# Dissertation

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**Oral examination:**

# Efficient Dendritic Cell Maturation and Initiation of a Strong T Cell Immune Response Requires B7-H1-Mediated Dendritic Cell 'Conditioning' During Interaction with T Cells.

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**Prof. Dr. Jianzhu Chen**

*In Loving Memory of my Father.*

*Science cannot solve the ultimate mystery of nature. And that is because, in the last analysis,we ourselves are part of the mystery that we are trying to solve.*

<div align="right">(Max Planck, 1858-1947)</div>

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# Abstracts

The immune system is composed of many interdependent cell types that collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumor cells. One of the prominent features of the adaptive immune system is the extensive interactions between T cells and dendritic cells (DCs). The T cell-DC interactions are essential for T cell development, homeostasis, and activation, and require two signals, known as 'The two signal model'. The first signal is initiated by T cell receptor (TCR) upon recognition of antigen in the form of peptides presented by the major histocompatibility complex (MHC) molecules. The second signal is mediated by engagement of co-regulatory molecules on naive T cells with its counterpart expressed by mature DCs. Depending on the nature of the regulatory signal both, DCs and T cells are affected. In this PhD work, we have shown the dual function of the co-regulatory B7-H1 (PD-L1) molecule.

The first part of this study demonstrated that B7-H1, expressed on the lung tissue, inhibits T cell response and promotes apoptosis of antigen-specific effector T cells in the periphery, most likely by binding to the inhibitory PD-1 receptor on T cells.

More importantly, we demonstrated a novel stimulatory function of the B7-H1 molecules that are expressed on T cells, regulating DC development and maturation. Flow cytometric analysis revealed that, following WSN-SIY influenza infection, dendritic cell maturation in B7-H1$^{-/-}$ mice was defective and as a consequence CD8 T cell response was diminished. The adoptive transfer of B7-H1-expressing T cells however completely restored DC maturation in the draining lymphnode and enhanced CD8 T cell response, such as activation and proliferation. Moreover, maturation-defective DCs in T and B cell-deficient RAG-1 knock-out mice after influenza infection were also rescued by B7-H1$^{+}$ T cells. Further analyses demonstrated rescue of T cell response in B7-H1$^{-/-}$ mice by the transfer of immature DCs from either B7-H1$^{+/+}$ wild type mice or RAG-1$^{-/-}$ mice that had previously received B7-H1$^{+}$ T cells.

The findings in this part of the study illustrated a novel molecular requirement for T cell-conditioned DC maturation that is mediated by B7-H1 and described the cellular interactions that promote efficient T cell responses to microbial infection.

# Zusammenfassung

Einer der bedeutenden Eigenschaften des adaptiven Immunsystems ist das ausgiebige Zusammenspiel von T-Zellen und Dendritischen Zellen (DZ). Die T-Zell-DZ Interaktion ist für die Entwicklung, die Homöostase und Aktiverung von T-Zellen grundlegend und benötigt zwei Signale, auch bekannt als das „Zwei-Signale Modell". Das erste Signal wird durch das T-Zell Rezeptor eingeleitet nachdem es das Antigen in Form eines Peptides durch das Major-Histo-Kompatibilitäts-Komplex erkennt. Das zweite Signal wird durch die Bindung von Kostimulatoren auf T-Zellen und DZ ausgelöst. Abhängig davon welche Moleküle interagieren, wirkt sich das Signal auf DZ oder T-Zellen aus.

In dieser Arbeit haben wir gezeigt, dass das koregulatorische B7-H1 (PD-L1) Molekül eine duale Funktion besitzt. Expression von B7-H1 in peripheren Organen, wie z.B. in der Lunge während infektionaler Höchstbelastung, beeinflusste die Antigen-spezifische T-Zellantwort negativ, höchstwahrscheinlich durch die Bindung an das PD-1 Rezeptor auf T-Zellen. Diese Ergebniss weist darauf hin, dass inhibitorische T-Zellen:PD-1 Interaktion eine Möglichkeit darstellt um das exzessive Anhäufung von Effektor T Zellen in der Lunge zu vermeiden.

Noch wichtiger war die Entdeckung einer neuen positiven Funktion von B7-H1 Molekülen das auf T-Zellen exprimiert wird und Einfluss auf DZ Entwicklung und Maturation hat. Analysen haben gezeigt dass nach einer WSN-SIY Influenza Infektion in B7-H1$^{-/-}$ Mäusen DZ defekt sind und daraus resultierend die T-Zellantwort geschwächt ist. Jedoch konnten DZ Maturation und T-Zellantwort, insbesondere Aktiverung und Proliferation, duch den Transfer von B7-H1-exprimierenden T-Zellen wieder hergestellt werden. Zusätzlich konnte gezeigt werden, dass DZ Maturation, dass in RAG-1$^{-/-}$ Mäusen ursprünglich defekt ist, auch durch Transfer von B7-H1$^{+}$ T-Zellen hergestellt wurde. Weitere Analysen demonstrierten die Wiederherstellung von T Zellantwort in B7-H1$^{-/-}$ Mäusen durch den Transfer von DZ aus B7-H1$^{+/+}$ Wildtyp oder aus RAG-1$^{-/-}$ Mäusen, die zuvor mit B7-H1$^{+}$ T-Zellen transferiert worden waren. Diese Ergebnisse zeigen die molekularen Voraussetzungen fuer eine T-Zellabhängige DZ Maturation und beschreiben die Zellulären Wechselwirkungen das eine effiziente T-Zellantwort gegenüber mikrobielle Infektionen begünstigen. Es ist bedeutend dass DZ, eingeleitet durch B7-H1, während Interaktion mit T-Zellen in einen konditionierten Zustand gebracht wird um effiziente DZ Maturation und eine anschliessende zu ermöglichen.

# Abbreviations

| | |
|---|---|
| Abs | Antibodies |
| ACK | Ammonium-Chloride - Potassium buffer |
| APCs | Antigen presenting cells |
| BAL | Broncho alveolar lavage |
| Bcl-XL | B cell lymphoma-XL |
| BCR | B cell receptor |
| BSA | Bovine serum albumin |
| BTLA | B and T lymphocyte attenuator |
| °C | Celsius |
| CDC | Center for disease control |
| CFSE | Carboxyfluorescein |
| CRNA | Complementary Ribonucleic acid |
| CTLA-4 | Ccytotoxic T lymphocyte antigen-4 |
| CTLs | Cytotoxic lymphocytes |
| DCs | Dendritic cells |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylendiamin-tetraacetat |
| ER | Endoplasmatic reticulum |
| FACS | Fluorescence activated cell sorting |
| FBS | Fetal bovine serum |
| FCS | Fetal calf serum |
| G | Gauge |
| Gal | Galactose |
| HA | Hemagglutinin |
| HBSS | Hanks buffered salt solution |
| Hi | High |
| ICOS | Inducible costimulator |
| ICOS-L | Inducible costimulator ligand |
| IFN- γ | Interferon-gamma |
| Ig | Immunoglobulin |

| | |
|---|---|
| IL-2 | Interleukin-2 |
| IMDM | Iscoves Modified Dulbecco Medium |
| I.v. | Intravenous |
| I.n. | Intranasal |
| I.p. | Intraperitoneal |
| LPS | Lipopolysaccharide |
| M1 | Matrix protein 1 |
| M2 | Matrix protein 2 |
| MAb | Monoclonal antibody |
| MACS | Magnetic cell sorting |
| MDCK | Madine Darbey canine kidney - cells |
| MHC | Major histocompatibility complex |
| Min | Minutes |
| MLN | Mediastinal lymphnode |
| MLRs | Mixed leukocyte |
| NA | Neuraminidase |
| NeuAc | N-acetylneuraminic acid |
| NKT | Natural killer T cells |
| NP | Nucleoprotein |
| NS | Non-structural proteins |
| NFAT | Nuclear factor activated cells |
| PA | Polymerase protein A |
| PAMP | Pathogen-associated molecular patterns |
| PB1 | Polymerase protein B1 |
| PB2 | Polymerase protein B2 |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD-1 | Programmed death-1 |
| PD-L1 | Programmed death ligand 1 (B7-H1) |
| PD-L2 | Progammed death ligand 2  (B7-DC) |
| PH | Pondus hydrogenii |
| PI | Propidium iodide |
| PLN | Peripheral lymphnode |
| PMHC | Peptide Major histocompatibility complex |

PRR              Pattern recognition receptors

RAG             Recombination activating gene

RBC             Red Blood Cell - lysis buffer

RNA             Ribonucleic acid

RNP             Ribonucleoprotein

Rpm             Rounds per minute

RT               Room temperature

Pfu              Parts forming unit

S.c.             Subcutaneous

SIY              SIYRYYGL

Ss-RNA         Single-stranded RNA

TCR             T cell receptor

TLR             Toll-like receptor

Th               T helper cells

VRNA           Viral ribonucleic acid

WHO            World Health Organization

WSN            Waisman Syndrome

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# 1. Introduction

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## 1.1. Influenza virus A infection

Influenza viruses are members of the family *Orthomyxoviridae.* There are three types of influenza: A, B and C (Krug and Lamb, 2001). Influenza type A virus is amongst the most serious infectious threats for humans, due to the severity of the disease, the variability of the virus envelope proteins and its ability for a rapid and global spread. Recent outbreaks of highly pathogenic influenza A virus infections in poultry and humans (known as "birdflu") and the potential threat of influenza pandemics with high mortality demand a better understanding of the pathogenesis of this disease.

The virus mainly attacks cells of the upper respiratory tract including the nose, throat and the bronchial tree. The disease is characterized by the sudden onset of high fever, sore throat, rhinitis, cough and headache. While infected individuals recover within a week, influenza poses a serious risk for the very young and elderly, potentially resulting in pneumonia and death (CDC Website). Estimates by the World Health Organization (WHO) depict three to five million severely ill, and 250,000 to 500,000 deaths related to influenza per year. To date vaccination with inactivated viruses or subunits of viruses are only partially effective. After a primary infection or vaccination with a certain virus strain, the epitope-specific neutralizing antibodies produced by B cells protect against reinfection with the same or closely-related viruses. However, the protection is reduced or lost for infection with variant viruses caused by genetic changes due to mutation and reassortment (antigenic shift) in influenza viruses (Carrat and Flahault, 2007, Bush et al., 1999; Fitch et al., 1997; Smith et al., 2004). Consequently, vaccine compositions need to be adjusted annually to include the most recent circulating influenza strains. Therefore, research has started to explore other arms of the immune system including innate immune cells involved in the first line of defense, as well as T cell-mediated immunity. Key participants, such as dendritic cells (DCs) play an important role in the initiation of the immune response by stimulation of the T cell- and B cell-mediated adaptive immunity that results in the resolution of infection and protection against reinfection.

### 1.1.1. Influenza virus A and its life cycle

Influenza type A viruses are enveloped viruses with a segmented single-stranded RNA (ss-RNA) genome (Krug and Lamb, 2001). For influenza A viruses, subtyping is based on the antigenicity of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins (**Figure 1-1**). HA and NA are responsible for virus attachment and penetration into cells as well as release of progeny virus from the infected cell. Influenza viruses encode also for M2 (integral membrane ion-channel protein), proteins comprising the ribonucleoprotein complex NP (nucleoprotein associated with RNA), and the polymerase proteins PA, PB1 and PB2. The matrix protein M1 is associated with both the ribonucleoprotein and the viral envelope and underlies the lipid bilayer. NS1 and NS2 represent non-structural proteins.
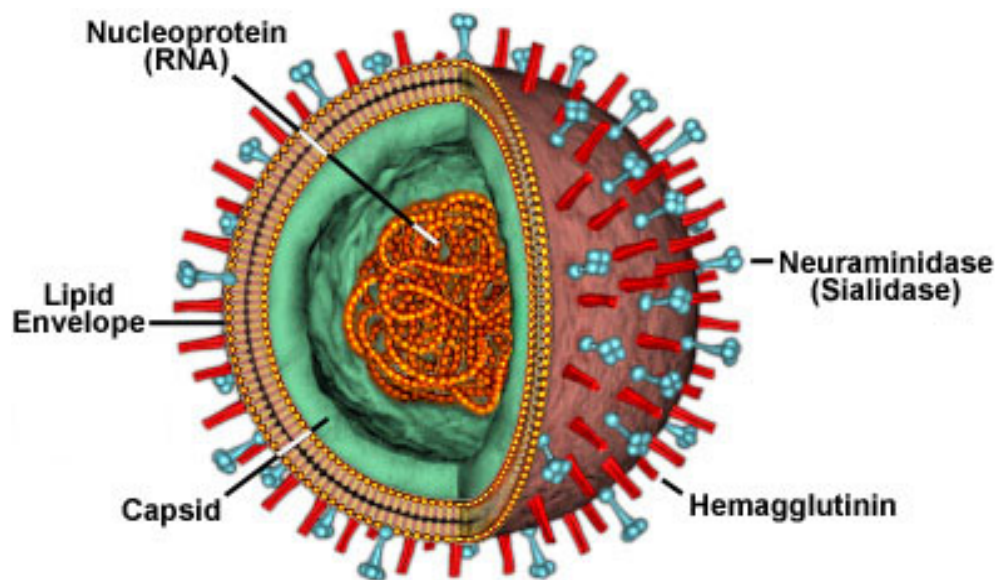


**Figure 1-1: Schematic diagram of the structure of an influenza A virus**

(Molecular Expressions, National High Magnetic Field Laboratory, The Florida State University, Tallahassee, Florida 32306)

In humans, the HA surface glycoprotein mediates virus binding to host-cell receptors containing sialyloligosaccarides terminated by N-acetyl sialic acid linked to galactose

(Gal) by an α (2,6)-linkage (NeuAc α 2,6Gal). Mice were shown not to express the α (2,6)-linked sialic acid receptors, but share with humans the expression of α (2,3)-type receptors (Ibricevic et al., 2006). Of note, avian viruses preferentially recognize NeuAc α-2,3Gal linkages (Matrosovic et al., 2004). The specificity of human trachea, which contains mainly α (2,6)-linked carbohydrate chains, is a key determinant in restricting the transfer of influenza virus directly from avian species that contain mainly α (2,3)-linkages on epithelial cells in the trachea.

After binding, the virus is internalized by endocytosis, followed by the fusion between the viral envelope and the endosomal membrane, which allows for delivery of the viral ribonucleoproteins into the cytosol and further transport into the nucleus. Among the RNA viruses, influenza virus is unique, as all of its RNA synthesis – transcription and replication – takes place in the nucleus of the infected cell (**Figure 1-2**). In the nucleus, the viral negative strand RNA (vRNA) serves as a template for the synthesis of capped, polyadenylated viral messenger RNA and of full-length positive-strand RNA or complementary RNA (cRNA). The cRNA serves as a template for the synthesis of new vRNA molecules. Viral RNA polymerase enters the host cell nucleus with the viral RNP complex and catalyzes all three of these reactions. For export, vRNA is packaged into ribonucleoprotein (RNP) complexes containing two viral proteins, NP and M1. Following protein synthesis, the three viral integral membrane proteins, HA, NA and M2 enter the host endoplasmic reticulum (ER), where all three are folded and HA and NA are glycosylated. HA, NA and M2 are transported to the Golgi apparatus where cysteine residues on HA and M2 are palmitoylated. All three proteins are directed to the virus assembly site on the apical plasma membrane. The release of influenza virus particles after separation of the virus and infected cell membrane is an active process. During the budding process, HA on the surface of the newly budding virion binds to cell surface molecules containing sialic acid residues as seen during attachment. The NA glycoproteins neuraminidase activity is essential to cleave the link between the HA and sialic acid on the surface of the host cell from which the budding virus is emerging from. Thus the NA mediated cleavage of sialic acid residues terminally linked to glycoproteins and glycolipids is the final step in releasing the virus particle from the host cell. This essential role of NA in release of virus particle has been demonstrated with the use of NA inhibitors (Palese, 1976; Luo, 1999; Garman, 2004), NA mutant viruses (Palese, 1974) and with viruses lacking NA activity (Liu, 1995). In all cases, viruses remained bound to the cell surface in clumps in the absence of NA enzymatic activity, resulting in

loss of infectivity. Addition of exogenous sialidase results in virus release and recovery of infectivity. The sialidase activity of the NA is also important for removing sialic acid from the HA on virus particles, if this is not removed, virus particles aggregate.
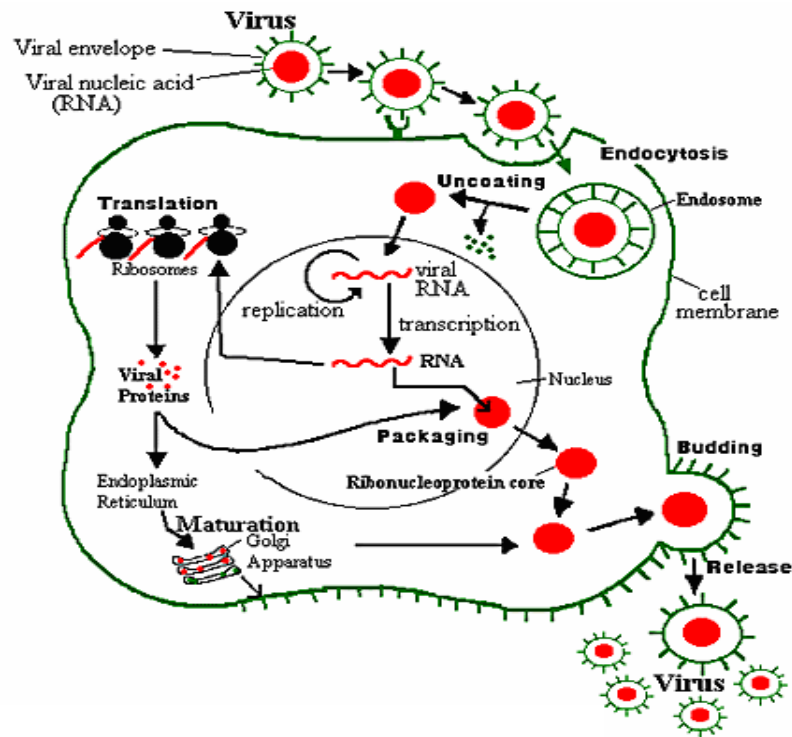


**Figure 1-2: Influenza replication -> Lifecycle**

(Virology Lectures to 3rd Year Medical Students, by Linda Stannard, 1995)

### 1.1.1.1 WSN-SIY virus

In our influenza mouse model, a mouse specific recombinant WSN-SIY influenza virus (H1N1) strain encoding the SIY epitope in the neuraminidase stalk was engineered by plasmid-based reverse genetics (Shen et al., 2008) (**Figure1-3**). This virus is recognized by SIY-specific CD8 T cells when the epitope is presented on $K^{b+}$ APCs or virus infected target cells.
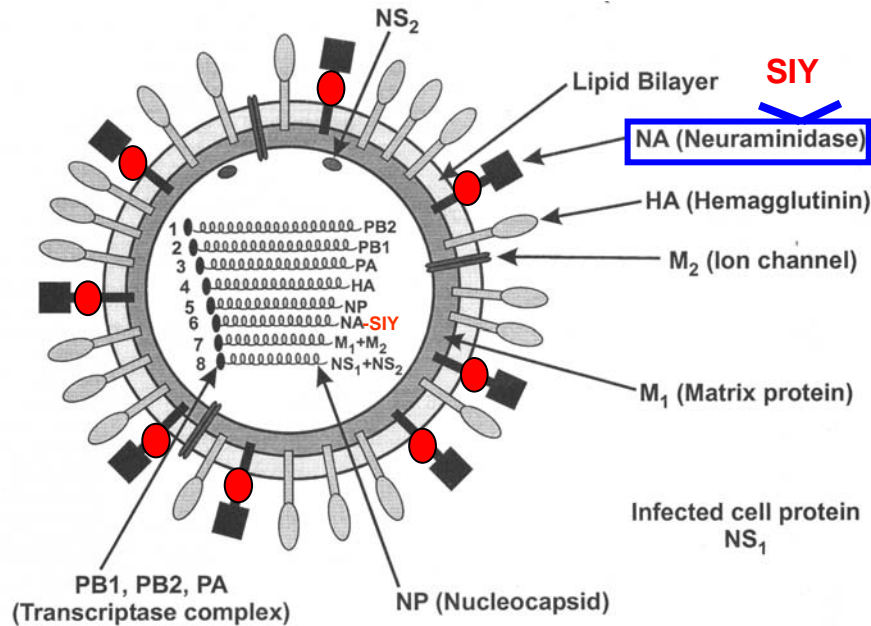
**Figure 1-3: Schematic representation of the murine WSN-SIY Influenza A virus.**

The CD8 epitope SIYRYYGL (SIY) is integrated into the neuroaminidase (NA) gene of a WSN influenza virus. The red circles represent the integrated SIY peptides that are expressed together with the neuroaminidase protein on the virus surface.


## 1.1.2 Influenza virus pandemics

Three times in the last century, the influenza A viruses have undergone major genetic changes mainly in their H-component, resulting in global pandemics and large tolls in terms of both disease and deaths. The most infamous pandemic was "Spanish Flu" which affected large parts of the world population and is thought to have killed at least 40 million people in 1918-1919. More recently, two other influenza A pandemics occurred in 1957 ("Asian influenza") and 1968 ("Hong Kong influenza") and caused significant morbidity and mortality globally. In contrast to current influenza epidemics, these pandemics were associated with severe outcomes also among healthy younger persons, however not on such a dramatic scale as the "Spanish flu" where the death rate was highest among healthy young adults. Most recently, limited outbreaks of a new influenza subtype A (H5N1) directly transmitted from birds to humans (bird flu) have

occurred mainly in East Asia but it was also detected in some parts of Europe and the Middle East.

## 1.2 The immune system

The immune system is a complex and highly developed system, which has evolved to protect the host against the attack of foreign pathogens as well as tumors. In vertebrates, it consists of the innate and the adaptive immunity. The non-specific component of the immune system (innate immunity) is a set of mechanisms that are not specialized for a particular pathogen. Adaptive immunity, on the contrary, displays a higher degree of specificity in recognizing foreign antigens than innate immunity as well as the property of memory. Typically, an adaptive immune response against an antigen is raised five or six days after the initial exposure to that antigen. Subsequent exposure to the same antigen results in a memory response, which is faster, stronger, and often more effective in neutralizing or clearing the pathogen than the first one. Due to this attribute, the immune system can confer life-long immunity to many infectious agents after an initial encounter. Adaptive and innate immunity, however, do not operate independently of each other but rather function as a highly interactive and cooperative system.

### 1.2.1. Immunological resistance to influenza virus infections

Specific immune mechanisms have evolved that recognize and, in most cases, eliminate infectious agents within a few hours (Ada and Jones, 1986). Specialized classes of immune cells contribute to responses resulting in protection against a possible reinfection by the same or closely-related viruses. The immune response occurs in three phases (as shown in **Figure 1-4**).

Components of the "first line of defense" are the mucus as a trap for the influenza virus, cytokines and cells of the innate immune system, such as DCs and macrophages that make contact with the invading virus and do not require a prolonged period of induction (Murphy and Webster, 1996; Tamura and Kutara, 2004). Tissue-resident cells can rapidly recruit other early effectors such as granulocytes and NK cells. However, innate immune cells are unable to confer antigen-specific actions and immune memory.

If the virus escapes the first line of early defense, it is detected and eliminated by the adaptive immune mechanism, where T and B cells and their products function as antigen-specific effectors. When activated, CD8 T cells differentiate into cytotoxic killer cells (CTL) that destroy virus-infected target cells. CD4 T cells differentiate into T helper (Th) cells. Th cells activate other immune cells as well as B cells that become plasma cells, which secrete antibodies (Abs). T and B lymphocytes also give rise to antigen-specific memory cells that can respond faster and more effectively than naïve cells in a secondary infection.

| **Innate Immunity** (immediate: 0-4 hrs) | **Early induced response** (early: 4-96 hrs) | **Adaptive Immunity** (late: >96 hrs) |
|---|---|---|
| Invading Organisms | Invading Organisms | Invading Organisms |
| Recognition by effector cells of innate immune system | Recruitment of effector cells | Antigen travels to lymphoid organs |
| Removal of microorganism | Identification by and activation of effector cells | Recognition by lymphocytes |
| | Removal of microorganism | Expansion and differentiation of effector cells |
| | | Removal of microorganism |

**Figure 1-4 Three phases of an immune response against infection.**
The first two phases rely on recognition of pathogens by innate receptors and occur early after infection. The adaptive immunity involves antigen-specific receptors and occurs at later times, because rare T and B cells specific for an antigen must undergo clonal expansion and acquire effector functions before participating in the fight against the invading microbe. (Adapted from Janeway et al., Immunobiology, 5th edition)

## 1.2.2 The innate immune system

The innate immune system is a collection of distinct subsystems that appeared at different stages of evolution including anatomic, physiologic, phagocytic and inflammatory barriers that prevent the entrance and establishment of infectious agents. Innate immune recognition is also known as pattern recognition and is based on the detection of molecular structures unique to microorganisms. The innate immune system senses pathogens through pattern-recognition receptors (PRR), which trigger the activation of antimicrobial defenses. The targets of PRR are named pathogen-associated molecular patterns (PAMP), although they are present on both pathogenic and non-pathogenic microorganisms. Bacterial PAMP are often components of the cell wall, such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acids and cell-wall lipoproteins. β-glycan is an important fungal PAMP, which is present on the fungal cell wall. The detection of these structures by the innate immune system can signal the presence of microorganisms. Moreover, the recognition of viruses also follows this principle; however, since viral components are synthesized within the host cells, the main targets of innate immune recognition in this case are the viral nucleic acids. In addition, the innate immune system is able to discriminate between self and viral nucleic acids based on specific chemical modifications unique to viral RNA and DNA, as well as on the cellular compartments where viral, but not host-derived, nucleic acids are found (Medzhitov, 2007). There are several classes of PRR, the best characterized being Toll-like receptors (TLR), which can elicit inflammatory and antimicrobial responses after activation by their microbial ligands (Akira et al., 2006). The innate immune response depends on the coordinate activity of several effector cells, like natural killer cells, monocytes/macrophages, DC and granulocytes.

