Tolerogenic $CD4^{-}8^{-}$ Dendritic Cells and their Conversion into Immunogenic Ones via TLR 9 Signaling

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ABSTRACT

It is clear that dendritic cells (DCs) are essential for priming of T cell responses against tumors. However, the distinct roles DC subsets play in regulation of T cell responses *in vivo* are largely undefined. In this study, we investigated the capacity of ovalbumin (OVA)-presenting $CD4^-8^-$, $CD4^+8^-$, or $CD4^-8^+$ DCs (OVA-pulsed DC (DC_{OVA})) from mouse spleen in stimulation of OVA-specific T cell responses. Our data show that each DC subset stimulated proliferation of allogeneic and autologous OVA-specific $CD4^+$ and $CD8^+$ T cells *in vitro*, but that the $CD4^-8^-$ DCs did so only weakly. Both $CD4^+8^-$ and $CD4^-8^+$ DC_{OVA} induced strong tumor-specific $CD4^+$ Th1 responses and fully protective $CD8^+$ cytotoxic T lymphocyte (CTL)-mediated antitumor immunity, whereas $CD4^-8^-$ DC_{OVA}, which were less mature and secreted substantial transforming growth factor (TGF-β) upon coculture with T cell receptor (TCR)-transgenic OT II $CD4^+$ T cells, induced the development of interleukin-10 (IL-10)-secreting $CD4^+$ T regulatory 1 (Tr1) cells. Transfer of these Tr1 cells, but not T cells from cocultures of $CD4^-8^-$ DC_{OVA} and IL-$10^{-/-}$ OT II $CD4^+$ T cells, into $CD4^-8^+$ DC_{OVA}-immunized animals abrogated otherwise inevitable development of antitumor immunity. Taken together, $CD4^-8^-$ DCs stimulate development of IL-10-secreting $CD4^+$ Tr1 cells that mediated immune suppression, whereas both $CD4^+8^-$ and $CD4^-8^+$ DCs effectively primed animals for protective $CD8^+$ CTL-mediated antitumor immunity.

Different DC subsets play distinct roles in immune responses. $CD4^-8^-$ DCs secreting TGF-β stimulate $CD4^+$ regulatory T type 1 (Trl) cell responses leading to inhibition of CD8 CTL responses and antitumor immunity. In this study, we explored the potential effect of three stimuli CpG, lipopolysaccharide (LPS) and anti-CD40 antibody in

conversion of $CD4^-8^-$ DC-induced tolerance. We demonstrated that when $CD4^-8^-$ DCs were isolated from overnight culture and cultured for another 8 hrs in AIM-V plus recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF) (15-20 ng/ml) and OVA (0.1 mg/ml) with CpG (5 ug/ml), LPS (2 ug/ml) and anti-CD40 antibody (10 ug/ml), their phenotype became more mature compared with the freshly isolated ones. CpG is the only agent that stimulates the DCs to secrete significant level of interleukin-6 (IL-6) and interleukin-15 (IL-15); DNA array analyses also indicate that CpG stimulates higher expression of IL-6 and IL-15 mRNA. CpG treatment most efficiently converts the tolerogenic DCs into immunogenic ones which stimulated the OTII $CD4^+$ T cell to become T helper type 1 (Th1) and T helper type 17 (Th17) rather Tr1, while the other two stimulator-treated DCs could not induce Th17 response. Their vaccination also induced the strongest antitumor CTL responses and protective immunity against tumor cell challenge. When $CD4^-8^-$ DCs were isolated from IL-6 knock out ($IL\text{-}6^{-/-}$) mice, CpG-treated DC_{OVA} vaccination almost completely lost their animal protection capacity. Wild type B6 DC_{OVA}-vaccinated IL-15 receptor knock out ($IL\text{-}15R^{-/-}$) mice can only provide up to 30% protection against tumor challenge. Those results indicate that IL-6/ IL-l5-induced Th17 plays a critical role in their conversion. Taken together, our findings indicate that CpG treatment is the most efficient agent that can convert tolerogenic DCs into immunogenic ones and induce long-lasting antitumor immunity.

We previously demonstrated that the nonspecific $CD4^+$ T cells can acquire antigen-specific DC-released exosomes (EXO) and these $CD4^+$ T cells with acquired exosomal MHC I peptide complex (pMHC I) can stimulate antigen-specific $CD8^+$ CTL responses. In my project we have found that $CD4^-8^-$DCs could induce regulatory T cell type 1(Tr1) response, thus it would be very necessary to know whether regulatory T cells

would change their antigen specificity if they got the membrane complex from DC through coculture or DC-derived exosome pulsing. During the beginning of my regulatory T cell project, we found that $CD8^+CD25^+$ Tr were much more easily expanded, while $CD4^+CD25^+$ Tr usually began to die just after 3 days in vitro culture and it's very hard to get enough cells for further research. Therefore, $CD8^+CD25^+$ were used as a model Tr cells in the following project. To assess whether the nonspecific $CD8^+CD25^+$ Tr cells can acquire antigen-specificity via acquired exosomal pMHC I, we purified $CD8^+CD25^+$ Tr cells from wild-type C57BL/6 mice and OVA-pulsed DC_{OVA}-released EXO_{OVA} expressing pMHC I complexes. We demonstrated that the nonspecific $CD8^+CD25^+$ Tr cells expressing forkhead box P3 (Foxp3), cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), perforin and granzyme B inhibited *in vitro* T cell proliferation and *in vivo* OVA-specific $CD4^+$ T cell-dependent and independent $CD8^+$ CTL responses and antitumor immunity. $CD8^+CD25^+$ Tr cells' suppressive effect is possibly mediated through its inhibition of DC maturation, down-regulation of secretion of Th1 polarization cytokines by DCs and its induction of T cell anergy via cell-to-cell contact. The nonspecific $CD8^+CD25^+$ Tr cells acquired antigen specificity by uptake of DC_{OVA}-released EXO_{OVA} expressing pMHC I and enhanced its effect on inhibition of OVA-specific $CD8^+$ T cell responses and antitumor immunity by 10-folds. The principles elucidated in this study may have significant implications not only in antitumor immunity, but also in other sectors of immunology (e.g, autoimmunity and transplantation).

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DEDICATION

To my family,

who offered me unconditional love and support throughout the course of this thesis. I thank my dear mother **Yufang Li** and late father **Jiaming Zhang** for raising me and supporting my higher education pursuit. I deeply thank my wife **Xiaojing Cheng** and my lovely little daughter **Huimin Zhang** for their unending and sincere support.

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Ab	Antibody
AC	adenyl cyclase
Ag	Antigen
APC	Antigen presenting cells
ATCC	American type culture collection
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
CD40 L	Co-stimulatory molecule 40 ligand
CFSE	Carboxyfluoroscein diacetate succinimidyl ester
CpG	Cytosine phosphate guanosine
CPM	Counts per minute
CPP	Cell-penetrating peptides
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
Cy	Cyclophosphamide
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule 3 (ICAM-3)–grabbing nonintegrin
Dectin-1	Dendritic cell-associated C-type lectin-1
Dectin-2	Dendritic cell-associated C-type lectin-2
DMEM	Dulbecco's modified Eagle’s medium
DMSO	Dimethylsulfoxide
DNGR-1	NK lectin group receptor-1
ECD	PE-Texas Red

ELISA	Enzyme linked immunosorbent assay
α-MEM	α-Minimal essential medium
EP	Electroporation
EXO	Exosomes
FasL	Fas ligand
FCS	Fetal cattle serum
FITC	Fluorescein isothiocyanate
Flt3-L	Fms-like tyrosine kinase 3-ligand
FoxP3	Fork-head/winged helix transcription factor
FR-4	Folate receptor 4
GA	Glatiramer acetate
GATA-3	GATA transcription factor 3
GITR	Glucocorticoid-induced TNF receptor-related protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp100	glycoprotein-100
Her-2/neu	Tyrosine kinase-type receptor/ human EGF receptor
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
i.v.	intravenous
IFN-α	Interferon-α
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL-1	Interleukin 1

IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17	Interleukin 17
IL-18	Interleukin 18
IL-21	Interleukin 21
IL-23	Interleukin 23
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factors
JNK	c-jun-N terminal kinase
KLH	Keyhole limpet hemocyanin
LC	Langerhans cell
LN	Lymph node
LOX-1	Lectin-like oxidized low-density lipoprotein receptor-1
LPS	Lipopolysaccharide
McAb	Monoclonal antibody
MAGE	Melanoma antigen
MART-1	Melan-A/melanoma antigen recognized by T cell
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex

MUC-1	Mucin-1
MyD88	Myeloid differentiation factor 88
NK	Natural killer
NTA_3-DTDA	3(nitrilotriacetic acid)-ditetradecylamine
NY-ESO-1	A member of cancer /testis antigen
OVA	Ovalbumin
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood monouclear cells
PBS	Phosphate buffered saline
PE	R-Phycoerythrin
pMHCI	peptide-MHC complex (SIINFEKL-H-$2K^b$ complex)
PMV	Plasma membrane vesicles
PSA	Prostate specific antigen
PSMA	Prostate specific membrane antigen
PTD	Protein transduction domain
RGS5	Regulator of G protein signaling 5
SAPK	Stress-activated protein kinase
s.c.	subcutaneous
ScFV	Single chain full-length variable Ab fragments
TAA	Tumor associated antigen
TAK1	Transforming growth factor beta-activated kinase 1
TCR	T cell receptor
Tg	Transgenic
TGF-β	Transforming growth factor-beta
Th	T helper

Th1	T helper type I
Th2	T helper type II
Th17	T helper type 17
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TLR-1	Toll-like receptor 1
TLR-2	Toll-like receptor 2
TLR-3	Toll-like receptor 3
TLR-4	Toll-like receptor 4
TLR-5	Toll-like receptor 5
TLR-6	Toll-like receptor 6
TLR-7	Toll-like receptor 7
TLR-8	Toll-like receptor 8
TLR-9	Toll-like receptor 9
TLR-10	Toll-like receptor 10
TLR-11	Toll-like receptor 11
TLR-12	Toll-like receptor 12
TLR-13	Toll-like receptor 13
TMB	3,3' ,5,5' -tetramethylbenzidine
TNF-α	Tumor necrosis factor α
Tr	Regulatory T cells
TRAG-3	Taxol resistance associated gene-3
TRAF	TNF Receptor Associated Factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule

TRIF	TIR-domain-containing adapter-inducing interferon-β
TRP	Tyrosinase-related protein
TSA	Tumor specific antigen
2-ME	2-mercaptoethanol

1.0 LITERATURE REVIEW

1.1 Introduction on dendritic cells

Dendritic cells (DCs), first recorded as Langerhans cells (LCs) in the skin in 1868 and initially characterized about 35 years ago as accessory cells for antibody production, are now found to be the strongest and unique antigen-presenting cells (APC) ever reported. Their ability to recognize, acquire, process and present antigen to naïve, resting T cells for the induction of antigen-specific immune responses surpass that of any other kind of APCs including macrophage, B cells, other leukocytes (1-5). DCs can even directly kill some tumor cells by expressing Fas ligand (FasL), granzyme B, perforin, tumor necrosis factor alpha (TNF-α) and TNF-related apoptosis-inducing ligand (TRAIL) (6-9); Very recently a new type of DCs called interferon-producing killer DCs, which share some characteristics of DC and natural killer (NK) cells and produce substantial amounts of type I interferons (IFN) and interleukin (IL)-12 or IFN-gamma (IFN-γ), depending on activation stimuli, was identified (10-13), although later this kind of cells were further confirmed to be activated natural killer (NK) cells (14, 15). Although DCs comprise only a small proportion (0.1-1%) of the cells in different lymphoid and non-lymphoid tissues (5), they are widely distributed in the body and divided into numerous subsets. Currently they are classified as conventional (c)DC ($CD11c^{high}$ and $MHCII^{high}$) and plasmacytoid (p)DC ($CD11c^{int}MHCII^{low}CD11b^{-}CD205^{-}CD123^{+}$) (16), with conventional

DC further grouped into $CD8\alpha^+$, $CD8\alpha^-$, dermal, monocyte-derived DC based on their function and marker differences (17). A third population of DCs, uniquely present in the lymph node but absent from the spleen or thymus, corresponds to migratory DCs including Langerhans cells and interstitial DCs that migrate from peripheral tissues through the lymphatics (18). DCs were first grouped as myeloid and lymphoid DCs according to whether they have markers related to lymphocytes (1). However, this lineage-based classification system has been discarded because both $CD8\alpha^+$ and $CD8\alpha^-$ DCs can arise from clonogenic common myeloid progenitors in both thymus and spleen (19, 20). $CD8^-CD11c^+$ precursor in the spleen could be committed to $CD8\alpha^+$ dendritic cells (21, 22), and DC subsets with function similar to spleen $CD8^+$ and $CD8^-$ dendritic cell could be induced in bone marrow cultures with Fms-like tyrosine kinase 3 ligand (Flt3 L), not just isolated from spleen (23). Both plasmacytoid and conventional dendritic cell subtypes could be developed from single precursor cells derived *in vitro* and *in vivo* (24, 25). Cross-priming, the ability of certain antigen-presenting cells to take up, process and present extracellular antigens with MHC class I molecules to CD8 T cells, has been credited to conventional myeloid DCs with the exception of the pDCs, meaning that DCs have the capacity to take up, process, and present exogenous antigens in association with MHC class I molecules (4). pDCs have been reported to have little or no T cell–stimulatory ability (26), but to promote the generation of regulatory T cells (27, 28), depending on the time after activation that pDC function is assessed. However, other contradictory results have shown that pDCs have the same capacity to stimulate anti-virus $CD4^+$ and $CD8^+$ T cells responses (29) and that pDCs use 'ready-made' stores of major histocompatibility complex (MHC) class I to rapidly present exogenous antigen to $CD8^+$ T cells. This meant that pDCs can also do the cross presentation (30). Even the antigen specific B cells has also been reported to cross-present immune-stimulating complex-associated cognate antigen (31) and cross

present the CpG-OVA to naive CD8 T cells (32); which means that cross-priming is widely existed among different APCs. Traditionally DCs have been considered to be an end-stage cells which only survive several days after activation and maturation (33), but this concept has been challenged by the report that culture-generated mature DCs can further differentiate under the influence of stromal cells (34).

DCs are the most potent antigen presenting cells that exist in virtually every tissue. However, most DCs that reside in lymphoid organ are phenotypically and functionally immature, most likely for the purpose of maintaining peripheral tolerance (35, 36) . Langerhans cells (LCs), which were the first characterized DC, capture antigens in the periphery during their immature stage, migrate to the lymphoid organs and present those captured antigens during their mature stage. When DCs are immature, they express very low level of MHC class II molecules, and costimulatory and adhesion molecules such as CD40, CD54 [inter-cellular adhesion molecule 1 (ICAM-1)], CD80 (B7.1) and CD86 (B7.2), etc. After meeting with exogenous and endogenous stimulation, they become mature and up-regulate the expression of above –mentioned antigen presentation machinery. They interact with antigen specific CD4 and CD8 T cells through the binding of MHC peptide with TCR, CD80/86 with /CD28 (37-40), CD54 with lymphocyte function-associated antigen-1 (LFA-1) (41, 42), CD40 with CD40L (43-46), OX40 with OX40L (47), 4-1BB with 4-1BBL (5, 48), and CD70 with CD27 (49-54), etc, and activate the naive T cells with the help of cytokines such as IL-2, IL-4, IL-12 and many more.

For the activation of naive $CD8^+$ T cells by APC, currently there are two popular mechanisms: cross-priming and direct priming. The major differences between cross-priming and direct priming are the source of Ag and the cell type that presents the Ag to the responding $CD8^+$ T cells. In cross-priming, professional APCs, such as DCs acquire exogenous Ag by endocytosis, pinocytosis and phagocytosis, process the Ag into

peptides and load them onto MHC I, then finally present them to the antigen specific $CD8^+$ T cells. In direct priming, the APCs, which may or may not be DC, synthesize, process their endogenous Ag, and present it by themselves to $CD8^+$ T cells. Recently a third mechanism, called cross-dressing was reported (55). DC directly acquire MHC class I-peptide complexes generated by other dead donor cells by a cell contact-mediated mechanism, and present the intact complexes to naive $CD8^+$ T cells. The activated $CD8^+$ T cells are restricted to the MHC class I genotype of the donor cells and are specific for peptides generated by the donor cells. *In vivo* studies demonstrate that the cooperation between cross-priming and cross-dressing are required for the optimized priming of $CD8^+$ T cells. Thus, cross-dressing might be an important mechanism by which DCs process inefficiently cross-presented antigens for priming of naive $CD8^+$ T cells. When DCs were injected *in vivo*, people found that a majority of resident DCs expressed donor MHC molecules and that a proportion of injected DCs acquired host MHC molecules. The bidirectional MHC molecule exchange between migratory and resident DCs (56) might provide a new clue for how DCs exchange antigen-MHC complexes and amplify the antigen presentation *in vivo*, and also showed that DC could acquire MHC class I-peptide complex from live cells, contrary to the Dolan et al's conclusion (55).

1.2 Mouse dendritic cells

Mouse DCs were first discovered in mouse spleen and named as DCs based on their unique morphology (57). However, because DCs are normally present in extremely small numbers in the circulation and tissue, their research didn't make significant progress for almost two decades until the development of *in vivo* and *in vitro* expansion of DCs. Recent advances in DC biology have allowed the development of

methods to generate large numbers of these cells *in vitro* (58-65). Because preparing dendrite cells preparation from bone marrow (BM) was the most efficient and popular way, most studies on DCs were based on this type of DCs. At the beginning of DC *in vitro* cultures, people used a cocktail of McAbs including anti-CD4, CD8, B220 and Ia antibody and rabbit complement to eliminate lymphocytes and I-a positive cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was used as the only growth factor to promote the formation of DC from bone marrow progenitors. After 1 week, more than 5 x 10^6 DCs from precursors within the large hind limb bones of a single animal can be produced; however, other cells such as granulocytes and macrophages were also generated and the DCs yield was relatively low (58). Scheicher et al first used anti-Ia antibody coated beads to isolate the cultured DC with low concentration of GM-CSF and cell purity up to 95% could be obtained (63). Lutz et al improved upon the above standard method to propagate 1–3 x 10^8 BM-DCs per mouse at 90–95% purity after 10–12 days. The achievement of higher cell yields is based on several modifications of the standard culture conditions: i) Avoid any antibody and complement treatment to preserve more precursors, ii) Reduce the plating density of bone marrow cells, iii) Extend the culture period to 10–12 days, iv) Decrease the concentration of GM-CSF from 200U/ml to 30-100U/ml from day 8 or 10 onwards to reduce granulocyte contaminations (60). Garrigan et al first used GM-CSF and IL-4 to culture mouse bone marrow DCs and found that BM DCs were superior to spleen DC in processing native protein (66). Labeur et al compared different combination of cytokines to culture BM DCs and found that DCs cultured in GM-CSF alone were immature. They found that lipopolysaccharide (LPS) and CD40L can make cells become mature, while DCs under GM-CSF+IL-4 with or without Flt3 ligand (Flt3L) exhibited intermediate maturation. When cells were further treated with CD40L, this group exhibited strongest antitumor capacity (67), which indicated generation of tumor

immunity by bone marrow-derived DCs is closely related to the DC maturation stage. DCs derived in GM-CSF plus IL-4 express cell-surface antigens typically associated with DCs, including DEC205, MHC class II, CD80, and CD86, and demonstrate potent allo-stimulatory activity (67). Son et al directly used BM cells without removing those attached cells as Labeur did (67) and did not use antibody and complement to remove other cells (58, 68). Culturing BM precursors using GM-CSF+IL-4 and six-well plates, around 30–40 x 10^6 /mouse DCs with 85–95% purity and more mature phenotype were obtained on day 7 (65). Although bone marrow cultures in the presence of GM-CSF with or without IL-4 resulted in the differentiation of DCs with phenotypical and functional characteristics that are similar to those described for human monocyte-derived DCs, there are still differences. In order to get data directly related to the human monocyte-derived DCs, research on DCs from mouse peripheral blood was very much needed, but there are very few reports because of limited amount of mouse blood. One paper claimed that from the blood of one mouse $1x10^6$ DC can be generated with GM-CSF (69). Another paper compared the mouse monocyte-derived DCs (MODC) prepared with GM-CSF and IL-4 as growth factors with bone marrow-derived DC and found that they displayed similar morphology, phenotype and immunostimulatory activity (70). The isolation and characterization of mouse MODC will provide more information on fundamental aspects of DC biology and which DC subsets are most suitable to induce anti-tumor immunity.

Mouse pDCs express low level of CD11c along with B220 and Gr1, and after treatment with Flt3L, mouse pDCs can also become CD123 positive(71, 72). They have also been found in the spleen, BM, and LN of naive animals and their number could be increased by the *in vivo* injection of Flt3L or Flt3L and GM-CSF. Mouse $CD11c^+B220^+$ pDCs could be cultured with BM cells cultured in medium supplemented with 200 ng/ml recombinant human FL (23, 73) or M-CSF (74). They are immature

APCs with very low level expression of MHC class II and activation markers; they don't express IL3Rα(CD123) which is reported to be expressed by human pDCs (75). Although being poor T cell stimulators, they could differentiate into cDCs when cultured in medium containing CpG plus CD40L plus GM-CSF (73). Thus, the *in vitro* generation of murine pDCs may serve as a useful tool to further investigate pDC biology as well as the potential role of these cells in viral immunity and other settings.

A few researches have been done to use Flt3L as a single growth factor to culture mouse BM DCs and produce different subsets similar to those isolated from spleen (61). Although the expression of CD4 and CD8 markers were not detected before adding maturation modulators, function and systemic phenotype analysis including mRNA and protein level indicated that $CD45RA^{high}$ plasmacytoid DC, $CD24^{high}$ and $CD11b^{high}$ cDC subsets were generated in the culture. The last two subsets functionally correspond to $CD8^+$ and $CD8^-$ DCs in the spleen, indicating that we can culture spleen DC subsets using BM precursors without the need of time-consuming and cost ineffective isolation from the spleen (23).

For the studies on the DCs from tissues such as thymus, spleen, lymph node and liver, collagenase and/or DNase digestion were exploited to separate the DCs. During the purification, some added EDTA in the buffer to keep the cells from attaching to each other. However, some DC subsets might be very sensitive to the EDTA treatment and may become damaged. After red blood cells lysis, people use short time plate culture and prewarmed PBS washing to crudely separate the splenic DCs from most T and B cells. They harvest the attached cells and purify $CD11c^+$ DCs by using MACS beads technology, or by directly staining the cells and using the FACS sorter to isolate the $CD11c^+$ DCs or different subsets (76-78).

One way of increasing the number of DCs in the tissue is *in vivo* injection of the DC growth factors, such as GM-CSF, G-CSF, progenipoietin-1 (ProGP-1), or Flt3L into the

mice (78-80). Among the above growth factors, ProGP-1 is the strongest factor in increasing the overall DC numbers. The effects on the ratio change of DC subsets among spleen depend on the specific growth factor; ProGP-1 and Flt3L selectively expanded $CD8^+$ DCs, whereas GM-CSF mainly increased the number of $CD8^-$ DCs. However, all the growth factors except G-CSF expanded DCs have some functional differences compared with its counterparts in the wild type mice (80).

By studying the relationship of $CD8^+$ and $CD8^-$ DCs, It was found that both common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) could differentiate into $CD8^+$ and $CD8^-$ DCs, thus DCs could be derived from two distinct differentiation pathways-myeloid or lymphoid (19). Various cytokines or growth factors such as GM-CSF, IL-4, TNF-α, and Flt3L have been reported to be involved in DC development. The first three were later found to be dispensable, while Flt3L remained critical to the development of DCs (81). GM-CSF and stem cell factor (SCF) were essential growth factors for the generation of DCs from CMP, whereas IL-7 was an essential cytokine for inducing the differentiation of CLPs into DCs (20). DCs located in peripheral lymphoid organs are renewed from circulating bone-marrow-derived precursors while Langerhans cells are derived from proliferating precursors that are present in the skin. However inflammatory changes in the skin can induce the recruitment of blood-borne progenitors with Langerhans-cell differentiation potential (82). Induction of T-cell immunity against microbial infection involves the recruitment of DC precursors to lymphoid tissues and their local differentiation into DCs (83). Recently more evidences have indicated that DCs could differentiate from either lymphoid or myeloid precursors depending on the environmental conditions, such as the location and the presence of different exogenous and endogenous stimuli (23, 83). Now the early precursors for all DC subtypes are thought to be within the BM $Flt3^+$ precursor populations, regardless of their lymphoid or myeloid lineage orientation (84). In mouse

blood, a $CD11c^+$ MHC class-II^- DC-restricted precursor population, which can fully reconstitute splenic $CD8^-$, $CD8^+$ and plasmacytoid $B220^+$ DC subpopulations, and lack lymphoid- or myeloid-differentiation potential, has been recently described (85). More recently a clonogenic common DC precursor (CDP) in mouse bone marrow was identified for the first time. The CDPs can develop and become, at a single-cell level, cDCs and pDCs, but not other cell lineages, *in vitro*. Phenotypically, CDPs express the receptor for Flt3L, the receptor for macrophage colony-stimulating factor (M-CSFR) and the receptor for stem cell factor (c-Kit), but they don't express specific lineage markers (Lin^-), CD11c and MHC-II. After transfer to nonirradiated 'steady-state' mice, CDPs develop exclusively into $CD8^+$ DCs, $CD8^-$ DCs and pDCs in the peripheral lymphoid organ such as spleen and in the lymph node. Under *in vitro* culture condition, Flt3 ligand alone could make CDPs differentiate into DCs and pDCs, but M-CSF, a cytokine that is essential for macrophage differentiation(18), could not support the survival or differentiation of CDPs. In addition, when Flt3L were co-injected into the animal, CPDs expand and differentiate into all DC subsets *in vivo* (25). A clonal DC precursor (pro-DC) that closely resembles the CDP phenotypically and functionally was also identified in the mouse bone marrow lately. Pro-DCs are defined phenotypically as $Lin^-CD11c^-MHC\text{-}II^-Flt3^+M\text{-}CSFR^+c\text{-}Kit^+$ similar to CDPs. Pro-DCs give rise at a single-cell level to pDCs and cDCs *in vitro*, whereas pro-DCs injected into nonirradiated mice give rise to pDCs, $CD8^+$ and $CD8^-$ DCs not only in peripheral organs such as spleen, but also in the bone marrow (24). Therefore, the direct precursors of DCs were identified, which might solve the dilemma of the identities of haematogenous DC precursors.

For the mouse DC research, two phenotypically and functionally distinct subpopulations- $CD8^-$ and $CD8^+$ DCs were identified at the beginning. They are located at different areas in spleen and Peyer's patches, where $CD8^-$ DCs reside in antigen uptake

zone and $CD8^+$ DCs stay in T cell area. Only $CD8^-$ DCs could induce B-cell activation and plasmablast differentiation (86, 87). Although only $CD8^+$ DCs could internalize apoptotic cells (88), $CD8^-$ DCs have higher capacity of endocytosis and pahgocytosis than $CD8^+$ DCs (89). *In vivo* $CD8^+$ DCs secrete much higher level of IL-12 to induce strong Th1 response, while $CD8^-$ DCs mainly elicit Th2 response (90, 91). However, other studies have found that the ability of driving Th1 and Th2 response is not totally depending on specific DC subsets. Both subsets reside in the spleen as immature cells and become mature upon culture *in vitro* in GM-CSF-containing medium or *in vivo* in response to lipopolysaccharide. Only after maturation can DCs acquire their T-cell priming capacity. Matured $CD8^+$ DCs and $CD8^-$ DCs can activate naïve $CD4^+$ and $CD8^+$ T cells with equal potency and efficiency; thus environmental factors may have the ability to influence the Th responses of DC subsets qualitatively (92). These factors include the activation state of the DCs, the nature of the antigen, the concentration of antigen, the type of receptor that is responsible for antigen uptake and specific cytokines, such as IL-10 and IFN-γ (83, 93). For example, $CD8^-$ DCs could produce IL-12 and induce the production of Th1 cytokines under defined experimental conditions, such as in the absence of IL-10 or after CD40L-mediated activation. At low antigen doses $CD8^+$ DCs induced the production of Th2 cytokines, and at high antigen dose, $CD8^-$ DCs could also induce Th1 response like $CD8^+$ DCs (94, 95). In the spleen, exogenous antigen is preferentially presented by $CD8^+$ DCs to $CD8^+$ T cells and by $CD8^-$ DCs to $CD4^+$ T cells. $CD8^-CD11b^-$ DCs in mesenteric lymph node (MLN) and $CD8^+CD11b^+$ DCs in peripheral lymph nodes (PLN) mainly cross-present OVA to $CD8^+$ T cells in their respective tissues in MLN and other PLN. Regardless of tissue origin, $CD8^-CD11b^+$ DCs generally present OVA to CD4 T cells. $CD8^-CD11b^+$ DCs in MLN and $CD8^+CD11b^+$ DCs in PLN present OVA to both $CD4^+$ T and $CD8^+$ T cells. Therefore, DCs' anatomical environment as well as their

phenotypes might also determine the antigen-presenting capacity of each distinct DC subset (96).

In spleen, DCs comprise around 1% of the total splenocytes. Among them, approximately 80% are cDCs and the remaining 20% are pDCs. Those cDCs have been classified into three subsets based on CD4 and CD8α expression on their surface and are named as $CD4^+8^-$ DC, $CD4^-8^+$ DC and $CD4^-8^-$ DC (76) ; each represents about 50%, 25% and 20-25% of the spleen cDCs, respectively. $CD4^-8^+$ DC are located in T cells areas and require interferon response factor (IRF8/ISCBP) and Id2 for their normal development and function, while $CD4^+8^-$ DC reside in non-T cell zone and need IRF4, IRF2, Relb and PU.1 for their normal development and function (83, 97, 98).

$CD4^-8^+$ DCs have been reported as variably effective stimulators of allogeneic $CD8^+$ T cell responses and $CD4^-8^-$ and $CD4^+8^-$ DCs as more effective in stimulating $CD4^+$ T cell responses (99, 100). When splenic $CD8\alpha^+$ DCs isolated from mice infected with Chlamydia muridarum were transferred into the naive mice, they induced better protective immunity than $CD8\alpha^-$ DC (101). $CD4^-8^+$ and $CD4^-8^-$ DCs can efficiently prime male Ag-specific CTLs, whereas $CD4^+8^-$ DCs do so only weakly (99). The $CD4^-8^+$DC makes itself unique among cDCs by having the exquisite cross-presentation ability to constitutively present exogenous cell-associated or soluble proteins very efficiently in the context of MHC I (100, 102). However, when antigen was presented as immune complex and DCs were activated via FcR ligation, $CD8^-$ DCs could also do the cross presentation (102). Different subsets have also been reported to play some inhibitory role in certain type of immune response. $CD4^+8^-$ DCs mediate tolerance or bystander suppression against diverse T cell specificities and support effective suppression of autoimmunity (103), while $CD4^-8^+$ DCs can induce tolerance to tissue-associated Ags and inhibit the transplantation rejection (104, 105). However, the

differences in inducing antitumor immunity among the three DC subsets remain to be explored.

During my research on this specific area, we found that bulk spleen DCs induced lower antitumor immunity when compared with the $CD4^+8^-$ DC and $CD4^-8^+$ DC subsets indicating that there are some interactions among those three subsets. The $CD4^-8^-$ DCs were found to be less mature even after overnight culture, and primed $CD4^+$ T regulatory 1(Tr1) cells to suppress the antitumor immunity (106), despite reports that spleen DCs will mature after culturing *in vitro* for 6 hrs or longer (66, 107, 108). The other two subsets stimulated strong Th1 response and resulted in immunoprotection from tumor challenge when those DC subsets were pulsed with low, physiological similar concentrations of OVA protein. The above data strongly suggested that DCs should be made functionally homogeneous and mature in order to be used as vaccine against tumor challenge or cancer treatment.

1.3 Human dendritic cells

Human DCs mostly originate from bone marrow-derived leukocytes (109); but Follicular DCs are of stromal origin and don't belong to leukocytes. Follicular DCs have no role in T cell, NK cell, or NKT cell immunity like conventional or plasmacytoid DCs. They mainly activate B cells in the germinal center without the need of T cell help through engagement of CD21 in the B-cell coreceptor complex by complement-derived CD21 ligand (110).

Different human DCs might have different phenotypes or functions depending on the culture conditions including the growth factors and time *in vitro*. At least four types of human DCs have been described such as conventional or "myeloid" DCs: i) $CD14^+$ blood

monocyte-derived DCs (MODCs); ii) dermal DCs or interstitial DCs (DDC-IDCs); ii) Langerhans cells (LCs); and iv) plasmacytoid DCs (31). Another special blood $CD11c^+$ DCs could be prepared by cell sorting from peripheral blood mononuclear cells and cultured just overnight in RPMI 1640 medium supplemented with autologous or pooled AB serum without adding any specific growth factors. Those DCs are lineage negative, $CD11c^+$, $CD86^+$, and HLA-DR^{bright} and express CD83 after activation by brief overnight culture; unlike MODC, they don't express CD209. Blood $CD11c^+$ DCs present low dose of antigen more efficiently and induce stronger Th1 response than MODC; therefore, blood DCs may be a better DC choice for clinical trials (111).

Because of the limitation of the quantity of human DCs, many studies on *in vitro* cultures have been carried out. Sallusto et al first combined interleukin-4 (IL-4) and GM-CSF to culture human peripheral blood mononuclear cells (PBMC) to prepare DCs and found that this kind of DCs can efficiently present soluble antigen, which means DCs were further activated and enriched by the presence of IL-4 (112). Functional human DCs were even generated from adherent peripheral blood monocytes by CD40 ligation in the absence of granulocyte-macrophage colony-stimulating factor (113). Later, immunomagnetic selection using a semi automated clinical scale immunomagnetic column was used to purify $CD14^+$ monocytes from PBMC as more specific source for human DC cultures (114, 115). Other have used human $CD34^+$ haematopoietic progenitor cells of the bone marrow and peripheral blood (116) cultured with c-kit ligand, GM-CSF, and TNF-α (117) or with Flt3L, c-Kit Ligand, GM-CSF, IL-4, and TNF-α (118) and adherent cells of cord blood by culture with GM-CSF, IL-4, and TNF-α (119) to generate functional antigen-presenting cells resembling mature monocyte-derived DCs.

Recently people are very interested in short-term culture DCs (FastDC). This type of mature DCs derived from human $CD14^+$ monocytes could be prepared within 48 hours: first 24hrs with GM-CSF(1000 U/ml) and IL-4 (500 U/ml), and next 24 hrs with addition of proinflammatory mediators (TNF-α, IL-1β, IL-6, PGE2) (120, 121), or uses calcium-mobilizing agents to drive differentiation of fully mature DCs from $CD14^+$ monocytes in only 2 days (122, 123). In the beginning, people were doubtful about short term culture DCs' ability to induce antitumor immunity; however, many studies have since confirmed that they could be as powerful as standard cultures monocyte-derived DCs (121, 124, 125). As such, the cost of research and clinical trials could be significantly decreased.

Human plasmacytoid pre-DCs (pDCs) are lineage-negative $CD11c^-CD33^-IL3R^+$ DCs with high power of IFN-α production. Their precursors in the circulation were identified as lineage negative, HLA-DR ^{bright}, $BDCA\text{-}2^+$, $BDCA\text{-}4^+$ and $CD123^{bright}$ cells (75, 126). $CD11c^+B220^+$ pDCs express TLR7 and TLR9, while classical $CD11c^+B220^-$ DCs preferentially express TRL2, TLR3, and TLR4. Although being poor T cell stimulators, they could differentiate into cDCs when cultured in medium containing IL-3 and CD40 ligand (CD40L) (75).

1.4 Dendritic cell maturation

DCs exist *in vivo* mostly as immature cells expressing very low level of CD80/86, CD40, and MHC II. Only under certain conditions such as inflammation can DCs become mature. When DCs are immature, they have a strong capacity of phagocytosis, and can therefore efficiently uptake antigen. However, at this stage, they have a very weak ability to present antigen, and thus usually induce tolerance. In order to provoke

effective immune responses such as anti-intracellular pathogen and tumor immunity, it's very necessary for DCs to become mature and develop strong antigen presenting machinery by increasing the expression of co-stimulatory molecules (CD40, CD80/86), adhesion molecules (CD54) and MHC II. They also need to secrete type I cytokines such as IL-12 to induce Th1 response resulting in high levels of antigen specific CTL to finally eliminate the pathogen or tumor cells.

DCs express the broadest repertoire of TLRs through which they can recognize a plethora of microbial compounds. After challenge with microbial or inflammatory stimuli, immature DCs undergo a complex process of maturation, resulting in their migration from tissues to secondary lymphoid organs and up regulation of MHC and costimulatory molecules that are essential for naïve T cell priming (127, 128). There are many different environmental stimuli that can mature DCs in different ways. Microbial products including lipid, polysaccharide, DNA, RNA, and protein are now recognized as various TLR ligands. They can react with different TLRs on both plasmacytoid and conventional DCs and induce maturation (127). CD40L (CD154), either expressed by activated T cells or as a multimeric recombinant protein, or agonistic anti-CD40 antibody can also mature DCs (129-131). Special formulas for culturing the matured and Th1-polarized DCs (Th1 DC) have been also explored. IL-1β (25 ng/ml), TNFα (50 ng/ml), IFNγ (1,000 units/ml), IL-6 (1,000 units/ml), PGE_2 (10^{-6} mol/L), poly-I:C (20 μg/ml), IFNα (3,000 units/ml), and LPS (250 ng/ml) were added to the PBMC culture started with *rhu* GM-CSF and IL-4 (both 1,000 IU/ml) at day 6 until day 8. A single round of *in vitro* sensitization with the above DCs induced up to 40-fold higher numbers of long-lived CTLs against melanoma-associated antigens (132). Another Th1 DC cocktail [CpGs (1 μmol/L), poly(I:C) (20 μg/ml), and/or IFN-γ (20 ng/ml)] was added to 5-day cultured BM DCs with GM-CSF and IL-4 for culturing another 18 hrs. Those DCs secreted high level of IL-12p70 and its maturation and polarization were not

affected even coculture with live B16 melanoma tumor cells, and tumor-loaded DCs induced delayed-type hypersensitivity responses *in vivo*. When DCs were loaded with B16 melanoma cells and injected into tumor-bearing mice, Th1-skewed tumor-specific $CD4^+$ T cells and a significant reduction in tumor growth were observed (133). One notable point is that IL-12 secretion level is not always related to the Th1 and CTL response, because data *in vitro* and *in vivo* have demonstrated that robust immune response could be induced with DCs matured in the presence of PGE2, even though PGE2 matured DCs secrete much lower level of IL-12 than CD40L matured ones (134, 135). However, the vaccination of cancer patients with DCs matured in the presence of IL-1β, TNFα, IL-6, and PGE_2 can expand their $FOXP3^+$ Treg cell population (136). Recent findings suggest that DCs matured with cocktail containing PGE_2 will have stable Tr-attracting properties mediated by CCL22, which could be further elevated after secondary stimulation of DCs in a neutral environment. IFNα, a mediator of acute inflammation, could restore the ability of the PGE_2-exposed DCs to secrete the Th1-attracting chemokines: CXCL9, CXCL10, CXCL11, and CCL5 by decreasing the secretion of CCL22 inudced by PGE_2; therefore, any formula for culturing MODC better contain IFN-α in order to reduce the ability to attract and induce Tr cells (137).

