex in a phagocytosis-dependent manner while carbon nanotubes trigger the complement system (89). Adjuvants vary in the quality and quantity of signals 1, 2 and 3 they deliver to T cells. These attributes of adjuvants become especially important when they are used in vaccination with antigens that possess very little, if any, inherent adjuvant activity, such as the minimally defined peptide epitopes typically used in peptide vaccines. Here we discuss some adjuvants that are commonly used in peptide-based cancer vaccines.

Incomplete Freund's adjuvant (IFA): IFA is a water-in-oil emulsion, identical to Complete Freund's Adjuvant (CFA) but without the heat-killed mycobacteria (e.g. *M tuberculosis*) to avoid acute granulomatous lesions at vaccination sites. It has previously been shown that IFA

promotes long-term retention and slow release of emulsified antigen at the inoculation site (90, 91). Likely as a result of this, IFA induces strong humoral and cellular immune responses. Clinical-grade IFA (Montanide™ oil series, SEPPIC Corp.) has been widely used clinically in cancer vaccines (92). Recently, our group showed that IFA-based peptide vaccines can induce potent cytotoxic CD8 T cell responses in mice, followed by T cell retention, exhaustion and deletion at the vaccination site, due to excessively long-term peptide Ag retention and chronic release by the poorly biodegradable IFA emulsion (93). Mechanistically, the long-term antigen presentation and consequent T cell recognition and cytokine release at the vaccination site induced chronic tissue inflammation and chemokine production that attracted and retained effector T cells, preventing them from reaching the tumor site. Eventually, persistent antigen stimulation at the vaccination site resulted in T cell exhaustion and Fas/FasL-mediated T cell apoptosis. Of note, this observation was obtained using vaccines based on minimal epitope-sized short peptides which can be presented by any MHC Class I-positive, nonprofessional APC (94). In contrast, long peptides require to be trimmed and efficiently presented by DC, which are the main APC type expressing optimal co-stimulatory molecules and cytokines for efficient priming of naïve T cells (95). Indeed, long peptides emulsified in IFA induced minimal T cell trafficking to vaccination sites and greatly reduced contraction of T cell levels (93). It is also proposed that long peptides which contain helper T cell epitopes will induce Th response to further enhance the CTL response (95). However, in 2 separate clinical trials using IFA, Th epitopes mixed with short CTL epitopes failed to improve CTL response in patients with metastatic melanoma (96, 97). This might be due to a difference in the nature of the antigens: virus-derived long peptides containing both Th and CTL epitopes vs. melanocyte self-antigen-derived short CTL epitope peptides mixed with short Th epitope peptides. Given the clear

benefit of CD4+ T cell responses in the generation and intratumoral function of CD8+ T cells (98, 99), further studies are needed to reconcile this discrepancy.

Aluminum adjuvants: Generally referred to as alum, both aluminum hydroxide (Alhydrogel™) and aluminum phosphate (Adjuv-phos™) adjuvants are widely used in human vaccines such as those against influenza, tetanus, diphtheria, pertussis, poliomyelitis, and HPV (100). During vaccine preparation, antigens are adsorbed to preformed aluminum adjuvants, hence their name aluminum-adsorbed vaccines. Aluminum adjuvants are known to promote Th2 response making them less suitable for vaccines against intracellular bacteria such as *M. tuberculosis*, which require a Th1-type immune response dominated by IFN- γ (101). When combined with MPL (a detoxified form of lipopolysaccharide, LPS), a TLR4 agonist, as in the AS04 adjuvant system (Glaxo SmithKline), alum-based vaccines induce Th1 responses with production of IFN- γ and IgG2a. Alum adjuvants were found to activate the NALP3 inflammasome in DC (102). Inflammasome activation leads to the production of proinflammatory cytokines including IL-1 β and IL-18 which promote the adaptive cellular (Th1/Th17/Th2) and humoral responses (103). IL-1 β promotes Th1 and Th17 while IL-18 serves as coactivator for other cytokines. In the presence of IL-12 and IL-15, IL-18 contributes to Th1 response via promoting IFN- γ production. In the absence of IL-12, IL-18 induces IL-4 which drives Th2 response (104). Thus, adjuvants that activate the inflammasome, including alum, can induce different types of T cell response, depending on tissue- or adjuvant-driven cytokine context.

Micro/nano particles: Micro- and nano-particles are attractive antigen/drug delivery systems because of several desired characteristics. First, the particles protect their cargo from serum/tissue peptidases/proteases and other degrading factors, thus increasing the half-life of

encapsulated Ag and immunomodulators *in vivo*. Second, particles can be engineered to target specific cell types or organs (such as lymph node) (105, 106). These features help reduce both the drug dose and off-target side effect. For example, Ag encapsulated in poly(lactic-co-glycolic acid) (PLGA) particles induced similar T cell responses with a 1000-fold lower dose compared to free Ag (89).

There are two basic ways to engineer particles for enhanced uptake by APC. Passive targeting relies on the size, charge and rigidity of the particle while active targeting depends on added ligands on the particle surface. Vaccine particles with size range from 500 nm to 2000 nm are preferentially trapped by tissue APC at the injection site (which may then traffic to LN), while 20 to 200 nm particles drain passively to LN where they are taken up by resident APC. Beside their role as Ag/drug carrier which increases signal 1, micro and nanoparticles can also enhance signals 2 and 3. PLG and polystyrene particles are thought to participate in inflammasome activation by enhancing the IL-1 β secretion by DC in a phagocytosis-dependent manner (107). Carbon nanotube particles, on the other hand, activate the complement system and subsequent inflammatory responses via binding to C1q (108). Materials used to make micro and nanoparticles include liposomes, synthetic polymers such as polystyrene, poly(lactide-co-glycolide) PLG, poly(lactic acid) PLA, PLGA or natural polymers such as gelatin, collagen and chitosan. The choice of material depends on the desired biocompatibility, half-life, hydrophobicity and polarity. For example, liposome particles are very versatile, allowing combination of Ag and cytokines like IL-2 or GM-CSF, to form a single particle to provide better immune response and protection (109). However, major drawbacks are the rapid clearing from the blood and accumulation of those particles in the liver. Coating a liposome with

polyethylene glycol (PEG) or other biocompatible polymers can reduce rapid systemic clearing and thus extend its half-life in vivo (110).

To improve the accumulation of a liposome to targeted tissue or organ, its surface can be decorated with receptors (e.g. antibodies) for target cell/tissue ligands and such modified liposomes are called immunoliposomes. Micro- and nanoparticles such as hydrophilic poly(DL-lactide-co-glycolide) microspheres and poly(propylene sulfide) nanoparticles have been designed to target the DC in draining LN (105, 106). A different approach is to attract DC to vaccination site. Recent reports showed that incorporating GM-CSF, CpG and tumor antigens in PLG matrices efficiently attracted and stimulated both conventional DC (CD11c+CD11b+ and CD11c+CD8a+) and plasmacytoid DC, resulting in superior immune responses (Th1 and CTL) against B16 melanomas in mice (111, 112). A very high concentration of GM-CSF (3000 ng) prolonged the DC retention in situ, resulting in suboptimal DC trafficking to draining LN and the subsequent inferior T cell priming and protection against tumor. This observation suggests that delivery systems inducing DC attraction *in situ* can promote T cell responses, but only if they do not prevent the DC from ultimately reaching the LN where T cell priming typically occurs.

The immunopotentiators

When vaccinologists moved from whole cell vaccines (live, attenuated or dead pathogens) to recombinant subunit vaccines for reasons of safety and manufacturing, they learned that these vaccines typically evoked weaker immunity and protection. The discovery of how our body senses pathogens via a family of highly conserved pattern recognition receptors (PRR) called Toll-like receptors (TLR) heralded the era of the specific receptor-mediated activation of innate immunity (113–115). Since then, other innate immune receptors have

been discovered including NOD-like receptors (NLR), C-type lectin receptors and retinoic acid inducible gene (RIG)-I-like receptors (RLR) and most recently cyclic GMP-AMP synthase (cGAS). Within the past few decades, numerous adjuvants have been developed to target these innate receptors. Signaling mechanisms of these receptors have been thoroughly discussed elsewhere (116–119); here we focus on the adjuvants that target these receptors, in particular those that have entered clinical trials of cancer vaccines.

Adjuvants targeting Toll-like receptors

TLR2 agonists

TLR2 is expressed on the surface of different immune cells like DC, macrophages and lymphocytes and recognizes bacterial lipopeptides. Upon engaging its ligands, TLR2 activates NF- κ B via the MYD88 signaling pathway. There are two common strategies to engage TLR-2 through vaccines: conjugating the antigen to bacterial lipopeptides or to palmitic acid. Bacterial lipopeptide MALP-2 and its synthetic analogues like Pam₂Cys and Pam₃Cys are most frequently used. The peptide-lipopeptide construct were shown to induce DC maturation, pro-inflammatory cytokine (IL-12, TNF- α , IFN- γ) secretion, B cell activation and enhanced CTL responses (120). Most current clinical trials of TLR-2 based adjuvants are for vaccination against infectious diseases such as HIV, HBV and Lyme disease. In 2014, vaccine using TLR-2 ligand (Pam₃CSK₄) conjugated with long synthetic peptide showed very promising results in a preclinical melanoma model (121). Interestingly, Pam₃CSK₄-peptide conjugated (but not the mixture of) Pam₃CSK₄ with peptide, induced robust T cell response and protection against tumor. This is in line with the cis-activation model showed by Desch *et al* (122), which essentially posits that signal 1 and 2 should be delivered by same APC for optimal T cell

priming.

TLR3 agonists

TLR3 is expressed in the endosomal compartment of conventional dendritic cells (cDC), macrophages and on the surface membrane of non-immune cells like epithelial cells (123). TLR3 is activated by double-stranded RNA or its synthetic analog polyinosine-polycytidylic acid (poly I:C) (124). TLR3 does not use the MyD88 signaling pathway but triggers TRIF signaling leading to activation of NF- κ B, MAP kinases and IRF3, which in turn induce the production of inflammatory cytokines, type 1 interferons (IFNs) and the subsequent upregulation of costimulatory molecules (125).

Poly I:C can enhance antigen cross-presentation by DC to CD8 T cells. Because of its rapid degradation by serum nucleases in primates, poly I:C has limited anti-tumor efficacy in humans (120). More stable derivatives of poly I:C were made to overcome its limitation, including poly ICLC (known as Hiltonol) and poly I:C₁₂U (126). In a phase 1 ovarian cancer trial, addition of poly ICLC to a vaccine consisting of NY-ESO1 long overlapping peptides in IFA dramatically induced rapid and efficient CD4 and CD8 T cell responses, compared to the vaccine alone (127). A recent study in monkeys showed that poly ICLC in combination with agonistic CD40 antibody significantly enhanced both CD4 and CD8 responses compared to either adjuvant alone (128). This is some of the first primate data confirming the multitude of mouse studies that indicated strong synergy when different classes of immunopotentiators are used together in vaccine adjuvants (88, 125, 126). I:C₁₂U and poly ICLC have entered clinical trials for other cancer including glioma, melanoma, carcinoma (poly ICLC) and HER-2 positive breast cancer (120).

TLR4 agonists

TLR4 is expressed on the surface of immune cells including cDC and macrophages as well as non-immune cells such as fibroblasts and epithelial cells. Triggering TLR4 will activate both MyD88 and TRIF dependent pathways leading to NF- κ B and IRF3/7 activation. TLR4 activation strongly promotes Th1 response through IL-12p70 induction (131). Due to its high toxicity, LPS has been replaced by the less toxic derivative, monophosphoryl lipid A (MPL), as vaccine adjuvant. The adjuvanticity of MPL has been studied extensively in several clinical trials (120). MPL is used in combination with aluminum (AS04) to skew the typical Th2 response induced by alum to a Th1 response (132). MPL as a vaccine adjuvant, in combination with tumor antigens, has entered into several clinical trials for melanoma, lung, and prostate cancer (133–135).

TLR7/8 agonists

Localizing within the endosomal compartments, both TLR7 and 8 can recognize single stranded (ss) RNA as they are structurally related (123). In human, TLR7 is predominately expressed in plasmacytoid dendritic cells (pDC) and to a lesser extent in B cells and monocytes/macrophages while TLR8 is mainly expressed in monocytes/macrophages and cDC (136). TLR7/8 signal through the MyD88 pathway, leading to upregulation of co-stimulatory molecules (CD80/86, CD40), production of cytokines (IFN- α , TNF- α , IL-12) and migration of DC from skin to lymph nodes. TLR8 (but not TLR7) is expressed on BDCA3⁺ cDC subset which is most potently responsible for cross-priming of CD8⁺ T cells (137). Therefore, preferential TLR7 agonists may exert weaker adjuvant activity than TLR8 or TLR7/8 agonists when used in CD8⁺ T cell-inducing vaccines. TLR7/8 can also activate B cells to produce antibody and cytokines such as IL-6 and TNF- α , and T cells to proliferate and produce cytokines including IFN- γ and IL-2 (138, 139). TLR7/8 can be activated by synthetic imidazoquinolines including imiquimod

(mostly acts on TLR7) and resiquimod (TLR7 and 8). Imiquimod (Aldara cream) has been approved to treat basal cell carcinoma and genital warts (140, 141). Several clinical trials of imiquimod as vaccine adjuvant in different cancers including chronic myeloid leukemia (CML), vulval intraepithelial neoplasia (VIN), prostate cancer and melanoma have been conducted (142–145). Overall, all vaccines induced both humoral and cellular responses in a major fraction of patients. In vaccinated patients with VIN, infiltration of both CD4 and CD8 T cells into tumor sites was shown to correlate with tumor clearance (143).

TLR9 agonists

TLR9 is expressed by human B cells and pDC and localizes in endo-lysosomal compartment (123). Its role is to detect unmethylated CpG motifs which are often found in bacterial, but not host cell DNA. Upon activation, TLR9 induces production of pro-inflammatory and Th1 cytokines (such as IL-12) by APC. There are 3 classes of synthetic CpG oligonucleotides (ODN) being used in preclinical and clinical studies. CpG A is a mix of phosphodiester/phosphorothioate backbone with palindromic sequences and poly G tail, a potent pDC activator and IFN- α inducer (146). CpG B only has phosphorothioate backbone. CpG B strongly activates B cells and promotes pDC and monocyte maturation (147). CpG C is a hybrid of the two above (148). CpG has been used in clinical trials of therapeutic cancer vaccines against melanoma, breast/lung/ovarian cancers, sarcoma and glioblastoma (149–153). Overall, the vaccines induced both humoral and cellular responses, but clinical benefit remained uncommon.

