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碩士論文

Department and Graduate Institute of Microbiology
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探討不同劑量的流感病毒感染以及施打疫苗對調節性 T 細胞生成的影響

The effects of the viral dose and immunization on induction of influenza virus antigen-specific regulatory T cells

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中文摘要

現時的流感疫苗主要是針對 B 細胞免疫來進行設計，使宿主產生強而有效的中和性抗體以阻止病毒的入侵。由於流行性感冒病毒的表面抗原具有高度變異性，導致流感疫苗需每年重新施打，相對地，可辨認具有高度一致性的病毒內部蛋白質的 T 細胞免疫將會成為新一代流感疫苗設計的思考方向。因此，我們實驗室主要針對探討哪些因子影響對抗流感病毒的 T 細胞免疫，希望能藉此設計出有效的 T 細胞疫苗。而最近的研究證明在急性流感病毒感染中會產生具有流感病毒特異性的調節性 T 細胞，但這些具有抗原特異性的調節性 T 細胞在急性流感病毒感染時所參與的免疫作用以及角色到目前還不明確。我們在這篇論文裏利用一株帶有卵白蛋白 (Ovalbumin, OVA) 抗原決定部位的 A 型流感病毒，PR8-OVA_{II}，作為感染模式，去研究具有抗原特異性的調節性 T 細胞在免疫作用時以及感染不同劑量的流感病毒中的角色是如何。根據我們的研究結果顯示，感染低劑量的病毒比感染高劑量的流感病毒產生較多的抗原特異性的調節性 T 細胞，而施打疫苗，同樣是一個較弱的免疫刺激，亦會促進具有抗原特異性的調節性 T 細胞的產生。因此我們認為，在一個較弱的免疫刺激之下較容易產生抗原特異性的調節性 T 細胞，這些產生出來的具有抗原特異性的調節性 T 細胞對後續對抗流感病毒的免疫反應會造成什麼影響是我們下一步想要去探討的。

關鍵詞：調節性 T 細胞、流感病毒、疫苗、不同病毒劑量

Abstract

Current influenza vaccine mainly focuses on inducing a strong B cell immunity based on neutralizing antibody to prevent the influenza virus infection. Due to the high mutation rate of influenza surface antigens, annual vaccination is necessary. T cell immunity targeting the conserved internal proteins is thus a candidate for designing a new type of influenza vaccine against a broad spectrum of viral strains. Therefore, we are interested in the factors that which affect T cell immunity against influenza virus. Recent studies have demonstrated that influenza virus-specific regulatory T (Treg) cells can be induced during acute influenza virus infection, but little is known about the role of Treg cells in regulating the immune response during acute influenza virus infection. Here, using OVA epitope-containing influenza A virus (PR8) as a model, we studied the role of viral antigen-specific Treg cells during immunization and different doses of influenza virus infection. We found that, low-dose infection promoted the induction of viral antigen-specific Treg cells. In addition, immunization, another suboptimal immune stimulation, also induced antigen-specific Treg cells. Understanding how these viral antigen-specific Treg cells affect T cell immunity will help us design better vaccine strategy against the infection of highly mutated and emerging pandemic strains of

influenza virus.

Key words: Regulatory T cells, influenza virus, vaccine, viral dose

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Chapter 1: Introduction

1.1 Influenza virus

Influenza A viruses belongs to the Orthomyxoviridae family. The genome of influenza A virus contains eight segmented, negative-sense RNAs which encode at least 12 viral proteins, including structure and non-structure proteins (Medina and Garcia-Sastre, 2011). Structure proteins can be divided into surface proteins and internal proteins. The former are hemagglutinin (HA), neuraminidase (NA) and membrane ion channel protein (M2). The internal proteins include matrix protein (M1), ribonucleoprotein complex: polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and nucleocapsid protein (NP), PB1-F2 which is involved in the induction of cell death (Chen et al., 2001). Non-structure protein 1 (NS1) and non-structure protein 2 (NS2), also known as NEP, which is a nucleus export protein (O'Neill et al., 1998).

Influenza A virus can be divided into different subtypes based on the antibody responses to hemagglutinin (HA) and neuraminidase (NA). To date, there are 16 HAs (H1-H16) and 9 NAs (N1-N9) (Wilks et al., 2012), but only H1, H2 and H3 influenza A

virus can efficiently infect human (Medina and Garcia-Sastre, 2011). Since HA is a binding protein targeting the host cells, it determines the host tropism. The receptor of human influenza A virus HA is α-2,6-linked sialic acid, whereas the receptor of avian influenza A virus HA is α-2,3-linked sialic acid. As pigs have both α-2,3 and α-2,6-linked sialic acid, it can be simultaneously infected by the human and avian influenza viruses, and serve as an intermediate host in which the reassortment of two different strains of influenza A virus occurs (Medina and Garcia-Sastre, 2011). This often leads to the emergence of a novel strain of influenza A virus.

As an RNA virus, the polymerase of influenza A virus lacks proofreading ability and is error-prone (Ishihama et al., 1986). As a result, mutations accumulate in viral surface proteins HA and NA, and thus escape the protection of neutralizing antibody. This is so-called antigenic drift and often occurs to seasonal influenza A virus. Antigenic shift is caused by genetic reassortment of two or more different strains of influenza A viruses co-infecting the same host cells. This creates a new strain of influenza A virus acquiring the HA and NA from different subtypes (Medina and Garcia-Sastre, 2011). Since the human population lack the protective immunity against

the novel strain of influenza virus, it spreads rapidly and widely and causes severe morbidity and mortality, which is known as pandemic (Ghendon, 1994).

1.1.1 Pandemics of influenza A virus

In the past century, there were several pandemics that caused a high morbidity. During the pandemic of 1918, the global outbreak of H1N1 influenza virus (Spanish flu) killed 20 to 40 million people all over the world, and sickened about one-quarter to one-half of the entire human population (Cox, 2000; Ghonden Y., 1994).

The influenza A virus H2N2 caused the Asian flu pandemic in 1957. Its HA and NA genes originated from avian influenza A virus and reasserted with the circulating human influenza A virus (H1N1). H2N2 influenza A virus had been dominating in the human society until pandemic Hong Kong flu (H3N2) occurred (Cox and Subbarao, 2000; Ghendon, 1994).

In 1968, the H3N2 influenza A virus caused the Hong Kong flu pandemic. Its HA and PB1 genes were derived from avian influenza virus and the other gene segments remained the same as that of the contemporarily circulating H2N2 virus (Cox and Subbarao, 2000; Ghendon, 1994). The H3N2 influenza A virus dominated among the

human population until the emergence of Russian flu (H1N1) which was almost identical to the H1N1 strains circulating in early 1950s (Cox and Subbarao, 2000; Ghendon, 1994). Instead of totally replacing the prior circulating strain of influenza A virus, this H1N1 strain co-circulated with H3N2 in human population until the outbreak of the pandemic influenza A virus (H1N1) in 2009.

1.1.2 Immune response during influenza infection

The innate immune system forms the first line defense against virus infection, and responds rapidly to control the virus replication. The negative-sense RNA genome of influenza virus can be recognized by several types of pattern-recognition receptors (PRRs), including the TLR family member, TLR7 (Diebold et al., 2004; Lund et al., 2004), the RIG-I like receptor (RLR) family, RIG-I (Kato et al., 2005), and the NOD like receptor (NLR) family, NLRP3 (Ichinohe et al., 2009). In addition, the alveolar macrophages are activated after phagocytosis of the influenza virus-infected cells and limit viral spread (Wijburg et al., 1997). After uptaking the influenza virions, the conventional dendritic cells, situated underneath the airway epithelium barrier and above the basal membrane, move to the draining lymph node, present the influenza

virus-derived antigens to naïve T cells, activate them, and eventually induce the development of the influenza-specific T cell immunity.

It is thought that after primary influenza A virus infection, the host induces rapid responses of highly protective and long-lasting memory B cells in respiratory tract and secondary lymphoid tissue. The memory B cells secrete antibody that blocks the virus entry and prevents the infections of the same influenza virus strains. However, due to the high mutation rate of the HA surface protein, antibody-mediated immune response fails to target and neutralize different strains of influenza viruses and finally cause sickness (Waffarn and Baumgarth, 2011)

$CD4^+$ and $CD8^+$ T cells are activated by mature DCs in response to infection. They essentially contribute to the clearance of virus-infected cells, but also induce pulmonary inflammation and tissue damage in the infected host. Lung effector $CD4^+$ and $CD8^+$ T cells use multiple mechanisms to eliminate influenza-infected cells. Conventionally, $CD8^+$ T cells are thought to clear the influenza virus infection via Fas/FasL and perforin-dependent mechanisms (Topham et al., 1997). $CD4^+$ T cells are believed to participate in the antiviral response through secreting cytokines that help the differentiation and maturation of $CD8^+$ T and B cells during primary infection and

eventually promote viral clearance (Boyden et al., 2012; Brown et al., 2004; Johnson et al., 2009).

Different from B cells that specifically target the surface antigens of preexisting strains of influenza viruses, T cell immunity may protect or limit the severity of influenza virus-mediated sickness where the host has no existence of protective antibody (Lee et al., 2008; Wilkinson et al., 2012), this may be due to the cross-protective property of T cells. Unlike the variable surfaces antigen of influenza A virus, the internal influenza viral proteins are highly conserved across strains (Wilkinson et al., 2012). Hence, although T cell immunity cannot prevent the infection of influenza virus, it recognizes the internal proteins of different strains of influenza viruses and limits disease severity (McMichael et al., 1983).

1.1.3 The challenge of current inactivated influenza virus vaccine

The pandemic of influenza results in infections of millions of people worldwide and causes significant health and economic burdens. The influenza vaccine is so far the best strategy to control the spread of influenza virus infection. Current influenza vaccines for preventing influenza virus infection are live attenuated vaccine and inactivated vaccine, and both of them are primarily based on the generation of

neutralizing antibody to block the influenza infection (Gomez Lorenzo and Fenton, 2013). However, frequent changes in the surface antigens of influenza virus allow it to escape antibody-mediated immunity. In contrast, T cells recognize endogenous conserved epitopes derived from the 6 internal genomes of influenza virus (Jameson et al., 1998), so they can provide cross-protection between different strains or even subtypes of influenza viruses. Therefore, T cell vaccine becomes an attractive approach for the universal influenza vaccine design. To develop effective T cell vaccine, it is critical to understand the mechanisms regulating the T cell immunity against influenza virus.

1.2 Regulatory T cells

About two decades ago, it was discovered that $CD4^+CD25^+$ T cells contribute to maintain self-tolerance by down-regulating immune responses to self and non-self antigens (Sakaguchi et al., 1995). Currently, we know that this T cell subset is defined as regulatory T (Treg) cells. Treg cells are critical to maintain immune homeostasis (da Silva Martins and Piccirillo, 2012) and prevent severe immunopathology caused by excessive immune responses (Kullberg et al., 2002). Abnormality of Treg cells function

impairs the peripheral tolerance and causes autoimmune diseases. The forkhead-winged helix transcription factor family member Foxp3 is the key transcription factor that determines the differentiation, function and identity of Treg cells (Brunkow et al., 2001; Fontenot et al., 2003; Fontenot and Rudensky, 2005; Ramsdell, 2003). However, there are some subsets of Treg cells that do not express Foxp3. For example, both Th3 and Tr1 cells secrete inhibitory cytokines and can suppress immune response, but they do not express Foxp3 (Battaglia et al., 2006; Weiner, 2001). The suppressive function of Treg cells with deficiency of Foxp3 is almost lost. Lack of Foxp3 expression in both human and mice leads to the development of autoimmune disease. The dysfunction of Foxp3 in human causes a rare X-linked fatal autoimmune disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome and the phenotype is similar to the lymphoproliferative disease observed in the “scurfy” mice which has spontaneous Foxp3 frameshift mutation (Brunkow et al., 2001). This particular strain of mice is a natural mouse model for IPEX characteristic of several autoimmune diseases, such as dermatitis, enteritis and diabetes, etc. The autoimmune lesions accompany massive lymphocyte infiltration, increase the induction of multiple cytokines, and very early death (Smyk-Pearson et al., 2003). This phenomenon can be rescued by

transferring sorted $CD4^+CD25^+$ T cells, preventing or limiting the disease progress, and finally prolonging the survival (Smyk-Pearson et al., 2003).

1.2.1 Subsets of regulatory T cell

There are two main types of Treg cells: thymus-derived $CD4^+CD25^+Foxp3^+$ Treg cells, also called natural Treg (nTreg) cells and $CD4^+CD25^+Foxp3^+$ Treg cells induced from naïve $CD4^+CD25^-$ T cells, which are called adaptive or inducible Treg (iTreg) cells (Curotto de Lafaille and Lafaille, 2009). Both nTreg and iTreg require IL-2 and TGF-β to drive the Foxp3 induction (Chen et al., 2003; Malek et al., 2008).

Naturally occurring $CD4^+CD25^+Foxp3^+$ regulatory T cells (nTreg) are the Treg subset which matures in the thymus. During TCR chain selection, they have high affinity for self-peptides (Jordan et al., 2001). Inducible regulatory T (iTreg) cells are derived from peripheral naïve $CD4^+CD25^-$ T cells, and under some circumstances, they go on to acquire a suppressive function (Kang et al., 2007). Both of them have the ability to suppress aberrant or excess immune responses and maintain homeostasis, preventing the autoimmune syndrome (Rudensky, 2011; Sakaguchi et al., 2008). While all of them play an important role in prevention of autoimmune disease, there is

evidence showing the functional differences between nTreg and iTreg cells. Only transfer of nTreg can prevent disease lethality but not suppress chronic inflammation and autoimmunity. On contrary, acute depletion of the iTreg cells results in weight loss and inflammation (Haribhai et al., 2011), showing that nTreg and iTreg cells play different roles in immune response.

1.2.2 The mechanisms of suppression by Treg cells

The mechanisms of Treg cells suppressing the inflammatory responses involve the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β. In addition, inhibitory receptors, cytotoxic T lymphocyte antigen 4 (CTLA-4) and lymphocyte-activation gene 3 (LAG-3), can be expressed on the cell surface and through a cell-cell contact to suppress the effector functions of immune cells (Campbell and Koch, 2011). Besides, interfering the interaction between DC and conventional T (Tconv) cells causes the reduction of TCR signaling and T cell activation (Tadokoro et al., 2006; Yamaguchi et al., 2011). In addition, due to Treg cells are constitutively expressing high levels of IL-2 receptor and produce no IL-2, they are able to deprive IL-2 produced by Tconv cells, inhibiting the activation of Tconv cells (Thornton and Shevach, 1998).