Very recently, people have been paying more attention to what maturation stage is better for the induction of antitumor immunity *in vivo*. Through directly analyzing their IL-12 secretion or by using microarray, people found that after *in vitro* activation by LPS, or by poly(I)-poly(C), and TNF-α plus IL-1β, DCs produced IL-12, a cytokine directly involved in the generation of CTL responses (138). This occurred only for a limited period with a peak between 5 and 8 h, and after 18 h there is no more IL-12 secretion from DCs. Those DCs become resistant to further stimulation by CD40L, and later this phenomenon was called DC exhaustion (139, 140). Animal studies also further support the above conclusion. 3 h anti-CD40 or 8 h LPS stimulated BM DCs induced 100%

protection, while 24 h anti-CD40 or 48 h LPS DCs didn't induced any protection at all (141-143).

In addition, suitably matured DCs represent a critical source of interleukin 6 (IL-6), IL-12, IL-15, IL-18, IL-23 and IFN-α – all of which are key members in innate immune responses and drive T helper type 1 (Th1) polarization (144-146). Under certain conditions, IL-12 secretion might not be the key polarization cytokine in these specific DC subsets (145, 147, 148). Macrophage-derived DCs have strong Th1-polarizing potential mediated by beta-chemokines rather than IL-12 (149). IL-12 production by DCs is tightly controlled, as it requires first a priming signal provided by microbial products or IFN-γ and then an amplifying signal provided by T cells through CD40 ligand (CD40L). IL-6 has been found to inhibit the function of regulatory T cells (150, 151); IL-6, IL-15 and IL-23 are among those key cytokines involved in the development and maintenance of a new T cell subsets Th17 (115, 152). Although Th17 has been confirmed to be the major player in autoimmunity and anti-bacterial infection instead of Th1 (153), there are still reports that Th17 need to synergize with Th1 to induce EAE (154). Th17, which secrete IL-21 (155), has been indirectly linked to antitumor immunity (156, 157), suggesting that Th1 and Th17 might cooperate in antitumor immunity. Very recently, Tumor-specific Th17-polarized cells have been found to be able to eradicate large established melanomas, providing direct evidence of Th17 involvement in antitumor immunity (158). Thus, DCs are capable of integrating signals from pathogens, cytokines and T cells, leading to the generation of an adaptive immune response of the appropriate class (144).

1.5 Antigen loading onto dendritic cells

With the availability of large number of cultured DCs from bone marrow precursor, $CD14^+$ peripheral blood monocyte and $CD34^+$ progenitor cells, various ways of antigen loading onto DCs have been tested in animal studies and clinical trials.

1.5.1 Synthetic CTL peptide

Tumor-antigen(s)-pulsed DCs have been demonstrated to induce MHC-class I- and class II-specific T cell responses *in vitro* and *in vivo*. DCs pulsed with peptide antigen *in vitro* and administered to animal induce antigen-specific, cytotoxic T lymphocyte (CTL)-mediated protection against a lethal tumor challenge and sustained regression of established tumors (159). Peptide-pulsed DCs can also convert the *in vivo* CTL-tolerizing potential of the peptide into specific immunostimulation (160).

1.5.2 Tumor lysate/acid-eluted peptides

Most cancer patients lack an identified tumor antigen and/or cannot provide sufficient tumor tissue for antigen preparation. They would lose the chance of treatment with cancer vaccines based on using either specific tumor antigens or mixtures of tumor-derived antigens in the form of peptides or proteins isolated from tumor cells. Compared with vaccination strategies directed against a single tumor antigen peptide, tumor lysates or acid-eluted tumor peptide as a better source of antigen could induce wide range of epitope-specific CTL and might be another choice for those tumors which antigen have not been characterized yet. Vaccines using antigens present in tumor cell lysates induce protective immunity with strong memory against distantly related tumor variants; however, it's possible to induce autoimmunity at the same time. The existence of a class of antigens shared among tumor variants provides an attractive target for

vaccine development (161). Electroporation (EP) has been used to achieve the safe, consistent, and efficient loading of DCs with whole tumor lysate and induced much better animal protection than conventionally coincubation protocols (162).

1.5.3 Tumor RNA

In addition to tumor peptides and lysates, DCs can also be pulsed or transfected with ribonucleic acid (RNA) coding for a tumor-associated antigen or whole tumor RNA to induce potent antigen and tumour-specific T-cell responses directed against multiple epitopes. Antigen in form of RNA carries the advantage of encoding multiple epitopes for many HLA alleles, thus permitting the induction of CTL response among many cancer patients independent of their HLA repertoire. Vaccination with the mRNA of tumor cells would extend the scope of vaccination to this group of patients as well because tumor mRNA, isolated from murine tumor cell lines or from primary human tumor cells microdissected from frozen tissue sections, can be amplified without loss of function (163-165). Conventional RNA/DC co-culture or liposome mediated loading for RNA transfection leads to very low ratios of transfected DCs. However, the recent developed square-wave electroporation technique for RNA transfection has significantly improved its efficiency with up to 90-99% of the viable cells transfected (166, 167). Proteasomal targeting of mRNA encoding cotranslationally ubiquitinated antigen was found to enhance intracellular degradation of target protein, and result in more efficient priming and expansion of TAA-specific $CD8^+$ T-cells (168).

1.5.4 Apoptotic tumor cells

Another way of pulsing DCs is using the apoptotic tumor cells induced *in vitro* with drugs or irradiation, because DCs express specific receptors for recognizing and uptaking

dying cells, such as αVβ5, CD36, or phosphatidylserine receptors for apoptotic cells (169, 170) and CD91 receptor for heat shock proteins exposed on necrotic cells (171, 172). After phagocytosing apoptotic/necrotic tumor cells *in vitro*, DCs can become mature and efficiently induce antitumor immunity (173). The advantage of using dying tumor cells as a source of tumor antigens are: (i) that DCs can present or cross-present both MHC class I and II epitopes of a defined tumor antigen (174)) or multiple tumor antigens (e.g., MAGE3 and gp100 of melanoma tumors) (169, 175); and (ii) unlike the case with peptide-pulsed DCs, this type approach is independent of HLA halotype and can thus be applied equally to all patients. Although the apoptotic cells are processed and presented through classical pathways for MHC II presentation to $CD4^+$ T cells and also are cross-presented through a phagosome-cytosolic pathway or released into the cytosol for presentation to $CD8^+$ T cells, uptake and ingestion of apoptotic cells mainly promotes an immunosuppressive environment that avoids inflammatory responses to self-antigens (176). It appears that the continuous presentation of self-peptides is required for active maintenance of T cell tolerance and prevents the occurrence of autoimmunity (177).

1.5.5 Tumor and dendritic cell fusion

DC/tumor fusions, like the tumor RNA pulsed DCs, might express a broad array of tumor Ags presented in the context of DC-mediated costimulation. One concern regarding the DC/tumor cell fusions is that tumor cells in the vaccine preparation may interfere the function of DCs as APC. People have recently found that fusions of breast carcinoma with DCs expanded regulatory T cells. They needed to add exogenous IL-12, and CpG during their DCs and T cells coculture to decrease the formation of Tr and to promote the

expansion of activated tumor specific T cells (178). This indicate that even after fusion with tumor, DCs still need the Th1 adjuvant to help them to form a Th1 response, although fusioned DCs have a matured phenotype and even secrete high amount of IL-12.

1.5.6 Targeting antigen to the dendritic cells

Recently DCs modified with genes such as tumor (associated) antigens and /or immunomodulatory proteins, and combined with cytokines further improved their capacity to promote the antitumor response (179). The more promising advance in DC vaccine against cancer is to target antigen directly to *in vivo* DCs or their specific subsets. This strategy is directly exploiting *in vivo* antigen presentation cells without the need of expansion and other treatment of APCs *in vitro*. Antibody response has been known to enhance the specific T cell response by promoting the opsonization of antigen via Fc receptor (FcR)-mediated recognition by APCs. Accordingly, targeting antigen to the APC's surface molecules is another potential route to enhance the immune response mediated by T cell such as antitumor and intracellular pathogen immunity. Several targeting delivery methods have been reported. One way is using the bispecific antibody which can bind the specific antigen and the receptor on the APC simultaneously (180). Other methods similar to this procedure use genetic methods to: (i) express the single chain antibody against APC surface molecules and antigen as a fusion protein (181, 182), (ii) to conjugate the antibody and antigen together (183-185), and (iii) to insert the antigen peptide into the constant region of the single chain antibody (186, 187). Apart from Ab targeting technology, cell-penetrating peptides (CPP) such as HIV TAT protein transduction domain (PTD), HSV-1 VP22 (188) and adenylate cyclase (CyaA) (189) have also been used to conjugate with antigen peptides or generate fusion protein to

target DCs (190, 191). People have compared the CPP and antibody targeting strategy using *in vitro* study and found that there is no advantage of cell-penetrating peptides over receptor (DC-SIGN)-specific antibodies in targeting antigen to human DCs for cross-presentation (192). Another strategy is to directly express antigen only in DCs by using mouse dectin-2 gene promoter to construct a lentivector. Potent $CD8^+$ and $CD4^+$ T-cell responses were also effectively induced after immunization with the optimal dose of the dectin-2 lentivector (193). The first eight targeting methods need the help of adjuvants to promote the maturation of DCs and induce desired Th response; the other four methods don't need the help of adjuvants to elicit effective immune response, though the response could be enhanced by simultaneous administration of them.

1.5.6.1 CD11c

A variety of different receptors on APCs have been exploited for targeting antigens, which may allow optimization for specific immune responses. The complement receptor CD11c/CD18, a surface receptor expressed almost exclusively on DCs and related to antigen internalization, is believed to play a crucial role in the process of antigen capture and presentation. A novel chelator-lipid, 3(nitrilotriacetic acid)-ditetradecylamine (NTA_3-DTDA) anchoring histidine-tagged forms of single chain full-length variable Ab fragments (ScFv), which target the CD11c of DCs , onto either tumor-derived plasma membrane vesicles (PMV) or onto antigen and LPS/IFN-γ-containing stealth liposomes were used to immunize mice, strong antigen-specific CTL responses in splenic T cells and a marked protection against tumor growth were obtained (194). When antigen was linked with anti-CD11c antibody and used for vaccination, fast and high antibody response were induced as well (195).

1.5.6.2 DEC205 (CD205)

DEC-205 is an endocytic receptor with 10 membrane-external, contiguous C-type lectin domains and is expressed at high levels on $CD8^+$ DCs in the T cell areas of lymphoid organs. It mediates the efficient processing and presentation of antigens on MHC class II products *in vivo*. Small doses (<1 μg/mouse) of anti-DEC-205 antibodies can be used to target antigens for presentation by DCs *in vivo*. When an antigenic peptide from hen egg lysozyme was engineered into form a fusion protein with anti-DEC-205 antibody, the antibody and peptide is selectively delivered to DCs (181). When protein antigen was targeted to the DC receptor DEC-205 in the steady state, antigen was presented through major histocompatibility complex class I and leads to peripheral $CD8^+$ T cell tolerance (183). However, if agonistic anti-CD40 antibody was injected into mice to mature DCs at the same time with targeted antigen, a single low dose of antibody-conjugated ovalbumin initiated immunity from the naive $CD4^+$ and $CD8^+$ T cell repertoire, and antigen presentation on MHC I was extended up to 2 weeks. These immunized mice showed better resistance to an established, rapidly growing tumor challenge and showed therapeutic effects on grown tumor with size of 0.7-1cm in diameter. Improved resistance to viral infection at a mucosal site was also seen. This suggests that antibody-mediated antigen targeting via the DEC-205 receptor increased the efficiency of stimulating T cell mediated immunity, including systemic and mucosal resistance in disease models (184). When the melanoma antigen tyrosinase-related protein (TRP)-2 was conjugated to anti-DEC-205 antibodies, mice immunized with these conjugates together with DC-activating oligonucleotides (CpG) showed long-lasting antitumor immunity (185).

1.5.6.3 MR (CD206)

The mannose receptor (MR; CD206) is a member of the calcium-dependent lectin receptor (CLR) family which have characteristic carbohydrate recognition domains with selective binding to specific glycans. Because of its strong role in endocytosis and phagocytosis, MR has been suggested to play a dual role in host defense and homeostasis. *In vivo* MR is primarily expressed on tissue macrophages (Mϕ) and lymphatic and hepatic endothelia in humans and mice, and also by subsets of DCs, especially interstitial DCs. *In vitro* cultured DCs from human monocytes and mouse BM also express this type glycan receptor, which indicates that this type of receptors could be used as a target for *in vivo* antigen delivery. By using hMR transgenic (hMR Tg) mice, anti-hMR antibody(B11)-OVA(B11-OVA) fusion proteins were found to be efficiently presented to OVA-specific $CD4^+$ and $CD8^+$ T cells in MR Tg, but not in non-Tg, mice. TLR agonist, CpG, significantly enhanced effector differentiation of responding T cells in MR Tg mice. Administration of both CpG and B11-OVA to hMR Tg mice induced OVA-specific tumor immunity while unimmunized WT mice remained unprotected. This confirmed that antigenic targeting of the human mannose receptor induced strong antitumor immunity (196).

1.5.6.4 Langerin (CD207)

Langerin/CD207 is a type II transmembrane protein, which was first detected on the Langerhans cells (197). Later its expression was also found at lower levels on DCs, especially $CD8^+$ $DEC205^+$ DCs in spleen and skin draining lymph nodes, although different species have varying expression levels (198). Langerin recognizes various

sugars such as mannose and sulfated sugars and are involved in endocytosis (197). Abs to Langerin can be internalized, which indicated that langerin could be used as a target for antigen delivery to DCs. Very recently, a fusion protein containing antibody against extracellular domain of langerin and OVA (model antigen) selectively and systemically targets appropriate DC subsets in draining lymph nodes and spleen. When injected *in vivo*, naive $CD8^+$ and $CD4^+$ T cells undergo 4–8 cycles of division in 3 days which indicated that OVA is efficiently presented. Compared with DEC205 antibody and OVA hybrid, antigen presentation detected by *in vivo* T cell proliferation could last a little longer, around 14 days, and is more efficient on MHC II (199). This means langerin can effectively mediate Ag presentation and is another candidate for targeting antigen to the DCs *in vivo*. However, there is no data on whether DC maturation stimuli are needed for better antigen presentation.

1.5.6.5 DC-SIGN (CD209)

DC-specific intercellular adhesion molecule 3 (ICAM-3) – grabbing nonintegrin (DC-SIGN) is a member of the type II C-type lectin family and an endocytic receptor mediating antigen presentation. In humans, DC-SIGN is exclusively and highly expressed on professional APCs such as DCs residing in lymphoid tissues, skin, mucosal surfaces, and also on specialized macrophages in placenta and lung (200, 201). This makes DC-SIGN a good candidate for targeting antigen to APCs *in vivo*. When a humanized antibody, hD1V1G2/G4 (hD1), directed against DC-SIGN was cross-linked to a model antigen, keyhole limpet hemocyanin (KLH), people found that the chimeric antibody-protein complex (hD1-KLH) bound specifically to DC-SIGN and was rapidly internalized and translocated to the lysosomal compartment. TLR ligands such as LPS were required to stimulate hD1-KLH pulsed DCs to become mature because the

conjugates themselves could not induce DC maturation. Autologous DCs pulsed with hD1-KLH induced 100-fold stronger proliferation of immunized patient peripheral blood leukocytes (PBLs) than KLH-pulsed DCs. hD1-KLH–targeted DCs also induced proliferation of naive $CD4^+$ and $CD8^+$ T cells, thus, targeting of antigen to DCs via anti-DC-SIGN antibody effectively induced antigen-specific primary and secondary response (201). Because of the significant differences between human and mouse homologue [CD209a (CIRE)], no animal study data is available (202).

1.5.6.6 Dectin-1

Dendritic cell-associated C-type lectin-1 (Dectin-1) is an NK cell receptor-like C-type lectin-like receptor (CLR), originally thought to be DC specific, but subsequently demonstrated to be expressed on other cells, including certain macrophage populations, neutrophils, and monocytes. Dectin-1 was shown to be expressed on $CD8\alpha^-CD4^-CD11b^+$ DCs found in spleen and lymph nodes and dermal DCs present in skin and s.c. lymph nodes. In human, Dectin-1 has been found to be involved in uptake and cross-presentation of cellular antigens (203). Injection of Ag-anti-Dectin-1 conjugates with poly I:C induced $CD4^+$ and $CD8^+$ T cell and Ab responses at low doses where free Ag failed to elicit a response. Compared with antibody targeting Ag to CD205, anti-Dectin-1 conjugates stimulated a much stronger $CD4^+$ T cell response and a much weaker $CD8^+$ T cell response, whereas anti-CD205 conjugates stimulated no antibody response. This indicated that Dectin-1 might be a better potential targeting molecule for immunization to promote Th2 or antibody response (204).

1.5.6.7 Dectin-2

Dectin-2 is a C-type lectin-like receptor encoded in the natural killer complex of C-type lectin genes and is expressed on cells of the myeloid lineage including spleen DCs and peritoneal macrophages. It recognizes high-mannose structures and acts as a pattern recognition receptor for fungi. A lentivector expressing the human melanoma antigen NY-ESO-1 under the control of dectin-2 promoter was constructed and injected into the HLA-A2 transgenic mice. Most of the cells expressing the model antigen were $CD11c^+$ DCs. A NY-ESO-1-specific $CD8^+$ T-cell response was primed and a $CD4^+$ T-cell response to a newly identified NY-ESO-1 epitope presented by H2 $I\text{-}A^b$ was also induced simultaneously. Optimized immunization with the dectin-2 lentivector can induce similar responses like those stimulated by a lentivector containing a strong constitutive viral promoter, thus, targeting antigen expression to DCs can provide another way of antigen preparation and direct delivery to the APCs *in vivo* (193).

1.5.6.8 DNGR-1

NK lectin group receptor-1 (DNGR-1) is a C-type lectin of the NK cell receptor group expressed in mice at high levels by $CD8^+$ DCs, at low levels by plasmacytoid DCs, and not by other hematopoietic cells. Its expression is also restricted to a small subset of human blood DCs that have similarities to mouse $CD8\alpha^+$ DCs. *In vivo* study, antigen epitopes covalently coupled to an antibody specific for mouse DNGR-1 were found to be selectively cross-presented by $CD8\alpha^+$ DCs and induced potent CTL responses. It also prevented development or mediated eradication of B16 melanoma lung pseudometastases when given with adjuvants. Because of its selective expression pattern and endocytic activity, DNGR-1, a novel, highly specific marker of mouse and human DC subsets,

could be used as a specific candidate for antigen targeting to DCs and be exploited for CTL cross-priming and tumor therapy (205).

1.5.6.9 CD36

CD36 is a Type BI scavenger receptor family member, which was thought to be expressed exclusively on the $CD8\alpha^+$ subset of blood-derived conventional DCs (206, 207), is also expressed by other APC including monocytes/macrophages and B lymphocytes (208). CD36 is involved in phagocytosis of apoptotic bodies and presentation of antigen to the $CD8^+$ T cells (209), and probably presentation of Ags derived from apoptotic bodies on MHC class II molecules (210). CD36 was originally reported to be unessential for MHC class I cross-presentation of cell-associated antigen by $CD8\alpha^+$ murine DCs (206, 207). When antibody against CD36 and OVA protein hybrid (anti-CD36-OVA) was tested in Ag presentation assays *in vitro* and *in vivo*, people found that anti-CD36-OVA was capable of delivering exogenous Ags to the MHC class I and MHC class II processing pathways. After immunization with anti-CD36-OVA, strong naive $CD4^+$ and $CD8^+$ Ag-specific T lymphocytes activation and the differentiation of primed $CD8^+$ T cells into long-term effector CTLs were induced. Compared with anti-DEC205-OVA, anti-CD36-OVA was much better at inducing long-term persistence of effector CTLs and did not require the addition of exogenous maturation stimuli. Anti-DEC205-OVA was however more efficient in inducing early events of naive $CD8^+$ T cell activation. Vaccination with anti-CD36-OVA also provides protection against Ag-specific tumor challenge, which is mediated through both humoral and cell-mediated immunity (182). The results indicated that CD36 could be a novel and better potential target receptor for delivery antigen to $CD8\alpha^+$ DCs.

1.5.6.10 LOX-1

LOX-1(Lectin-like oxidized low-density lipoprotein receptor-1) is one of the scavenger receptors for HSP binding on human DCs. A neutralizing anti-LOX-1 McAb has been reported to inhibit Hsp70 binding to DCs and Hsp70-induced antigen cross-presentation. Using anti-LOX-1 McAb to target tumor antigen to LOX-1 *in vivo* could completely prevent the tumor growth without administration of additional maturation stimuli. Thus, the scavenger receptor LOX-1 is another promising candidate for targeting tumor antigen to *in vivo* DCs for cancer immunotherapy (211)

1.5.6.11 Mac-1

Mac-1 is present on myeloid DCs, macrophages, neutrophils, and NK cells. Bordetella pertussis adenylate cyclase (CyaA) delivers directly its N-terminal catalytic (adenyl cyclase (AC)) domain into the cytosol of eukaryotic cells bearing the $\alpha_M\beta2$ integrin (CD11b/CD18) (Mac-1). Both *in vitro* and *in vivo* studies have shown that $CD8^+$ and $CD4^+$ T cell epitopes inserted into the AC domain of a genetically detoxified CyaA could be delivered into the cytoplasm of $CD11b^+$ DCs (212, 213). This kind of vaccine could induce robust Th1 type responses (214). $CD8^+$ T cell activation does not require $CD4^+$ T cell help, nor the CD40 signalling (212), but TLR ligands (CpG, poly I:C and polyuridine, not R484) and cyclophosphamide enhance the therapeutic efficacy of CyaA-E7 fusion protein on advanced tumors. Thus, CyaA appears to be a safe and potent vehicle for *in vivo* Ag delivery to $CD11b^{high}$ DCs, leading to Th1 polarized CD4 and $CD8^+$ T cell priming (189, 213).

1.5.6.12 Other targets

Using chemokine ligands to target antigen to the DCs has also been reported. 37-kDa immature laminin receptor protein (OFA-iLRP) and MIP3α/CCL20 or β-defensin mDF2β (chemoattractant ligands of CCR6) fusion protein could induce antitumor immunity even without additional DC maturation stimulation (215). By preparing immune-complexes (IgG-antigen) (IC) to target the FcγR on the DCs, mouse DC maturation and efficient MHC class I/II-restricted presentation of peptides from exogenous, IgG-complexed antigens were induced (216). ICs should efficiently sensitize DCs for priming of both $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes *in vivo*.

1.6 Dendritic cell polarization

For cancer immunotherapy, Th1 response is preferred because it promotes CTL formation and finally kills the tumor cells. In order to induce Th1 immune responses, DCs must be first polarized meaning that they must be treated to become optimally mature, otherwise Th2 or Tr response will be induced, inhibiting antitumor immunity. During the process of DC maturation, interactions between CD40 (DC) and CD40L (T cells) and/or TLR and TLR ligands play a critical role in the polarization of DCs and their induced immune response. Therefore, CD40L or agonistic anti-CD40 Ab, TLR ligands, and their combination have been extensively explored to promote BM DCs maturation/polarization and induction of Th1 type antitumor immunity.

1.6.1 CD40 ligand and agonistic anti-CD40 antibody

CD40, the receptor for CD40 ligand (CD40L), is expressed on APCs such as B cells, macrophages, and DCs with its expression increasing with maturation. CD40L is a member of the TNF family and is expressed primarily on activated Th cells. The CD40 and CD40L interaction induces the maturation and activation of DCs, and helps TLR ligands to stimulate DCs to secrete cytokines such as IL-12, which are essential for the development of the Th1 response (217, 218). Agonistic anti-CD40 Ab can function as CD40L and modulate DCs to drive naive $CD8^+$ T cells, while ligation of CD40 on DCs restores CTL activity in $CD4^+$ depleted mice. In addition, mice treated with anti-CD40L Ab had impaired induction of Ag-specific CTLs. These data indicate that CD40-CD40L interactions play a key role in modulating APC function so that these cells can prime $CD8^+$ T cells *in vivo* (219).

1.6.2 Toll-like receptor ligands

Different TLRs share the same or exploit distinct signaling pathways and result in various cellular responses. TLR3 induce IFNF-β only through TRIF- and IKK-related kinase and activation of TRAF3 and IRF3 and seems to be independent of MyD88 pathway. Other TLRs such as TLR1, 2, 4, 7/9 induce cytokine production and activation of molecules via MyD88 recruited IRAKs and TRAF6. The TRAF6 resulted in activation of TAK1 that phosphorylates and activates the IKK-complex, causing NF-κB to be released and translocated to the nucleus. At the same time, TAK1 activates stress-activated protein kinases (SAPK) pathways and c-Jun-NH2-kinases (JNK) and

p38, thus culminating in the activation of the transcription factor, activator ptrotein-1(AP-1), which plays a crucial role in the induction of inflammatory-response genes. TLR4 also activates cells after binding LPS through MyD88-independent pathway via TRAM and TRIF. This makes it a unique TLR and results in some special physiological or pathological distinction (220, 221). Another important recent discovery on TLR signaling is that MyD88-dependent pathway also activates some IRFs; when plasmacytoid DCs react with TLR7/9 ligands, IRF7 were activated, while when conventional DCs react with their own TLR9 ligands, IRF1 were activated. IRF5 might be activated during the reaction of TLR3,4,7/8 ligands with all kinds of DCs (222).

Toll-like receptors (TLRs) are characterized as type I transmembrane protein with leucine-rich repeats in the extracellular domain and a cytoplasmic Toll/interleukin (IL)-1 receptor homology (TIR) domain. As innate receptors, they sense microbial products and trigger DC maturation and cytokine production, thus effectively bridging innate and adaptive immunity. Around 10 to 13 TLRs have been proposed to be existed in most mammalian species. Different TLRs recognize one or more PAMPs (pathogen-associated molecular patterns), including lipopolysaccharide (LPS), lipid A, monophosphoryl lipid A (MPL), and HSPs (heat shock protein) (by TLR4); Mycobacterial cell wall fractions enriched for lipoarrabinomannan, Mycobacteria-derived monoacylated muramyl dipeptide derivatives, bacterial lipoproteins and lipoteichoic acids (by TLR2+TLR1, or TLR2+TLR6); peptidoglycan (by TLR2); flagellin (by TLR5); herpes simplex virus, the unmethylated CpG DNA of bacteria and viruses, insect DNA and vertebrate DNA in liposomes (by TLR9); double-stranded RNA (by TLR3); single-stranded viral RNA (by TLR7 and TLR8) and Toxoplasma profilin (by TLR11). Some synthetic ligands can also be recognized by certain TLRs, such as tripalmitoyl-cysteinyl-seryl-(lysyl)3-lysine (Pam_3CSK_4) by TLR1/2, Imidazoquinoline compounds [resiquimod (R-848), imiquimod, and oxoribine], 7-Thia-8-oxoguanosine and 7-deazaguanosine, ANA975, an

oral prodrug of isatoribine, SM-360320 S-27609, 3M-01 and 3M-03 by TLR7; 3M-01 and 3M-02, but especially 3M-02 and Poly-G10 by TLR8, Poly(I:C) and Ampligen (poly I:poly C12U) by TLR3, and CpG ODN, CpG-containing ODN, i.e., IMOs, and dumbbell-like covalently closed ODN (dSLIM-30L1) by TLR9. TLR 10, 12 and 13 ligands have not been identified or confirmed (127, 223).

Unlike other TLRs, TLR5 is not expressed on conventional DCs or macrophages in mice, but is expressed mainly on intestinal $CD11c^+$ lamina propria cells (LPC). $CD11c^+$ LPC detected pathogenic bacteria and secreted proinflammatory cytokines in a TLR5-dependent way (224). TLR10 is expressed in humans but not in mice, TLR8 has no function in mice, and TLRs 11, 12 and 13 are only identified in mice. TLRs 1, 2, 4, 5 and 6 (present on the cell surface) seem to specialize in the recognition of mainly bacterial products that are unique to bacteria and not made by the host. Their detection therefore affords a straightforward self-non-self discrimination. TLRs 3, 7, 8 and 9 (present in the endosomal compartment), in contrast, specialize in viral detection and recognize nucleic acids, which are not unique to the microbial world (144, 221, 223, 225-227). Like the TLR2 forms complex with TLR1/TLR6, TLR8 can interact with TLR7 or TLR9, and TLR9 can also interact with TLR7. However these interactions resulted in antagonistic effects. The TLR9-TLR7 interaction reduces TLR7 signaling, while the TLR8 ligands decrease the TLR7 and 9 induced signaling cascade (228). This indicated that not all the TLR ligands combinations can be used for enhancing APC maturation. Although most TLR ligands will activate DCs to stimulate Th1 response, it‘s also very important to remember that some TLR ligands will induce Th2 response. TLR2 and TLR5 agonists can provoke Th2 response under certain conditions and LPS at low doses will induce Th2 immune responses (229). Among the spleen DCs, our specific DC subset $CD4^-8^-$DCs express TLRs from TLR1 to TLR 9, but $CD4^-8^+$ DCs and $CD4^+8^-$ DCs don’t have TLR7 and TLR3, respectively (226), which means that almost

every kind of TLR ligands could be used to trigger and induce the maturation of $CD4^-8^-$DCs.

In vitro studies of the above mentined have also been confirmed by *in vivo* experiments. Murine renal cell carcinoma (RENCA)-pulsed CpG-ODN-treated DCs were able not only to significantly reduce tumor growth but also to prevent tumor implantation in 60% of mice (230). Poly(I:C) and CpGs treated DCs, which secrete very high levels of IL-12, loaded with B16 melanoma cells and injected into tumor-bearing mice induced Th1-skewed tumor-specific $CD4^+$ T cells and a significant reduction in tumor growth (133). When CpG or plus anti-IL-10 were injected inside the tumor, the tumor infiltrated immature DCs matured and induced the regression of established tumor. If the tumor cells were transfected with CCL20 (MIP-3α) or CCL16, chemokines for immature DCs, the antitumor immunity was even stronger than that to those tumors without transfection of those chemokine genes (231, 232). Another *in vitro* study showed that tumor-infiltrating DCs (TIDCs) were able to present tumor-associate antigen (TAA) in the context of major histocompatibility complex class I but that they were refractory to stimulation with the combination of lipopolysaccharide, interferonγ, and anti-CD40 antibody. However, TIDC paralysis could be reverted by *in vitro* or *in vivo* stimulation with the combination of a CpG immunostimulatory sequence and an anti-interleukin 10 receptor (IL-10R) antibody. CpG plus anti–IL-10R treatment enhanced the TAA-specific immune response, triggered de novo IL-12 production, resulted in robust antitumor therapeutic activity exceeding by far that of CpG alone, and elicited antitumor immune memory (233). Tumor necrosis factor alpha and CD40 ligand combination could also antagonize the inhibitory effects of interleukin 10 on T-cell stimulatory capacity of DCs (234)

1.6.3 Cooperation among CD40L and TLR ligands

The cooperation of TLR ligands and CD40L or anti-CD40 in induction of DCs to secrete IL-12 is very important in generating CTL from naïve polyclonal $CD8^+$ T cells *in vitro* (219). CpG and PPD need the help of CD40L to stimulate the spleen DC mixture, $CD4^-8^-$DCs and $CD4^-8^+$DCs, but not $CD4^+8^-$DCs to secrete IL-12. Peptide antigen could also enhance the IL-12 induction capacity of PPD (235). LPS could not efficiently stimulate bone marrow or monocyte-derived DCs to secrete IL-12 without the help of CD40L or T cell help (217, 236). Various TLR ligand combinations such as R848+LPS, R848+poly (I:C), LPS+CpG, and LPS+ poly(I:C), which react with TLRs on the cell surface and inside cellular compartments at the same time but separately, have been reported to stimulate higher level of IL-12 secretion than single ligand and reach the similar level of IL-12 induced by TLR ligands +IFN-γ (144, 237). DCs activated simultaneously through TLR-7 (and TLR 2/6,3,4, 7, 9 to some extent) and anti-CD40 displayed 10-fold increases in their ability to stimulate $CD8^+$ T cell responses over DCs activated through each individual receptor alone (238). Synergistic activation of macrophages via CD40 and TLR9 also results in T cell independent antitumor effects (239). Simultaneous activation of TLR-3 and TLR-9 on macrophages induced synergistic levels of nitric oxide, IL-12, TNF-α, and IL-6 production (240).

1.6.4 Antigen dose

There have been reports that the Th1 or Th2 response might not depend on the type of DCs but rather on the antigen concentration; higher doses stimulate Th1 response, while lower doses stimulate a more Th2 response. After TLR ligand stimulation, more Th1 responses were induced (93). We also found that high concentration of OVA could

switch the $CD4^-8^-$DC induced response from Tr to Th1 (241). However, such high concentrations are not practical for clinical trials.

1.7 Dendritic cell vaccine

Since DCs have the most powerful capacity to present antigen via MHC I to activate the CD8 T cells to form CTL and attack intracellular pathogen and tumor cells (3, 242, 243), DCs pulsed with various antigens and modification as described above have been used in animal study and/or put into clinical trials (2, 3, 179, 244, 245).

With the availability of large numbers of DCs cultured *in vitro* and identification of tumor specific or associated peptides, DCs have been pulsed with various human and mouse tumor peptide antigens to induce antitumor immunity. Various specific MHC-restricted synthetic peptides derived from tumor-associated antigens such as adipophilin protein (246), bcr-abl chimeric nonapeptide [GFKQSSKAL] (247, 248), carcinoembryonic antigen (CEA) (249-251), endogenous retroviral gene products gp70/p15E (252, 253), folate binding protein (FBP) (254, 255), Heparanase (256), hepatocellular carcinoma-associated antigen HCA587 (257), HER-2/neu oncoprotein (258-266), HPV 16 E7 (267), melanoma related antigen MART-1/ Melan A, MAGE-1, MAGE-3, gp100 (268), or tyrosinase (269-276), MUC-1 (277-280), multiple myeloma (281), NY-ESO-1 (282), ovarian tumor antigen stratum corneum chymotryptic enzyme (283), prostate-specific membrane antigen (PMSA) (284-288), regulator of G protein signaling 5 (RGS5) (289), survivin (290-292), and TRAG-3 (293) have been identified and/or used for pulsing the DCs for animal study or clinical trials. Various CTL responses and even temporary regression of diseases have been recorded. However, the disadvantage is that specific peptide for certain protein antigens must be first identified

and peptide-pulsed DCs induced CTL responses usually are very weak. Other antigen such as autologous lysates may be more effective (275), or DC preparation should be improved (276).

For those tumors in which antigenic peptides were not identified, a lot of studies have been carried out with tumor lysate. DCs coincubated with whole-tumor lysate can elicit specific antitumor T-cell responses. DCs pulsed with the tumor lysate from fibrosarcomas and mammary carcinoma (294), hepatocellular carcinoma BNL 1ME A.7R.1 (BNL) (295, 296), melanoma (297), pancreatic carcinoma (298), renal cell carcinoma (162, 299), and syngeneic GL261 gliomas (300, 301) have been tested in mice models. DCs pulsed with lysates from human breast carcinoma (302), malignant brain tumor (303, 304), myeloma (305), ovarian cancer (306, 307), and pancreatic carcinoma (308) have been demonstrated to induce tumor antigen-specific CTL responses *in vitro*, and the T cell response can be enhanced by KLH. Vaccination of patients with cancer using DCs pulsed with tumor lysates were also shown to be effective for advanced breast (309, 310), advanced gynaecological malignancies (311), hormone-refractory prostate carcinoma (312), malignant melanoma (269, 313), parathyroid carcinoma (314, 315), pediatric solid tumors (316, 317), renal cell carcinoma (RCC) (318-321), and uterine serous papillary cancer (322).

The mRNA from allogeneic prostate whole tumor (323), autologous melanoma (324), whole renal cell carcinoma (325, 326), whole pediatric brain tumor (327), and whole neuroblastoma (328); and synthetic mRNA from lung cancer and breast cancer(CEA) (329), prostate[prostate specific antigen(PSA) (330) and telomerase (331) have also been used to transfect monocyte-derived DCs for clinical trials. All the above trials indicated that this form of vaccination is feasible and safe, but elicited T cell responses varied in different trials from 0 to 100% of tested patients. Cytokine patterns after cancer

vaccination may be more complex than indicated by the classic Th1/Th2 dichotomy (332, 333).

In animal models, murine multiple myeloma, mastocytoma, breast carcinoma and renal cancer cell fusion with DCs have been used to vaccinate the animal and could protect against tumor lethal challenge and effectively eradicate established tumor (178, 334-337). Fusions of patient-derived breast carcinoma cells and DCs stimulated the formation of antigen specific CTL and lyse the autologous tumor cells *in vitro* (338). In a clinical trial for patients with metastatic breast carcinoma, clinical responses were only observed in a few of patients, although most patients developed some kind of antitumor immunity after vaccination with autologous DC/tumor fusions (337). However, most of the ongoing presently clinical studies using multiple rounds of immunizations have been reported to induce regulatory $CD4^+$ T cell response (339). Immature DCs or even mature DCs have also been claimed to expand the regulatory T cells and induce immunosuppression (340-344). Thus, the best conditions for carrying out clinical trials still need to be further explored, and more basic researches in this area are in great demand.