STING agonist

TLR-independent antiviral responses (i.e. type 1 interferon induction) were shown to be induced by double stranded (ds) DNA in the cytosol (154). Later, dsDNA was found to activate the transcription factor NF- κ B and IRF3 via an endoplasmic reticulum adaptor called STING (stimulator of interferon genes) (155). Subsequently, the receptor for cytosolic DNA, the cyclic GMP-AMP synthase or cGAS, was discovered (156). Upon binding to cytosolic DNA, cGAS catalyzes the synthesis of cGAMP which in turn binds to and activates the adaptor protein STING. Recent results indicate that spontaneous T cell priming against tumor antigen requires STING-dependent type I IFN induction (157). Promising results from preclinical studies with STING agonists injected directly into tumors in the aggressive B16 melanoma model led to high excitement for their application in clinical trials (158). Recent results also indicate that STING agonists can function as adjuvant in a setting of whole-cell tumor cell vaccine (159). It will be interesting to learn how STING agonists perform in animal models and clinical trials as compared with other TLR agonists as adjuvants for peptide vaccines, and whether their combination offers additional benefit, given their different intracellular signaling pathways.

Cytokines as adjuvants

IL-2

Among cytokines extensively used for immunotherapy, IL-2 was initially described as a T cell growth factor (TCGF) responsible for the clonal expansion, differentiation and survival of T cells (160), and later of activated B cells and natural killer (NK) cells (161, 162). Although CD4 T cells are the major source of IL-2 *in vivo*, CD8 T cells, NK cells and DC can also produce IL-2 (163–166). IL-2 was FDA-approved for the therapy of metastatic renal cell carcinoma in 1992 and metastatic melanoma in 1998 (17, 167). IL-2 mediates anti-tumor activity by activating

tumor-specific T cells and NK cells. In mice, addition of IL-2 to experimental cancer vaccines can greatly increase the therapeutic efficacy (93, 129). IL-15 signals through the same IL-2 R $\beta\gamma$ complex also used by IL-2, and can promote peptide-induced T cell proliferation, especially in T cells with low-affinity TCRs (168). In patients with melanoma, addition of an experimental gp100 peptide/IFA vaccine to IL-2 gave a higher clinical response rate than observed in patients receiving IL-2 alone, and also higher than previously observed for gp100 peptide vaccine alone, suggesting IL-2 can function as a vaccine adjuvant in humans (169). However, IL-2 can expand immunosuppressive regulatory T cells (Treg) which may dampen the immune response or anti-tumor efficacy (170). Because Treg express both IL-2R α and IL-2R $\beta\gamma$ while CTL express only the latter, blocking IL-2R α when using IL-2 preferentially expands CTL (171). Recently, a mutant form of IL-2 (IL-2 mutein) was reported to have higher antitumor efficacy with reduced proliferation induction on Treg, possibly due to preferential binding to IL-2R $\beta\gamma$ but not IL-2R α (172). Similarly, IL-2 pre-complexed with IL-2-specific antibodies, and IL-2 covalently modified with polyethylene glycol have shown selective binding to IL-2R $\beta\gamma$ but not IL-2R α , favoring selective effects on CD8⁺ T cells (173, 174). If these modifications can lower the toxicity of IL-2, which may be partly mediated by IL-2R α , these IL-2-based compounds may make a comeback in cancer immunotherapy, including as vaccine adjuvants (171).

Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF has been used as a cancer vaccine adjuvant with limited success. GM-CSF can be produced by many cell types including myeloid cells, lymphocytes, fibroblast, endothelial/epithelial/mesothelial cells and certain tumor cells (175). The production of GM-CSF is induced by bacterial toxin and inflammatory cytokines such as IL-1, IL-6, and TNF α (176).

GM-CSF receptor is found on myeloid cells and non-hematopoietic cells such as endothelial cells. In vaccine settings, GM-CSF has been shown to initiate the recruitment and maturation of DC as well as activation of macrophages, neutrophils, and NK cells, indicating that it is a potential vaccine adjuvant (177, 178). Combination of GVAX (irradiated tumor cell expressing GM-CSF) and CTLA-4/PD-1 was very promising in preclinical studies, especially in combination with anti-CTLA-4 and anti-PD-1 checkpoint blockade, leading to the first clinical trials of checkpoint blockade in patients with cancer. Recombinant GM-CSF has been used in peptide vaccine trials, where it has had varying success in raising T cell responses. This may be partially due to a balance between pro- and anti-inflammatory properties of GM-CSF depending on its dose (111). In addition, there appear to be complex interactions between GM-CSF and other factors in the tumor-conditioned microenvironment that influence its ability to either enhance or reduce vaccine-induced T cell responses (179–182). Several positive peptide/protein vaccine trials have incorporated GM-CSF (96, 183). However due to the lack of a vaccine arm without GM-CSF, its exact impact on clinical outcome remains unknown (184, 185).

Interferons (IFNs)

IFNs are of great interest for adjuvant development, owing to their pleiotropic effect on different immune cells such as DC, B cells and T cells as well as non-immune cells. IFN- α and IFN- β promote DC maturation, including the up-regulation of MHC and costimulatory molecules. In virus-infected cells, type I IFNs prevent virus replication by halting transcriptional and translational machineries, accelerating RNA degradation by inducing RNase L and inducing apoptosis (186). IFN- α and pegylated IFN- α have been approved for advanced renal cell carcinoma and chronic hepatitis C treatment, respectively, and both are given after surgical

resection of primary melanoma to reduce the chance of recurrence (187). Preclinical studies showed direct adjuvant efficacy of type I IFN in a peptide-based anti-melanoma vaccine, where it promoted T cell numbers, longevity and effector function, resulting in improved tumor control (82). In contrast to type I IFN, IFN- γ (the sole type II IFN) is typically only produced by specialized immune cells including T cells, NK cells and NKT cells (188). Recombinant IFN- γ (or genetically engineered IFN- γ 1b) is approved to treat chronic granulomatous disease (189). In cancer immunotherapy, a phase III clinical trial combining chemotherapy with IFN- γ for patients with advanced ovarian and peritoneal carcinomas was terminated due to serious adverse effects (190).

CD40 agonist antibody

CD40 costimulatory receptor belongs to tumor necrosis factor receptor (TNFR) superfamily. It is expressed on B cells, DC, monocytes, macrophages as well as non-hematopoietic cells such as fibroblast, epithelial and endothelial cells (80). The engagement of CD40 to CD40L leading to the recruitment of adapter proteins called TNFR-associated factors (TRAF). TRAF in turn activates multiple signaling pathways including NF κ B, MAPK, PI3K and PLC γ signaling pathways. On B cells, CD40 activation induces antibody (Ab) isotype class switching, somatic hyper mutation (which leads to Ab affinity maturation) and memory differentiation (191, 192). Collectively, these effects of CD40 activation on B cells are responsible for T cell (or thymus) dependent humoral response because the ligand of CD40, CD40L, is primarily expressed on activated T cells. On DC, CD40 activation not only upregulates CD70 and CD80/CD86 which are ligands for CD27 and CD28 receptors, respectively, on T cells

but also increases antigen-MHC complex stability and DC life span (193–196). Accordingly, CD40-CD40L signaling plays a critical role in cellular response via activating DC.

III. L-tyrosine as a potential adjuvant for peptide based vaccine

Our lab previously showed that for peptide based vaccines, the choice of antigen delivery system can affect the ensuing anti-tumor immune response (93). Specifically, long-lived water-in-oil emulsions of incomplete Freund's adjuvant (IFA), which greatly prolongs Ag presentation time, diminished immune responses when used as adjuvant for short antigenic peptides. Specifically, T cells became sequestered at the persisting, antigen-rich vaccination site, where they underwent apoptosis without reaching the tumor. While a very short-lived, water-based formulation showed no T cell sequestration and consequently improved anti-tumor activity, it is possible that it was actually cleared too quickly to allow maximal T cell priming. If this is true, Ag delivery systems may be designed to extend Ag presentation time long enough for optimal CTL response, but not long enough to induce sequestration at the vaccination site.

L-tyrosine is an amino acid with poor water-solubility and the capacity to entrap grass and tree allergens during pH change-induced flash-precipitation which was first described as an immunological adjuvant for allergy vaccine in 1982 (197). Specifically, L-tyrosine was shown to have a short-term depot effect when formulated with grass pollen. Such property of L-tyrosine makes it an interesting candidate for our quest of an adjuvant, specifically Ag delivery system, which has an intermediate Ag presentation: longer than saline but shorter than IFA. One additional advantage of L-tyrosine is that it has been used clinically in treating pollen allergy and its safety profile has been well documented (198). However, there is no report on

the ability of L-Tyrosine to function as a vaccine adjuvant for short peptides to induce antigen specific T (particularly CD8+) cell responses.

To compare the potency of L-tyrosine (particulate) versus saline (aqueous) versus IFA (water-in-oil based), in terms of CD8 T cell response, I chose hgp100₂₅₋₃₃ peptide (KVPRNQDWL) (here after referred to as gp100) and the pmel-1 T cell model. Briefly, C57BL/6 (here after referred to as B6) mice were adoptively transferred with naïve pmel-1 T cells expressing a gp100-specific TCR and the congenic Thy1.1 marker which serves to distinguish the pmel-1 T cells from Thy1.2 expressing host lymphocytes. These mice were subsequently immunized with gp100 peptide in different formulations (i.e. L-tyrosine, saline, and IFA) plus covax. Covax is a molecularly defined adjuvant consisting of agonistic anti-CD40 antibody, the Toll-Like Receptor (TLR)7 agonist, imiquimod, and IL-2 which together dramatically improve vaccination-induced CD8+ T cell responses as previously demonstrated by our group (93). The level of gp100 specific T cell responses (% of total CD8 T cells) in the peripheral blood were followed based on their Thy1.1 marker (figure 6A). Their function was assessed basing on the capability of producing IFN- γ and granzyme B. Vaccination with gp100/L-tyrosine induced superior T cell numbers in peripheral blood compared to the equivalent dose (50 μ g/mouse) of gp100 in saline and IFA formulations (figure 6B). Functionally, pmel-1 T cells induced by gp100/L-tyrosine produced comparable IFN- γ to gp100/saline but higher than gp100/IFA (figure 6C). However, granzyme B production by pmel-1 T cells after L-tyrosine vaccine was the lowest, compared to saline and IFA groups (figure 6D). At late memory phase (day 300 post vaccination) pmel-1 T cells were detected in mice immunized with peptide in saline and L-tyrosine but not IFA formulation (figure 7A). Although gp100 specific T cell levels of L-tyrosine vaccinated mice were higher than that of saline group, T cells of both groups displayed similar

central memory phenotype (CD27⁺CD62L⁺) (figure 7B). Since all amino acids (except glycine) have L- and D- forms, I sought to determine whether peptide formulated in different optical isomers (i.e. L- and D- forms) of tyrosine induced different T cell responses. I found L- and D- tyrosine induced very similar gp100 specific T cell responses, suggesting the ability of tyrosine to form microparticle was more important for its vaccine adjuvant activity than its possible pharmacological activity (Figure 8). Although different allergens formulated with L-tyrosine have been shown to induce antibody production, it was unclear whether short peptide formulated with L-tyrosine could induce antigen specific T (particularly CD8⁺) cell responses. I next compared CD8⁺ T cell responses after vaccination with L-tyrosine and saline, in the presence or absence of covax, to determine the potency of L-tyrosine if used as single adjuvant without covax. I found that short peptide gp100 formulated with L-tyrosine without covax induced a very modest but detectable T cell response, indicating the importance of covax as part of the peptide vaccination regimen (figure 9).

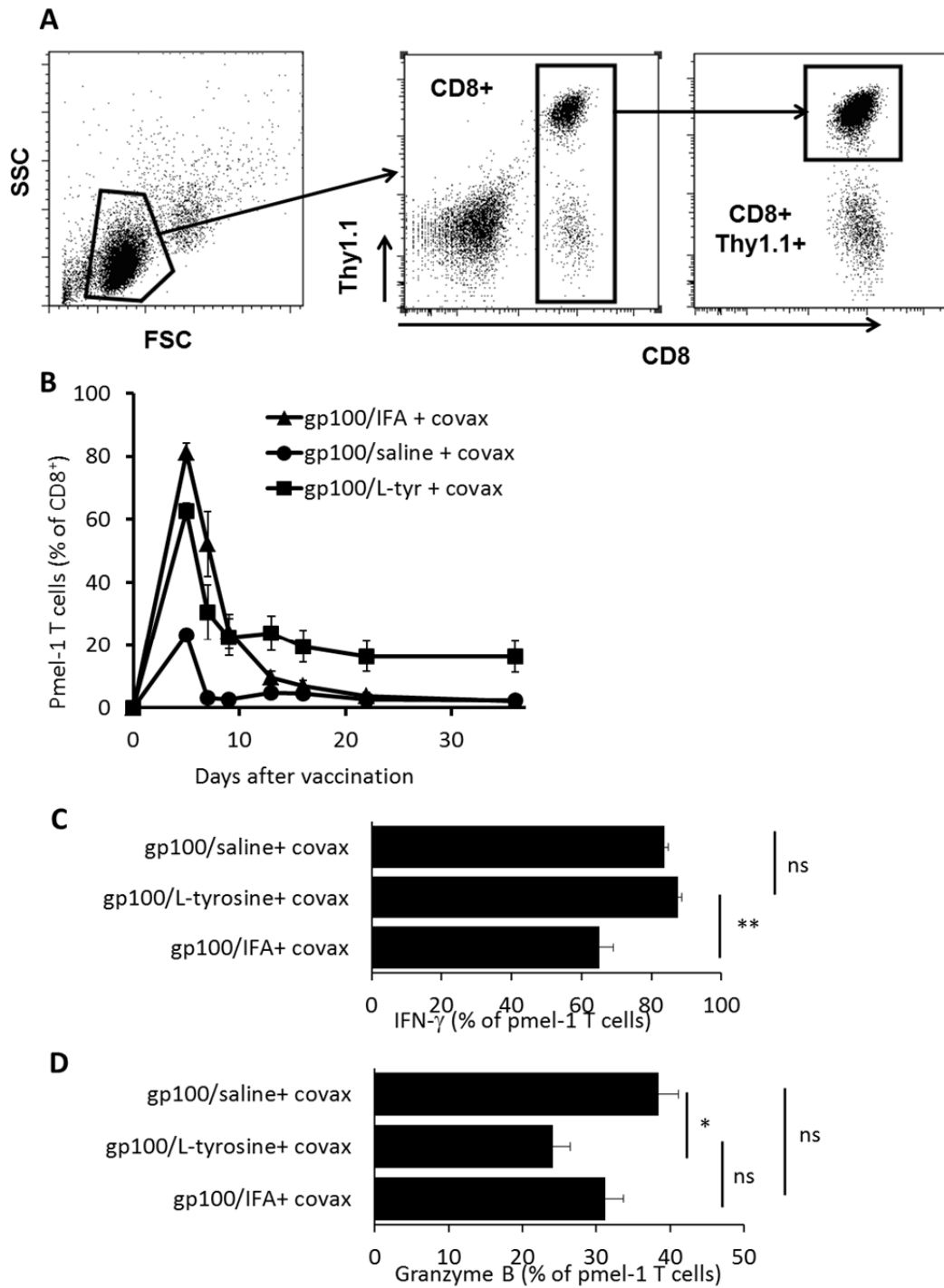


Figure 6. L-tyrosine is a potent vaccine adjuvant for the induction of CD8⁺ T cell responses. A) Gating strategy for gp100 specific T cell (pmel-1) detection in peripheral blood: cells from lymphocyte population basing their forward and side scatter pattern (FSC and SSC, respectively) were subjected to subsequent analysis with CD8 and Thy1.1 markers. Pmel-1 T

cells were positive for both CD8 and Thy1.1. B) Pmel-1 T cell level as a percentage of CD8 + T cells in the blood of mice at different time points. C) IFN- γ and D) granzyme B production by pmel-1 T cells. Mice received 8×10^5 pmel-1 T cells and indicated treatments on day 0. Peptide dose was 50 μ g per mouse. Data are shown as the mean \pm s.e.m. Statistical differences between the two groups were determined by student t-test. n = 5 mice per group per experiment. Data are representative of 3 independent experiments. *: P < 0.05, **: P < 0.01, ns: not significant.