A recent study has showed that Treg cells, like the regular effector $CD4^+$ T cells, can undergo contraction and form a memory phenotype after acute viral infection (Brincks et al., 2013; Sanchez et al., 2012). These memory Treg cells display almost entirely $CD62L^{lo}$ population, which corresponding to a TEM phenotype. Following the secondary infection, these memory Treg cells can respond and expand rapidly, and secret high levels of IL-10 to suppress the local inflammation (Brincks et al., 2013).

During the viral infection, Treg cells contribute to limiting excessive inflammation and cytokine secretion, reducing tissue damage and immunopathogenesis in inflammation sites, and limiting the responses of pathogen-specific T cells (Brincks et al., 2013; Suvas et al., 2003; Toka et al., 2004). Since Treg cells can eliminate the capability of effector T cells to cure the infection, they promote the pathogen persistence and finally cause a chronic infection (Belkaid et al., 2002; Li et al., 2008).

Although a lot of evidences has shown the important role of Treg cells during infection, and the majority of previous studies focus on chronic infection. In contrast, the role of Treg cells in acute infections remains controversial. A few of recent studies have demonstrated that during the acute viral infection, including herpes simplex virus (HSV), respiratory syncytial virus (RSV), dengue virus, coronavirus, rotavirus and

influenza virus, Treg cells controlled the excess of immune response and prevented the immunopathology, but reduced the antiviral immune responses (Betts et al., 2012; Dejnirattisai et al., 2008; Fulton et al., 2010; Kim et al., 2008; Lee et al., 2010; Suvas et al., 2003; Trandem et al., 2010). In addition, pathogen-specific Treg cells can also be identified. For instance, by adoptively transferring naïve HA-specific Treg cells into B10.D2 mice, the activation and expansion of HA-specific Treg cells were found 7 days after the infection with VV (vaccinia virus)-HA (Sanchez et al., 2012). Also, by using MHC class II tetramers specific for the influenza epitope ($NP_{311-325}/IA^b$), it was demonstrated that the antigen-specific Treg cells responded to the primary and secondary influenza virus infection (Brincks et al., 2013).

1.3 Factors that promote the generation of iTreg cells

Many studies focus on investigating the relationship between Treg cells and tumors. There accumulates a lot of evidence that a large number of $CD4^+CD25^+Foxp3^+$ Treg cells infiltrate into the tumor and impair immune responses to tumor cells (Nishikawa and Sakaguchi, 2010). Removal of $CD4^+CD25^+Foxp3^+$ Treg cells enhances antitumor responses and inhibits tumor growth (Litzinger et al., 2007). In addition to the tumor environment, infection of pathogens can also induce Treg cells. However,

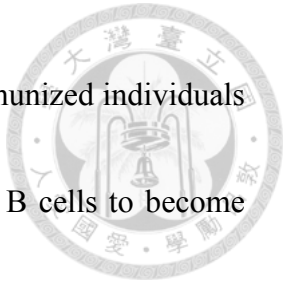
previous Treg cell studies mainly focus on chronic infection. During chronic infection, Treg cells accumulate in the infection site and the draining lymph node (LN) and play a dominant role in limiting immunopathology mediated by pathogen-specific T cells (Alatrakchi and Koziel, 2009; Dolganiuc and Szabo, 2008). In addition to chronic infection, Treg cells can be induced by parasites and bacteria infection (Gomez-Escobar et al., 2000; Kandulski et al., 2008) and play an important role in regulating immune responses to pathogens. In some cases, Treg cells promote pathogen persistence, and finally lead to a chronic infection.

Recent studies have demonstrated that during acute viral infection, Treg cells can be induced and play a critical role in regulating T cell immunity. During the infection of HSV (Suvas et al., 2003), RSV (Fulton et al., 2010; Lund et al., 2008) and lymphocytic choriomeningitis virus (LCMV) (Lund et al., 2008), depletion of Treg cells enhances pathogen-specific CD8 T cell immunity. However, all of these studies analyzed the effect of the whole population of Treg cells, but failed to dissect the function of nTreg and iTreg cells during viral infection. Recently, Betts R. J. et al. have demonstrated the existence of influenza virus-induced Treg cells by using the *in vitro* proliferation assay (Betts et al., 2012). In addition, by using MHC II tetramer (NP_{311}/I-A^{b+}), influenza

virus-specific Treg cells can be detected following acute influenza virus infection of mice (Brincks et al., 2013). Although it is getting clear that pathogen-specific Treg cells can be induced after infection, little is known about how Treg cells are induced and maintained following acute viral infection.

A previous study found that the concentration of the peptide required to stimulate the activation of $CD4^+CD25^+$ T cells is 10 to 100 fold lower than that required for triggering the proliferation of $CD4^+CD25^-$ T cells (Takahashi et al., 1998). This finding is matched for *in vivo* administrating low dose HA antigen and without agonistic antibody FGK 45.5 (anti-CD40), promote to generate antigen-specific Treg cells *in vivo* (Kretschmer et al., 2005). These data imply that low dose of antigen and subimmunogenic environment promote the induction of iTreg cells.

Since epithelial mucosa are frequently exposed to foreign antigen, and are susceptible to immune-mediated damage, the mechanisms to prevent unnecessary immune responses are required. It has been known that administration of foreign antigens by oral route or inhalation through airway can promote tolerance by inducing antigen-specific Treg cells (Chen et al., 1995).



Immunization against certain antigens or diseases allow the immunized individuals to quickly respond to a second encounter by educating T cells and B cells to become memory immune cells. In addition to induction of the robust immunity against foreign antigens, immunization may also induce antigen-specific Treg cells. After immunizing $Foxp3^{EGFP}$ mice with CFA, Treg cells got a vigorous proliferation (Haribhai et al., 2007). Treated the BALB/c mice with SRP299, a killed Mycobacterium vaccae, the BALB/c mice gave rise to IL-$10^{+}CD4^{+}CD45RB^{Lo}$ regulatory T cells and reduced airway inflammation (Zuany-Amorim et al., 2002). All the evidence suggests that vaccination may also induce antigen-specific Treg cells.

1.3.1 Homeostasis of lung

Influenza virus primarily infects the epithelial cells of the respiratory tract. Lung is one of the organs belonging to the mucosal immune system. The function of lung is to carry out gas exchange. It is a route that often exposes to a broad range of antigens, most of which are harmless. It is important to maintain homeostasis of lung and prevent excessive immunopathogenesis (Holt et al., 2008).

Based on above discussion, Treg cells can play an important role in acute viral infection. We know that the antigen-specific Treg cells can be induced during acute

viral infection. Depleting of the Treg cells by PC61 antibody can enhance the antigen-specific T cell immunity. Interestingly, a previous study showed that the mice primed with lower dose of influenza virus results in reducing survival rate in the lethal secondary infection (Marois et al., 2012). However, little is known about the relationship between infectious dose, Treg and Teff cells.

We thus hypothesize that low-dose influenza virus tends to induce pathogen-specific Treg cells during acute viral infection. Repeated administration of influenza vaccine induces and expands pathogen-specific Treg that may reduce the T cell immunity against influenza virus infection.

Chapter 2: Specific aim

This study aims to investigate the dose effects of influenza A virus infection and immunization on the induction of antigen-specific Treg cells and to study the role of antigen-specific Treg cells in establishing the T cell immunity during acute influenza virus infection.

Chapter 3: Materials and Methods

3.1 Mice

C57BL/6 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and the National Taiwan University College of Medicine Laboratory Animal Center (Taipei, Taiwan). C57BL/6 OT-II Thy1.2^{+} mice was provided by Dr. M.H. Tao (Academia Sinica, Taipei, Taiwan). Foxp3^{gfp} mice was a gift from Dr. Alexander Y. Rudensky (Howard Hughes Medical Institute and Immunology Program, Memorial Sloan-Kettering Cancer Center, New York, NY). OT-II and Foxp3^{gfp} mice were then crossed to generate OT-II x Foxp3^{gfp} mice. All mice used in these studies were 6-8 weeks old and were bred and maintained at the National Taiwan University College of Medicine Laboratory Animal Center (Taipei, Taiwan) in the specific pathogen-free conditions.

3.2 Plasmid

The eight genome segments of PR8-containing plasmids were a gift from Dr. Shin-Ru Shih (Research Center for Emerging Viral Infections, Chang Gung University, Taiwan, ROC).

3.3 Cell culture and virus

Madin Darby Canine Kidney (MDCK) cells and human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (GPS, Gibco), and were grown at 37℃, 5% CO_2. Influenza viruses A/PR8 (PR8, H1N1 subtype) and A/PR8-OVA_{II} (PR8-OVA_{II}) were generated by reverse genetics.

3.4 Determining the viral titers by plaque assay

At 1 day prior to determine the viral titer, 1.2 x 10^6 MDCK were seeded in the 6-well plates. After formation of a monolayer of MDCK cells, 0.2 ml of each diluted virus was added into the well. The plate was incubated at 37℃, 5% CO_2, 1 hour for the

virus absorption. Mixture of 0.3% agarose gel, DMEM (Hyclone) supplement with 1x NEAA (Gibco), 1 mM sodium pyruvate (Gibco) and 0.3% BSA (Gibco), 10 mM HEPES (Gibco) and 2 μg/ml TPCK (Sigma, cat. T1426) were added to the cell monolayers. Plates were incubated for 2 days at 37℃, 5% CO_2. The cells were fixed with 5% formaldehyde at room temperature for at least 1 hour. The plaques were visualized and counted after stained with 0.5% crystal violet.

3.5 Reverse genetics for generation of recombinant influenza A virus

MDCK and 293T were seeded in a ratio of 1:1 at 3.5-cm dish containing DMEM (Hyclone) supplemented with 10% FBS and were grown at 37℃, 5% CO_2 for 24 hours. Plates were washed with Opti-MEM (Gibco) twice, and added with the mixture of lipofectamin 2000 and 8 plasmids which containing the whole genome of influenza A virus (pHW-PR8-HA, pHW-PR8-NA, pHW-PR8-PA, pHW-PR8-PB1, pHW-PR8-PB2, pHW-PR8-NP, pHW-PR8-NS, pHW-PR8-M). Twenty-four hours post transfection, the culture medium was then replaced by the infection medium which contained DMEM (Hyclone) supplemented with 1x NEAA (Gibco), 1 mM sodium pyruvate (Gibco) and 0.3% BSA (Gibco), 10 mM HEPES (Gibco) and 2 μg/ml TPCK (Sigma). The supernatants were collected after 48 hours.

3.6 Generation of bone marrow-derived dendritic cells

After removal of the muscle from the femurs and tibias collected from 4-6 weeks old C57BL/6 mice, the bones were placed at 75% alcohol for sterilizing, and washed with RPMI 1640 (Gibco) supplemented with 10% FBS twice sequentially. Both ends of the bone were cut with scissors, and a 25-gauge needle and 2 ml RPMI 1640 (Gibco) supplemented with 10% FBS were used to flush the marrow out. The suspended cells were passed through 100-μm-pore-size cell strainer (BD, falcon) to obtain a single-cell suspension. Red blood cells (RBCs) were lysed using Ammonium-Chloride-Potassium (ACK) (150 mM NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA in pH 7.2) lysis buffer. At day 0, 2×10^6 cells were seeded into 10-cm petri dish (Alpha Plus). The cell culture medium contained 10 ml RPMI 1640 (Gibco) supplemented with 10% FBS, 100 unit/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (GPS, Gibco), 50 μM 2-mercaptoethanol (2-ME) and 2000 U recombinant murine GM-CSF (granulocyte - macrophage colony-stimulating factor) (Peprotech, cat. 315-03). At day 3, another 10 ml culture medium was added. At days 6 and 8, half of the culture medium was collected, and centrifuged. The supernatant was then removed and replaced with 10 ml fresh culture medium to resuspend the cell pellet. The resuspended cells containing the

fresh culture medium were put back to the original dish. At day 10, the non-adherent cells were collected by gently pipetting and about 60% of them were $CD11b^+$ dendritic cells.

3.7 Adoptive transfer of $CD4^+$ T cells to the recipient mice

Cells were isolated from the spleens of OT-II mice. $CD4^+CD25^-$ T cells were purified using CD4 T lymphocyte enrichment set (BD Bioscience, cat. 558131) with biotin-conjugated anti-CD25 antibody (BD Bioscience, cat. 553070). Cells were labeled with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) at the final concentration of 5 μM. About 0.5-1.5 × 10^6 naïve $CD4^+$ T cells were transferred to $Thy1.2^+$ C57BL/6 mice by tail vein injection. One day after the adoptive transfer, recipient mice were infected with different doses of influenza A virus. Seven days post infection, spleens, draining lymph nodes (dLNs), bronchoalveolar lavage (BAL) and lung samples were collected. In some experiments, iTreg cells were generated and sorted for GFP-positive $CD4^+$ cells on a FACSAria flow cytometer (BD biosciences) to obtain purities of >95%. One day after the adoptive transfer, recipient mice were infected with different strains of influenza A virus. Seven days post-infection, spleens, draining lymph nodes (dLNs), bronchoalveolar lavage (BAL) and lung samples were

collected.

3.8 Mice immunization

OVA was purchased from Sigma (cat. A5503) and dissolved in PBS. Imject alum (Pierce Biochemicals, cat. 77161) contains aluminum hydroxide and magnesium hydroxide. Immunogen was mixed with Imject Alum at a ratio of 1:1, followed by continuously mixing for 30 minutes. One day after adoptively transferring CFSE-labeled naive OT-II naïve CD4 T cells into the recipient mice, we intraperitoneally immunized the recipient mice with PBS, 10 μg OVA or 10 μg OVA plus alum (OVA-alum) in a total volume of 30 μl. To compare the immunization route-related variation in the immune response, we performed intraperitoneal or intramuscular immunization of the recipient mice with OVA-alum. All the injections were done by using 26-gauge needles.