## 1.2.3 The adaptive immune system

The two main components of the adaptive immune system are B and T lymphocytes. B cells are part of the humoral-mediated response which is specialized in the recognition and elimination of the extracellular pathogens, while T cells generate cell-mediated immune responses which target the intracellular pathogens. B lymphocyte maturation takes place within the bone marrow and is accompanied by the acquisition of a unique antigen-binding receptor on their surface, called the B cell receptor (BCR); the BCR

constitutes a membrane-bound antibody. Upon antigen encounter, B cells differentiate into memory and effector cells, called plasma cells; the latter produce antibodies in a secreted form. T lymphocytes also arise in the BM but, subsequently, migrate to the thymus for their final maturation. During their maturation, T cells express a unique antigen binding molecule, the T cell receptor (TCR). Both the BCR and TCR are products of somatic gene rearrangement during maturation. The genes encoding these receptors are assembled from variable and constant fragments through recombination activation gene (RAG) protein-mediated somatic recombination. However, the TCR in contrast to the BCR can only recognize antigen bound to MHC molecules

Besides the conventional B and T cells, adaptive immunity includes also the so-called innate-like lymphocytes (i.e. the B1 cells, the marginal-zone B cells, natural killer T (NKT) cells and subsets of γδ T cells); the diversity of antigen receptors expressed by these cells is restricted and not entirely random and their specificities are skewed towards a predefined set of ligands (Bendelac et al., 2001).

## 1.3. Dendritic cells as a link between innate and adaptive immunity

Dendritic cells, as professional antigen presenting cells (APCs), represent a heterogenous cell population residing in most peripheral tissues, particularly at sites of interface with the environment (skin and mucosae), where they represent 1% - 2% of the total cell numbers (Banchereau J, Steinman RM, 1998; Steinman, 1991). In mice, at least five different subsets have been identified. All DCs express CD11c (the integrin $\alpha_x$-chain), and are further subdivided depending on their expression of CD11b (the integrin $\alpha_M$ - chain), CD4, CD8 and CD205.

In the absence of ongoing inflammatory and immune responses, dendritic cells constitutively patrol through the blood, peripheral tissues, and secondary lymphoid organs (Vermaelen and Pauwels, 2005). In peripheral tissues, dendritic cells take up self and nonself antigens. Internalized antigens are then processed into proteolytic peptides, and these peptides are loaded onto MHC class I and II molecules. This process of antigen uptake, degradation, and loading is known as antigen presentation (**Figure 1-5**).
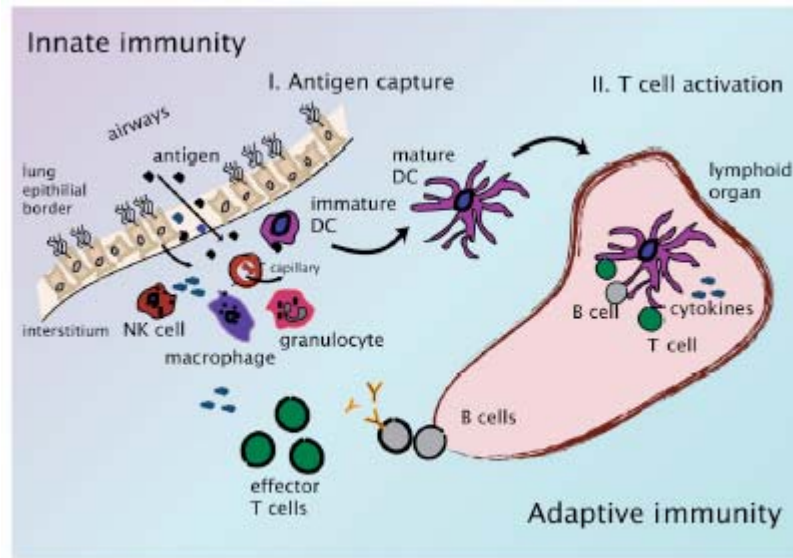
**Figure 1-5: Paradigm of innate and adaptive immunity following lung infection.**

Pathogen invasion initiates a cascade of processes that control a coordinated immune response. After antigen uptake by pulmonary dendritic cells and macrophages, their maturation and migration to lymph nodes result in encounter of antigen-specific T cells, which are subsequently activated. After differentiation, effector T cells migrate to the site of infection and combat together with NK cells infected cells and microbe progeny. B cells are activated to produce antibodies, which protect against reinfection with the same virus.

Constitutively, however, peripheral dendritic cells present antigens quite inefficiently. A signal from pathogens, often referred to as a danger signal, induces dendritic cells to enter a developmental program, called maturation, which transforms dendritic cells into efficient antigen presenting cells and T cell activators (Lambrecht et al., 2001). Bacterial and viral products, as well as inflammatory cytokines and other self-molecules, induce dendritic cell maturation through direct interaction with specific dendritic cell surface receptors. T lymphocytes, through CD40-dependent and -independent pathways, and endothelial cells contribute to the final maturation of dendritic cells through direct cell-to-cell contact and the secretion of cytokines (Bell et al., 1999).

### 1.3.1 Dendritic cell activation and maturation

Soon after encountering a danger signal, the efficiency of antigen uptake, intracellular transport and degradation, and the intracellular traffic of MHC molecules are modified (Thery and Amigorena, 2001). Peptide loading as well as the half-life and delivery to the cell surface of MHC molecules is increased. The surface expression of adhesion molecules and costimulatory molecules such as B7.1 (CD80) and B7.2 (CD86), which bind both CD28 and CTLA-4 on T cells, is increased. These modifications increase the T cell priming ability of dendritic cells. Thus, dendritic cells become the most potent APCs, and the only ones capable of activating naive T lymphocytes and of initiating adaptive immune responses (Kaspenberg, 2003).

To interact with T cells, however, dendritic cells also need to migrate out of the tissues to reach secondary lymphoid organs. Concomitant with the modifications of their antigen presentation abilities, maturation also induces massive migration of dendritic cells out of peripheral tissues. Modifications in the expression of chemokine receptors and adhesion molecules, as well as profound changes of the cytoskeleton organization, contribute to the migration of dendritic cells, through the lymph, toward secondary lymphoid organs. By linking antigen uptake, peptide loading, and cell migration to the encounter of a danger signal (Gallucci and Matzinger, 2001), dendritic cells restrict antigen presentation to those antigens internalized during maturation, thus favoring the stimulation of T cells specific for potentially pathogenic antigens. It is important that, in addition to presenting antigens, dendritic cells also influence the outcome of immune responses (Banchereau and Steinman, 1998). Different dendritic cell subsets and dendritic cells at different maturation stages express distinct surface molecules and secrete different cytokines; thus they determine selectively the type of induced immune response.

The extraordinary efficiency of dendritic cells for T cell stimulation prompted many research groups around the world to undertake dendritic cell-based active immunotherapy protocols (Nestle et al., 2001). As the fundamental mechanisms underlying antigen presentation in dendritic cells are being characterized, the information is used to optimize the preparation of dendritic cells, their sensitization with antigens, and the routes of injection.

## 1.3.2 Dendritic cell mediated activation of T cells

DCs have been called "nature's adjuvant" due to their ability to coordinate many protective immune functions in response to infections. As already mentioned DCs have the unique capacity to stimulate naïve CD8 T cells and are thereby essential for the induction of primary immune response. The high capacity of DCs, compared to other antigen-presenting cells (i.e. macrophages, B cells), to induce T cell proliferation was first demonstrated in primary mixed lymphocyte reactions (MLRs) (Steinman and Witmer, 1978; Nussenzweig and Steinman, 1980; Green and Jotte, 1985). DCs were shown to be approximately 100 times more effective at inducing T cell proliferation than macrophages and B cells. Furthermore, removal of DCs dramatically reduced the MLR stimulatory capacity of splenocytes (Steinman et al., 1983).

DCs efficiently cluster with T cells (Pope et al., 1994), which is probably a vital feature for efficient induction of T cell responses. In line with their higher expression of MHC and costimulatory molecules, mature DCs are superior to immature DCs at inducing T cell proliferation (Steinman and Nussenzweig, 2002). Furthermore, mature DCs can rapidly polarize immune response with different effector functions depending on the cytokines they secrete (Maldonado-Lopez et al., 1999). DC secretion of Interleukin 12 (IL-12) has proven to be a key cytokine for successful activation of cytotoxic T cell responses against viral infections (D'Andrea et al., 1992; Macatonia et al., 1995). In addition, the interaction between CD40L and CD40 is critical to bring DCs to a state of maturation to efficiently induce T cell priming, which is called licensing or conditioning of DCs (**Figure 1-6)**. However, it has been also reported that DCs require a preceding contact with T cells in order to efficiently mature upon antigen encounter (Kitajima et al., 1996, Flores-Romo, 1999). In addition to their central role in innate and adaptive immunity, DCs can also induce tolerance. While the immunostimulatory functions require mature DCs, an increasing number of studies demonstrate that DCs that are not fully mature may induce T cell tolerance and can dampen the immune response and protect the host from autoreactive T cells (Steinman et al., 2003).
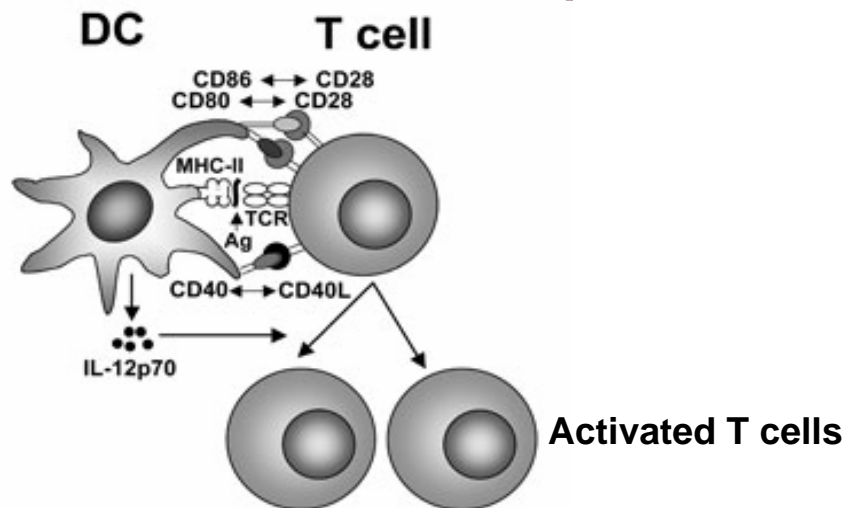
**Figure 1-6: Naïve CD8 T cell priming by mature dendritic cells.**

In addition to antigen presentation, costimulatory signals and cytokine production (IL-12) by dendritic cells are necessary to efficiently prime naïve CD8 T cells. (Nashimul Ahsan; Chronic Allograft Failure: Natural History, Pathogenesis, Diagnosis and Management, 2008)

## 1.4 T lymphocytes and the immune response

### 1.4.1 The two signal model of T cell activation

Peripheral T cell responses to a given antigen involve the clonal expansion of a small subset of T cells that possess unique TCRs but have common antigen specificity. As a result, the overall response to antigen encounter is a direct consequence of factors influencing the activation, expansion, and regulation of individual T cells. TCR engagement of peptide/MHC (pMHC) ligands on professional antigen presenting cells is critical for the initiation of T cell mediated immune responses (Davis et al., 1988). TCR recognition of cognate pMHC, referred to as signal one, is required for T cell activation and determines the antigen specificity of T cells involved in normal immune responses. In addition, the TCR is differentially responsive to subtle changes in its ligand and can mediate signals that result in diverse biological responses (Kersh and Allen, 1996). TCRs possess inherent affinity for self-pMHC as a result of positive selection during

thymic development. Central tolerance mechanisms restrict the affinity of interactions between TCRs and self-pMHC that are involved in peripheral immune homeostasis, and serve to prevent unwarranted lymphocyte activation. In the absence of additional signals, TCR engagement by a MHC-antigen complex is reported to result in anergy, apoptosis, or inefficient T cell activation (Wells et al., 2001). The requirement for an additional signal to optimize T cell activation has proven critical for the maintenance of peripheral self-tolerance and the initiation of productive immune responses.

The 'two-signal' model of lymphocyte activation states that optimal lymphocyte responses require an initial signal resulting from TCR interaction with APC-expressed pMHC (peptide-major histocompatibility) complexes, coupled with a secondary antigen-independent signal termed costimulation (Laffery et al., 1974)(**Figure 1-7**).

CD28 is the most well-known costimulatory molecule and is constitutively expressed by >90% of human CD4 T cells, and >50% of human CD8 T cells. CD28 interaction with B7 molecules on APCs provides signal two and results in enhanced activation, accelerated cell cycle progression, T cell survival, and clonal expansion (Salomon and Bluestone, 2001; Acuto and Micel, 2003). The hallmark of CD28 costimulation is enhanced interleukin-2 (IL-2) production and bcl-XL upregulation, which promote T cell expansion and support the development of an effective immune response (Blair et al., 1998). CD28 enhances T cell activation by diverse mechanisms that serve to enable TCR signaling and promote prolonged T cell-APC interaction. CD28 engagement amplifies membrane-proximal signaling initiated by the TCR, but also transduces a unique signal reportedly necessary for the stabilization of some RNA transcripts and efficient upregulation of numerous genes including IL-2 and bcl-XL (Frauwirth and Thompson, 2002; Diehn et al., 2002).
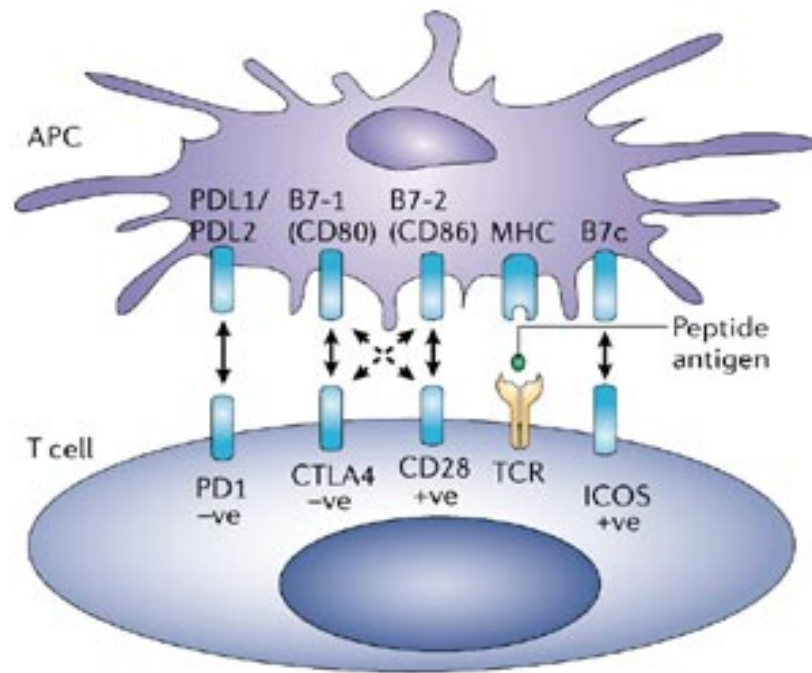
**Figure 1-7 The 'two-signal' model of lymphocyte activation**

T cells are activated through stimulation of the T-cell receptor (TCR). Co-stimulatory and co-inhibitory molecules that are also present at the T-cell surface set thresholds for this activation, and thereby regulate the degree of activation as well as the development of peripheral T-cell tolerance. CD28 and ICOS are activating receptors that bind members of the B7 family of ligands on antigen-presenting cells (APCs) such as macrophages and dendritic cells. The cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is inhibitory on most T cells (with the exception of regulatory T cells), and, like CD28, can bind both CD80 and CD86, but with varying affinities (indicated by solid and dashed interaction lines). This can result in cross competition for CD28 co-stimulation. The programmed cell death 1 (PD-1) protein is also inhibitory for T-cell activation, and can bind two related ligands, B7-H1 (PD-L1) and B7-DC (PDL2). –ve: negative; +ve: positive (Gregersen and Behrens, *Nature Reviews Genetics*)

In addition, CD28 signaling potentiates the macromolecular reorganization of the cell membrane initiated by TCR ligation, resulting in aggregation of numerous multimolecular signaling complexes at the site of T cell/APC contact (Wulfing et al., 2002; Huang et al., 2002; Wetzel et al., 2002). In fact, recent work suggests that CD28:B7 interactions in the absence of TCR triggering are sufficient for cell polarization and lipid raft aggregation

(Kovacs et al. 2005). The resulting structure, termed the 'immunological synapse' (IS), stabilizes the intercellular contact for as much as 24 hours, and is reportedly necessary for full commitment to activation (Lanzavecchia and Sallusto, 2001). However, the temporal parameters governing CD28 costimulation have not been well defined. While CD28 functions primarily to enhance lymphocyte activation, it also plays a prominent role in the maintenance of peripheral tolerance. CD28 signaling is obligatory for the development and maintenance of regulatory T cells (Boden et al., 2003), upregulates immune attenuators including CTLA-4, and skews T cell responses towards an autoimmune protective Th2 phenotype (Bour-Jordan and Blueston, 2002). CD28-mediated effects on TCR signaling pathways have been well characterized, but the molecular and biochemical mechanisms mediating qualitative effects of CD28 costimulation are poorly understood and the distal effectors of CD28 signaling have not been fully elucidated.

## 1.4.2 The B7/CD28-CTLA-4 pathway

Members of the B7/CD28 family of receptors regulate diverse aspects of the adaptive immune system and are key determinants of T cell responsiveness and effector function. Signaling pathways initiated by B7/CD28 family members are essential components of mechanisms that simultaneously promote and sustain T cell responses to pathogens and serve to maintain tolerance to self-antigen by inhibition or attenuation of T cell responses. CD28 and CTLA-4 are the critical costimulatory receptors regulating antigen responsiveness of mature peripheral T cells, and provide positive and negative second signals to T cells, respectively. CD28 transmits signals important for T cell survival and proliferation, while CTLA-4 inhibits T cell responses and regulates peripheral tolerance. CTLA-4 and B7 share B7 ligands, B7.1 and B7.2. Regulation of CTLA-4 and CD28 signaling is effected by precise control of surface expression, availability of ligands, differential ligand-binding characteristics, and unique signaling properties. Additional members of the CD28 family include ICOS, PD-1, and BTLA. While the ligand for the inhibitory receptor BTLA remains undiscovered, both ICOS and PD-1 are known to bind B7 family members. While PD-1 induces negative signals, ICOS results in positive and negative regulation of T cell responses. However, CD28 and Ctla-4 are unique in their ability to regulate primary T cell responses and provide dominant signals controlling T cell activation in naïve lymphocytes.

CD28 costimulation is essential for normal immune function. CD28-deficient mice or mice treated with antagonists of CD28-B7 interaction manifest profoundly defective responses to allograft antigens (Salomon and Bluestone 2001), infectious pathogens (Sahinian et al., 1993; King et al., 1996; Mittrucker et al., 2001; Compton and Farrell, 2002), graft versus host disease (Via et al., 1996), and induction of contact hypersensitivity (Kondo et al., 1996). Similarly, absence of CD28 costimulation reduces T cell proliferation in vitro and in vivo in response to TCR stimulation (Green et al., 1994; Lucas et al. 1995; Gudmundsdottir et al., 1999). More globally, CD28 deficiency results in diminished germinal center formation and defective isotype class switching in B cells, and diminished CD4-dependent CD8 T cell responses (Lane et al., 1994; Ferguson et al. 1994; Prilliman et al., 2002). CD28 is uniquely capable of synergizing with TCR-mediated signals to activate transcription factors controlling proliferation, differentiation, and cell death including NFκB (Kane et al., 2002), NFAT (Diehn et al. 2002), and AP-1 (Rincon and Flavell, 1994). While receptor-ligand interactions mediating costimulatory signals are diverse, CD28 is unique in its ability to integrate signals with the TCR and to centrally determine the outcome of antigen receptor engagement in naïve T cells (Zuckerman et al., 1998; Zhou et al., 2002). Moreover, CD28 is unique in its ability to provide signals required for cellular activation, cell cycle entry, and efficient cytokine production in lymphocytes, and results in greater enhancement of immune responses than other costimulatory receptor interactions (Van der Merwe and Davis, 2003). An essential component of CD28 costimulation is regulation of distal determinants of cell death, proliferation, and differentiation including cytokines interleukin 2 (IL-2), interferon gamma (IFN-γ), and interleukin 4 (IL-4) (McAdam et al., 1998); chemokines Mip-1alpha/CCL3 (Herold et al., 1997); cytokine receptors CD25, IL-12R, and CXCR5 (Park et al., 2001; Walker et al., 2000); and APC counter-receptors CTLA-4/CD152, CD154/CD40L, ICOS, CD134/OX40, and 4-1BB (review by Sharpe and Freeman, 2002). Thus, CD28 orchestrates the regulation of genes that shape the phenotype of responding cells in ways that determine not only their immediate response, but also their ability to respond to and provide intercellular signals in subsequent days.

CTLA-4 is the second well-characterized member of the CD28-like receptor family. CTLA-4 surface expression is induced approximately after two days of T cell activation following CD28 interaction (Perkins, Wang et al. 1996). The recognition that CD28 and CTLA-4 share the ligands B7.1 (CD80) and B7.2 (CD86) suggest that CTLA-4 may

compete with CD28 for ligands in situations where APC-expressed B7 is limiting. CTLA-4 binds to B7.1 and B7.2 with a much higher affinity than CD28 (Leung, Bradshaw et al. 1995). The engagement of CTLA-4 delivers a negative signal to the activated T cell, opposing the CD28 mediated costimulation: CTLA-4 inhibits IL-2 synthesis, leads to cell cycle arrest and thus counteracts and terminates the T cell response. Mice expressing a mutant form of CTLA-4 that lacks the cytoplasmic domain are long-lived and show no evidence of lymphocytic infiltration of peripheral tissues, but did exhibit lymphadenopathy, increased numbers of activated T cells, and a predominantly Th2 phenotype. Therefore, CTLA-4 is considered to be a crucial control of T cell proliferation and for peripheral T cell tolerance.

B7.1 and B7.2, the first members of the B7-family, which have been identified, are expressed on APC (reviewed in Bluestone 1995). B7.2 is constitutively expressed at low levels on professional APC and is upregulated within hours after APC–T cell interaction. In contrast B7.1 is absent on resting APC and is induced on the cell surface 3-4 days after T cell activation (Walunas, Lenschow et al. 1994). It has been suggested therefore that B7.2 is substantial for initial T cell activation, B7.1 being required for sustained T cell activation.

### 1.4.3 ICOS-ICOS-ligand pathway

Inducible costimulatory signal (ICOS) is the second molecularly described member of the CD28-family. Like CD28 and CTLA-4 it is encoded on the 2q33-34 locus suggesting that all three molecules most likely arose by gene duplication (Hutloff et al., 1999; Mages et al., 2000). Therefore it is not surprising that ICOS resembles CD28/CTLA-4 structure in many ways. The predicted mature ICOS protein is a 199 amino acid type I transmembrane molecule belonging to the Ig-superfamily. The expression pattern of ICOS differs strikingly to CD28 and CTLA-4. As implicated by its name, ICOS is not constitutively expressed but induced in CD4 and CD8 T cells upon TCR engagement and following T cell activation (Hutloff et al., 1999; Beier et al., 2000; Mages et al., 2000). Regarding the CD4 subset, ICOS expression can be induced on naïve TH0 cells, as well as previously activated TH1 and TH2 cells. Cell surface levels of ICOS remain high on TH2 cells and fall rapidly on TH1 cells after the initial phase of T cell activation. A constitutive baseline expression can be detected in TH2 clones and $CD4^+CD44^{hi}CD69^{low}$ cells (memory cells) but not on TH1 clones. In the course of T cell activation high levels

of cell surface expression of ICOS is detected especially in the late stage of activation. Experiments in murine models show no effect of B7.1-blocking and a minor impact of CD28-blocking on ICOS upregulation whereas blockade of B7.2 leads to significant impaired ICOS expression. Immunhistochemical studies showed that ICOS$^+$ cells are mainly distributed in the cortical thymus and the apical light zone of germinal centers in lymph nodes (Mages et al., 2000). Elevated levels of ICOS mRNA can be detected already one hour after TCR engagement, followed by surface expression within 12 hours. Protein expression reaches a maximum after 48 hours and declines then slightly (Coyle et al., 2000; Mags et al., 2000). Although ICOS expression is supported by CD28 engagement, it is not absolutely CD28 dependent. The fact that ICOS upregulation can be detected under conditions where the TCR is engaged but not CD28 is a strong indicator for the independent costimulatory potency of ICOS.

## 1.4.4 B7-H3 and B7-H4: new members of the B7 family

B7-H3 is a recently discovered member of the B7-family. It is mainly expressed on immature DC, but is absent on B cells. Its expression is down regulated upon stimulation with LPS (Chapoval et al. 2001). B7-H3 is able to stimulate CD4 and CD8 cells and induces in both populations IFN-γ production. B7-H3 fusion proteins binds to activated and not to resting T cells what indicates the upregulation of the B7-H3 counter-ligand after T cell activation (reviewed in Sharpe and Freeman 2002).

B7-H4 is expressed on professional APC as well as in non-lymphoid tissues. It controls T cell immunity by the inhibition of T cell proliferation, cytokine production (both the production of TH1 and TH2 cytokines) and cell cycle progression (2003; Sica et al. 2003; Loke et al. 2003). Therefore, B7-H4 may play a role in negative regulation of T cell immunity. B7-H4 putatively interacts with BTLA, which is expressed during T cell activation. BTLA-expression remains on TH1 but not TH2 cells. BTLA modestly inhibits antigen-induced production of IL-2 (Watanabe et al. 2003).