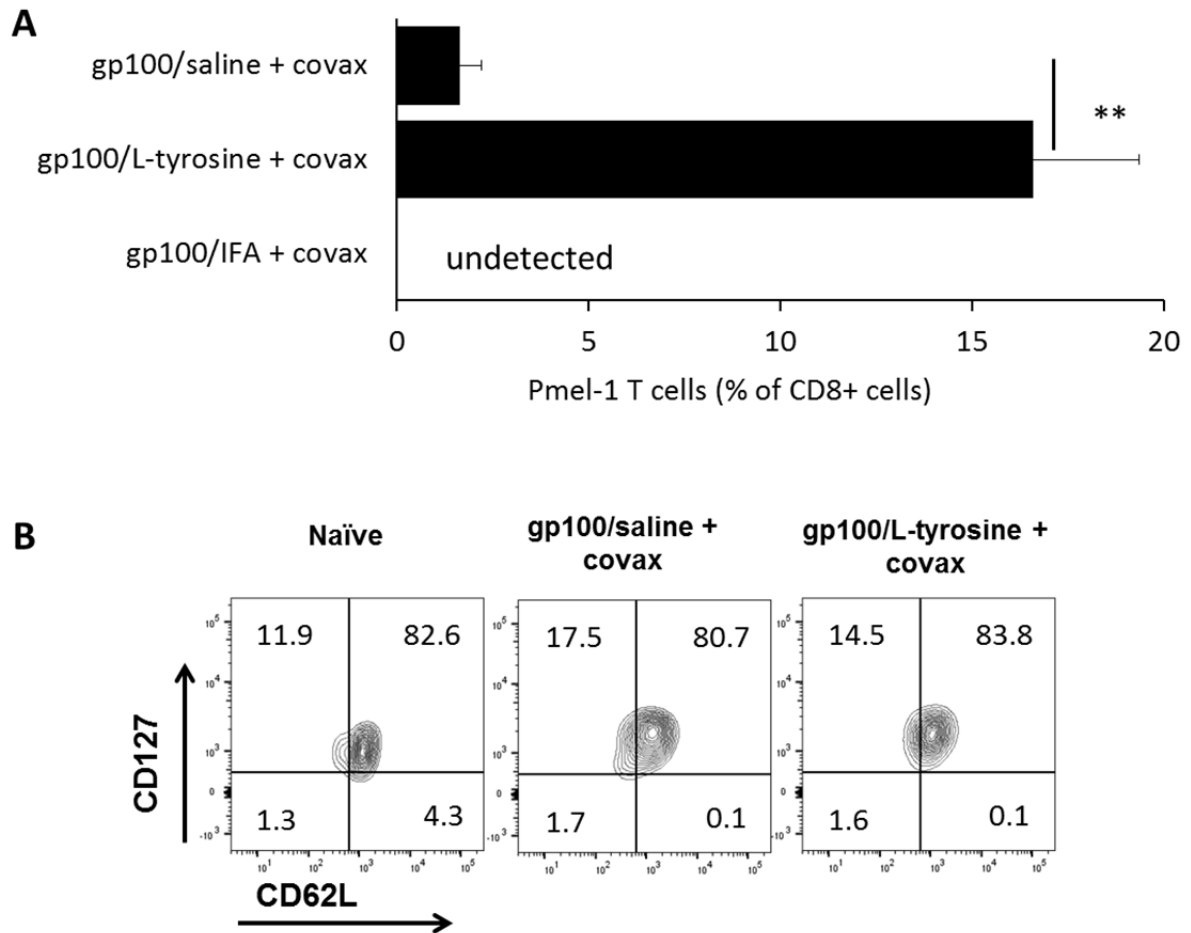


Figure 7. Memory phenotypes of pmel-1 T cells in late memory phase. A) Pmel-1 T cell level in peripheral blood on day 300 post vaccination and B) their memory phenotypes. Naïve: CD8 T cell from naïve pmel-1 mouse. Mice received 8×10^5 pmel-1 T cells and indicated treatments on day 0. Data are shown as the mean \pm s.e.m. Statistical differences between the two groups were determined by student t-test. $n = 5$ mice per group per experiment. Data are representative of 2 independent experiments. *: $P < 0.05$, **: $P < 0.01$, ns: not significant.

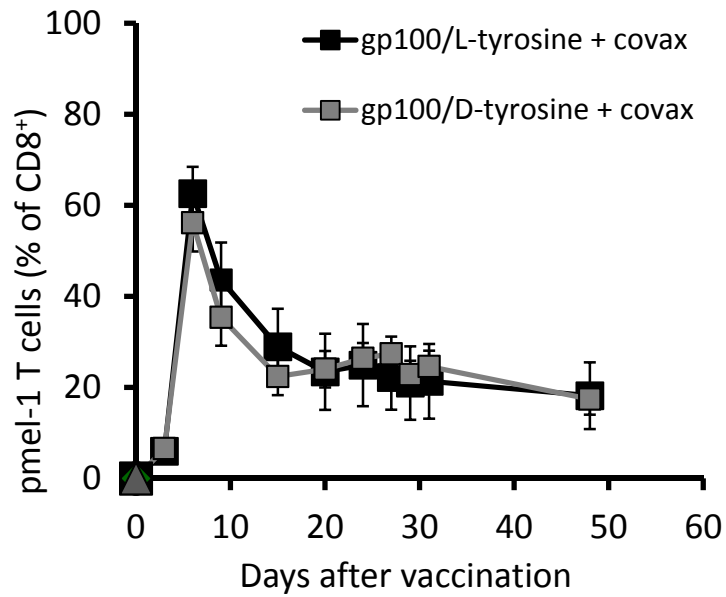


Figure 8. L- and D-tyrosine induced similar T cell responses. Mice received 8×10^5 pmel-1 T cells and indicated treatments on day 0. Peptide dose was 50 μg per mouse. Data are shown as the mean \pm s.e.m. Statistical differences between the two groups were determined by student t-test. $n = 5$ mice per group per experiment. Data are representative of 2 independent experiments. *: $P < 0.05$, **: $P < 0.01$, ns: not significant.

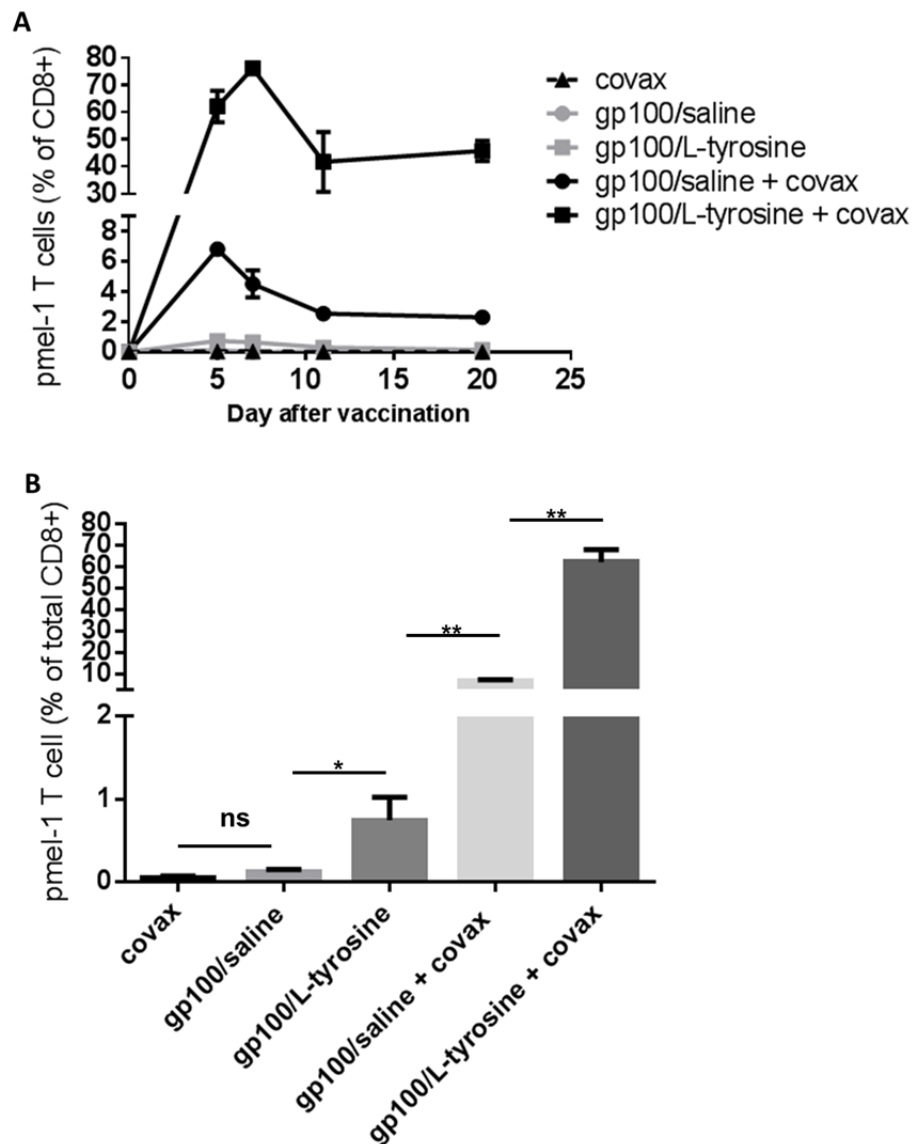


Figure 9. Peptide formulated with L-tyrosine without induced modest but detectable T cell response. A) Dynamics of gp100 specific T cell responses (pmel-1) in peripheral blood after different vaccines and B) pmel-1 T cell number in peripheral blood on day 5 post vaccination of the same experiment. Mice received 8×10^5 pmel-1 T cells and indicated treatments on day 0. Data are shown as the mean \pm s.e.m. Statistical differences between the two groups were determined by student t-test. n = 5 mice per group per experiment. Representative data from 2 independent experiments. *: P < 0.05, **: P < 0.01, ns: not significant.

CHAPTER 2 – OBJECTIVES, HYPOTHESIS AND SPECIFIC AIMS OF CURRENT WORK

The main objective of my dissertation project is to dissect the mechanism(s) of action of L-tyrosine as an adjuvant for **peptide based vaccine**. I hypothesized that L-tyrosine enhances antigen specific CD8 T cell response by acting as an **immunopotentiator** and/or **antigen delivery system**. To address this hypothesis, I propose 2 specific aims.

Aim 1. Determine the immunostimulating properties of L-tyrosine.

I hypothesize that L-tyrosine can activate the innate immune system. To detect the activation of the innate immune system in our vaccine model, I will measure the induction of different cytokines and chemokines as well as the recruitment of different cell types to vaccination site. Furthermore, I will investigate the contribution of each of these events to the enhanced T cell responses after L-tyrosine vaccine.

Aim 2. Determine antigen presentation time of peptide in formulation with L-tyrosine.

I hypothesize that L-tyrosine can extend the antigen presentation duration. To address this hypothesis, I will use antigen specific T cells to detect the persistence of antigen.

CHAPTER 3 – THE IMMUNOSTIMULATING PROPERTIES OF L-TYROSINE

I. Background

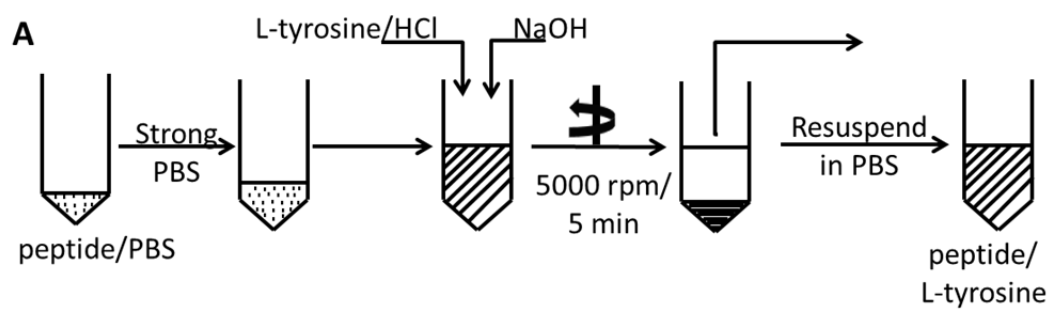
The use of L-tyrosine as vaccine adjuvant has been reported by a number of publications (198–200). In formulation with different allergens (including pollen and dust mite (*D. pteronyssinus*)) as well as ovalbumin protein, L-tyrosine induces antibody (IgG but not IgE) production, making it suitable for allergy desensitization. The humoral response induced by allergens/L-tyrosine suggests L-tyrosine can act as an immunostimulant. Our preliminary data (figure 6 and 7) showed that gp100 peptide formulated with L-tyrosine could also induce a robust cellular response in provision with covax. In the absence of covax, L-tyrosine induced modest but detectable cellular response (figure 9). Such observations underscore the need for better understanding the immunostimulating role of L-tyrosine, particularly in the context of peptide based vaccine, which has not been documented. Accordingly, in this chapter, I will characterize immunostimulating properties of L-tyrosine. First, I will characterize cell types recruited to vaccination site and their activation state. Second, cytokines/chemokines induction by L-tyrosine will be investigated. Finally, I will seek to determine the impact of changes induced by L-tyrosine to cellular response in peptide based vaccine settings.

II. Results

1. Preparation and quantification of peptide in L-tyrosine

The preparation of peptides with L-tyrosine was adapted from the protocol for pollen allergens as previously reported (199). Details of the adapted protocol were described in section of experimental procedures. The formulation of peptide with L-tyrosine is depicted in figure 10A. Size of L-tyrosine microparticles was shown in figure 10B. Mass spectrometry

analysis (MS/MS) was used to quantify the retention rate of different peptides in L-tyrosine to determine if different peptide will behave differently in formulation with L-tyrosine. While gp100₂₅₋₃₃ (KVPRNQDWL) (here after referred to as gp100), OVA₂₅₇₋₂₆₄ (SIINFEKL) (referred to as OVA-I) and P15E₆₀₄₋₆₁₁ (KSPWFTTL) peptides show similar in cooperation rates with L-tyrosine particles, sTRP2₁₈₀₋₁₈₈ (SVYDFVWL) stands out with nearly 100 percent of initial input amount retained within L-tyrosine particles (table 1). I speculated that the adsorption to L-tyrosine correlates with the hydrophobicity of the peptide, indicated by GRAVY (grand average hydropathicity) score (table 2). GRAVY score of each peptide was calculated based on their amino acid sequences. Peptides with more positive score are considered more hydrophobic and *vice versa*. Further studies including more peptides are needed to fully elucidate the pattern of peptide adsorption rate to L-tyrosine microcrystals.