3.9 Tissue harvest

Mice were sacrificed at the indicated times and the spleen, draining lymph node, lung and bronchoalvelar lavage (BAL) samples were collected. BAL samples were collected by three 0.5 ml phosphate-buffered saline (PBS) instillation through the

trachea of mice, the BAL fluid after each instillation was pooled together. The spleen, draining lymph node and lung were pushed through the metallic mesh and then passed through a 40-μm-pore-size cell strainer (BD, falcon) to obtain a single-cell suspension. Cells were treated with ACK lysis buffer for RBC lysis. Lung samples were further processed with ficoll-hypaque density gradient centrifugation to enrich lymphocytes. Cells were washed with PBS buffer twice. The cell viability and number were determined by counting and trypan blue exclusion.

3.10 *In vitro* generation of iTreg cells

Mice were sacrificed and the spleen samples were collected. After isolation of splenocyte, $CD4^+$ $CD25^-$ T cells were purified by using CD4 T lymphocyte enrichment set (BD Bioscience, cat. 558131) with biotin-conjugated anti-CD25 antibody (BD Bioscience, cat. 553070). For generating non-Ag-specific Treg cells, the flat-bottomed 6-well plate was pre-coated with 10 μg/ml anti-CD3 (BD Pharmingen, cat. 553057) in a volume of 800 μl for 1.5 hours at 37℃. Naïve $CD4^+$ T cells isolated from C57BL/6 mice were added and cultured with 5 ng/ml of recombinant human TGF-β (R&D Systems, cat. 240-B-002), 2 μg/ml anti-CD28 (BD Pharmingen, cat. 553294) and 100

U/ml recombinant human IL-2 (Peprotech, cat. AF-200-02) at 37℃ for 5 days. These cells were examined by flow cytometry, and about 30-40% inducible Treg could be generated.

3.11 *In vitro* suppression assay

We used CD4 T lymphocyte enrichment set (BD Bioscience, cat. 558131) with biotin-conjugated anti-CD25 antibody (BD Bioscience, cat. 553070) to purify naïve $CD4^+$ T cells and follow by CFSE label. The purified 5 x 10^4 CFSE labeled $CD4^+CD25^-$ T cells were cultured in the flat-bottom 96-well plate which was pre-coated with 0.25 μg/mL anti-CD3 antibody (BD Pharmingen, cat. 553057). For coculture, 5 x 10^4 BMDCs and the indicated number of iTreg cells were added to the well in RPMI 1640 culture medium, 50 μM 2-ME, 0.5 μg/ml anti-CD28 (BD Pharmingen, cat. 553294) and 50 U/ml recombinant human IL-2 (Peprotech, cat. AF-200-02) at 37℃ for 3 days.

3.12 Antibody and flow cytometry

The antibodies for staining were PE-Cy5-, APC-conjugated anti-CD4 (clone: RM4-5, cat. 15-0042; 17-0042), FITC-conjugated anti-CD3e (clone: 145-2C11, cat. 11-0031), PE-conjugated anti-CD8a (clone: 53-6.7, cat. 12-0081), PE-conjugated anti-Foxp3 (clone: NRRF-30, cat. 12-4771) and FITC-conjugated anti-Vα2 (clone:

B20.1, cat. 11-5812), all of these antibodies were purchased from eBioscience. PE-conjugated anti-Vβ5.1, 5.2 (clone: MR9-4, cat. 139503) was purchased from BioLegend. APC-conjugated anti-IFN-γ (clone: XMG 1.2, cat. 554413) was purchased from BD pharmingen. For tetramer or pentamer staining, single-cell suspensions were stained with influenza nucleoprotein ($NP_{366-374}/D^b$) pentamer or acid polymerase ($PA_{224-233}/D^b$) tetramer for 15 min at room temperature. The pentamer of $NP_{366-374}/D^b$ was purchased from ProImmune and the $PA_{224-233}/D^b$ tetramer was obtained from the National Institutes of Health Tetramer Core Facility followed by staining of cell surface molecules. Cells with single-cell suspension were incubated with the indicated antibody for 30 min on ice, and were then washed with FACS buffer (PBS with 1% FBS) twice. For intracellular staining, cells were fixed and permeabilized by BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (cat. 555028) by following the manufacturer's protocol. Briefly, the cells in a single-cell suspension were incubated with the fixation/permeabilization buffer (BD Cytofix/Cytoperm™) for 18 min on ice, washed with perm/wash buffer (BD Cytofix/Cytoperm™) twice, stained by the indicated antibody for 30 min on ice, and were finally washed with perm/wash buffer (BD Cytofix/Cytoperm™) twice. The flow cytometry data was examined by

FACSCalibur (BD bioscience). All events were analyzed by using FlowJo software (Tree Star).

3.13 Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad Software). Statistical significance between different groups was determined by Student t test. The *p* values <0.05 was considered significant.

Chapter 4: Results

4.1 The generation of PR8-OVA_{II}

To establish a system with trackable antigen-specific $CD4^+$ T cells during the acute influenza virus infection, we first engineered a particular influenza A virus strain PR8-OVA_{II} containing a specific T cell epitope $OVA_{323-339}$ recognized by OT-II $CD4^+$ T cells isolated from OT-II TCR-transgenic (-Tg) mice. A similar strategy has been used to generate WSN-OVA_{II} (Chapman et al., 2005). Briefly, the amino acid (a.a.) 43-58 of neuraminidase were replaced by $OVA_{323-339}$. This resulting NA-OVA_{II} of PR8 contains a slight longer NA stalk (Fig. 2A). We then utilized the reverse genetics system (Hoffmann et al., 2000) to generate PR8-OVA_{II} (Fig. 2B) and measured the replication capability of PR8-OVA_{II} *in vivo*, since changes of NA stalk might affect the efficiency of influenza virus replication (Castrucci and Kawaoka, 1993). Mice were challenged intranasally with different doses of PR8-OVA_{II} and exhibited different degrees of body weight loss. Compared to PR8, PR8-OVA_{II} in mice was less virulent (Fig. 3, 4). The minimum lethal dose (4×10^4 PFU) of PR8-OVA_{II} in mice was much higher than PR8 (40 PFU), approximately 1000-fold higher than PR8 (Fig. 3). In contrast to PR8, the same infectious level of PR8-OVA_{II} caused a slight body weight loss.

We chose PR8-OVA_{II} model for testing the role of antigen-specific Treg cells in viral infection, because OVA is a well characterized antigen model system. Fusing $OVA_{323-339}$ peptide to NA of PR8 allow us to conveniently track the antigen-specific Treg cells *in vivo*, particularly when MHC II tetramers are not easy to design and the frequency of antigen-specific $CD4^+$ Treg cells is very low (Nepom, 2012). A number of recent studies have highlighted the potential use of OVA system to study the immunity during influenza virus infection (Robertson et al., 2006). We thus took advantage of this antigen-specific T cell/influenza virus system that was based on adoptive transfer of OVA-specific $CD4^+$ T cells from TCR transgenic OT-II mice followed by infection of PR8-OVA_{II} to examine our hypothesis (Fig. 1).

4.2 Infection of PR8-OVA_{II} specifically induces the proliferation of OT-II $CD4^+$ T cells

To assesse whether the OVA epitope can be presented during PR8-OVA infection, we purified naïve $CD4^+CD25^-$ T cells from splenocytes of OT-II or wild-type (WT) C57BL/6 mice. After staining with CFSE, a total of 1 x 10^6 cells were adoptively transferred into the recipient WT C57BL/6 mice. One day later, the recipient mice were infected intranasally with 10 PFU PR8 or 1000 PFU PR8-OVA_{II} (Fig. 5A), and the

body weight of the mice were monitored on a daily basis (Fig. 5B). Seven days postinfection, the mice were sacrificed, and cells from spleen, dLN, lung and BAL were assessed for the proliferation of CFSE-labeled donor cells. Only the group of mice that were adoptively transferred with naïve OT-II $CD4^+$ T cells and then infected with PR8-OVA_{II} induced a strong proliferation of CFSE-labeled donor cells (Fig. 5C). Quantification of dividing cells of total $CFSE^+CD4^+$ T cells revealed a higher proliferation rate in the group of mice adoptively transferred with OT-II naïve $CD4^+$ T cells and infected with PR8-OVA_{II} (Fig. 5D). Therefore, the OVA epitope of PR8-OVA_{II} could be presented *in vivo* and specifically induced the proliferation of OVA-specific $CD4^+$ T cells.

4.3 Low-dose influenza virus infection promotes the induction of viral antigen-specific regulatory T cells

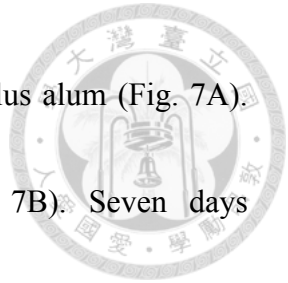
After verifying that the model of PR8-OVA_{II} worked, we further investigated the dose effects of viral antigens on the induction of viral antigen-specific Treg cells. Since 4 x 10^4 PFU is the minimal lethal dose of PR8-OVA_{II} in C57BL/6 mice, we chose one quarter of it as a high dose infection. Based on the loss of body weight of mice infected with different doses of PR8-OVA_{II} (Fig. 4B), we found no obvious body weight loss

when mice were infected with 50 PFU PR8-OVA_{II} which we chose as a low-dose for infection. We also chose 1 x 10^3 PFU for moderate dose infection. WT mice were adoptively transferred with 1.5 x 10^6 CFSE-labeled OT-II $CD4^+CD25^-$ T cells and one day later were challenged with low, moderate and high doses of PR8-OVA_{II} (Fig. 6A). The body weight of these mice were monitored daily. Seven days postinfection, cells from spleen, dLN, lung and BAL were harvested and then stained with anti-CD4 and anti-Foxp3 antibody. $CD4^+CFSE^+$ T cells in the recipient mice infected with 1 x 10^4 PFU PR8-OVA_{II} showed strong proliferation in spleen, dLN and the lung sample pooled with BAL. Based on the dilution of CFSE, almost all the $CFSE^+CD4^+$ T cells were dividing, and few $CFSE^+$ $Foxp3^+$ (antigen-specific Treg) cells were observed in all samples. The group of mice infected with moderate dose (1 x 10^3 PFU) of PR8-OVA_{II} showed a middle level of $CFSE^+CD4^+$ T cell proliferation, and about 8.4% $CFSE^+$ $Foxp3^+$ among the donor cells, indicating induction of viral antigen-specific Treg cells during a moderate dose of influenza A virus infection. $CFSE^+CD4^+$ T cells of the mice infected with low dose of PR8-OVA_{II} exhibited slight proliferation. The conversion of $CFSE^+CD4^+Foxp3^-$ to $CFSE^+CD4^+Foxp3^+$ viral antigen-specific Treg cells was about 12.4%. These data were consistent with the previous report that low-dose antigen

promotes induction of Foxp3^{+} Treg cells (Kretschmer et al., 2005; Long et al., 2011). Taken together, our finding shows a quantitative correlation of infectious dose and the induction of viral antigen-specific Treg. Low-dose influenza A virus infection promotes the induction of antigen-specific Treg.

4.4 Immunization without adjuvant promoted the introduction of antigen-specific Treg cells

After demonstrating that low-dose influenza infection promoted the induction of antigen-specific Treg cells, we then investigated whether immunization, the way to mimic human immunize influenza vaccination, can also induce the antigen-specific Treg cells. As some of current influenza vaccines do not contain any adjuvants, we further wanted to compare the effect of adjuvant to induce the antigen-specific Treg cells. Since human vaccination mainly works through an intramuscular route, we also compare the effects of intramuscular and intraperitoneal injection on the induction of Treg cells. We adoptively transferred 1.5 x 10^{6} CFSE-labeled OT-II CD4^{+}CD25^{-} T cells into WT recipient mice. One day later, the recipient mice were intraperitoneally immunized with 10 μg OVA and 10 μg OVA with alum. Mice injected with PBS served as a negative control. To compare the effects of different routes of immunization, we



also performed intramuscular injection of mice with 10 μg OVA plus alum (Fig. 7A). The body weight of these mice were monitored daily (Fig. 7B). Seven days postimmunization, cells from spleen, dLN , lung and BAL were harvested. After staining with anti-CD4 and anti-Foxp3 antibody, cells were then analyzed by flow cytometry. All the groups of donor cells, except the negative control, showed a strong dilution of CFSE. About 2 to 4% of donor cells converted to antigen-specific Treg cells in the groups which were immunized by 10 μg OVA with alum. Interestingly, the mice immunized with 10 μg OVA only showed the highest induction of antigen-specific Treg cells, about 24% of donor cells (Fig. 7C). As the data showed here, low-dose infection and immunization without adjuvant promoted the induction of antigen-specific Treg cells.

4.5 *In vitro* generated iTreg cells are functional to suppress the proliferation of $CD4^+$ T cells

Knowing that low-dose acute influenza A virus infection promoted the induction of viral antigen-specific Treg, we further examined the physiological function of viral antigen-specific Treg cells. Since Treg cells constitute only 5-10% of peripheral $CD4^+$ T cells, and no markers can distinguish iTreg and nTreg cells, it is not convenient to

isolate iTreg cells *in vivo*. To overcome this hurdle, we used a well-established method to generate inducible regulatory T cells (iTregs) *in vitro*, so we could acquire enough iTreg cells for adoptive transfer. We first tested whether *in vitro* generated iTreg had suppressive function. Briefly, the iTreg cells were converted from $CD4^+CD25^-$ naive T cells after being stimulated with anti-CD3, anti-CD28, TGF-β and human IL-2. By this protocol, about 30-40% cells became $Foxp3^+$ (Fig. 8) after 5 days culture. We sorted the $CD3^+CD4^+GFP^+$ iTreg by FACSAria flow cytometer and then cocultured with CFSE labeled $CD4^+CD25^-$ T cells in a culture medium containing anti-CD3 and anti-CD28 antibody for 3 days. *In vitro* generated iTreg cells were able to suppress anti-CD3 induced proliferation of $CD4^+CD25^-$ T cells (Fig. 9).