1.8 Exosomes

DCs process exogenous antigens (Ags) in endosomal compartments such as multivesicular endosomes (345) that can fuse with plasma membrane, thereby releasing Ag presenting vesicles called "exosomes" (EXO) (346, 347). Exosomes (EXO) are 50–90 nm diameter vesicles containing Ag presenting (MHC class I, class II, CD1, hsp70–90), tetraspan (CD9, CD63, CD81), adhesion (CD11b, CD54) and costimulatory (CD80 and CD86) molecules (348, 349), i.e. the necessary machinery required for eliciting potent

$CD4^+$ and $CD8^+$ T cell responses. Moreover, exosomes pulsed with tumor peptides have been reported to provoke T cell-dependent antitumor effects, and TLR-3 or -9 ligands could significantly increase the intensity of the induced immune response (346, 350). Mature BM DCs pulsed with exosomes could more efficiently induce OVA-specific cytotoxic T-lymphocyte responses, antitumour immunity and $CD8^+$ T-cell memory *in vivo* than exosomes themselves or DC pulsed with OVA protein. Moreover, exosome plused DC could also more efficiently eliminate established tumor (351).

Both human and mouse tumor cells constitutively secreted these membrane vesicles which are similar to DC-derived exosomes in their morphology, density and expression of certain membrane markers (MHC I, LAMP1, tetraspanins, HSP70-80). Tumor derived exosomes have been confirmed to contain whole native tumor antigens and could induce CTL *in vitro.* They also promote T cell-dependent cross-protection against syngenic and allogenic tumors in mice, thus this type of exosomes might be a source of tumor rejection antigens and could be used for pulsing DCs to vaccinate the related recipients. One clinical trial has found that autologous MODC pulsed with autologous ascites purified exosomes could expand tumor specific lymphocytes from peripheral blood cells. However, some data suggest that tumor-derived exosomes might have negative effects on the host's immune system. Tumor derived exosomes might suppress T cells response through their inhibitory components such as FAS-L (352), NKG2D ligands (353) or HLA-G (354), and they even could promote the tumor growth by dampening NK function (355). Other data indicated that exosomes released by human tumor can skew IL-2 responsiveness in favor of regulatory T cells and away from cytotoxic cells (356) and promote the differentiation of monocytes to myeloid-derived suppressor cells, thus support tumoral growth and immune escape (357), therefore exosomes released by tumors themselves should not be used alone as tumor vaccine.

1.9 T cell subsets (Th1/Th2/Th17/Tfh/Tr)

1.9.1 T helper cell subsets (Th1, Th2, Th17 and, Tfh)

Among the immune cells, different T cell subsets play a cooperative or inhibitory role on one another. $CD4^+$T cells play central roles in the regulation of immune responses by activating or suppressing immune cells and tissue cells. Originally, the adaptive immune system is thought to be controlled by two subsets of T helper (Th) cells, termed Thl and Th2, which preferentially promote cellular and humoral immunity, respectively, with their own specific cytokine profiles (358-360). Thl cells make IL-2, IFN-γ, and TNF-α, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Thl cells proliferate in response to IL-2 in an autocrine fashion, and Th2 cells proliferate similarly in response to IL-4. Mostly Th1 and Th2 regulate each other negatively. IL-4 and IL-10 inhibit Thl differentiation and proliferation, respectively, while IFN-γ is able to inhibit Th2 differentiation. Th1 cells enhance the innate and Th1-dependent immune responses of NK and $CD8^+$ T cells by secreting IFN-γ, ultimately leading to the elimination of intracellular pathogens and tumor cells. Th1 cells also play a significant role in systemic pathology such as autoimmune disease and delayed-type hypersensitivity responses. Th2 cells produce cytokines like IL-4, IL-5, and others which are the major helpers for B cell and Ab responses. These cells might also activate eosinophils (IL-5) to kill the parasite and may have a role in allergy and asthma. Recently a new type of Th17 were identified to play crucial roles in regulating tissue inflammation and the development of disease in several animal models of autoimmunity; the former culprit for autoimmune disease was cleared of its involvement (361). Th17 cells could be differentiated by culturing $CD4^+$ T cells with TGF-β and IL-6. A very recent study using serum-free medium concluded that human Th17 like its mouse counterpart also needs the presence of TGF-β for

differentiation (362), thus solving the dilemma whether there is any difference in growth factors for human and mouse Th17. IL-23 is necessary for the maintenance of the Th17 response (152).All the above three kinds of Th cells have their own differentiation mediated lineage-specific transcription mechanism. IL-12 activates the transcription factor signal transducer and activator of transcription 4 (STAT4) to regulate Th1-cell differentiation. TCR cross-linking and IL-12 induced signaling cascades finally lead to expression of the transcription factor T-bet, which enhances the secretion of IFN-γ and decreases the production of Th2 cytokines. T-bet is thus the master regulator of Th1-cell differentiation (363). By contrast, IL-4 promotes Th2-cell differentiation through the action of STAT6. This up-regulates expression of GATA-binding protein 3 (GATA3), which is both necessary and sufficient for Th2-cell development. GATA-3 is the key regulator of Th2-cell differentiation (364). Both Th1- and Th2-cell-specific transcriptional regulators (STAT1 and T-bet, STAT6 and GATA3, respectively) inhibit Th17 cell differentiation (365). For the Th17 cells, TGF-β and IL-6 activate STAT3and STAT4(366, 367) and then upregulated the expression of retinoic acid receptor-related orphan receptor gamma-T (RORγt) to differentiate naive T cells into Th17. RORγt is the Th17-specific transcriptional regulator, because expression of RORγt in naive T cells was both necessary and sufficient to induce IL-17A, IL-17F and IL-23R expression (368). $Rorc^{-/-}$ mice are not responsive to IL-23 and possess lower numbers of Th17 cells. RORα seems to synergize with RORγt to promote differentiation and function of Th17 cells, because *Rora-Rorc* double-mutant mice harbor few Th17 cells and are more resistant to inflammatory diseases (367).

Another major subset of nonpolarized effector T cells that provides help to B cells has been identified through microarray analysis and was named as T follicular helper (T_{fh}) cells. They home to the B cell areas of secondary lymphoid tissue, through interactions mediated via the chemokine receptor CXCR5 and its ligand CXCL13 (369). They

have their own specific CD markers such as CD84 and CD200, and the transcription factor BCL6. Tfh cells secrete cytokine IL-10 and IL-21. Tfh cell generation was regulated by ICOS ligand (ICOSL) expressed on B cells and was dependent on IL-21, IL-6, and signal transducer and activator of transcription 3 (STAT3). They provide cognate help to B cells for high-affinity antibody production in germinal centers (GC) through IL-21. IL-21 was identified as a key cytokine that promotes the development of these specialized effector cells. T cell-derived IL-21 promotes themselves expression of CXCR5, which directs these cells to interact with B cells in the follicular regions and germinal centers (370-372).

1.9.2 Regulatory T cells

The specific capacity of the immune system to recognize and eliminate various foreign invaders by the enormous diversity of antigen-specific receptors on B and T lymphocytes has to be balanced by mechanisms preventing reactivity against self-antigens. Several tolerance mechanisms are operating in parallel under physiological conditions for the silencing of T cells during their development in the thymus or in the periphery. Although cell-intrinsic processes leading to deletion or inactivation of autoreactive T cells were regarded as the most important tolerance mechanisms before 1990, regulatory T cells (Tr) are now considered to play a more dominant role in tolerance, controlling autoreactive T cells that have escaped deletion in the thymus. Those Tr cells are classified into naturally occurring Tr cells and those that can be induced by suboptimal antigenic stimulation (373). Naturally occurring $CD4^+CD25^+$ and $CD8^+CD25^+$ Tr cells are believed to be generated in the thymus, possibly by recognizing self-antigen on epithelial cells (374). The presence of the respective self-antigen has

been shown to be necessary for their survival in the periphery (373). Other types of Tr such as TCRαβ^{+} CD4^{-}CD8^{-} double negative regulatory cells (375), and TCRαβ^{+} CD4^{+}CD8^{+} double positive CD25 T regulatory cells (376) which maintain immune homeostasis by secreting granzyme B and perforin were also reported.

1.9.2.1 CD4^{+}CD25^{+} Tr, Tr1 and Th3

Contrary to Th cells, regulatory T cells (Tr), which suppress the immune system to prevent overactive responses and inflammation rather than help the response, have been getting more and more attention recently since naturally occurring thymic-derived CD4^{+}CD25^{+} Tr cells were isolated based on their expression of CD25 under steady state (377). CD4^{+}CD25^{+} Tr cells are a T cell population with immunosuppressive properties that constitutes 5–10% of the total peripheral CD4^{+} T cells. Besides the expression of CD25, they constitutively express other several activation markers, such as the glucocorticoid-induced tumor-necrosis factor (TNF) receptor-related protein (GITR), OX40 (CD134), L-selectin (CD62 ligand (CD62L)), neuropilin-1, and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152). Other markers have also been reported to be associated with CD4^{+}CD25^{+} Tr cells, such as folate receptor 4 (FR-4) (378), and CD39 and CD73 (379). The transcription factor forkhead box P3 (FOXP3) have been identified as a crucial transcription factor for the development and functionality of CD4^{+}CD25^{+} Tr cells and also a specific intracellular marker for the identification of Tr cells (380) (381); however, Foxp3 expression is not always related to human CD4^{+}CD25^{+} Tr cells (382). Notch and TGF-β signalling pathways cooperatively regulate Foxp3 expression and regulatory T cell maintenance both *in vitro* and *in vivo* (383), although Notch has been reported to be related to Th1. Currently three kinds of CD4^{+} Tr cells have been proposed; the naturally occurring CD4^{+}CD25^{+} Tr cells secrete

high level of IL-10 and express TGF-β, $CD4^+$ regulatory T cells of type 1 (Tr1) express high levels of IL-10 and moderate levels of IL-5, IFN-γ, and TGF-β, but no IL-2 and IL-4 (384), and T helper 3 (Th3) regulatory T cells express high levels of TGF-β (385). The last two Tr cells usually are induced by antigen specific stimulation, thus they are considered antigen specific. The first $CD4^+CD25^+$ Tr cells are non-antigen specific and need to be activated to exhibit its optimal inhibitory function (386, 387). Neuropilin-1 contributes to the prolonged interaction of regulatory T cells with DCs (388). CTLA-4, TGF-β and IL-10 were the mediators that are reported to be involved in $CD4^+CD25^+$ Tr cells induced immune suppression, and under most conditions, cell-contact was necessary for the $CD4^+CD25^+$ Tr cells to exhibit their inhibition. Other membrane molecules or soluble factors, however, need to be further explored. The proliferation test of naive $CD4^+$ and $CD8^+$ T cells stimulated with T cells-depleted splenocytes and anti-CD3 antibody has become a classic assay for identifying Tr cells. $CD4^+CD25^+$ could facilitate the induction of T cell anergy, which T cells lost the ability to produce IL-2 and proliferate (389), and express CD73 and FR-4. Naturally occurring $CD4^+CD25^+$ Tr cells cells have also been reported to directly induce conventional $CD4^+CD25^-$ cells to become suppressive cells by infectious tolerance and the newly formed $CD4^+$ suppressor cells inhibited proliferation of $CD4^+$ T cells either via IL-10 or TGF-β production, not via cell-contact (390, 391). $CD4^+CD25^+$ Tr cells could inhibit the BM DCs stimulated T cell to secrete IL-2, but could not prevent the stimulated proliferation (392). Human $CD4^+CD25^+$ Tr cells could increase the B7-H4 expression of human monocyte-derived DCs by enhancing its own secretion of IL-10 (393). Contrary to the naive T cells which promote the secretion of IL-6 and decrease the production of IL-10 after culture with DCs, $CD4^+CD25^+$ Tr cells have the opposite effects (394). Auto-antigen specific $CD4^+CD25^+$ Tr cells also could inhibit the naive T cells induced mouse spleen DC maturation, though spontaneous maturation of DCs was slightly increased (395).

Human $CD4^+CD25^+$ Tr cells reduced the expression of costimulatory molecules such as CD83, CD80, CD86, but not CD40 on DCs. They promoted DCs to secrete IL-10 directly , thus restraining the maturation and antigen presenting function of the DCs (396). When anti-CD25 antibody were injected into the mice to deplete the CD25 expressing $CD4^+CD25^+$ Tr cells, several progressively tumor growth were suppressed (397, 398). In addition, when tumor specific $CD8^+$ T cells were co-transferred with $CD4^+CD25^+$ Tr cells into the mice, their antitumor immunity was eliminated (399, 400). When low dose of cyclophosphamide was used to deplete the Tr cells *in vivo*, this treatment markedly enhance the magnitude of secondary but not primary CTL responses induced by DC derived exosome vaccines, and also improved immune response in other animal tumor models (401-403) and even in patients with metastatic melanoma (404). All the above data indicated that a direct link between Tr cells and reduced tumor immunity.

1.9.2.2 $CD8^+$ Tr cells

The existence of regulatory $CD8^+$ T cell subsets as an effectors of suppressor in models of autoimmune diseases, transplantation, and in protection against cancer has been proposed in 1970s and early 1980s (405, 406). However, because of lack of defining markers and difficulties in isolating these cells, those earlier reports proposed suppressive mechanisms have been discredited or ignored. Recently, with the discovery of $CD4^+CD25^+$ regulatory T cells as naturally existed regulatory cells and more advanced cell purification techniques, $CD8^+$ Tr cells again have attracted more and more attention among immunologists.

The formation of $CD8^+$ Tr cells has been reported to be naturally occurring or induced by *in vitro* cultures, almost using the same mechanism as that of $CD4^+CD25^+$ Tr

cells. Although $CD8^+$ Tr cells were proposed to play a very important role in *in vivo* inhibition and infectious immunosuppression as T suppressor, the first phenotypically characterized CD8 Tr cells were $CD8^+CD28^-$ (407) and Qa1-depencent $CD8^+$ regulatory cells (408). Multiple priming of human T cells in mixed lymphocyte cultures induced allospecific and xenospecific Ts which were $CD8^+CD28^-$, not $CD8^+CD28^+$. These $CD8^+CD28^-$ cells can specifically recognize the MHC class I antigens expressed by antigen-presenting cells (APC) used for *in vitro* immunization and prevent target APC to increase expression of CD80 and CD86, thus interfering with the CD28-B7 interaction required for T helper (Th) activation. Immature myeloid and CD40L primed plasmacytoid DCs were reported to induce the formation of $CD8^+$ Tr cells, which secrete IL-10, but mainly rely on cell-contact (340) or IL-10 (27) to carry out their inhibitory function. Haptenated apoptotic cells can induce $CD8^+$ suppressor T cells without priming $CD4^+$ T cells for immunity (409). Tolerogenic treatment of lupus mice with consensus peptide induces Foxp3-expressing, apoptosis-resistant, TGF-β-secreting $CD8^+$ T cell suppressors (410). When transgenic $CD8^+$ T cells were activated with OVA expressing antigen-presenting cell (APC) in the presence of exogenous transforming growth factor, 5-15% OT-1 T cells express FoxP3 and could mediate linked suppression of primary immune responses and cardiac allograft rejection (411). In rat, $CD8^+$ $CD45RC^{low}$ T cells were also naturally formed and had regulatory properties. They produce mainly IL-4, IL-10, and IL-13 cytokines upon *in vitro* stimulation and expresses Foxp3 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). However, they are not cytotoxic against allogeneic targets (412). Intestinal epithelial cells (IECs) are able to induce the proliferation of a small fraction of $CD8^+$ peripheral T cells. The $CD8^+CD28^-$ subset of IEC-activated $CD8^+$ T cells, which express CD101 and CD103, interact with IECs through gp180 and have regulatory functions (413). Vasoactive intestinal peptide generates human tolerogenic DCs that induce $CD4^+$ and $CD8^+$ Tr cells (414). Stimulation of OT-1

$CD8^+$ cells with OVA_{257}+IL-4+IL-12+dexamethasone (DEX) induced IL-10-secreting $CD8^+$ T cells. IL-4/IL-12/DEX can even induce an equivalent $IL\text{-}10^+$ phenotype from freshly isolated $CD44^{high}$ memory or $CD44^{low}$ naive OT-1 population (415).

In 1970, the suppressor T cells were thought to be $CD8^+$ T cells, but the purification of this type of T cells was impossible so there is almost no phenotype data about these specific T cells. During the past ten years, the progress in cell isolation techniques and the discovery of $CD4^+CD25^+$ regulatory T cells make it possible for us to know more about the phenotype of this rare type of T cells. Most cell surface and intracellular markers on $CD4^+CD25^+$ regulatory T cells such as CTLA-4, TGF-β, PD-1,CD25 were also detected on $CD8^+$ regulatory cells, and the relation to their function *in vitro* and *in vivo* were similar. $CD8^+$ regulatory T cells also have some specific markers which are related to their specific inhibition mechanism. The first phenotypically characterized CD8 regulatory cells was $CD8^+CD28^-$; by *in vitro* culture with alloantigen stimulation, $CD8^+$ T cells were differentiated into $CD8^+CD28^+$ and $CD8^+CD28^-$; the $CD28^-$ type T cells can inhibit the proliferation of the $CD28^+$ ones, which indicated that CD28 is a specific markers for identifying the regulatory cells from the mixed T cells culture. CD103 ($\alpha_E\beta_7$ integrin) was initially described to be expressed on both murine and human $CD8^+$ T lymphocytes localized in intestine, bronchoalveolar fluid, and allograft tissues. Recently, CD103 was shown to be a target of FoxP3 and was also found to be expressed on $CD4^+$ Tr cells. Now CD103 are found to be specifically expressed on alloantigen-induced $CD8^+$ regulatory T cells, not $CD8^+$ effector cells (416). CD122 is another marker which could be used to select the $CD8^+$ Tr cells for IL-2 receptor β chain deficient mice. $CD8^+CD122^+$ have been found in very young mice because of their role in prevention of abnormal T cells in CD122-deficient mice (417, 418). Although $CD4^+CD25^+$ T cells exist in this kind of mouse, they don't show any inhibitory function here (417). Some $CD8^+$ Tr cells were found to express TRAIL, granzyme, and

perforin (419), which are rarely reported ever on $CD4^+CD25^+$ Tr cells. A novel population of Qa-1-restricted $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ T cells was identified to be one of the $CD8^+$ Tr cells. They express higher level of CD69, CD40, CD94, TL-tetramer and TL-168(peptide), lower levels of CD122 compared with $CD8\alpha\beta^+$ OT-1, and express similar level of CD44, CD25 and CD28. Among them, CD94 is an anti-apoptosis molecule (420). Foxp3 were found to be expressed on naturally occurring $CD8^+$ Tr cells such as $CD8^+CD25^+$ and $CD8^+CD28^-$, those *in vitro* induced ones are not always having this specific transcription factors, which means that Foxp3 to $CD8^+$ regulatory T cells is not as important as that to $CD4^+CD25^+$ regulatory T cells. Although mRNA for GITR are detected in human $CD8^+CD25^+$ regulatory T cells, those molecules were not found to be expressed on their surface. With different models and different purification methods, phenotypes among those reported $CD8^+$ Tr cells varied significantly. Until now there is no single marker expressed on all reported $CD8^+$ Tr cells.

With the discovery of induced $CD8^+$ Tr cells, more efforts have been made to find the counterparts of $CD4^+CD25^+$ Tr cells. In human thymus, $CD8^+CD25^+$ thymocytes sharing phenotype, functional features, and mechanism of action with $CD4^+CD25^+$ T regulatory cells (421) were found. $CD8^+CD25^+$ Tr cells could be expanded by TCR stimulation with modified anti-CD3 McAb in diabetic patients (422). In old (423) and MHC $II^{-/-}$ (424) mice, $CD8^+CD25^+$ regulatory cells were found in thymus and peripheral lymph organs such as spleen and lymph nodes with higher ratios than younger mice and/or normal mice. The discovery of $CD8^+CD25^+$ Tr cells in human thymus and old mouse spleen would indicated that *in vivo* the development of Tr cells shares the same pathway, no matter the type of T cells is $CD4^+$ or $CD8^+$. CD25 marker should be suitable for identifying and purifying the Tr cells from the naive animals (421, 423). Although human thymus $CD4^+CD25^+$ and $CD8^+CD25^+$ don't proliferate and secrete cytokine after activation treatment, both show stronger inhibition on Th1 rather than Th2 (374); Mouse

thymic $CD8^+CD25^+$ Tr cells secrete various cytokines after activation and seems to be different from human $CD8^+CD25^+$ Tr cells (424). Another study reported that $CD8^+CD25^+$ T cells might be a type of memory cells in aged mice (425). With regards to its inhibitory mechanism, both human and mouse $CD8^+CD25^+$ Tr cells inhibit the naive T cell proliferation through cell-contact, but detailed studies are needed to be done whether this Tr cells share all the features with $CD4^+CD25^+$ Tr cells or they have their own special mechanism such as killing activity like Qa-1-restricted $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ T cells which kill the "antigen specific" $CD4^+$ T cells.

Based on antigen specificity, Tr cells can also be classified into another two groups. One is naturally occurring with no antigen specificity while the other is antigen induced and shows antigen specificity. However, some termed naturally antigen specific Tr cells have also been isolated (373, 426-428). The debates over origins of the antigen specificity are still unsolved (429, 430). Trogocytosis is a new name for "fast,cell to cell contact-dependent uptake of membranes and associated molecules". This phenomenon has been reported in almost every kind of cells, such as antigen-presenting cells(APC) and T cells. Cell-to-cell transfers of HLA-G make effector T cells act as regulatory cells (431). Double negative (regulatory) T cells in human being and mice could function antigen specifically to induce apoptosis and suppress proliferation of antigen-specific CTLs by acquiring peptide-MHC complexes (375, 432). $CD4^+$ and $CD8^+$ T cells can also acquire MHC-peptide complex from DCs to act as antigen specific APC to stimulate other T cells (433-435). We are interested in whether Tr cells can acquire the MHC-peptide complex to become antigen specific, and what molecules are key players in this antigen specificity transferring. $CD8^+CD25^+$ Tr cells (423) (424), which are found to be easily amplified *in vitro* by us, will be the model cells in our project.

2.0 HYPOTHESIS AND OBJECTIVES

RATIONALE

Since DCs have the most powerful capacity to present antigen via MHC I and activate the CD8 T cells to form CTL and attack intracellular pathogen and tumor cells (3, 242, 243), DCs pulsed with various antigens and modifications have been used in animal studies and/or advanced into clinical trials (2, 3, 179, 244, 245). However, most of the presently ongoing clinical studies using multiple rounds of immunizations have been reported to induce regulatory $CD4^+$ T cell response (339). Immature or even mature DCs have also been reported to expand the regulatory T cells and induce immunosuppression (340-344). Therefore, determing how to make the DC vaccine become more efficient in inducing Th1 response and inhibiting the Tr cell formation is the key research direction in cancer immunotherapy. There are three subsets of DC based on their expression of CD4 and CD8 in spleen. They are named as $CD4^+8^-$ DCs, $CD4^-8^+$ DCs, and $CD4^-8^-$ DCs. Inconsistent reports about their functional differences in inducing allogeneic T cell response, various immune tolerance and cross-presentation have been published (see review above). The ability of murine DC subsets to direct T helper cell differentiation is also dependent on microbial signals (436), which means that all three DC subsets could induce Th1 or Th2 under certain conditions. Antigen dose could also switch the direction of induced Th responses (93). Thus spleen DCs is a good model to study the differences in antitumor immunity induced by different types of DCs.

The data from this thesis is from the culmination of two research projects, broken into Part 1 and 2. The first part relates to spleen DCs subsets. It studies their functional differences in antitumor immunity and how to convert the tolerogenic $CD4^-8^-$DCs into immunogenic one and induce Th1 response. The second part is based on our discovery that $CD4^-8^-$DCs induce Tr1 rather than Th1 response. We are interested to know

whether and how Tr cells can acquire their antigen specificity by getting the membrane complex (exosomes) from antigen pulsed DCs.

2.1 Part 1: Phenotypic and functional characterization of spleen DC subsets and conversion of tolerogenic $CD4^-8^-$ DCs into immunogenic ones

HYPOTHESIS: Although there are a lot of papers published on spleen DCs, none of them studied the differences in antitumor immunity among the three subsets. Because different subsets have been reported to induce Th1 or Th2 response, some differences in inducing CTL response which lead to kill tumor cells might exist. By knowing the differences, we can explore various ways to make them become functionally homogenous and more efficacious, thus develop a new type of DC-based vaccine against cancer.

OBJECTIVES: In this thesis, part 1 focuss on: i) Setting up the methods for purifying splenic DCs and their subsets with MACS technology. ii) Phenotype and functional analysis of the three DC subsets and fresh splenic DCs. iii) Animal study using CD4 and CD8 knock out mice to study the role of $CD4^+$ and $CD8^+$ T cells in different DC subsets induced antitumor immunity. iv) Phenotype and functional analysis of $CD4^-8^-$ DCs subsets stimulated with different stimuli. v) Analysis of activated CD4 T cells induced by different DC subsets and differentially stimulated $CD4^-8^-$ DCs. vi) DNA array analysis of the differences between CpG and LPS treated $CD4^-8^-$ DC_{OVA}. vii) Study on IL-6 and IL-15 effects on the formation of Th17 and Th1 induced by CpG treated $CD4^-8^-$ DC_{OVA} using *in vitro* and *in vivo* assay.

2.2 Part 2: Inhibitory mechanism of $CD8^+$ $CD25^+$ regulatory T cells

HYPOTHESIS: Membrane transfer and exosomes release and acquisition are found between many cells. What happens after one cell receives the membrane molecules is attracting more attention. T cells that acquire membrane complexes from DCs can behave like APCs and even neutrophil can become APCs after absorbing antigen-MHC complex from exosomes. What would happen if the regulatory T cells get the antigen-MHC complex from antigen pulsed DCs? Will they become a DC-like T cells or will they become antigen-specific regulatory T cells enhancing its immunosuppressive function?

OBJECTIVES: In this thesis, part 2 focuses on: i) Purification and expansion of $CD8^+CD25^+$ T cells from naive mouse. ii) Characterization of this type T cells and confirmation that it's a type of regulatory T cells (Tr). iii) Study its effects on splenic DCs and naive T cells. iv) Purification and characterization of exosomes from DC pulsed with OVA (model tumor antigen). v) Study its effects of Tr cells after being pulsed with exosomes on CTL formation and function and compare with unpulsed Tr cells. vi) Further confirm the above results by doing animal studies.

3.0 MATERIALS AND METHODS (Methods, see details in manuscripts)

3.1 Reagents and Suppliers

Table 3.1 lists the reagents used in the experiments presented in this thesis. All of the reagents used were molecular biology or research grade. Table 3.2 lists the commercially available kits used in this study

Table 3. 1: List of reagents and suppliers

Reagents	Suppliers
Ammonium chloride	EM Sciences
Anit-Biotin MACS Beads	Miltenyi Biotec
Anti-CD4 MCAS beads	
Anti-CD8 MCAS beads	
BSA	Sigma
CFSE	Molecular Probes
Collagenase IV	Worthington Biochemical Corp
CpG 1826(TCCATGA*CG*TTCCTGA*CG*TT)	Synthesized by Operon
DMEM	Invitrogen
DMSO	Sigma
Dynal CD4 beads	Invitrogen
Dynal CD8 beads	
FCS	Hyclone, Invitrogen
Gentamicin Solution	Invitrogen
Glutamine solution	Sigma
G418	Invitrogen
GM-CSF	Peprotec
Hydrochloric acid	EM Sciences
IL-2	Peprotec
ODN 1892(TCCAGGACTTCTCTCAGGTT)	Synthesized by Operon
OVA I(SIINFEKL)	Synthesized by Multiple Peptide Systems
OVA II(ISQAVHAAHAEINEAGR)	
Mut-1(FEQNTAQP)	

RPMI 1640	Invitrogen
Sodium chloride	EM Sciences
Streptavidin-ECD	Beckman Coulter
Streptavidin-FITC	BD Biosciences
Streptavidin-PE	
Tris	EM Sciences
Trypsin-EDTA	Invitrogen
Tween 20	Bio-Rad
2-mercaptoethanol	Bio-Rad

Table 3. 2: Commercially available kits used in this study

Commercial Kits		Suppliers
ELISA Kits for	IFN-γ	BD Biosciences
	IL-2	
	IL-4	
	IL-6	
	IL-10	
	IL-12	
	IL-15	eBioscience
	IL-17	
	TGF-β	R&D Systems
	TNF-α	BD Biosciences
Fixation and Permeabilization Solution Kit with BD GolgiStop™		BD Biosciences
FoxP3 intracellular staining kit		eBioscience
PE-labeled H-2K^{b}/ SIINFEKL tetramer		Beckman Coulter
TMB Substrate Kit		BD Biosciences

3.2 Antibodies

Table 3.3 lists the various Abs and their respective suppliers used in the presentation of this thesis. Abs were labelled with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), or biotin.

Table 3. 3: Antibodies and their suppliers

Antibodies	Suppliers
Anti-mouse B7-H1	eBioscience
Anti-mouse B7-DC	
Anti-mouse B7-H3	
Anti-mouse B7-H4	
Anti-mouse CTLA-4	BD Biosciences
Anti-mouse H-2K^{b}	
Anti-mouse I-A^{b}	
Anti-mouse IFN-γ	
Anti-mouse IL-6	eBioscience
Anti-mouse IL-10	BD Biosciences
Anti-mouse IL-15	eBioscience
Anti-mouse IL-17	BD Biosciences
Anti-mouse CD4	eBioscience
Anti-mouse CD8	
Anti-mouse CD11c	BD Biosciences
Anti-mouse CD25	
Anti-mouse CD28	
Anti-mouse CD30	
Anti-mouse CD40	
Anti-mouse CD54	
Anti-mouse CD62L	
Anti-mouse CD69	
Anti-mouse CD73	eBioscience
Anti-mouse CD80	BD Biosciences
Anti-mouse CD86	

Anti-mouse FR-4	eBioscience
Anti-mouse FasL	BD Biosciences
Anti-mouse GITR	
Anti-human granzyme B	Serotec
Anti-mouse perforin	BD Biosciences
Anti-pMHCI	Dr. Xiang's Lab
Anti-mouse TGF-β	
Anti-mouse TLR-4	eBioscience
Anti-mouse TLR-9	
Anti-mouse TRAIL	BD Biosciences
Anti-rat IgG	Jackson ImmunoResearch
Anti-hamster IgG	

4.0 MAUSCRIPTS

4.1 $CD4^{-}8^{-}$ Dendritic Cells Prime $CD4^{+}$ T Regulatory 1 Cells to Suppress Antitumor Immunity

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Running Title: $CD4^{-}8^{-}$ DCs prime Tr1-mediated immune suppression

Key Words: $CD4^{-}8^{-}$ DC subset, TGF-β, Tr1, immune suppression, antitumor immunity

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Abbreviation: DC_{OVA}, OVA-pulsed DC.

4.1.1 ABSTRACT

It is clear that DCs are essential for priming of T cell responses against tumors. However, the distinct roles DC subsets play in regulation of T cell responses *in vivo* are largely undefined. In this study, we investigated the capacity of OVA-presenting $CD4^-8^-$, $CD4^+8^-$, or $CD4^-8^+$ DCs (OVA-pulsed DC (DC_{OVA})) in stimulation of OVA-specific T cell responses. Our data show that each DC subset stimulated proliferation of allogeneic and autologous OVA-specific $CD4^+$ and $CD8^+$ T cells *in vitro*, but that the $CD4^-8^-$ DCs did so only weakly. Both $CD4^+8^-$ and $CD4^-8^+$ DC_{OVA} induced strong tumor-specific $CD4^+$ Th1 responses and fully protective $CD8^+$ CTL-mediated antitumor immunity, whereas $CD4^-8^-$ DC_{OVA}, which were less mature and secreted substantial TGF-β upon coculture with TCR-transgenic OT II $CD4^+$ T cells, induced the development of IL-10-secreting $CD4^+$ T regulatory 1 (Tr1) cells. Transfer of these Tr1 cells, but not T cells from cocultures of $CD4^-8^-$ DC_{OVA} and IL-$10^{-/-}$ OT II $CD4^+$ T cells, into $CD4^-8^+$ DC_{OVA}-immunized animals abrogated otherwise inevitable development of antitumor immunity. Taken together, $CD4^-8^-$ DCs stimulate development of IL-10-secreting $CD4^+$ Tr1 cells that mediated immune suppression, whereas both $CD4^+8^-$ and $CD4^-8^+$ DCs effectively primed animals for protective $CD8^+$ CTL-mediated antitumor immunity.

4.1.2 INTRODUCTION

DCs efficiently collect, transport, and present Ags to T cells. Although all DCs express multiple surface markers related to their Ag processing and T cell stimulatory functions (e.g., MHC class II, CD40, CD80), they in fact comprise heterogeneous subsets that differ substantially in other surface markers, as well as their developmental origin and physiology. Murine splenic $CD11c^+$ DCs were originally characterized as being either $CD8^+$ or $CD8^-$ cells, with both reportedly priming allogenic $CD4^+$ and $CD8^+$ T cells *in vitro* (90) and Ag-specific $CD4^+$ T cells *in vivo* (90). Splenic $CD8^+$ DCs reportedly elicit Th1 and CTL responses via an IL-12-dependent mechanism *in vivo* (437), whereas $CD8^-$ DCs stimulate Th0/Th2 responses through their elaboration of IL-10 (90, 95). $CD8^+$ and $CD8^-$ DCs from Peyer's patches similarly induce Th1 and Th2 responses, respectively (438).

More recently, splenic $CD8^-$ DCs have been subdivided into $CD4^-$ and $CD4^+$ populations (76). Thus, $CD11c^+$ splenic DCs comprise three distinct subsets, with $CD4^+8^-$ cells representing ~50% of splenic DCs and $CD4^-8^+$ and $CD4^-8^-$ DCs each ~25% of the total population (76, 439). $CD4^-8^+$ DCs have been reported as variably effective (99, 100) stimulators of allogeneic $CD8^+$ T cell responses and $CD4^-8^-$ and $CD4^+8^-$ DCs as more effective in stimulating $CD4^+$ T cell responses (99, 100). *In vivo*, $CD4^-8^+$ and $CD4^-8^-$ DCs can efficiently prime male Ag-specific CTLs, whereas $CD4^+8^-$ DCs do so only weakly (99). Splenic DCs can also express tolerogenic phenotypes; $CD4^+8^-$ DCs reportedly mediate tolerance or bystander suppression against diverse T cell specificities (103), while $CD4^-8^+$ DCs can induce tolerance to tissue-associated Ags (104). The

reasons for the different (i.e., immunogenic vs tolerogenic) results observed using the different DC subsets are at present unclear.

Based on the known critical roles of DCs in induction of primary immune responses, these cells have been used in DC-based cancer vaccines (179). However, given the discrepant immunological functions of the various DC subsets, with their potentials for adverse effects in the context of tumor immunity, we thought it important to critically test the capacity of each DC subset to prime antitumor responses. In this study, we addressed the distinct roles of each of these DC subsets in stimulation of OVA-specific $CD4^+$ and $CD8^+$ T cell responses *in vitro* and in priming of OVA-specific CTLs as well as antitumor immune responses in vivo.

4.1.3 MATERIALS AND METHODS

4.1.3.1 Cell lines, Abs, cytokine, peptides, and animals

The OVA-transfected MO4 cell line was kindly provided by Dr. P. Srivastava (University of Connecticut School of Medicine, Farmington, CT). EL4 T cell lymphoma, EG7 (i.e., OVA-transfected EL4 cells) and LB27 B cell hybridoma expressing I-a^b were obtained from the American Type Culture Collection. MO4 and EG7 tumor cells were maintained in DMEM plus 10% FCS and 0.5 mg/ml G418 (Invitrogen Life Technologies). Biotin- and FITC-conjugated monoclonal anti-mouse I-A^b (AF6-120.1), CD4 (GK1.5), CD8 (53-6.7), CD11c (HL3), CD54 (3E2), CD80 (16–10A1), CD86 (GL1), F4/80 (MCAP497), biotin-conjugated anti-CD25 (7D4), CD69 (H1.2F3), $V_{\alpha}2V\beta 5^+$TCR (MR9-4) Abs, and those related FITC-conjugated isotype Abs described above were obtained from BD Pharmingen. FITC- and R-PE-conjugated streptavidin were obtained from Caltag Laboratories. Recombinant mouse GM-CSF and IL-4 were obtained

from R&D Systems. The H-2K^b- and I-A^b-specific OVAI ($OVA_{257–264}$, SIINFEKL) and OVAII ($OVA_{323–339}$, ISQAVHAAHAEINEAGR) peptides(440) were synthesized by Multiple Peptide Systems. Wild-type, CD_4, and IL-10 knockout (KO) C57BL/6, wild-type BALB/c, and the OVA-specific TCR-transgenic OT I and OT II mice(440) were obtained from The Jackson Laboratory. Homozygous IL-10$^{-/-}$ OT II mice were generated by backcrossing the IL-10$^{-/-}$ mice onto the OT II background for three generations; homozygosity was confirmed using mouse genomic DNA by PCR according to the company's protocol. All animal experiments were conducted according to the guidelines of the Canadian Council for Animal Care.

4.1.3.2 Isolation of spleen DCs

This protocol is a modified version of that described by Livingstone and Kuhn(441). Briefly, spleens were injected with DMEM containing 1 mg/ml collagenase (Worthington Biochemical), cut into small fragments, and digested in the above-described enzyme solution for 45–60 min at 37°C. Single-cell suspension was prepared by pressing the digested tissues through a stainless mesh. After the RBC were lysed with Tris-NH_4Cl, the spleen cells were washed once in PBS and resuspended in RPMI 1640 plus 10% FCS and 50 μM 2-ME (complete medium) and incubated at 37°C in 100 x 20-mm petri dishes (one spleen equivalent per dish). After 90 min at 37°C, nonadherent cells were removed by gentle washing three times with prewarmed normal saline. Adherent cells were harvested by using trypsin/EDTA (Invitrogen Life Technologies). Spleen DCs were further purified from these adherent cells by incubating them with the biotin-conjugated anti-CD11c Ab and then the anti-biotin Ab-coupled microbeads, and then passing them over a MACS LS column (Miltenyi Biotec) according to the company's instruction. The microbead-bound cells were fresh CD11c$^+$ spleen DCs. For OVA protein pulsing, adherent cells were cultured overnight in AIM V medium (serum-free lymphocyte medium; Invitrogen Life

Technologies) plus GM-CSF (15–20 ng/ml) and OVA protein (0.1 mg/ml) (Sigma-Aldrich). The next day, nonadherent cells were harvested and high-density cells were removed by using Histopaque 1083 (Sigma-Aldrich). The cells were washed twice in 0.5% BSA in PBS and used for isolation of spleen DC subsets.