## 1.4.5 The PD-1-B7-H1/B7-DC pathway

PD-1 is another member of the CD28-like receptor family (Agata et al. 1992). Although it shares some structural homologies with CD28, CTLA-4 and ICOS (like the single extracellular V-like Ig domain) it differs from them in many ways (reviewed in Sharpe and Freeman 2002). Its gene is encoded on the 2q37 locus (instead of CD28, CTLA-4 and ICOS on the 2q33 locus) (Shinohara, Taniwaki et al. 1994). PD-1 is expressed on activated T cells, B cells and Monocytes (Agata et al. 1996). PD-1 has been suggested to be a main modulator regulating TCR thresholds of activation (Nishimura, Honjo et al. 2000). It further more inhibits the production of effector cytokines and blocks cell cycle progression. PD-1 is upregulated within 48 hrs after T cell activation. PD-1-deficient mice of the C57BL/6 strain develop a late onset, progressive arthritis and lupus-like glomerulonephritis with high levels of IgG3 deposition (Nishimura et al., 1999).

The B7-H1 (PD-L1) and B7-DC (PD-L2) ligands exhibit distinct patterns of expression; B7-H1 is expressed more broadly than is B7-DC (Freeman et al., 2000; Dong et al., 1999; Latchman et al., 2001). B7-H1 is expressed on resting and upregulated on activated B, T, myeloid, and dendritic cells (Liang et al., 2003). B7-H1 is expressed on CD4+ CD25+ T cells, but whether it has a role in function of this regulatory population in not yet clear. In contrast to B7-1 and B7-2, B7-H1 is expressed in nonhematopoietic cells including microvascular endothelial cells and in nonlymphoid organs including heart, lung, pancreas, muscle, and placenta (Roding et al., 2003; Ishida et al. 2002; Petroff et al., 2003). The expression of B7-H1 within nonlymphoid tissues suggests that it may regulate self-reactive T or B cells in peripheral tissues and/or may regulate inflammatory responses in the target organs. However, the roles of B7-H1 on T cells, APCs, and host tissues are not yet clear. There are many potential bidirectional interactions between B7-H1 and PD-1 owing to the broad expression of B7-H1 and the expression of PD-1 on T cells, B cells, and macrophages. In contrast, B7-DC is induced by cytokines only on macrophages and DCs. The relatively greater role of IFN-$\gamma$ in stimulating B7-H1 expression and IL-4 in stimulating B7-DC expression suggests that B7-H1 and B7-DC may have distinct functions in regulating Th1 and Th2 responses (Loke and Allison, 2003).

## 1.4.5.1 B7-H1 (PD-L1)

B7-H1 has been described to be inducible in a variety of organs, including nonlymphoid tissues such as the heart, lung and placenta (Liang et al., 2003, Freeman et al., 2000). Ligation of cognate receptor(s) on T cells by B7-H1 was first reported to predominantly stimulate interleukin 10 production (Dong et al., 1999; Tamura et al., 2001), which might negatively regulate cell-mediated immunity (Moore et al., 2001). An overwhelming number of studies supported the role of B7-H1 as a negative regulator of T cell responses in vitro using a polyclonal activator of T cells (immobilized anti-CD3 mAb) and B7-H1.Ig (Dong et al., 2003; Carter et al., 2002;). Similar conclusions were drawn in studies using antibodies that blocked B7-H1 signaling on human endothelial cells (Mazanet and Hughes, 2002; Rodig, et al., 2003), dendritic cells (Selenko-Gebauer et al., 2003; Brown et al., 2003), liver non parenchymal cells (Iwai et al. 2003), and glioma cells (Wintterle et al., 2003). In vivo the injection of B7-H1-Ig promoted cardiac allograft survival and protected against chronic rejection (Ozkaynak et al., 2002). In addition, B7-H1-Ig plus anti-CD154 (anti-CD40L) mAb treatment induced long-term islet allograft survival (Gao et al., 2003).

Since B7-H1 is expressed in a number of nonlymphoid tissues and shown in several studies that it primarily inhibited T cell responses, possibly by signaling through PD-1, it was suggested that this B7 family member promotes peripheral tolerance at the effector phase, i.e., when T cells migrate into target tissues (Nishimura and Honjo, 2001).

In contrast to these conclusions, it has been shown that the transgenic expression of B7-H1 on mouse pancreatic islets promotes diabetes pathogenesis and islet allograft rejection in a minor mismatch setting. Consistent with this result, both soluble and membrane-bound forms of B7-H1 augment T cell proliferation in vivo (Subudhi et al., 2004). Another study found that the development of chronic intestinal inflammation is inhibited by blocking B7-H1 signals with an anti-B7-H1 mAb (Kanai et al., 2003). These results suggest that B7-H1 can costimulate T cell responses.

So far, the cellular mechanism by which B7-H1 and B7-DC promote diametric outcomes remains elusive. Although many of the model systems that examined the role of B7-H1 in vivo have used similar reagents (e.g., B7-H1-Ig and anti-B7-H1 mAb), the results from these studies have clearly yielded conflicting conclusions. This suggests that in certain in vivo settings B7-H1 costimulates T cell responses, and that in other circumstances it co-inhibits these responses. One possibility for how B7-H1 and B7-DC

can both enhance and inhibit T cell responses is that these ligands interact with an additional receptor other than the inhibitory receptor PD-1. In fact several independent groups have provided strong evidence suggesting that another putative receptor exists, permitting B7-H1 and B7-DC to costimulate T cell responses independently of PD-1 (Subudhi et al., 2004; Liu et al., 2003; Shin et al., 2003; Wang et al., 2003). For instance, it has been demonstrated that B7-H1 mediated costimulation can occur in the absence of PD-1 signaling using two different experimental approaches (Subudhi et al., 2004). The identification of this receptor became a task for many research groups in this field.

In summary, it seems that the dual nature of B7-H1 allows positive and negative regulation of T cell response which helps to keep activated T cells in balance.

# 2. Aim of this Thesis

To date, a large number of studies have investigated and demonstrated the regulatory function of B7-H1 (PD-L1) with respect to T cell immune reactions. Depending on the experimental set up, costimulatory or co-inhibitory functions of B7-H1 was suggested, but the signals that enhance or suppress T cell response was assumed to be induced by complementary binding partners, such as PD-1 and B7.1 (CD80). Therefore, little is known about the functions of the B7-H1molecules that are expressed on T cells.

The aim of this PhD work was to analyze and determine the regulatory functions of the B7-H1 (PD-L1) in T cell immune response and to show the underlying molecular mechanisms, following infection with the influenza virus in our influenza mouse model.

1) In the first part of this thesis, the effect of B7-H1 in particular on T cell activation and proliferation in the draining lympnode (DLN) was examined during the early phase of infection by comparing antigen-specific T cell response in B7-H1$^{-/-}$ mice and WT. Furthermore, response and survival of activated antigen-specific effector T cells in various tissues, including the peripheral tissues such as the lung and the bronchoalveolar lavage (BAL) was determined.

2) In the second part of the thesis, the influence of B7-H1 on T cell priming by antigen-presenting cells (APCs) was examined by analyzing dendritic cell maturation and dendritic cell-mediated T cell activation during influenza infection.

3) In the final part of this thesis, the attempt was made to identify the counterpart molecule that interacts with B7-H1.

# 3. Materials and Methods

## 3.1 MATERIALS

### 3.1.1 Laboratory equipment

(*listed in alphabetical order*)

| | |
|---|---|
| Aluminum Foil (Heavy) | (Reynolds) |
| Centrifuge (micro) Allegra 6 series | (Beckman Coulter) |
| Centrifuge (micro) Allegra X-15xr | (Beckman Coulter) |
| Centrifuge 5415 table top | (Eppendorf) |
| Centrifuge (ultra) | (Beckman Coulter) |
| Conical tubes | (Falcon) |
| Dialysis cassette | (Thermo scientific) |
| Dissection tools | (Roboz) |
| FACS cytometer, FACScalibur | (BD) |
| FACS cytometer, LSRII | (BD) |
| FACS tubes | (BD Biosciences) |
| Freezer (-20ºC) | (Thermo) |
| Freezer (-80ºC) | (Forma Scientific) |
| Fridge (4ºC) | (Marvel) |
| Hoods | (Sterile Guard) |
| Ice maker | (Hoshizaki) |
| Incubator Shaker | (New Brunswick scientific) |
| Kimwipes | (Kimberly Clark) |
| Latex Gloves | (Microflex) |
| MACS cell separation kits | (Miltenyi Biotec) |
| MACS cell separation columns | (Miltenyi Biotec) |

Microscope                                          (Nikon)

Microscope                                          (Vista vision)

Microscpoe glas slides                              (VWR)

Microwave                                           (GE)

Needle (27-G and 28-G)                              (BD)

Neubauer Hematocytometer                            (Hausser Scientific)

Papertowel                                          (Kimberly Clark)

Parafilm                                            (Parafilm)

PCR tubes                                           (Falcon)

PH meter                                            (Beckmann)

Pipet aid                                           (Drummond)

Pipetman                                            (Gibson)

Pipet tips                                          (Neptune)

Plastic bottles                                     (Nalgene)

Power supply Power                                  (Biorad)

Reaction tubes                                      (Greiner Bio-One)

Scale                                               (Metler Toledo)

Scale (micro)                                       (Metler Toledo)

Seal wrap                                           (Fisher)

Stirrer                                             (Corning)

Syringe (0.5 and 1ml)                               (UltiCare)

Waterbath                                           (Precision)

Waterbath                                           (VWR)

Vortex genie                                        (Fisher)


## 3.1.2 Cell culture products

Standard tissue culture flasks - 75cm2             (Greiner Bio-One)

96-well U-bottom with lid – Standard TC             (BD)

96-well V-bottom with lid – Standard TC             (BD)

96-well flat-bottom with transwell insert          (Corning)

12-well flat-bottom with lid – Standard TC         (BD)

6-well flat-bottom with lid – Standard TC               (BD)

50ml conical tubes                                      (Falcon)

15ml conical tubes                                      (Falcon)

5 ml round-bottom polypropylene test tube               (BD)

5 ml round-bottom polystyrene test tube with

cell strainer                                           (BD)

0,6 ml round-bottom test tube                           (Greiner)

Eppendorf tubes                                         (Eppendorf)

70 µm cell strainer Falcon                              (BD)

40 µm cell strainer Falcon                              (BD)

Cryovial®, 2ml sterile                                  (Roth)

NalgeneTM Cryo 1°C Freezing Container                   (Nunc)


### 3.1.3 Cell culture media


RPMI 1640 (1x) w/o L-Glutamine                          (made at MIT)

D-MEM (1x) (High Glucose) with L-Glutamine,

mg/L D-Glucose, w/o sodium pyruvate                     (made at MIT)

D-PBS (1x) w/o Ca, Mg, sodium bicarbonate               (made at MIT)

IMDM Iscoves Modified Dulbecco Medium (1x)              (GIBCO-Invitrogen)

Fetal Bovine Serum,                                     (GIBCO-Invitrogen)

Penicillin/Streptomycin-Solution 10000 U/ml

penicillin, 10000 µg/ml streptomycin                    (GIBCO-Invitrogen)

L-Glutamine 200 mM (100x), 29.2 mg/ml                   (GIBCO-Invitrogen)

β-mercaproethanol 50mM                                  (GIBCO-Invitrogen)

Trypsin -EDTA (1x) HBSS w/o $Ca_2$+/$Mg_2$+ w/ EDTA     (GIBCO-Invitrogen)

Dimethylsulphoxide   (DMSO)                             (Sigma-Aldrich)

Cell Dissociation Solution Non-enzymatic 1x             (Sigma-Aldrich)

DME HEPES                                               (Gibco-Invitrogen)

2x F12                                                  (Invitrogen)

Agar                                                    (USB Corp)

BSA                                                     (Sigma-Aldrich)

1% DEAE Dextran                                                    (BioChemica)

## 3.1.4 Solutions

ACK (RBC) lysis buffer (in 100ml $H_2O$)          0.829 g NH4Cl

                                                          0.1 g $KHCO_3$

                                                           0.38 mg EDTA, pH 7.3

FACS buffer (1x PBS)                              0.02 % (v/v) $NaN_3$ in PBS

                                                           1% FCS

                                                           0.5 mM EDTA

Freezing medium (1x FCS)                          10% DMSO

MACS buffer (1x PBS)                              1% FCS

                                                           0.5 mM EDTA

                                                           0.1% $NaN_3$

Primary cell culture medium                       10% FCS
(1x RPMI)                                         2 mM L-glutamine

                                                           100 U/ml Penicillin

                                                           100 µg/ml Streptomycin

                                                           1 mM Sodium Pyruvate

                                                           1x Non-essential amino acids

                                                           0.25 mM β-mercaptoethanol

PBS (10x)                                         1.37 M NaCl

                                                           27 mM KCl

                                                           100 mM $Na_2HPO_4$ (anhydrous)

                                                           20 mM $KH_2PO_4$

| | |
|---|---|
| HBSS buffer | 0.137M NaCl |
| | 5.4mM KCl |
| | 0.25mM $Na_2HPO_4$ |
| | 1.3mM $CaCl_2$ |
| | 1.0mM $MgSO^4$ |
| | 4.2 mM $NaHCO_3$ |

## 3.1.5 Chemicals

| | |
|---|---|
| Avertin (2,2,2 ribromomethanol) | (Sigma-Aldrich) |
| Carboxyfluorescein (CFSE) | (Invitrogen) |
| Collagenase A and D | (Roche Diagnostics) |
| DNAse I | (New England Biolabs [NEB]) |
| Percoll | (Sigma-Aldrich) |
| Trypan blue | (Sigma-Aldrich) |

## 3.1.6. Antibodies and peptide

| Specifity: | Conjugate: | Isotype: | Source: |
|---|---|---|---|
| 1B2 (2C TCR specific Ab) | Biotinylated | rat-IgG | MIT |
| Annexin V | PE | | eBiosciences |
| B7-H1 | PE | rat-IgG2a | eBiosciences |
| CD4 | FITC and PE | rat-IgG2b | BD |
| CD8 | FITC and PE | rat-IgG2b | BD |
| CD11c | APC | Armenian Hamster-IgG | BD |
| CD25 | PE | rat-IgM | BD |
| CD40 | PE | rat-IgG2a | eBiosciences |
| CD69 | PE | rat-IgG1 | BD |

| | | | |
|---|---|---|---|
| CD80 | PE | rat-IgG2b | eBiosciences |
| CD86 | PE | rat-IgG2b | eBiosciences |
| Fc Blocking Ab (CD16/CD32) | | | BD |
| Mouse IgG$_1$ | PE | | eBiosciences |
| PD-1 | PE | Armen. Hamster-IgG | eBiosciences |
| Vβ11 (F5 TCR specific) | FITC | rat-IgG | BD |
| H-2K$^b$-Ig Fusion protein | | mouse IgG | BD |
| Soluble SIYRGYYGL (SIY)-peptide | | | (MIT Biopolymers) |

### 3.1.7 Cell lines

Madin-Darby canine kidney (MDCK) cell line was used for virus production and plaque assay.

### 3.1.8 Magnetic cell sorting (MACS) beads and columns

| | |
|---|---|
| B cell isolation kit | Miltenyi Biotec |
| CD4 T cell isolation kit | Miltenyi Biotec |
| CD8 T cell isolation kit | Miltenyi Biotec |
| CD11$^+$ DC isolation kit | Miltenyi Biotec |
| LS Column | Miltenyi Biotec |
| Collecting tubes (13ml) | Miltenyi Biotec |

### 3.1.9 Viruses

For intranasal infection (i.n.), recombinant WSN-SIY influenza ($6 \times 10^4$ pfu/ml) encoding the SIY epitope in the neuroaminidase stalk was constructed as described previously and for intraperitoneal infection (i.p.), influenza A/WSN/33 (WSN) ($5 \times 10^6$ pfu/ml) virus was used. Aliquots of both viruses were stored at -80°C.

## 3.2 MICE

Six-week-old male C57BL/6 mice (B6) were purchased from Taconic Farms. B7-H1 knock out mice was kindly provided by Prof Lieping Chen (Maryland, MD). 2C TCR transgenic mice on the RAG1$^{-/-}$ and C57BL/6 background, expressing transgenic TCR on CD8 T cells that recognizes the SIYRYYGL peptide (SIY), were maintained in the animal facilities at Massachusetts Institute of Technology (MIT). The 2C B7H1$^{-/-}$ mice were obtained by backcrossing between 2C TCR transgenic mice and B7-H1$^{-/-}$ mice. RAG-deficient F5 transgenic mice (13) express T cell receptor for influenza nucleoprotein (NP) peptide 366-374. CD80$^{-/-}$ (B7.1$^{-/-}$) mice on B6 background were purchased from Taconic.Mice were used at the age of 8-12 weeks. All mice were maintained at the MIT animal facilities and used according to the guidelines of the MIT Committee on Animal Care.

## 3.3. METHODS

### 3.3.1 Virus production and Plaque Assay

6-well plates were seeded with $7x10^5$ MDCK cells/well and incubated over night at 37°C and 5% $CO_2$. The next day, the cells were washed once with PBS. Virus supernatant was diluted in a 1:10 dilution series in serum-free DMEM/F12 medium. 250 µl of virus dilution was added per well. The cells were incubated with the virus for 60 min at room temperature (RT) to allow virus adsorption. The medium was removed and the cells were washed once with PBS. Each well was overlayed with 3 ml of melted oxoid agar. Once the agar had solidified, the plates were incubated for 2-3 days at 37°C and 5% $CO_2$.

## 3.3.2 Cell culture methods

MDCK cells were grown in DMEM supplemented with 4.5 g/l glucose, 25 mM HEPES, 2 mM glutamine, 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin, and cultured at 37°C in 5% CO2. Depending on the purpose, cells were grown on plastic dishes with different sizes (Greiner, Germany), or on 24 mm Transwell (Costar, Cambridge, MA) polycarbonate filters (0.4 µm pore size) when experiments with polarized cells were performed. For conditioning of cells, serum-free medium (DMEM, 0.1-0.005% (w/v) BSA, antibiotics) was used. Cells were washed two times with PBS and starved in serum-free medium for 2 h at 37°C. After removal of the media, conditioning was carried out with fresh medium for 24-72 h at 37°C.

## 3.3.2.1 Determination of cell number (counting)

An aliquot of cell suspension was diluted 1:1 or 1:10 (if from spleen and liver) with trypan blue solution (0.05 % w/v) to distinguish dead cells and live cells were counted with a Neubauer counting chamber (0.1 mm depth). The number of live cells per ml is calculated as following:

*Number of cells per ml = Number of cells counted per mm$^2$ X dilution X 10$^4$*

## 3.3.2.2 Splitting of adherent cells

For the splitting of adherent cells, the culture medium was removed from the flasks, the cells were washed once with pre-warmed PBS, and Trypsin-EDTA or Cell Dissociation Solution was added in sufficient amount to cover the cell layer. Cells were then incubated at 37°C for ~5 min, checking in parallel the progress of detachment under the microscope. After complete detachment, culture medium was added to the flasks, cells

were collected in Falcon tubes and centrifuged (1200 rpm, 10 min, RT). Cells were subsequently splitted at the appropriate ratio into new flask or used in experiments.

### 3.3.3 Isolation of organs and preparation of single cell suspensions

### 3.3.3.1 Lung

Mice were euthanized by an atraumatic method such as anesthetic overdose by $CO_2$ narcosis. Before isolating the lung, fluid from the bronchoalveolar lavage (BAL), containing lymphnodes, was collected by cutting a small hole into the trachea and inserting a catheter. The BAL was flushed with RPMI medium three times using a 1ml insuline syringe.

To isolate lung lymphoid cells, the chest of the mouse was opened, the lung vascular was excised avoiding the peritracheal lymphnodes, then washed twice with PBS. The Lung were then cut into little pieces, and incubated in RPMI + 5% FCS, containing 2mg/ml collagenase A and 1 g/ml DNase (Type I). A volume of 25 ml was used for four to six sets of lungs. After incubation for 60 min at 37°C on a rotary agitator (approximately 60 rpm) at 37°C, any remaining intact tissue was disrupted by passage through a 21-gauge needle. Tissue fragments and majority of the dead cells were removed by a 250-mm mesh screen, and cells were collected by centrifugation. The cell pellet was suspended in 4 ml of 40% Percoll and layered onto 4 ml of 70% Percoll, then centrifuged (2,500 rpm, 4°C, 30 min). Cells from the interphase was collected and resuspended in RPMI + 5% FCS.

### 3.3.3.2 Liver

Mice were euthanized with overdose by $CO_2$ narcosis. Then the abdomen was opened rapidly, the intestine to the mouse's left was displaced and the hepatic portal vein was cannulated with a 27-G needle on a 5-ml syringe. The portal vein was perfused with 5 ml ice-cold PBS and drained by cutting the inferior vena cava above the liver until it turned

pale. Subsequently, the liver was excised, the gall bladder was removed and cut into segments with scissors, then homogenized using a sieve and plunger. The slurry from a single liver was transferred to a 50-ml centrifuge tube. To each liver slurry, 40 ml ice-cold RPMI + 5% FCS was added and centrifuged for 10 min at 300 × g (1200 rpm), 4°C. Then, the pellet was resuspended in 10 ml medium containing collagenase A (2mg/ml) and placed in water bath for 40 min at 37°C. After digestion, 10ml of 70% percoll (v/v) was added and centrifuged at 1500 × g (2500 rpm), 4°C, allowing a density gradient separation of the cells. Lymphocytes from the interphase was collected and resuspended in 10ml RPMI + 5% FBS.

### 3.3.3.3 Spleen and lymphnodes

Spleen and peripheral lymphnodes were excised using sterile forceps and scissors and kept in ice-cold PBS + 5% FCS medium. Single cell suspensions were obtained by mincing the spleen or LN through a 40 μm cell strainer or using frosted glass slides. To remove large debris, cell suspension was pipetted through a Nylon mesh filter (0.2μm).

### 3.3.3.4 Red blood cell (RBC) lysis

For the lysis of erythrocytes (red blood cells), splenocytes and cell cells from the lung and liver were treated with buffered ammonium chloride potassium phosphate solution (ACK-buffer) for 3 mins at RT and then washed with RPMI + 5% FCS at 350 x g (1400 rpm, 10 min, 4°C). Cells were resuspended in RPMI + 5% FCS.

### 3.3.4 Adoptive cell transfer and infection

Unless otherwise mentioned, 1 x $10^6$ cells were washed once with cold HBSS buffer and resuspended in 100μl HBSS buffer. Mice were anaesthetized using Avertin and cells were injected (i.v.) retro-orbital with a 27-G insulin needle. On the following day (24 hrs

later), mice were again anaesthetized and infected intranasal with influenza virus diluted in PBS. For the infection of RAG-1$^{-/-}$ mice, virus was injected intraperitoneal.

### 3.3.4.1 CFSE labeling of T cells

For the proliferation assay, donor cells were labeled with 5µM CFSE at room temperature (in the dark) for 10 minutes and then washed twice with PBS before adoptively transferred into mice.

### 3.3.4.2 CFSE labeling of DCs (in situ CFSE labeling)

A stock solution of 25mM CFSE (Invitrogen) in DMSO was prepared and stored in -20°C until use. For experimental use, the CFSE stock solution was diluted in Iscove's Media to a concentration of 8mM and given (50µl/mouse) i.n. to the mice, 6 hours before infection.

### 3.3.4.3 Transfer of CD4, CD8 and B cells

Mice were anesthetized with 2,2,2 Tribromoethanol (Avertin) and 100 µl of T cell or B cell suspension was injected intravenous (i.v.) into mice, one day before infection. For the F5 CD8$^+$ T cell transfer, a single cell suspension from the lymphnodes of F5 transgenic mice were isolated and purified using the CD8$^+$ isolation kit (Miltenyi Biotec). Various numbers of purified F5 CD8$^+$ T cells were injected i.v. into mice one day before infection.

### 3.3.4.4 Transfer of dendritic cells

Dendritic cells (DCs) from freshly isolated spleens of F5 mice or B7-H1$^{-/-}$ mice were treated with 2µg/ml collagenase D and 25µg/ml DNAse I and purified (purity ≥90%) by magnetic sorting with anti-CD11c MACS beads. For adoptive transfer, 3-5x10$^5$ dendritic

cells were suspended in 100µl HBSS and injected subcutaneous (s.c.) into both hind footpads (50µl each) of mice, one day before infection.

### 3.3.5 Infection of mice

Mice were anesthetized with 2,2,2 Tribromoethanol (Avertin) and infected either intranasal (i.n.) with 100pfu WSN-SIY virus in 50µl PBS or intraperitoneal (i.p.) with $10^6$ pfu WSN virus in 1ml PBS. For the CFSE instillation experiment, mice were infected i.n. with 1000pfu WSN-SIY virus

### 3.3.6 Fluorescence activated cell sorting (FACS)

### 3.3.6.1 FACS staining and analysis

For staining of cells, 1-5 x $10^6$ cells were washed once with FACS buffer 350 x g (1400 rpm, 10 min, 4°C) and incubated with Fc blocking (CD16/CD32) antibody for 10 min on ice to block Fc receptors. Subsequently, the primary antibodies were added and cells were further incubated for 30 min at 4°C. For biotinylated antibodies, cells were washed once with FACS buffer and stained with conjugated streptavidin for additional 30 min at 4°C. After staining, cells were washed once, resuspended in FACS buffer + PI (propidium iodide) to stain dead cells and fluorescence was assessed by a FACSCalibur or LSRII flow cytometer and data were analyzed with the CellQuest software.

## 3.3.7 MACS sorting

## 3.3.7.1 T cell isolation

CD8$^+$ T cell purification:

The CD8$^+$ T cells are indirectly magnetically labeled with a biotin-conjugated antibody cocktail binding to non-CD8$^+$ cells and allowing the isolation of untouched CD8$^+$ T cells. Up to 2x10$^9$ Cells from murine lymph nodes or spleens were resuspended in MACS buffer and incubated with a biotin-conjugated antibody cocktail for 10 mins (4°C) in the dark. Then anti-biotin microbeads were added and incubated for an additional 15 mins (4°C). Magnetic labeled cells were washed once with MACS buffer and separated using the MACS separator. The negative (unlabelled) fraction of the cells was collected. This fraction contains usually >90% CD8$^+$ T cells.

CD4$^+$ T cell purification:

The CD4$^+$ T cells are indirectly magnetically labeled with a biotin-conjugated antibody cocktail binding to non-CD4$^+$ cell. Up to 2x10$^9$ Cells from murine lymph nodes or spleens were resuspended in MACS buffer and incubated with a biotin-conjugated antibody cocktail for 10 mins (4°C) in the dark. Then anti-biotin microbeads were added and incubated for an additional 15 mins (4°C). Magnetic labeled cells were washed once with MACS buffer and separated using the MACS separator. The negative (unlabelled) fraction of the cells was collected. This fraction contains usually >90% CD4$^+$ T cells.