4.6 *In vitro* generated iTreg cells showed no suppressive function *in vivo*

To investigate the effects of the viral antigen-specific Treg cells during influenza A virus infection *in vivo*, we adoptively transferred 5 x 10^5 *in vitro* generated OVA-specific iTreg cells into WT mice. Mice transferred with non-specific iTreg cells or RPMI 1640 medium only served as negative controls. One day later, the recipient mice were then challenged with 10 PFU PR8 or 1000 PFU PR8-OVA_{II} (Fig. 10A). The body weight of mice were monitored on a daily basis, but no significant difference

among groups was found (Fig. 10B). Seven days after postinfection, we harvested the cells from spleen, dLN, lung and BAL and then stained $CD3^+CD8^+$ cells with MHC I pentamer or tetramer specific for two immunodominant influenza A virus epitopes, nucleoprotein ($NP_{366-374}/D^b$) and acid polymerase ($PA_{224-233}/D^b$) to measure the influenza virus-specific CD8 T cell immunity. The percentage of $CD3^+CD8^+tetramer^+/pentamer^+$ T cells shows no different in the groups which were adoptively transferred with OVA-specific iTreg cells and followed by PR8-OVA_{II} infection. Similar results were observed by using influenza virus-infected BMDCs to stimulate the IFN-γ production of influenza-specific $CD8^+$ T cells, and the percentage of $CD3^+CD8^+IFN\text{-}\gamma^+$ cells showed no different among groups. Together, these data demonstrate that *in vitro* generated iTreg cells have no suppressive function *in vivo*.

Chapter 5: Discussions

5.1 Regulatory T cells in acute viral infecion

Treg cells are known to play a suppressive role in regulating the immune response through secreting inhibitory cytokines, such as IL-10 or TGF-β, to suppress the responses of effector T cells (Belkaid et al., 2002; Chen et al., 1994). They can also reduce the immunostimulatory activity of dendritic cells through expressing the inhibitory receptors, including CTLA4, or by downregulating the expression of co-stimulatory molecules CD80 and CD86 on their surface, and finally inhibit the priming of effector T cells (Wing et al., 2008). Many prior studies have demonstrated that Treg cells play an important role in chronic infection, autoimmune disease and tumor, but the role of Treg cells in acute viral infection are relatively unclear until recently (Betts et al., 2012; Dejnirattisai et al., 2008; Fulton et al., 2010; Kim et al., 2008; Lee et al., 2010; Suvas et al., 2003; Trandem et al., 2010). During acute viral infection, the immune system usually responds quickly to control the invasion of pathogens, so it is generally assumed that Treg cells play a minor role in modulating the immune response induced by acute viral infection. Recent studies have showed that

Treg cells accumulated in lung and BAL during RSV (Fulton et al., 2010) and influenza virus (Betts et al., 2012) infection. However, these previous studies only examined the frequency of total Treg cells. Recently, using MHC II tetramer staining, Brincks et al. have clearly demonstrated that viral antigen-specific Treg cells could be induced during acute influenza virus infection (Brincks et al., 2013).

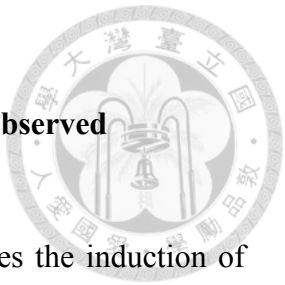
5.2 Model of PR8-OVA$_{II}$

It has been challenging to study the influenza virus antigen-specific Treg cells *in vivo*. To demonstrate the existence of viral antigen-specific Treg cells, Betts et al. isolated Treg cells from the whole lung of mice infected with influenza A virus, and then cocultured them with BMDCs infected with influenza A virus. They found that about 37% of Treg cells in lung responded and proliferated, indicating that these Treg cells are viral antigen-specific (Betts et al., 2012). However, we followed the same method, but failed to show the existence of influenza viral antigen-specific Treg cells, primarily because the number of Treg cells in the whole lung was very rare. Besides, Sanchez et al. used the recombinant vaccinia virus encoding HA (VV-HA) to infect the mice by adoptive transfer of Treg cells isolated from 6.5 HA-TCR Foxp3-GFP mice and

found that about 50-fold expansion of the transferred Treg cells compared with mock-infected control mice (Sanchez et al., 2012), demonstrating the antigen specificity of Treg cells in response to viral infection. However, this system utilized recombinant vaccinia virus, and is not an authentic influenza virus infection model. Brincks EL et al. published a study using MHC class II tetramers to track viral antigen-specific Treg cells, and showed the memory response of influenza virus-specific Treg cells during acute influenza virus infection (Brincks et al., 2013). Since we do not have the influenza virus-specific MHC class II tetramers, we generated an PR8 NA-OVA_{II} influenza system to study and track viral antigen-specific Treg cells *in vivo* during acute viral infection. Combining PR8-OVA_{II} and adoptive transfer of the monoclonal OVA-specific TCR transgenic $CD4^+$ T cells overcome the challenge that the number of antigen-specific Treg cells is too rare to be clearly monitored and measured.

In this study, we found that viral antigen-specific Treg cells can be induced during acute influenza virus infection, consistent with the previous studies (Betts et al., 2012; Brincks et al., 2013). Interestingly, we discovered the correlation between the infectious dose of influenza A virus and viral antigen-specific Treg cells following acute influenza A virus infection, suggesting that low-dose infection promotes more production of viral

antigen-specific Treg cells (fig. 6C). Consistent with our findings, previous studies have demonstrated that low-dose foreign antigen or subimmunogenic stimulation promotes antigen-specific Treg cells in a non-infection condition. The difference between previous studies and ours is that we used an infectious model, not just foreign antigen stimulation. The antigen dose of the infection model amplifies along with the viral replication, but that of the foreign antigen stimulation model does not. Besides, viral infection can activate several pattern recognition receptors (PRRs) and cause inflammatory response, so it is often believed that Treg cells are not induced during acute viral infection. Here, our results clearly proved that low-dose virus infection promotes the induction of viral antigen-specific Treg cells. This indicates that the inverse dose effects of antigens on induction of antigen-specific Treg cells can be observed in both infectious virus and non-proliferating antigens. It is likely that a general mechanism regulates the induction of antigen-specific Treg cells for both of them in dose-dependent manner, given that the dose of antigens are below certain levels, probably below the level in which strong immune responses are induced.



5.3 No suppressive function of *in vitro* generated iTreg cells was observed

Here, our finding proved that low-dose virus infection promotes the induction of antigen-specific Treg. Although we failed to demonstrate that the *in vitro* generated iTreg cells suppressed antiviral T cell immunity *in vivo*, the CFSE-based *in vitro* suppression assay indicated that these *in vitro* generated iTreg cells were functional. This discrepancy maybe due to the number of adoptively transferred iTreg cells was not enough to suppress the antiviral CD8 T cell immunity after influenza virus infection. Alternatively, the function of the *in vitro* generated iTreg cells is probably not stable (Polansky et al., 2008; Xu et al., 2007). The prior study showed that the *in vitro* generated iTreg cells conversed to Foxp3$^-$ Tconv after adoptive transfer into the recipient mice (Xu et al., 2007; Zhou et al., 2009a; Zhou et al., 2009b) so these transferred iTreg cells may eventually lose their suppressive function. Another possibility is that the preexisting endogenous Treg cells in attenuated the effects of transferred antigen-specific Treg cells on the antiviral T cell immunity.

Previously, we found repeated low-dose influenza A virus infection reduced the influenza virus-specific CD8 T cell immunity although we did not know the underlying

mechanisms regulating the antiviral immune response then. Here, we proved the induction of viral antigen-specific Treg cells by low-dose influenza A virus infection, providing a plausible explanation for the infection dose effects on antiviral T cell immunity. In addition, whether repeated low-dose influenza virus infection expands the viral antigen-specific Treg cells and thus reduces the influenza virus-specific CD8 T cell immunity requires further investigation. However, to study this issue needs a long period of time to track the donor cells *in vivo*, so we have to use some cell lineage marker, such as Thy1.1 or CD45.1, for the tracking purpose in the future.

5.4 Conclusion

During the seasons of influenza virus endemics, repeated exposure to low-dose influenza virus is quite possible. Therefore, the influenza virus-specific iTreg cells may accumulate in the lung. Besides, administration of influenza vaccine annually is also likely to induce or boost the production of influenza virus-specific Treg cells. The effects of these two factors on shaping the immune system against influenza virus infection remains largely unknown and are the central interest of this study. We have established a PR8-NA-OVA_{II} influenza virus/OT-II T cells system to investigate this

question. This unique system is designed to mimic a real infectious environment where the host frequently exposes to low-dose influenza virus and receives vaccination during the seasonal influenza epidemics. The robustness of this system should allow us to explore the complex interaction between the influenza A virus and viral antigen-specific Treg cells, and their effects on establishment of antiviral T cell immunity.

In conclusion, we have demonstrated that antigen-specific Treg cells can be induced after a single dose of influenza infection. We further prove that low-dose influenza virus infection promotes the induction of viral antigen-specific iTreg cells. Also, immunization without adjuvant induced the antigen-specific iTreg cells. The kinetics and balance of antigen-specific Treg cells and effector T cells are important issues for establishment of effective antiviral immune responses. Understanding how antigen-specific Treg cells and effector T cells are generated and balanced should help us develop better vaccination strategy against virus infection and also learn how to manipulate the immune response against acute viral infection.

Chapter 6: Figures

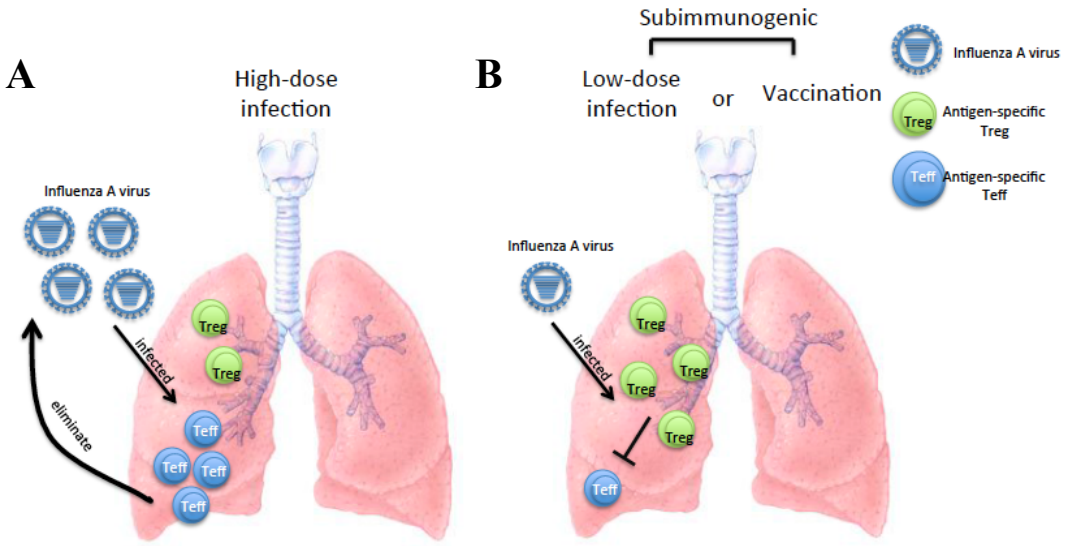


Figure 1. The hypothetical model for induction of influenza-specific Treg cells and Teff cells by different doses of influenza virus infection.

(A) High-dose of influenza A virus infection elicits severe inflammatory responses. In such a situation, both Treg cells and Teff cells replicate and expand, but replication of Teff cells surpasses that of Treg cells, resulting in the dominance of Teff cells and eventual elimination of the invading virus. (B) Under the low-dose viral infection or a subimmunogenic environment, viral antigen-specific Treg cells respond and proliferate more rapidly than Teff cells, leading to the dominance of viral antigen-specific Treg cells.

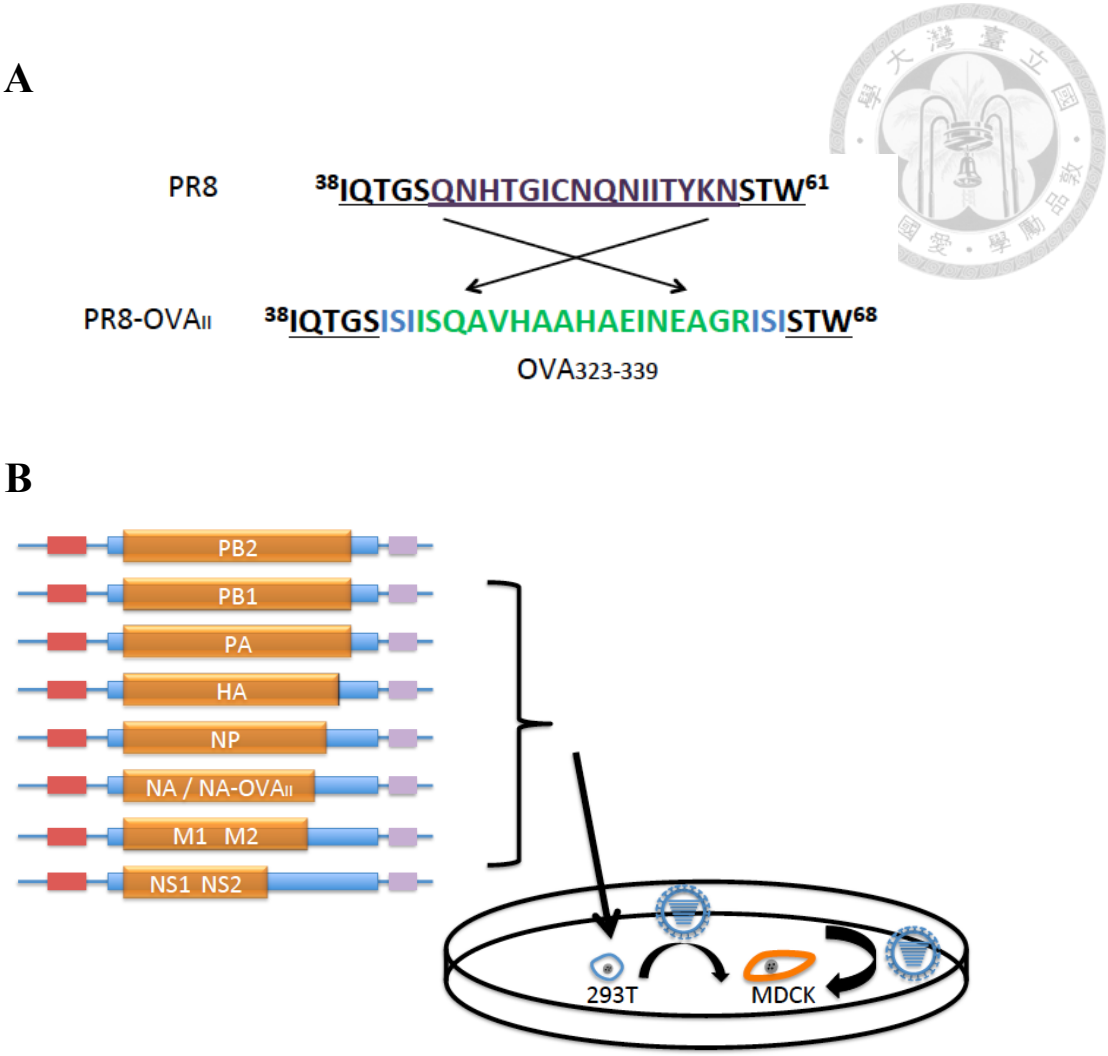


Figure 2. The generation of PR8 and PR8-OVA$_{II}$ by using reverse genetics.