4.1.3.3 Purification of spleen DC subsets

DCs subsets were purified as previously described (90) with modification. Briefly, purified overnight cultured nonadherent cells were incubated with the biotin-conjugated anti-CD8 Ab and then with anti-biotin Ab-coupled microbeads, and passed over a MACS LS column (Miltenyi Biotec) according to the company's instruction. The microbead-bound cells were $CD4^{-}8^{+}$ spleen DCs. The flow-through cells were then incubated with biotin-conjugated anti-CD4 Ab and then repeated the same procedure as described above for isolation of $CD8^{+}$ spleen DCs. The bound cells were $CD4^{+}8^{-}$ spleen DCs. The flow-through cells were then passed through a LD column to completely remove any residual cells expressing CD4 and CD8 markers. The final flow-through cells were incubated with the biotin-conjugated anti-CD11c Ab, and the procedure described above was repeated. The bound cells were $CD4^{-}8^{-}$ spleen DCs. The purified $CD4^{+}8^{-}$, $CD4^{-}8^{+}$, and $CD4^{-}8^{-}$ spleen DCs were used for the following studies. DCs pulsed with OVA protein were termed as OVA-pulsed DC (DC_{OVA}).

4.1.3.4 Preparation of DC_{OVA}-activated $CD4^{+}$ T cells

Naive OVA-specific $CD4^{+}$ T cells from OT II or OT II/IL-$10^{-/-}$ mice were obtained by passage of splenocytes through nylon wool columns (173). The T cells were fractionated by negative selection using anti-mouse CD8 (Ly2) paramagnetic beads (Dynal Biotech) according to the manufacturer's protocols to yield populations that were >98% $CD4^{+}/V_{\alpha}2V_{\beta}5^{+}$. For activation, the naive $CD4^{+}$ T cells (2 x 10^{5} cells/ml) were

stimulated for 5 days with purified DC_{OVA} subsets (1 x 10^5 cells/ml) in the presence of IL-2 (10 U/ml) or anti-TGF-β Ab (0.5 μg/ml; R&D Systems) and then isolated from Ficoll-Paque (Amersham Biosciences) density gradients(173).

4.1.3.5 Analyses of phenotype and cytokine profile

All fresh spleen DCs, purified overnight-cultured spleen DC subsets, and T cells were analyzed by flow cytometry using marker-specific and isotype-matched control Abs as noted (173). The values of mean fluorescence intensity (MFI) for the control and the sample were measured by flow cytometry. The expression index (EI) representing the degree of molecule expression was calculated by dividing the sample MFI with the respective control MFI. For assessment of cytokine production, DC subsets were cultured in the presence of GM-CSF (20 ng/ml) and LPS (1 μg/ml) (218), while DC_{OVA} subset-stimulated $CD4^+$ T cells were restimulated with irradiated (10,000 rad) and OVAII peptide-pulsed LB27 tumor cells (442). One day subsequently, the supernatants were assayed for IFN-γ, IL-4, IL-10, TNF-α, and TGF-β using ELISA kits (R&D Systems) (173).

4.1.3.6 T cell proliferation assays

Standard T cell proliferation assays were conducted using naive $CD4^+$ or $CD8^+$ T cells purified from BALB/c, OT I, or OT II mice as responder cells and irradiated (4000 rad) DCs and DC_{OVA} subsets were used as stimulators, respectively (173).

4.1.3.7 CTL assay

Splenic lymphocytes (5 x 10^6) from mice vaccinated with the various DC_{OVA} subsets were cocultured for 4 days in 24-well plates with irradiated (10,000 rad) EG7 cells (2 x

10^5). The activated T cells were harvested and used as effector cells against radiolabeled EG7 or EL4 target cells in ^{51}Cr release assays (173).

4.1.3.8 Animal studies

For investigation of antitumor immunity, C57BL/6 mice (n = 8/group) were vaccinated s.c. with 1 x 10^6 $CD4^+8^-$, $CD4^-8^+$, or $CD4^-8^-$ DC_{OVA} and then challenged s.c. 10 days later with 1 x 10^5 MO4 tumor cells. When investigating the role of $CD4^+$ and $CD8^+$ T cells in antitumor responses, $CD4^+$ and $CD8^+$ T cells were depleted by i.v. injection of 0.5 mg anti-CD4 and anti-CD8 Ab at days –3, 0, and 3 relative to the DC immunization, respectively (173). In all experiments, irrelevant isotype-matched rat Abs were used as controls, and target cell depletion was confirmed by FACS analysis of the circulating T cells. To demonstrate that the anti-CD4 Ab effects (above) were not dependent on a coincidental targeting of $CD4^+$ DCs, in some experiments CD4 KO C57BL/6 mice were used in place of the anti-CD4 Ab-treated mice.

In the studies to confirm that regulatory T (Tr) cells generated *in vitro* and *in vivo* were functional *in vivo*, OT II or OT II/IL-$10^{-/-}$ $CD4^+$ T cells, which had been activated by coculture with freshly purified DC_{OVA}, and $CD4^+$ T cells, which were purified from mice immunized with $CD4^-8^-$ DC_{OVA} for 7–9 days by using the method described above for purification of $CD4^+8^-$ DCs with MACS beads, were injected i.v. into mice (2 x 10^6/mouse) vaccinated with a fully protective dose (i.e., 1 x 10^6) of $CD4^-8^+$ DC_{OVA} 9 days before. OT II and normal $CD4^+$ T cells activated *in vitro* and *in vivo* by $CD4^+8^-$ and $CD4^-8^+$ DC_{OVA} were used as controls. These mice were then challenged s.c. with 1 x 10^5 MO4 tumor cells 1 day later. Animal mortality was monitored daily for up to 10 wk; for humanitarian reasons, all mice with tumors that achieved a size of 1.5 cm in diameter were sacrificed.

4.1.4 RESULTS

4.1.4.1 Phenotypic characterization of spleen DCs and DC subsets

Fresh spleen DCs were prepared as described in *Materials and Methods* and then analyzed for their cell surface expression of a panel of immunologically important molecules by flow cytometry. As shown in Fig.4.1. 1*A*, they mostly express DC marker CD11c, indicating their nature of DCs. They displayed a very low expression of MHC class II (I-A^b) (EI, 2.6) and costimulatory molecule CD80 (EI, 1.6) and CD86 (EI, 1.4), which are closely associated with DC maturation, indicating that they are immature DCs. Three subsets of spleen DCs were purified from the overnight-cultured spleen DCs as described in *Materials and Methods*. As shown in Fig. 1*B*, three purified subsets of DCs mostly (>90%) expressed CD11c and all expressed high amounts of I-A^b, CD80, and CD86 molecules, indicating that they are mature DCs. There were two distinct peaks of I-A^b, CD54 and CD80 expression, indicating that the differentiation stages of DC subsets are heterogeneous. There was ~6–7% of the CD3-positive T cell population, but no F4/80-positive macrophages (443), within the above DC subset preparations as examined by flow cytometry (data not shown). Among the three DC subsets, the $CD4^-8^-$ DC subset had less expression of MHC class II (I-A^b) (EI, 8.3) and costimulatory molecules CD80 (EI, 10.7) and CD86 (EI, 4.0) compared with the other two DC subsets with a 2- to 3-fold higher EI (Fig.4.1. 1*B*), indicating that it is a relatively less mature form of mature DCs. As shown in Fig. 4.1. 2, cytokine secretion profiles among DC subsets are significantly different. After stimulation with LPS, both $CD4^+8^-$ and $CD4^-8^+$ DC subsets secreted a higher level of IFN-γ (0.5–1 ng/ml) and TNF-α (0.2 ng/ml) but a very low level of TGF-β. On the contrary, $CD4^-8^-$ DC subset secreted a much higher level of TGF-β (0.55

ng/ml) (Student's *t* test; $p < 0.01$ vs $CD4^+8^-$ DCs or $CD4^-8^+$ DCs) and a very low level of IFN-γ and TNF-α.

4.1.4.2 Stimulation of allogeneic T cells *in vitro* by DC subsets

DCs are potent stimulators of primary MLRs and are able to induce the proliferation of allogeneic T cells *in vitro* (173). Thus, we compared the ability of three DC subsets in stimulation of primary 3-day MLRs against allogeneic T cells. All three DC subsets demonstrated the ability to stimulate purified $CD4^+$ and $CD8^+$ T cells from BALB/c mice in the 3-day MLRs. Among them, the $CD4^-8^+$ DC is the most effective type of DCs in stimulation of proliferation of unfractionated allogeneic T cells, whereas both $CD4^+8^-$ and $CD4^-8^-$ DC subsets showed similar stimulation activity (data not shown). When testing on T cell subsets, $CD4^-8^+$ DCs are also the most effective in stimulation of both allogeneic $CD4^+$ and $CD8^+$ T cells, whereas $CD4^+8^-$ DCs are only more effective in stimulation of $CD8^+$ T cells than $CD4^-8^-$ DCs (Fig. 4.1. 3, *A* and *B*).

4.1.4.3 Stimulation of tumor Ag-specific T cells *in vitro* by DC subsets

We next checked and compared their abilities in presentation of tumor Ag OVA instead of alloantigens. For this purpose, we used spleen DCs subsets pulsed with OVA protein for stimulation of transgenic OT II $CD4^+$ and OT I $CD8^+$ T cells *in vitro* in the 3-day T cell proliferation assay. Accordingly, we found that the results were very similar to those of MLRs. The $CD4^-8^+$ DC subset had a higher capacity of stimulating $CD4^+$ and $CD8^+$ T cell proliferation than the other two subsets in the 3-day T cell proliferation assay (Student's *t* test; $p < 0.05$). Again, the $CD4^-8^-$ DC subset is the weakest inducer of T cell proliferation, probably due to its relatively low maturity. In addition, we also found that the efficiency of T cell proliferation by both $CD4^+8^-$ and $CD4^-8^-$ DC subsets peaked at day 3 of stimulation, whereas the efficiency of T cell proliferation by the $CD4^-8^-$ DC

subset peaked at day 5 of stimulation. Interestingly, the $CD4^-8^-$ DC subset became the strongest inducer in stimulation of both OT II $CD4^+$ and OT I $CD8^+$ T cells *in vitro* in the 5-day T cell proliferation assay (Fig. 4.1. 3, *C* and *D*).

4.1.4.4 $CD4^+8^-$ and $CD4^-8^+$ DCs prime CTL-mediated antitumor immune responses *in vivo*

Next, we wished to test the ability of the various DC subsets for *in vivo* CTL priming. Splenic lymphocytes from mice immunized with DC_{OVA} subsets were cocultured with irradiated EG7 tumor cells expressing OVA for 4 days and harvested. These T cells are called CTLs. To assess their cytotoxic activities, we conducted a chromium release assay by using these CTLs as effector cells and the ^{51}Cr-labeled EG7 tumor cells as target cells. As shown in Fig. 4.1. 4*A*, CTLs derived from mice immunized with $CD4^+8^-$ and $CD4^-8^+$ DC_{OVA} showed similar cytotoxic activity against EG7 tumor cells. At an E:T ratio of 50, the specific killing activities are all ~50%, but essentially none of the OVA-negative EL4 cells. In contrast, T cells derived from mice immunized with $CD4^-8^-$ DC_{OVA} did not show any OVA-specific cytotoxic activity, indicating that the tumor-specific CTL responses in this group of mice were inhibited.

To examine whether DC subsets are capable of inducing antitumor immunity, we vaccinated mice with DC_{OVA} subsets and 10 days later challenged the mice with MO4 tumor cells (1×10^5 cells per immunized mouse). As shown in Fig. 4.1. 4*B*, MO4 tumor cell challenges were invariably lethal within 4 wk after implantation for the control mice vaccinated with PBS, whereas two of eight mice vaccinated with unfractioned splenic DC_{OVA} were protected against MO4 tumor challenge. Most (seven of eight) animals immunized with either $CD4^+8^-$ or $CD4^-8^+$ DC_{OVA} were protected for at least 9 wk,

whereas all of the mice immunized with $CD4^-8^-$ DC_{OVA} died within 5 wk of tumor inoculation.

To study the immune mechanisms in the protective immunity, immunized mice were depleted of their $CD4^+$ T cells using the anti-CD4 Ab before MO4 tumor challenge. As shown in Fig. 4.1. 5, *A* and *B*, the immune protections against MO4 tumor challenge dramatically dropped from 100% to 25% and 0%, respectively, in $CD4^+$ T cell-depleted mice immunized with $CD4^+8^-$ and $CD4^-8^+$ DC_{OVA}. Because the use of anti-CD4 Ab for $CD4^+$ T cell depletion *in vivo* may also affect and eliminate $CD4^+8^-$ DCs, we then repeated the $CD4^+8^-$ DC_{OVA} immunization experiment in CD4 KO mice. As shown in Fig. 4.1. 5*B*, the immune protection became 25% in this group of mice. These results clearly indicate that $CD4^+$ Th cells play an important role in $CD4^+8^-$ and $CD4^-8^+$ DC-primed OVA-specific immune responses. In addition, the immune protection against MO4 tumor challenge dramatically dropped from 100% to 0% in $CD8^+$ T cell-depleted mice immunized with $CD4^+8^-$ and $CD4^-8^+$ DC_{OVA}, indicating that $CD8^+$ CTLs are the major effector cells in the antitumor immunity derived from $CD4^+8^-$ and $CD4^-8^+$ DCs.

4.1.4.5 $CD4^-8^-$ DCs prime tolerant immune responses against tumors *in vivo*

In contrast, $CD4^-8^-$ DC vaccination did not show any immune protection against the challenge of 0.1 x 10^6 MO4 tumor cells. All of the mice immunized with $CD4^-8^-$ DC_{OVA} died within 5 wk after tumor inoculation (Fig. 4.1. 5*C*), confirming that the tumor-specific CTL responses in this group of immunized mice were lacking. To investigate whether $CD4^+$ Tr cells induced by $CD4^-8^-$ DCs are responsible for the lack of CTL responses, we conducted the above animal studies in $CD4^+$ T cell-depleted mice by using the anti-CD4 Ab. Surprisingly, we found that 88% of mice significantly prolonged their survival (Student's *t* test; $p < 0.05$) and 50% of mice were eventually protected from

tumor challenge in $CD4^+$ T cell-depleted mice immunized with $CD4^-8^-$ DC_{OVA}, compared with the wild-type mice. These results were further confirmed by using CD4 KO mice, which showed that 75% of the immunized mice were protected from tumor challenge. These results clearly indicate that $CD4^+$ Tr1 cells induced by $CD4^-8^-$ DCs are most likely responsible for the *in vivo* immune suppression against MO4 tumor cells.

4.1.4.6 $CD4^-8^-$ DCs induce $CD4^+$ Tr1 differentiation *in vitro*

Since DC subsets may be specialized to prime different $CD4^+$ T cell responses (444), we examined the pattern of cytokine secretion of $CD4^+$ T cells activated by OVA protein-pulsed DC subsets by ELISA. We first confirmed that these activated T cells were CD4 positive and displayed the clonotypic $V_\alpha2V_\beta5$ OVA TCR, as well as the T cell activation markers CD25 and CD69 (data not shown). We then examined their cytokine profiles. We found that the activated $CD4^+$ T cells from the $CD4^+8^-$ or $CD4^-8^+$ DC_{OVA} cocultures secreted similarly high levels of IFN-γ, but relatively little or no IL-4 or IL-10 (Fig. 4.1. 6*A*), indicating a Th1 phenotype. However, the $CD4^+$ T cells activated by TGF-β-secreting $CD4^-8^-$ DC_{OVA} secreted high levels of IL-10 (1.75 ng/ml) and substantial amounts of IFN-γ (2.1 ng/ml), indicating a Tr1 phenotype.

4.1.4.7 $CD4^+$ Tr1 cells inhibit the antitumor immunity through IL-10

Because Tr1 cells suppress immune responses (445), we next assessed whether they also inhibit the antitumor immunity in our animal model. We injected these Tr1 cells into $CD4^-8^+$ DC_{OVA}-immunized mice before tumor challenge. As shown in Fig. 4.1. 6*B*, $CD4^-8^-$ DC_{OVA}-induced OT II $CD4^+$ Tr1 cells completely inhibited the immune protection of $CD4^-8^+$ DC_{OVA} immunization, whereas $CD4^+8^-$ or $CD4^-8^+$ DC_{OVA}-activated Th1 cells showed enhanced protection against MO4 tumor challenge when compared with the $CD4^-8^+$ DC_{OVA} control. To confirm the above findings, we also isolated the $CD4^+$ T

cells from $CD4^-8^-$ DC_{OVA}-immunized immunized mice and repeated the same experiment as that for *in vitro*-activated Tr1 cells. As we expected, we found almost identical results to those for *in vitro*-cultured Tr1 cells (data not shown), confirming that $CD4^+$ Tr1 cells induced by $CD4^-8^-$ DC_{OVA} are most likely responsible for the *in vivo* immune suppression against MO4 tumor cells. To assess whether IL-10 was involved in the inhibition of $CD4^-8^+$ DC_{OVA}-induced antitumor immunity, OT II $CD4^+$ T cells ($IL\text{-}10^{-/-}$) were used to coculture with the $CD4^-8^-$ DC_{OVA}. After 5 days of culture, the Tr1 ($IL\text{-}10^{-/-}$) cells were injected into $CD4^-8^+$ DC_{OVA}-immunized mice. As shown in Fig. 4.1. 6*B*, most of the immunized mice were protected against the MO4 challenge and no inhibition was observed, while the normal Tr1 cells completely inhibited the antitumor immunity of $CD4^-8^+$ DC_{OVA}.

4.1.4.8 TGF-*β* is partially responsible for the induction of Tr1 by $CD4^-8^-$ DC_{OVA}

It has recently been reported that LPS-stimulated B cells expressing TGF-β induced T cell anergy via activation of Tr cells (446). To explore the mechanism of Tr1 induction, we added anti-TGF-β Ab to the coculture of $CD4^-8^-$ DC_{OVA} and OT II $CD4^+$ T cells and then tested the cytokine profile of activated CD4 T cells. Interestingly, their cytokine profile became similar to that of $CD4^-8^+$ DC_{OVA}-activated Th1 (Fig.4.1. 6*A*). Their IL-10 secretion was decreased nearly 10 times when compared with their controls. Furthermore, these T cells were used in *in vivo* study as described above. No inhibition, but a little enhancement of antitumor immune response, was observed in the $CD4^-8^+$ DC_{OVA}-immunized mice (Fig.4.1. 6*B*), indicating that TGF-β was involved in promoting the formation of Tr1 cells.

4.1.5 DISCUSSION

Because of the critical roles DCs have in induction of primary immune responses, they have been extensively used for DC-based cancer vaccines. It has been shown that DCs, when pulsed with tumor-derived MHC class I-restricted peptides and tumor Ags, are able to induce significant CTL-dependent antitumor immune responses (179). Because the distinct immunological functions of DC subsets may have detrimental effects on the host's immune responses, it is important to assess the capacity of these DC subsets in priming antitumor immunity.

Splenic DCs have been classified into three subsets ($CD8^+4^-$, $CD8^-4^+$, and $CD4^-8^-$ DCs). Although the phenotypic and functional differences between $CD8^+$ and $CD8^-$ DC subsets have been extensively studied (90) (95, 437, 438), the differences between $CD4^+$ and $CD4^-$ populations within the $CD8^-$ DC subset have been less investigated. In this study, we conducted a systemic study on the phenotypic characteristics and the functional differences in stimulation of T cells among the three DC subsets. For the first time, our data showed that $CD4^-8^-$ DCs secreted marked levels of TGF-β and represent a characteristic consistent with a tolerogenic phenotype (446). In contrast, the $CD4^+8^-$ and $CD4^-8^+$ DCs secreted moderate and high levels of IFN-γ relative to the $CD4^-8^-$ DCs, respectively, but little or no TGF-β. We then investigated the capacity of three DC subsets pulsed with OVA protein in priming OVA-specific antitumor immune responses *in vivo*. Our data showed that only $CD4^+8^-$ DC_{OVA} and $CD4^-8^+$ DC_{OVA} could induce the antitumor response and protect the immunized mice from tumor challenge, whereas $CD4^-8^-$ DC_{OVA} could not stimulate any protective immune response against tumor, indicating distinct *in vivo* antitumor immune responses derived from vaccination of three DC subsets.

There is now compelling evidence that $CD4^+$ T cells, specialized in suppressing immune responses, play a critical role in immune regulation. Three major populations of Tr cells have been identified based on their distinct phenotype ($CD4^+CD25^+$) or cytokine profile (Tr1 and Th3). The $CD4^+CD25^+$ Tr subset mediates immune suppression in a non-Ag-specific manner, whereas the latter Tr1 and Th3 mediate immune inhibition via production of IL-10 and TGF-β, respectively, but both in an Ag-specific way (447). In this study, the cytokine profiles of $CD4^+$ T cells activated by DC_{OVA} subsets differed markedly. $CD4^+8^-$ or $CD4^-8^+$ DC_{OVA} induce Th1 phenotype response, whereas T cells activated by TGF-β-secreting $CD4^-8^-$ DCs secreted high levels of IL-10 and substantial amounts of IFN-γ, a characteristic of Tr1 cells (447). Tr1 cells can also be induced by addition of exogenous IL-10 to primary murine T cell cultures or by coculturing T cells with TGF-β/IL-10-expressing "tolerogenic" DCs (445, 448, 449) or by immature DCs (342). In addition, others have also shown that the IL-10-stimulated $CD11c^{low}$ $CD45RB^{high}$ tolerogenic DCs, which have a phenotype similar to our $CD4^-8^-$ DCs, also induced Tr1 cell differentiation and immune tolerance *in vivo* (450). LPS-stimulated B cells expressing membrane-bound TGF-β have recently been shown to induce T cell anergy (446) via the activation of Tr cells (451). Interestingly, in this study, the addition of anti-TGF-β Ab to $CD4^-8^-$ DC_{OVA} coculture with OT II T cells converted the cytokine profile of these $CD4^+$ T cells from the Tr1 phenotype to the Th1 phenotype, indicating the critical role of TGF-β of $CD4^-8^-$ DCs in stimulation of the Tr1 response.

These Tr1 cells also distinct from Th1 or Th2 cells in that they produce high levels of IL-10 and no IL-4, and proliferate poorly upon TCR ligation, suppress immune responses *in vitro* and *in vivo* through secreted IL-10 (452). In this study, we also characterized the functional effect of these Tr1 cells in induction of immune suppression *in vivo*. The $CD4^+$ Tr1 cells from $CD4^-8^-$ DC_{OVA}/OT II cocultures or purified from $CD4^-8^-$

DC_{OVA}-immunized mice were transferred into $CD4^-8^+$ DC_{OVA}-immunized mice and abolished the $CD4^-8^+$ DC_{OVA}-induced antitumor immunity. Unlike the wild-type Tr1 cells (above), the IL-$10^{-/-}$ CD4 OT II T cells activated with $CD4^-8^-$ DC_{OVA} had little impact on $CD4^-8^+$ DC_{OVA}-driven antitumor immunity, clearly implicating the IL-10-producing Tr1 cells as central to the tolerance observed in this model system, and this is consistent with previous reports (452) (451). As expected, when we transferred the control Th1 cells from $CD4^+8^-$ or $CD4^-8^+$ DC/OT II $CD4^+$ T cell cocultures or T cells from coculture with $CD4^-8^-$ DC_{OVA} in the presence of anti-TGF-β Ab into $CD4^-8^+$ DC-vaccinated animals, we observed augmented tumor protection, confirming the critical role of TGF-β of $CD4^-8^-$ DCs in stimulation of the Tr1 response.

$CD4^+$ Th and Tr cells play an important role in modulation of immune responses by enhancement and suppression of $CD8^+$ CTL responses, respectively (453). In this study, we showed that depletion of $CD4^+$ Th and Tr cells significantly reduced and enhanced antitumor immune responses in $CD4^+8^-$ or $CD4^-8^+$ DC- and $CD4^-8^-$ DC-immunized mice, respectively. Surprisingly, the immune protection in $CD4^+$ T cell-depleted $CD4^-8^-$ DC-immunized mice is much stronger than that in $CD4^+$ T cell-depleted $CD4^+8^-$ or $CD4^-8^+$ DC-immunized mice. The immune mechanism behind this phenomenon is currently unknown. The enhanced immunity seen in $CD4^+$ T cell-depleted $CD4^-8^-$ DC-immunized mice may be partially derived from the capacity of $CD4^-8^-$ DCs making more IL-12p70 than $CD4^+8^-$ DCs when stimulated appropriately (235), which is associated with stimulation of CTL responses (454). It has been reported that different spleen DC subsets when pulsed with MHC class I-restricted viral peptide can induce antiviral immunity mediated by Th-independent CTL responses (455). In this study, we also found that our three DC subsets when pulsed with OVAI peptide can all induce

protective immunity against MO4 tumor cells, among which the $CD4^{-}8^{-}$ DC subset stimulates the strongest immunity (data not shown), also supporting the above finding.

Discrepant results regarding immune priming vs tolerance have previously been reported for $CD4^{+}8^{-}$ (100, 103) and $CD4^{-}8^{+}$ DCs (90, 104, 456). In addition, our results of tolerogenic $CD4^{-}8^{-}$ DCs in antitumor immunity are also in contrast to a previous report wherein $CD4^{-}8^{-}$ DCs efficiently stimulated H-Y Ag-specific CTL responses (99). It has been reported recently that the environmental conditions or stimuli under which DCs stimulate T cells critically affect the type of immune responses that ensue (173, 436, 456). This suggests that the discrepancies between our results and those observed in other systems (e.g., autoimmunity, allotransplantation, or antitumor immunity) would likely be attributable to the varying antigenic and environmental conditions in each model system, where different TLRs are stimulated (457).

Taken together, our data unequivocally confirmed that the different DC subsets can function either as stimulators or inhibitors of immune responses. $CD4^{-}8^{-}$ DC stimulated IL-10-secreting $CD4^{+}$ Tr1-mediated immune suppression, whereas both $CD4^{+}8^{-}$ and $CD4^{-}8^{+}$ DC induced $CD8^{+}$ CTL-mediated antitumor immunity. This information has very substantial implications for DC-based approaches to the design of cancer vaccines.

Note: This manuscript has been published in 2005 in Journal of Immunology (Zhang, X., H. Huang, et al. (2005). "$CD4^{-}8^{-}$ DCs prime $CD4^{+}$ T regulatory 1 cells to suppress antitumor immunity." J Immunol **175**(5): 2931-7.), the format was modified to fit the current version of thesis.

4.1.6 Figure legends for Manuscript 4.1

Figure 4.1.1: Phenotpye analyses of spleen DC subsets.

A. Fresh spleen DCs were purified with biotin-anti-CD11c and anti-biotin microbeads after harvesting those attached cells after 90 min incubation as described in Materials and Methods. They expressed very low level of CD80, CD86 and I-A^b. B. DC subsets were purified as described in. FITC-directed labeled Abs against mouse CD54, CD80, CD86, and MHC class II were used to stain the cells described above; corresponding isotype Abs were used as controls (thin lines). The values of MFI for the control and the sample are shown in the upper right corners. $CD4^-8^-$DCs had a lower level of CD54, CD80, CD86 and I-A^b compared with other two subsets.

Figure 4.1.2: Cytokine secretion assay for three DC subsets.

Three purified dendritic cell subsets were cultured with LPS (1 ug/ml) and rmGM-CSF (20ng/ml) for 24 hours and DC subset supernatants were measured for IFN-γ, TNF-α, and TGF-β secretion by ELISA. All those kits were purchased from BD biosciences (Mississauga, ON, Canada). One representative experiment of three is shown.

Figure 4.1.3: *In vitro* allogeneic and autologous T cell proliferation assays.

Irradiated (4000 rad) DC_{OVA} subsets (0.1 x 10^6 cells/well) and their 2-fold dilutions were cultured with a constant number (0.1 x 10^6 cells/well) of allogeneic BALB/c $CD4^+$ (A) and $CD8^+$ (B) T cells or autologous OT II $CD4^+$ (C) and OT I $CD8^+$ (D) T cells, respectively. After 48 h, thymidine incorporation was determined by liquid scintillation

counting. *, $p < 0.05$ vs cohorts of $CD4^+8^-$ and $CD4^-8^-$ DCs (Student's t test). One representative experiment of three is depicted.

Figure 4.1.4: *In vitro* cytotoxicity and *in vivo* animal studies.

A, Activated $CD8^+$ T cells from $CD4^-8^+$, $CD4^+8^-$, and $CD4^-8^-$ DC_{OVA}-immunized mice, respectively, were used as effector cells, whereas ^{51}Cr-labeled EG7 or irrelevant EL4 cells were used as target cells in 6-h ^{51}Cr release assay. Each point represents the mean of triplicate cultures. One representative experiment of three is shown. B, Mice (n = 8) were s.c. vaccinated with unfractionated DC_{OVA} or $CD4^+8^-$, $CD4^-8^+$, and $CD4^-8^-$ DC_{OVA} subsets, respectively, and then s.c. challenged, 10 days later, with 1 x 10^5 MO4 tumor cells. Animal mortality was monitored daily for up to 10 wk. The data are representative of two experiments with similar results.

Figure 4.1.5: $CD4^+$ T cells were responsible for $CD4^-8^-$DC induced immunosuppression.

$CD4^+$ and $CD8^+$ T cell subset depletion experiments were performed in mice by i.v. injection of anti-CD4 and anti-CD8 Ab on days –3, 0, and 3 relative to $CD4^-8^+$ (A), $CD4^+8^-$ (B), and $CD4^-8^-$ (C) DC_{OVA} immunizations, respectively. In addition, CD4 KO mice were used for replacement of $CD4^+$ T cell-depleted mice in $CD4^+8^-$ (B) and $CD4^-8^-$ (C) DC_{OVA} immunization groups. Animal mortality was monitored daily. The data are representative of two experiments with similar results.

Figure 4.1.6: $CD4^-8^-$DCs induced Tr1 to inhibit antitumor immunity.

A, The $CD4^+$ T cell supernatants were measured for IFN-γ, IL-4, IL-10, and TNF-α secretion by ELISA. One representative experiment of three is shown. B, Mice were immunized with $CD4^-8^+$ DC_{OVA} (•) or PBS (○). Mice vaccinated with $CD4^-8^+$ DC_{OVA} 9

days before were further i.v. injected with OT II $CD4^+$ T cells activated *in vitro* by $CD4^-8^-$ (OTII ($CD4^-8^-$ DC_{OVA}); ◇), $CD4^+8^-$ (OTII ($CD4^+8^-$ DC_{OVA}); ■), and $CD4^-8^+$ (OTII ($CD4^-8^+$ DC_{OVA}); □) DC_{OVA}, respectively, or OT II/IL-$10^{-/-}$ $CD4^+$ T cells activated by $CD4^-8^-$ DC_{OVA} (OT II/IL-$10^{-/-}$ ($CD4^-8^-$ DC_{OVA}); △) or OT II $CD4^+$ T cells activated by $CD4^-8^-$ DCs in the presence of anti-TGF-β Ab (OTII ($CD4^-8^-$ DC_{OVA}/anti-TGF-β); *). Ten days subsequent to DC_{OVA} immunization, these mice were then challenged s.c. with 1 x 10^5 MO4 tumor cells. Animal mortality was monitored daily. The data are representative of two experiments with similar results.

Figure 4.1. 1: Phenotpye analyses of spleen DC subsets.

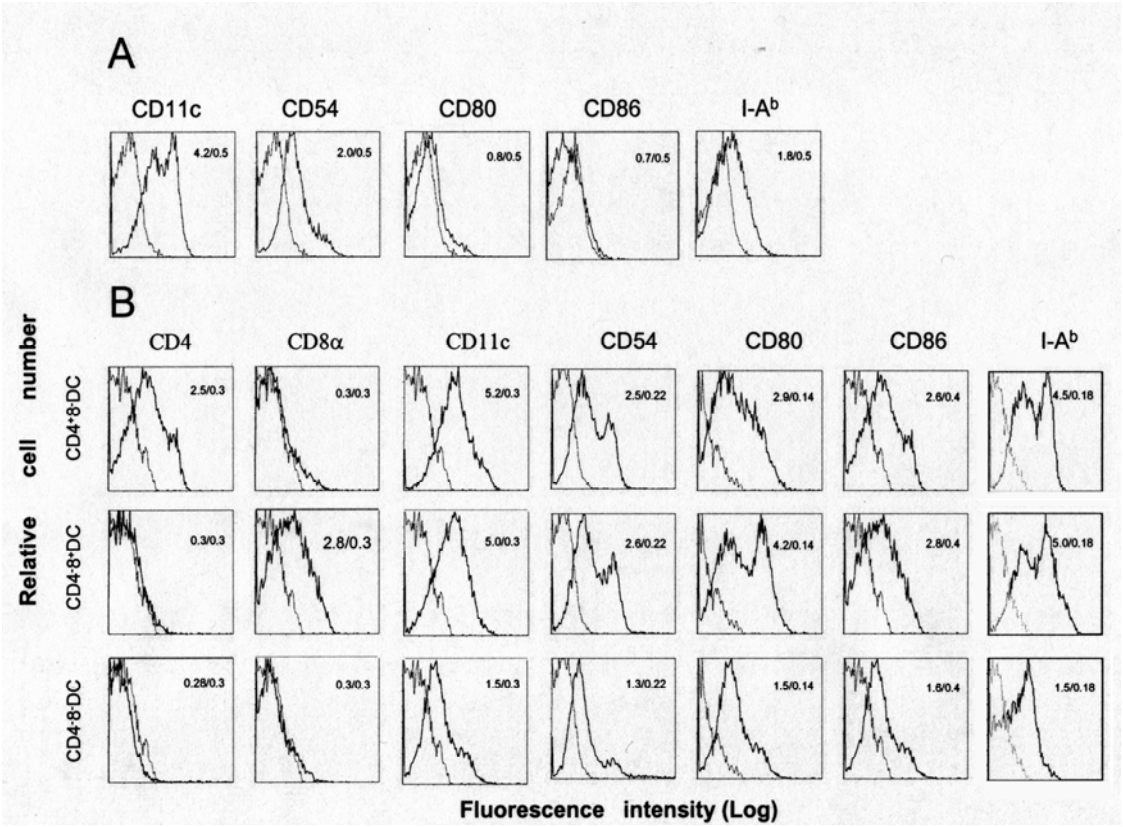


Figure 4.1. 2: Cytokine secretion assay for three DC subsets.

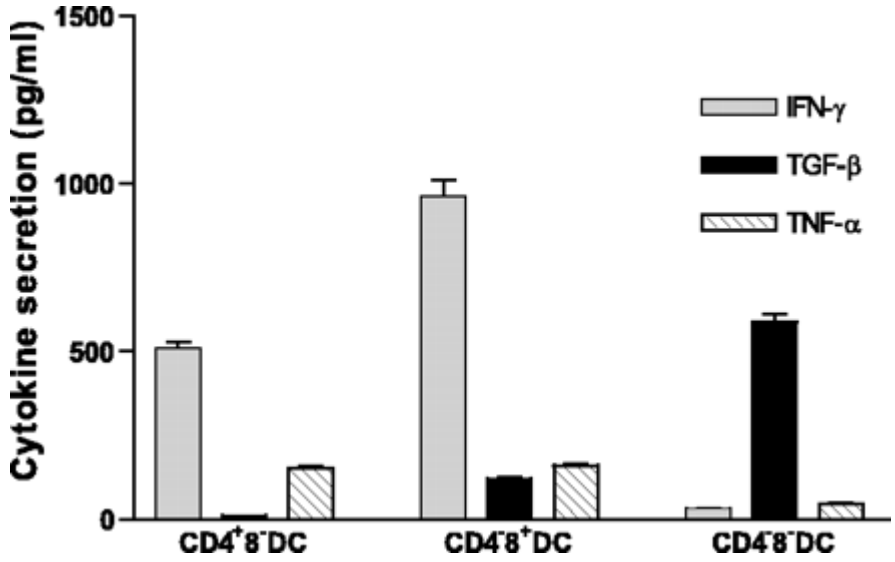


Figure 4.1. 3: *In vitro* allogeneic and autologous T cell proliferation assays.

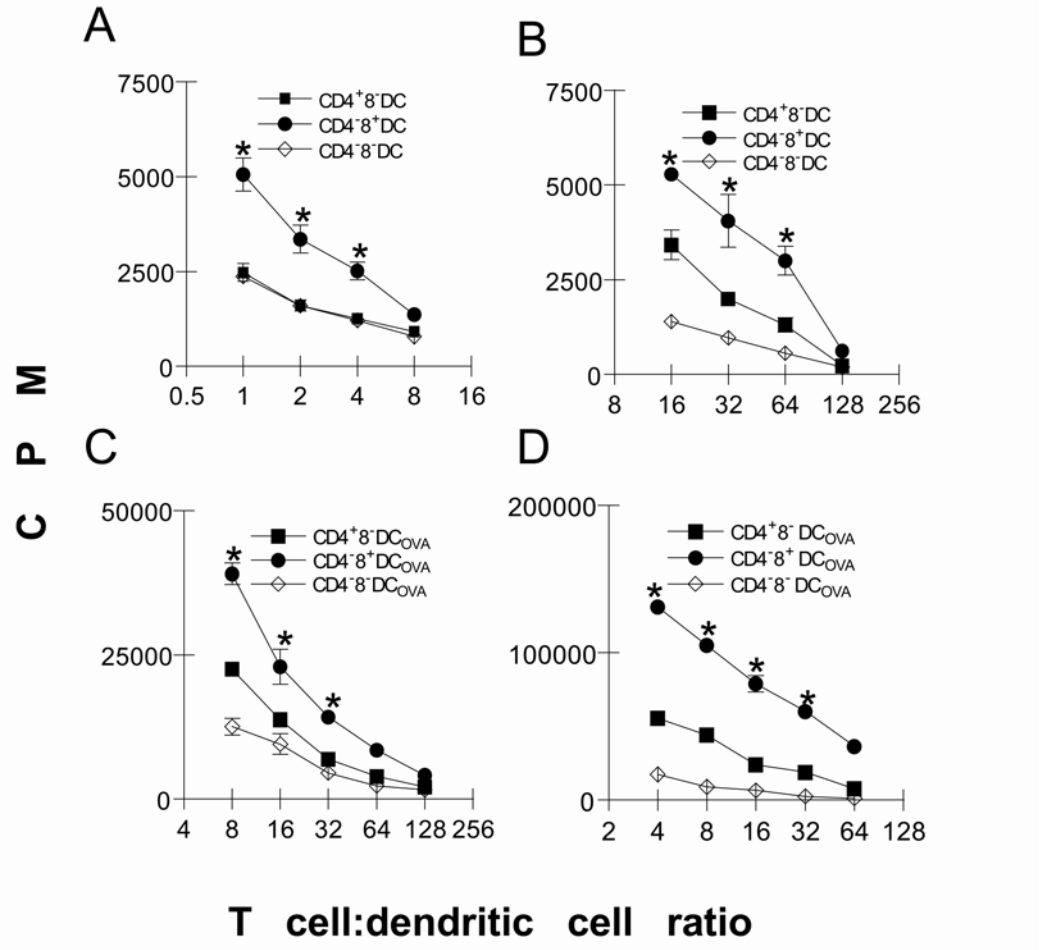


Figure 4.1. 4: *In vitro* cytotoxicity and *in vivo* animal studies.