B



Figure 10. Preparation of L-tyrosine microparticles. A) Schematic of formulating peptide with L-tyrosine. (B) L-tyrosine microparticles under microscope with a scale bar.

| Table 1: Percentage of peptide trapped by L-tyrosine particles after preparation process. | | | | |
|---|---------------------------------------|--------------------------------------|---|--|
| | gp100 ₂₅₋₃₃ (KVPRNQDWL) | OVA ₂₅₇₋₂₆₄ (SIINFEKL) | sTRP2 ₁₈₀₋₁₈₈ (SVYDFFVWL) | P15E ₆₀₄₋₆₁₁ (KSPWF TTL) |
| Amount of peptide trapped from initial input (mean \pm s.e.m) | 24.0 \pm 3.6% | 25.0 \pm 2.1% | 99.0 \pm 1.0 % | 34.6 \pm 2.2 % |

| Table 2: Hydrophobicity of different peptides as depicted by GRAVY (grand average of hydropathicity) score. | | | | |
|---|---------------------------------------|--------------------------------------|---|--|
| | gp100 ₂₅₋₃₃ (KVPRNQDWL) | OVA ₂₅₇₋₂₆₄ (SIINFEKL) | sTRP2 ₁₈₀₋₁₈₈ (SVYDFFVWL) | P15E ₆₀₄₋₆₁₁ (KSPWF TTL) |
| GRAVY SCORE (the more positive the more hydrophobic) | -1.4889 | 0.4875 | 1.2556 | -0.2500 |

2. L-tyrosine-based vaccination induces inflammatory reaction at vaccination site but not the induction of co-stimulatory molecules on dendritic cells.

Successful vaccination requires the induction of local inflammation, resulting in recruitment and/or activation of antigen presenting cells (APC) such as dendritic cells (DC). I hypothesized that L-tyrosine recruits APC and induces inflammatory cytokines and chemokines at vaccination sites and vaccine draining lymph nodes. To address this hypothesis I characterized chemokine induction and leukocyte recruitment at the cutaneous vaccination site. L-tyrosine microparticles did not induce DC to upregulate co-stimulatory molecules including CD86 and CD40 (figure 11A and 11B) but triggered a massive infiltration of neutrophils to the vaccination site (figure 11C). Consistent with the neutrophil recruitment, inflammatory cytokines and chemokines including G-CSF, IL-6, CXCL-1 (MIP-2), CCL3 (MIP-1 α) and CCL4 (MIP-1 β) were found elevated at injection site after L-tyrosine vaccine (figure 12). In fact, these cytokines and chemokines have been shown to positively regulate the differentiation, activation and mobilization of neutrophil (201–205). To determine whether the recruitment of neutrophils to the vaccination site was important for CD8 T cell priming by L-tyrosine formulated vaccine, I depleted neutrophils and measured pmel-1 T cell levels in peripheral blood. Ly6G depleting antibody (clone 1A8) was chosen over Gr-1 because Gr-1 depleting antibody targets both Ly6G and Ly6C which potentially eliminate not only neutrophils but monocytes, DC and activated lymphocytes (206). I found no difference between Ly6G-depleted and control groups (figure 13A), suggesting that the particulate nature of L-tyrosine vaccine triggers the influx of phagocytes, which however do not participate in the enhanced T cell priming. The efficiency of Ly6G depletion was confirmed in the blood and skin (figure 13B and 13C, respectively).

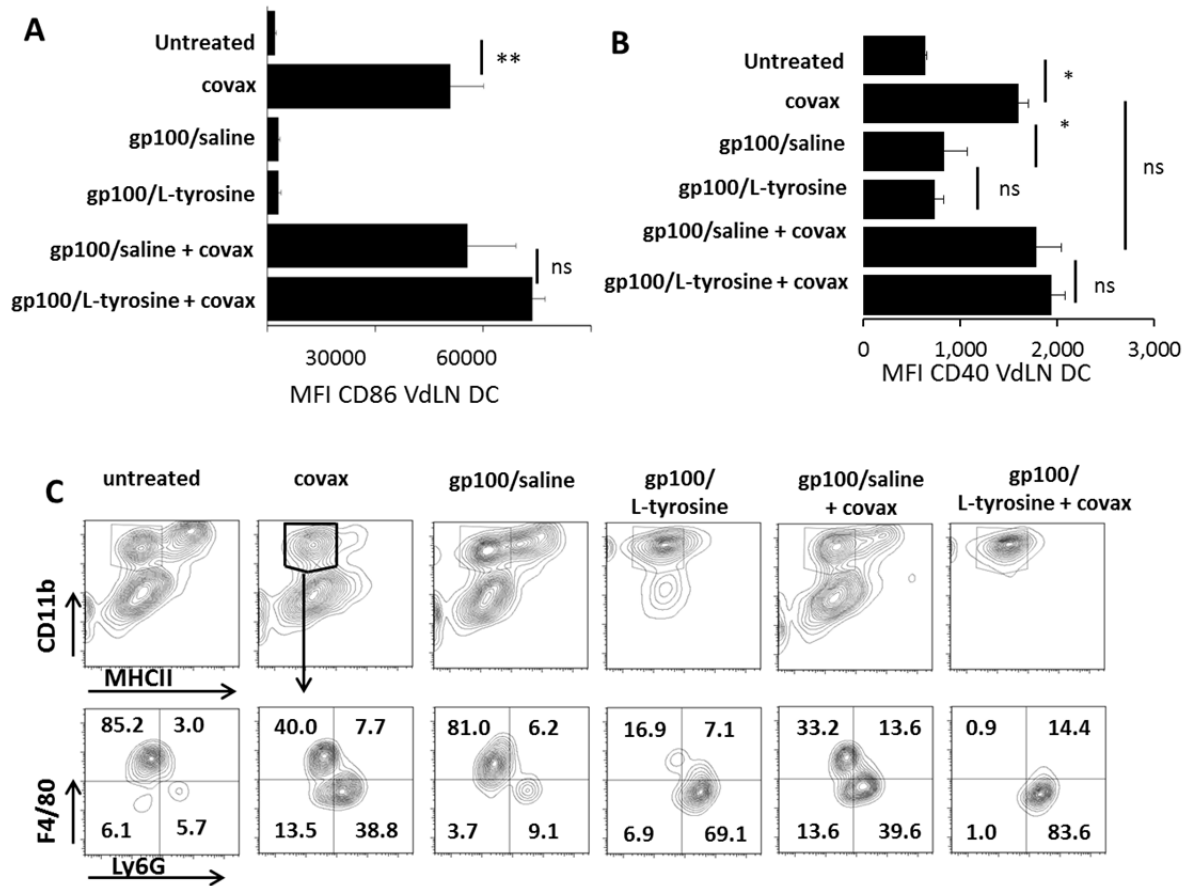


Figure 11. Local inflammatory responses to L-tyrosine-based vaccination. L-tyrosine did not induced CD86 and CD40 upregulation on dendritic cells at VdLN (A and B) but massive neutrophil (Lin⁻,CD11b⁺,MHCII⁻,F4/80⁻, Ly6G⁺) infiltration to vaccination site (C). Statistical differences between the two groups were determined by the unpaired two-tailed t-test. n = 3 - 4 mice per group. Data are representative of 2 independent experiments. *: P < 0.05, **: P < 0.01, ns: not significant. VdLN: vaccine draining lymph node, DC: dendritic cell.

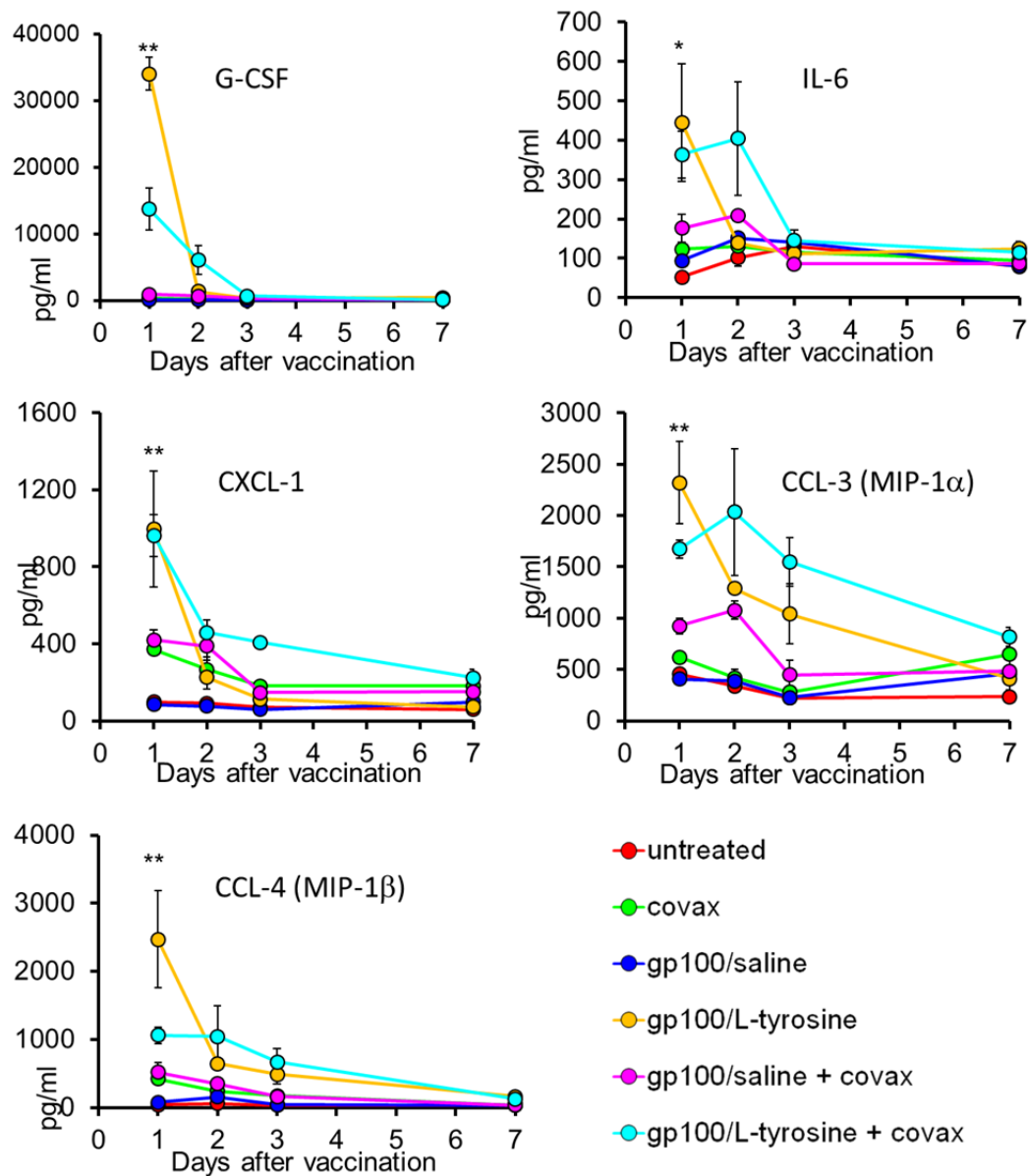


Figure 12 Inflammatory cytokines and chemokines induced by L-tyrosine at vaccination site. Mice were immunized with indicated treatments. At indicated time points, cytokines and chemokines from at vaccination sites (supernatant from homogenized skins) were measured using Luminex® assay. Statistical differences between the groups were determined by Kruskal-Wallis test. $n = 3 - 4$ mice per group. Data are representative of 2 independent experiments. *: $P < 0.05$, **: $P < 0.01$.

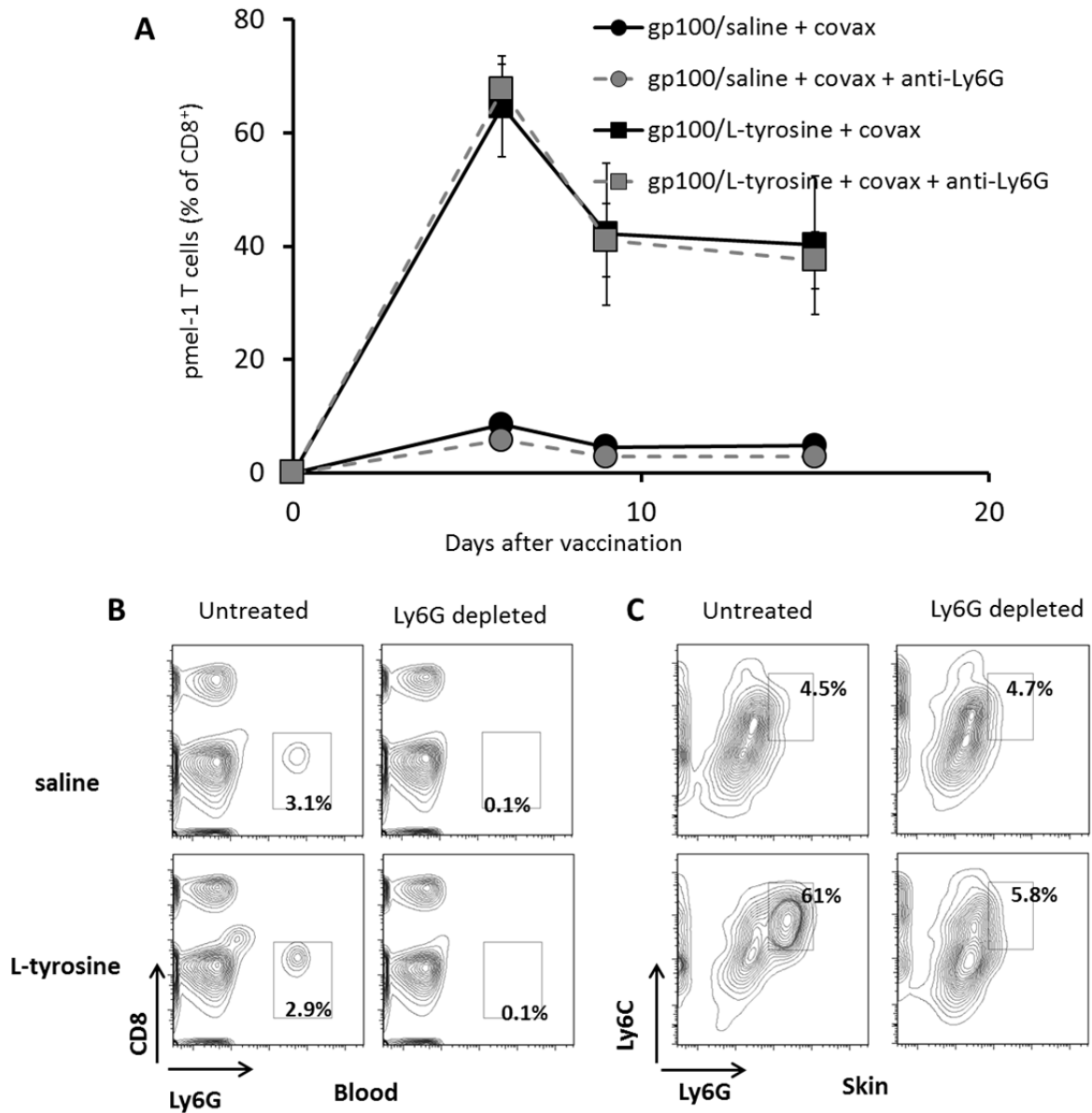


Figure 13. Neutrophils do not contribute to T cell priming after L-tyrosine vaccine. Anti-Ly6G Ab was given 2 days prior to vaccination and every 3 days afterward. Mice received 8×10^5 pmel-1 T cells and indicated treatments on day 0. A) Pmel-1 T cells level in the blood after indicated treatments. The depletion of Ly6G population was confirmed in peripheral blood (B) and the skin at vaccination sites (C) on day 4 after vaccination. $n = 5$ mice/group. Data are representative of 2 independent experiments.