(A) Map of the gene of PR8 influenza virus neuramindase (NA) of PR8 influenza A virus and the modified PR8-OVA$_{II}$. The amino acids sequence of NA$_{43\text{-}58}$ were replacing by OVA$_{323\text{-}339}$ sequence. (B) An 8-plasmid reverse genetics system was used to produce PR8 and PR8-OVA$_{II}$ influenza A viruses. The procedures were described in the section of Materials and Methods.

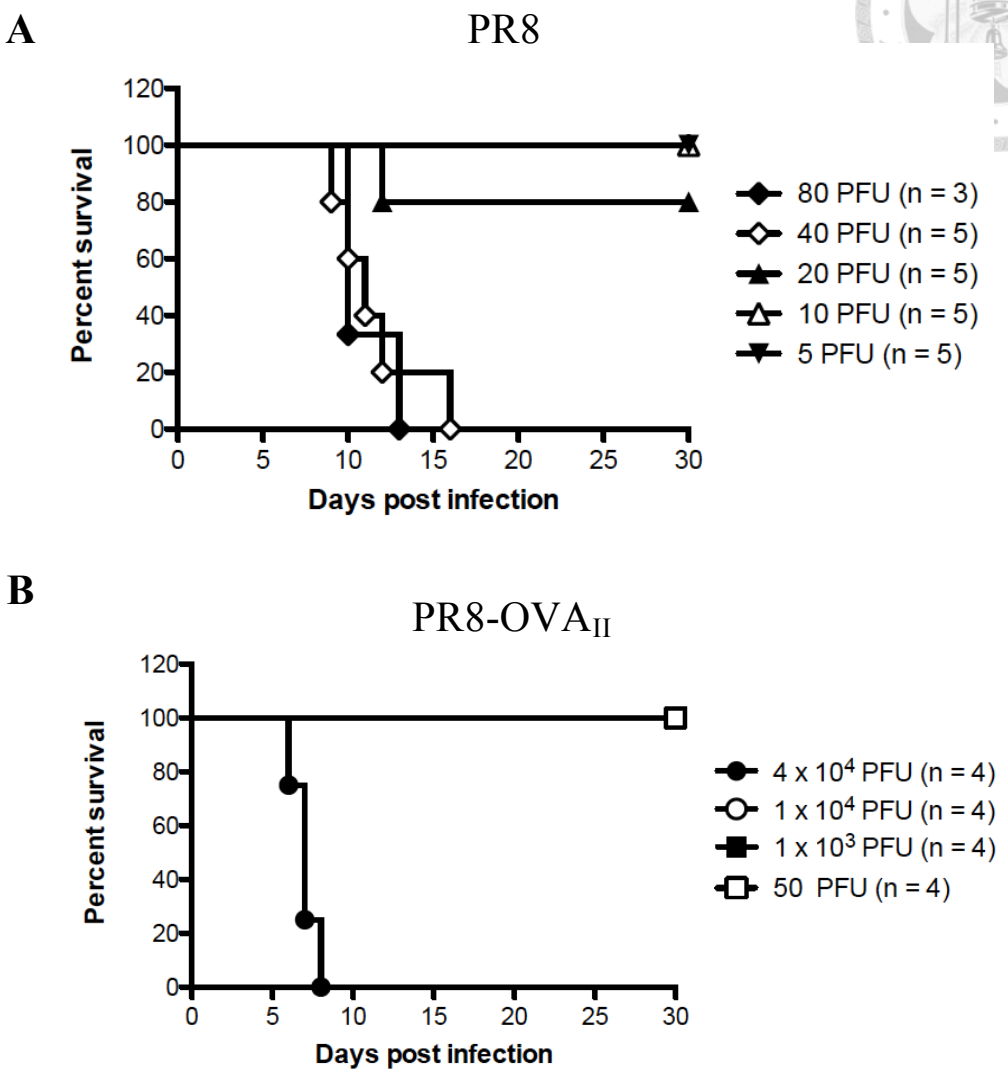


Figure 3. The survival curve of mice infected with PR8-OVA$_{II}$ and PR8.

(A) The survival curve of mice infected with PR8-OVA: Six to eight-week-old mice were infected with 80 PFU, 40 PFU, 20 PFU, 10 PFU or 5 PFU and then monitored daily postinfection. The number within the parenthesis indicated the number of mice in each group. (B) The survival curve of mice infected with PR8-OVA$_{II}$: The survival of mice was monitored post-PR8-OVA$_{II}$ on a daily basis.

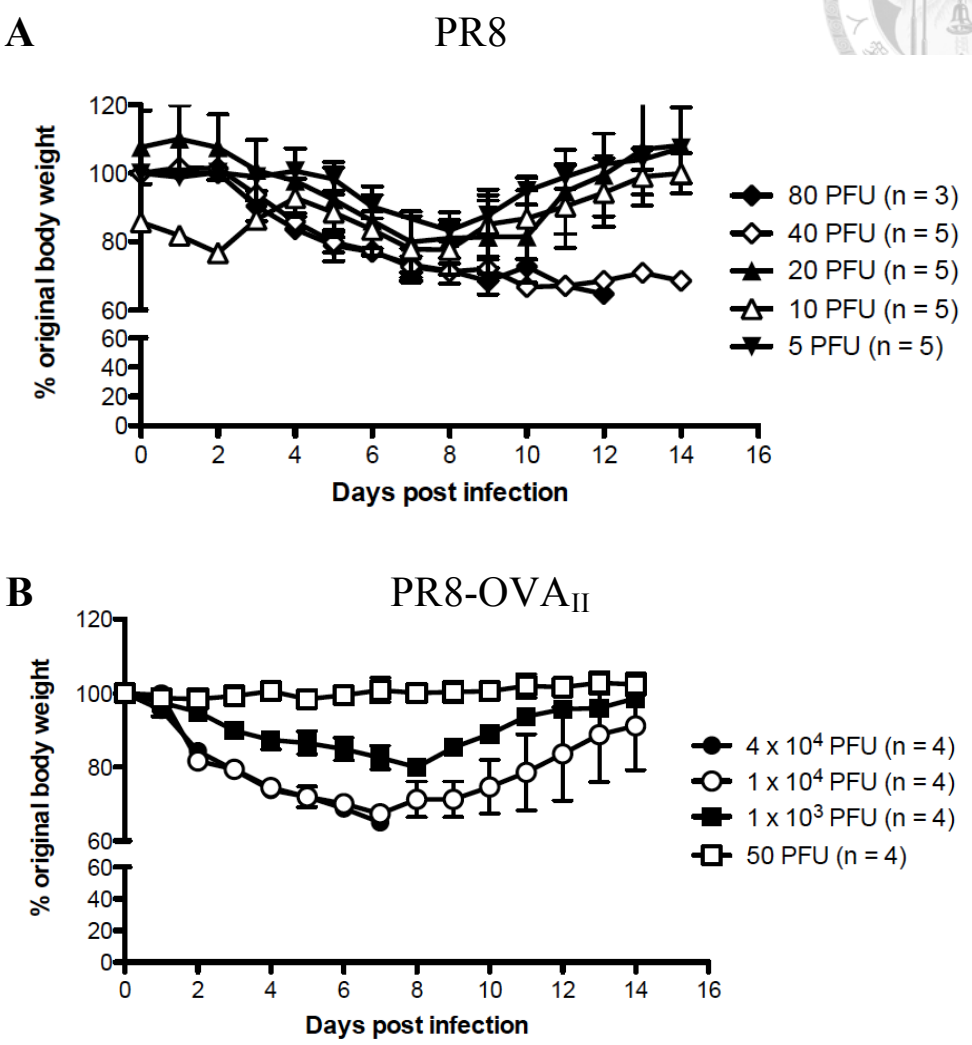
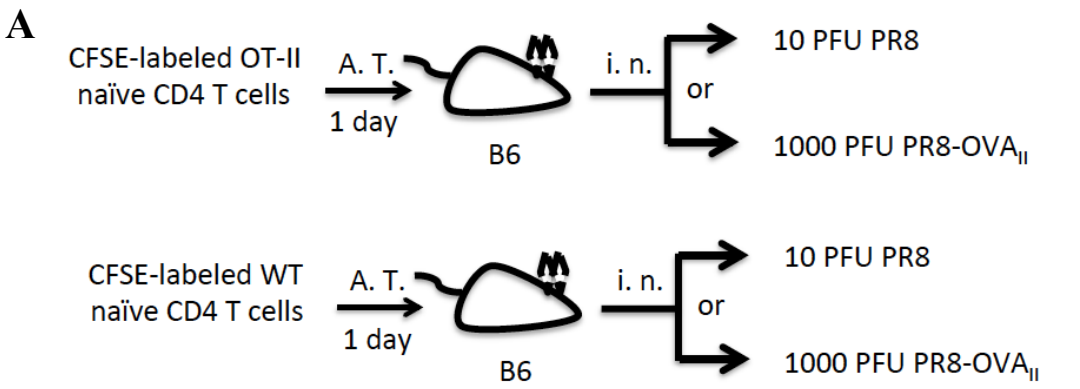
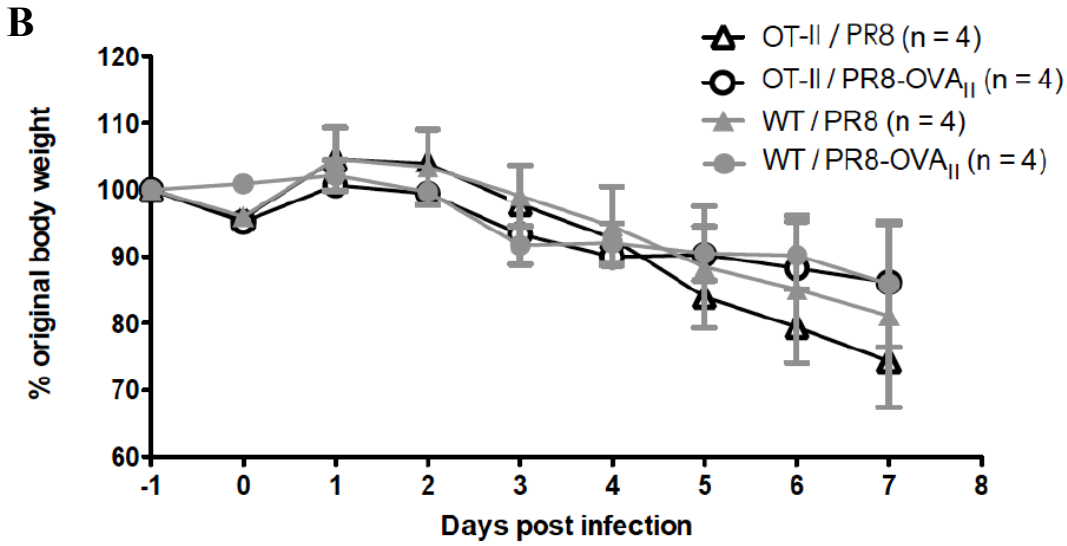
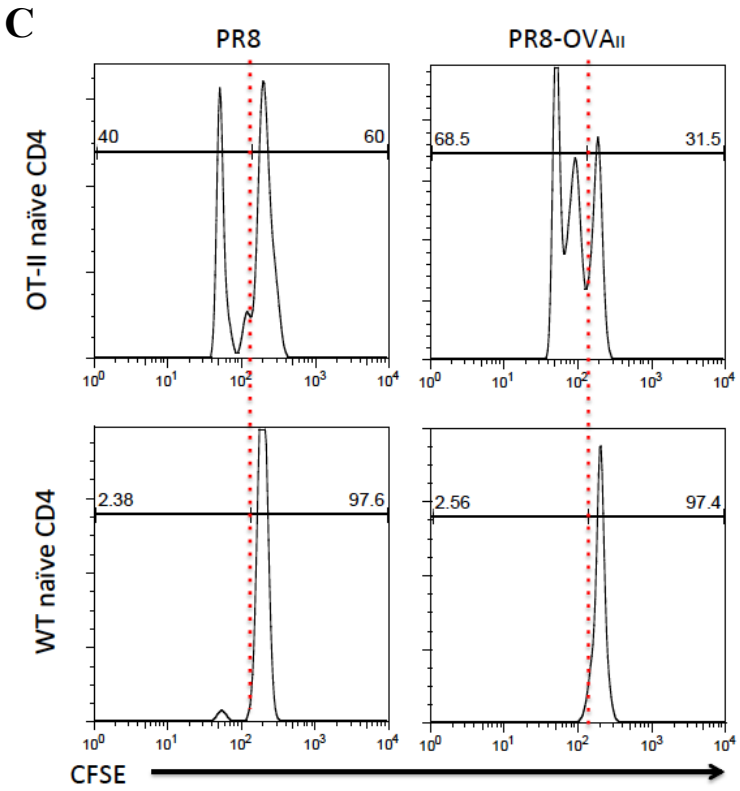


Figure 4. Body weight loss of mice infected with PR8 and PR8-OVA$_{II}$.

(A) Body weight was monitored daily after PR8 infection. Six to eight-week-old mice were infected with 80 PFU, 40 PFU, 20 PFU, 10 PFU, 5 PFU PR8. (B) Body weight was monitored daily after different doses of PR8-OVA$_{II}$ infection. The number within the parenthesis indicated the number of mice in each group.