## 3.3.7.2 B cell isolation

Cell suspensions from murine spleen are labeled with B cell-specific antibodies coupled to magnetic beads and passed over an isolation column. The positive fraction is retained as purified B cells. B cells then were adoptively transferred into mice.

### 3.3.7.3 Dendritic cell isolation

The CD11c$^+$ DCs cells are directly magnetically labeled with a biotin-conjugated antibody specific to CD11c$^+$ DC cells. Up to 2x10$^9$ Cells from murine lymph nodes or spleens were resuspended in MACS buffer and incubated with CD11c-microbeads for 15 mins (4°C) in the dark. Magnetic labeled cells were washed once with MACS buffer and separated using the MACS separator. The positive fraction of the cells was collected. If needed, the separation step was repeated. This fraction contains usually >95% CD11c$^+$ DCs.

### 3.3.8 Statistical analysis

Differences between groups were calculated using standard Student's *t* test. Values of $p \leq 0.05$ were considered to be statistically significant.

# 4. Results

# 4. Results

## 4.1 Murine WSN and WSN-SIY influenza virus

### 4.1.1 Production and titration of the recombinant influenza virus

In order to determine the function of B7-H1 in regulation of CD8 T cell immune response in this study, we utilized influenza virus infection in our mouse models. Different mouse strains were infected primarily with a recombinant WSN strain of influenza A virus (H1N1), also known as A/WSN/33 (WSN) strain, that expresses a CD8 epitope SIYRYYGL (SIY) (referred to as WSN-SIY). Then the response of SIY-specific transgenic and endogenous CD8 T cells to this virus was analyzed.

As infection of mice with influenza virus is an essential part in most of the experiments done in this study, a sufficient quantity of the WSN-SIY and WSN virus (needed for some experiments) was produced and the viral titer for both viruses was determined by performing a plaque assay. In order to produce a large quantity of the viruses, MDCK (<u>M</u>adin <u>D</u>arbey <u>C</u>anine <u>K</u>idney) cells (7 x $10^5$ cells per well) were infected with different concentrations of the WSN and WSN-SIY virus for one hour at room temperature (RT) before the cells were washed and covered with a thin layer of melted oxoid agar. The solidified agar permits live viruses to infect only neighboring cells and to form visible plaques. The infected MDCK cells were then incubated at 37ºC for 2-3 days until clear plaques were visible. By counting the plaques and multiplying the number by the associated dilution factors, we determined the viral titer (pfu/ml) and collected the newly produced live viruses. The maximum concentration of the WSN-SIY virus (8 x $10^4$ pfu/ml) was yielded two days post infection (dpi) and dropped almost by half (4.4 x $10^4$ pfu/ml) on day three. Similarly, the concentration of the WSN-virus was maximal on day two post infection (about 5 x $10^6$ pfu/ml) and dropped dramatically on day three. Only viruses that were collected on day two post infection were used in the course of this work, as vitality and viability of viruses collected at later time points may be impaired.

## 4.2 The role of B7-H1 in T cell immune response against influenza virus

In this first part of the study, the immunoregulatory role of B7-H1 in the acute T cell response, following influenza infection, was determined by comparing the responses of both endogenous and adoptively transferred CD8 T cells in B7-H1-knockout (B7-H1$^{-/-}$) and B7-H1$^{+/+}$ wildtype (WT). Moreover, by backcrossing B7-H1 null mice with 2C-TCR transgenic mice we were able to characterize the function of T cell-expressed B7-H1 during the early phase of the immune reaction. Overall, the results that were obtained in this part of the thesis demonstrated that B7-H1 expression in the lung negatively influences T cell response and promotes apoptosis in T cells. This finding supported the scientifically documented inhibitory role of peripheral B7-H1 expression. However, further experiments examining the immune response of endogenous as well as transgenic B7-H1$^{-/-}$ T cells have revealed a novel stimulatory function of B7-H1 molecules that are expressed on T cells.

### 4.2.1 Enhanced transgenic CD8 T cell response in B7-H1$^{-/-}$ mice

After antigen encounter, T cells generate cell-mediated immune responses which target the intracellular pathogens. Efficient T cell immune response underlies a well balanced interplay of co-regulatory molecules. CD8 T cells from the lymphnodes of 2C TCR (T cell receptor) (2C) transgenic RAG-1$^{-/-}$ (recombination activating gene-1) mice on the C57BL/6 (B6) background were isolated, RBC (red blood cells) were lysed and CD8$^+$ T cells were purified using antibody-linked magnetic beads. 1 x 10$^6$ naïve 2C CD8 T cells were adoptively transferred i.v. (intravenous) into wildtype C57BL/6 (WT) mice and B7-H1$^{-/-}$ mice. On the following day, all mice were infected i.n. (intranasally) with a sublethal dose (100pfu) of WSN-SIY virus. Seven, 14, and 30 dpi (days post infection), the frequencies and numbers of 2C CD8 T cells in both lymphoid and non-lymphoid organs were determined by flow cytometry staining with antibodies (Ab) for CD8 and 2C TCR.

By seven days post infection, when the CD8 T cell response to influenza has reached the peak level, both frequency and the number of 2C CD8 T cells in the respiratory tissues, including bronchoalveolar lavage (BAL) and the lung parenchyma,

were significantly higher in B7-H1$^{-/-}$ mice than in WT mice, as shown in **Figure 4-1**. The response of 2C CD8 T cells in the other tissues that we have analyzed was not as dramatic but 2C CD8 T cell response in the liver and spleen of B7-H1$^{-/-}$ mice were also somewhat higher than in WT mice.
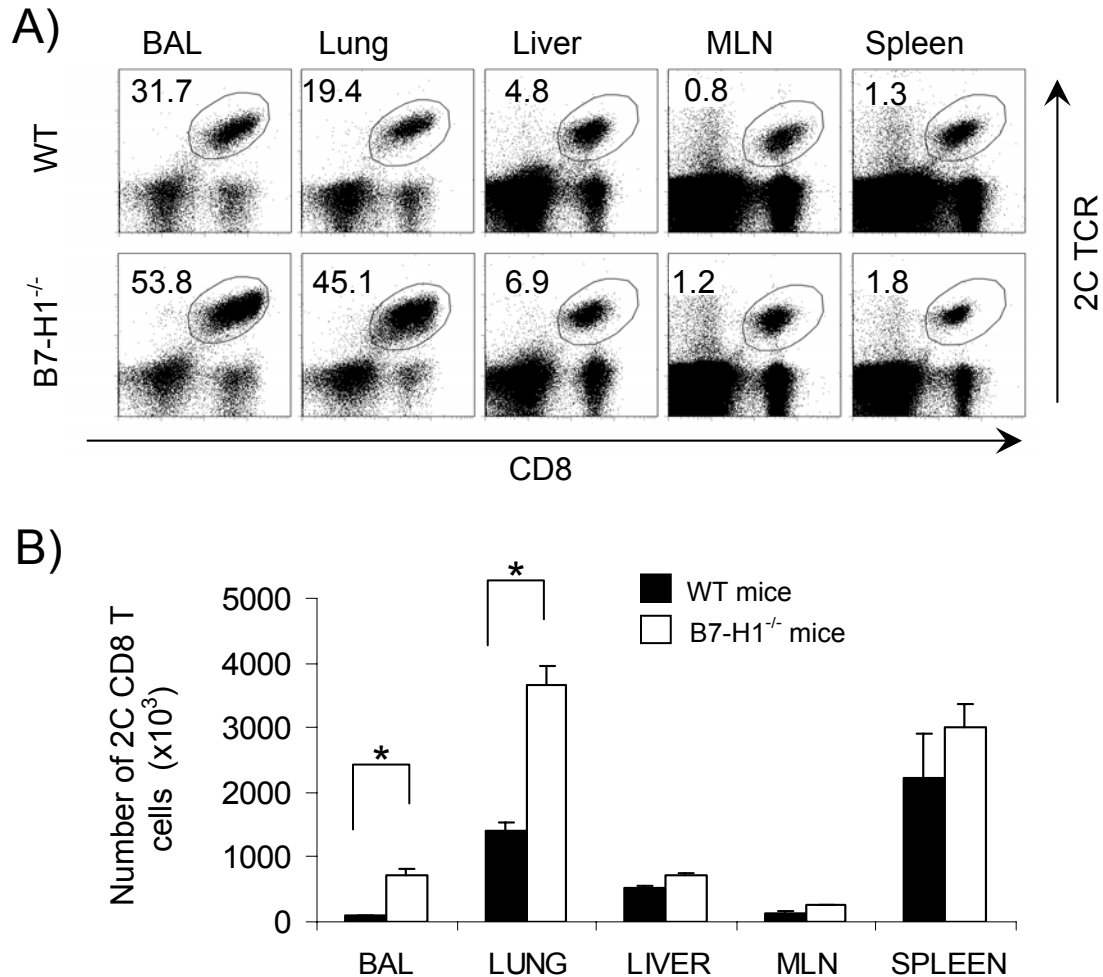


**Figure 4-1: Enhanced expansion of 2C CD8 T cells 7 dpi in B7-H1$^{-/-}$ mice.**

$10^6$ naïve 2C CD8 T cells were transferred into WT mice and B7-H1$^{-/-}$ mice infected with 100pfu WSN-SIY virus on the following day. 2C CD8 T cells from various tissues were assayed 7 dpi by staining with anti-CD8 and 1B2 antibody (2C TCR staining). A) 2C TCR versus CD8 staining profiles of live cells from the various tissues from WT mice (top row) and B7-H1$^{-/-}$ mice (bottom row) are show The numbers indicate the percentage of CD8$^+$ 2C TCR$^+$ cells among all live cells. B) The number of 2C CD8 T cells in different tissues, calculated by multiplying the total cell number with percentage of CD8$^+$ 2C TCR$^+$ cells in specific tissue. Values represent the mean of three mice ± SEM (Standard error of the mean) (n=3). *p<0.05 (Student's t-test)

The experimental procedure from above was repeated and 2C CD8 T cell response in B7-H1$^{-/-}$ and WT mice was analyzed, 14 and 30 days post infection. Although number and frequency of 2C CD8 T cells dropped 14 dpi in both mice, the frequency and the number of 2C CD8 T cells in the BAL and the lung parenchyma were still significantly higher in B7-H1$^{-/-}$ than WT mice (**Figure 4-2**).
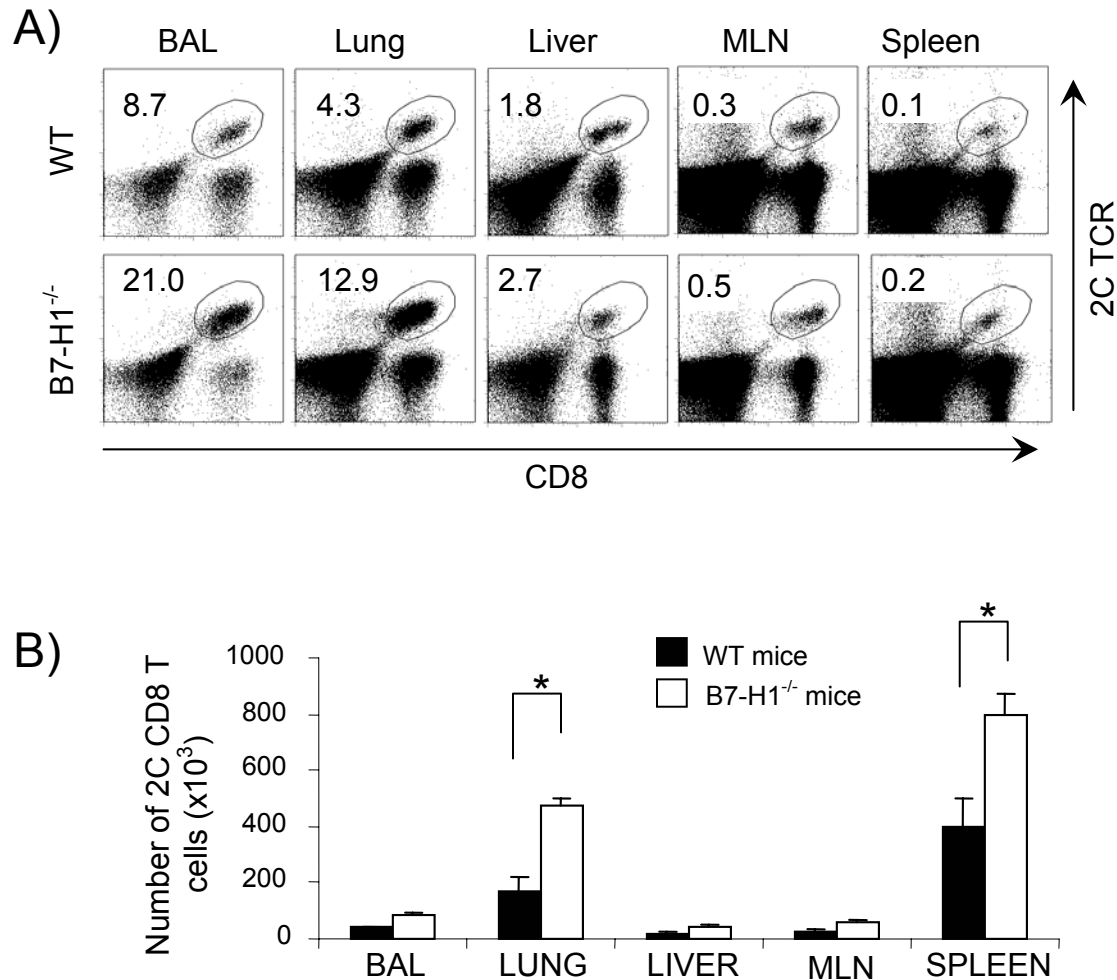


**Figure 4-2: Increased frequency and number of 2C CD8 T cells 14 dpi in B7-H1$^{-/-}$ mice.**
WT mice and B7-H1$^{-/-}$ mice were infected with 100pfu WSN-SIY virus and 2C CD8 T cells from various tissues were assayed 14 dpi by staining with anti-CD8 and 1B2 antibody. A) 2C TCR versus CD8 staining profiles of live cells from the various tissues from WT mice (top row) and B7-H1$^{-/-}$ mice (bottom row). The numbers indicate the percentage of CD8$^+$ 2C TCR$^+$ cells among all live cells. B) The number of 2C CD8 T cells in different tissues, calculated by multiplying the total cell number with percentage of CD8$^+$ 2C TCR$^+$ cells in specific tissue. Values represent the mean of three mice ± SEM (n=3). * p<0.03.

Even 30 dpi where most of the responding T cells have died, the frequency and the number of the remaining 2C CD8 T cells in BAL and the lung parenchyma were still higher in B7-H1$^{-/-}$ mice than in WT mice (**Figure 4-3**). However, due to very low numbers of 2C CD8 T cells that could be isolated from both mice, the variations of the 2C CD8 T cell frequencies within the samples were somewhat high.
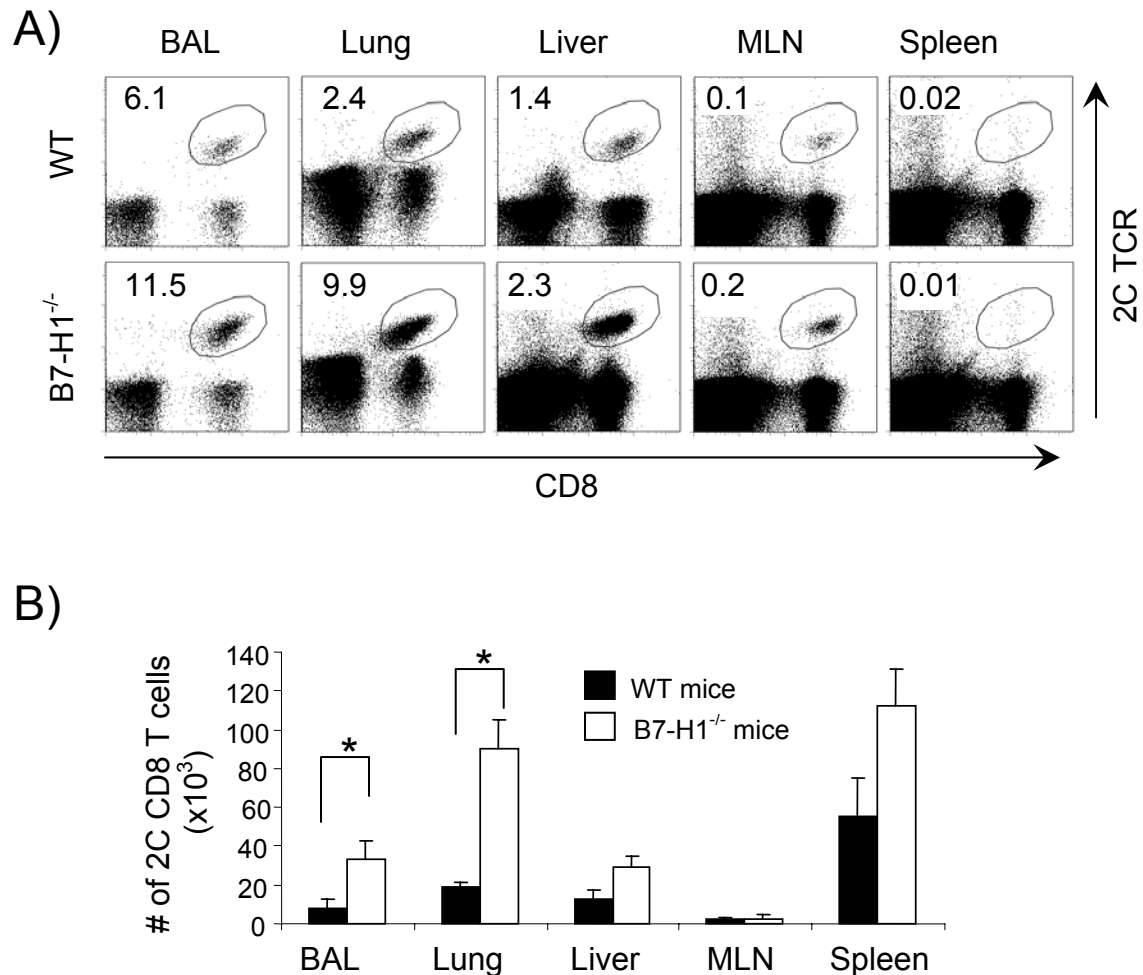


**Figure 4-3: Increased frequency and number of 2C CD8 T cells 30 dpi in B7-H1$^{-/-}$ mice.**
WT mice and B7-H1$^{-/-}$ mice were infected with 100pfu WSN-SIY virus and 2C CD8 T cells from various tissues were assayed 30 dpi by staining with anti-CD8 and 1B2 antibody. **A)** 2C TCR versus CD8 staining profiles of live cells from the various tissues from WT mice (top row) and B7-H1$^{-/-}$ mice (bottom row). The numbers indicate the percentage of CD8$^{+}$ 2C TCR$^{+}$ cells among all live cells. **B)** The number of 2C CD8 T cells in different tissues, calculated by multiplying the total cell number with percentage of CD8$^{+}$ 2C TCR$^{+}$ cells in specific tissue. Values represent the mean of three mice ± SEM (n=3).  * p<0.05.

These results demonstrated a tissue-specific enhanced 2C CD8 T cell expansion in B7-H1$^{-/-}$ mice after viral challenge. To further dissect the role of B7-H1 in the increased 2C CD8 T cell frequency and number in B7-H1$^{-/-}$ mice and to illustrate the detailed function of B7-H1 in this process, activation and proliferation of 2C CD8 T cells was analyzed during the early phase of infection. Again, 1 x 10$^6$ 2C CD8 T cells were transferred i.v. into WT mice and B7-H1$^{-/-}$ mice and infected with 100 pfu WSN-SIY virus 24 hours later. Four, 5, 6 and 7 days post infection, CD8 T cells from the MLN was isolated and assayed for CD25 (a marker for T cell activation) upregulation. The expression levels of CD25 for 2C CD8 T cells in both mice were similar for all four time points. On day 4 and 5 post infection, expression of CD25 was upregulated but dropped significantly on day 6 and was very low by day 7 (**Figure 4-4**).
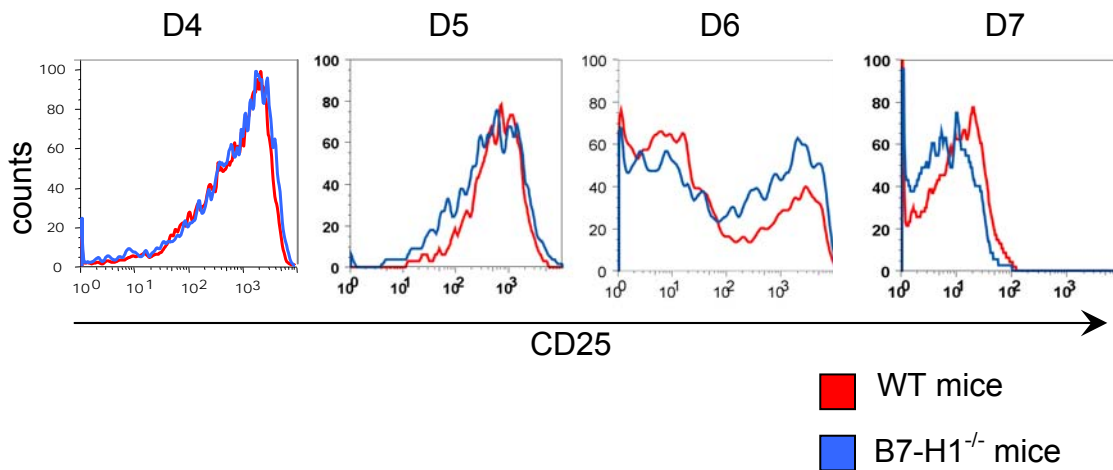


**Figure 4-4: Similar levels of CD25 expression for 2C CD8 T cells in B7-H1$^{-/-}$ and WT mice.**
WT mice and B7-H1$^{-/-}$ mice were infected with 100pfu WSN-SIY virus and 2C CD8 T cells from mediastinal lymphnode (MLN) were assayed on various dpi by staining with anti-CD8, 1B2 antibody and anti-CD25. The histograms show the intensity of CD25 expression of 2C CD8 T cells. Various mice were pooled for D4 and D5 and individual mice were used for D6 and D7.

Although the expression of CD25 (and presumably cellular activities) in both mice did not differ, it was still possible that the proliferation of 2C CD8 T cells in both mice have different intensities. To visualize proliferation, 2C CD8 T cells were labeled with the

CFSE (Carboxyfluorescein succinimidyl ester) dye and transferred i.v. into WT and B7-H1$^{-/-}$ mice. On the following day all mice were infected with 100 pfu WSN-SIY virus. Proliferation intensity of the cells was determined 3, 4 and 5 dpi, by analyzing CFSE dilution in 2C CD8 T cells, isolated from pooled MLNs. Furthermore, the number of the 2C CD8 T cells in both mice was calculated by multiplying the percentage of 2C TCR positive T cells with the total cell number. Both, cell number of the proliferating 2C CD8T cells as well as proliferation intensity were similar in both mice. These results demonstrated that there was no significant difference in initial activation and proliferation of 2C CD8 T cells in WT and B7-H1$^{-/-}$ mice (**Figure 4-5**).
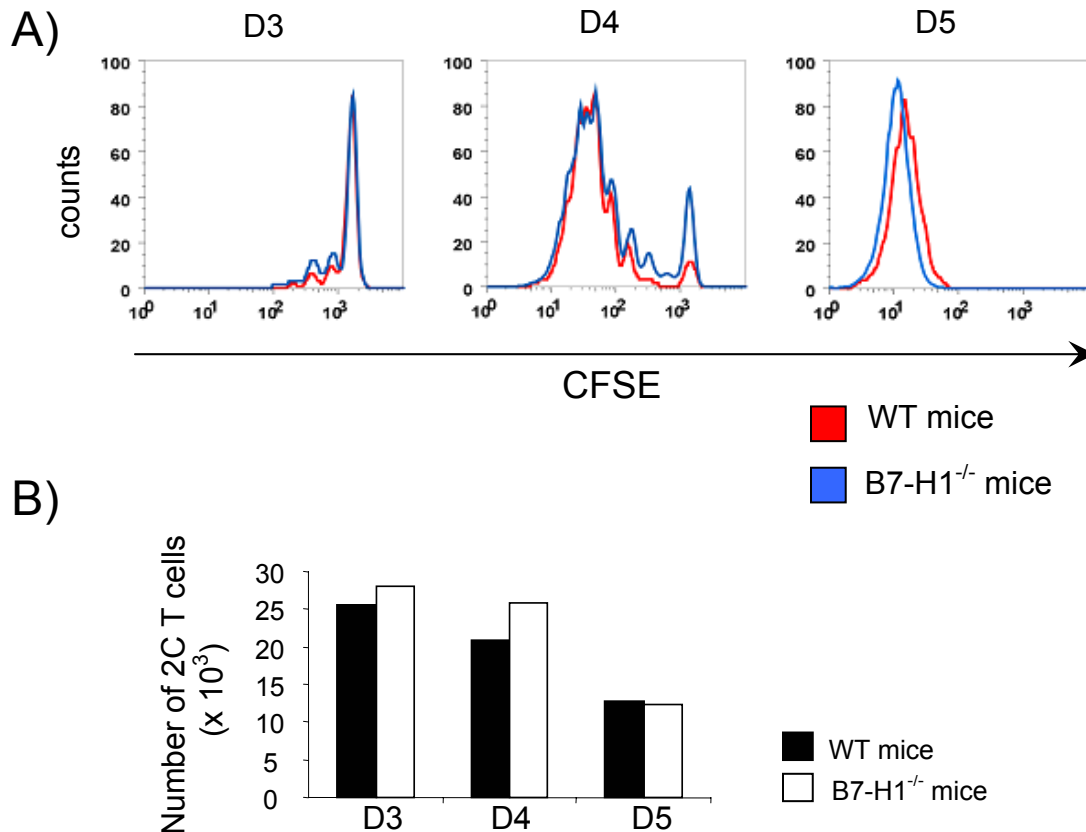


**Figure 4-5: Proliferation and 2C CD8 T cell number is similar in B7-H1$^{-/-}$ and WT mice.**
WT and B7-H1$^{-/-}$ mice were infected with 100 pfu WSN-SIY virus and CFSE labeled 2C CD8 T cells from MLN were assayed on various dpi by staining with anti-CD8, 1B2-Ab. A) The histograms show the CFSE dilution of 2C CD8 T cells on the specified days. B) Number of 2C CD8 T cells, calculated by multiplying total live cells from the MLN with percentage of CD8$^+$ 2C TCR$^+$ cells. Three mice were pooled for D3, two mice for D4 and individual mice were used for D5.