3. Inflammasomes have no appreciable role in L-tyrosine vaccine activity

The induction of inflammatory cytokines and macrophage inflammatory proteins (MIP) such as CXCL-1 (MIP-2) CCL3 (MIP-1 α), CCL4 (MIP-1 β) and particularly the recruitment of neutrophils to vaccination site by L-tyrosine microparticles were strong evidences of local inflammation. Also, certain particulate materials have been documented to exert vaccine adjuvant activity through their activation of inflammasomes (207). Therefore, it was important to determine whether inflammasome activation was responsible for enhanced T cell response after L-tyrosine based vaccine. ASC is an adaptor protein which plays an essential role for the formation of different inflammasome complexes (103). The absence of ASC will prevent the formation and thereby inactivate inflammasome complexes. To determine the contribution of inflammasomes in our vaccine settings, pmel-1 T cell response after saline and L-tyrosine based vaccines in wild-type and ASC knock-out mice were compared. No difference in T cell response among wild-type and genetically inflammasome-deficient ASC-KO mice (figure 14) was found. This result suggests that superior T cell response after L-tyrosine based vaccine was inflammasome independent.

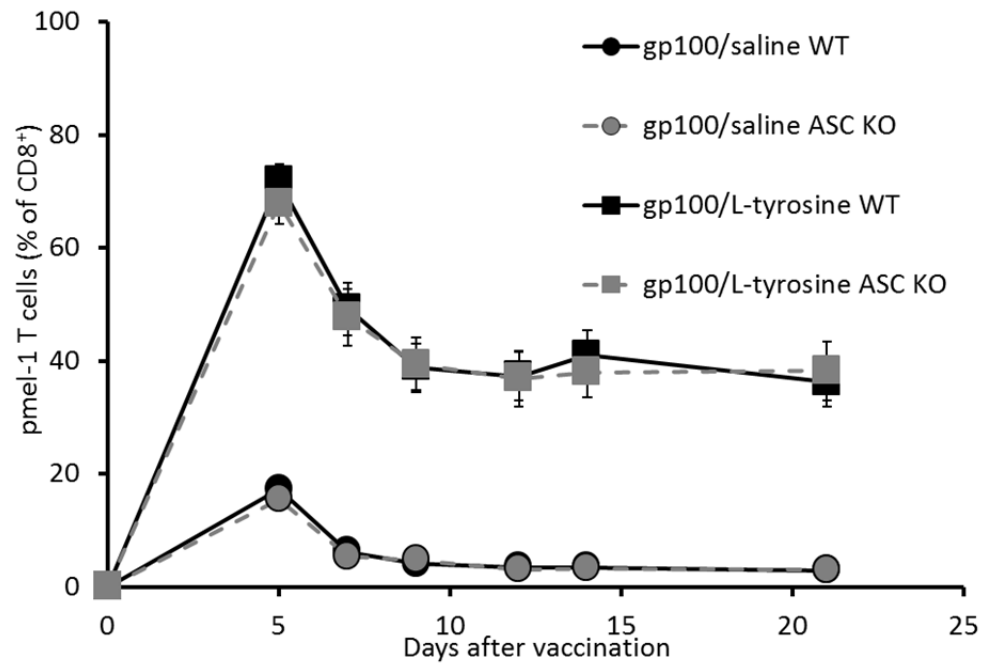


Figure 14. L-tyrosine adjuvant activity is inflammasome independent. No difference in T cell responses between wild-type and ASC KO groups was found, regardless of vaccine formulations. Mice received 8×10^5 pmel-1 T cells and indicated treatments on day 0. $n = 5$ mice per group. Data are representative of 3 independent experiments. WT: wild type, ASC KO: ASC knockout.

4. Gp100 peptide must be co-precipitated with L-tyrosine to induce superior T cell response

Although the mechanism of action of L-tyrosine in our vaccine settings was independent from inflammasome activation, it is crucial to determine the contribution of cytokines and chemokines induced by L-tyrosine, namely G-CSF, IL-6, CXCL-1, CCL3 and CCL4. Naturally, the total (compounded) effect of these cytokines and chemokines to the enhanced T cell response after L-tyrosine based vaccine should be investigated first. Subsequent studies of individual cytokine/chemokine should follow only when the total effect of these cytokines was established. One convenient way to study the total contribution of these cytokines and chemokines induced by L-tyrosine based vaccine was to use peptide in saline mixed with “empty” L-tyrosine particles (depicted in figure 15A). I hypothesized that if L-tyrosine induced cytokines/chemokines plays a major roles in enhanced T cell response, the peptide in saline mixed with empty L-tyrosine particles will give response as peptide in L-tyrosine and higher than peptide in saline. I found T cell response after peptide in saline mixed with L-tyrosine was very similar to that of peptide in saline. I conclude that L-tyrosine-induced inflammatory cytokines/chemokines (as well as neutrophil recruitment) were not contributing factors for superior T cell response (figure 15B). This finding also suggested that peptide needed to be co-precipitated with L-tyrosine, to presumably become entrapped in the microparticles and induce superior T cell response.

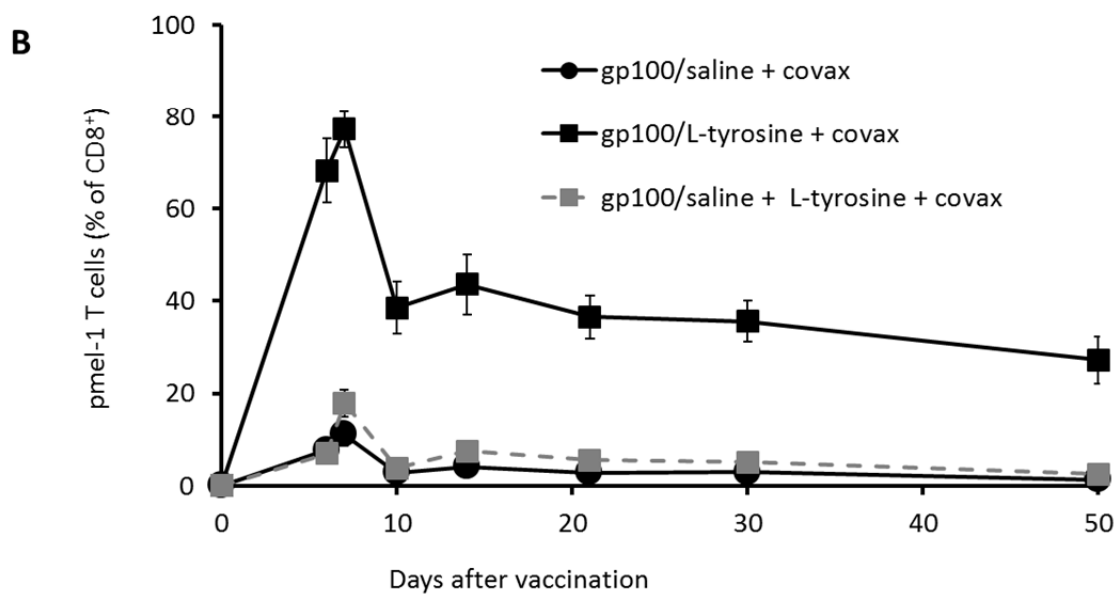
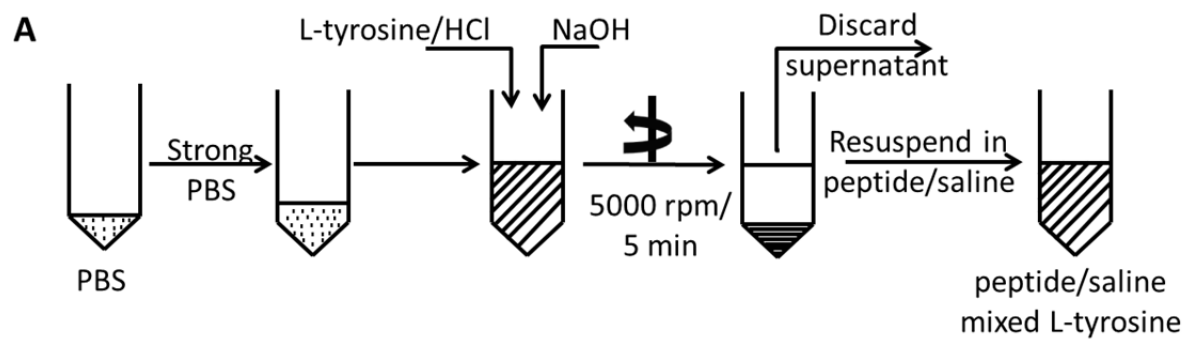


Figure 15. gp100 peptide must to be co-precipitated with L-tyrosine to induce superior CD8 T cell response. A) Schematic of the preparation of gp100/saline mixed with L-tyrosine. B) Pmel-1 T cell number (percentage of CD8 T cells) of after peptide in saline, L-tyrosine and saline mixed with L-tyrosine formulations. Mice received 8×10^5 pmel-1 T cells and indicated treatments on day 0. n= 5 mice per group per experiment. Data are representative of 2 independent experiments.

III. Discussion

Using Luminex® assay and flow cytometry, I found L-tyrosine induced local inflammation at injection site, as evidenced by the induction of G-CSF, IL-6, CXCL-1, CCL3 and CCL4, accompanied by the infiltration of neutrophils. G-CSF is a cytokine that control the mobilization of neutrophils from the bone marrow as well as the differentiation and activation of neutrophils (204, 205). IL-6 is a pro-inflammatory (also considered an anti-inflammatory in certain conditions) cytokine secreted by endothelial cells and immune cells (macrophages, dendritic cells and CD4 lymphocytes) driving the infiltration of neutrophils in acute phase of inflammation (203). CXCL-1, CCL3 and CCL4 are neutrophil chemoattractants (201). However, neither neutrophils nor these cytokines/chemokines were found to have a significant role in superior T cell induction after L-tyrosine based vaccine, as demonstrated by neutrophil depletion and peptide in saline mixed with L-tyrosine microparticles, respectively. Furthermore, enhanced gp100 specific T cell (pmel-1) response did not depend on inflammasome complexes' activation as shown by ASC KO experiment. Taken together, the local inflammation did not have a significant contribution toward dramatic T cell response after L-tyrosine formulation in our vaccine settings (with the provision of covax).

Importantly, although the upregulation of co-stimulatory molecules was not found on DC after L-tyrosine injection (in the absence of covax), the activation of DC cannot be ruled out. In fact, IL-6, CCL3 and CCL4 can be produced by activated DC (205, 206).

CHAPTER 4 – ANTIGEN PRESENTATION TIME OF PEPTIDE IN FORMULATION WITH L-TYROSINE

I. Background

When formulated with pollen, L-tyrosine has been suggested to possess short-term depot effect basing on two observations. First, there was a delayed release of radio labeled antigen (^{125}I -labeled ragweed antigen E) in formulation with L-tyrosine. Second, the half-life of L-tyrosine after subcutaneous injection is approximately 48 hours and that L-tyrosine almost disappeared by day 10 post injection (quantified by ^{14}C -tyrosine) (199). In our peptide based vaccine settings, although L-tyrosine can induce local inflammation at vaccination site, the inflammation does not contribute to remarkable T cell response as demonstrated in previous chapter. Taken together, all observations suggested that the major mechanism of action of L-tyrosine adjuvant in peptide based vaccine is to extend the Ag presentation time.

II. Results

1. L-tyrosine formulation extended the duration of peptide presentation

I hypothesized that L-tyrosine extends the duration of peptide exposure to naïve CD8+ T cells compared to free peptide delivered in saline. To test this hypothesis, I immunized mice with gp100 peptide in different vaccine formulations and gp100 specific (pmel-1) T cells labeled with CFSE to detect the functional availability (i.e. persistence) of the peptide *in vivo*. Method of using Ag specific T cells labeled with CFSE have been previously reported (210, 211). Briefly, mice were immunized at day 0 and received CFSE labeled naïve gp100-specific pmel-1 CD8 T cells at different time points after vaccination. 72 hours after each transfer, adoptive pmel-1 T cells from VdLN were harvested and analyzed for CFSE dilution using flow cytometry. The period of 72 hour post transfer was carefully chosen because pmel-1 T cells would barely

divide within 48 hours (data not shown) while giving the cells longer time (e.g. 96 hours) would allow them to exit the lymph node (212). I found that L-tyrosine extended gp100 peptide presentation approximately 4 day longer than that of saline, but not as long as IFA (figure 16). Thus, L-tyrosine microparticles functioned as a vaccine formulation that caused an intermediate duration of antigen presentation to T cells *in vivo*.