A
CFSE-labeled OT-II naïve CD4 T cells
A. T.
1 day
B6
i. n.
or
10 PFU PR8
1000 PFU PR8-OVAII
CFSE-labeled WT naïve CD4 T cells
A. T.
1 day
B6
i. n.
or
10 PFU PR8
1000 PFU PR8-OVAII


B
OT-II / PR8 (n = 4)
OT-II / PR8-OVAII (n = 4)
WT / PR8 (n = 4)
WT / PR8-OVAII (n = 4)
% original body weight
120
110
100
90
80
70
60
-1
0
1
2
3
4
5
6
7
8
Days post infection


C
PR8
PR8-OVAII
OT-II naïve CD4
WT naïve CD4
40
60
68.5
31.5
2.38
97.6
2.56
97.4
CFSE

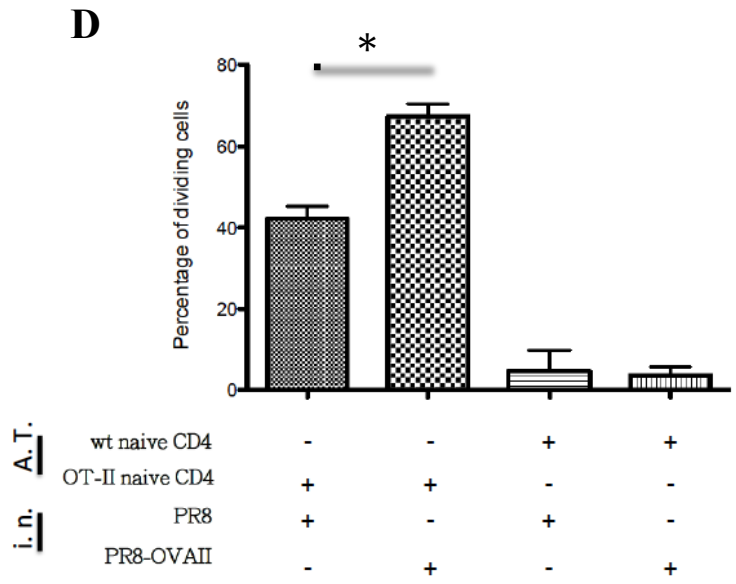
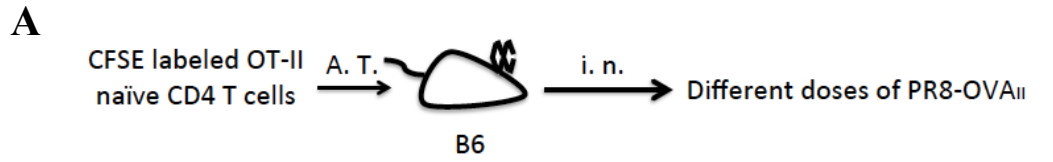
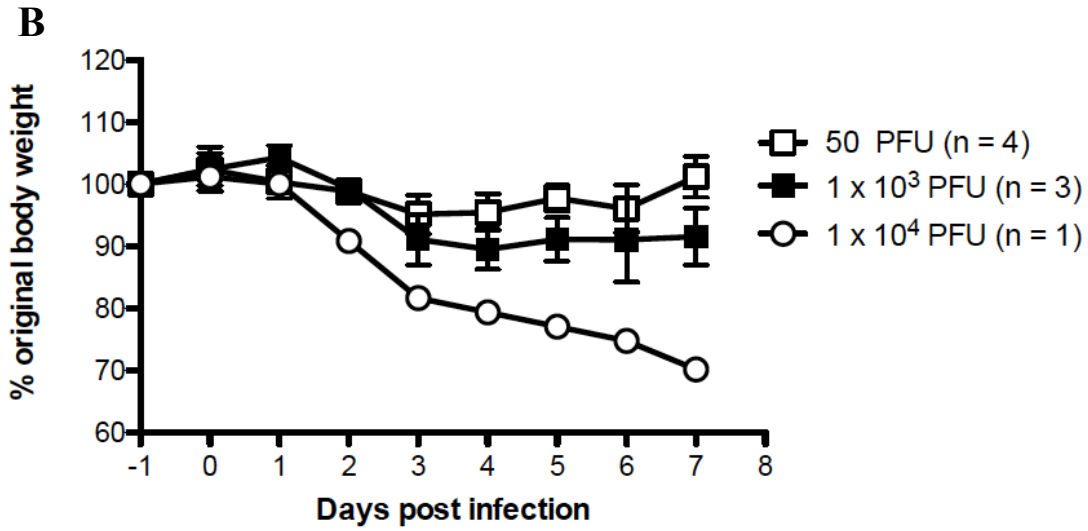
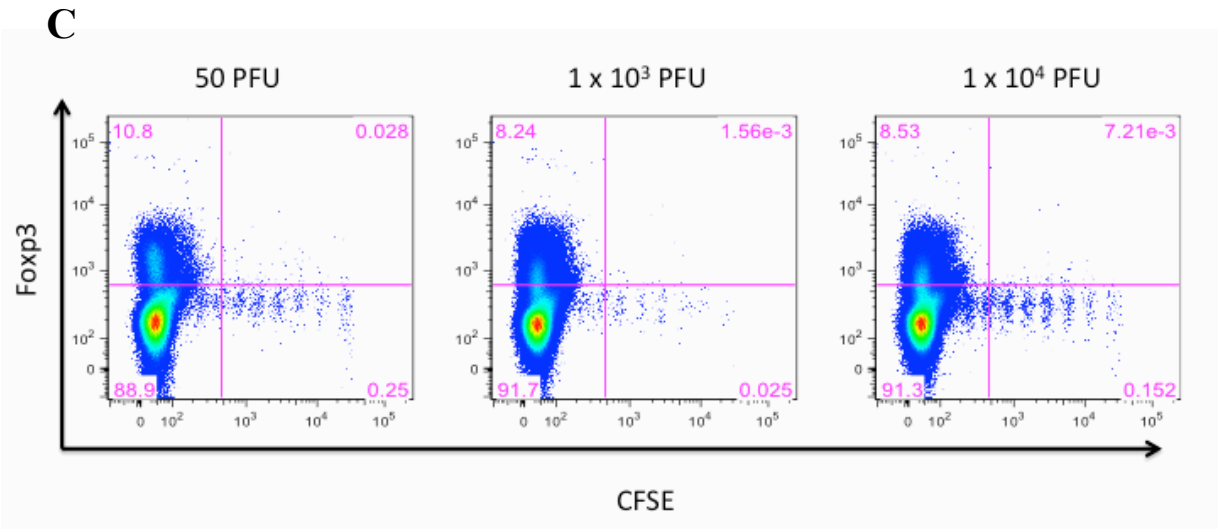


Figure 5. PR8-OVA$_{II}$ infection specially induced OT-II CD4$^+$ T cells proliferation.

(A) The experimental design for determining the antigen specificity of PR8-OVA$_{II}$ in stimulation of OT-II CD4$^+$ T cells. A total of 1 x 10^6 OT-II or WT CD4$^+$CD25$^-$ T cells were adoptively transferred (A.T.) into WT B6 mice. One day later, the recipient mice were infected intranasally (i.n.) with 10 PFU PR8 or 1000 PFU PR8-OVA$_{II}$ per mouse. Mice were sacrificed at 7 days postinfection. There are four mice per group. (B) The body weight of mice was monitored daily after adoptive transfer. (C) Representative histogram of lymphocyte in lymph node gated on CD4$^+$CFSE$^+$ cells at the 7 days postinfection after adoptive transfer of CFSE-labeled OT-II (top) and WT (bottom) CD4$^+$CD25$^-$ T cells, respectively. (D) The percentage of CD4$^+$CFSE^{low} T cell subset determined by flow cytometry.


A
CFSE labeled OT-II
naïve CD4 T cells
A. T.
B6
i. n.
Different doses of PR8-OVAII


B
% original body weight
120
110
100
90
80
70
60
-1
0
1
2
3
4
5
6
7
8
Days post infection
50 PFU (n = 4)
1 x 10^3 PFU (n = 3)
1 x 10^4 PFU (n = 1)


C
50 PFU
1 x 10^3 PFU
1 x 10^4 PFU
Foxp3
CFSE
10.8
0.028
88.9
0.25
8.24
1.56e-3
91.7
0.025
8.53
7.21e-3
91.3
0.152

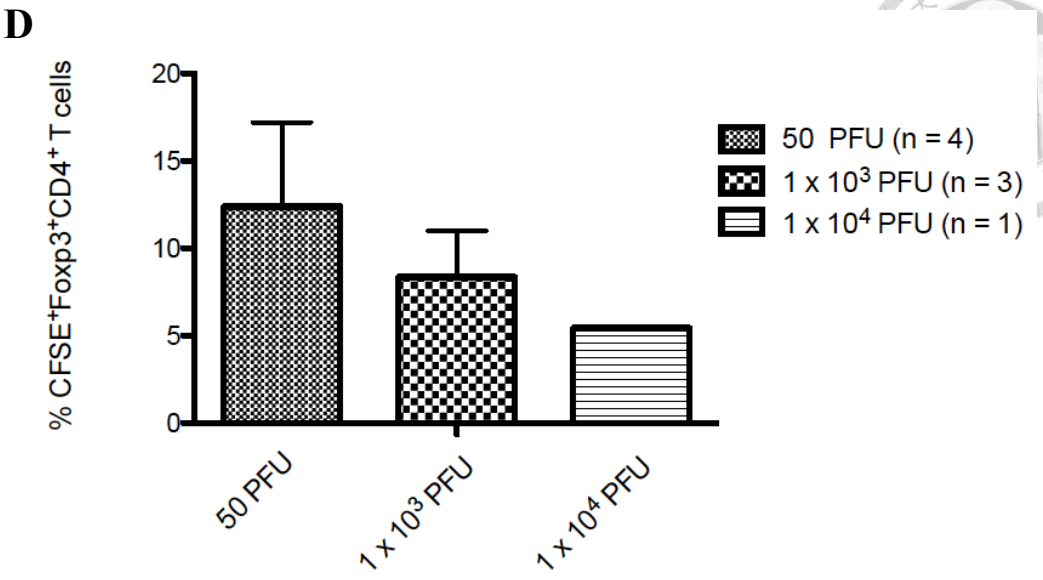
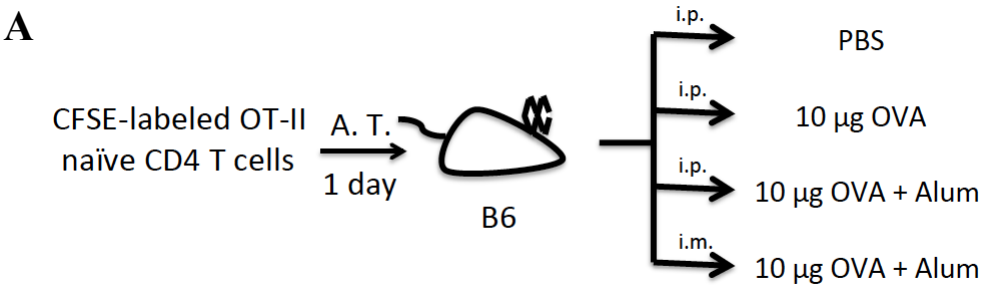
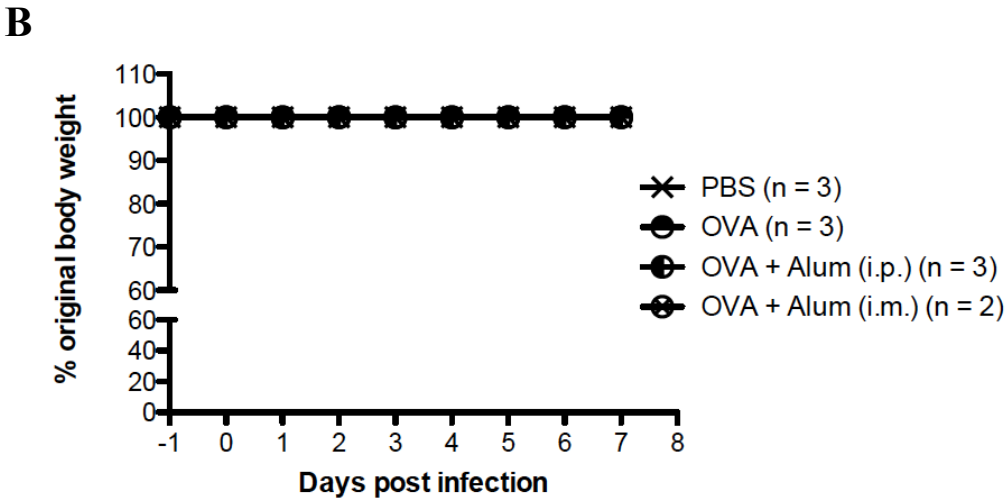
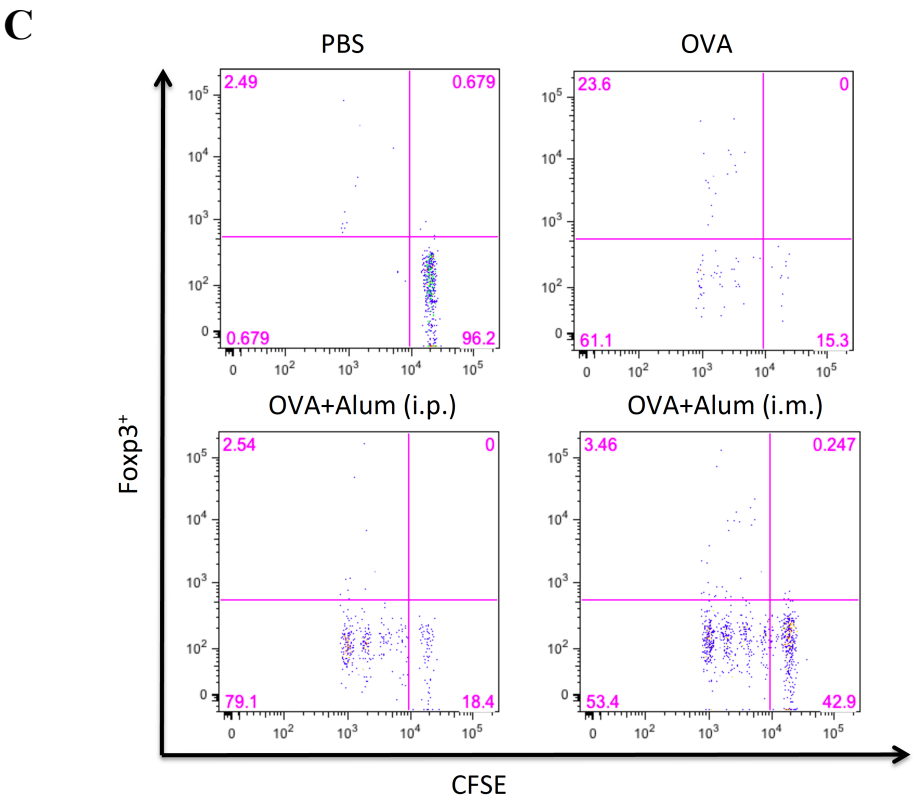


Figure 6. The induction of antigen-specific Treg cells by different doses of PR8-OVA$_{II}$ infection.