As 2C CD8 T cell activation and proliferation during early T cell immune response seems to be similar in both mice, other factors (e.g. cell survival, migration) might be affected by B7-H1, leading to enhanced 2C CD8 T cell expansion in B7-H1$^{-/-}$ mice.

PD-1 (Programmed death receptor-1) is a known receptor for B7-H1 and its expression is upregulated in activated T cells. Various published studies demonstrated PD-1-mediated inhibitory signaling and apoptosis after interaction with B7-H1 (Hori et al., 2006, Carter et al., 2002; Ha et al., 2008). Since interaction between B7-H1 and PD-1 in B7-H1$^{-/-}$ mice is impossible to occur, reduced PD-1 mediated apoptosis in these mice could explain our observations. To test this, 1 x 10$^6$ 2C CD8 T cells were transferred i.v. into WT mice and B7-H1$^{-/-}$ mice and infected with 100 pfu WSN-SIY on the following day. Seven dpi, cells from the lung (including the BAL), the MLN and the spleen were isolated and stained for Annexin V, a marker for apoptosis. Approximately 85-90% of 2C CD8 T cells residing in the lung of WT mice were positive for Annexin V, suggesting that the majority of these cells will most likely undergo apoptosis very soon. In contrast, a significant fraction (30-40%) of the 2C CD8 T cells in the lung of B7-H1$^{-/-}$ mice were negative for Annexin V, demonstrating prolonged survival and offering an explanation for the increased number of 2C CD8 T cells in these mice **(Figure 4-6)**. In both mice, the intensity of Annexin V staining for 2C CD8 T cells from the MLN and spleen were very low, indicating that apoptosis of 2C CD8 T cells occur predominantly in the lung tissue. This result was in accordance with other published data that have demonstrated the negative effect of B7-H1 on T cell response in peripheral organs, most likely by binding to the inhibitory PD-1 receptor that is upregulated on activated 2C CD8 T cells. Although prolonged survival of 2C CD8 T cells in B7-H1$^{-/-}$ mice seems to be one reason for enhanced T cell expansion, other factors (e.g. migration) may also be affected by B7-H1 and contribute to this phenomenon. But we have not further investigated these possibilities.
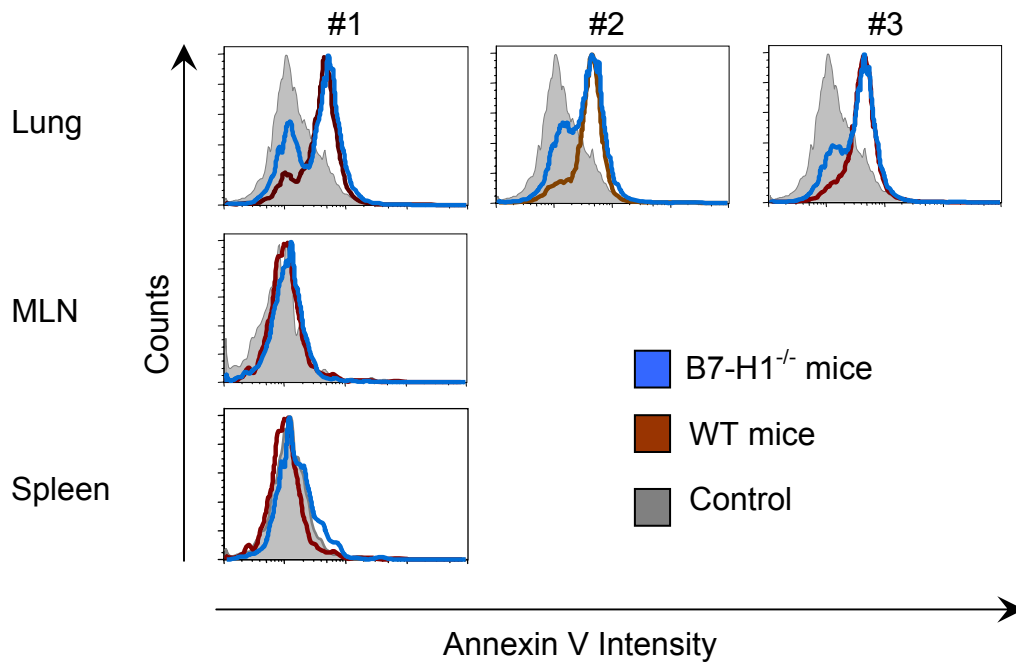
**Figure 4-6: 2C CD8 T cells in B7-H1^{-/-} show reduced apoptosis.**

WT and B7-H1^{-/-} mice were infected with 100pfu WSN-SIY virus and 2C CD8 T cells from the specified organs were assayed for apoptosis 7 dpi by staining with anti-CD8, 1B2-Ab and Annexin V. Histograms show the intensity of Annexin V in PI-negative live 2C CD8 T cells. As control, 2C CD8 T cells from uninfected WT mice were stained with Annexin V. (n=3). PI= propidium iodide (marker for dead cells).

## 4.2.2 Impaired response of antigen-specific endogenous CD8 T cells in B7-H1^{-/-} mice during initial phase of infection

To further probe the role of B7-H1 in the adaptive immune response we studied endogenous SIY specific CD8 T cells. These cells can be identifed by flow cytometry staining for CD8 and K$^b$:Ig fusion protein loaded with SIY peptide (SIY-K$^b$). We infected i.n. WT mice and B7-H1^{-/-} mice with 100 pfu WSN-SIY virus. Seven, 14, and 30 dpi, the frequency and number of SIY-specific CD8 T cells in both lymphoid and non-lymphoid organs were determined by flow cytometric analysis. In contrast to the response of 2C CD8 T cells, both the frequency and the number of SIY-specific CD8 T cells in the BAL and the lung parenchyma were significantly lower in B7-H1^{-/-} mice than in WT mice 7

days post infection. Although not as dramatic as the lung and the BAL, frequency and number of SIY-specific CD8 T cells in the spleen of B7-H1$^{-/-}$ mice were low as well. There was no significant difference in the frequency and number of SIY-specific CD8 T cells from the liver and MLN in both mice (**Figure 4-7**).
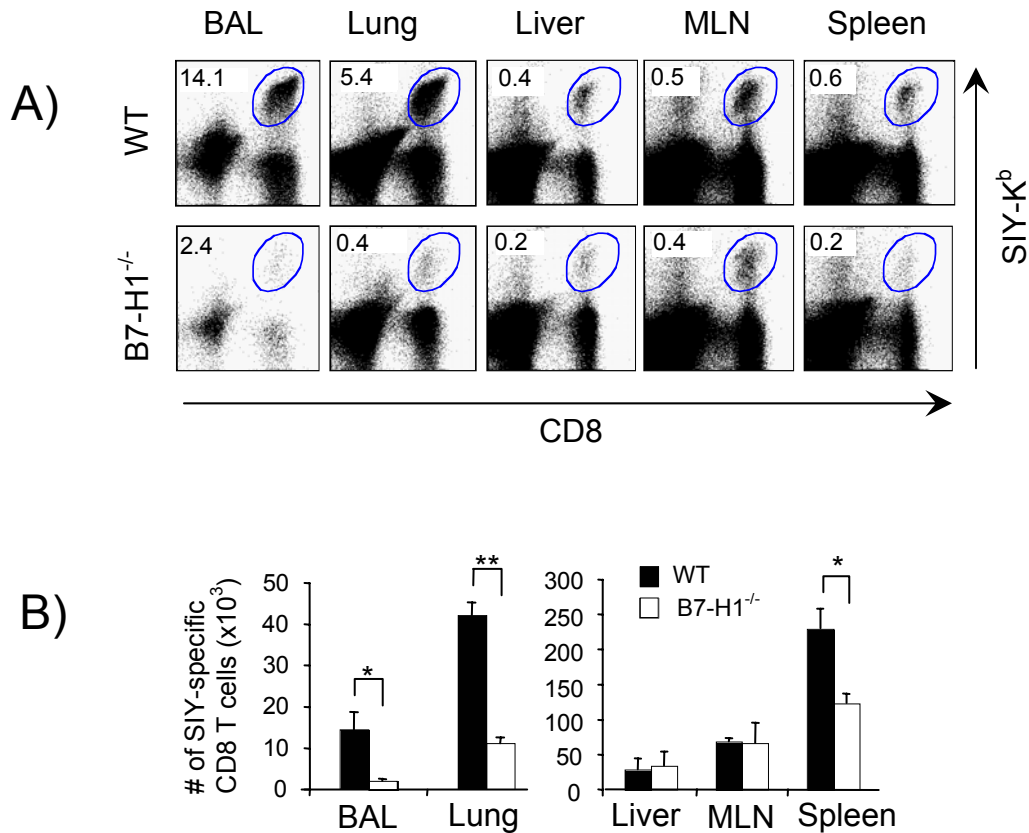


**Figure 4-7: Impaired expansion of endogenous SIY-specific CD8 T cells 7 dpi in B7-H1$^{-/-}$ mice.**
WT and B7-H1$^{-/}$ mice were infected with100 pfu WSN-SIY virus and 7 dpi SIY-specific CD8 T cells from various organs were assayed by staining with anti-CD8 and SIY-K$^b$. **A)** Representative SIY-K$^b$ versus CD8 staining profiles of live cells from the various tissues of WT (top row) and B7-H1$^{-/-}$ mice (bottom row). The numbers indicate percentages (n=5) of CD8$^+$ SIY-K$^{b+}$ T cells in the gated area among all live cells. **B)** Comparison of the number of SIY-specific CD8 T cells in different tissues, calculated by multiplying the total cell numbers with the percentage of CD8$^+$ SIY-K$^{b+}$ in the specific tissue. Values represent the mean ± SEM (n=5). * p<0.05, ** p<0.01.

By 14 days post infection, however, the frequency and the number of SIY-specific CD8 T cells in BAL, the lung parenchyma, and the spleen were similar between B7-H1$^{-/-}$ and WT mice (**Figure 4-8**).
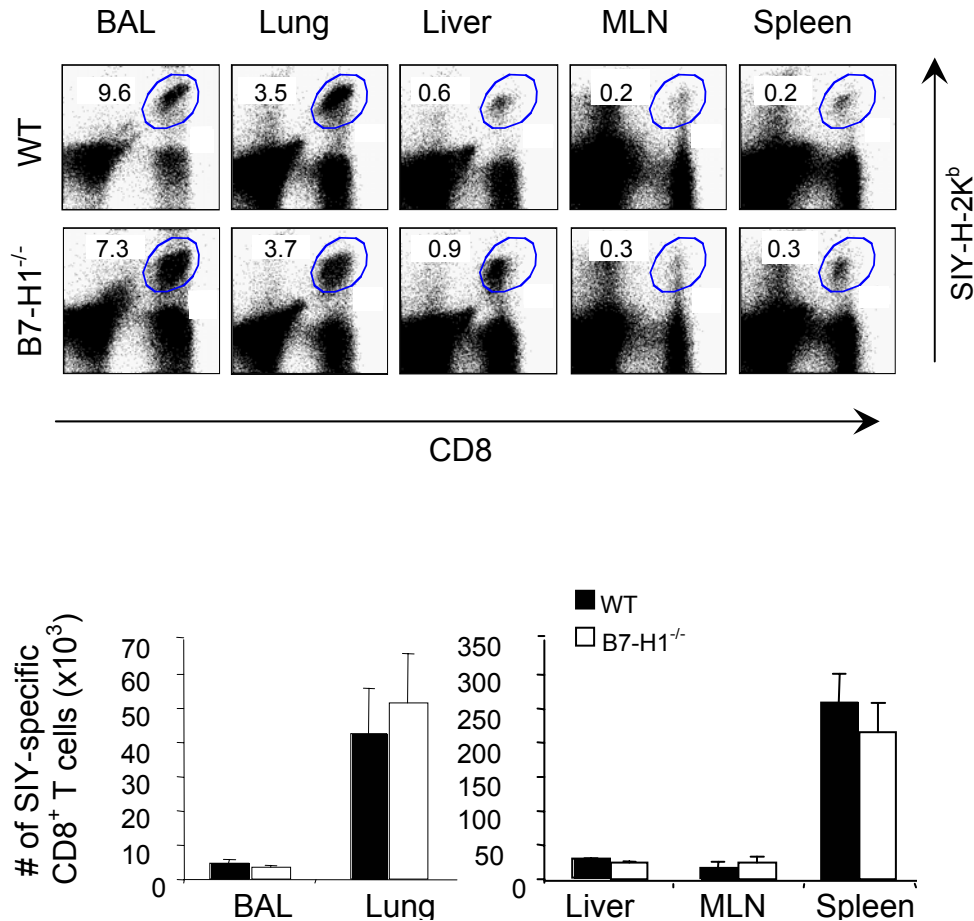


**Figure 4-8: SIY-specific CD8 T cell response in B7-H1$^{-/-}$ mice and WT mice is similar, 14 days post infection.**

WT mice and B7-H1$^{-/-}$ mice were infected with 100 pfu of WSN-SIY virus and 14 dpi, SIY-specific CD8 T cells from various tissues were assayed by staining with anti-CD8 and SIY-K$^b$. **A)** Representative SIY-K$^b$ versus CD8 staining profiles of live cells from the various tissues of WT (top row) and B7-H1$^{-/-}$ mice (bottom row) are shown. The numbers indicate percentages (n=3) of CD8$^+$ SIY-K$^{b+}$ T cells in the gated area among all live cells. **B)** Comparison of the number of SIY-specific CD8 T cells in different tissues, calculated by multiplying the total cell numbers with the percentage of CD8$^+$ SIY-K$^{b+}$ in the specific tissue. Values represent the mean ± SEM (n=3).

And by 30 dpi, the frequency and the number of SIY-specific CD8 T cells in the lung parenchyma and the BAL were somewhat higher again in B7-H1$^{-/-}$ than in WT mice. However, due to very low numbers of cells, the differences were significant only in the BAL (p<0.05) (**Figure 4-9**). There was no difference in SIY-specific T cell frequencies and numbers in other tissues.
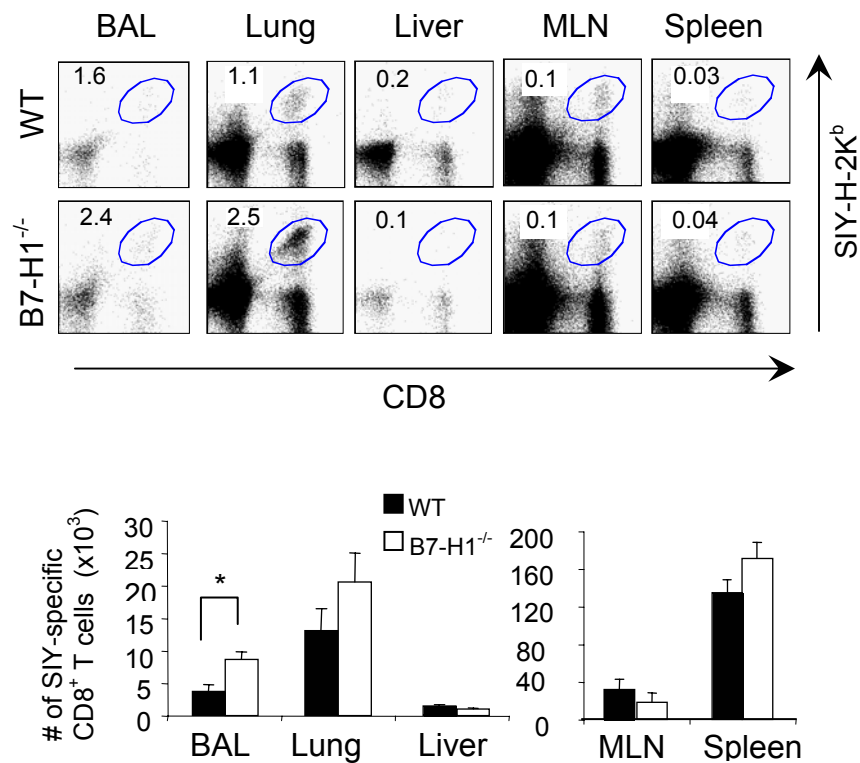


**Figure 4-9:  Antigen-specific CD8 T cell responses in B7-H1$^{-/-}$ mice are slightly increased 30 days post infection.**
WT mice and B7-H1$^{-/-}$ mice were infected with 100 pfu of WSN-SIY virus and SIY-specific CD8 T cells from various tissues were assayed by staining with anti-CD8 and SIY-K$^b$, 30 dpi. **A)** Representative SIY-K$^b$ versus CD8 staining profiles of live cells from the various tissues of WT (top row) and B7-H1$^{-/-}$ mice (bottom row) are shown. The numbers indicate percentages (n=3) of CD8$^+$ SIY-K$^{b+}$ T cells in the gated area among all live cells. **B)** Comparison of the number of SIY-specific CD8 T cells in different tissues, calculated by multiplying the total cell numbers with the percentage of CD8$^+$ SIY-K$^{b+}$ in the specific tissue. Values represent the mean ± SEM (n=3).  * p<0.05

These results demonstrated that the early (7 dpi) response of endogenous SIY-specific CD8 T cells to influenza infection in B7-H1$^{-/-}$ mice is, in contrast to 2C CD8 T cell response, impaired. However, at later time points of infection (14 dpi), percentage and number of endogenous CD8 T cells in B7-H1$^{-/-}$ mice began to increase and by 30 dpi, both percentage and number of SIY-specific endogenous CD8 T cells were higher in B7-H1$^{-/-}$ mice. Taken together, there is a profound difference in the response of 2C CD8 T cells and endogenous CD8 T cells in B7-H1$^{-/-}$ mice. This difference is most dramatic 7 dpi, when response of 2C T cells is enhanced but endogenous antigen-specific CD8 T cell response is impaired, as shown in the diagram in **Figure 4-10**. Interestingly, endogenous T cell response 30 dpi on the other hand is similar to 2C CD8 T cell response.
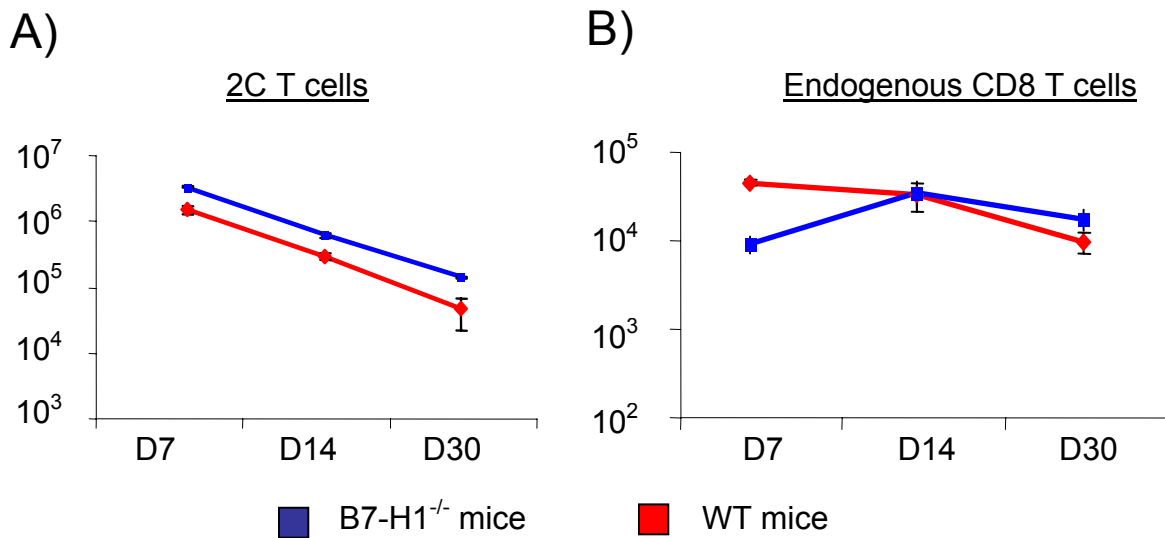


**Figure 4-10: Comparison between 2C T cell response and endogenous T cell response on different days post infection.**

A) As described earlier, WT and B7-H1$^{-/-}$ mice were transferred with 1 x 10$^6$ 2C T cells and infected 24 hrs later. 2C T cell response from the lung is shown. On various days post infection, cells were assayed for CD8 and 2C TCR. Diagram shows number of CD8$^+$ 2C TCR$^+$ cells on the specified days. B) SIY-specific endogenous CD8 T cell response. WT and B7-H1-/- mice were infected with WSN-SIY virus and cells from the lung were assayed for CD8 and SIY-K$^b$ on various day post infection. Diagram shows number of CD8$^+$ SIY-K$^{b+}$ cells.

## 4.2.3 Impaired response of transgenic B7-H1$^{-/-}$ CD8 T cells in B7-H1$^{-/-}$ mice during initial phase of infection

The different immune response between transgenic 2C CD8 T cells and endogenous antigen-specific CD8 T cells to the WSN-SIY virus in B7-H1$^{-/-}$ mice motivated us to compare the early response of both cells in detail. 2C CD8T cells originate from transgenic 2C TCR mice on B6 background that carry functional B7-H1 genes. Therefore, unlike endogenous CD8 T cells in B7-H1$^{-/-}$ mice, 2C CD8 T cells are capable to express B7-H1. It is possible that B7-H1 expression on T cells play a key role in T cell immune response. To test this, we established B7-H1$^{-/-}$ 2C CD8 T cells by backcrossing 2C TCR transgenic mice with B7-H1$^{-/-}$ mice (both on B6 background). In the first set of experiments, B7-H1$^{+}$ 2C CD8 T cells and B7-H1$^{-/-}$ 2C CD8 T cells were transferred i.v. into WT and B7-H1$^{-/-}$ recipient mice and infected with WSN-SIY virus 24 hours later. Seven dpi, 2C CD8 T cell response was determined by assaying CD8$^{+}$ 2C TCR$^{+}$ T cells in the lung (including both BAL and the lung parenchyma), MLN and the spleen. As shown in **Figure 4-11**, the frequency and number of responding 2C CD8 T cells in the lung and the spleen were significantly higher in B7-H1$^{-/-}$ recipients that were transferred with B7-H1$^{+}$ 2C CD8 T cells than B7-H1$^{-/-}$ recipients that were transferred with B7-H1$^{-/-}$ 2C CD8 T cells. Interestingly, the frequency and the number of B7-H1$^{+}$ and B7-H1$^{-/-}$ 2C CD8 T cells in the corresponding tissues were similar in WT recipients. These results indicate that B7-H1 plays a key role in the early activation of CD8 T cells and that B7-H1 expression on T cells was sufficient to compensate for an environmental deficiency.
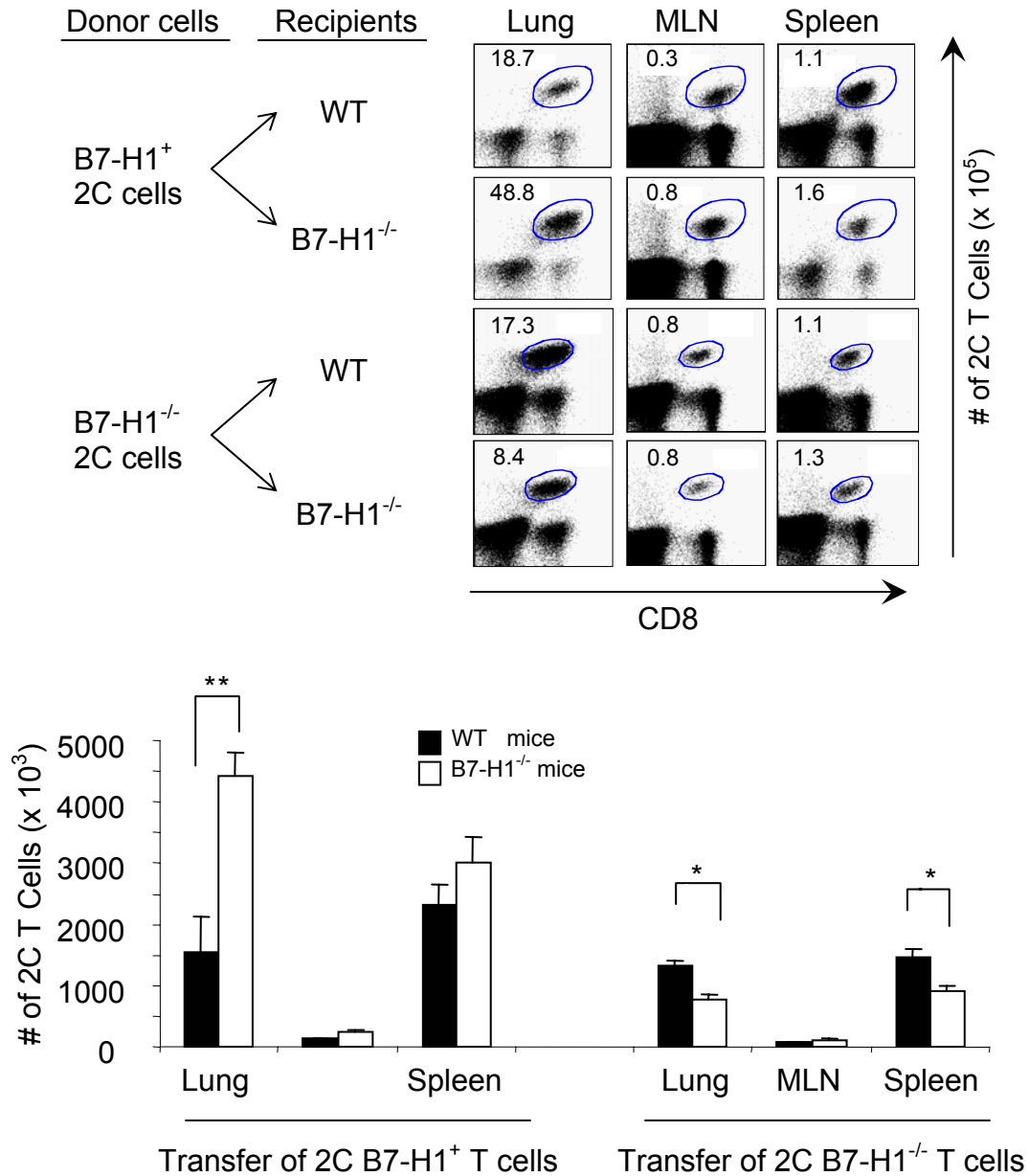
**Figure 4-11: Diminished CD8 T cell response by B7-H1$^{-/-}$ 2C T cells in B7-H1$^{-/-}$ mice.**

Naïve B7-H1$^{+/+}$ or B7-H1$^{-/-}$ 2C T cells were adoptively transferred into either WT or B7-H1$^{-/-}$ recipient mice (10$^6$ per mouse), followed by infection i.n. with WSN-SIY virus 24 hrs later. Seven dpi, cells from the indicated tissues were analyzed for CD8 and 2C TCR by flow cytometry. **A)** Representative CD8 versus 2C TCR staining profiles of total live cells in various tissues. The numbers indicate percentages of CD8$^+$ 2C TCR$^+$ cells among all live cells. **B)** The comparison of the numbers of B7-H1$^+$ and B7-H1$^{-/-}$ CD8$^+$ 2C TCR$^+$ cells among the lung, MLN and spleen of various recipient mice (top panel: transfer of B7-H1$^{+/+}$ 2C T cells; bottom panel: transfer of B7-H1$^{-/-}$ 2C T cells). Values represent the mean ± SEM (n=3). *p<0.05, **p<0.01.