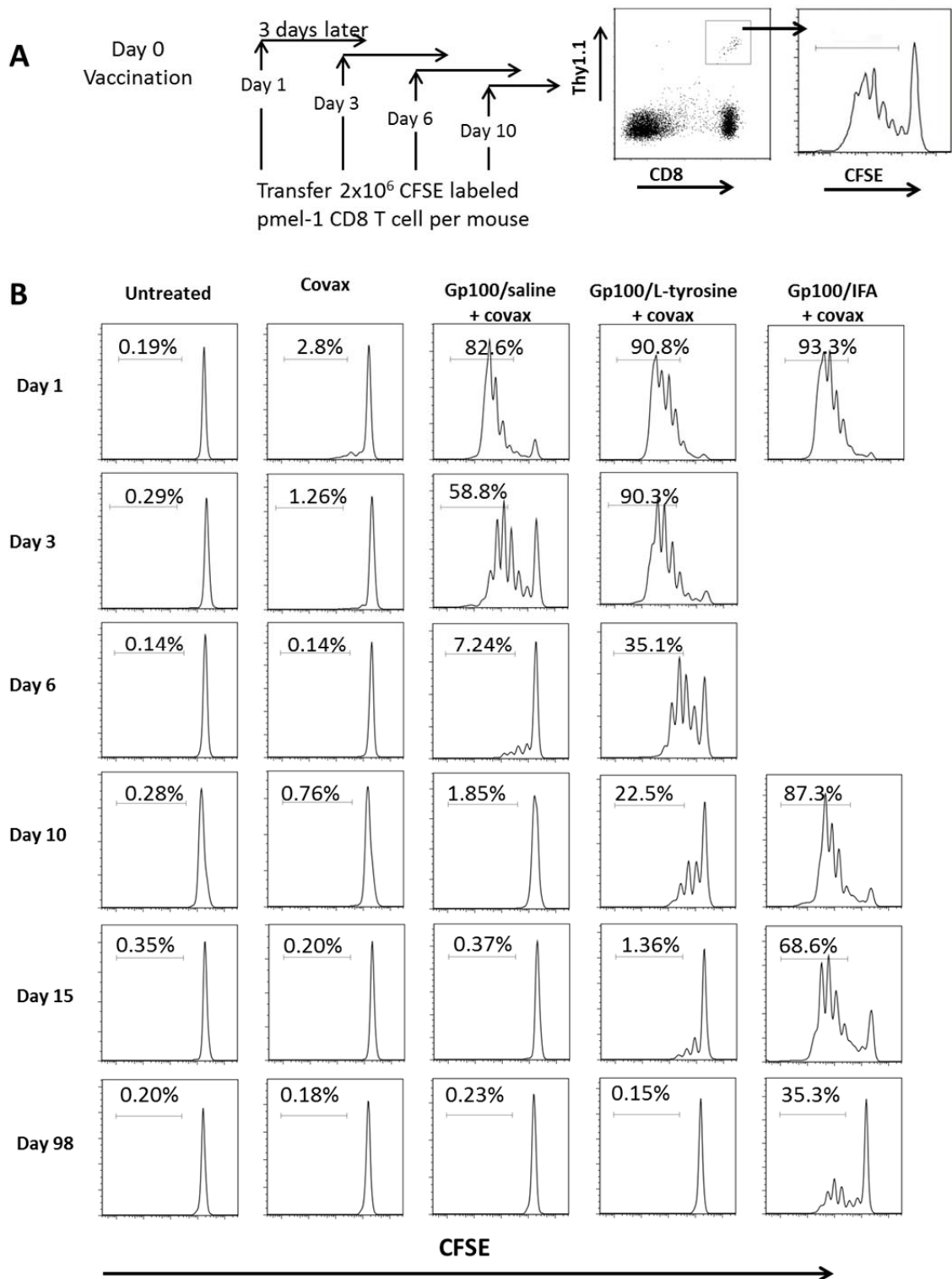


Figure 16. L-tyrosine extends antigen presentation time *in vivo*. A) Experiment design. All mice were treated on day 0. At indicated time point, 2×10^6 CFSE labeled pmel-1 CD8+ T cells were transferred to hosts. 72 hours later, vaccine draining lymph nodes were harvested for pmel-1 T

cells. CFSE dilution of adoptive pmel-1 T cells was measured by flow cytometry. $n = 5$ mice/group. Data are representative of 2 independent experiments.

2. Repeated injection of peptide in saline recapitulates the vaccine adjuvant effect of L-tyrosine

To further investigate whether the extended duration of Ag presentation was mainly responsible for the potent vaccine adjuvant effect of L-tyrosine formulation, I sought to determine if a similar result could be achieved by extending Ag presentation through repeated injections of peptide in saline. I first tested what was the optimal number of repeated injection (figure 17). As depicted, daily administration of peptide for 3 consecutive days induced optimal T cell responses while administering more than 3 injections had detrimental effect on T cell responses. Based on this observation, I performed a direct comparison of repeated injection of gp100 in saline versus gp100 in L-tyrosine in terms of T cell response and tumor rejection efficacy. Indeed, T cell levels were very comparable between groups receiving gp100/L-tyrosine and 3 doses of gp100/saline for 3 consecutive days (day 0, 1, 2) (figure 18A). Importantly, although repeated injections of saline for 3 consecutive days induced similar T cell level in peripheral blood to that of L-tyrosine, their function (IFN γ /TNF α production) was inferior to the function of L-tyrosine-induced T cells (figure 18B). The higher numbers but inferior T cell function after repeated injection may explain why tumor rejection by these T cells was improved compared to single gp100/saline injection but still not as efficient as gp100/L-tyrosine group (figure 18C).

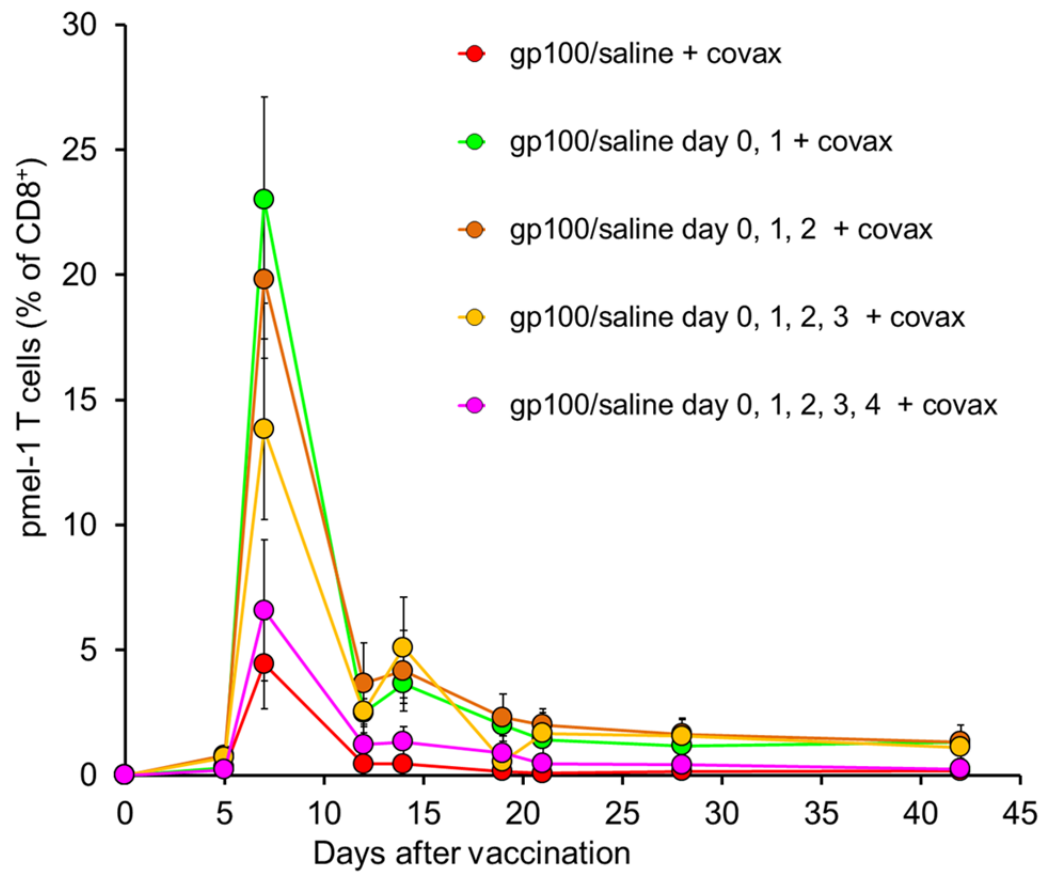


Figure 17. Titrating the number of injection of gp100 in saline for optimal T cell response. Mice received 8×10^5 pmel-1 T cells and indicated treatments on day 0. $n = 5$ mice per group per experiment. Data are representative of 2 independent experiments.

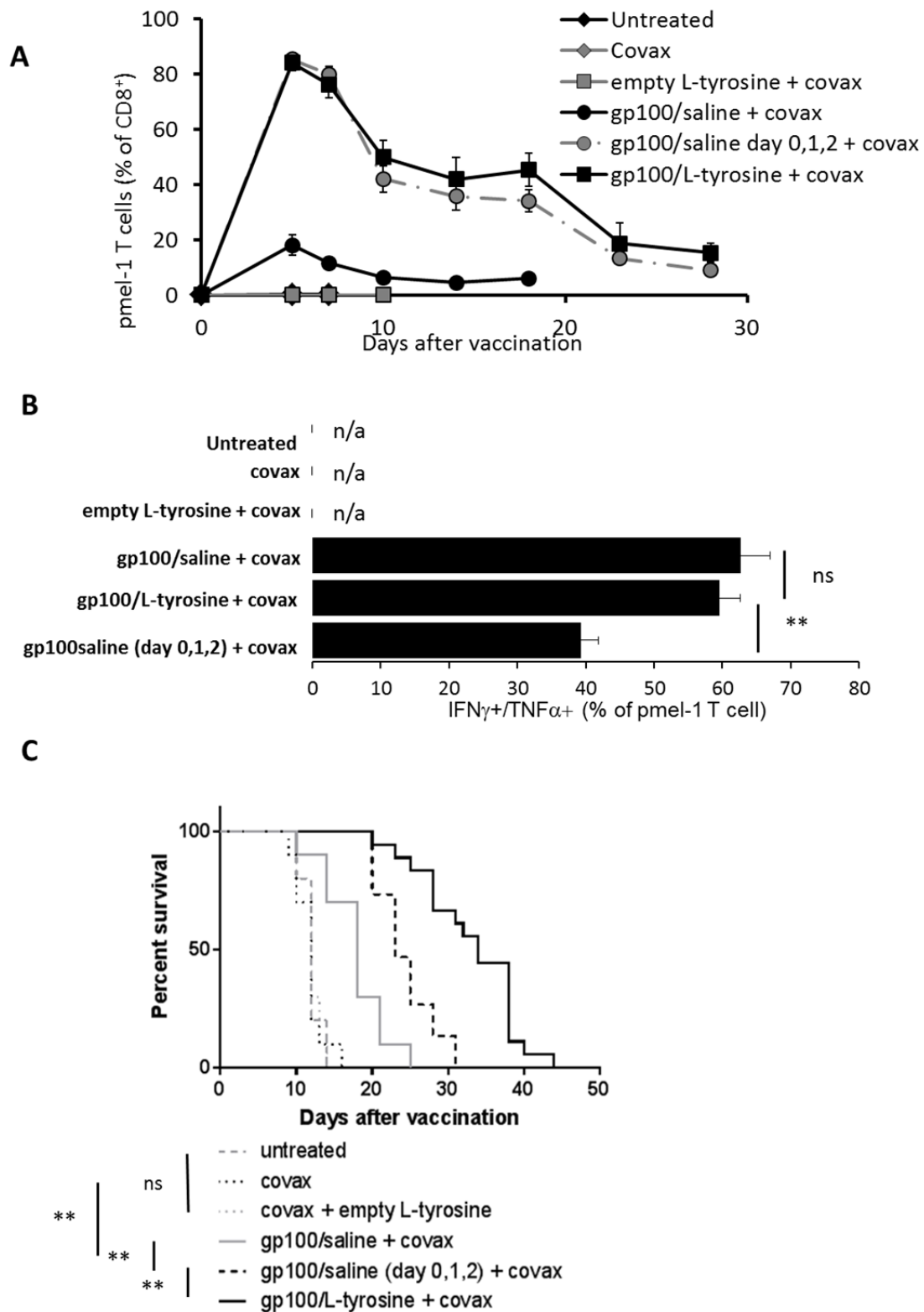


Figure 18. Levels of gp100 specific CD8⁺ T cell response directly correlate with anti-tumor efficacy. A) Pmel-1 T cell response after different vaccine formulations, including repeated

injection of peptide in saline. B) T cell function, including IFN- γ and granzyme B production at day 7 post vaccination (shown as the mean \pm s.e.m.). C) Anti-tumor efficacy of corresponding groups of same experiments. n= 10 – 20 mice/group. Data pooled from 2 independent experiments. *: P< 0.05, **: P< 0.01, ***: P < 0.001, ns: not significant, n/a: not available.

3. Repeated injection of gp100₂₅₋₃₃ and OVA₂₅₇₋₂₆₄ peptide enhances the endogenous T cell responses

To rule out the possibility that my findings applied uniquely to our specific model system based on gp100 antigen and gp100-specific, transgenic T cells, I examined endogenous T cell responses to gp100 and OVA-I₂₅₇₋₂₆₄ (SIINFEKL) peptides. When testing endogenous T cell responses to gp100, I observed a very low response after 1 vaccination, likely due to the reported very low T cell precursor frequency to this self-antigen (213). However, after 2 booster vaccination, mice receiving gp100/L-tyrosine showed a dramatically stronger gp100-specific CD8⁺ T cell response than mice receiving gp100/saline (figure 19). I then tested endogenous T cell responses to the unrelated non self-antigen, ovalbumin peptide OVA₂₅₇₋₂₆₄ (OVA-I) and observed a strong CD8⁺ T cell response after two vaccinations, with OVA-I/L-tyrosine and especially three successive daily OVA-I/saline vaccinations giving a clearly enhanced T cell response (figure 20). Overall, these results demonstrate that approaches that prolonged antigen presentation *in vivo* deserve further investigation in the development of human cancer vaccines.

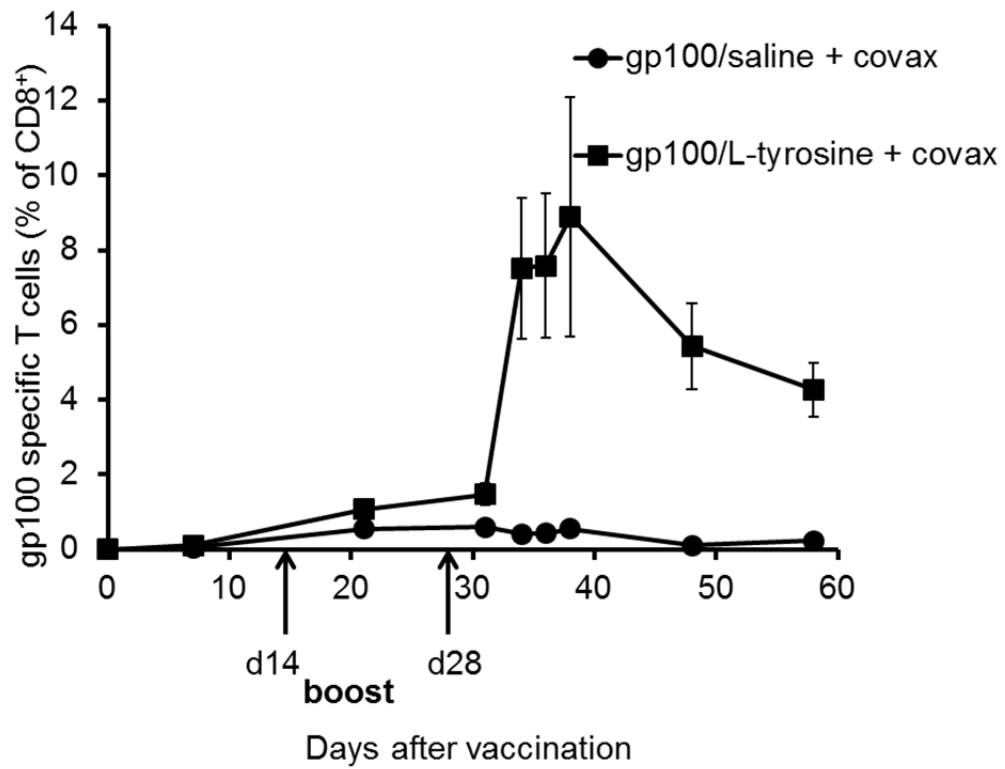


Figure 19. Endogenous gp100 specific T cell responses after saline and L-tyrosine vaccines, detected by IFN- γ positive CD8 T cells. Boosters were given as indicated times, with same dose and formulations as for priming. n = 5 mice per group. Data are representative of 2 independent experiments.