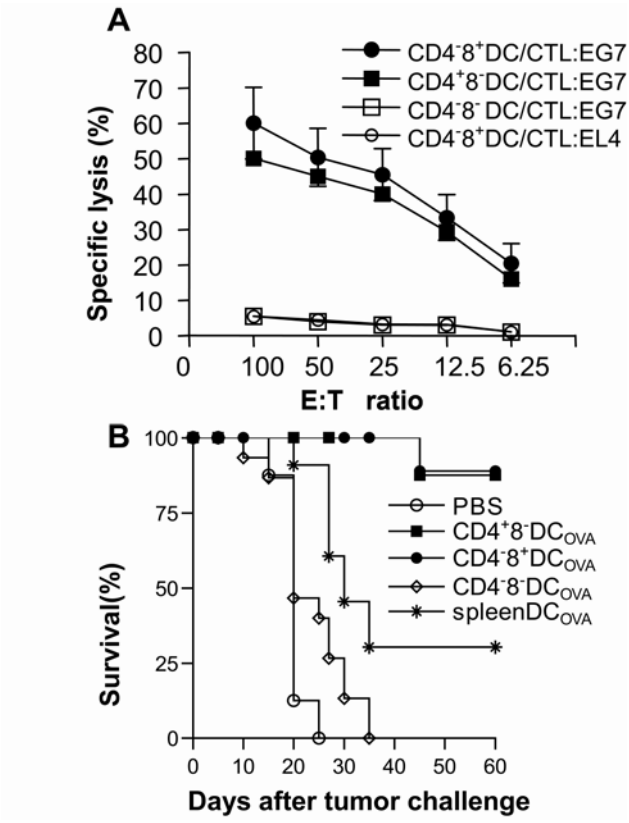


Figure 4.1. 5: $CD4^+$ T cells were responsible for $CD4^-8^-DC$ induced immunosuppression.

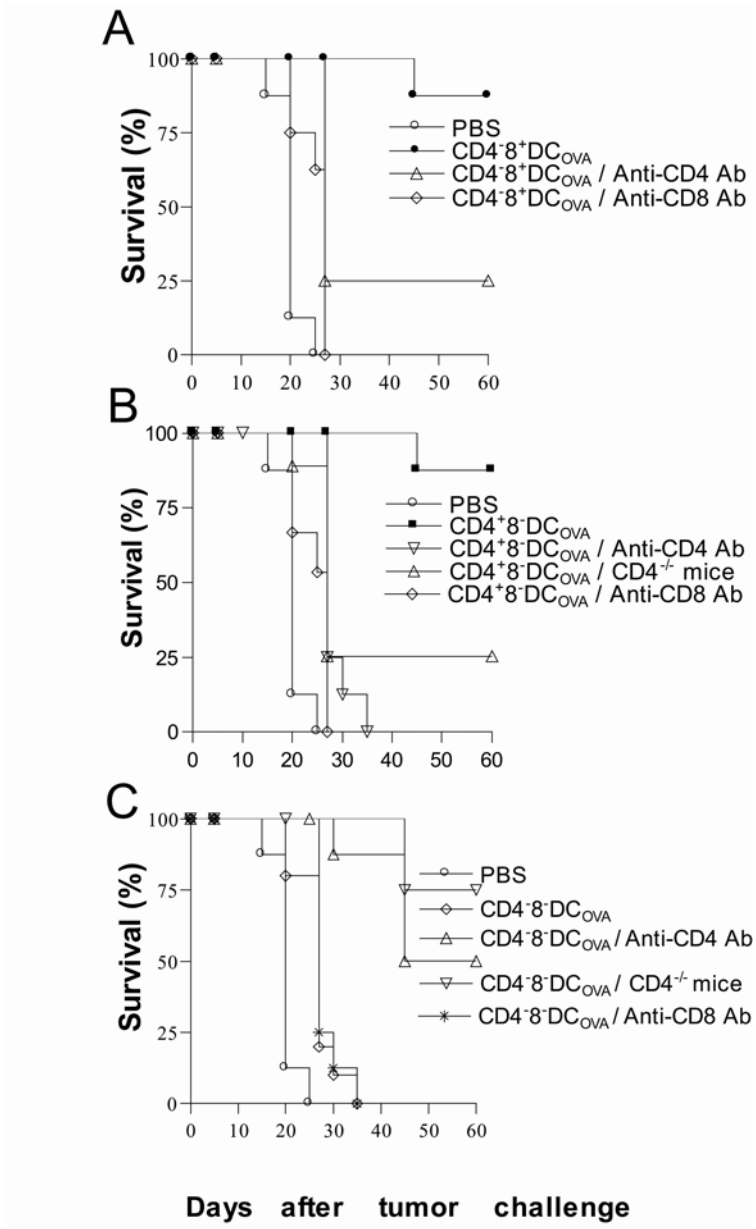
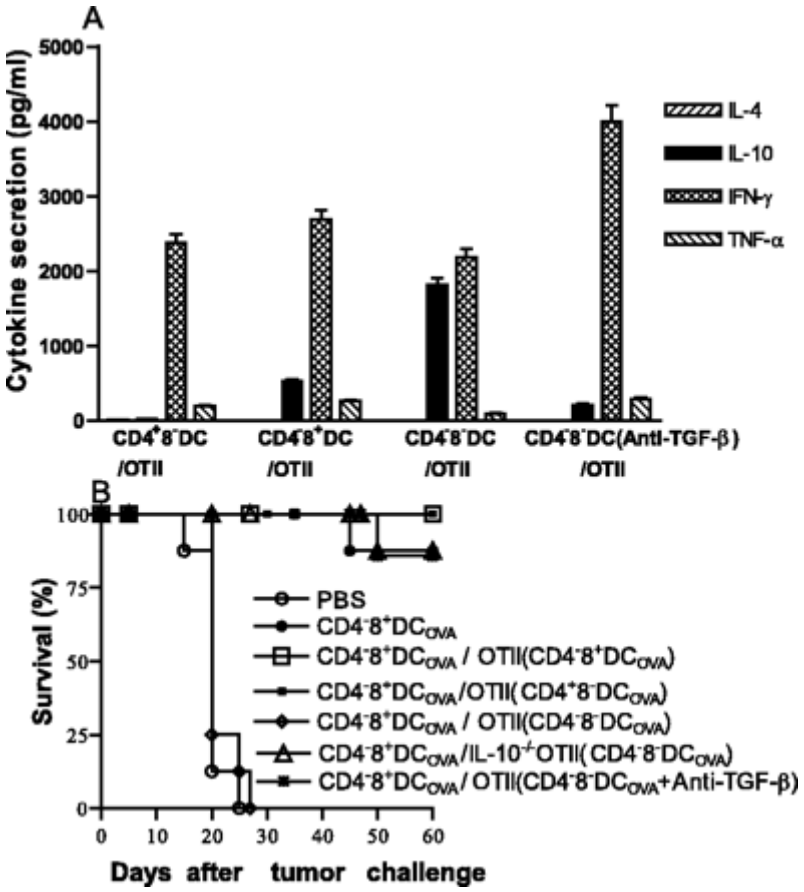


Figure 4.1. 6: CD4$^-$8$^-$DCs induced Tr1 response to inhibit antitumor immunity.



In the first manuscript, we found that $CD4^+8^-$ and $CD4^-8^+$ DCs subsets induced Thl cell responses leading to protective antitumor immunity, whereas $CD4^-8^-$ DC secreting TGF-β stimulated responses of Trl cells secreting IL-10 and IFN-γ leading to immune tolerance (106). Our research indicated that DCs are usually composed of different subsets that might posses different functions leading to antitumor immunity or tolerance. However, for the cancer patient, immunotolerance should be avoided or converted to improve the efficency of DC vaccine-based cancer immunotherapy. Thus DCs should be made functionally homogenous and mature in order to be used as vaccine against tumor challenge or cancer treatment. Research on how to convert the tolerogenic $CD4^-8^-$ DCs into immunogenic ones will shed light on the way of improving DC based cancer treatment.

4.2 TLR9 Signaling Converts Tolerogenic TGF-β-secreting $CD4^-8^-$ DCs into Immunogenic IL-6/1L-15-Secreting Ones Capable of Stimulating Antitumor Thl/Th17 Cell Responses

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4.2.1 ABSTRACT

Different DC subsets play distinct roles in immune responses. $CD4^-8^-$ DCs secreting transforming growth factor (TGF)-β stimulate $CD4^+$ regulatory T type 1 (Trl) cell responses leading to inhibition of CD8 cytotoxic T lymphocyte (CTL) responses and antitumor immunity. In this study, we explored the potential effect of three stimuli CpG, LPS and anti-CD40 antibody in conversion of $CD4^-8^-$ DC-induced tolerance. We demonstrated that when $CD4^-8^-$ DCs were isolated from overnight culture and cultured for another 8 hrs in AIM-V plus rmGM-CSF (15-20 ng/ml) and OVA (0.1 mg/ml) with CpG (5 ug/ml), LPS (2 ug/ml) and anti-CD40 antibody (10 ug/ml), their phenotype became more mature compared with the freshly isolated ones. CpG is the only agent that stimulates the DCs to secrete significant level of IL-6 and IL-15; DNA array analyses also indicate that CpG stimulates higher expression of IL-6 and IL-15 mRNA. CpG treatment most efficiently converts the tolerogenic DCs into immunogenic ones which stimulated the OTII $CD4^+$ T cell to become Th1 and Th17 rather Tr1, while the other two stimulator-treated DCs could not induce Th17 response. Their vaccination also induced the strongest antitumor CTL responses and protective immunity against tumor cell challenge. When $CD4^-8^-$ DCs were isolated from IL-$6^{-/-}$ mice, CpG-treated DC_{OVA} vaccination almost completely lost their animal protection capacity. Wild type B6 DC_{OVA}-vaccinated IL-$15R^{-/-}$ mice can only provide up to 30% protection against tumor challenge. Those results indicate that IL-6/IL-l5-induced Th17 plays a critical role in their conversion. Taken together, our findings indicate that CpG treatment is the most efficient agent that can convert tolerogenic DCs into immunogenic ones and induce long-lasting antitumor immunity.

4.2.2 INTRODUCTION

DCs are specialized antigen-presenting cells (APCs) that recognize, acquire, process and present antigen to naive, resting T cells for the induction of an antigen specific immune response (244). Immature DCs are widely distributed in the body, although they occupy only a small proportion (0.1-1%) of the cells in different lymphoid and non-lymphoid tissues (35, 36). Toll-like receptors (TLRs) are innate receptors that sense microbial products and trigger DC maturation, thus efficiently bridging innate and adoptive immunity (225). DCs express the broadest repertoire of TLRs through which they can recognize a plethora of microbial compounds. After challenging with microbial or inflammatory stimuli including endogenous (IL-1β, TNF-α, etc) and exogenous mediators (LPS and CpG) (2, 36, 340-342) in the micro environments, immature DCs undergo a complex process of maturation wherein they up-regulate MHC class II, CCR7 and co stimulatory molecules that are essential for naive T cell priming (127, 128) and migrate to T cell zones of lymph nodes to stimulate T cell responses (2, 36, 340, 341). In addition, DCs represent a critical source of IL-6, IL-12, IL-15, IL-1β, IL-23 or IFN-β, especially IL-12, which are key members in innate responses and drive T helper type 1 (Th1) polarization (145, 146, 458). IL-12 production by DCs is tightly controlled, as it requires first a priming signal provided by microbial products or IFN-γ and then an amplifying signal provided by T cells through CD40 ligand (CD40L) (217, 219). Therefore, CD40 and TLR4 or TLR9 signalings triggered by anti-CD40 Ab and LPS or CpG have been extensively explored to promote *in vitro* and *in vivo* DC maturation and induce Th1 type antitumor immunity (144, 217-219, 225, 458).

In addition to the micro environments wherein DCs acquire their Ag and different

stimuli determine their potential immunogenicities, the different DC subsets are also capable of inducing distinct Th or Tr cell-mediated immune responses (2, 36, 340-342, 459). In mouse spleens, DCs have been classified into three subsets based on CD4 and CD8 expression on their surface, which are named as $CD4^+8^-$ DC representing ~50% of splenic DCs and $CD4^-8^+$ and $CD4^-8^-$ DCs each ~25% of the total population (76, 99, 460). $CD4^-8^+$ DCs have been reported as variably effective stimulators of allogeneic $CD8^+$ T cell responses, and $CD4^-8^-$ and $CD4^+8^-$ DCs as more effective in stimulating $CD4^+$ T cell responses (99, 100). *In vivo,* $CD4^-8^+$ and $CD4^-8^-$ DCs can efficiently prime male antigen-specific CTL, whereas $CD4^+8^-$ DCs do so only weakly (99). Splenic DCs can also express tolerogenic phenotypes; $CD4^+8^-$ DCs reportedly mediate tolerance or bystander suppression against diverse T cell specificities (103), while $CD4^-8^+$ DCs can induce tolerance to tissue-associated Ags and heart transplant (104, 105). We have recently demonstrated that $CD4^+8^-$ and $CD4^-8^+$DCs subsets induced Thl cell responses leading to protective antitumor immunity, whereas $CD4^-8^-$ DC secreting TGF-β stimulated responses of Trl cells secreting IL-10 and IFN-γ leading to immune tolerance (106). In addition, we have also shown that Trl-induced immune tolerance is mediated by its secretion of immunosuppressive IL-10.

In this study, we investigated whether CD40 and TLR signalings can convert tolerogenic $CD4^-8^-$ DCs into immunogenic ones and what type of $CD4^+$ T cell responses is stimulated by the converted DCs. We stimulated $CD4^-8^-$ DCs with agonistic anti-CD40 Ab, LPS and CpG and phenotypically and functionally characterized treated DCs. We demonstrated that CpG treatment most efficiently converts the tolerogenic DCs into immunogenic ones capable of stimulating Thl and Thl7 cell responses leading to induction of efficient CTL responses and antitumor immunity.

4.2.3 MATERIALS AND METHODS

4.2.3.1 Cell lines, antibodies, cytokines, peptides and animals

The ovalbumin (OVA)-transfected B16 melanoma cell line BL6-10_{OVA} was generated in our lab (461). EL4 T cell lymphoma, EG7 (i.e., OVA-transfected EL4 cells) and LB27 B cell hybridoma expressing I-A^b were obtained from the American Type Culture Collection (Manassas, VA). BL6-10_{OVA} and EG7 tumor cells were maintained in DMEM plus 10% FCS and 0.5 mg/ml G418 (Invitrogen, Burlington, ON, Canada). Biotin- and FITC-conjugated monoclonal anti-mouse I-A^b (AF6-120.1), CD4 (GK1.5), CD8 (53-6.7), CDllc (HL3), CD54 (3E2), CD80 (16-10A1), CD86 (GL1), biotin- conjugated anti-CD25 (7D4), CD69 (H1.2F3), Vβ5.1,5.2 TCR (MR9-4), and those above related FITC-conjugated isotype antibodies were obtained from BD Biosciences (Mississauga, ON). Neutralization antibody against mouse IL-6 and IL-15 were purchased from eBioscience (San Diego, CA). Streptavidin-FITC was obtained from Caltag (Burlingame, CA). Phosphorothioate-modified CpG ODN 1826: 5'-TCCATGA*CG*TTCCTGA*CG*TT-3', and the control ODN 1982: 5'-TCCAGGACTTCTCTCAGGTT-3' were synthesized by Operon (Huntsville, AL) (462) and diluted in PBS. No endotoxin could be detected in ODN preparations (<0.03 EU/ml); BioWhittaker, Walkersville, MD). Agonistic anti-CD40 antibody was purified in our lab from the FGK 45.5 hybridoma cells (463) culture supernatant. LPS were purchased from Sigma (St. Louis, MO). Recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was obtained from R&D Systems Inc. (Minneapolis, MN). Wild-type C57BL/6 and OVA-specific T cell receptor transgenic OT I, OT II (440),

$CD4^{-/-}$ (464), $CD8^{-/-}$ (465), IL-$6^{-/-}$ (466) and IL-$15R^{-/-}$ (467) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). All animal experiments were carried out according to the guidelines of the Canadian Council for Animal Care.

4.2.3.2 Isolation of splenic DCs

This protocol is a modified version of that described by Livingstone et al (441). Briefly, spleens from wild type C57BL/6 or IL-6-/- mice were injected with DMEM containing 1 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ), cut into small fragments and digested in the above enzyme solution for 45-60 min at 37°C. Single cell suspension was prepared by press the digested tissues through a stainless mesh. After the red blood cells were lysed with Tris-NH_4Cl, the spleen cells were washed once in PBS and resuspended in RPMI-1640 plus 10% FCS and 50 μM 2-ME (CM) and incubated at 37°C in 100 x 20 mm petri dishes (one spleen equivalent per dish). After 90 min incubation, nonadherent cells were removed by gentle washing 3 times with pre-warmed normal saline. Adherent cells were cultured overnight in CM plus rmGM-CSF (15-20 ng/ml). For OVA protein pulsing, adherent cells were cultured overnight in AIM-V® medium (serum-free lymphocyte medium) (Invitrogen, Burlington, ON) plus rmGM-CSF (15-20 ng/ml) and indicated concentration of OVA (Sigma Chemical Corp., St. Louis, MO). The next day, the non-adherent DCs (DC_{OVA}) were harvested and used for isolation of spleen DC subsets.

4.2.3.3 Purification of splenic $CD4^-8^-$DC subset

DC subsets were purified as previously described (106) with modification. Briefly, the above nonadherent DCs were incubated with the biotin-conjugated anti-CD8 and anti-CD4 antibody and then with anti-biotin antibody-coupled MACS® microbeads (Miltenyi Biotech, Auburn, CA). All of the cell suspension was then loaded onto LD

column to completely remove any cells expressing CD4 and CD8 markers according to the company's instruction. The final flow-through cells were incubated with the biotin-conjugated anti-CD11 c antibody and then with biotin conjugated microbeads and passed over a MACS® LS column. The bound cells were freshly prepared splenic $CD4^-8^-$ DCs and were used for the following study.

4.2.3.4 Activation and protein pulsing of $CD4^-8^-$ DC subset

Purified $CD4^-8^-$ DCs were cultured in AIM-V containing rmGM-CSF (15-20 ng/ml), OVA protein (0.1 mg/ml), 2-ME (50 μM) with CpG (5 μg/ml), LPS (2 μg/ml), or anti-CD40 (10 μg/ml) for 8 hrs. Those DCs were harvested for flow cytometric analysis, T cell culture and animal study, or purification of the total RNA for DNA array analysis using the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada); their culture supernatants were collected for measuring IL-6, IL-10, IL-12, IL-15, IFN-γ and TNF-α using ELISA kits from BD biosciences, and TGF-β using ELISA kit from R&D System (Minneapolis, MN). When DCs were pulsed with OVA protein, they are termed as $CD4^-8^-$ DC_{OVA}.

4.2.3.5 Phenotypical characterization of stimulated $CD4^-8^-$DCs

Purified $CD4^-8^-$ DCs which were cultured as described above were analyzed by flow cytometry using a panel of Abs specific for MHC class II, CD40, CD54, CD80,CD86 as previously described (106).

4.2.3.6 DNA array analyses of differentially stimulated $CD4^-8^-$DCs

The DNA array analyses were conducted using commercial GEArray® S Series Mouse Dendritic & Antigen Presenting Cells Gene Array (Superarray Biosciences Corp., Frederick, MD), comprising the cDNAs of antigen presentation cells-related cytokines,

chemokines and their receptors, antigen uptake, antigen presentation, cell surface receptors, and signal transduction genes (described within the SuperArray website, www.supperarray.com) double-spotted on a 3.8x4.8 cm nylon membranes. Prior to hybridization, the membranes were prewetted with 5ml deionized water, then prehybridized for 1-2 h at 60°C with hybridization solution supplemented with 100 µg/ml heat-denatured salmon sperm DNA (SuperArray).

To generate the membrane probes for the analyses, total RNA was isolated from the activated DCs using RNeasy Mini kit (Qiagen Inc. Mississauga, ON). The concentration and quality of the RNA was determined by spectroscopy (OD_{260}) and denaturing agarose gel electrophoresis, respectively. The labeled cDNAs were generated according to the manufacturer's protocol (SuperArray) using AmpoLabeling-LPR Kit. Briefly, RT primer (1ul) were annealed to 5 µg of RNA (final volume, 10 µl) for 3 min at 70°C, and cool to 37°C and kept for 10 min; then incubated for 25 min at 37°C after adding Buffer BN 4 µ1, RNase-free H_2O 4 µ1, RNase Inhibitor 1 µl and Reverse Transcriptase 1 µl. RT products were then mixed with Mouse Dendritic & Antigen Presenting Cells Gene Array -specific primers 9 µl, Buffer L 18 µl, biotin-16-dUTP (Roche Diagnostics, Laval, Quebec) 2 µl and DNA polymerase 1 µl for Linear Polymerase Reaction(LPR) Labeling Reaction. After being treated at 85°C for 5 min. the LPR were done for 30 cycles using the following condition: 85°C, 1 min, 50°C, 1 min, 72°C, 1 min, and then 72°C for 10 min. The labeled cDNAs were denatured for 2 min at 94°C and quickly chilled on ice, then incubated with the prehybridized array membranes in roller bottles for overnight at 60°C. The probed arrays were washed twice in 2 x SSC/1%SDS for 1 hr at 60°C, and then in 0.1 x SSC/0.5%SDS at 60°C for 2hr. Prepare the blocking buffer by adding 0.5 ml of 20% SDS to 100 ml Odyssey Blocking Buffer (LI-COR, Cat #927-40000) for a final SDS concentration of 0.1 %, add 5 ml into the tube and gently shake at room temperature for a

minimum of 120 minutes. Dilute the streptavidin-IRDye™ 800CW conjugate with Blocking Buffer to a concentration of 1:70,000. Remove old blocking buffer and add 5 ml of diluted streptavidin-IRDye™ 800CW solution. Incubate 10-15 minutes at room temperature while shaking. Wash the blot 3 times in 1X PBST (0.1 % Tween-20) with shaking, for 10 min each, at room temperature. Follow with a rinse in 1X PBS, with shaking, for 5 minutes at room temperature. Results (dot signal density) were scanned using Odyssey® Infrared Imaging System and analyzed with the supplied software according to our previous description (468).

4.2.3.7 *In vitro* T cell proliferation assay

To assess the antigen presenting function of differentially stimulated $CD4^-8^-$ DC_{OVA}, we performed antigen specific T cell proliferation assay. Naive OVA-specific $CD4^+$ T cells from OT II mice were obtained by passage of splenocytes through nylon wool columns (173). The T cells were fractionated by negative selection using anti-mouse CD8 (Ly2) paramagnetic beads (Dynal Biotech) according to the manufacturer's protocols. OT I $CD8^+$ T cells were purified by adopting the same method as for OT II $CD4^+$, but using Dynabeads mouse CD4 (L3T4). The irradiated (3000 rad) $CD4^-8^-$ DC_{OVA}, and their twofold dilutions were cultured with a constant number of naive OT II $CD4^+$ and OT I $CD8^+$ T cells ($1x10^5$ cells/well), respectively. After culturing for 3 days, thymidine incorporation was determined by liquid scintillation counting (173).

4.2.3.8 Cytokine analysis of activated OT II $CD4^+$T cells

Naive OVA-specific $CD4^+$ T cells (2×10^5 cells/ml) from OT II were purified as described above and were stimulated for 3 days with freshly purified $CD4^-8^-$ DC_{OVA} from overnight culture or then further treated with the above culture conditions (1×10^5 cells/ml) in the presence of IL-2 (10 U/ml) and then isolated from Ficoll-Paque

(Amersham Biosciences) density gradients (173); cell culture supernatants were harvested and used for IL-17 analysis. Some cell culture was added with Golgistop® (BD biosciences) for the last six hours, cells were then harvested for intracellular staining of IL-17 with FITC-anti-IL-17 and PE-anti-CD4. The *in vitro* activated OT II T cells were further stimulated with LB27 pulsed with OVA II peptide for 24 h, and all the above culture supernatants were harvested for assay of IL-2, IL-4, IL-l0, IL-17, IFN-γ, TNF-α and TGF-β using ELISA kits from BD Biosciences and eBioscience (IL-17).

4.2.3.9 Tetramer staining assay (*In vivo* antigen specific CTL proliferation)

Six days after the immunization, 50 μL blood was taken from the tail of the immunized mice. Those blood samples were incubated with PE-conjugated H-2K^{b}/OVA$_{257-264}$ tetramer (Beckman Coulter, Mississauga, Ontario, Canada) for 30 min first and then with FITCconjugated anti-CD8 antibody for another 30 min at room temperature. The erythrocytes were then lysed using lysis/fixed buffer (Beckman Coulter) according to the company instruction. The cells were washed and analyzed by flow cytometry (469).

4.2.3.10 *In vivo* cytoxicity assay

The *in vivo* cytotoxicity assay was performed with modification as described by (470). Briefly, splenocytes were harvested from naive mouse spleens and incubated with high (3.0 μM, CFSE^{high}) or low (0.3 μM, CFSE^{low}) concentrations of CFSE to generate differentially labeled target cells. The CFSE^{high} cells were pulsed with OVA I peptide, whereas the CFSE^{low} cells were pulsed with Mut l peptide and served as internal controls. These peptide pulsed target cells were washed extensively to remove free peptides and then i.v.-coinjected at a 1: 1 ratio into the above immunized mice 7-9 days after immunization. Sixteen hours after the target cell delivery, the spleens of immunized mice

were removed, and their respective fluorescence intensity of residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the recipients' spleens were analyzed by flow cytometry. To calculate specific lysis, the following formulas were used: ratio = (percentage $CFSE^{low}$/percentage $CFSE^{high}$); percentage specific lysis = (1 (ratio naive/ratio immunized) x 100) (471).

4.2.3.11 Animal study

For investigation of antitumor immunity, wild type C57BL/6, $CD4^{-/-}$, $CD8^{-/-}$, or IL-$15R^{-/-}$ mice (n = 8/group) were vaccinated s.c. with 0.5-1 x $l0^6$ differently prepared and OVA pulsed $CD4^-8^-$ DCs from wild type B6 or IL-$6^{-/-}$ mice, respectively, and then challenged s.c. 10 days later with l x $l0^5$ BL6-10_{OVA} tumor cells. Tumor growth and mouse survival were monitored daily for 60 days after tumor cell inoculation (106); for humanitarian reasons, all mice with tumors that achieved a size of 1.5 cm in diameter were sacrificed.

4.2.4 RESULTS

4.2.4.1 CpG, LPS and anti-CD40 Ab treatment induce $CD4^-8^-$ DC maturation

The freshly prepared $CD4^-8^-$ DCs from C57BL/6 mice expressed lower level of MHC class II, CD40, CD54, CD80 and CD86, indicating that they are relatively immature DCs (106). However, after culturing with CpG, LPS, anti-CD40 Ab for 8 hours, $CD4^-8^-$ DCs up-regulated expression of the above molecules (Fig.4.2. lA), indicating that they become a more matured form of DCs upon CD40 and TLR signaling. In addition, all treatments completely blocked DC's TGF-β secretion (Fig.4.2. 1B). CpG treatment stimulated $CD4^-8^-$ DCs to secrete higher amount of IL-6 (380 pg/ml), IL-10 (130 pg/ml), IL-15 (280 pg/ml) and IFN-γ (130 pg/ml) than those for the other two stimuli. However, CD40 signaling induced the highest level of IL-12 (320 pg/ml) secretion (Fig.4.2. 1B).

4.2.4.1 DNA microarray analyses of gene expressions identified more Th1 polarization molecules among CpG treated $CD4^-8^-DC_{OVA}$

Total RNAs were purified from stimulated DCs with RNeasy Mini kit(Qiagen), GEArray® S Series Mouse Dendritic & Antigen Presenting Cells Gene Array (Superarray Biosciences Corp., Frederick, MD)(comprising the cDNAs of antigen presentation cells-related cytokines, chemokines and their receptors; antigen uptake; antigen presentation; cell surface receptors and signal transduction genes) were used to analyze the DC-related gene expression by using streptavidin-IRDye™ 800CW conjugate for detecting hybridization signal with Odyssey® Infrared Imaging System and methods as we described before (468).

Among the 151 DC related genes, 96 were expressed in LPS-stimulated $CD4^-8^-$ DC_{OVA} and the mRNA expression level of 25 genes were higher in LPS group. 140 were expressed in CpG-activated DCs and the mRNA expression level of 77 genes were higher in CpG group. 46 genes were expressed at a similar level in both groups (Table 4.2. 1, 4.2. 2, 4.2. 3). mRNA expression results confirmed our DC phenotype and cytokine profiles analyses. Although in LPS group, those genes expressed at higher level could not explicitly explain why this kind of stimulated DCs induced Th1 response much less efficiently than CpG-treated DCs, CpG stimulation did increase the expression of many of Th1/Th17 polarization molecules such IL-1, IL-6, IL-12β (IL-23β), IL-15, IFN-γ, CCL-19, OX40L, 4-1BBL. IL-1 and IL23 have been reported to be involved in the formation and/or maintenance of Th17 (472). 4-1BB costimulatory signals preferentially induce $CD8^+$ T cell proliferation and lead to the amplification *in vivo* of cytotoxic T cell responses (48). OX40 ligand (OX40L) strongly inhibited the generation of IL-10-producing Tr1 cells and their IL-10 production and strongly inhibited suppressive function of differentiated IL-10-producing Tr1 cells (473). CCL19 could promote IFN-γ-dependent antitumor responses in a lung cancer model (474) and as an adjuvant for DNA vaccination, it induced a Th1-type T-cell response and enhanced antitumor immunity (475). TRANCE, which is a DC-specific survival factor (476) and is beneficial to the formation of CTL *in vivo* (477), was also only expressed significantly in the CpG group, which further confirmed the previous report (478). This molecule's expression perfectly explained the dilemma of why the CpG stimulated group has higher cell survival after the same length of stimulation compared with LPS group (data not shown). DCIR expression was down-regulated by signals inducing DC maturation such as CD40 ligand, LPS, or TNF-alpha. Thus, DCIR is differentially expressed on DCs depending on their origin and stage of maturation/activation. DCIR represents a novel

surface molecule expressed by Ag presenting cells, and of potential importance in regulation of DC function (479).

4.2.4.3 CpG-treated $CD4^-8^-$ DCs induce a mixture of $CD4^+$ Th1/Th17 cell response

To assess the *in vitro* stimulatory effect, we performed T cell proliferation assays. 8 hr CpG-treated $CD4^-8^-$ DCs more efficiently stimulated $CD4^+$ and $CD8^+$ T cell proliferation than the freshly prepared $CD4^-8^-$ DCs ($p<0.05$), whereas the other two stimuli-treated ones had a similar stimulatory effect as the freshly prepared $CD4^-8^-$ DCs (Fig.4.2. 2A). In contrast to the freshly prepared $CD4^-8^-$ DCs capable of stimulating IL-10/IFN-γ-expressing $CD4^+$ Trl cell responses (106), $CD4^+$ T cells stimulated by all three stimuli maintained its IFN-γ secretion, but significantly down-regulated IL-10 expression ($p<0.05$) (Fig.4.2. 2B). Anti-CD40 Ab-treated DCs secreted TNF-α (500 pg/ml), whereas CpG-treated DCs induced $CD4^+$ T cells to secrete IL-17 (330 pg/ml) (Fig. 4.2. 2B). By analyzing the intracellular cytokine of $CD4^+$ T cells, we found that there were three distinct $CD4^+$ T cell populations that secreted IFN-γ only, IL-17 only, and both IL-17 and IFN-γ, respectively, indicating that CpG-treated DCs induced a mixture of $CD4^+$ Th1 and Th17 cell responses.

4.2.4.4 CpG-treated $CD4^-8^-$ DCs induce the most efficient $CD8^+$ CTL responses and antitumor immunity

We previously demonstrated that the freshly prepared tolerogenic $CD4^-8^-$ DC_{OVA} suppressed OVA-specific $CD8^+$ CTL responses and antitumor immunity (106). To assess the *in vivo* stimulatory effect, we performed tetramer staining assays using blood samples of mice immunized with the above treated DC_{OVA}. As shown in Fig.4.2. 3A, vaccination

of mice with the original $CD4^-8^-$ DC_{OVA} did not induce any OVA-specific $CD8^+$ CTL responses, whereas three stimuli-treated $CD4^-8^-$ DC_{OVA} all induced *in vivo* OVA-specific $CD8^+$ CTL proliferation, accounting for 1.50% , 0.66%, 0.42% of the total $CD8^+$ T cell population, respectively. Among them, CpG-treated $CD4^-8^-$ DC_{OVA} induced the highest $CD8^+$ CTL responses ($p<0.05$). To assess the effector function of $CD8^+$ T cells activated by these three stimuli-treated $CD4^-8^-$ DC_{OVA}, we performed *in vivo* cytotoxicity assays. As shown in Fig.4.2. 3B, vaccination of mice with the original $CD4^-8^-$ DC_{OVA} did not induce any OVA-specific $CD8^+$ CTL responses against OVA I-pulsed target cells, whereas CpG-, LPS- and anti-CD40 Ab-treated $CD4^-8^-$ DC_{OVA} all stimulated OVA-specific $CD8^+$ CTL responses leading to loss of OVA-specific target cells by 84% (CpG), 45% (LPS) and 37% (anti-CD40 Ab), respectively. This confirmed that CpG-treated $CD4^-8^-$ DC_{OVA} induce the strongest CTL effector function ($p<0.05$). In addition, our animal studies further confirmed the results derived from the above *in vivo* CTL proliferation and cytotoxicity assays. As shown in Fig. 4.2. 3C, mice with inoculation of BL6-10_{OVA} tumor cells in PBS-treated or freshly prepared tolerogenic $CD4^-8^-$ DC_{OVA} died within 25 days after tumor cell inoculation, similar to what we previously reported (106). However, the groups of mice immunized with three stimuli-treated OVA-pulsed $CD4^-8^-$ DC_{OVA} were all protected against OVA-expressing BL6-10_{OVA} tumor cell challenge to various extents. Among them, vaccination of mice with CpG-treated $CD4^-8^-$ DC_{OVA} provided the strongest protection with 10 out of 10 (100%) mice surviving ($p<0.05$), while LPS and anti-CD40 groups only provided around 50% and 45% protection, respectively. To study the immune mechanism in the antitumor immunity, we immunized $CD8^{-/-}$ and $CD4^{-/-}$ mice lacking of $CD8^+$ and $CD4^+$ T cells with CpG-treated $CD4^-8^-$ DC_{OVA} and then challenged the mice with tumor cell inoculation. As shown in Fig.4.2. 3D, we found that 100% and 75% of mice immunized the CpG-treated DC_{OVA} lost immune protection against tumor challenge, respectively ($p<0.05$); indicating that

both $CD8^+$ and $CD4^+$ T cells are involved in CpG-treated DC_{OVA} induced T cell responses. Taken together, our data indicate that CpG treatment completely converts the tolerogenic DCs into immunogenic ones capable of efficiently stimulating $CD4^+$ T cell-dependent OVA-specific $CD8^+$ CTL responses and antitumor immunity.

4.2.4.5 IL-6 and IL-15 play an important role in CpG-treated $CD4^-8^-$ DC_{OVA}-induced Th1/Th17 responses

It has been demonstrated that various cytokines including TGF-β, IL-6, IL-21 and IL-23 play a key role in inducing $CD4^+$ Thl/Th17 cell responses (480). To assess the potential effect of IL-6 and IL-15 in CpG-treated $CD4^-8^-$ DC-induced Thl/Th17 cell responses, we added anti-IL-6 and anti-IL-15 Abs to CpG-treated $CD4^-8^-$ DC_{OVA} culture, As shown in Fig. 4.2. 4A, interestingly, both IL-17 and IFN-γ secretion were decreased by around 50% for anti-IL-6 Ab group and 45% for anti-IL-15 Ab group ($p<0.05$), indicating that IL-6 and IL-15 secretion are involved in CpG-treated DC induced Thl/Thl7 immune responses. To assess whether Thl/Thl7 cells are involved in CpG-treated DC-induced antitumor immunity, as shown in Fig.4.2.4B, we used CpG-treated $CD4^-8^-$ DC_{OVA} derived from $IL6^{-/-}$ mice for immunization of wild-type C57BL/6 mice or used CpG-treated $CD4^-8^-$ DC_{OVA} derived from wild-type C57BL/6 mice for immunization of $IL\text{-}15R^{-/-}$ mice as recipient mice. We found that 2/3 mice immunized with $IL\text{-}6^{-/-}$ mice-derived DC_{OVA} lost animal protection compared with wild type DC, wild type mice derived DC_{OVA} immunized $IL\text{-}15R^{-/-}$ mice could only protect mice from tumor challenge up to 50% ($p<0.05$), indicating that IL-6 and IL-15 secretion is critical to CpG-treated DC_{OVA}-induced antitumor immunity.

4.2.5 DISCUSSION

DCs are professional APCs that play a crucial role as initiators or modulators of adoptive immune responses. Although DC-based vaccines have been used successfully to generate CTL activity against tumor Ags, evidence has accumulated that DCs have also a potent ability to tolerize T cells in an Ag-specific manner (459). Immature DCs can mediate immune tolerance, presumably by induction of regulatory T cells (341, 342). They can stimulate $CD4^+$ and $CD8^+$ Tr cells and suppress autoimmune diseases (340, 481, 482). Sometimes, mature DCs pulsed with specific antigen could expand the $CD4^+$ T cells *in vitro* to suppress the development of autoimmune diabetes and restore normoglycemia in diabetic NOD mice when the latter were administrated *in vivo* (343, 344).