Although our previous results demonstrated a stimulatory role of B7-H1 in CD8 T cell response, we repeated this experiment to determined the response of B7-H1$^{-/-}$ 2C CD8 T cells by transferring only 5000 cells into B7-H1$^{-/-}$ mice and WT mice. By transferring low copy numbers of B7-H1$^{-/-}$ 2C CD8 T cells we aimed to simulate the response of endogenous T cells in both mice. Seven dpi, 2C T cell response was determined by assaying CD8$^+$ 2C TCR$^+$ T cells in the lung, MLN and the spleen. Numbers of transferred cells seemed to be irrelevant, as the frequency and number of B7-H1$^{-/-}$ 2C CD8 T cells in the lung and spleen of B7-H1$^{-/-}$ recipients were again significantly lower than in WT mice (**Figure 4-12)**. There was again no difference in frequency and number of B7-H1$^{-/-}$ 2C CD8 T cells in the MLN. This result was in accordance with the previous data and illustrated that the number of virus-specific T cells is insignificant for T cell response.
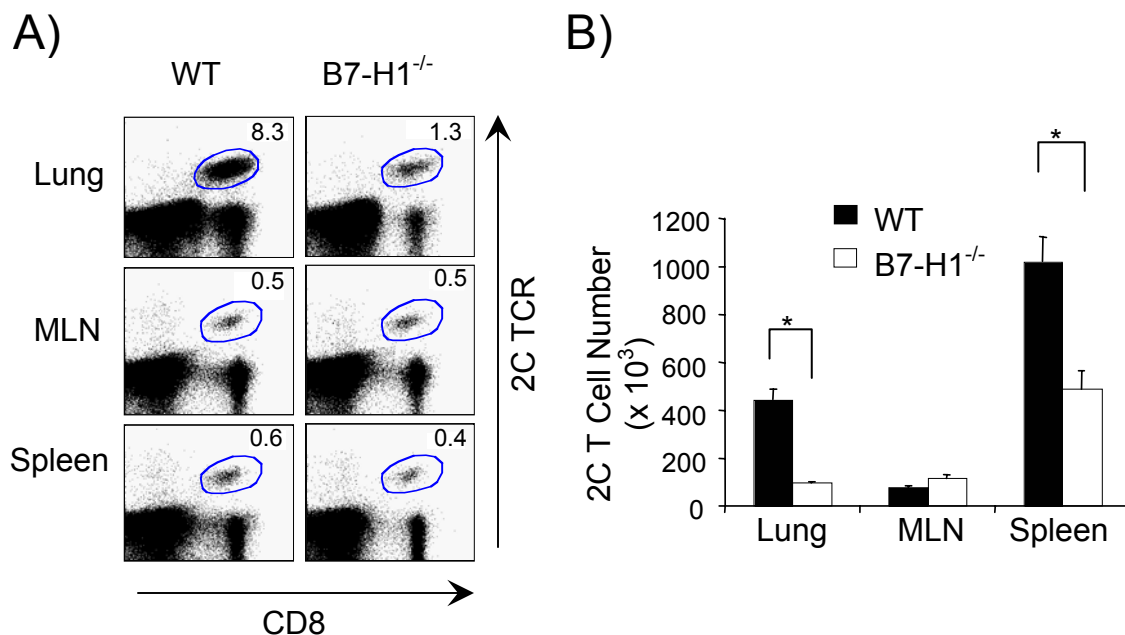


**Figure 4-12: Transfer of 5000 B7-H1$^{-/-}$ 2C CD8 T cells into WT and B7-H1$^{-/-}$ mice.**
Naive B7-H1$^{-/-}$ 2C CD8 T cells were adoptively transferred into either WT or B7-H1$^{-/-}$ recipient mice (5 x 10$^3$ per mouse), followed by infection with WSN-SIY virus 24 hrs later. Seven dpi, cells from the indicated tissues were analyzed for CD8 and 2C TCR by flow cytometry. **A)** Representative CD8 versus 2C TCR staining profiles of total live cells in various tissues. The numbers indicate percentages (n=3) of CD8$^+$ 2C TCR$^+$ cells among all live cells. **B)** Comparison of the numbers of CD8$^+$ 2C TCR$^+$ cells among the lung, MLN and spleen of various recipient mice Values represent the mean ± SEM (n=3). * p<0.01

To further dissect the difference in response to antigen, activation and proliferation of B7-H1$^+$ and B7-H1$^{-/-}$ 2C CD8 T cells in B7-H1$^{-/-}$ recipients following influenza infection was examined. Therefore, 1 x 10$^6$ CFSE labeled B7-H1$^+$ and B7-H1$^{-/-}$ 2C CD8 T cells were transferred i.v. into B7-H1$^{-/-}$ recipient mice and infected with 100 pfu WSN-SIY virus, 24 hours later. Two, 3, 4, 5 and 7 dpi, 2C T cells from MLN of recipient mice were assayed for CD69 and CD25 expression and CFSE intensity. Although CD69 was transiently upregulated by both B7-H1$^+$ and B7-H1$^{-/-}$ 2C CD8 T cells, the percentage of B7-H1$^+$ 2C CD8 T cells that upregulated CD69 was significantly higher than that of B7-H1$^{-/-}$ 2C T cells 3 days post infection (**Figure 4-13**). Similarly, the percentage of B7-H1$^+$ 2C CD8 T cells that upregulated CD25 was significantly higher than that of B7-H1$^{-/-}$ 2C CD8 T cells 4 dpi. Furthermore, B7-H1$^+$ 2C CD8 T cells proliferated earlier and more extensively than B7-H1$^{-/-}$ 2C CD8 T cells. Three dpi, nearly 50% of B7-H1$^+$ 2C CD8 T cells proliferated and by 5 dpi almost all 2C CD8 T cells have undergone multiple divisions as indicated by low CFSE intensity. In contrast, few B7-H1$^{-/-}$ 2C CD8 T cells divided 3 dpi and by 5 dpi many of them still had high CFSE intensity (**Figure 4-13**). This result demonstrated impaired activation and proliferation of B7-H1$^{-/-}$ CD8 T cells in B7-H1$^{-/-}$ recipient mice during the early phase of infection and provided an explanation for the difference in immune response between B7-H1$^+$ CD8 T cells and B7-H1$^{-/-}$ CD8 T cells following viral challenge.
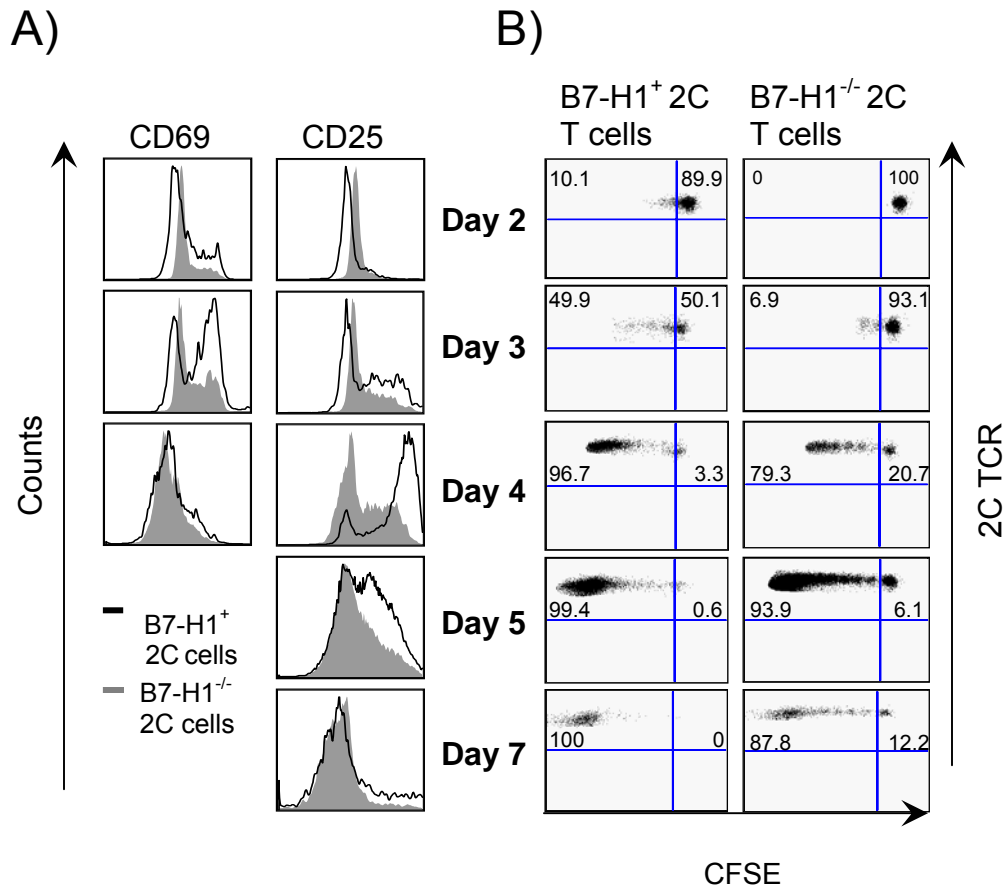
**Figure 4-13: T cell activation and proliferation of B7-H1$^{-/-}$ T cells are impaired in B7-H1$^{-/-}$ mice.**
Naïve B7-H1$^{+}$ or B7-H1$^{-/-}$ 2C T cells were labeled with CFSE and transferred into B7-H1$^{-/-}$ recipients (1 x 10$^{7}$ per mouse). 24 hrs later, mice were infected with 100 pfu WSN-SIY virus and 2-7 dpi cells from MLN were assayed for CD8, 2C TCR, CFSE plus CD69 or CD25. **A)** Comparison of CD69 and CD25 expression by B7-H1$^{+}$ and B7-H1$^{-/-}$ CD8$^{+}$ 2C TCR$^{+}$ cells between 2-7 dpi. **B)** CFSE intensity of B7-H1$^{+}$ and B7-H1$^{-/-}$ CD8$^{+}$ 2C TCR$^{+}$ cells between 2-7 dpi. The numbers indicate percentages of 2C T cells in the gated areas. Data for 2-4 dpi were from cells pooled from multiple mice and data for 5 and 7 dpi were from individual mice.

## 4.2.4 Transfer of B7-H1$^{+}$ T cells can rescue endogenous CD8 T cell response in B7-H1$^{-/-}$ mice

The enhanced response of B7-H1$^{+}$ 2C T cells in B7-H1$^{-/-}$ recipient mice suggests a stimulatory role of B7-H1 expression on T cells during early T cell response. However, it

was interesting to observe that B7-H1$^{-/-}$ 2C CD8 T cell response in wild-type mice was enhanced too. B7-H1$^{-/-}$ 2C CD8 T cells in WT mice are in an environment where they can interact with B7-H1$^{+}$ cells. To determine if the presence of B7-H1-expressing cells positively influences the response of endogenous antigen-specific CD8 T cells in B7-H1$^{-/-}$ mice and to obtain more direct evidence for the stimulatory function of B7-H1, different numbers of B7-H1$^{+}$ 2C T cells (5 x 10$^{3}$, 1 x 10$^{4}$ and 1 x 10$^{5}$) were adoptively transferred into B7-H1$^{-/-}$ mice. On the following day, recipient mice were infected i.n. with WSN-SIY virus, and the frequency of responding 2C CD8 T cells and endogenous SIY-specific CD8 T cells in the lung was analyzed seven days post infection using flow cytometry. As expected, without 2C CD8 T cell transfer, virtually no CD8$^{+}$ 2C TCR$^{+}$ cells were detected in the lung and frequency of endogenous SIY-specific CD8 T cell was very low (**Figure 4-14**). With increasing numbers of 2C CD8 T cell transferred, the frequencies of CD8$^{+}$ 2C TCR$^{+}$ cells in the lung increased from 0.5% to 12%. More interestingly, without 2C CD8 T cell transfer, the percentage of endogenous CD8 SIY-specific CD8 T cells in the lung was 1.2%. This percentage increased to 6.4% when 5000 B7-H1$^{+}$ 2C CD8 T cells were transferred, suggesting an enhanced response by endogenous SIY-specific CD8 T cells. However, as the number of transferred 2C CD8 T cells increased, the percentages of endogenous SIY-specific CD8 T cells in the lung decreased, probably due to competition between the 2C and endogenous SIY-specific T cells for the same SIY epitope.
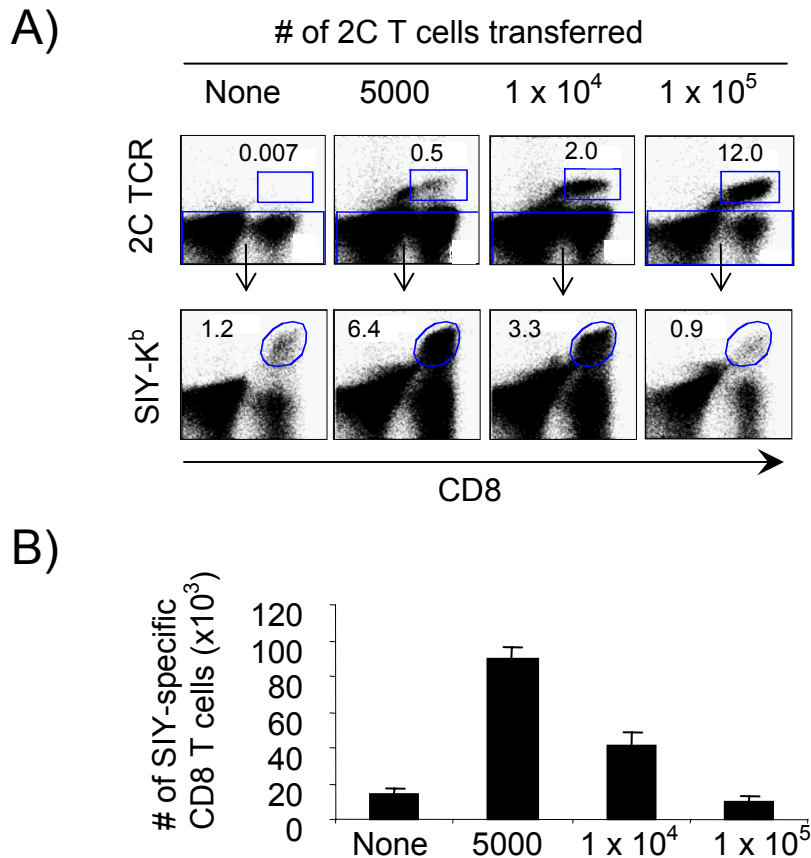
**Figure 4-14: Enhanced CD8 T cell response in the lung of B7-H1$^{-/-}$ mice by transferred B7-H1$^{+}$ T cells**
Different numbers of purified B7-H1$^{+}$ 2C T cells were transferred i.v. into B7-H1$^{-/-}$ mice. 24 hrs later, mice were infected i.n. with 100 pfu WSN-SIY virus. As control, B7-H1$^{-/-}$ mice without 2C cell transfer were infected at the same time. 7 dpi cells from the lung were assayed for 2C TCR, CD8 and SIY-K$^{b}$. **A)** Top row: representative 2C TCR versus CD8 staining profiles of live cells. The numbers indicate percentages (n=2) of CD8$^{+}$ 2C TCR$^{+}$ cells among all live cells. Bottom row: SIY-K$^{b}$ versus CD8 staining profiles of 2C TCR-negative cells (as gated in the top panel and shown by the arrows). The numbers indicate percentages of CD8$^{+}$ SIY-K$^{b+}$ cells among all live cells. **B)** The Numbers of CD8$^{+}$ SIY-K$^{b+}$ cells in the lung calculated by multiplying total cell numbers with percentage of CD8$^{+}$ SIY-K$^{b+}$ cells (n=2).

To avoid the possible complication by the transferred 2C T cells responding to the same epitope, B7-H1$^{+}$ CD8 T cells that express the F5 TCR (F5 T cells) but do not respond to WSN-SIY were utilized to determine endogenous SIY-specific CD8 T cell response. Different numbers of F5 T cells (1 x 10$^{4}$, 1 x 10$^{5}$ and 1 x 10$^{6}$) were adoptively transferred into either WT mice or B7-H1$^{-/-}$ mice. 24 hrs later, mice were infected with 100 pfu WSN-

SIY virus and 7 days post infection, the frequencies and numbers of endogenous SIY-specific CD8 T cells were determined in the lung. As expected, there was no difference in the frequency and the number of SIY-specific CD8 T cells in WT mice with or without F5 T cell transfer (**Figure 4-15**). In contrast, both the frequency and the number of SIY-specific CD8 T cells increased in the lung of B7-H1$^{-/-}$ mice with increasing numbers of F5 T cells transferred. When 1 x 10$^6$ F5 T cells were transferred, both the frequency and the number of SIY-specific CD8 T cells in the lung reached the same levels as in WT mice demonstrating enhanced endogenous T cell response in the presence of B7-H1 expressing T cells. Even if the T cells are bystanders (F5 T cells) the expression of the B7-H1 is sufficient to rescue the response of B7-H1-deficient CD8 T cells.
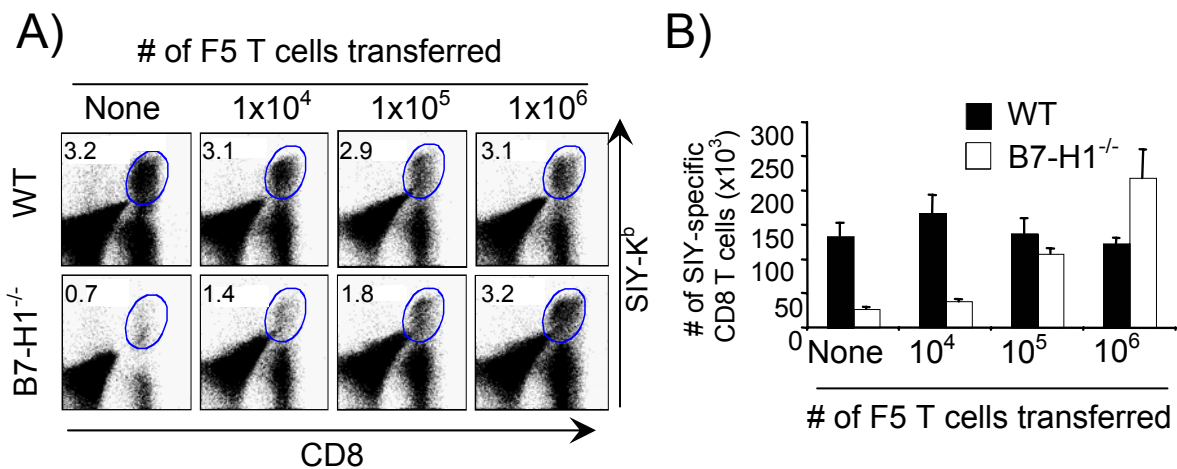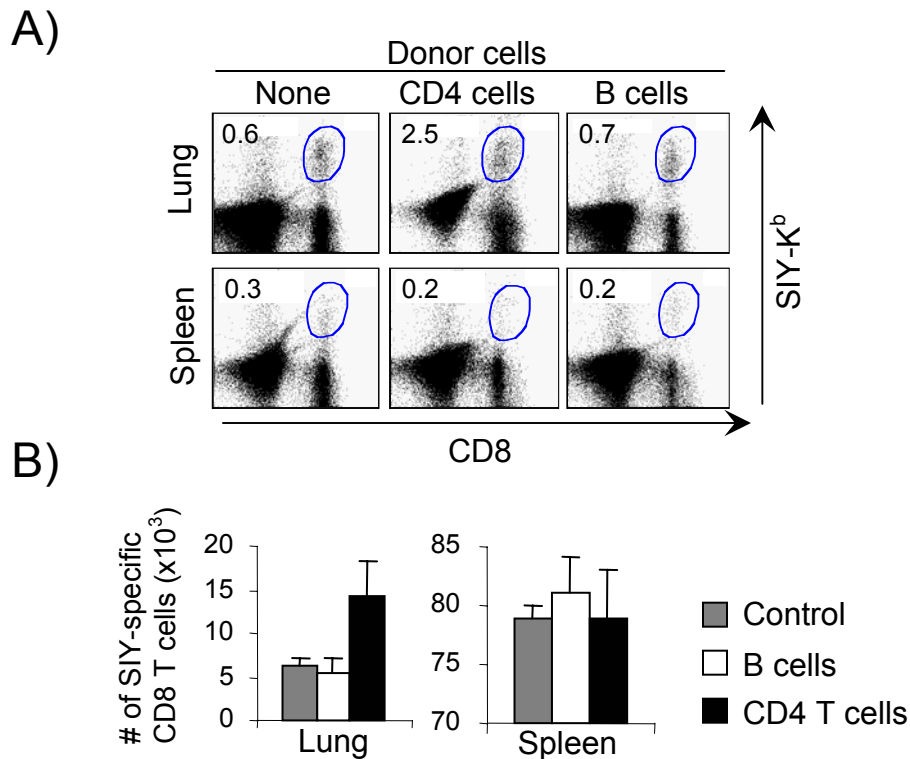


**Figure 4-15: Enhanced CD8 T cell response in B7-H1$^{-/-}$ mice by transferred B7-H1-positive T cells**
Different numbers of purified B7-H1$^+$ F5 T cells were transferred i.v. into WT and B7-H1$^{-/-}$ mice. 24 hrs later, mice were infected i.n. with 100 pfu WSN-SIY virus and 7 dpi cells from the lung were assayed for CD8 and SIY-K$^b$. **A)** Representative SIY-K$^b$ versus CD8 staining profiles of live cells are shown. The numbers indicate percentages of CD8$^+$ SIY-K$^{b+}$ cells among all live cells. **B)** Comparison of the numbers of CD8$^+$ SIY-K$^{b+}$ T cells in lung of WT and B7-H1$^{-/-}$ mice that were transferred with no or different numbers of F5 T cells. Values represent the mean ± SEM (n=3).

**4.2.5 Endogenous CD8 T cell response in B7-H1$^{-/-}$ mice is restored by only T cells**

Next we sought to determine whether transfer of B7-H1-expressing cells that are no CD8 T cells would also enhance endogenous CD8 T cell response. Therefore, purified CD4 T

cells and B cells (each 1 x 10$^6$ per mouse) were transferred i.v. into B7-H1$^{-/-}$ mice. Both cell types, which express similar levels of B7-H1 as CD8 T cells, were isolated from B6 mice and purified. 24 hrs later, recipient mice were infected with WSN-SIY and 7 dpi the frequency and number of SIY-specific CD8 T cells were determined in the lung and the spleen. As shown in **Figure 4-16**, an enhanced SIY-specific CD8 T cell response was observed in mice that had been injected with CD4 T cells, but not with B cells. Together, these results suggest that only transferred B7-H1-expressing T cells can enhance endogenous CD8 T cell response in B7-H1$^{-/-}$ mice.
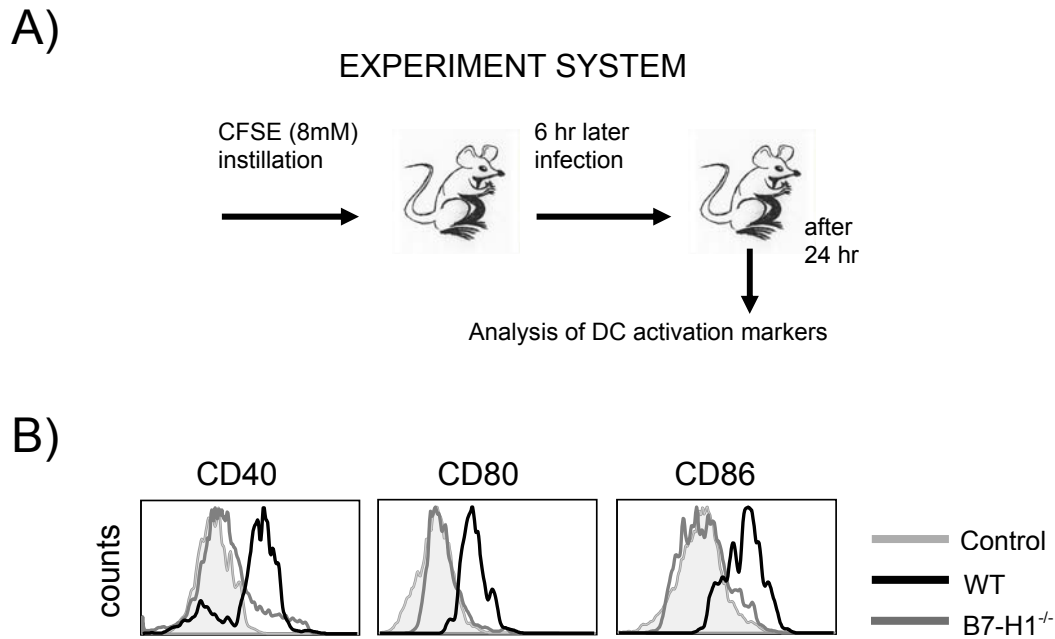


**Figure 4-16: Enhanced T cell response in B7-H1$^{-/-}$ mice by adoptive transfer of B7-H1$^+$ CD4 T cells**
CD4 T cells and B cells were purified from C57BL/6 mice (1 x 10$^6$ per mouse) and transferred i.v. into B7-H1$^{-/-}$ mice. 24 hr later, mice were infected i.n. with WSN-SIY virus. As control, B7-H1$^{-/-}$ mice that did not receive any cells were infected at the same time. Seven dpi, cells from the lung and spleen were assayed for SIY-K$^b$ and CD8. **A)** SIY-K$^b$ versus CD8 staining profiles of total live cells in the indicated tissues are shown. The numbers indicate the percentages (n=2) of CD8$^+$ SIY-K$^{b+}$ T cells among all live cells. **B)** Comparison of the numbers of CD8$^+$ SIY-K$^{b+}$ T cells in lung of WT and B7-H1$^{-/-}$ mice that were transferred with CD4 T cells or B cells. Values represent the mean ± SEM (n=2).