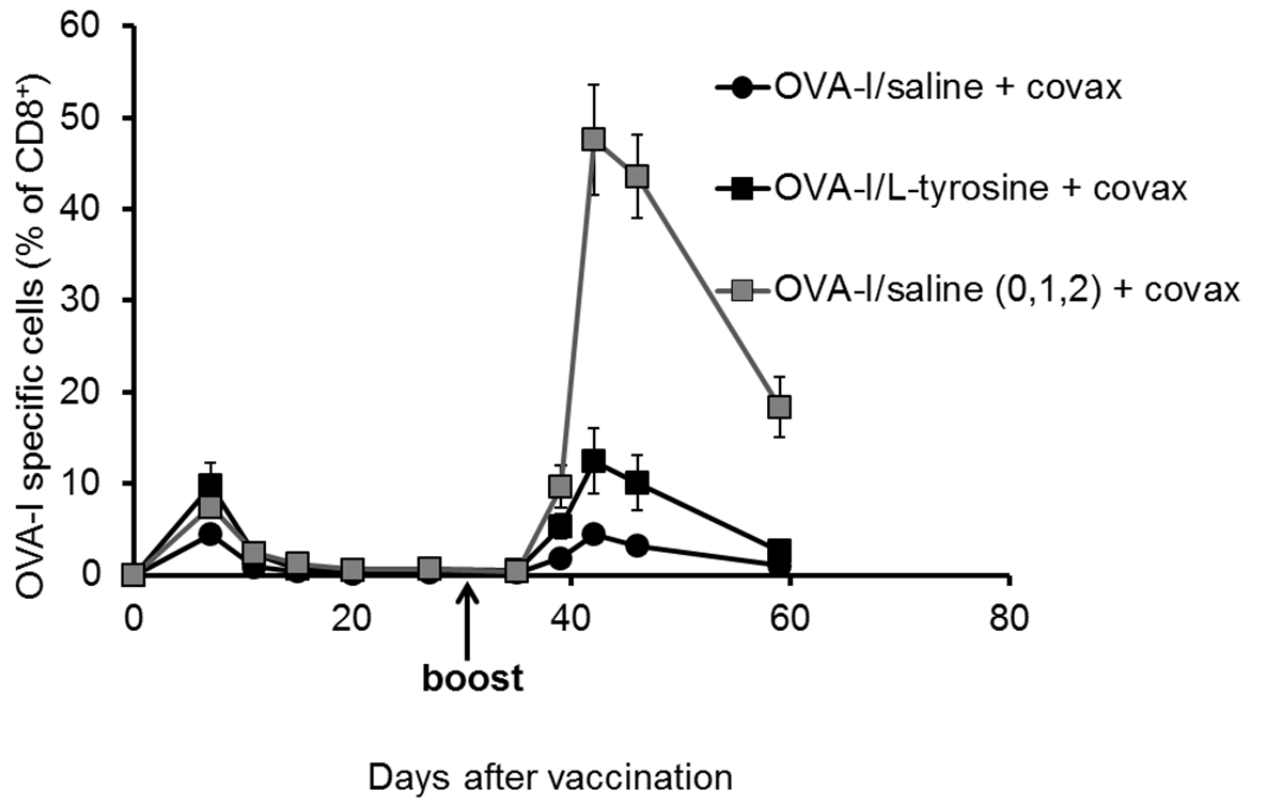


Figure 20. Endogenous OVA-I (OVA₂₅₇₋₂₆₄) specific T cell responses after saline and L-tyrosine vaccines, detected by OVA-I dextramer H2-Kb positive CD8 T cells. Booster was given as indicated time, with same dose and formulations as for priming. n = 5 mice per group. Data are representative of 2 independent experiments.

III. Discussion

Using CFSE-labeled gp100-specific pmel-1 T cells, I was able to follow the antigen presentation time of gp100 peptide after different vaccine formulations, including saline, L-tyrosine and IFA. As expected, peptide formulated in L-tyrosine had longer presentation duration than peptide in saline but not as long as peptide formulated in IFA. Daily administration of peptide in short-lived saline formulation induced a similar robust T cell number as induced by L-tyrosine, but with reduced function. In fact, multiple injections of peptide in saline induced lower IFN- γ /TNF- α production by Ag specific T cells. As a result, despite inducing similar T cell number in peripheral blood, multiple injection of peptide in saline did not induce a robust anti-tumor efficacy as peptide/L-tyrosine.

In clinical settings for therapeutic vaccine, adoptive transfer of antigen specific T cells is unlikely (except in combination with adoptive cell therapies which are currently not widely used). Therefore, it was critical to determine whether my findings with adoptive pmel-1 model applied to the endogenous T cell repertoire. In fact, I found the same pattern with endogenous T cell repertoire. Importantly, the induction of detectable Ag-specific T cell responses required booster(s), likely due to the low frequency of Ag-specific T cells from the endogenous repertoire. Nevertheless, my data with only 2 peptides is not exhaustive and far from providing a predictive model for wide variety of peptides: short versus long, MHC Class I vs MHC Class II-restricted and so on.

CHAPTER 5 – DISCUSSION

I. Summary

1. Summary of chapter 3

In this chapter, I investigated the innate immune stimulating properties of L-tyrosine. Our lab was the first to formulate L-tyrosine with small size (9 amino acids) short peptide. Previous studies suggested that L-tyrosine promotes Th1 response due to the induction of IgG2 Ab. However, further mechanisms of action of L-tyrosine were largely unstudied. My results suggest that L-tyrosine does activate the innate immune system, evidenced by the local inflammation at injection site. Particularly, neutrophil infiltration and neutrophil related cytokines and chemokines were dramatically induced by L-tyrosine particles. Nevertheless, the local inflammation did not contribute to superior T cell response after L-tyrosine vaccine, as demonstrated by a series of experiments. My findings are in line with the observation that separate injection antigen (allergen) and L-tyrosine (without adsorption) negates the adjuvanticity of L-tyrosine (198). The adsorption rate of pollen to L-tyrosine varies from 60 to 90 percent (199) while adsorption rate of short peptides (only 4, including gp100, OVA-I, P15E and TRP2) lies within the range of 25 to 99 percent.

2. Summary of chapter 4

The depot effect is defined as a sustained/delayed release of antigen. Past studies suggested the depot property of pollen formulated in L-tyrosine by the short-term in vivo persistence of the L-tyrosine particle itself (48 hour half-life) and a delayed release of ¹²⁵I-labeled ragweed antigen E. My results in chapter 3 showed little (if any) contribution of

induced inflammation to T cell response, strongly suggesting the depot effect of L-tyrosine as a mediator of its adjuvant in our peptide vaccine model.

To determine whether L-tyrosine can extend Ag presentation time to T cells, I used Ag-specific, CFSE-labeled T cells to measure T cell division. The T cell division, as a result of Ag, was reflected by CFSE dilution. I found that T cells can detect gp100 and respond by proliferating for approximately 3-4 days longer when gp100 is formulated with L-tyrosine vs. saline. When formulated with water-in-oil incomplete Freund's adjuvant (IFA), gp100 remains up to 100 days post vaccination which is consistent with previous finding by our group (93). Repeated injections of peptide in saline from 2 to 4 consecutive days induced the highest T cell responses while a single injection, or 5 injections on 5 consecutive days, induced suboptimal T cell numbers. Daily administration of peptide in saline for 3 consecutive days (day 0, 1, 2) was selected for a head-to-head comparison with L-tyrosine, in terms of T cell induction and anti-tumor efficacy. Although both vaccine regimens gave similar T cell responses, L-tyrosine vaccine conferred significantly better tumor protection.

I found L-tyrosine vaccine synergizes with covax to induce superior Ag-specific CD8 T cell responses. Most experiments were carried out using adoptive naïve pmel-1 T cells because of several advantages. First, the frequency of gp100-specific T cell from the host endogenous repertoire is low which requires at 2 boosters for gp100 and one booster for OVA-I in order to detect antigen specific CD8 T cells. Second, due to the aggressive nature of B16 melanoma in mouse model, using adoptive pmel-1 T cells allows a larger T cell response sooner, permitting anti-tumor immunity to suppress tumor growth before rapidly growing tumors have killed the mouse. Third, the congenic marker Thy1.1 of pmel-1 T cells allows them to be detected directly

by flow cytometry. Since this is a somewhat artificial system, I confirmed the activity of L-Tyrosine as a vaccine adjuvant with other peptides, drawing on the endogenous T cell repertoire. In short, I found that my findings apply to multiple peptides, and to T cell responses from the endogenous repertoire.

II. General discussion and future directions

1. The contribution of Ag exposure duration to T cell response

The remarkable CD8 T cell responses after L-tyrosine and repeated injection vaccines illustrate the critical importance of Ag exposure time for the activation and differentiation of T cells. Early *in vitro* studies suggested that a very brief Ag stimulation (~2 hours) was enough for CD8 T cells to enter clonal expansion (214, 215). Subsequent studies showed that longer antigenic stimulation (20 – 96 hours) was required for CD8 T cell to acquire full effector function and memory differentiation after expansion (216–219). After receiving Ag stimulation, although CD8 T cells can undergo autonomous (Ag independent) proliferation, the magnitude of the T cell response does continue to depend on further Ag (87, 220, 221). Extended Ag stimulation increases the magnitude of CD8 T cell response by driving further T cell proliferation and improving T cell survival (217, 221). It has further been postulated that the magnitude of T cell response by prolonged Ag stimulation may also be affected by the number of Ag-specific naïve T cells recruited to clonal expansion (222). However, Ag specific T cells were recruited to clonal expansion very efficiently in our vaccine settings (figure 21), regardless of vaccine formulations. This finding is consistent with previous reports which demonstrated that during infection, the majority of Ag-specific T cells are efficiently recruited into the Ag-containing lymph node, where they are primed and enter the expanding pool of

effector T cells (221, 223). Therefore, we conclude that the increased magnitude of CD8 T cell responses after L-tyrosine and repeated injection was caused by Ag driven – increased clonal expansion rather than enhanced recruitment of Ag-specific naïve T cells into proliferation.

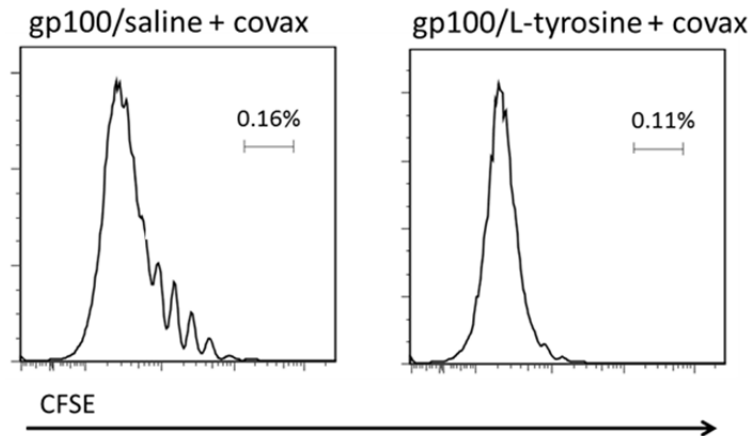


Figure 21. T cell recruitment to proliferation is very efficient in both saline and L-tyrosine vaccine. 2×10^6 CFSE labeled pmel-1 CD8⁺ T cells were transferred to hosts and mice were vaccinated as indicated. Seven days post transfer, vaccine draining lymph nodes were harvested and CFSE dilution of pmel-1 T cells was measured by flow cytometry. $n = 3$ mice per group.

2. The choice of peptides and tumor antigens

In this study, I primarily studied vaccination-induced T cell responses to gp100 peptide. The kinetics of Ag presentation are likely to be unique for each peptide, based on its solubility, length, susceptibility to proteases and peptidases, and affinity for the MHC Class I binding groove, which protects bound peptides from degradation.

Historically, due to the time consuming and labor intensive nature of cancer antigen discovery process, differentiation antigens which are shared among patients were favored over unique antigens which are not shared between patients. However, with the speed of cancer neo-antigen discovery assisted by powerful computational biology, as well as recent findings which suggest neo-antigens may out-perform differentiation antigens as targets for anti-tumor T cells, the paradigm of therapeutic cancer vaccine is shifting from shared to personalized antigens. This poses additional challenges to peptide-based vaccines, particularly how to accurately and rapidly identify truly antigenic peptides from the vast number of potential peptides. Recently, a very exciting report described a computational model that may help predict T cell response basing on antigen affinity and dose (224). This model will be tremendously helpful, particularly when many peptides are used in a single vaccine cocktail. Nevertheless, in formulation with L-tyrosine, the kinetics of Ag presentation of an individual peptide will depend on the efficiency to co-precipitate with L-tyrosine and on how long the peptide/L-tyrosine complex will persist *in vivo*.

Future refinements in peptide vaccine adjuvants may include formulations that are relatively insensitive to the physicochemical nature of the antigenic peptide, allowing for standardized peptide incorporation efficiency. Since most Ag-specific immunotherapies

(including vaccines) rely on a single Ag, tumor escape through loss of Ag expression is likely to occur (184, 225). Combining multiple antigenic peptides into a cancer vaccine may help prevent such tumor escape (226).

3. Clinical relevance of this study and the contribution of therapeutic cancer vaccines toward cancer treatment

Cancer vaccines are widely explored as a mean to induce tumor-specific T cells, but thus far clinical success has been limited (227, 228). One reason is a profound lack of potent vaccine adjuvants available for the potentiation of cancer vaccines aimed at inducing robust tumor-specific T cell responses. Given recent progress in the design of therapeutic vaccines, including tumor Ag selection, Ag delivery platform, and immunopotentiators, the development of effective therapeutic vaccine for cancer is accelerating. I have shown here that the duration of antigen presentation is an important parameter that can be controlled through vaccine formulation to drive effective anti-cancer T cell responses for the therapy of patients with cancer. I devised a flowchart to illustrate the standing of cancer vaccine in cancer immunotherapies as well as cancer treatment in a broad perspective (figure 22). To be noticed, therapeutic cancer vaccines may not be a suitable option for patients with bulky tumor masses. Most likely, cancer vaccines are ideal to prevent tumor relapse or to treat multiple sporadic tumors which are unsuitable for surgical removal.