Toll-like receptor (TLR) ligands have been widely reported to promote the maturation of DCs and induce Th1-polarized immune response which would benefit cancer immunotherapy (457). Synthetic oligodeoxynuc1eotides (ODNs) that contain immunostimulatory CpG motifs bind to TLR 9 on DC and trigger an immunomodulatory cascade that skews the host's immune milieu in favor of Th1 responses (483-485). Vicari et al firstly demonstrated the successful reversal of intratumoral tolerogenic DC paralysis by CpG ODNs and anti-IL 10 receptor antibody (Ab) (233). However, single use of either CpG or anti-IlL-l0R Ab was inactive. Recently, Guiducci et al confirmed their results with the treatment of CpG ODNs and anti-IL-10R Ab in another animal model (232). They demonstrated that treatment of CpG ODNs and anti-IL-10R Ab can convert tumor-infiltrating tolerogenic type 2 macrophages (M2) secreting immunosuppressive IL-10, TGF-β and prostaglandin E2 (PGE_2) to immunogenic type 1 macrophages (M1) secreting inducible nitric oxide synthase, IL-12 and TNF-α, and thus eradicate established

tumors. When CpG or plus anti-IL-10 were injected inside the tumor, the tumor infiltrated immature DC could become matured and induced the regression of established tumor. If the tumor cells were transfected with CCL20 (MIP-3α) or CCL 16, chemokines for immature DC, the antitumor immunity were even stronger than just those treatments alone (231, 232). Murine renal cell carcinoma (RENCA)-pulsed CpG-ODN-treated DCs were able not only to significantly reduce tumor growth but also to prevent tumor implantation in 60% of mice (230). Poly(I:C)- and CpGs- treated DCs, which secrete very high level of IL-12, loaded with B16 melanoma cells and injected into tumor-bearing mice induce Th1-skewed tumor-specific $CD4^+$ T cells and a significant reduction in tumor growth (133). Among the spleen DCs, our specific DC $CD4^-8^-$ DCs express TLRs from TLR1 to TLR 9, but $CD4^-8^+$ DCs and $CD4^+8^-$ DCs don't have TLR7 and TLR3, respectively (226). This means that almost every kind of TLR ligands could be used to trigger and induce the maturation of $CD4^-8^-$ DCs.

CD40, the receptor for CD40L (CD154), is expressed on APCs such as B cells, macrophages, and DCs with its expression increasing with maturation. CD40L is a member of the TNF family and is expressed primarily on activated Th cells. CD40 and CD40L interaction induces the maturation and activation of DCs and help TLR ligands to stimulate DCs to secrete cytokines such as IL-12, which is essential for the development of the Th1 response (217, 218). Agonistic anti-CD40 Ab can function as CD40L and modulate DC to drive naive $CD8^+$ T cells, while ligation of CD40 on DC restores CTL activity in $CD4^+$ depleted mice. In addition, mice treated with anti-CD40L Ab had impaired induction of Ag-specific CTLs. These data indicate that CD40-CD40L interactions play a key role in modulating APC function so that these cells can prime $CD8^+$ T cells *in vivo* (219). CD40 ligand and TNF-α combination could antagonize the inhibitory effects of interleukin l0 on T cell stimulatory capacity of DCs (234).

The cooperation of TLR ligands and CD40L or anti-CD40 in induction of DCs to secrete IL-12 is very important in generating CTL from naive polyclonal $CD8^+$ T cells *in vitro* (219). CpG and PPD need the help of CD40L to stimulate the spleen DC mixture, $CD4^-8^-$ DCs and $CD4^-8^+$ DCs, but not $CD4^+8^-$ DCs to secrete IL-12. Peptide antigen could also enhance the IL-12 induction capacity of PPD (226). LPS could not efficiently stimulate bone marrow or monocyte-derived DCs to secrete IL-12 without the help of CD40L or T cell help (217, 436). Various TLR ligands combination such as R848+LPS, R848+poly (I:C), LPS+CpG, and LPS+ poly(I:C), which react with TLRs on the cell surface and inside cellular compartments at the same time but separately, have been reported to stimulate higher level of IL-12 secretion than single ligands and reach the similar level of IL-12 induced by TLR ligands +IFN-γ (144, 237, 486). DCs activated simultaneously through TLR-7 (and TLR 2/6,3,4, 7, 9 to some extent) and anti-CD40 displayed 10-fold increases in their ability to stimulate $CD8^+$ T cell responses over DCs activated through each individual receptor alone (53, 238). Synergistic activation of macrophages via CD40 and TLR9 also results in T cell independent antitumor effects (239). Simultaneous activation of TLR-3 and TLR-9 induced synergistic levels of nitric oxide, IL-12, TNF-α, and IL-6 production (240).

$CD4^-8^-$ DCs has been reported to be tolerogenic DCs and induce Trl response to inhibit $CD8^+$ CTL response, while the other two subsets $CD4^-8^+$ DC and $CD4^+8^-$ DC are immunogenic and stimulate strong antitumor response (106). In this study, for the first time, we demonstrated that CpG, LPS and anti-CD40 Ab can all convert tolerogenic $CD4^-8^-$ DC subset secreting immunosuppressive TGF-β into immunogenic ones secreting IL-12. The Th1 cell response is favored when pulsed with low concentration of OVA (0.1 mg/ml*)* compared with the freshly isolated $CD4^-8^-$ DCs. However, if the protection efficiency against tumor challenge among those groups were carefully analyze in detail,

we found that the IL-12 secretion level is not always proportional to the antitumor immunity; anti-CD40 group DCs boast highest level of IL-12, however, they only provided around 50% protection, while the CpG group DCs produce only 1/3 of IL-12 compared with the above group but provided 100% protection (please refer to Fig.4.2. 3C). Antigen presentation assay and *in vivo* CTL formation and cytotoxicity detection all indicated that CpG-treated $CD4^{-}8^{-}$ DCs is the strongest Thl inducer among those three group stimuli (see Fig.4.2. 3, A and B).

IL-6 has been found to inhibit the function of regulatory T cells (150, 151); IL-6, IL-15, IL-21 and IL-23 are among those key cytokines involved in the development and maintenance of a new T cell subset, Th17 (115, 152, 480, 487). Th17 has been confirmed to be the major player in autoimmunity and anti-bacterial infection instead of Th1 (153), however, other reports found that Th17 still needs to synergize with Th1 to induce EAE (488) and Th1 and Th17 T cells are independently capable of inducing disease in two established models of autoimmunity(489, 490); Th17, which secrete IL-21 (155), has been indirectly linked to antitumor immunity, which indicate that Th1 and Th17 might cooperate in antitumor immunity. Hsp70-mediated induction of Th17 autoimmunity had been exploited to reject established prostate tumors (157). Very recently, tumor-specific Th17-polarized cells have been reported to eradicate large established melanomas (158). Although IL-12 is considered to a classic type I cytokine, dendritic cells-derived IL-12 might not be critical for generation of CTLs *in vivo* under certain conditions whereas other cytokine(s) such as IL-15, IL-18 and IL-23, etc might be involved (145). LPS has been described to induce the $CD8^{-}$ DC to activate MyD88 dependent Delta 4 Notch-like ligand expression and promote Th1 response independent of IL-12 (147).

We checked IL-6 and IL-15 secretion among those DCs and only the CpG group secretes these two cytokines. IL-6 has been reported to inhibit the development of

regulatory T cells and promote the formation of Th17 T cells (480). Our neutralization study found that IL-6 and IL-15 were necessary for the formation of Th17 T cells, because IL-17 secretion from those CpG-stimulated DC activated OT II $CD4^+$ T cells were decreased more than 30-50% when either of the neutralization antibody were added to the coculture of dendritic and T cells. IFN-γ secretion was also reduced, indicating that both cytokines were also involved in the Thl induction. *In vivo* IL-6 blockade has also been reported to inhibit the induction of myelin antigen-specific Th17 cells and Thl cells in experimental autoimmune encephalomyelitis (491). Then we used IL-$6^{-/-}$and IL-$15R^{-/-}$ mice to carry out animal studies. Compared with the wild type mice, in the absence of IL-6 secretion from DCs or impaired reaction to the DC derived IL-15, vaccinated mice lost 75% and 50% protection, respectively. When CD4 KO mice were used for animal study, 75% of those immunized mice lost their tumor growth inhibition capacity, which indicated that Th response is a major part of DC induced antitumor immunity. Our results provide the first direct evidence that Th17 cooperate with Thl to induce antitumor immunity and CpG is the best candidate for converting the tolerogenic $CD4^-8^-$ DC into immunogenic ones stimulating Thl and Th17 mixed antitumor immune response.

This manuscript will be submitted to the Journal of Immunology.

4.2.6 Figure legends for Manuscript 4.2

Figure 4.2.1: Phenotype and cytokine analyses of differentially stimulated and fresh $CD4^-8^-$DCs.

A, $CD4^-8^-$DCs were isolated as described in Material and Methods and cultured in AIM-V containing 10 ng/ml rmGM-CSF and 0.1 mg/ml with CpG (5 ug/ml), LPS (2 ug/ml) or anti-CD40 (10 ug/ml) alone. After 8 hrs, cells were harvested and stained with FITC labeled anti-CD40, CD54, CD80, CD86, and I-Ab, and analyzed by flow cytometry, FITC labeled isotype IgGs (dotted line) were used as control. A representative example of three independent experiments is shown. B, $CD4^-8^-$DCs were cultured as above, The supernatants were harvested after their culture. IL-6, IL-10, IL-12, IL-15, IFN-γ,TNF-α and TGF-β were measured using related ELISA kits from BD Biosciences, eBiosciences and R & D Systems. One of three similar experiments is shown.

Figure 4.2.2: *In vitro* autologous T cell proliferation assays and activated $CD4^+$ T cell cytokine secretion.

A, Irradiated (4000 rad) differentially treated $CD4^-8^-DC_{OVA}$ (0.1×10^6 cells/well) and their 2-fold dilutions were cultured with a constant number (0.1×10^6 cells/well) of autologous OT II $CD4^+$ and OT I $CD8^+$ T cells, respectively. After 72 h, thymidine incorporation was determined by liquid scintillation counting. $*$, $p < 0.05$ vs cohorts of LPS- and anti-CD40 Ab- treated $CD4^-8^-$ DCs (Student's *t* test). One representative experiment of three is depicted. B, OT II $CD4^+$ T cells were cultured *in vitro* for 3 days with $CD4^-8^-$ DC_{OVA} treated by CpG, LPS or anti-CD40 antibody for 8hrs, respectively; then activated T cells were re-stimulated with LB27-OVAII for 48 hrs. All

the above culture supernatants were measured for IL-2, IL-4, IL-10, IL-17, IFN-γ, TNF-α, and TGF-β secretion by ELISA. *, $p<0.5$ vs cohorts of LPS-, anti-CD40 Ab-treated and fresh $CD4^-8^-$DCs activated T cells. One representative experiment of three is shown.

Figure 4.2.3: *In vivo* CTL proliferation and cytotoxicity assay, and animal study.

A, After vaccination s.c. with differentially treated $CD4^-8^-$ DC_{OVA}, collect 50 ul of mouse tail vein blood into 1.5 ml eppendorf tubes containing 50 ul 2 x heparin and gently mix well. Add 1.5 ul H2-K^b OVA I tetramer-PE reagents each sample and incubate at room temperature for 30 min in the dark place, and then add 1.5 ul anti-CD8-FITC for another 30 min, add 500 ul lysis buffer and 12.5 ul fixative and lyse RBC for 30-60 min. Wash the cells with PBS for two times and suspend the cells in 500 ul PBS and run flow to detect the CD8 and tetramer double positive cells. The experiment shown is representative of two additional experiments. B, Differentially labeled $CFSE^{high}$ and $CFSE^{low}$ naïve C57B/L splenocytes were pulsed with OVAI peptide and DMEM only, respectively, then injected by tail vein into the PBS(○) and stimulated DC immunized mice 6-7 days before. 18-20 hrs later, the splenocytes of mice were collected to analyze the specific lysis of OVA I labeled $CFSE^{high}$ cells, as determined by live cells gated flow cytometry. C, Mice were vaccinated with freshly isolated $CD4^-8^-$ DCs or cultured in media containing 15 ng/ml rmGM-CSF and 0.1 mg/ml OVA with CpG (5 ug/ml) (■) , LPS (2 ug/ml) (▲), or anti-CD40 antibody (10 ug/ml) (♦) for 8 hours, respectively; and ten days later were challenged s.c. with $1x10^5$ $BL6\text{-}10_{OVA}$ tumor cells. Animal mortality was monitored daily up to 8 weeks. D, CpG-treated $CD4^-8^-$ DC_{OVA} were used to immunize the CD4 (▼) and CD8 (▲) knock out mice as above, normal B6 mice (■)

were used as positive control. Then the animals were challenged and observed as above. The data are representative of three experiments with similar results for b and c.

Figure 4.2.4: IL-6 and IL-15 were the crucial cytokine for inducing Th1/Th17 antitumor immunity.

A, OT II $CD4^+$ T cells were cultured *in vitro* for 3 days with CpG-treated $CD4^-8^-$ DC_{OVA} only, or with 10 ug/ml anti-IL-6, or anti-IL-15 neutralization Ab, respectively; All the above culture supernatants were measured for IL-2, IL-4, IL-10, IL-17, IFN-γ, TNF-α, and TGF-β secretion by ELISA. One representative experiment of three is shown. B, CpG-treated $CD4^-8^-$ DC_{OVA} isolated from IL-$6^{-/-}$ mice (□) were used to vaccinate normal B6 mice and wild type B6 derived CpG-treated $CD4^-8^-$ DC_{OVA} were used to immunize IL-15Rα$^{-/-}$ mice(Δ) and normal B6 mice(■) , respectively. Ten days later were challenged s.c. with $1x10^5$ $BL6\text{-}10_{OVA}$ tumor cells. Animal mortality was monitored daily up to 8 weeks. The data are representative of three experiments with similar results.

Figure 4.2. 1: Phenotype and cytokine analyses of differentially stimulated and fresh CD4$^-$8$^-$ DCs.

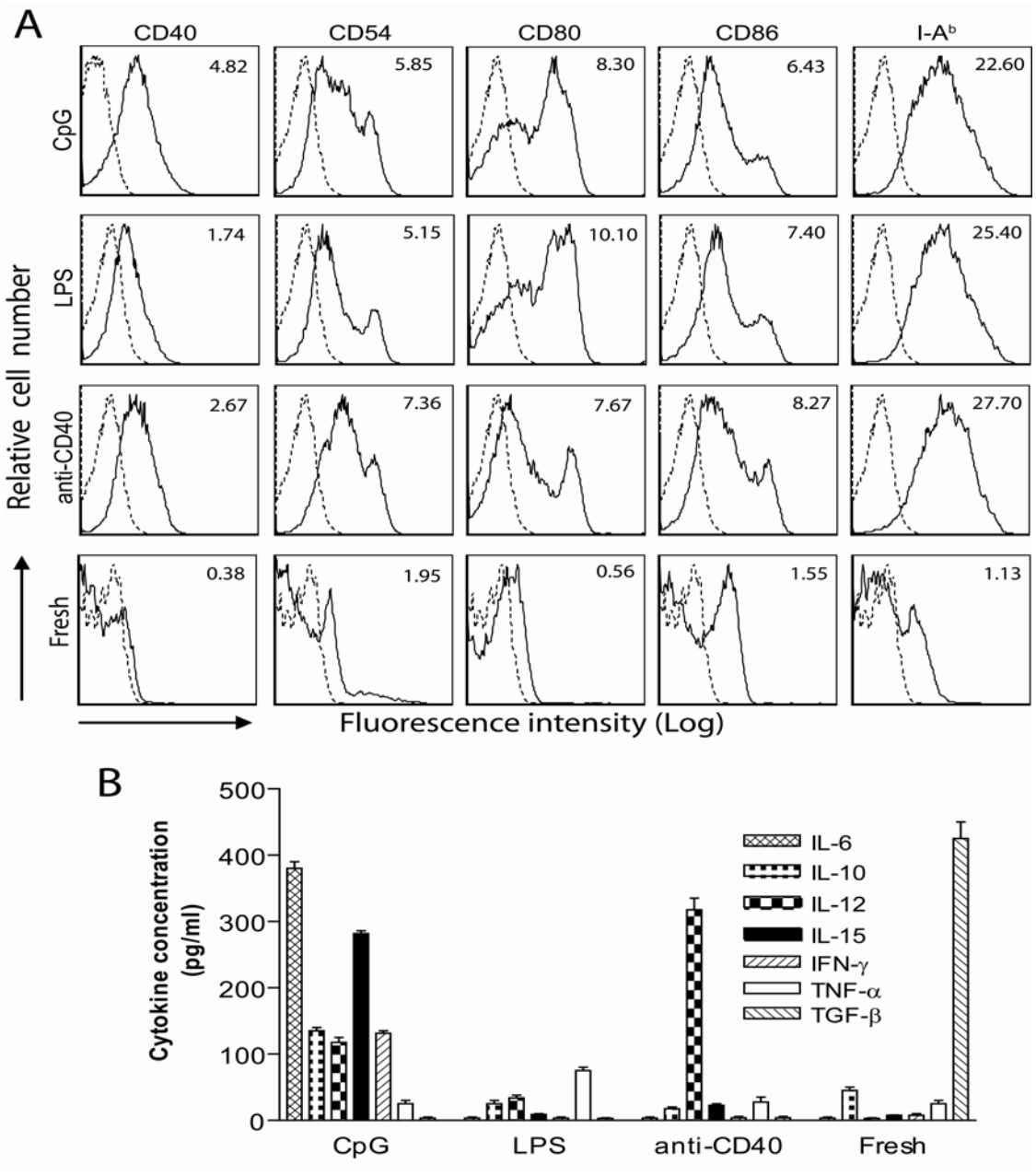


Figure 4.2. 2: In vitro autologous T cell proliferation assays and activated $CD4^+$ T cell cytokine secretion.

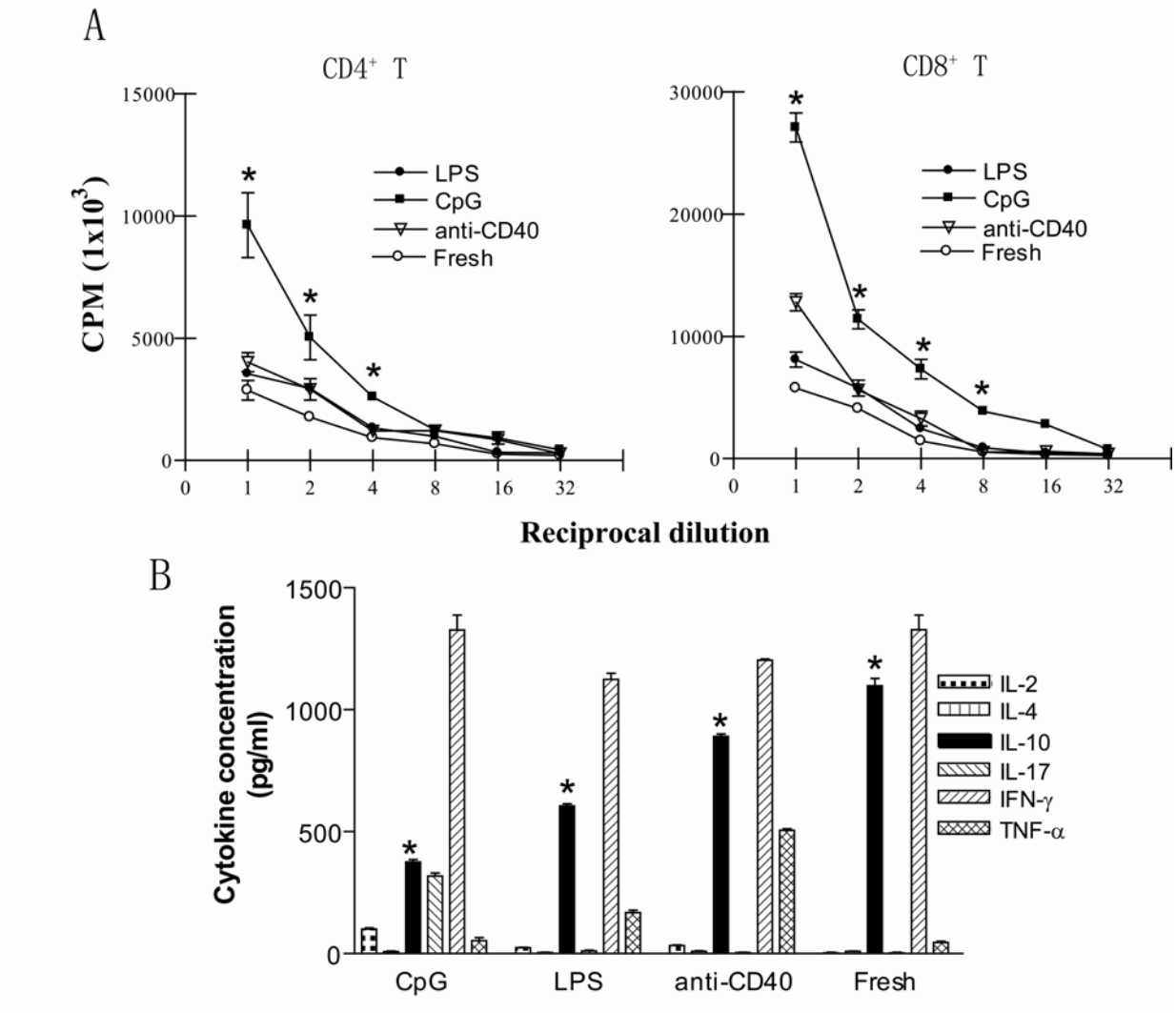


Figure 4.2. 3: In vivo CTL proliferation and cytotoxicity assay, and animal study.

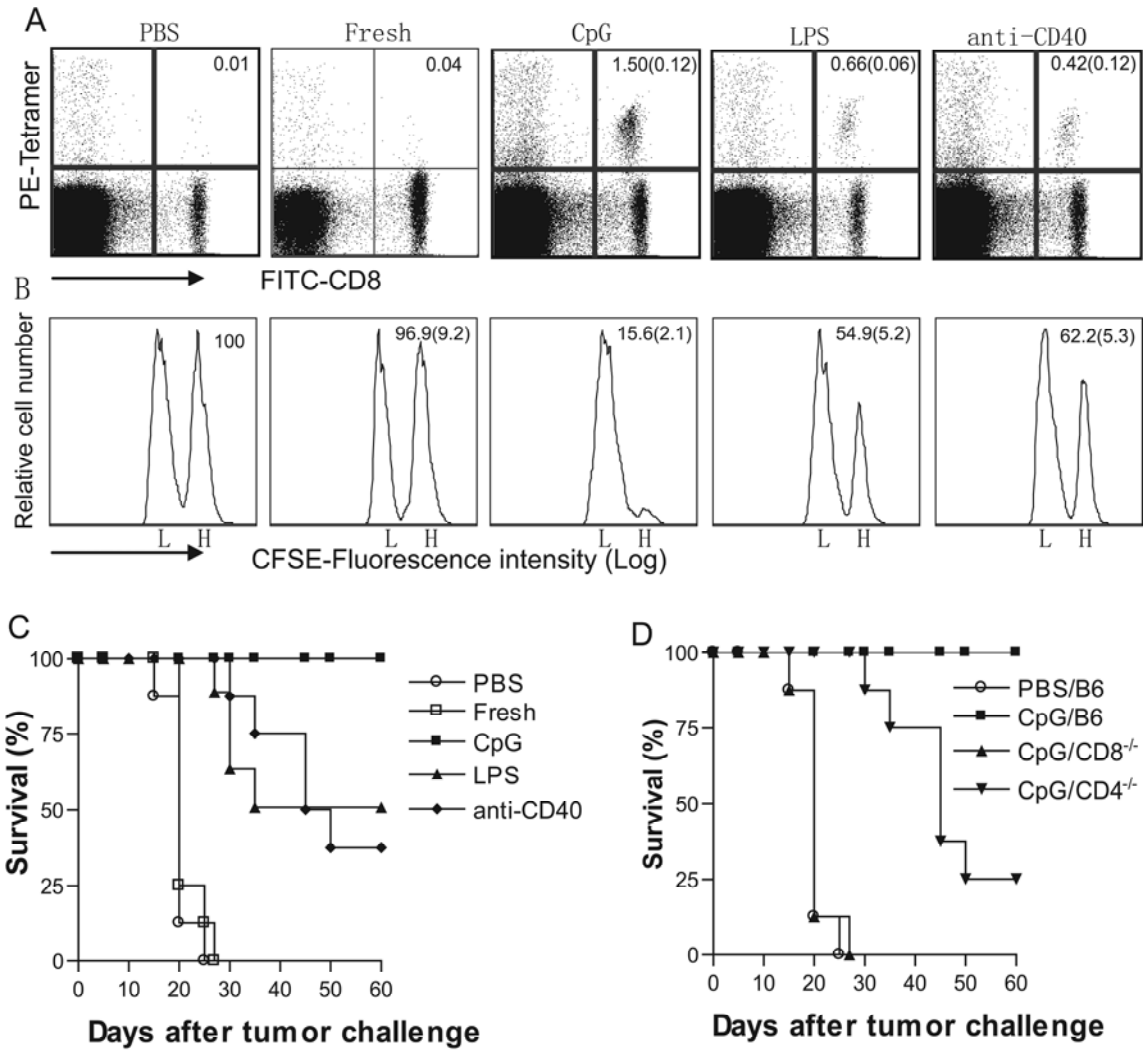
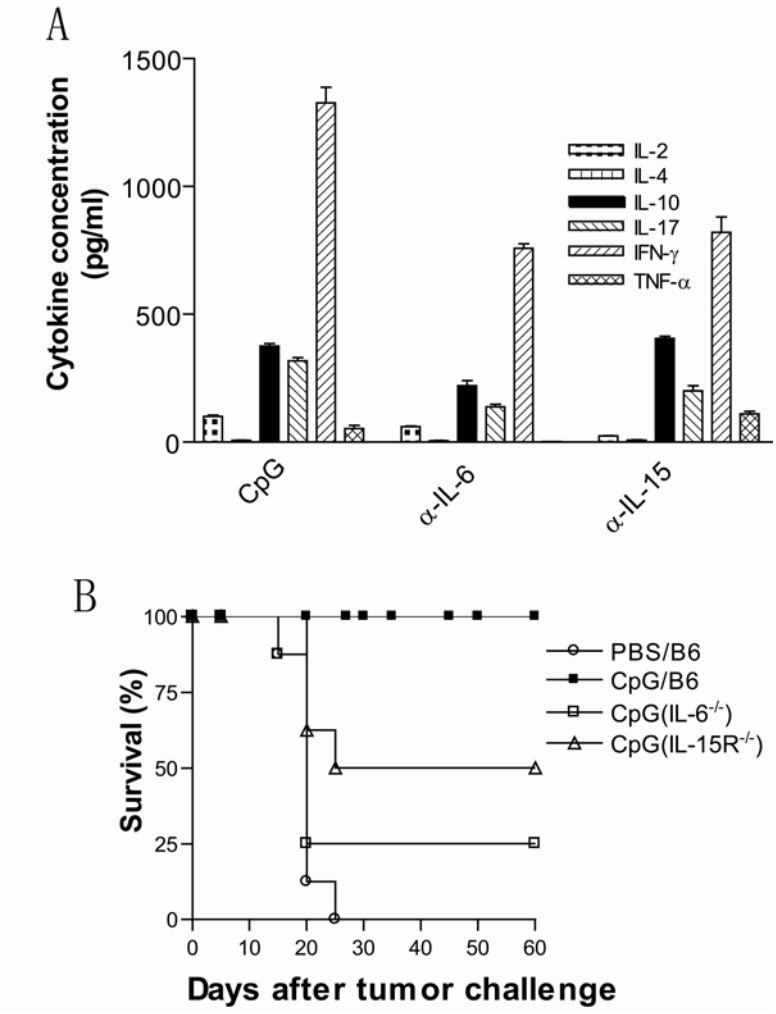


Figure 4.2. 4: IL-6 and IL-15 were the crucial cytokines for inducing Th1/Th17 antitumor immunity.



4.2.7 Tables for Manuscript 4.2

Table 4.2. 1: List of genes that were expressed at similar levels in CpG- and LPS-treated $CD4^{-}8^{-}$ DCs

Gene	GeneBank Acession No.	CpG Group (EL)	LPS group(EL)	DE
Cytokines, Chemokines and Their Receptors				
CCL3	NM_011337	5.89	4.83	<2
CCL5	NM_013653	11.27	10.73	<2
CCR2	NM_009915	1.07	1.37	<2
CCR7	NM_007719	3.39	5.43	<2
IFN-α1	NM_010502	6.46	3.68	<2
IFN-γR	NM_010511	0.87	1.27	<2
IL-18	NM_008360	0.93	0.88	<2
TNF-α	NM_013693	1.26	1.72	<2
Antigen Uptake				
CDC42	NM_009861	3.06	5.55	<2
DCP1B	XM_284227	1.39	1.77	<2
Icosl	NM_015790	1.37	2.38	<2
Igsf6	NM_030691	1.54	2.61	<2
Marcks	NM_008538	2.49	1.83	<2
Pfn1	NM_011072	3.36	5.86	<2
Prg	NM_011157	4.95	7.51	<2
Tral	NM_011631	5.32	3.03	<2
Antigen Presentation				
CD52	NM_013706	7.82	7.63	<2
CD80	NM_009855	1.63	0.86	<2
Clecsf8	NM_010819	1.29	1.99	<2
DC-LAMP	NM_177356	1.55	1.52	<2
H-2DMa	NM_010386	0.79	1.19	<2
H-2DMb2	NM_010388	7.91	4.53	<2
Cell Surface Receptors				
CD47	NM_010581	1.45	1.33	<2
DEC205	NM_013825	1.70	1.23	<2
FcεR1A	NM_010814	2.99	3.54	<2
Fcγr2b	NM_010187	1.17	0.81	<2
FcγR3(CD16)	NM_010188	2.63	1.69	<2
Lrp1	NM_008512	1.91	1.97	<2
TLR1	NM_030682	2.58	1.90	<2
TLR2	NM_011905	1.17	1.38	<2
Signal Transduction				
CD11b	NM_008401	1.48	2.41	<2
CREB-2	NM_009716	1.57	2.57	<2
EBi3	NM_015766	1.85	2.03	<2
Ifi30	NM_023065	4.02	3.58	<2
IFI44	NM_133871	2.75	3.26	<2
Ifit1	NM_008331	2.25	2.59	<2
Ifit3	NM_010501	2.67	2.12	<2
Isg15	NM_015783	2.43	1.97	<2
M-CSFR	NM_007779	15.23	19.85	<2
Mst1	NM_021420	1.68	2.34	<2
NF-κB1	NM_008689	4.84	3.30	<2
RELA	NM_009045	5.23	3.03	<2
S100a4	NM_011311	4.39	4.52	<2
S100b	NM_009115	3.89	2.88	<2
Vcl	NM_009502	1.30	1.43	<2

The expression level (EL) value represents the normalized signal to background (S/B) ratio of each DNA array signal. The differential expression (DE) value represents the fold change of gene between CpG- and LPS- activated DCs. CpG/LPS- activated $CD4^-8^-$ DC_{OVA} value of <2 are considered to be similar expression in the two groups of T cells.

Table 4.2. 2: List of genes that were expressed at higher levels in LPS- than CpG-treated $CD4^-8^-$ DCs

Gene	GenBank Acession No.	LPS group (EL)	CpG group (EL)	DE
Cytokines, Chemokines and Their Receptors				
IL-12a	NM_008351	3.70	0.49	7.61
CXCR4	NM_009911	6.38	2.09	3.04
CCR3	NM_009914	1.25	-	
CCR5	NM_009917	2.33	-	
CCR6	NM_009835	2.96	-	
Antigen Uptake				
ICAM-2	NM_010494	1.43	0.09	14.66
Abcb3(Tap2)	NM_011530	3.35	0.24	13.86
Pim2	NM_145737	4.27	0.51	8.33
Lip1	NM_021460	5.39	1.38	3.91
DCTN2	NM_027151	3.68	1.24	2.98
ICAM-1	NM_010493	2.83	1.25	2.27
Pnrc1	XM_131355	2.71	1.25	2.17
Abcb2(Tap1)	NM_013683	2.39	1.11	2.15
ICOS	NM_017480	3.37	-	
CD68	NM_009853	0.75	-	
Antigen Presentation				
CD86	NM_019388	6.02	2.14	2.81
Cell Surface Receptors				
Plaur	NM_011113	2.24	-	
Signal Transduction				
Isg20	NM_020583	1.69	0.56	3.03
F13a	NM_028784	4.69	1.60	2.93
CDKI(p21)	NM_007669	4.03	1.72	2.34
Btg1	NM_007569	6.79	2.98	2.28
Casper(Flip)	NM_009805	5.19	2.33	2.23
CD18	NM_008404	4.69	2.20	2.13

Up-regulated gene expression was based on that the DE value for the LPS-activated DCs relative to the CpG-activated DCs was ≥2, or that the LPS-activated DCs expressed the designated gene, while the CpG-activated DCs did not (indicated as "-", below the detection limits for EL). A total of 25 genes are up-regulated in the LPS-activated DCs, but those with EL values of less than 0.70 are not listed in the above table

Table 4.2. 3: List of genes that were expressed at higher levels in CpG- than LPS-treated $CD4^{-}8^{-}$ DCs

Gene	GenBank Acession No.	CpG group (EL)	LPS group (EL)	DE
Cytokines, Chemokines and Their Receptors				
CCL19	NM_011888	2.18	0.07	31.18
IL-1β	NM_008361	1.35	0.09	14.75
CX3CL1	NM_009142	1.57	0.12	13.67
IFN-β	NM_010510	1.46	0.23	6.29
IL-6	NM_031168	2.87	0.61	4.67
IL-16	NM_008360	2.48	0.54	4.56
IL-1α	NM_010554	2.72	0.62	4.39
CCL22	NM_009137	1.05	0.24	4.28
L-17	NM_010552	0.96	0.25	3.86
IL-10	NM_010548	3.09	0.91	3.40
CXCL16	NM_023158	5.11	1.98	2.58
IL-12β	NM_008352	2.59	-	
OX40L	NM_009452	2.22	-	
IL-2	NM_008366	1.89	-	
HVEM-1	NM_019418	1.63	-	
IFN-γ	NM_008337	1.56	-	
IL-15	NM_008357	1.32	-	
TRANCE(TNFSF11)	NM_011613	1.25	-	
IL-4	NM_021283	1.09	-	
4-1BBL	NM_009404	1.07	-	
CCL17	NM_011332	0.76	-	
Antigen Uptake				
Rac1	XM_132485	3.74	0.25	14.96
Pscdbp	NM_139200	3.97	0.83	4.74
NADPH P450	NM_008898	1.73	0.84	2.06
CD44	XM_130536	3.75	-	
Nr3c2	XM_356093	2.53	-	
Mx1	NM_010846	2.52	-	
Mlp	NM_010807	2.05	-	
Rnase6	XM_127690	1.82	-	
MIF	NM_010798	1.79	-	
B3Bwg0562e	NM_177664	1.57	-	
Prkra	NM_011871	1.47	-	
Map4k3	XM_128800	1.34	-	
SOD2	NM_013671	1.17	-	
Mx2	NM_013606	1.02	-	
Mtrf1	NM_145960	0.80	-	
Antigen Presentation				
CD1d1	NM_007639	2.13	0.84	2.53
CD83	NM_009856	15.38	7.16	2.15
CD-207	NM_144943	1.17	0.58	2.00
Dectin	NM_ 020008	4.89	-	
DCIR	NM_011999	1.60	-	
CD2	NM_013486	0.86	-	
CD-209a	NM_133238	0.79	-	
Cell Surface Receptors				
CD40	NM_011611	2.27	0.19	11.69
CD36	NM_007643	1.64	0.65	2.52
TLR9	NM_031178	2.18	-	
TLR5	NM_016928	1.77	-	
TLR4	NM_021297	1.55	-	
TLR3	NM_126166	1.46	-	
Fcrl3	NM_144599	1.34	-	
FcεR2A(CD23)	NM_013517	1.27	-	

FcεR1γ	NM_010185	1.09	-	
FcγR1	NM_010186	1.07	-	
Signal Transduction				
Gbp2	NM_010260	2.78	0.02	174.55
IFP35	NM_027320	1.68	0.07	24.48
Cystatin C	NM_009976	2.20	0.19	11.07
Zfp398	NM_173034	1.94	0.29	6.71
Arhgdib	NM_007486	10.05	2.65	3.79
Gbp3	NM_018734	1.49	0.59	2.51
NF-κB2	NM_019408	4.18	2.01	2.08
AIM2-est	XM_357160	6.13	-	
Cct6a	NM_009838	3.82	-	
CHD4	NM_145979	2.46	-	
Ifi204	NM_015766	1.97	-	
Fscn3	NM_019569	1.90	-	
Fragilis	NM_025378	1.32	-	
Acpp	NM_019807	1.17	-	
Ifit2	NM_008332	1.11	-	
C1qrf	NM_011795	1.01	-	
Adar	NM_019655	0.73	-	

Up-regulated gene expression was based on that the DE value for the CpG-activated DCs relative to the LPS-activated DCs was ≥2, or that the CpG-activated DCs expressed the designated gene, while the LPS-activated DCs did not (indicated as "-", below the detection limits for EL). A total of 77 genes are up-regulated in the CpG-activated DCs, but those with EL values of less than 0.70 are not listed in the above table.

Our first two manuscripts indicated that DCs are not functionally homogeneous and contain different subsets and induce antitumor immunity or immunotolerance. Our results also demonstrated that one way of provoking immunotolerance is through formation of regulatory T cells (Tr). However, the mechanism for Tr antigen specificity acquisition is still controversial. Some reports indicated that Tr might get the antigen specificity during their development. Other results have shown that Tr antigen specificity is induced in vitro or during the immune response in vivo. Our lab recently found that CD4+ and CD8+ can acquire pMHC I (OVA I peptide and MHC I) complex to become antigen specific APC. We are interested in whether pMHCI could confer the antigen specificity to Tr. During our study, CD8+CD25+ Tr were found to be easily expanded in vitro and rarely studied, thus this type of Tr was used as a model cell in our project.