## 4.3 The role of B7-H1 in dendritic cell maturation

In the first part of this thesis, a dual regulatory role of B7-H1 in CD8 T cell immune response following influenza infection was shown. In addition to the inhibitory effect of peripheral expressed B7-H1 on T cell survival in the lung, it was also shown that B7-H1 expression on T cells positively influences T cell activation and proliferation during early immune response. B7-H1-deficient mice therefore exhibited impaired CD8 T cell response to antigen. In the second part of this thesis, the detailed mechanism underlying impaired T cell response was examined. Since efficient T cell activation requires adequate stimulation by mature dendritic cells (DC), the role of B7-H1 on DC maturation was investigated.

### 4.3.1 Impaired CD8 T cell response in B7-H1$^{-/-}$ mice is due to defective dendritic cell maturation and can be recovered by B7-H1$^{+}$ T cells

Following intranasal influenza infection, dendritic cells in the lung capture antigens, mature and migrate to the MLN where they activate naïve T cells to respond to the infection. To determine whether the impaired early CD8 T cell activation and proliferation in B7-H1$^{-/-}$ mice is due to compromised DC migration and/or maturation, number and maturation status of DCs in MLN following infection with high dose WSN-SIY virus (1000 pfu) were determined. In order to identify and track DCs that migrate from the lung to the MLN, WT mice and B7-H1$^{-/-}$ mice were instilled with the esterified vital dye CFSE intranasally and infected with 1000 pfu WSN-SIY i.n. 6 hr later. 24 hr post-infection (30 hr post CFSE instillation), CD11c$^{high}$ CFSE$^{+}$ DCs from the MLN were enumerated and assayed for activation markers CD40, CD80 and CD86. The numbers of DCs recovered from MLN of infected WT mice (2290±165) and B7-H1$^{-/-}$ mice (2400±274) were similar suggesting that migration of antigen-loaded DCs from the lung to the MLN is not affected by B7-H1. As expected, DCs from the MLN of WT mice upregulated CD40, CD80 and CD86. However, as shown in **Figure 4-17**, upregulation of CD40, CD80 and CD86 on DCs was diminished and thus DC maturation in B7-H1$^{-/-}$ mice was defective.

A)

EXPERIMENT SYSTEM



B)



**Figure 4-17: Defective dendritic cell maturation underlies the diminished CD8 T cell response in B7-H1$^{-/-}$ Mice**

**A)** Schematic description of the experiment. WT mice and B7-H1$^{-/-}$ mice were instilled i.n. with the esterified CFSE. 6 hrs later, mice were infected with 1000 pfu WSN-SIY virus and 24 hr post infection, MLN were pooled from 4 mice and digested with collagenase D/DNase I. The resulting single cell suspension was assayed for CD11c, CFSE plus CD40, CD80 or CD86. **B)** The histograms show CD40, CD80 and CD86 staining intensity of CD11c$^+$ CFSE$^+$ DCs. As control, CD11c$^+$ CFSE$^-$ DCs from spleen of WT mice were used.

As transfer of B7-H1$^+$ T cells into B7-H1$^{-/-}$ recipient mice restored response of endogenous CD8 T cells, we tested if the transfer of B7-H1$^+$ T cells will also restore DC maturation. Therefore, B7-H1$^{-/-}$ mice were instilled i.n. with CFSE and some of the B7-H1$^{-/-}$ recipients received i.v. B7-H1$^+$ F5 CD8 T cells (1 x 10$^6$) at the same time. Six hours later, all mice were infected i.n. with WSN-SIY virus and 24 hrs post infection, CD11c$^{high}$ CFSE$^+$ DCs from the MLN were assayed for activation markers CD40, CD80 and CD86. As expected, while DCs in the MLN of B7-H1$^{-/-}$ mice that did not receive F5 T cells were again low for all three activation makers, DCs from B7-H1$^{-/-}$ mice that had received B7-H1-expressing F5 CD8 T cells upregulated CD40, CD80 and CD86 to a

level similar to DCs in WT mice (**Figure 4-18**). These results demonstrate that once more that maturation of DCs in B7-H1-deficient mice is defective. However, the fact that DC maturation in B7-H1$^{-/-}$ recipients was almost completely restored after B7-H1-expressing T cells were transferred suggests that B7-H1 expression on T cells influences DC maturation.
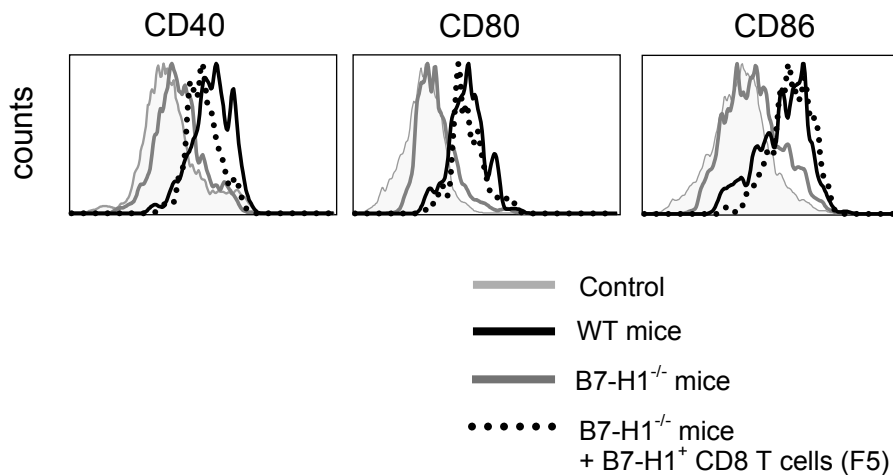


**Figure 4-18: Defective dendritic cell maturation in B7-H1$^{-/-}$ mice is restored by transfer of B7-H1$^{+}$ CD8 T cells**

B7-H1$^{-/-}$ mice were instilled i.n. with CFSE. Some of the B7-H1$^{-/-}$ mice were also given i.v. 1 x 10$^{6}$ purified B7-H1$^{+}$ CD8 T cells (F5) at the time of CFSE instillation. 6 hr later mice were infected with 1000 pfu WSN-SIY virus and 24 hr post infection MLN were pooled from 4 mice and digested with collagenase D/DNase I. The resulting single cell suspension was assayed for CD11c, CFSE plus CD40, CD80 or CD86. The histograms show CD40, CD80 and CD86 staining intensity of CD11c$^{+}$ CFSE$^{+}$ DCs. As control, CD11c$^{+}$ CFSE$^{-}$ DCs from spleen of WT mice were used.

Although previous experiments suggested that DC maturation that are defective in B7-H1$^{-/-}$ mice can be rescued by B7-H1-expressing T cells, it was not entirely clear whether this restoration is B7-H1-dependent or caused solely by the process of cell transfer that might have triggered immune reaction in these mice. In order to further confirm that rescue of DC maturation is indeed B7-H1-dependent, activation of DCs in

B7-H1$^{-/-}$ mice was compared after transferring either B7-H1$^+$ or B7-H1$^{-/-}$ T cells. In the first set of experiments, B7-H1$^{-/-}$ mice were injected with either B7-H1$^+$ T cells (from B6 mice) or B7-H1$^{-/-}$ T cells (from B7-H1$^{-/-}$ mice), instilled with CFSE, infected with WSN-SIY virus and analyzed as above. As shown in **Figure 4-19**, only DCs from mice that were injected with B7-H1$^+$ T cells but not B7-H1$^{-/-}$ T cells upregulated the activation markers. This first experiment provided evidence that the process of cell transfer did not trigger immune reaction altering DC maturation.
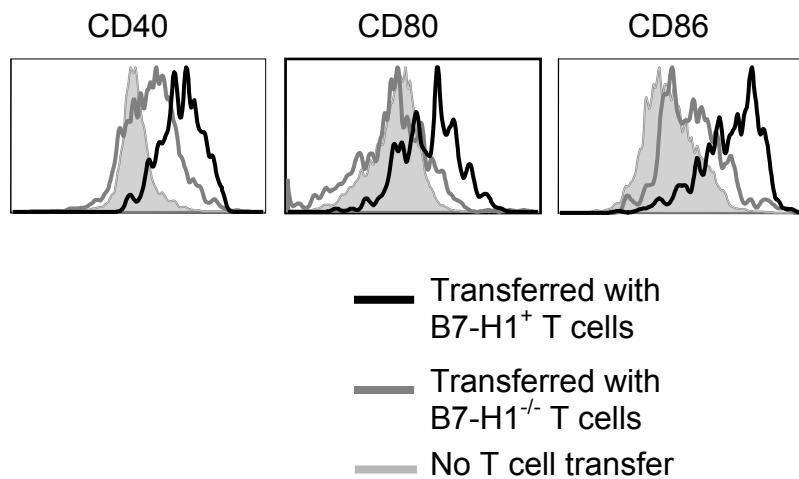


**Figure 4-19: No recovery of DC maturation in B7-H1$^{-/-}$ mice after transfer of B7-H1$^{-/-}$ CD8 T cells.**
CD8 T cells were purified from either WT or B7-H1$^{-/-}$ mice and transferred i.v. into B7-H1$^{-/-}$ mice. Immediately after transfer, the mice were instilled i.n. with CFSE. 6 hr later, mice were infected i.n. with 1000 pfu WSN-SIY virus. As control, B7-H1$^{-/-}$ mice without cell transfer were infected at the same time. 24 hrs post infection, cells from MLN were assayed for CD11c, CFSE plus CD40, CD80 or CD86. The histograms show CD40, CD80 or CD86 staining intensity of CD11c$^+$ CFSE$^+$ DCs. Data were from cells pooled from 4 mice.

In the second set of experiments, purified CD8 T cells from either WT mice (B7-H1$^+$) or B7-H1$^{-/-}$ mice (1 x 10$^6$ per mouse) were transferred i.v. into RAG-1$^{-/-}$ mice, that are T and B cell-deficient due to the lack of the recombination activating gene-1 (RAG-1). Previous studies have shown that DCs in RAG-deficient mice are defective. Therefore we tested if B7-H1-expressing T cells will have an influence of DC maturation in these mice.

However, RAG-1$^{-/-}$ mice have tiny MLN which makes it almost impossible to isolate dendritic cells from this tissue. To overcome this problem, 24 hours post cell transfer, recipient mice were infected with a high dose of WSN virus intraperitoneally (i.p.) (into the peritoneal cavity). As a negative control, some RAG-1$^{-/-}$ mice without T cell transfer were infected at the same time. While IP infections with influenza are not productive and the virus cannot replicate in the peritoneal cavity, it still causes activation, migration and antigen presentation of peritoneal resident APCs. 24 hrs post-infection, maturation status of DCs in the draining lymphnodes (mesenteric lymph nodes), were determined by flow cytometric analysis. As expected, the level of CD40, CD80 and CD86 was low on DCs from RAG-1$^{-/-}$ mice that were infected but did not receive any T cells. Similarly, the activation markers were not upregulated if RAG-1$^{-/-}$ mice were given B7-H1$^{-/-}$ T cells. In contrast, the level of CD40, CD80, and CD86 was significantly upregulated on DCs from RAG-1$^{-/-}$ mice that were given B7-H1$^{+}$ T cells, as shown in **Figure 4-20**. These results clearly demonstrate that T cells can rescue DC maturation in both B7-H1$^{-/-}$ mice and RAG1$^{-/-}$ mice in a B7-H1-dependent manner.
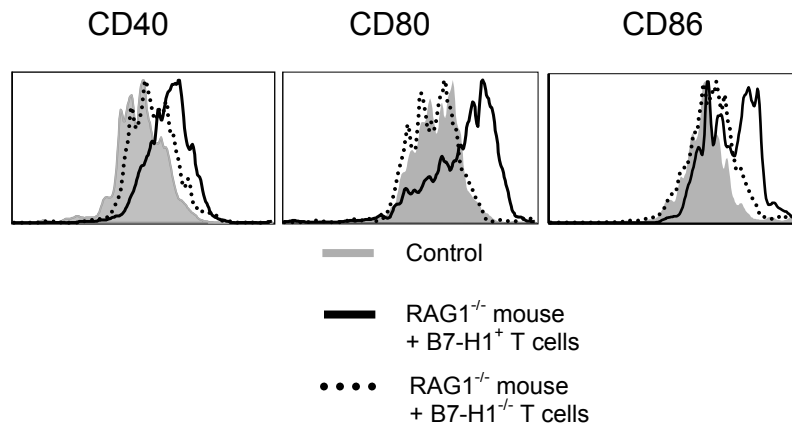


**Figure 4-20: Defective dendritic cell maturation in RAG-1$^{-/-}$ mice is restored by B7-H1$^{+}$ T cells**

Restoration of DC maturation in RAG1$^{-/-}$ mice by transferred B7-H1$^{+}$ but not B7-H1$^{-/-}$ T cells. Purified B7-H1$^{+}$ or B7-H1$^{-/-}$ 2C T cells (1 x 10$^{6}$) were transferred i.v. into RAG1$^{-/-}$ mice. 24 hrs later, mice were infected intraperitoneally (i.p.) with 1 x 10$^{6}$ pfu WSN virus. As control, RAG1$^{-/-}$ mice without T cell transfer were infected at the same time. 24 hrs post infection, cells from the draining mesenteric lymph node were isolated and assayed for CD11c plus CD40, CD80, or CD86. The histograms show CD40, CD80 and CD86 staining intensity of CD11c$^{+}$ DCs.

To further determine if defective DC maturation underlies the impaired CD8 T cell response in B7-H1$^{-/-}$ mice, we tested if transfer of B7-H1$^{+}$ DCs can directly rescue CD8 T cell response in B7-H1$^{-/-}$ mice. Thus, DCs from either WT mice or B7-H1$^{-/-}$ mice were isolated and purified (>98% CD11c$^{high}$) (**Figure 4-21**) and injected into the hind footpad of either WT or B7-H1$^{-/-}$ recipient mice (5 x 10$^{5}$ per mouse). 24 hrs later, mice were infected with WSN-SIY virus i.n. and 7 dpi the frequency and the number of SIY-specific CD8 T cells was determined in the lung. Both the percentage and number of SIY-specific CD8 T cells increased significantly in WT mice upon transfer of either B7-H1$^{+}$ or B7-H1$^{-/-}$ DCs. In contrast, the frequency and number of SIY-specific CD8 T cells in B7-H1$^{-/-}$ recipients increased only when the mice were given B7-H1$^{+}$ DCs (**Figure 4-21**). These results provided clear evidence that the impaired CD8 T cell response in B7-H1$^{-/-}$ mice results from defective DC maturation.
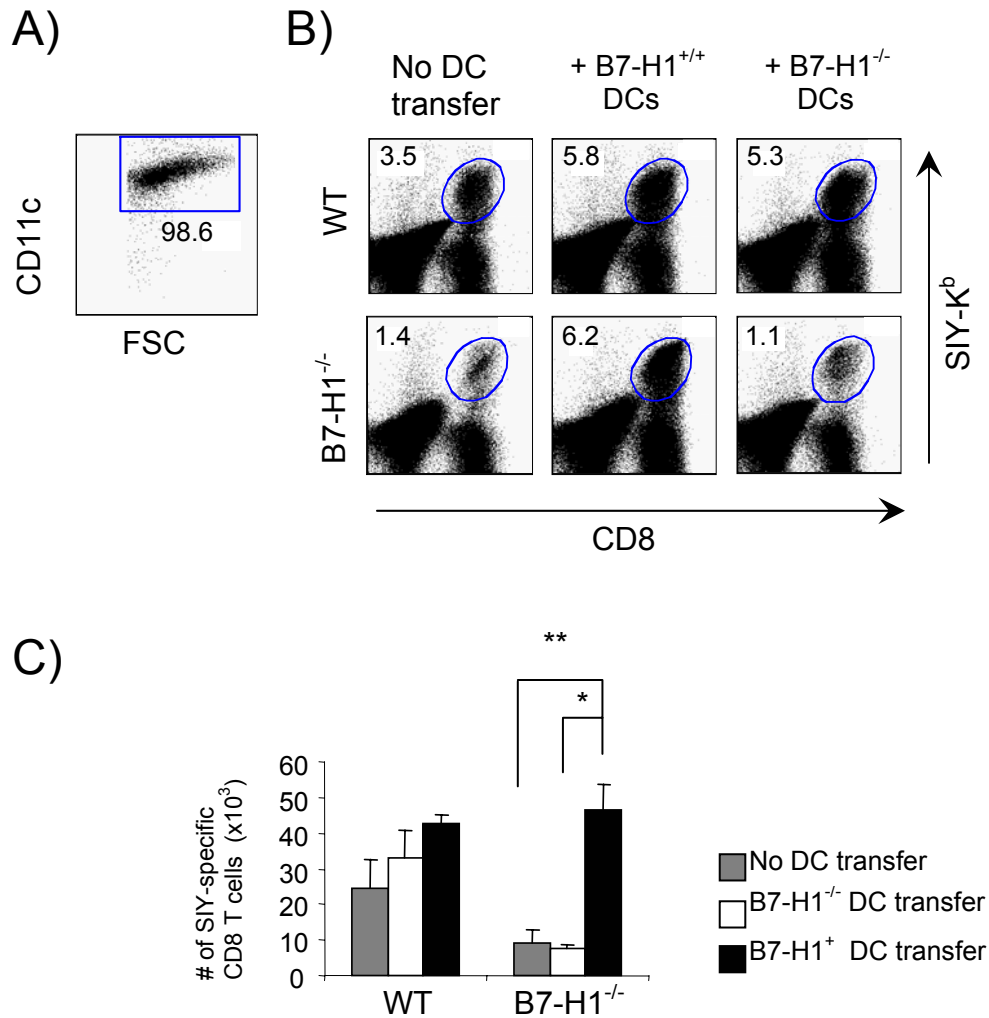
**Figure 4-21: Restoration of CD8 T cell response in B7-H1$^{-/-}$ mice by transferred B7-H1$^{+}$ DCs but not B7-H1$^{-/-}$ DCs.**

DCs from spleen of either WT or B7-H1$^{-/-}$ mice were purified and transferred into the hind footpad of either WT mice or *B7-H1$^{-/-}$* mice (5 x 10$^5$ per mouse). 24 hr later, mice were infected with 100 pfu WSN-SIY virus and 7 dpi cells from the lung were assayed for CD8 and SIY-K$^b$. **A)** Purity (> 98.5%) of CD11c$^{high}$ DCs using MACS beads. **B)** Representative SIY-K$^b$ versus CD8 staining profiles of live cells are shown. The numbers indicate percentages (n=3) of CD8$^+$ SIY-K$^{b+}$ cells among all live cells. **C)** Comparison of the number of SIY-specific CD8 T cells in the lung of WT mice and B7-H1$^{-/-}$ mice, calculated by multiplying the total cell numbers with the percentage of CD8$^+$ SIY-K$^{b+}$ cells. Values represent the mean ± SEM (n=3). *p<0.01, **p<0.05

### 4.3.2 T cell-DC interaction induces B7-H1-mediated DC conditioning

The restoration of DC maturation by transferred B7-H1$^+$ T cells suggested that T cells may provide signals that normally condition DCs to enable efficient maturation following antigen challenge. This signaling is most likely missing in B7-H1$^{-/-}$ mice resulting in defective DC maturation. Because naïve T cells continue to form physical contacts with DCs in the lymphoid organs, the B7-H1-dependent DC conditioning could occur prior to infection (inflammation). This could be an explanation why B7-H1$^{-/-}$ mice have defective DCs and impaired CD8 T cell response. However, compared to naïve DCs from RAG-1$^{-/-}$ mice, we did not detect any differences in CD40, CD80 and CD86 expression by DCs from RAG-1$^{-/-}$ mice that were adoptively transferred with B7-H1$^+$ T cells in the absence of influenza infection (**Figure 4-22**).
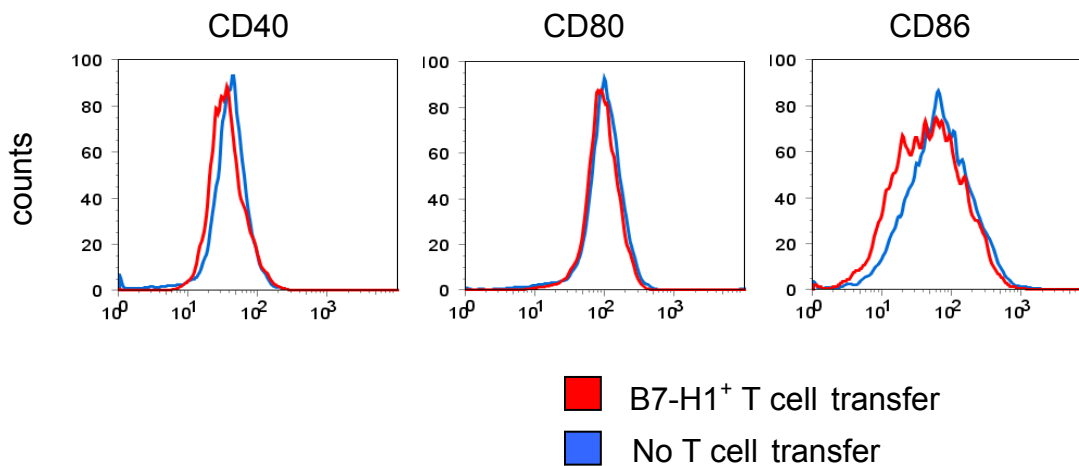


**Figure 4-22: Levels of DC activation marker did not change when RAG-1$^{-/-}$ recipient received B7-H1$^+$ T cells in the absence of influenza infection.**

Purified naïve B7-H1$^+$ T cells (1 x 10$^6$ ) were i.v. transferred into RAG-1$^{-/-}$ mice and infected i.p. with 1 x 10$^6$ pfu WSN virus, 24 hrs later. As control, RAG-1$^{-/-}$ mice that did not received T cells were infected at the same time. After 24 hrs post infection, CD11c$^{high}$ DCs from the MLN were isolated and assayed for activation markers (CD40, CD80 and CD86). The histograms show CD40, CD80 and CD86 staining intensity of CD11c$^{high}$DCs.

To further demonstrate that B7-H1$^+$ T cells can condition DCs in the absence of infection directly, B7-H1$^+$ 2C CD8 T cells or B7-H1$^{-/-}$ 2C CD8 T cells were purified and adoptively transferred  i.v. into RAG-1$^{-/-}$ mice (1 x 10$^6$ per mouse) without infecting the mice. Three days later, DCs from both recipient mice were isolated, purified and transferred into the hind footpad of B7-H1$^{-/-}$ mice (5 x 10$^5$ per mouse), followed by i.n. infection with 100 pfu WSN-SIY virus 24 hrs later. As control, B7-H1$^{-/-}$ mice with no DC transfer were infected at the same time. Seven dpi, the frequency and number of endogenous SIY-specific CD8 T cells were determined in the lung, MLN and spleen. As shown in **Figure 4-23**, both the frequency and number of SIY-specific CD8 T cells were increased by approximately 3-fold in the lung of B7-H1$^{-/-}$ recipients that were given DCs from RAG-1$^{-/-}$ mice that had received B7-H1-expressing T cells (conditioned DC). In contrast, transferring DC from RAG-1$^{-/-}$ mice that received B7-H1$^{-/-}$ T cells (unconditioned DC) did not increase either the frequency or the number of SIY-specific CD8 T cells. Taken together, these results demonstrate that the interaction between T cells and DC via B7-H1 prior to infection conditions DCs for efficient maturation upon antigen stimulation, and therefore stimulate a robust CD8 T cell response.