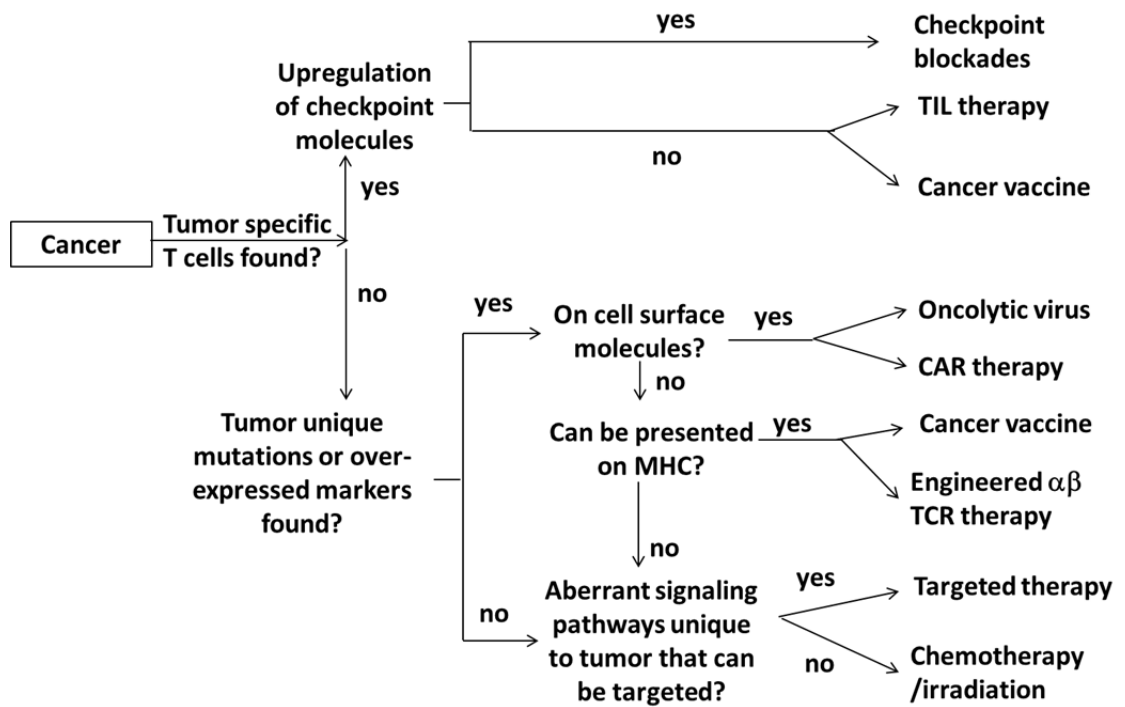


Figure 22. The contribution of cancer vaccine to cancer treatment.

4. Future directions

Since several questions remained unanswered in this study, I would like to propose some possible directions for future study.

Although L-tyrosine was shown to extend Ag presentation time, which is likely due to the depot property of L-tyrosine for peptide based vaccine, further studies are needed for a rigorous confirmation. First, the vaccination site can be removed at different time points post vaccination, to see if immune responses are diminished by cutting short the time of peptide release and antigen presentation. Second, peptide can be labeled with fluorescence or radioisotopes to allow temporal and spatial tracking of peptide *in vivo*. However, labeling approaches bear limitations that should be considered. The presence of fluorescence/radioisotope may not be a *bona fide* marker for presence of peptide, because degraded peptides and free amino acids are not recognized by T cells, but would still give a fluorescent/radioactive signal. In other words, the detection of signal would not necessarily reflect the functional availability of the antigen. Nevertheless, when combined with functional availability assay of Ag (using CFSE labeled T cells), this method will provide a solid evidence of depot effect. Third, with the advances in proteomics, peptide can be detected by mass-spectrometry. Yet, this approach, together with labeling approaches cannot tell whether detected peptide is functionally presented on an APC, and/or recognized by T cells.

Although repeated injection of peptide and L-tyrosine formulation induced similar tumor-specific T cell levels, the resulting anti-tumor efficacy of L-tyrosine was more potent. This discrepancy could imply that L-tyrosine has other, undiscovered impacts on T cell responses and/or tumor growth besides the extended Ag presentation effect we describe

here. In fact, meta- and ortho- tyrosine have been shown to mediate a form of concomitant tumor resistance, a phenomenon where primary tumor suppresses the growth of distant secondary tumors (229). L-tyrosine used in our vaccine, however, was para-L-tyrosine, whose tumor suppressive activity was not detected (206). It appears more likely that 3 daily injections of peptide in saline and 1 injection of peptide in L-tyrosine microparticles do not produce identical kinetics of Ag release and presentation by APCs, resulting in a difference in the quality of the ensuing T cells response, as we observed. This indicates that there may be more effective regimens of repeated Ag injection, for example a gradual increase of Ag dose followed by a gradual decrease in a time span of several days, more closely mimicking the kinetics of Ag production during a viral or bacterial infection; these permutations remain to be explored. Last but not least, the persistence of peptide *in vivo* can be achieved without using Ag carrier/deliver system. Indeed, peptide can be constructed to form macromolecular nanofiber structures that appear to persist much longer *in vivo* than individual nonamer peptides(230). Another approach is to chemically modify the peptides to increase their resistance to proteolysis (231), although these approaches require careful evaluation to ensure the immunogenic peptide is productively liberated and recognized from its chemically modified form.

CHAPTER 6 – EXPERIMENTAL PROCEDURES

Reagents and Chemicals. The synthetic, high-affinity H-2D^b-restricted heteroclitic mouse gp100_{25–33} peptide (KVPRNQDWL), H-2K^b-restricted chicken OVA_{257–264} (OVA-I) peptide (SIINFEKL), P15E_{604–611} (KSPWFTTL), sTRP2_{180–188} (SVYDFVWL) were purchased from Peptides International (Louisville, KY) at a purity >95%. Optima-Grade acetonitrile, methanol, and water were purchased from Thermo-Fisher Scientific (Waltham, MA). Mass Spectrometry-grade formic acid (Fluka; 98%) was purchased from Sigma Aldrich (St. Louis, MO). 1X Phosphate Buffered Saline was purchased from Mediatech Inc. (Manassas, VA). Sodium hydroxide (molecular biology grade) and hydrochloric acid (36.5% v/v) were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium chloride, sodium phosphate monobasic, sodium phosphate dibasic, and L-tyrosine (cell culture grade) were purchased from Sigma (St. Louis, MO). Mouse cytokine/chemokine milliplex kit (catalog # MXMCY70KPX25MGBK) was purchased from EMD Millipore (Massachusetts, USA). OVA-I dextramer H2-Kb was purchased from Immudex (Fairfax, VA).

Mice. All mouse protocols were reviewed and approved by the Institutional Animal Care and Use Committee at The University of Texas - M.D. Anderson Cancer Center. Pmel-1 TCR transgenic mice on C57BL/6 background (the Jackson Laboratory, ME) were crossed with CD90.1 congenic mice to yield pmel-1^{+/+}CD90.1^{+/+} mice (hereafter referred as pmel-1 mice). C57BL/6 (B6) mice were purchased from Charles River (Wilmington, MA). B16-F10 melanoma bearing mice were established by injecting 300,000 B16 cells in the volume of 0.1 ml subcutaneously. Tumor bearing mice received treatments on day 6 after tumor injection when

the average tumor size was approximately 30 mm². ASC knock-out mice were the gift from Dr. Thirumala-Devi Kanneganti from St. Jude Children's Research Hospital.

Cell culture. B16-F10 melanoma cell line, from ATCC (Manassas, VA), was cultured in complete medium including RPMI 1640 with 10% FBS, 100 µg/ml streptomycin and 100 µg/ml penicillin (Life Technologies, Carlsbad, CA.)

Vaccination. Peptides in saline and IFA were prepared as previously described (93), at equivalent dose to that in L-tyrosine. Preparation of peptide with L-tyrosine was adapted from a protocol for grass pollen/L-tyrosine described by Wheeler *et al.* (197). A peptide solution was prepared by dissolving 4 mg of peptide in 2 mL of 1x-PBS. After dissolution, 0.667 mL of strong PBS (0.83 M Na₂HPO₄, 0.25 M NaH₂PO₄, 0.137 M NaCl) was added to the solution. Next, 0.667 mL each of 3.2 M sodium hydroxide and 1.3 M L-tyrosine in 3.9 M hydrochloric acid were added simultaneously, mixed, resulting in a final solution volume of 4 ml. The suspension was centrifuged and supernatant was discarded. Remaining peptide/L-tyrosine pellet was dissolved in PBS to make up a volume of 4 ml and ready for injection. Final peptide concentration was approximately 0.25 mg/ml. Each mouse received 200 µl vaccine (100 µl x 2 vaccination sites). For the quantification of tumor specific CD8⁺ T cells, pmel-1 splenocytes were intravenously transferred to B6 mice in the same day with vaccination. Covax, including anti-CD40 antibody (clone FGK4.5/ FGK45, BioXcell, New Hampshire), IL-2 (TECIN, Hoffman LaRoche) and Imiquimod cream 5% (Fougera, Melville, NY) was given on the same day with peptide vaccination. Anti-CD40 dose: 50 µg subcutaneously; IL-2: 100,000 IU, once on day 0 and twice on day 1 and 2 intraperitoneally; imiquimod cream: 50 mg, applied topically on vaccination site.

Adoptive transfer of pmel-1 T cells. Each mouse received approximately 80,000 pmel-1 CD8 T cells from pmel-1 mouse via tail i.v. injection. For *in vivo* antigen detection experiment, pmel-1 CD8 T cells were purified using CD8 T cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) then labeled with CFSE as described elsewhere (232). Each mouse received 2×10^6 CFSE labeled pmel-1 CD8 T cells i.v.

FACS analysis. Mice were tail-bled on the indicated days. Extracellular staining was performed using FACS buffer containing 2% FBS. Intracellular cytokine staining was performed using the cytofix/cytoperm kit from BD Biosciences (San Jose, CA) basing on the manufacturer's recommendation. Granzyme B staining was done without stimulation while IFN- γ staining was done after 4 hours of stimulation with 1 μ M hgp100₂₅₋₃₃ peptide. Intracellular transcription factor staining was done following Foxp3 staining protocol by eBioscience (San Diego, CA). Antibodies were either purchased from eBioscience or BD Biosciences: CD8a (clone 56-6.7), CD4 (GK1.5), CD90.1 (HIS51), IFN- γ (XMG1.2), TNF- α (MP6-XT22), Granzyme B (NGZB), CD19 (eBio1D3), CD3e (145-2C11), NK1.1 (PK136), CD44 (IM7), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), F4/80 (BM8), CD62L (MEL-14), CD27 (A7R34) MHCII (M5/114.15.2), CD40 (HM40-3), CD86 (GL-1), Ly6G (1A8), Ly6C (AL-21).

Cytokine/chemokine assay. On day 1, 2, 3 and 7 post vaccination, skins at vaccination site were hair-removed, weighted, mechanically disrupted in ice cold PBS (1 ml/sample) and centrifuged for supernatant collection. The cytokines/chemokines in the supernatant were measured using Milipex mouse cytokine/chemokine panel (Millipore) according to the manufacturer's instructions. Fluorescence signal was measured on Luminex 100/200 system

and data were analyzed using Excel software. Final cytokine/chemokine readouts were normalized by sample weight.

Quantification of peptide in L-Tyrosine formulation. After the peptide/L-tyrosine co-precipitation (as described in vaccination section), the final volumes of the supernatant and crystal fractions were determined to be 2.85 mL and 1.15 mL, respectively. The individual fractions were stored at 4 °C until analysis. Peptide stock (2.49 mg/mL) and intermediate (100 µg/mL) solutions were prepared in water, and were stored at 4 °C until analysis. The intermediate solution was used to prepare calibration standards at 50.0, 25.0, 10.0, 2.00, and 1.00 µg/mL concentrations in water. Prior to sample processing, the peptide loaded particle and supernatant fractions were warmed to room temperature. The peptide-loaded L-tyrosine particles contained in the crystal fraction were dissolved by an addition of 4 mL of formic acid followed by gentle vortex-mixing. Once the particles were completely dissolved, an additional 1.88 mL aliquot of water was added to the sample to increase the final sample volume to 7.00 mL. In prior to analysis, three individual sample dilutions were prepared at 10x, 50x, and 100x in water. Because the supernatant fraction remained free from particles after short-term refrigerated storage, the only processing required was the three individual sample dilutions prepared at 10x, 50x, and 100x in water.

LC-MS/MS System Conditions: Sample analysis was performed on an Agilent 1290 Infinity Binary UHPLC coupled to an Agilent 6460 tandem-mass spectrometer. Mobile phase A (MPA) and mobile phase B (MPB) used for this study were 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The chromatographic column used was an Agilent Zorbax RRHD Eclipse Plus C18 (2.1 x 50 mm, 1.8 micrometer; dead volume: ~0.12 mL; dead time: ~0.60

min at 0.200 mL/min). The column was heated to 40 °C, and the chromatographic flow rate was 0.200 mL/min. The gradient elution program was set as follows: dwell at initial conditions of 90:10 MPA:MPB for 1.5 minutes post-injection; ramp to 20:80 MPA:MPB at 4.0 minutes post-injection; ramp to 0:100 MPA:MPB at 5.0 minutes post-injection; and ramp back to initial conditions (90:10 MPA:MPB) at 5.5 minutes post-injection until the gradient stops at 6.5 minutes post-injection. The overall cycle-time for a single injection was approximately 7.0 minutes. The mass spectrometer acquisition source parameters were as follows: source: Agilent Jet Stream ESI source; gas temperature: 275°C; gas flow: 6 L/min; nebulizer: 40 psi; sheath gas temperature: 325 °C; sheath gas flow: 9 L/min; capillary voltage: +3,750 V; nozzle voltage: 0 V. The molecule specific acquisition parameters were as follows: precursor to product transition: m/z 490.n2 to m/z 848.4; MS1 and MS2 were set to unit resolution; dwell time: 250 ms; fragmentor voltage: 100 V; collision energy voltage: 10 V; cell acceleration voltage: 7 V; and the source polarity was set to positive mode.

Statistical analysis. All results are expressed as mean \pm s.e.m (standard error of the mean). For in vivo experiments, group size was n = 5 except for tumor challenge experiments whose group size was n = 10. Data were analyzed using unpaired two-tailed t-test where p value < 0.05 would be considered as significant. All experiments were repeated at least once with comparable results.

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