4.3 $CD8^+CD25^+$ regulatory T cell's suppressive effect is mediated through inhibition of dendritic cell maturation and induction of T cell anergy and enhanced via acquired exosomal pMHC I complexes

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Running Title: Augmentation of $CD8^+CD25^+$ regulatory T cell suppression via acquired pMHC I complexes

Key Words: $CD8^+$ Tr cells, DC maturation, T cell anergy, pMHC I complex, cell contact, CTL, tumor immunity

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4.3.1 ABSTRACT

Natural nonspecific $CD8^+CD25^+$ regulatory T (Tr) cells play important roles in maintenance of self-tolerance and control of autoimmunity. We previously demonstrated that the nonspecific $CD4^+$ T cells can acquire antigen-specific DC-released exosomes (EXO) and these CD4+ T cells with acquired exosomal pMHC I can stimulate antigen-specific $CD8^+$ CTL responses. To assess whether the nonspecific $CD8^+CD25^+$ Tr cells can acquire antigen-specificity via acquired exosomal pMHC I**,** we purified $CD8^+CD25^+$ Tr cells from wild-type C57BL/6 mice and OVA-pulsed DC_{OVA}-released EXO_{OVA} expressing pMHC I complexes. We demonstrated that the nonspecific $CD8^+CD25^+$ Tr cells expressing Foxp3, CTLA-4, GITR, FasL and granzyme B inhibited *in vitro* T cell proliferation and *in vivo* OVA-specific $CD4^+$ T cell-dependent and independent $CD8^+$ CTL responses and antitumor immunity. $CD8^+CD25^+$ Tr cells' suppressive effect is possibly mediated by its inhibition of DC maturation, down-regulation of secretion of Thl polarization cytokines by DCs and its induction of T cell anergy via cell-to-cell contact. The nonspecific $CD8^+CD25^+$ Tr cells acquired antigen specificity by uptake of DC_{OVA}-released EXO_{OVA} expressing pMHC I and enhanced its effect on inhibition of OVA-specific $CD8^+$ T cell responses and antitumor immunity by 10- folds. The principles elucidated in this study may have significant implications not only in antitumor immunity, but also in other sectors of immunology (e.g, autoimmunity and transplantation).

4.3.2 INTRODUCTION

Natural self-antigen (Ag)-reactive $CD4^+CD25^+$ and $CD8^+CD25^+$ regulatory T (Tr) cells expressing forkhead box P3 (Foxp3) (380, 412, 421, 424) play important roles in maintenance of self-tolerance and control of autoimmunity (492). They develop in the thymus and then enter peripheral tissues, where they suppress the activation of self-reactive T effector cells in a non-antigen-specific manner (493, 494). The mechanisms by which $CD4^+CD25^+$ Tr cells exert their suppressive effect have been extensively reported. It has been shown that $CD4^+CD25^+$ Tr cells' suppressive effect is dependent upon the production of suppressive cytokines such as interleukin-10 (IL-10) and transformation growth factor-beta (TGF-β) (495-500). However, in contrast to these findings, most reports indicated that $CD4^+CD25^+$ Tr cells exerted their immune suppression via cell-to-cell contact fashion, in which the Tr cell-surface cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) was involved (501-503). In comparison, the mechanisms responsible for $CD8^+CD25^+$ Tr cells' inhibition of the immune responses are less well studied (504).

DCs process exogenous antigens (Ags) in endosomal compartments such as multivesicular endosomes (345) which can fuse with plasma membrane, thereby releasing Ag presenting vesicles called "exosomes" (EXO) (346, 347). Exosomes (EXO) are 50-90 nm diameter vesicles containing Ag presenting (MHC class I, class II, CD1, hsp70-90), tetraspan (CD9, CD63, CD81), adhesion (CDllb, CD54) and co stimulatory (CD80 and CD86) molecules (348, 349), i.e. the necessary machinery required for generating potent immune responses. Kennedy et al previously demonstrated that $CD4^+$ T cells can acquire antigen presenting cell (APC) membrane molecules *in vivo,* and induce

memory cytotoxic T lymphocyte (CTL) responses (433). We have recently demonstrated that $CD4^+$ T cells derived from (ovalbumin) OVA-specific T cell receptor (TCR) transgenic OT II mice uptook DC_{OVA} released EXO_{OVA} via MHC/TCR and CD54/LFA-1 interactions, and these $CD4^+$ T cells with acquired exosomal pMHC I and co stimulatory molecules stimulated central memory $CD8^+$ CTL responses (469, 477). Interestingly, the stimulatory effect of $CD4^+$ T cells was found to be specifically targeted to $CD8^+$ T cells *in vivo* via acquired exosomal pMHC I complexes. However, whether the non-specific $CD8^+CD25^+$ regulatory T cells can also get Ag specificity via uptake of Ag-specific DC released EXO remains illusive.

In this study, we investigated the suppressive effect of $CD8^+CD25^+$ Tr cells and its molecular immune mechanisms responsible for its suppressive effect in a well-established OVA-specific animal model. We first purified $CD8^+CD25^+$ Tr cells from wild-type C57BL/6 mice. We then investigated its suppressive effect on *in vitro* T cell proliferation and DC maturation and *in vivo* OVA-specific $CD8^+$ T cell responses and antitumor immunity. In addition, we also investigated whether the non-specific $CD8^+CD25^+$ Tr cells can get their OVA Ag specificity via uptake of OVA-specific DC_{OVA}-released EXO_{OVA} leading to enhancement of its inhibition in OVA-specific $CD8^+$ CTL responses and antitumor immunity.

4.3.3 MATERIALS AND METHODS

4.3.3.1 Reagents, cell lines and animals

Ovalbumin (OVA) was obtained from Sigma (St. Louis, MO). OVA I (SIINFEKL) and OVA II (lSQAVHAAHAEINEAGR), which are OVA peptides specific for H-$2K^b$

and I-A^b, respectively (440, 505). Mutl (FEQNTAQP) peptide is specific for H-$2K^b$ of an irrelevant 3LL lung carcinoma (506). All peptides were synthesized by Multiple Peptide Systems (San Diego, CA). Biotin-labeled, PE or FITC-Iabeled antibodies (Abs) specific for H-$2K^b$ (AF6-88.5), lab (AF6-120.1), CD4 (GK1.5), CD8 (53-6.7), CDllc (HL3), CD25 (7D4), CD28 (37.51), CD30 (mCD30.1), CD40 (3/23), CD54 (3E2), CD62L (MEL-14), CD69 (I:I1.2F3), CD80 (16-10Al), FasL (CDI78, MFL3), GITR (DTA-1), CTLA-4 (9H10), perforin (δG9), and Va2V~5+ TCR (MR9-4) as well as FITC-conjugated streptavidin were all obtained from BD Biosciences(Mississauga, ON, Canada).Biotin-labeled antibodies for TLR-4 (UT41), FITC-anti-mouse FR4 (Folate receptor, 12A5), PE–anti-CD73 (TY/11.8) and mouse regulatory T cells staining kit (88-8815) were purchased from eBiosciences (Cornerstone Court West, San Diego, CA). PE anti-granzyme B (GBll) was bought from AbD Serotec (Raleigh, NC). Rat anti-TGF-β McAb was purified from culture supernatant of related hybridoma in our lab. PE anti-mouse TRAIL (CD253) (N2B2) was bought from Cedarlane Laboratories Limited, Burlington, Ontario, Canada) The anti-LFA-l Ab, the cytotoxic T lymphocyte-associated Ag (CTLA)-4/Ig fusion protein, the recombinant mouse IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from R&D Systems Inc (Minneapolis, MN). Dynal mouse CD3/CD28 T cell expander were bought from Invitrogen (Burlington, ON, Canada). The highly lung metastasis OVA-transfected BL6-10_{OVA} melanoma cell line was generated in our own laboratory (461). Female OVA-specific TCR-transgenic OT I and OT II mice, H-$2K^b$ and I-A^b gene knockout (KO) mice were obtained from the Jackson Laboratory (Bar Harbor, MA). All mice were treated according to animal care committee guidelines of the University of Saskatchewan.

4.3.3.2 Spleen APC_{OVA} preparation

This protocol is a modified version (106) of that originally described by Livingstone and Kuhn (441). Briefly, spleens were injected with Dulbecco's modified Eagle's medium (DMEM) containing 1 mg/ml collagenase (Worthington Biochemical, NJ), cut into small fragments, and digested in the above-described enzyme solution for 45-60 min at 37°C. Single-cell suspension was prepared by pressing the digested tissues through a stainless mesh. After the red blood cells were lysed with Tris-NH_4CI, the spleen cells were washed once in phosphate buffered saline (PBS) and re-suspended in AIM-V® medium (serum-free lymphocyte medium; Invitrogen Life Technologies) plus OVA protein (Sigma-Aldrich) (0.2 mg/ml), recombinant mouse granulocyte macrophage-colony stimulating factor (rmGM-CSF) (10 ng/ml) and 50 μM 2-ME, and incubated at 37°C in 100 x 20-mm petri dishes (one spleen equivalent per dish). After 90 min at 37°C, nonadherent cells were removed by gentle washing three times with pre-warmed normal saline, the used medium were kept for next step. Adherent cells were cultured for 3-4 hrs in the above AIM-V medium. Nonadherent cells were harvested by vigorously pipetting, washed with PBS for two times and used for animal study. Those cells were termed spleen antigen presentating cells ($SpAPC_{OVA}$).

4.3.3.3 Spleen and Bone marrow dendritic cells preparation

For spleen DCs, the first several steps were the same as $SpAPCo_{VA}$ preparation, only OVA were omitted and medium were replaced with 10%FCS-RPMI (Roswell Park Memorial Institute Media) with 50 μM 2-ME (2-mercaptoethanol), and after first time incubation on plate, attached cells were harvested by vigorously pipetting with PBS and washed twice, and then cells were incubated with biotin-anti-CDllc and anti-biotin MACS® beads sequentially before loading onto LS for positively selecting $CD11c^+$

cells-splenic DCs (SpDC). The generation of bone marrow-derived mature DCs (mDC) under high-dose of GM-CSF/IL-4 (20 ng/ml) has been described previously (173). DCs at day 6 of culture were further pulsed with OVA protein (0.3 mg/ml) in fetal calf serum-free AIM-V medium (Invitrogen, Burlington, Canada) for overnight culture and termed DC_{OVA}. DC_{OVA} derived from H-$2K^b$ and I-A^b gene KO mice were termed ($K^{b-/-}$)DC_{OVA} and (I-$A^{b-/-}$)DC_{OVA}.

4.3.3.4 Exosome preparation

Preparation and purification of exosomes (EXO) derived from the culture supernatants of DC_{OVA} were previously described (351). EXO derived from DC_{OVA} were termed EXO_{OVA}. Similar to DC_{OVA}, EXO_{OVA} also expressed MHC class I (H-$2K^b$) and class II (I-A^b), CDllc, CD40, CD54, CD80 and pMHC I complex (OVA I peptide and H-$2K^b$), but in a less content, compared to DC_{OVA}(351). EXO derived from ($K^{b-/-}$)DC_{OVA} were termed EXO_{OVA}($K^{b-/-}$). To generate 5-carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labeled EXO, DC_{OVA} were stained with 10 μM CFSE at 37°C for 20 minutes (507) and washed three times with PBS, and then pulsed with OVA protein in AIM-V serum-free medium for overnight. The CFSE-labeled EXO (EXO_{CFSE}) was harvested and purified from the culture supernatants as previously described above (351).

4.3.3.5 $CD8^+CD25^+$ regulatory T cell preparation, expansion and characterization

Naive $CD8^+$ T cells were isolated from normal B6 mouse spleens, enriched by passage through nylon wool columns (C&A Scientific, Manassas, VA), and then purified by negative selection using anti-mouse CD4 (L3T4) paramagnetic beads (Invitrogen, Burlington, ON, Canada) to yield populations that were >98% $CD8^+$ (461). Naive $CD8^+$ T cells were used to purify $CD25^+$ cells by incubating with biotin-labeled anti-CD25 antibody (7D4) and anti-biotin MACS beads (Miltenyi Biotech, Auburn, CA)(508) and

pass through LS column. The positively selected T cells were naive $CD8^+CD25^+$ regulatory T cells (Tr), the range of yield is between 0.1 to 0.3 x 10^6 cells/mouse. Those T cells were cultured in RPMI 1640 medium containing IL-2 (20 U/ml) and CD3/CD28 T cell expander beads (1:1) for 5 to 7 days for expansion and activation, After removing CD3/CD28 beads, those T cells were used for FACS analysis and exosome pulsing for animal study; some cells were also re-stimulated with CD3/CD28 beads for 24-48 hrs, the supernatant were collected for cytokine profile analysis (including IL-2, IL-4, IL-I0, IFN-γ, TGF-β and TNF-α). Regulatory T cell staining kit #3 (PE-anti-CD25 (clone PC61.5), FITC-CD4 (clone GK1.5) and ECD-Foxp3 (clone FJK-16S)) (eBioscience, San Diego, CA), FITC-CD8 (53-6.7) and biotin-anti-CD25 (7D4) with PE-Streptavidin were used to analyze the regulatory T cell distribution in spleens.

4.3.3.6 Exosomal molecule uptake by $CD8^+CD25^+$ regulatory T cells

Firstly, the $CD8^+CD25^+$ Tr cells were incubated with EXO_{CFSE} [10-30 μg/1 x10^6 T cells in 100 μl of AIM-V serum-free medium containing IL-2 (20 U/ml)] at 37°C for 1, 2, 3, and 4hr, and then assessed by confocal fluorescence microscopy. To further determine the transfer of exosomal molecules to T cells, $CD8^+CD25^+$ T cells were incubated with EXO_{OVA}, and then analyzed for expression of H-$2K^b$, CD40, CD54, CD80 and pMHC I by flow cytometry. The $CD8^+CD25^+$ T cells co-cultured with EXO_{OVA} were Tr/E cells. For blocking assays, $CD8^+CD25^+$T cells were incubated with anti-H-$2K^b$, anti-I-A^b and anti-LFA-I Abs (50 μg/ml) or CTLA-4/lg (50 μg/ml), respectively, on ice for 30 min, then were co-cultured with EXO_{CFSE} for 4h at 37°C. The cells was harvested and analyzed for CFSE expression by confocal fluorescence microscopy.

4.3.3.7 Inhibition assay for T cell proliferation and spleen dendritic cell maturation

To assess the inhibition effect of $CD8^+CD25^+$ T cells, we performed *in vitro* T cell

proliferation assay. T cells depleted APC were harvested by pushing the cell out with a syringe plunger from splenocytes loaded nylon column after eluting the unattached T cells. $CD8^+CD25^+$ T cells (1 xl 0^5 cells/well) cells and their 2-fold dilutions were cultured with a constant number of 1 μM/ml CFSE labeled naive $CD4^+$ or $CD8^+$ T cells (l x 10^5 cells/well) in presence of T cells depleted APC derived from CS7BL/6 mice (2 x 10^5 cells/well) and anti-CD3 (2ug/ml). To examine the molecular mechanism, a panel of reagents including TGF-β (100 μg/ml), IL-10 and CTLA -4 (each 10 μg/ml) antibodies, and a mixture of isotype-matched irrelevant Abs (as control reagents) were added to the cell cultures, respectively. After culturing for 5 days, cell division and CD25 expression was detected by flow cytometry (424)*;* cell proliferation is detected by ^{3}H-incorporation assay using CD3/CD28 beads as stimulatant. For the DC maturation assay, freshly isolated immature $CD11c^+$ spleen DCs were incubated with $CD8^+CD25^+$ Tr with or without naive T cells for overnight, and then Tr were removed by incubating the cell suspension with biotin-anti-CD3 Abs and anti-biotin microbeads, respectively, and running through LS column, negatively purified DCs were stained with DC related markers and analysis by flow cytometry (509), or cultured overnight with 1 ug/ml LPS and rmGM-CSF in AIM-V medium and then their supernatants were harvested for cytokine analyses. In *in vivo* OVA-specific $CD8^+$ T cell proliferation inhibition assay, C57BL/6 mice (8 mice per group) were i.v. immunized with irradiated (4,000 rads) DC_{OVA} (l x 10^6 cells/mouse) or recombinant *Listeria monocytogenes* expressing OVA (rLmOVA) (510) alone or together with $CD8^+CD25^+$ Tr cells (3 x 10^6 cells/mouse). For evaluation of *in vivo* OVA-specific $CD8^+$ T cell proliferation, the tail blood samples derived from mice 6 days after immunization were incubated with PE-H-$2K^b$/OVAI tetramer and FITC-anti-CD8 Ab (Beckman Coulter) and analyzed by flow cytometry (351).

4.3.3.8 *In vivo* cytotoxicity inhibition assay

In vivo cytotoxicity assay was performed as previously described (461). Briefly, C57BL/6 mice (8 mice per group) were i.v. immunized with irradiated (4,000 rad) DC_{OVA} alone or together with $CD8^+CD25^+$ Tr cells (3 x 10^6 cells/mouse). Splenocytes were harvested from naive mouse spleens and incubated with either high (3.0 μM, $CFSE^{high}$) or low (0.3 μM, $CFSE^{low}$) concentrations of CFSE, to generate differentially labeled target cells. The $CFSE^{high}$ cells were pulsed with OVA I peptide, whereas the $CFSE^{low}$ cells were pulsed with unrelated peptide Mut1 or medium only and served as internal controls. These pulsed target cells were washed extensively to remove free peptides, and then i.v. co-injected at 1: 1 ratio into the immunized mice 7-10 days after immunization. Sixteen hrs after the target cells delivery, the spleens of injected mice were removed and residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the recipients' spleens were analyzed by flow cytometry.

4.3.3.9 Animal studies

To examine immunosuppression of antitumor protective immunity conferred by $CD8^+CD25^+$ T cells. Wild-type C57BL/6 mice (n=8) were injected i.v. with irradiated (4,000 rad) spleen APC_{OVA} (2 x 10^6 cells/mouse) with or without co-injection of $CD8^+CD25^+$ Tr cells (0.3-3 x 1 0^6 cells/mouse). The mice injected with PBS were used as a control. To assess the antitumor immunity, the immunized mice were challenged s.c. with 0.1 x 10^6 BL6-10_{OVA} tumor cells ten days subsequent to the immunization. Animal mortality was monitored daily for up to 10 wk; for humanitarian reasons, all mice with tumors that achieved a size of 1.5 cm in diameter were sacrificed.

4.3.4 RESULTS

4.3.4.1 $CD8^+CD25^+$ regulatory T cells express CD28, Foxp3, CTLA-4, GITR, perforin and granzyme B

We first analyzed the presence of $CD4^+CD25^+$ and $CD8^+CD25^+$ regulatory T (Tr) cells in wild-type C57BL/6 mice by using e-Biosciences Regulatory T cell kits. As shown in Fig. 4.3. 1A, $CD4^+CD25^+$ and $CD8^+CD25^+$ Tr cells accounted for 5.51% and 1.38% of the total T cell population in mouse splenocytes, respectively, which is consistent with some previous reports (424, 511). Both $CD4^+CD25^+$ and $CD8^+CD25^+$ Tr cells expressed Foxp3 with the former expressing higher amount of Foxp3 than the latter. These $CD8^+CD25^+$ Tr cells were expanded *in vitro* by using anti-CD3/CD28 Ab-coupled beads (512), and then analyzed by flow cytometry. Naive $CD8^+$ $CD25^-$ T cells did not express T cell activation markers CD25 and CD69, but displayed a high level of cell-surface CD62L and GITR, and intracellular CTLA-4 (Fig.4.3. 1B). $CD8^+CD25^+$ Tr cells, however, expressed a high level of CD25 and CD69, indicating that they are active T cells. They also expressed a high level of cell-surface CD28 and GITR, and intracellular CTLA-4 and Foxp3, but had low or no expression of TGF-β, CD30, CD62L and TLR-4, indicating that they are $CD8^+CD28^+$ Tr cells, but not $CD8^+CD28^-$ and TLR-4-expressing $CD4^+CD25^+$ Tr cells (513). Since $CD8^+$ Tr cells may have some characteristics that are attributed to cytotoxic T cells (405, 420), we also assessed expression of a number of cytotoxicity-related molecules such as FasL, TRAIL, perforin and granzyme B by flow cytometry. As shown in Figure 1b, $CD8^+CD25^+$ Tr cells expressed both the intracellular perforin and granzyme B, but not the cell-surface TRAIL and FasL. However, we found that $CD8^+CD25^+$ Tr cells derived from OVA-specific TCR transgenic OT I mice did not show any killing activity to OVA I-pulsed splenocytes or

OVA-expressing BL6-10_{OVA} tumor cells (data not shown). In addition, these $CD8^+CD25^+$ Tr cells secreted immune suppressive cytokines such as IL-10 (1.5 ng/ml/$l0^6$ cells/24 hr) and TGF-β (0.8 ng/ml/$l0^6$ cells/24 hr) as well as inflammatory cytokines such as IFN-γ (4.0 ng/ml/$l0^6$ cells/24 hr) and TNF-α (3.0 ng/ml/10^6 cells/24 hr), but no IL-4 (Fig.4.3. 1B), indicating that $CD8^+CD25^+$ Tr cells also share some features of Trl cells such as secretion of both IL-1 0 and IFN-γ (424).

4.3.4.2 $CD8^+CD25^+$ Tr cells inhibit dendritic cell maturation

Fresh immature mouse SpDCs become mature ones through overnight culturing in presence of GM-CSF (107, 108). $CD4^+CD25^+$ regulatory T cells have also been reported to play inhibitory role via reducing the DC maturation (396, 509). To assess the *in vitro* inhibitory effect of $CD8^+CD25^+$ Tr cells, we incubated splenic DCs with $CD8^+CD25^+$ Tr cells, and then analyzed DCs after incubation by flow cytometry. As shown in Figure 2a, DCs greatly down-regulated expression of DC maturation markers such as CD80, CD86, and $I\text{-}A^b$, but also down-regulated that of PD-L1 (B7-H1), PD-L2 (B7-DC) and only slightly increase that of B7-H3 and B7-H4 after incubation with $CD8^+CD25^+$ Tr cells, indicating that $CD8^+CD25^+$ Tr cells could inhibit spontaneous DC maturation. It has been demonstrated that presence of naive T cells(Tn) is necessary for mouse $CD4^+$ Tr cells to exert its immune suppressive effect, while Tr themselves weakly promote the DC maturation (509). To assess whether $CD8^+CD25^+$ Tr cells also inhibit naive T cell-induced DC maturation, we incubated DCs with $CD8^+CD25^+$ Tr cells in presence of naive $CD4^+$ T cells, and then phenotypically analyzed DCs by flow cytometry. We found that DCs also greatly down-regulated expression of DC maturation markers (Fig.4.3. 2A), indicating that $CD8^+CD25^+$ Tr cells could also inhibit naive T cell-induced DC

maturation.

4.3.4.3 $CD8^+CD25^+$ Tr cells induce dendritic cell tolerogenicity

To further characterize $CD8^+CD25^+$ Tr cell-treated DCs, we then analyzed the cytokine secretion of Tr-treated DCs. After stimulation with LPS, SpDC only secreted high level of IL-6 (840 pg/ml), IFN-γ (1069 pg/ml), and TNF-α (883 pg/ml), but very low level of IL-10 (80 pg/ml); in the presence of Tn, SpDC also secreted significant amount of the above mention cytokines (IL-6, 372 pg/ml; IFN-γ, 696 pg/ml; and TNF-α, 451 pg/ml), although with much lower level compared with SpDC only, but with a little higher level of IL-10 (200 pg/ml). However, the Tr-treated DCs secreted much lower level of IL-6, IFN-γ and TNF-α (all below 10 pg/ml), the IL-10 secretion change was only and almost doubly increased in DC-Tr only (152 pg/ml) culture group (Fig.4.3. 2B). , indicating that $CD8^+CD25^+$ Tr cells induce DC tolerogenicity. Our results were also indirectly supported by those reports that $CD4^+CD25^+$ Tr cells inhibited DCs by increasing its own secretion of IL-10 and decreasing IL-6 secretion (393, 394).

4.3.4.4 $CD8^+CD25^+$ Tr cells inhibit *in vitro* T cell proliferation via cell-to-cell contact

To assess the *in vitro* inhibition effect, we performed CFSE-labeled T cell proliferation assay by using $CD8^+CD25^+$ Tr cells. As shown in Figure 3a, $CD8^+CD25^+$ Tr cells suppressed the *in vitro* proliferation of both $CD4^+$ and $CD8^+$ T cells in a dose-dependent manner. At a ratio ($CD8^+25^+$ Tr cell and responder T cell) of 1:1, $CD8^+CD25^+$ Tr cells completely (100%) and prominently (75%) suppressed $CD4^+$ and $CD8^+$ T cell proliferation. We also found that their inhibition of T cell proliferation was not affected by the blocking reagents including CTLA-4-IgFc, anti-IL-10 and anti-TGF-β Abs (Fig.4.3. 3B), indicating that its inhibition is not dependent upon CD28/CD80 interactions and secretion of immune suppressive cytokines IL-10 and TGF-β, which is

consistent with some previous reports (503, 514). However, the *in vitro* transwell experiments suggest that the suppressive activity of $CD8^+CD25^+$ Tr cells requires cell-to-cell contact because $CD8^+CD25^+$ Tr cells separated by transwell membrane did not show any inhibitory effect. Our data are also consistent with some previous reports (503, 514).

4.3.4.5 $CD8^+CD25^+$ Tr cells induce *in vitro* T cell anergy

$CD4^+CD25^+$ regulatory T cells have also been reported to play inhibitory role via inducing T cell anergy (389). To assess the potential effect of $CD8^+CD25^+$ Tr cells, we purified naive $CD4^+$ T cells and pre-incubated them with $CD8^+CD25^+$ Tr cells, and then phenotypically characterized them by flow cytometry. As shown in Fig.4.3. 3C, they did not express T cell activation marker CD25, but express high level of CD73 and FR-4, indicating that $CD8^+CD25^+$ Tr cells are capable of inducing T cell anergy (515). To functionally characterize them, we tested $CD4^+$ T cell proliferation using CD3/CD28 beads as stimuli in ^{3}H-thymidine incorporation assay. As shown in Fig. 4.3. 3D, the primary naive T cells proliferated upon stimulation derived from CD3/CD28 beads in a dose-dependent manner. However, the naive T cells after incubation with $CD8^+CD25^+$ Tr cells lost their proliferative capacity.

4.3.4.6 $CD8^+CD25^+$ Tr cells inhibit both $CD4^+$ Th cell-dependent and -independent $CD8^+$ T cell responses *in vivo*

To assess whether $CD8^+CD25^+$ Tr cells inhibit $CD8^+$ T cell responses *in vivo*, we performed tetramer staining assay using blood samples of mice immunized with rLmOVA and DC_{OVA}. Both the bacteria rLmOVA and DC_{OVA} stimulated proliferation of H-$2K^b$/OVAI tetramer-positive $CD8^+$ T cells accounting for 12.02% and 1.53% of the total $CD8^+$ T cell population in wild-type C57BL/6 mice (Fig.4.3. 4A). However, only

the bacteria rLmOVA (8.23%), but not the DC_{OVA} (0.03%) stimulated *in vivo* OVA-specific $CD8^+$ T cell responses in I-A^b gene KO mice lacking $CD4^+$ Th cells, indicating that DC_{OVA} and bacteria rLmOVA stimulate $CD4^+$ Th cell-dependent and $CD4^+$ Th cell-independent $CD8^+$ T cell responses, respectively. To assess *in vivo* inhibitory effect of $CD8^+CD25^+$ Tr cells in $CD4^+$ Th cell-dependent and $CD4^+$ Th cell-independent $CD8^+$ CTL responses, we performed another tetramer staining assay by using the peripheral blood samples from mice immunized with both DC_{OVA}/ rLmOVA and $CD8^+CD25^+$ Tr cells. As shown in Fig.4.3. 4B, the stimulation of DC_{OVA}- and rLmOVA-induced proliferation of H-$2K^b$/OVAI tetramer-positive $CD8^+$ T cells in presence of $CD8^+CD25^+$ Tr cells in wild-type C57BL/6 mice significantly dropped from 3.05% and 11.98% to 1.74% and 6.05% of the total $CD8^+$ T cell population, respectively ($p<0.05$), indicating that $CD8^+CD25^+$ Tr cells are capable of suppressing both $CD4^+$ Th cell-dependent and $CD4^+$ Th cell-independent $CD8^+$ CTL responses.

4.3.4.7 $CD8^+CD25^+$ Tr cells inhibit *in vivo* effector $CD8^+$ CTL responses and antitumor immunity

To assess whether $CD8^+CD25^+$ Tr cells suppress $CD8^+$ T cell differentiation into effector $CD8^+$ cytotoxic T cells, we adoptively transferred OVA I peptide-pulsed splenocytes that had been strongly labeled with CFSE ($CFSE^{high}$), as well as the control peptide Mut1-pulsed splenocytes that had been weakly labeled with CFSE ($CFSE^{low}$), into the recipient mice that had been injected with DC_{OVA} alone or DC_{OVA} and $CD8^+CD25^+$ Tr cells, respectively. The mice immunized with DC_{OVA} had larger loss of $CFSE^{high}$ target cells (85%) (Fig.4.3. 4C), indicating that DC_{OVA} can most efficiently stimulate $CD8^+$ T cell differentiation into CTL effectors. As expected, the injection of $CD8^+CD25^+$ Tr cells significantly reduced the loss of $CFSE^{high}$ target cells (55%) in DC_{OVA}-immunized mice, indicating that $CD8^+CD25^+$ Tr cells efficiently inhibit *in vivo*

effector $CD8^+$ CTL responses. To assess whether $CD8^+CD25^+$ Tr cells also suppress antitumor immunity, we performed animal studies. As shown in Fig.4.3. 4D, all PBS control mice died of tumor within 4 weeks after inoculation of OVA-expressing BL6-$l0_{OVA}$ tumor cells. Most of $SpAPC_{OVA}$-immunized mice (75%) were protected from BL6-10_{OVA} tumor cell challenge. However, $CD8^+CD25^+$ Tr cell administration significantly reduced $SpAPC_{OVA}$-induced antitumor immunity and 4/8 (50%) of mice died of tumor ($p<0.05$), indicating that $CD8^+CD25^+$ Tr cells also suppress *in vivo* antitumor immunity.

4.3.4.8 $CD8^+CD25^+$ Tr cells uptake DC_{OVA}-released exsomes via TCR/MHC I and CD28/CD80 interaction

We previously demonstrated that $CD4^+$ T cells can uptake OVA-specific DC_{OVA}-released exosomes (EXO_{OVA}) expressing pMHC I (469, 477). In this study, Fig. 4.3. 5A demonstrates that EXO_{OVA} released from wild-type C57BL/6 (B6) DC_{OVA}, but not $EXO_{OVA}(K^{b-/-})$ released from H-$2K^{b-/-}$ mouse ($K^{b-/-}$)DC_{OVA} displayed pMHC I expression. To assess uptake of OVA-specific DC_{OVA}-released EXO_{OVA} by $CD8^+CD25^+$ Tr cells, $CD8^+CD25^+$ Tr cells derived from B6 mice were incubated with CFSE-Iabeled DC_{OVA}-released exosomes (EXO_{CFSE}) at different doses and for various times, and then analyzed by confocal microscopy. The CFSE dye was apparently detectable on $CD8^+CD25^+$ Tr cells by confocal microscopy after incubation with EXO_{CFSE}. As shown in Fig.4.3. 5A, the uptake of EXO_{CFSE} by $CD8^+CD25^+$ Tr cells increased with dose of EXO_{CFSE}. When $CD8^+CD25^+$ Tr cells incubated with EXO_{CFSE} at a concentration of 30 μg/ml, 82% of $CD8^+CD25^+$ Tr cells became CFSE-positive. The uptake of EXO_{CFSE} by $CD8^+CD25^+$ Tr cells also increased with incubation time and reached a maximal level (80% CFSE-positive cells) after 3 hr incubation (Fig.4.3. 5B). The CFSE-positive cells declined with the time when culturing them in medium, but were still detectable more

than 3 days in culture (Fig.4.3. 5B), indicating that the uptaken exosomal molecules on $CD8^+CD25^+$ Tr cells are quite stable, which is consistent with a previous report by Undale et al (516). Therefore, we regularly used EXO_{OVA}-pulsed $CD8^+CD25^+$ Tr cells by incubation of $CD8^+CD25^+$ Tr cells with EXO_{OVA} (30 μg/ml) for 3 hr in this study. To elucidate the molecular pathway involved in EXO uptake, we used a panel of reagents in the blocking assay. We demonstrated that the anti-H-$2K^b$ Ab and CTLA-4/Ig fusion protein, but not the anti-LFA-l and anti-I-A^b Abs were able to significantly reduce EXO_{CFSE} uptaking ($p<0.05$), indicating that the nonspecific $CD8^+CD25^+$ Tr cells uptake OVA-specific DC_{OVA}-released EXO_{OVA} via TCR-H-$2K^b$ and CD28/CD80 interactions, which is consistent with a previous report on exosome uptake of $CD8^+$ T cells by Hwang et al (517).

4.3.4.9 $CD8^+CD25^+$ Tr/E cells with uptake of exosomes acquire functional exosomal pMHC I

We previously demonstrated that OVA-pulsed DC_{OVA} and EXO_{OVA} released from DC_{OVA} also expressed pMHC I complexes (477). To assess whether $CD8^+CD25^+$ Tr cells with uptake of EXO_{OVA} also express exosomal pMHC I, we performed flow cytometric analysis. As shown in Fig.4.3. 5A, the original $CD8^+CD25^+$ Tr cells did not express pMHC I. However, they became pMHC I-positive after incubation of EXO_{OVA}, indicating that $CD8^+CD25^+$ Tr/E cells acquire the exosomal pMHC I complexes. To confirm it, we also incubated $CD8^+CD25^+$ Tr cells with $(K^{b-/-})DC_{OVA}$-released $EXO_{OVA}(K^{b-/-})$ without pMHC I expression and analyzed these $CD8^+CD25^+$ Tr/E$(K^{b-/-})$ cells by flow cytometry. In addition, As shown in Fig.4.3. 5A, these $CD8^+CD25^+$ Tr/E$(K^{b-/-})$ cells then became pMHC I negative, confirming that $CD8^+CD25^+$ Tr/E cells express acquired exosomal pMHC I complexes. To assess whether the acquired exosomal pMHC I complexes are functional, we performed IL-2 secretion assay. As shown in

Fig.4.3. 6A, pMHC I-expressing DC_{OVA} and $CD8^+CD25^+$ Tr/E cells stimulated OVA-specific TCR-expressing RF3370 cells to secreted IL-2, respectively. On the contrary, pMHC I-negative $CD8^+CD25^+$ Tr/E($K^{b-/-}$) cells derived from $CD8^+CD25^+$ Tr cells with uptake of ($K^{b-/-}$)DC_{OVA}-released EXO_{OVA}($K^{b-/-}$) failed in stimulation of RF3370 cells to secrete IL-2, indicating that the acquired exosomal pMHC I complexes on $CD8^+CD25^+$ Tr/E cells are functional.

4.3.4.10 $CD8^+CD25^+$ Tr/E cells with acquired exosomal pMHC I enhance its suppressive effect on $CD8^+$ T cell responses and antitumor immunity

We previously demonstrated that the nonspecific $CD4^+$ T cells can acquire antigen specificity via uptake of antigen-specific DC-released EXO (469). To assess whether the nonspecific $CD8^+CD25^+$ Tr cells can also become OVA Ag-specific Tr cells via uptake of OVA-specific DC_{OVA}-released EXO_{OVA}, we performed the tetramer staining, *in vivo* cytotoxicity assay and animal studies by using $CD8^+CD25^+$ Tr/E cells expressing acquired exosomal pMHC I. As shown in Fig.4.3. 6B, the stimulation of DC_{OVA}-induced proliferation of H-$2K^b$/OVAI tetramer-positive $CD8^+$ T cells in presence of $CD8^+CD25^+$ Tr/E cells accounted for only 1.32% of the total $CD8^+$ T cell population (Fig.4.3. 6B), which is significantly lower than that (1.78%) in presence of $CD8^+CD25^+$ Tr cells without pMHC I expression($p<0.05$) indicating that the acquisition of exosomal pMHC I greatly enhances the suppressive effect of $CD8^+CD25^+$ Tr cells. To our surprise, $CD8^+CD25^+$ Tr/E almost completely inhibited the rLmOVA induced *in vivo* CTL proliferation, indicated that $CD8^+CD25^+$ Tr/E cells have stronger capacity of inhibiting the $CD4^+$ T cell-independent CTL response. To confirm it, we repeated the above tetramer staining assay by using pMHC I-negative $CD8^+CD25^+$ Tr/E($K^{b-/-}$) cells. As shown in Fig4.3. 6B, the stimulation of DC_{OVA} induced proliferation of H-$2K^b$/OVAI tetramer-positive $CD8^+$ T cells resume to the level when $CD8^+CD25^+$ Tr cells were used,

confirming that the acquisition of exosomal pMHC I does greatly enhance the suppressive effect of $CD8^+CD25^+$ Tr cells. As expected, the injection of $CD8^+CD25^+$ Tr/E cells significantly reduced the loss of $CFSE^{high}$ target cells in DC_{OVA}-immunized mice, with 71 % of the target cells left alive, while the un-pulsed $CD8^+CD25^+$ Tr group only has around 44% left alive (Fig4.3. 6C), indicating that $CD8^+CD25^+$ Tr/E cells more efficiently inhibit *in vivo* effector CD8+ CTL responses. Again like the tetramer assay, $CD8^+CD25^+$ Tr/E($K^{b-/-}$) lost their superiority to the $CD8^+CD25^+$ Tr, with 43% target cells left alive, further confirmed that it's the pMHC I mediated antigen specificity enhanced the suppression power of $CD8^+CD25^+$ Tr cells in our model. Similarly in the animal studies, all mice died of tumor within 4 weeks after inoculation of OVA-expressing BL6-10_{OVA} tumor cells (Fig.4.3. 6D). $SpAPC_{OVA}$-immunized mice were mostly (75%) protected from BL6-10_{OVA} tumor cell challenge. However, $CD8^+CD25^+$ Tr/E cells administration significantly reduced $SpAPC_{OVA}$-induced antitumor immunity and 7/8 (87.5% verse 45% for $CD8^+CD25^+$ Tr) of mice died of tumor, indicating that $CD8^+CD25^+$ Tr/E cells also show stronger suppression *in vivo* antitumor immunity than its un-plused counterparts. We also did the dose curve study of this specific Tr (Fig.4.3. 6D) and found that 0.3 x $l0^6$ $CD8^+CD25^+$ Tr/E could reach the effects of 3 x 10^6 $CD8^+CD25^+$ Tr; 0.6 x $l0^6$ and more $CD8^+CD25^+$ Tr/E showed the same extent of inhibition of animal protection induced with $SpAPC_{OVA}$ vaccination in our model; while $CD8^+CD25^+$ Tr cells dose was decreased to 0.3 x 10^6, their inhibitory function could not be demonstrated here. The above study indicated that $CD8^+CD25^+$ Tr/E were at least 10 times more powerful than $CD8^+CD25^+$ Tr cells without acquisition of pMHC- I complex, indicating that the important role of acquired exosomal pMHC I complexes on $CD8^+CD25^+$ Tr/E cells in targeting Tr's suppression to $CD8^+$ T cells *in vivo*. In order to test whether IL-l0 of $CD8^+CD25^+$ Tr cells play any role in the inhibition, we compared IL-$10^{-/-}$ and wild type C57BL/6 mice derived $CD8^+CD25^+$ Tr cells (Fig.4.3. 6D), no

significant differences ($p>0.05$) were found between these two groups in this model.