(A) A total of 1 x 10^6 OT-II $CD4^+CD25^-$ T cells were adoptively transferred into WT B6 mice. One day later, the recipient mice were infected intranasally with 50 PFU, 1 x 10^3 PFU and 1 x 10^4 PFU PR8-OVA$_{II}$ per mouse. Mice were sacrificed at 7 days postinfection. Each group had four mice per group. (B) The body weight of mice was monitored daily after adoptive transfer until they had to be sacrificed. (C) Representative dot plot of lymphocytes in lymph node gated on $CD4^+$ cells at the 7 days postinfection after adoptively transferred of CFSE-labeled OT-II $CD4^+CD25^-$ T cells. (D) The quantification of the percentage of $Foxp3^+$ cells among donor cells in (C).


A
CFSE-labeled OT-II
naïve CD4 T cells
A. T.
1 day
B6
i.p.
PBS
i.p.
10 μg OVA
i.p.
10 μg OVA + Alum
i.m.
10 μg OVA + Alum


B
% original body weight
110
100
90
80
70
60
60
40
20
0
-1
0
1
2
3
4
5
6
7
8
Days post infection
PBS (n = 3)
OVA (n = 3)
OVA + Alum (i.p.) (n = 3)
OVA + Alum (i.m.) (n = 2)


C
PBS
OVA
OVA+Alum (i.p.)
OVA+Alum (i.m.)
2.49
0.679
0.679
96.2
23.6
0
61.1
15.3
2.54
0
79.1
18.4
3.46
0.247
53.4
42.9
Foxp3+
CFSE

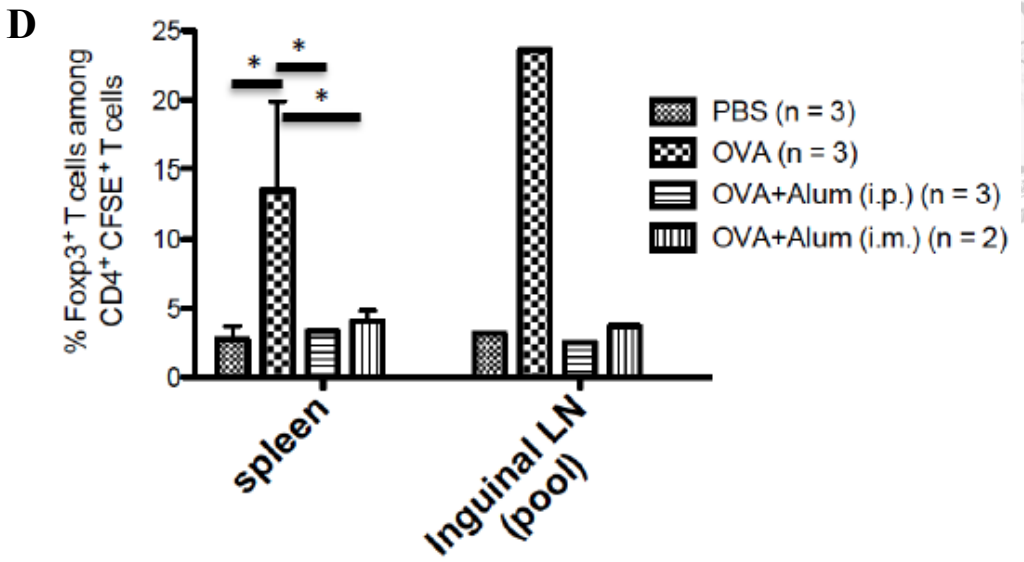


Figure 7. The induction of antigen-specific Treg cells by immunization of cognate antigen.

(A) A total of 1.5 x 10^6 OT-II $CD4^+CD25^-$ CFSE-labeled T cells were adoptively transferred into WT recipient mice. One day after adoptive transfer, the recipient mice were intraperitoneally immunized with PBS (as a negative control), or 10 μg OVA. The group immunized by 10 μg OVA with alum was administered by intraperitoneally or intranmuscular injection. Mice were sacrificed at seven days after immunization. (B) The body weight of mice was monitored daily until the day to sacrificed. (C) Representative dot plots of lymphocytes in lymph node gated on $CD4^+$ cells at the seven days after immunization. (D) The bar graphs represented the quantification of the percentage of $Foxp3^+$ T cells among donor cells in (C).

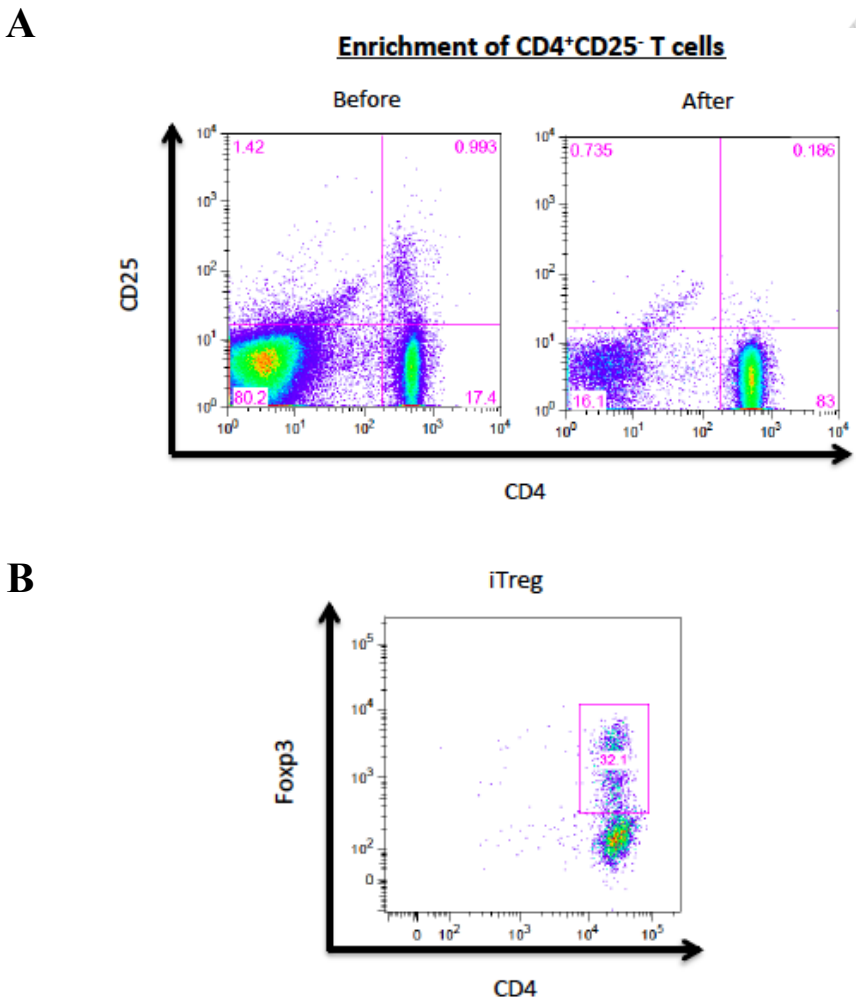


Figure 8. *In vi*tro generation of inducible Treg cells.

(A) Enrichment of $CD4^{+}CD25^{-}$ T cells was done using CD4 enrichment kit with CD25-biotin antibody (BD bioscience). The left panel shows the dot plot of $CD4^{+}CD25^{+}$ cells in OT-II splenocytes before enrichment. The right panel shows the dot plot of $CD4^{+}CD25^{+}$ cells in OT-II splenocytes after $CD4^{+}CD25^{-}$ enrichment. (B) iTreg cells were generated *in vitro* using the protocol described at the section of Materials and Methods. The dot plot was gated on $V\beta5.1^{+}CD4^{+}Foxp3^{+}$ T cells after five days of *in vitro* culture.

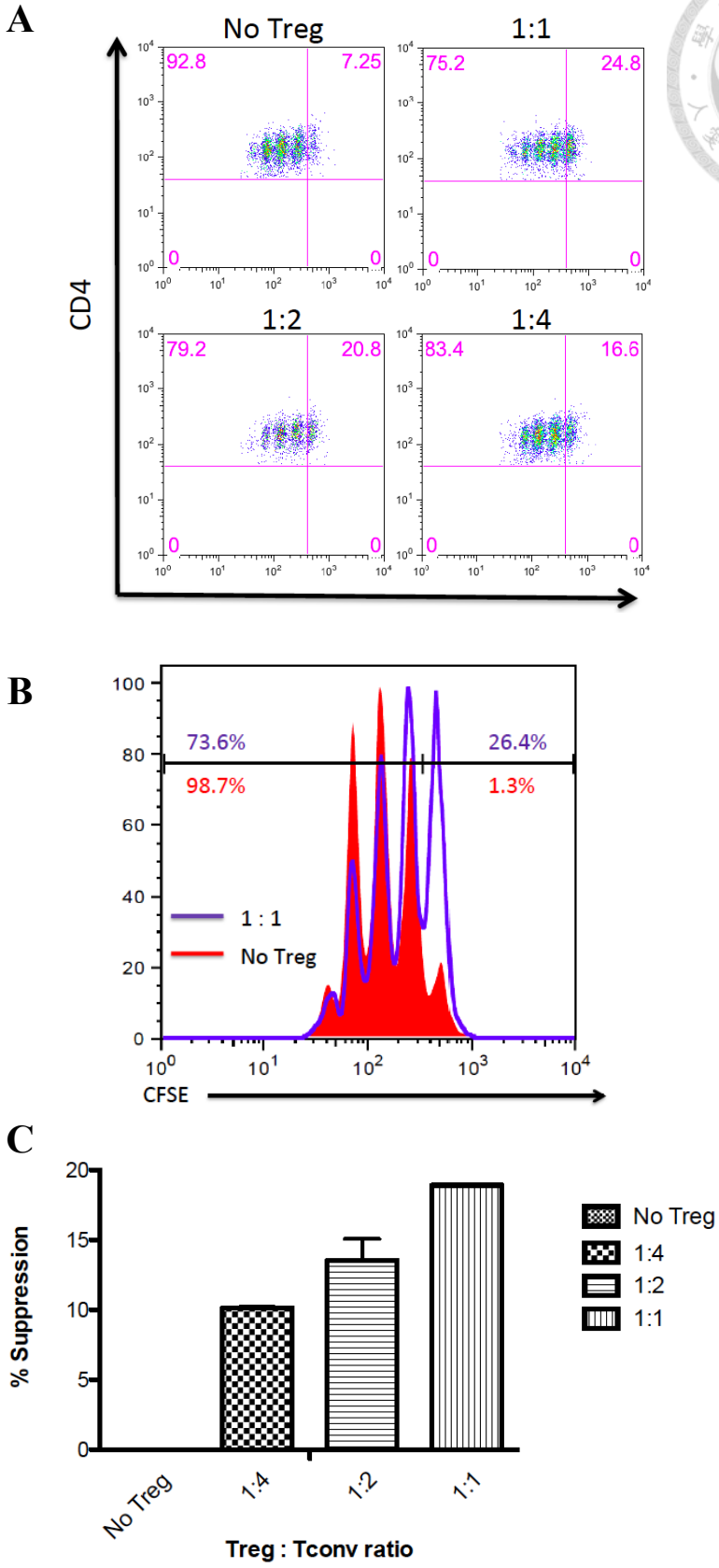
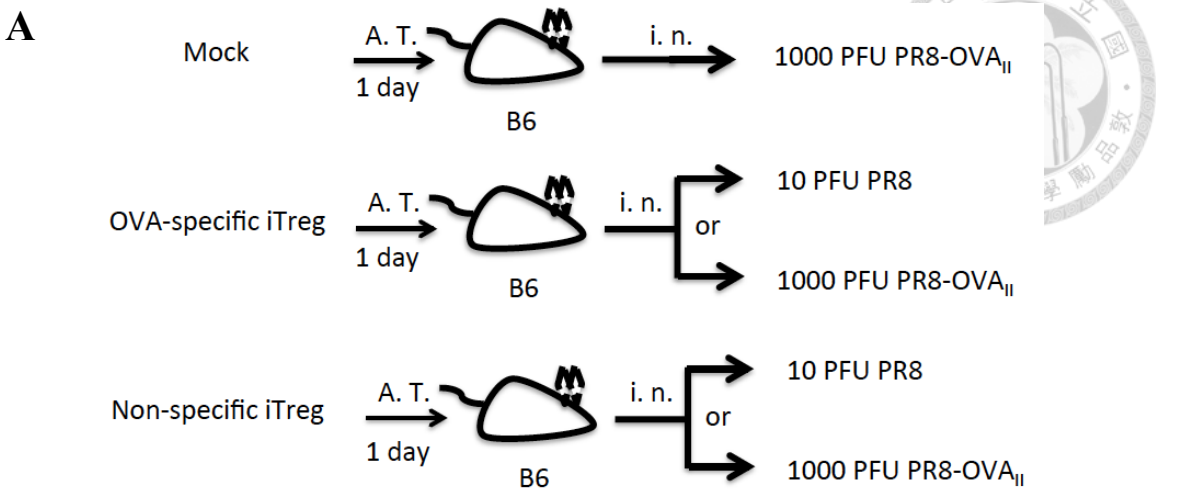
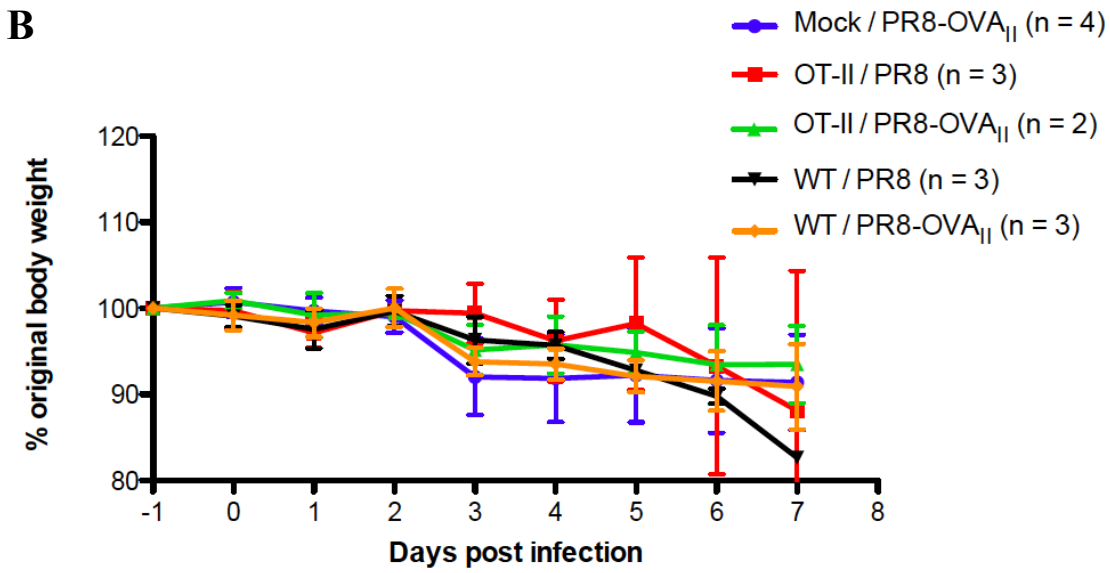