4.3.5 DISCUSSION

$CD8^+$ Tr cells have been originally found in models of autoimmune diseases, transplantation and cancer in 1970s and early 1980s (405, 406). However, because of lacking defining markers and difficulties in isolating these cells, the suppressive mechanisms in these earlier reports have been discredited or ignored. Recently $CD8^+$ Tr cells have been primarily reported to be essential *in vivo* in prevention of experimental autoimmune encephalomyelitis and in participation of oral tolerance (518-520). In human both natural self-antigen (Ag)-reactive $CD4^+CD25^+$ and $CD8^+CD25^+$ Tr expressing forkhead box P3 (Foxp3) (380, 421) play a unique role in restoring immune homeostasis and in maintaining immune privileged sites (502, 521). They develop in the thymus and then enter peripheral tissues, where they suppress the activation of self-reactive T effector cells in a non-Ag-specific manner (427). In contrast to the natural nonspecific $CD8^+CD25^+$ regulatory T cells derived from thymus, adoptive $CD8^+$ Tr cells developed in the peripheral tissues have been reported to be antigen specific. Mouse $CD8^+CD25^+$ Tr have been reported in I-$A^{b-/-}$ gene KO mouse thymus and in mouse periphery during ontogeny (424). In this study, we demonstrated that there were small amount of $CD8^+CD25^+$ Tr cells accounting for 1.35% of the total $CD8^+$ T cell population in the spleen of wild-type C57BL/6 mice compared to a larger amount (5.51 %) of $CD4^+CD25^+$ Tr cells in total $CD4^+$ T cells.

The first fully characterized $CD8^+$ Tr cells were $CD8^+CD28^-$ by *in vitro* culture peripheral blood mononuclear cell with multiple rounds of stimulation with allogenic, xenogenic or antigen-pulsed syngenic APCs (522). However, the mechanisms responsible for $CD8^+$ Tr cell's inhibition of the immune responses are still controversial

(504). Some kinds of $CD8^+$ Tregs involved soluble TGF-β and IL-10 in their inhibition of T cell proliferation (27, 523), whereas most other reports indicated that cell-contact is important in $CD8^+$ Tr cell's inhibitory effect through CTLA-4, membrane-bound TGF-β and granzyme B or even tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (409, 504). $CD8^+CD28^-$ Tr up-regulated the expression of immunoglobulin-like transcript (ILT) 3 and ILT4 on monocytes and DCs, rendering these APCs tolerogenic with reduced expression of CD80 and induction of antigen-specific unresponsiveness in $CD4^+$ T helper cells (524). The human $CD8^+CD25^+$ Tr cells isolated from thymus suppress the proliferation of autologous $CD25^-$ T cells via cell-contact through CTLA-4 and TGF-β (421). In this study, we found that $CD8^+CD25^+$ Tr cells significantly inhibited *in vitro* $CD4^+$ and $CD8^+$ T cell proliferation via cell-contact mechanism. Unlike the human thymus-derived $CD8^+CD25^+$ Tr cells, which don't proliferate and secrete cytokines (374), the mouse peripheral $CD8^+CD25^+$ Tr cells proliferate vigorously *in vitro* under stimulation of CD3/CD28 T cell expander beads, and express CTLA-4 and secrete high level of IL-10 and TGF-β. However, none of them is involved in the mechanism of their suppression. Mouse $CD4^+CD25^+$ Tr cells which slightly promoted DC maturation could suppress the naive T cell-induced DC maturation (509). In this study, we found that $CD8^+CD25^+$ Tr cells could not only impaired DC spontaneous maturation, but also inhibited the naive T cell-induced DC maturation, indicating that $CD8^+CD25^+$ Tr cells are superior to $CD4^+CD25^+$ Tr cells in suppressing DC maturation. In addition, they also induced CD4 T cell anergy. Although $CD4^+CD25^+$ Tr cells could increase expression of the inhibitory molecule B-7H4 on monocyte-derived DC through up-regulating their own secretion of IL-10 (393), or down –regulating their secretion of IL-6 (394), we didn't find any $CD8^+CD25^+$ Tr cells-mediated change on IL-10 and B7-H4 expression. However we did find that $CD8^+CD25^+$ Tr cells almost completely inhibited secretion of the two Thl-polarizing cytokines (IFN-γ and TNF-α) besides down-regulation of IL-6

secretion, indicating that $CD8^+CD25^+$ Tr cells might employ even more wide inhibitory mechanism.

Peripheral adaptive $CD8^+$ Tr cells, which are antigen specific, are induced by unique antigen presenting cells during viral infections or tumor development (521). Glatiramer acetate (GA)-induced $CD8^+$ Tr cells which are cytotoxic can directly kill $CD4^+$ T cells in a GA-specific manner by cell contact-dependent mechanisms (525). The killing was enhanced by pre-activation of the target $CD4^+$ T cells and may depend on presentation of GA through HLA - E. Tang et al demonstrated that $CD8\alpha\alpha^+$ Tr cells can kill $CD4^+$ T cells with a dynamic expression of TCR peptide/Qa-l complexes within a narrow window of time (420). Several other groups reported the acquisition of antigen specificity by $CD8^+$ Tr cells via MHC molecules such as HLA-G (431), Qa-l (420) and HLA-A2 (375). We have recently demonstrated that $CD4^+$ T cells derived from OVA-specific T cell receptor (TCR) transgenic OT II mice uptook DC_{OVA} released EXO_{OVA} via MHC/TCR and CD54/LFA-I interactions, and these $CD4^+$ T cells with acquired exosomal pMHC I and costimulatory molecules stimulated central memory $CD8^+$ CTL responses (469, 477). Interestingly, the stimulatory effect of $CD4^+$ T cells was found to be specifically targeted to $CD8^+$ T cells *in vivo* via acquired exosomal pMHC I complexes. In this study, we found that the nonspecific $CD8^+CD25^+$ Tr cells acquired antigen specificity by uptake of antigen-specific exosomal pMHC I and enhanced its inhibitory effect by l0-folds, confirming the Ag-specific targeting role of acquired exosomal pMHC I complexes on $CD8^+CD25^+$ Tr cells.

Taken together, our data demonstrated that nonspecific $CD8^+CD25^+$ Tr cells expressing Foxp3, CTLA-4, GITR, perforin and granzyme B inhibited *in vitro* T cell proliferation via cell-to-cell contact and *in vivo* OVA-specific CD4+ T cell-dependent and -independent $CD8^+$ CTL responses and antitumor immunity. $CD8^+CD25^+$ Tr cells'

suppressive effect is mediated by its inhibition of DC maturation, induction of DC tolerogenecity and T cell anergy. The nonspecific $CD8^+CD25^+$ Tr cells acquired OVA antigen specificity by uptake of DC_{OVA}-released EXO_{OVA} expressing pMHC I and enhanced its effect on inhibition of OVA-specific $CD8^+$ T cell responses and antitumor immunity by 10-folds. The principles elucidated in this study may have significant implications not only in antitumor immunity, but also in other sectors of immunology (e.g, autoimmunity and transplantation).

This manuscript will be submitted to the Journal of Leukocyte Biology.

4.3.6 Figure legends for Manuscript 4.3

Figure 4.3.1: Characterization of $CD8^+CD25^+$ Tr(Tr) in spleen.

A, Distribution of $CD4^+CD25^+$ and $CD8^+CD25^+$ Tr. Naïve splenocytes were harvested by pressing the spleen through mesh to get the single cell suspension and lysed the RBC with 0.83% NH4Cl-Tris, and were stained with FITC-anti-CD4 or -CD8, anti-CD25-Biotin(7D4)/ streptavidin-PE, and anti-Foxp3-ECD(FJK-16S); double positive $CD4^+CD25^+$ and $CD8^+CD25^+$ were gated and analyzed for the expression of Foxp3 by flow cytometry. B, Cytokine profile of $CD8^+CD25^+$ Tr. *In vitro* cultured $CD8^+CD25^+$ Tr were restimulated with CD3CD28 dynal beads for 24 hrs. The supernatants were harvested after their culture. IL-2, IL-4, IL-10, IFN-γ, TNF-α, and TGF-β were measured using related ELISA kits from BD Pharmingen and R & D Systems. One of three similar experiments is shown. C, Phenotypic analyses of activated $CD8^+CD25^+$ Tr. Purified $CD8^+CD25^+$ Tregs and naive CD8 Tcells were culture in medium containing the same number of CD3CD28 beads as the cells for 5-7 days. The cells were stained with Anti-CD8, CD25, CD28, CD30, CD62L, CD69, CD80, CTLA-4, Foxp3, FasL, GITR, granzyme B, perforin, TLR-4, TRAIL, TGF-β, and their corresponding control were prepared. A representative example of three independent experiments is shown. Note: the dotted lines were the corresponding control.

Figure 4.3.2: Influences of $CD8^+CD25^+$ Tr (Tr) on splenic DCs.

A, Tr inhibit the maturation of splenic DC. Fresh immature splenic DCs were purified as described in Materials and Methods and cultured with same number of Tr, and/or with naive T cells for overnight; splenic DCs were negatively purified with biotin-anti-CD3 and anti-biotin MCAS beads and stained for CD80, 86, $I\text{-}A^b$, B7-H1, B7-DC, B7-H3, and

B7-H4, respectively. Numbers in graph were their expression level as MFI. B, Tr inhibit Th1 polarization cytokine secretion. The above cocultured splenic DC were stimulated with LPS overnight and their supernatants were harvested for analyses of IL-6, IL-10, IL-12, IFN-γ, TNF-α, and TGF-β secretion.

Figure 4.3.3: Influences of $CD8^+CD25^+$ Tr (Tr) on naive T cells.

A, Dose-dependent suppression of T cell proliferation by Tr. CFSE labeled naïve $CD4^+$ or $CD8^+$ T cells (1×10^5/well) were cultured with 2 μg/ml anti-CD3 Ab in the presence of 2×10^5 irradiated T cell depleted splenocytes and decreasing amounts of activated Tr (at indicated ratios). After 5 days of culture, proliferation was measured based on CFSE signal. A representative CFSE profile from one experiment of three is shown. B, Tr cells mediate suppression by cell contact, not by CTLA-4, IL-10 and TGF-β. For the mechanism study, the ratio of T cells to Tr is from1:1 to1:8 and all the results were similar; only 1:1 results were shown. C, Tr induced T cell anergy. After culturing with Tr overnight, CD4 T cells were purified for CD73 and FR-4 expression, and simulated with different ratios of CD3CD28 dynal beads for 3 days, ^{3}H-tymidine was added for last 12 hrs of culture for tritium incorporation assay, then cells were analyzed for their CD25 expression by flow cytometry or proliferation by β-counter.

Figure 4.3.4: $CD8^+CD25^+$ Tr (Tr) effects on antitumor immunity.

A, DC_{OVA} and rLm_{OVA} induced $CD4^+$-dependent and independent CTL response, respectively. The tail-vein blood was collected after vaccination of wild type and $I\text{-}A^{b\text{-}/\text{-}}$ B6 mice with DC_{OVA} and Lm_{OVA} for 6 and 9 days, respectively, and stained with PE-H-2-K^b/OVAI tetramer and FITC-CD8 Ab for flow cytometry analysis. Double positive T cells were OVAI-specific CTL. B, Tr inhibit the DC_{OVA} and Lm_{OVA} induced CTL response. B6 mice were treated with vaccination only, $3x10^6$Tr only, or with Tr (iv),

OVAI specific CTL were detected as described above. C, Tr inhibited DC_{OVA} induced *in vivo* cytotoxicity. Splenocytes from C57BL/6 mice were pulsed with OVA I peptide and strongly stained with CFSE ($CFSE^{high}$), or pulsed with control Mut1 peptide and weakly stained with CFSE ($CFSE^{low}$). These labeled cells were then i.v. injected at ratio of 1:1 into mice after immunization for 8-10 days, 16-20 hrs later, the relative proportions of $CFSE^{high}$ and $CFSE^{low}$ cells remaining in the spleens of the recipient mice were assessed by flow cytometry. The values in each panel represent the percentage of $CFSE^{high}$ cells (+/- the standard deviation) and $CFSE^{low}$ cells remaining in the spleens. D, Tr inhibited $SpAPC_{OVA}$ induced anti-tumor immunity. The naïve B6 mice (4 per group) were immunized with 0.2 mg/ml OVA pulsed SpAPC or with $3x10^6$ Tr cells, 10 day later, mice were challenged with $0.1x10^6$ $BL6\text{-}10_{OVA}$ tumor cells s.c., and were sacrificed when their tumor growth reached 1-1.2cm in diameter. One representative result of three experiments (for a, b, c, and d) is shown.

Figure 4.3.5: Exosome uptake by $CD8^+CD25^+$ Tr (Tr) cells.

A. BM DC_{OVA} derived exosomes express pMHCI. EXO_{OVA}, $EXO_{OVA}(K^{b-/-})$, Tr, Tr/E, and Tr/E($K^{b-/-}$) were stained with biotin-anti-pMHCI and FITC-streptavidin, and analyzed by flow cytometry(solid dark line for pMHCI, dotted line for the isotype control, Tr/E means Tr pulsed with related exosomes). B, Kinetic study of exosomes uptake by Tr cells. Activated Tr cells (30×10^6 cells/3 ml) were incubated with CFSE labeled exosomes (EXO_{CFSE}) (30 μg/1×10^6 cells) in AIM-V containing IL-2 (10U/ml) at 37°C, 5×10^6 cells were taken out after 1, 2, 3, and 4 hrs of incubation, respectively; cells were washed and fixed right away. CFSE-positive T cells were detected by confocal microscopy and the average percentages of CFSE-positive T cells were calculated from three different areas. C, Dose curve. Tr were pulsed as above, but with different concentration, respectively, and detected as b. D, Blocking assay. 5×10^6 cells were

used for each experiment under the same condition as above with 50 ug/ml of each reagent, cells were treated as above after 4 hrs culture. d. Stability assay. Activated Tr cells with incubation of EXO_{CFSE} for 4 h were cultured in AIM-V containing IL-2 (10 U/ml) at 37°C for different times after extensively washing. CFSE-positive T cells were detected by confocal microscopy and the percentages of CFSE-positive T cells were calculated after different period of culture. Note:

Figure 4.3.6: Characterization of exosome pulsed $CD8^+CD25^+$ Tr (Tr/E).

A, MHC I presentation of OVA I to RF3370 hybridoma cells by Tr/E. The amount of IL-2 secretion of stimulated RF3370 in examined wells was substracted by the amount of IL-2 in wells containing DC_{OVA}, Tr/E, Tr/E($K^{b-/-}$), or RF3370, respectively. B, In OVA-specific $CD8^+$ T cell proliferation inhibition assay, the tail blood samples from mice immunized with irradiated DC_{OVA}, and rLmOVA, either alone or together with Tr , Tr(IL-$10^{-/-}$), Tr/E or Tr/E($K^{b-/-}$) cells (3×10^6 cells per mouse) were stained with PE-H-$2K^b$/OVAI tetramers and FITC-anti-CD8 Ab, and then analyzed by flow cytometry. C, *In vivo* $CD8^+$ T cell cytotoxicity assay. Splenocytes from C57BL/6 mice were pulsed with OVA I peptide and strongly stained with CFSE ($CFSE^{high}$), or pulsed with control Mut1 peptide and weakly stained with CFSE ($CFSE^{low}$). These labeled cells were then i.v. injected at ratio of 1:1 into mice immunized with irradiated DC_{OVA}, or DC_{OVA} plus various Tr cells as in panel 6b (above). Sixteen hours later, the relative proportions of $CFSE^{high}$ and $CFSE^{low}$ cells remaining in the spleens of the recipient mice were assessed by flow cytometry. The values in each panel represent the percentage of $CFSE^{high}$ cells (The value in parentheses represents the standard deviation) and $CFSE^{low}$ cells remaining in the spleens. *, representing $p<0.05$ versus cohorts of immunized mice treated with Tr1, Tr1(IFN-$\gamma^{-/-}$) and Tr1(vivo) cells, respectively, (Student's t test). D, and E, Tr/E showed stronger inhibition on *in vivo* antitumor immunity induced by

$SpAPC_{OVA}$ than Tr itself. Wild-type C57BL/6 mice (n=8) were either injected s.c. with $SpAPC_{OVA}$ alone or in conjunction with i.v.-injected 3×10^6 or other number of Tr, Tr/E, Tr(IL-$10^{-/-}$), or Tr/E($K^{b-/-}$) cells, respectively. 9-11 days later the mice were given 0.1×10^6 BL6-10_{OVA} tumor cells s.c., then mouse survival was monitored daily for up to 6 weeks. One representative experiment of three for b, c, d, and e is depicted. Note: The number after Tr or Tr/E means number of million cells. Tr(IL-$10^{-/-}$): Tr from IL-10 knock out mice.

Figure 4.3. 1: Characterization of $CD8^{+}CD25^{+}$ Tr (Tr) in spleen

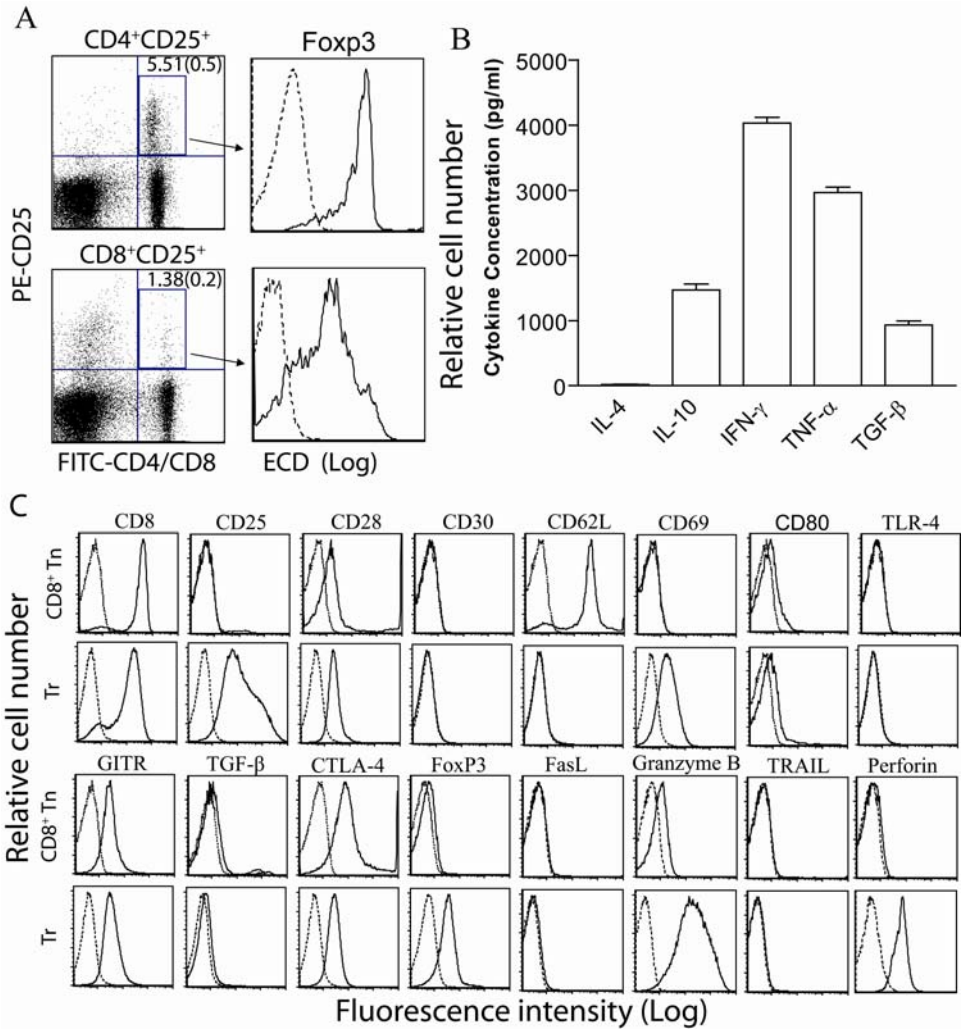


Figure 4.3. 2: Influences of $CD8^{+}CD25^{+}$ Tr (Tr) on splenic DCs.

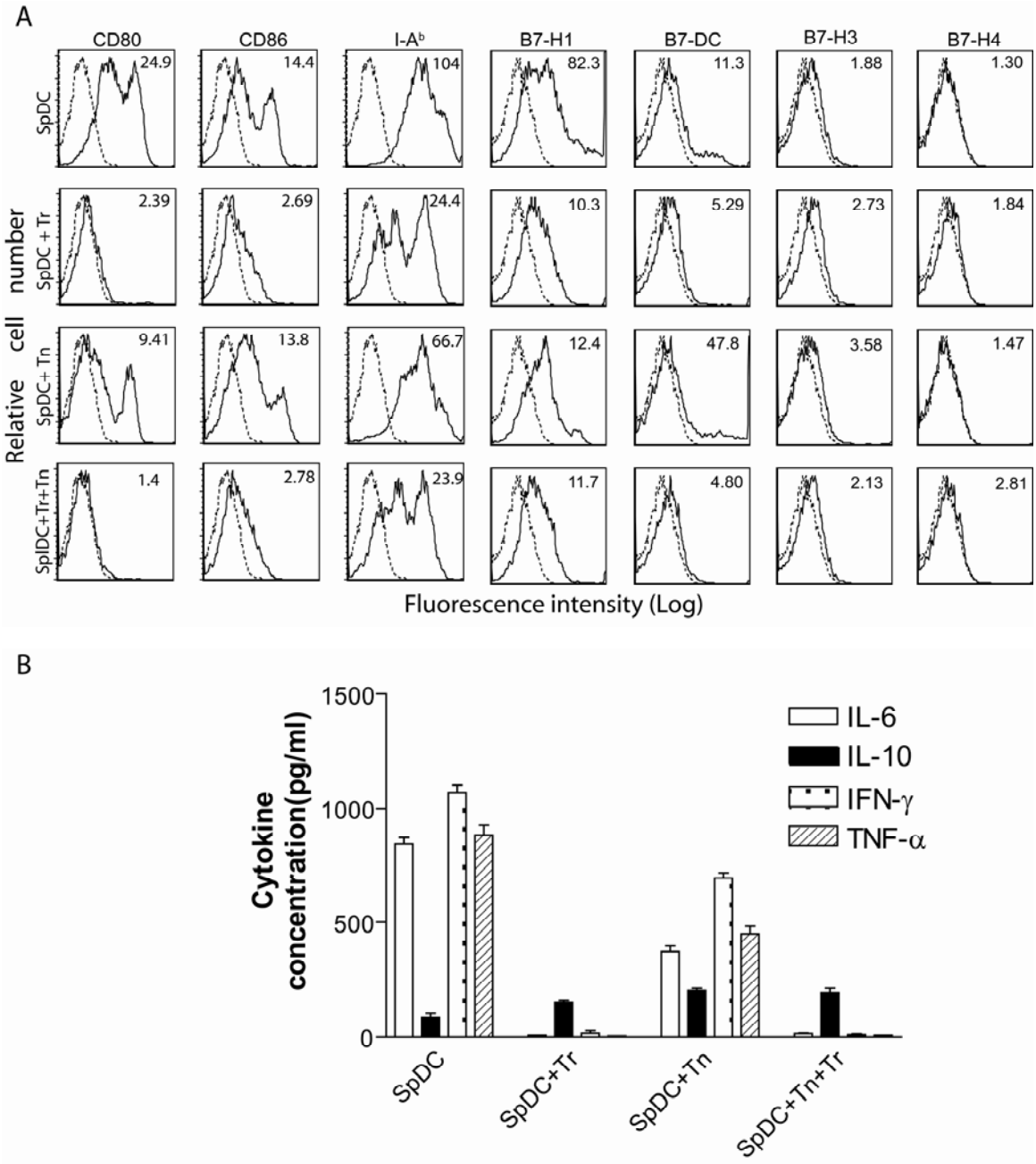


Figure 4.3. 3: Influences of $CD8^{+}CD25^{+}$ Tr (Tr) on naive T cells.

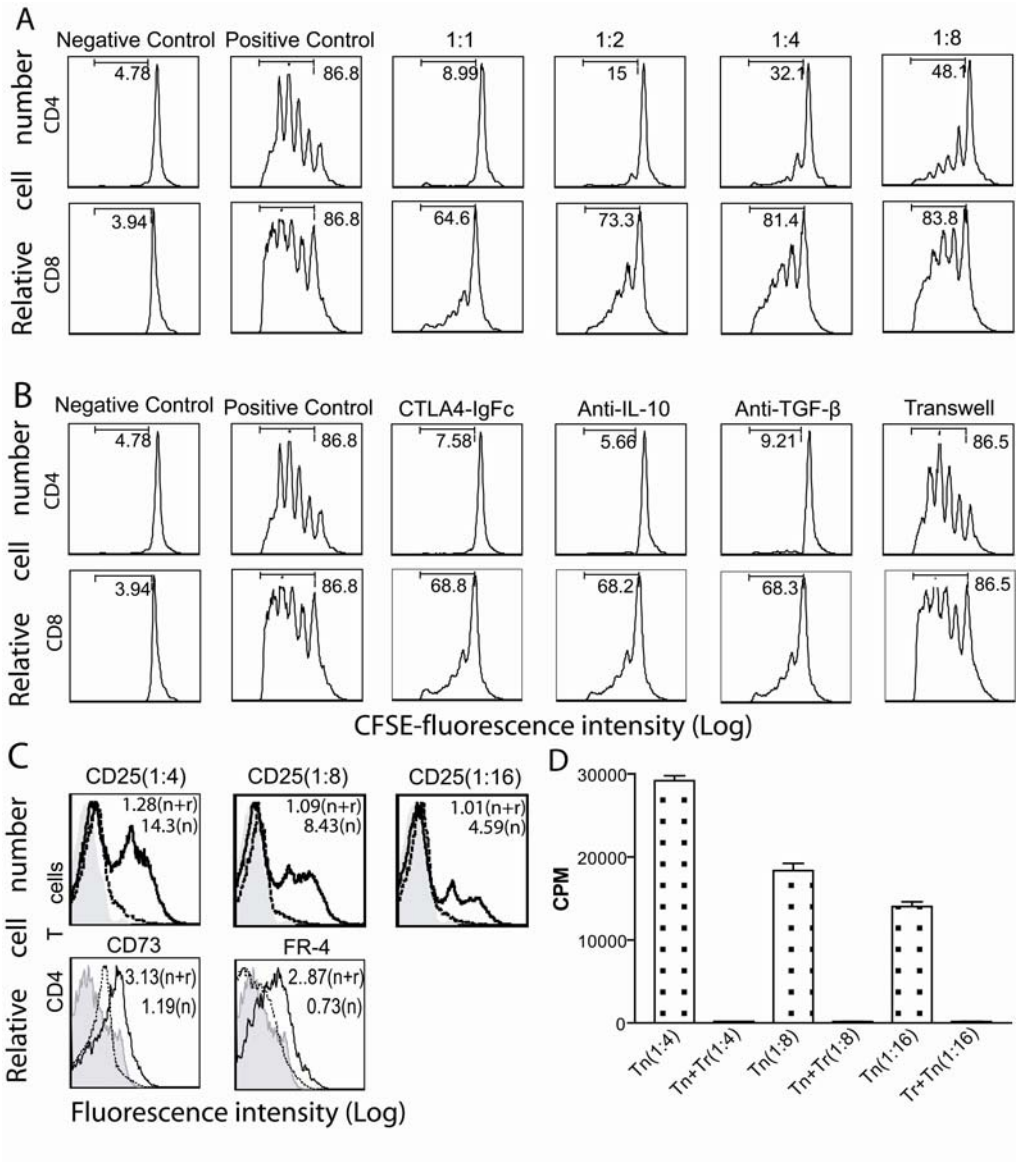


Figure 4.3. 4: $CD8^+CD25^+$ Tr (Tr) effects on antitumor immunity.

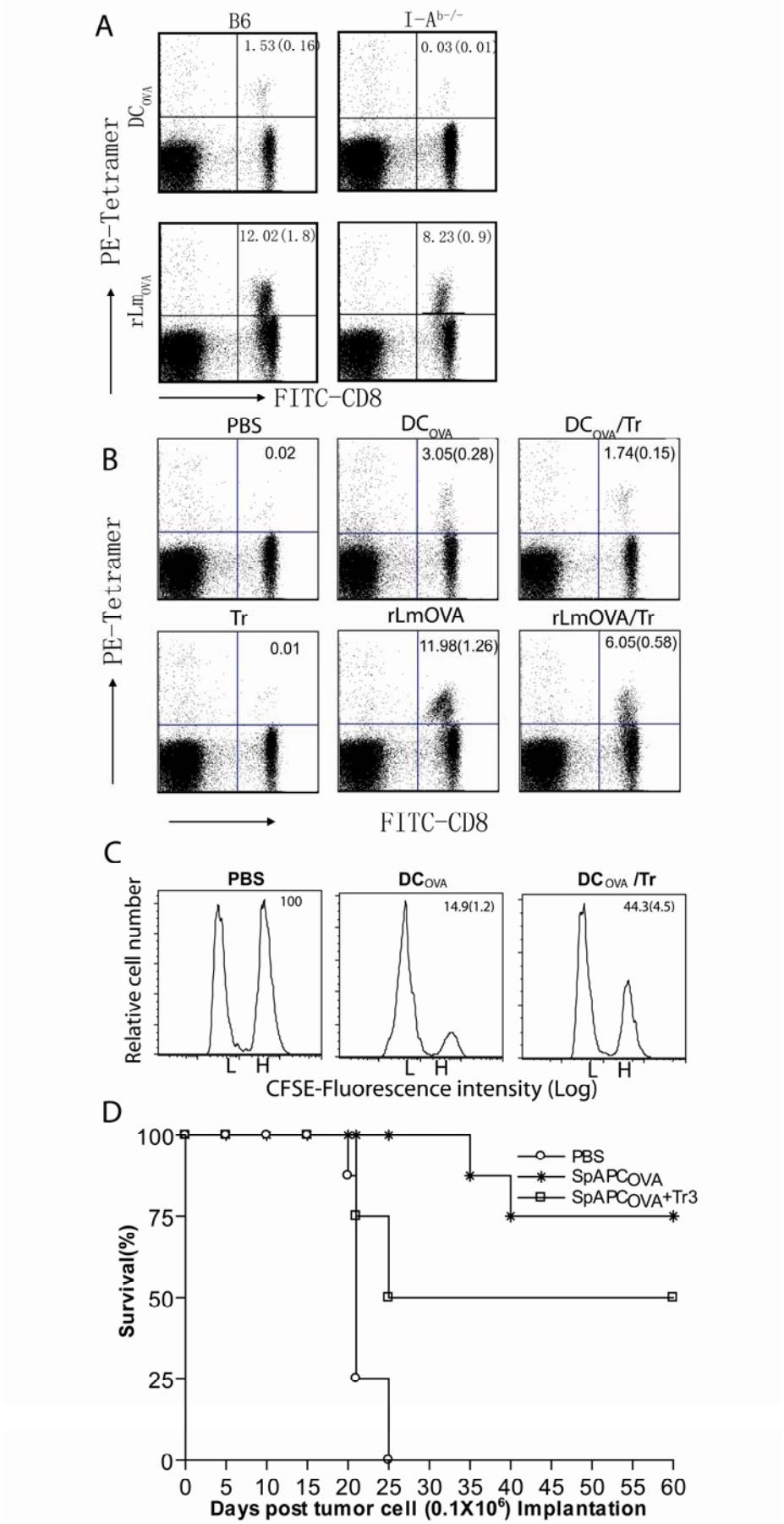


Figure 4.3. 5: Exosome uptake by $CD8^{+}CD25^{+}$ Tr (Tr) cells.

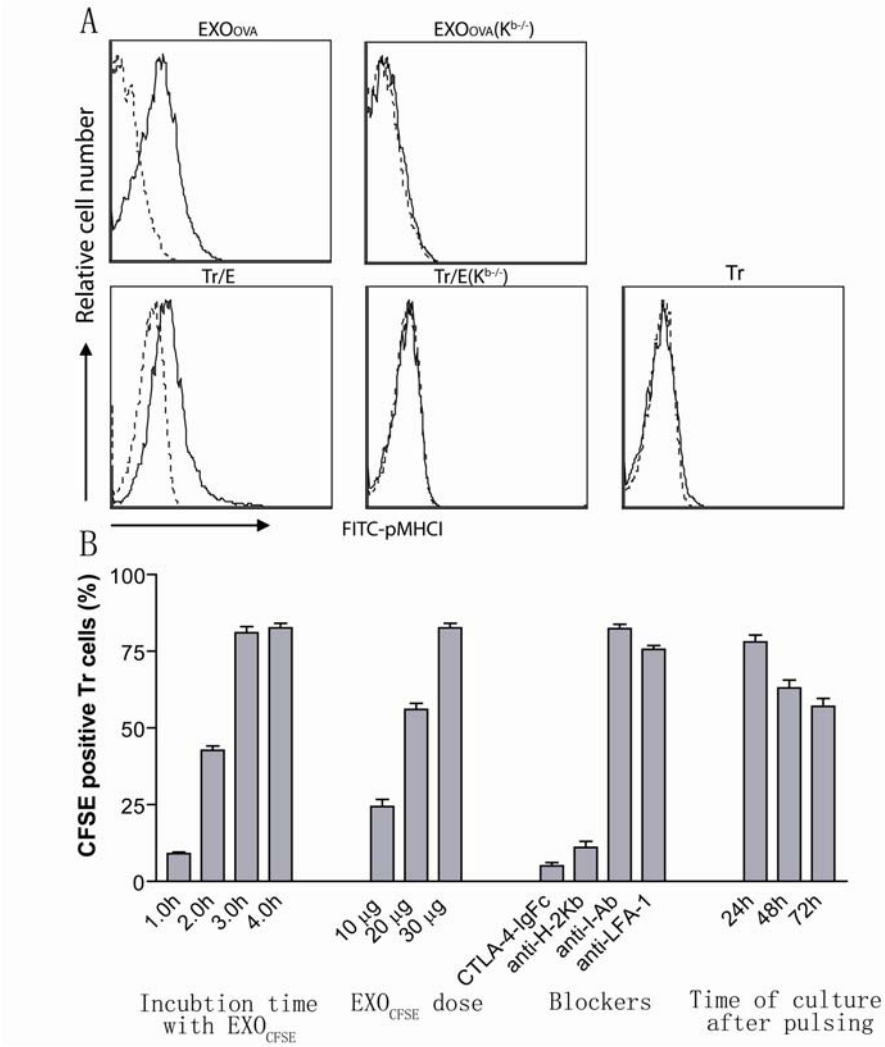
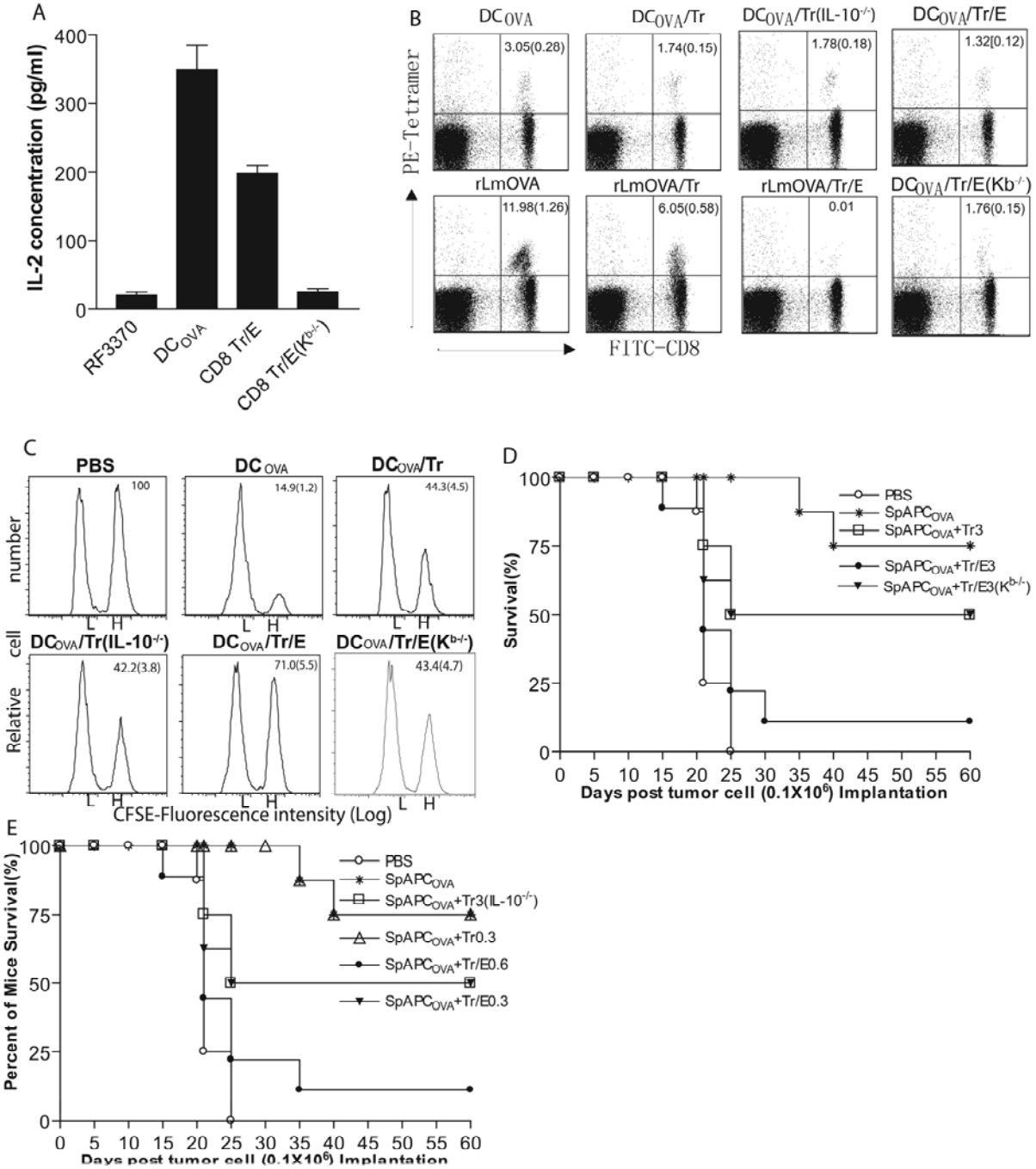


Figure 4.3. 6: Characterization of exosome pulsed $CD8^+CD25^+$ Tr (Tr/E).



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