A
No Treg
1:1
1:2
1:4
92.8
7.25
75.2
24.8
79.2
20.8
83.4
16.6
CD4
B
73.6%
26.4%
98.7%
1.3%
1 : 1
No Treg
CFSE
C
% Suppression
No Treg
1:4
1:2
1:1
Treg : Tconv ratio

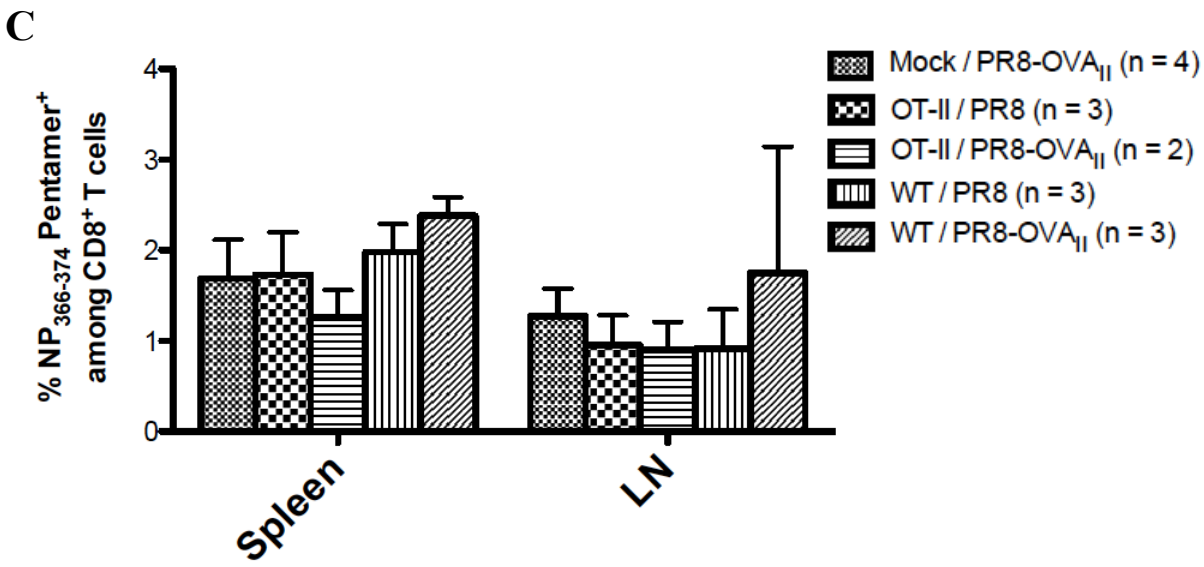
Figure 9. *In vitro* generated iTreg cells are functional to suppress the proliferation of $CD4^+$ T cells.

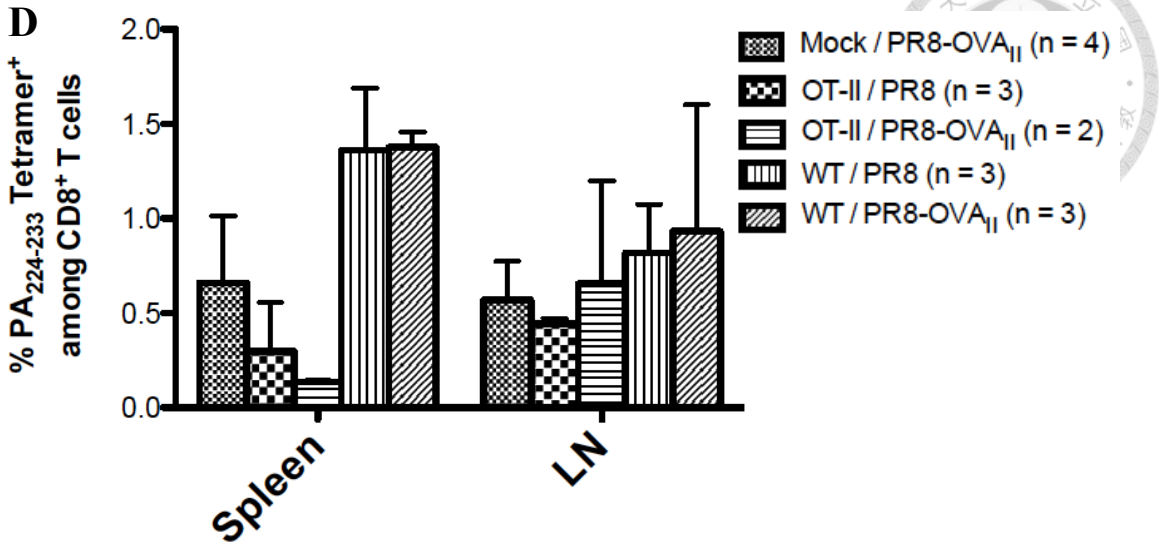
The suppressive ability of Treg cells were determined by the percentage of $CD4^+CFSE^{low}$ T cells using the anti-CD3 stimulated CFSE-based suppression assay which was described in details at the Materials and Methods section.

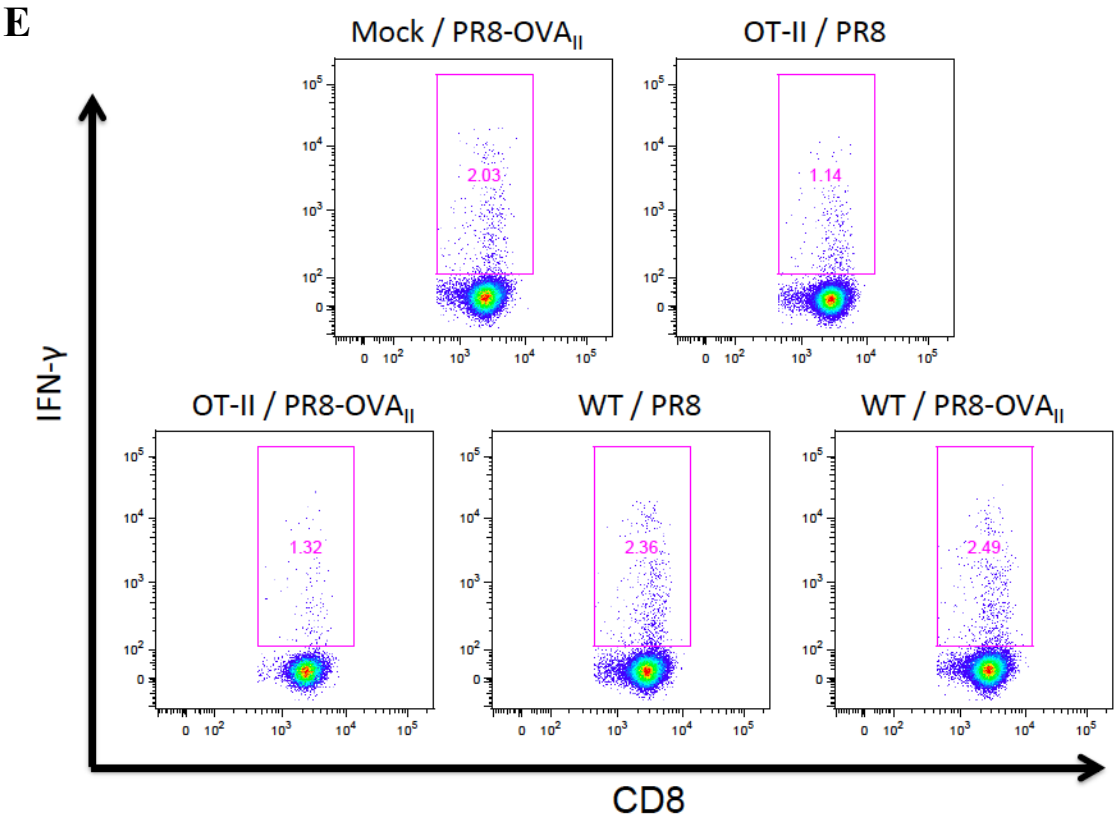
Anti-CD3 stimulated WT CFSE-labeled $CD4^+$ T cells were coculture with different Treg cells at different ratio (1:4, 1:2, 1:1) or without Treg cells. The representative dot plots were shown in (A). The number in each plot indicates the proliferating ($CD4^+CFSE^{low}$) cells and non-proliferating ($CD4^+CFSE^{high}$) cells. The response without suppressors (No Treg) was used as a negative control. (B) Combination of histograms of stimulated $CD4^+$ T cells in the presence of Treg at a ratio of 1:1. The red line represents the histogram of stimulated $CD4^+$ T cells without Treg. (C) The bar graph represented the suppression ratio of $CD4^+CFSE^+$T cell subsets in (A).


A
Mock
A. T.
1 day
B6
i. n.
1000 PFU PR8-OVAII
OVA-specific iTreg
A. T.
1 day
B6
i. n.
or
10 PFU PR8
1000 PFU PR8-OVAII
Non-specific iTreg
A. T.
1 day
B6
i. n.
or
10 PFU PR8
1000 PFU PR8-OVAII


B
Mock / PR8-OVAII (n = 4)
OT-II / PR8 (n = 3)
OT-II / PR8-OVAII (n = 2)
WT / PR8 (n = 3)
WT / PR8-OVAII (n = 3)
% original body weight
120
110
100
90
80
-1
0
1
2
3
4
5
6
7
8
Days post infection


C
Mock / PR8-OVAII (n = 4)
OT-II / PR8 (n = 3)
OT-II / PR8-OVAII (n = 2)
WT / PR8 (n = 3)
WT / PR8-OVAII (n = 3)
% NP366-374 Pentamer+ among CD8+ T cells
4
3
2
1
0
Spleen
LN


D
% PA224-233 Tetramer+ among CD8+ T cells
2.0
1.5
1.0
0.5
0.0
Spleen
LN
Mock / PR8-OVAII (n = 4)
OT-II / PR8 (n = 3)
OT-II / PR8-OVAII (n = 2)
WT / PR8 (n = 3)
WT / PR8-OVAII (n = 3)


E
Mock / PR8-OVAII
OT-II / PR8
OT-II / PR8-OVAII
WT / PR8
WT / PR8-OVAII
2.03
1.14
1.32
2.36
2.49
IFN-γ
CD8

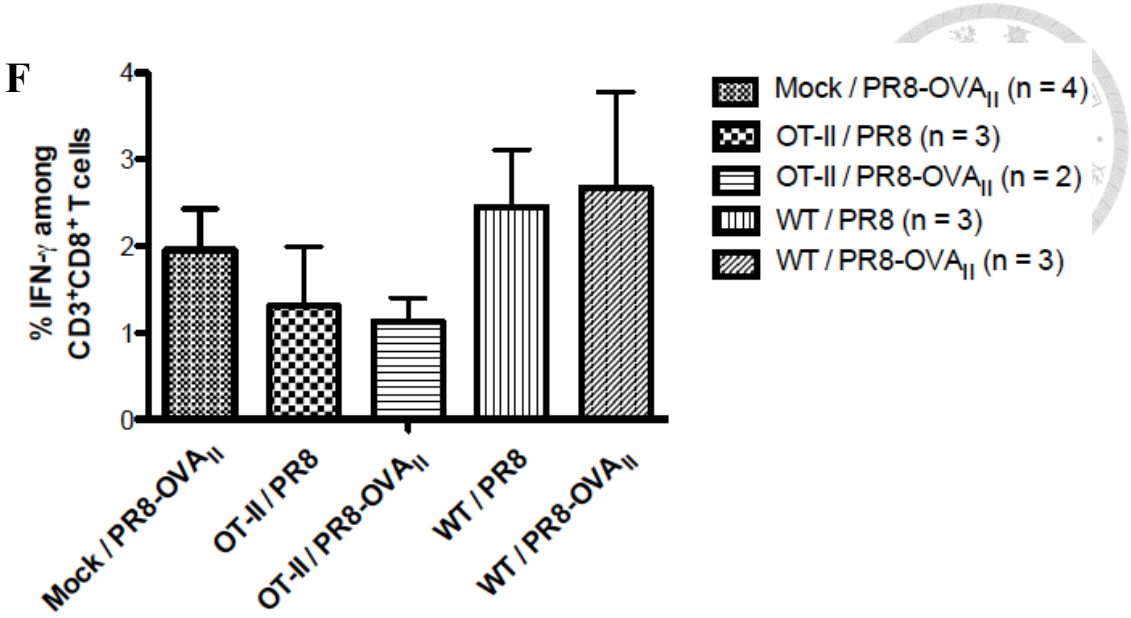


Figure 10. *In vitro* generation of iTreg cells showed no suppressive function *in vivo*.

(A) The experimental design for determining the suppressive ability of iTreg cells *in vivo*. *In vitro* generated OVA-specific iTreg or non-specific iTreg cells (0.5 x 10^6) were sorted and then injected intravenously into WT mice. Mice were also injected with medium only (RPMI 1640) as a control. One day later, the recipient mice were infected with 10 PFU PR8 or 1000 PFU PR8-OVA_{II}. (B) The body weight of the mice was monitored daily after postinfection until they were sacrificed. (C, D) Spleen and lymph node were harvest after seven days postinfection and analyzed the percentage of $FluNP_{366}/D^b$ pentamer$^+$ or PA_{224}/D^b tetramer$^+$ cells among total $CD3^+CD8^+$ T cells were analyzed. (E, F) Representative dot plots and the percentage for IFN-γ production by $CD3^+CD8^+$ T cells isolated from spleen after simulation with PR8-infected BMDC.

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