A)



B)



- ☐ (grey) B7-H1⁻ᐟ⁻ mice without DC transfer
- ☐ B7-H1⁻ᐟ⁻ mice transferred with non-conditioned DCs
- ■ B7-H1⁻ᐟ⁻ mice transferred with conditioned DCs
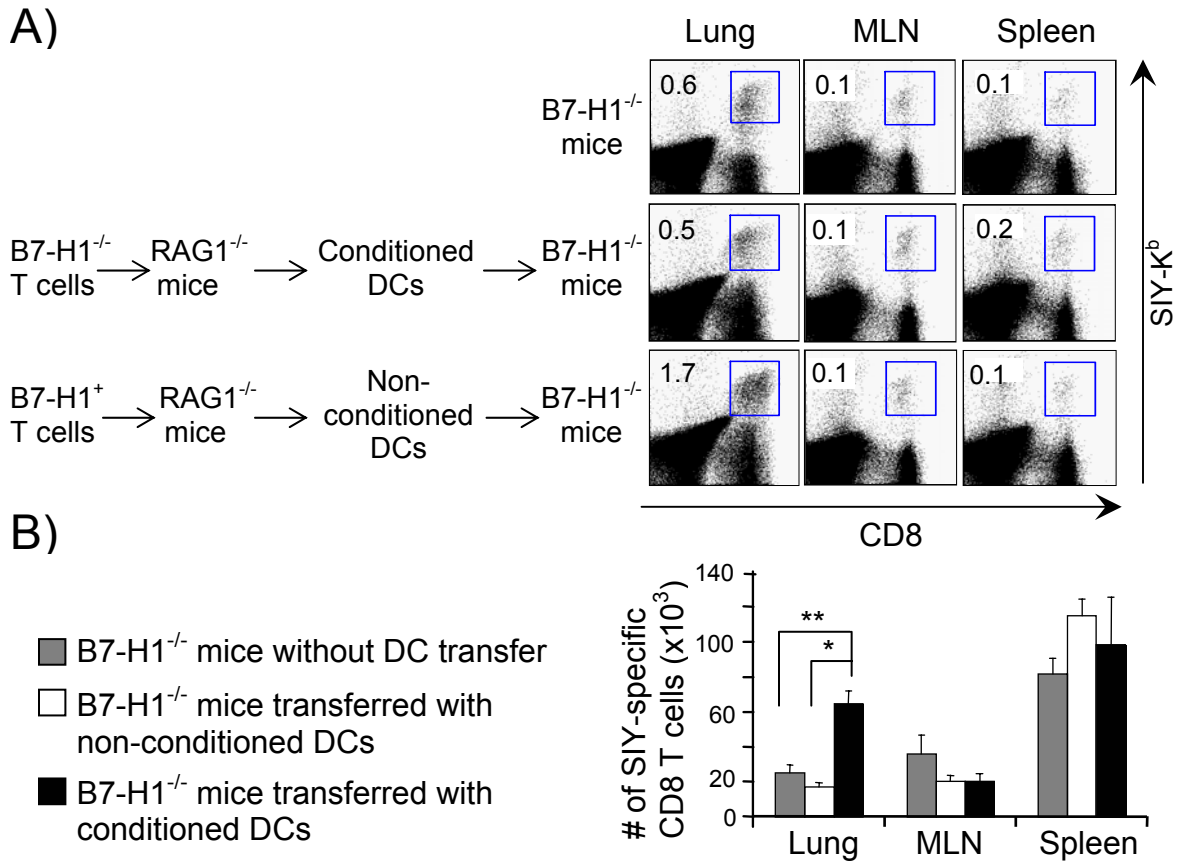
**Figure 4-23: CD8 T cell response in B7-H1$^{-/-}$ mice is rescued by conditioned DCs from RAG-1$^{-/-}$ mice**
Purified B7-H1$^{+}$ or B7-H1$^{-/-}$ T cells were transferred i.v. in RAG1$^{-/-}$ mice (1 x 10$^{6}$ per mouse). Three days later, DCs were purified from RAG1$^{-/-}$ mice and injected into the hind footpad of B7-H1$^{-/-}$ mice (5 x 10$^{5}$ per mouse). 24 hrs following DC transfer, mice were infected i.n. with 100 pfu of WSN-SIY virus. As control, B7-H1$^{-/-}$ mice without DC transfer were infected at the same time. 7 dpi, cells from the lung, MLN and spleen were assayed for CD8 and SIY-K$^{b}$. **A)** Experimental scheme and representative SIY-K$^{b}$ versus CD8 staining profiles of live cells in the indicated tissues. The numbers indicate percentages of CD8$^{+}$ SIY-K$^{b+}$ cells among all live cells. **B)** Comparison of the number of SIY-specific CD8 T cells in different tissues, calculated by multiplying the total cell numbers with the percentage of CD8$^{+}$ SIY-K$^{b+}$ in the specific tissue. Values represent the mean ± SEM (n=3). *p<0.005, **p<0.01.

### 4.3.3 B7-H1 mediated DC conditioning is B7.1 independent

In the previous experiments, we have shown that B7-H1 expression on T cells mediate DC conditioning during T cell-DC interaction. However, the molecule that is expressed on DCs and trigger DC conditioning upon binding to B7-H1 is not known. B7-H1 is traditionally known to interact with the PD-1 receptor. Because PD-1 is not expressed in dendritic cells, B7-H1 likely interacts with another molecule in mediating DC conditioning. A recent study reported the interaction between B7-H1 and the co-stimulatory ligand B7.1 (CD80) (Butte et al, 2007). As B7.1 is expressed by DCs, we examined whether T cell conditioning of DCs is through B7-H1–B7.1 interaction. First, we determined if DCs in B7.1$^{-/-}$ mice can mature properly following influenza virus infection. Following CFSE instillation and influenza infection, CD11c$^{high}$ CFSE$^{+}$ DCs from the MLN of B7.1$^{-/-}$ mice upregulated CD40 and CD86, suggesting normal DC maturation (**Figure 4-24)**.  Since DC maturation seemed to be normal in B7.1$^{-/-}$,  we determined if B7-1$^{-/-}$ DCs can restore CD8 T cell response in B7-H1$^{-/-}$ mice.  DCs were purified from B7.1$^{-/-}$ mice (>98% CD11c$^{high}$) and transferred into the hind footpad of B7-H1$^{-/-}$ mice, followed by i.n. infection with WSN-SIY virus 24 hr later. As control, B7-H1$^{-/-}$ mice with no DC transfer were infected at the same time. Seven dpi, the frequency and number of endogenous SIY-specific CD8 T cells were determined in the lung, MLN and spleen. Compared to control mice, both the frequency and number of SIY-specific CD8 T cells were increased by approximately 3-fold in the lung of B7-H1$^{-/-}$ recipients that were given DCs from B7.1$^{-/-}$ mice (**Figure 4-24**). These results clearly demonstrate that B7.1$^{-/-}$ DCs can rescue CD8 T cell response in B7-H1$^{-/-}$ mice, suggesting that B7.1 is not required for T cell conditioning of DCs.

Unfortunately, during the course of this PhD work, we were unable to invest additional time in order to experimentally identify and analyze the unknown receptor.
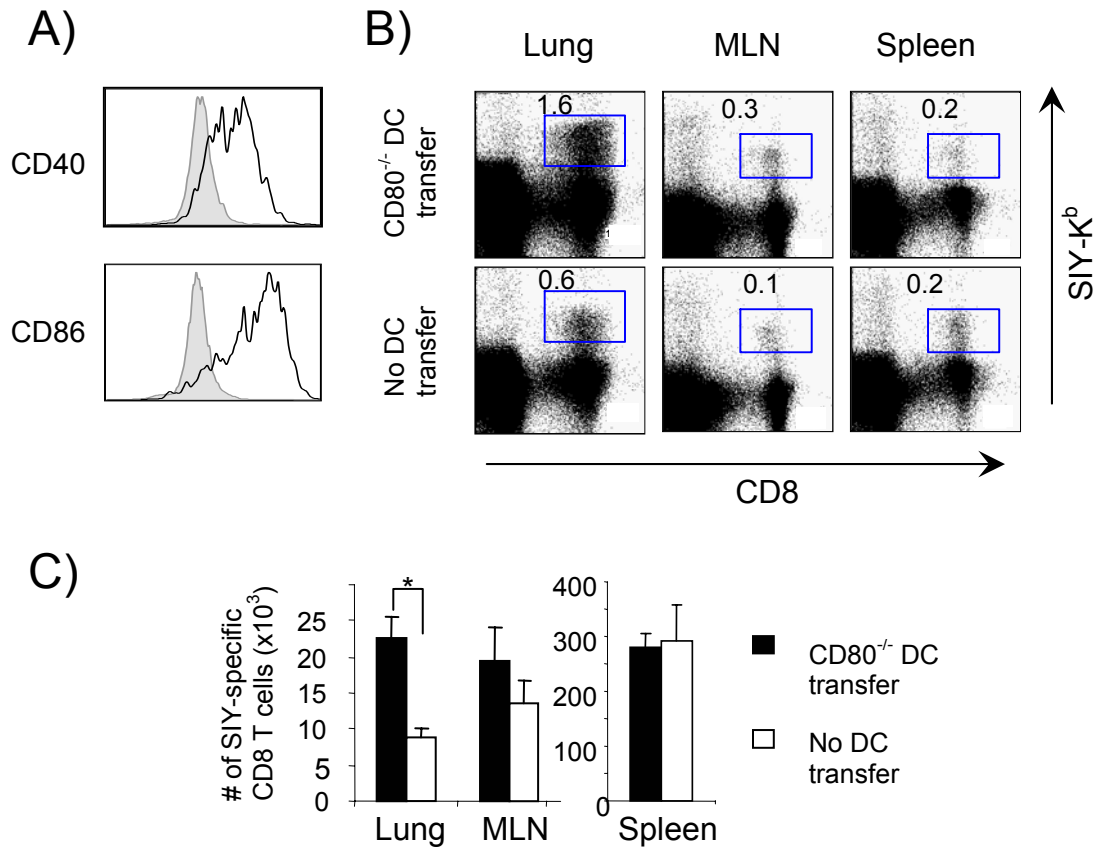
**Figure 4-24: B7-H1-mediated dendritic cell maturation is CD80-independent.**

**A)** Normal DC maturation in CD80$^{-/-}$ mice. CD80$^{-/-}$ mice were instilled with CFSE and 6 hr later, infected with WSN-SIY virus. 24 hrs post infection, cells from MLN and spleen were assayed for CD11c, CFSE plus CD40 and CD86. CD40 and CD86 expression is compared between CD11c$^{hi}$ CFSE$^{+}$ DCs from MLN (black line) and CD11c$^{hi}$ CFSE$^{-}$ DCs from the spleen (shaded area). **B** and **C**. DCs were purified from B7.1$^{-/-}$ mice and injected into the hind footpad of B7-H1$^{-/-}$ mice (5 x 10$^{5}$ per mouse). 24 hrs later, mice were infected with WSN-SIY virus. As control, B7-H1$^{-/-}$ mice without DC transfer were infected at the same time. Seven dpi, cells from the lung, MLN and the spleen were analyzed for CD8 and SIY-K$^{b}$ dimmer. **B)** SIY-K$^{b}$ versus CD8 staining profiles are shown for the live cells in the indicated tissues. The numbers indicate the percentages (mean ± SEM, n=3) of CD8$^{+}$ SIY-K$^{b+}$ T cells among all live cells. **C)** The number of SIY-specific CD8 T cells in different tissues, calculated by multiplying the total cell numbers with percentage of CD8$^{+}$ SIY-K$^{b+}$ in the specific tissue. Values represent the mean ± SEM (n=3). *p<0.05.

# 5. Discussion

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The adaptive immune system is a composition of highly specialized, systemic cells and processes that eliminate or prevent pathogenic challenges. Within the adaptive immune system, T cells play an important role in the effective defense against pathogens. However, adequate activation and regulation of antigen-specific T cells is an important requirement for a strong and effective immune response. In addition to antigen presentation by antigen-presenting cells (APCs), signals induced by co-stimulatory molecules play an essential role in this process. The number of newly identified and analyzed co-regulatory molecules has risen in the past few years and more are expected to be discovered in the future.

The object of this work was to identify the functions and the detailed mechanisms of the co-regulatory B7-H1 (PD-L1) molecule during T cell immune response against influenza virus infection in mice. Over the past decade, the functions of B7-H1 has been the focus for many studies and depending on the experimental system used in the respective studies, co-inhibitory or co-stimulatory properties of the B7-H1 molecule have been described. However, despite the numerous and intensive studies on B7-H1, the detailed immunoregulatory role of this molecule when expressed on T cells is still not fully understood.

In the first part of this work, a dual regulatory characteristic of the B7-H1 molecule in T cell immune response following influenza infection was demonstrated by assaying activation, proliferation and survival of virus-specific CD8 T cells. It was also shown that stimulatory and inhibitory functions of B7-H1 are temporally as well as spatially separated and are likely to involve different counter molecules. Subsequent experiments then aimed to identify the detailed mechanisms in how B7-H1 positively influences T cell response.

In the second part of this thesis, the direct influence of B7-H1 on dendritic cell maturation was illustrated and a B7-H1-mediated "conditioning" of DCs was shown. For

efficient DC maturation and strong T cell priming, a preceding DC conditioning was required that occurred during T cell-DC interaction in the absence of infection.

In the final part of this work, an attempt was made to identify the complementary molecule(s) that is interacting with B7-H1.

## 5.1 The role of B7-H1 in T cell response against Influenza virus infection

### 5.1.1 Model system: T cell immune response after WSN-SIY infection

B7-H1, a member of the B7 family of co-regulatory molecules, provides a mechanism for balance between stimulatory and inhibitory signals needed for an effective immune response. Since both negative and positive regulatory functions of the B7-H1 molecule has been described in different experimental systems, the aim of this work was to elucidate the detailed function of B7-H1 on T cell immune response following an acute respiratory infection with the influenza virus (Webster et al., 1999). In our mouse model, infection with the recombinant murine influenza A/WSN virus that expresses a CD8 epitope SIYRYYGL (SIY) (WSN-SIY) provided a well-developed experimental system to analyze T cell–mediated immunity in vivo and to determine the influence of B7-H1 on the activation, proliferation as well as survival of antigen-specific CD8 T cells.

Consequently, the production of sufficient amounts of the WSN and WSN-SIY viruses became the first task. MDCK cells were infected with different dilutions of the viruses and the concentration of the newly produced viruses was determined by using a well-established method, known as the plaque assay (Gaush and Smith, 1968). For an unknown reason, WSN-SIY viruses repeatedly infected MDCK cells much slower than WSN viruses and therefore the maximum yield of WSN-SIY virus was significantly lower then that of WSN. However, the concentration of both viruses was still very high and enough to allow detailed analyses throughout this thesis.

## 5.1.2 Inhibitory function of peripheral B7-H1 on 2C CD8 T cell response during infection

The immune-regulatory characteristics of B7-H1 in mice was first examined by comparing the response of 2C TCR transgenic cells (2C) in B7-H1-deficient (B7-H1$^{-/-}$) mice and WT mice, following WSN-SIY virus infection. The use of transgenic 2C CD8 T cells has the advantage of being easily detectable and allows the isolation of sufficient numbers of 2C CD8 T cells for further analysis. The influence of B7-H1 on 2C CD8 T cell response was examined at three different time points (7, 14 and 30 dpi) by assaying frequency and number of 2C CD8 T cells in both mice.

The "early" acute phase of infection is very critical and requires efficient and strong T cell response in order to successfully battle the viral challenge. A weak T cell response may lead to a chronic infection or even end fatal (Wherry et al. 2005; Shin and Rehermann, 2006). At later time points of infection, during memory T cell development and homeostasis, the influence of B7-H1 on the persistence of adoptively transferred virus-specific CD8 T cells, that might protect against the re-infection with the same or a related virus strain, was tested. Flow cytometric analysis in all three time points revealed significantly increased frequency and number of 2C T cells in B7-H1$^{-/-}$ mice, suggesting an inhibitory function of B7-H1 in 2C CD8 T cell response against WSN-SIY. However, as indicated by the T cell numbers and frequencies, B7-H1-mediated inhibition seemed to be limited only to the respiratory tissues (bronchoalveolar lavage and lung). Previously published in vitro studies demonstrated a negative effect of B7-H1 on T cell activation, proliferation and survival by using agonistic antibodies and anti-CD3 stimulation (Brown et al, 2003). Furthermore, the severe graft-versus-host-like disease that develops in 2C TCR transgenic PD-1$^{-/-}$ mice, together with the inhibitory effects of transfectants expressing B7-H1 and MHC class I on 2C TCR CD8 T cell responses, suggested that B7-H1 has an important role in regulating CD8 T cell responses in the periphery (Honjo et al, 1999; Carter et al, 2002).

In this work, the mechanism for enhanced 2C CD8 T cell expansion in B7-H1-deficient mice was further examined by analyzing early T cell activation and proliferation. Interestingly, T cell activation, as shown by CD25 staining, an early marker for T cell activation, and proliferation, as indicated by the CFSE dilution, revealed no significant difference in B7-H1$^{-/-}$ and WT mice between 3 to 7 dpi. This suggested that priming of 2C CD8 T cells was not affected by B7-H1 expression. In other studies, B7-H1 has been described to have negative effects on T cell survival by inhibiting stimulatory signals and

promoting apoptosis after interacting with the PD-1 receptor (Barber et al, 2006; Koup et al, 2006). The fact that ligation between PD-1 and B7-H1 in B7-H1$^{-/-}$ mice is impossible, strengthened the possibility that activated 2C CD8 T cells in these mice survive for a longer period of time than 2C CD8 T cells in B7-H1$^{+}$ (WT) mice. Flow cytometric staining of 2C CD8 T cells for the apoptotic marker Annexin V 7 dpi demonstrated that a significant fraction of 2C CD8 T cells in B7-H1$^{-/-}$ mice exhibited low Annexin V expression. This observation indicated reduced T cell apoptosis in B7-H1-deficient mice and provided an explanation for the enhanced 2C CD8 T cell response. However, elevated T cell apoptosis was observed primarily in the lung of B7-H1$^{+}$ WT mice suggesting a local initiation of B7-H1-mediated apoptosis. It has been shown that inhibitory signals that come from B7-H1 ligation play a crucial role in peripheral tolerance by preventing auto-reactive T cell development (Latchman at al, 2004). As B7-H1 expression has also been detected in non-lymphoid organs such as heart, placenta, lung, and pancreas in both human and mouse tissues (Liang et al., 2003), it is very likely that the regulatory function of B7-H1 expression in the lung is to prevent the excessive accumulation of active effector T cells after the virus is cleared.

## 5.1.3 Stimulatory influence of B7-H1 on endogenous CD8 T cell response during infection

Although adoptive transfer of monoclonal T cells is widely used to examine their characteristics in a specified environment, the analysis of endogenous CD8 T cell response in B7-H1$^{-/-}$ mice was preferred in this study. Endogenous T cells are unmodified naïve T cells that allow us a more significant study of the role of B7-H1. To this end, we determined the response of endogenous antigen-specific CD8 T cells to WSN-SIY virus infection in B7-H1$^{-/-}$ mice and WT mice seven, 14 and 30 dpi. Unexpectedly, the response of SIY-specific endogenous CD8 T cell in B7-H1$^{-/-}$ mice was profoundly diminished 7 days post infection. This result suggested, contrary to the previous observed results for 2C CD8 T cell response, that the expression of B7-H1 is beneficial for endogenous CD8 T cell response. However, impaired response of endogenous CD8 T cells was predominantly found in the respiratory tissues and was restricted to the early phase of infection (day 7 post infection).

One major difference between both cell types (2C T cells and endogenous T cells) is their origin. While 2C T cells, derived from 2C TCR transgenic mice on B6 background

(B7-H1$^{+/+}$), are capable to express B7-H1 on their cell surface, endogenous T cells in B7-H1$^{-/-}$ mice do not express B7-H1. Previous published studies suggested that reverse signaling through co-regulatory ligands, such as B7-H1 and B7-DC, may deliver direct signals into cells and influence their immunological functions (Nguyen et al., 2002; Dong et al., 2003). To test if the discrepancy between 2C CD8 T cell and endogenous CD8 T cell response in B7-H1$^{-/-}$ mice was caused due to expression of B7-H1 on 2C CD8 T cells, activation and proliferation of B7-H1-expressing and B7-H1-deficient T cells during early phase of infection was analyzed. The problem of very low numbers of endogenous antigen-specific CD8 T cells during early phase of infection was overcome by utilizing B7-H1-deficient 2C CD8 T cells from 2C TCR transgenic mice that were backcrossed with B7-H1$^{-/-}$ mice (both on B6 background). Isolation of B7-H1$^{-/-}$ 2C CD8 T cells from these double transgenic mice and transfer into B7-H1$^{-/-}$ recipients allowed us to examine activation and proliferation of B7-H1$^{-/-}$ T cells even during early point of infection. The impaired response of B7-H1$^{-/-}$ 2C CD8 T cells in B7-H1$^{-/-}$ mice 7 dpi, compared to B7-H1$^{+}$ 2C CD8 T cells, suggested that B7-H1 expression on CD8 T cells positively influences T cell immune response to WSN-SIY virus. This result motivated us to perform a more detailed comparison of B7-H1$^{+}$ vs. B7-H1$^{-/-}$ 2C CD8 T cell response to WSN-SIY infection in B7-H1$^{-/-}$ recipient mice by assaying T cell activation and proliferation. The significantly enhanced upregulation of the early activation markers CD69 and CD25 demonstrated that B7-H1 expression on T cells indeed positively affects T cell activation. Similarly, proliferation of B7-H1-expressing 2C CD8 T cells in B7-H1$^{-/-}$ mice was more vigorous and more distinctive throughout the course of infection. These novel results indicated that B7-H1 plays an important key role in the early activation of CD8 T cells and that B7-H1 expression on T cells is sufficient to compensate for an environmental deficiency.

Based on these results, it is tempting to postulate that B7-H1$^{+}$ 2C CD8 T cells may receive direct signals through their own B7-H1 ligands that promote activation and proliferation after antigen presentation. However, this seems to be unlikely as expansion of B7-H1-deficient 2C CD8 T cells in WT mice occurred normal and at a level comparable to B7-H1$^{+}$ 2C CD8 T cells. Although we cannot completely rule out that reverse B7-H1 signaling into CD8 T cells additionally contributes enhanced T cell response, this effect is presumably insignificant in our experimental system.

The results we have obtained so far, by analyzing the response of B7-H1$^+$ and B7-H1$^{-/-}$ T cells following challenge with influenza virus, demonstrated a dual regulatory function of B7-H1 that is temporally and spatially separated. Additionally to previously published studies that illustrated inhibition of T cell activation and reduced survival mediated by peripheral B7-H1 expression, our results also demonstrated that B7-H1 expression in the lung promotes apoptosis of virus-specific effector CD8 T cells that have migrated to the lung to clear the virus. The apoptotic signal hereby is most likely triggered when B7-H1 binds to the PD-1 receptor on activated T cells. Expression of PD-1 is not constitutive but upregulated on activated T cells, 48-72 hrs post infection (Agita et al., 1996). Therefore, the delay in PD-1 upregulation explains why B7-H1-mediated apoptosis did not negatively affect T cell expansion during the first few days of infection. However more interestingly, we demonstrated a novel function of B7-H1 molecules that are expressed on T cells. Expression of B7-H1 on T cells positively influences early T cell activation and proliferation in the draining lymphnode (MLN) of infected mice.

## 5.1.4 Recovery of T cell response by transfer of B7-H1-expressing T cells

As already illustrated, immune response of B7-H1$^{-/-}$ 2C CD8 T cells in B7-H1$^{-/-}$ recipients are impaired. However, in the same experiment, B7-H1$^{-/-}$ 2C CD8 T cells that were transferred into WT mice exhibited normal immune response. The frequency and numbers of these cells were similar to that of B7-H1$^+$ 2C CD8 T cells. This observation suggested that B7-H1-deficient T cells are capable to undergo normal activation and proliferation in the presence of B7-H1 expressing cells, which is the case in WT mice.

By transferring various numbers of B7-H1-positive CD8 T cells (2C) into B7-H1$^{-/-}$ recipient mice we determined the influence of B7-H1-expressing cell in endogenous CD8 T cell response. The results of the first set of experiments presented a significantly enhanced endogenous CD8 T cell response to WSN-SIY infection in B7-H1$^{-/-}$ mice that had received B7-H1$^+$ 2C T cells. Interestingly, transfer of low copy numbers of B7-H1$^+$ 2C CD8 T cells provided the best stimulus for enhanced endogenous T cell response. With increasing 2C CD8 T cell numbers, endogenous T cell response declined, most likely due to competition for the antigen. However, this experiment was not optimal since both cell types responded to the SIY- antigen. To avoid a possible complication, B7-H1-expressing CD8 T cells from F5 TCR transgenic mice were transferred into B7-H1$^{-/-}$

mice, as these cells do not respond to the SIY epitope. It was irrelevant whether quiescent F5 CD8 T cells or antigen-specific 2C CD8 T cells were transferred. In both cases, SIY-specific endogenous CD8 T cell responses were clearly enhanced, indicating recovery of impaired T cell response by B7-H1 expressing cells.

In both experiments, B7-H1-expressing CD8 T cells restored or improved response of endogenous antigen-specific CD8 T cells in B7-H1$^{-/-}$ mice. However, it is important to note that the B7-H1 molecule is expressed in many different cell types within the lymphatic tissue, including CD4 T cells, B cells, DCs, Macrophages and many more. Many of these cells may interact with each other during inflammation and exchange signals. Therefore, the positive effect of B7-H1-expression on other cell types was examined by transferring purified CD4 T cells and B cells into B7-H1$^{-/-}$ mice. Both CD4 T cells and B cells express B7-H1 on their cell surface at levels comparable to CD8 T cells but only T cells interact directly with DCs (review Sharpe, 2007; Yamazaki et al., 2002). Our results revealed that only B7-H1-positive CD4 T cells but not B cells restored endogenous T cell response. This suggested that only B7-H1-expressing T cells are capable to positively influence endogenous T cell stimulation. Moreover, considering that the stimulatory feature of B7-H1 is induced exclusively by B7-H1-positive T cells strengthens the possibility that antigen presenting cells (APCs) such as dendritic cells might be the target of these cells.

## 5.2 The role of B7-H1 in dendritic cell maturation following infection with influenza virus

### 5.2.1 B7-H1 influences dendritic cell maturation

It is well established that effective T cell activation requires adequate signaling by functional mature dendritic cells that have been stimulated by antigens (Steinman 1991, Norbury et al., 2002). DCs that are immature lack the ability to efficiently initiate T cell activation, and mice with defective DC maturation exhibit poor immune responses (Ignatius et al., 2000). Therefore, we determined if impaired endogenous CD8 T cell response in B7-H1$^{-/-}$ mice underlies defective DC activation. Indeed, maturation of DCs in B7-H1$^{-/-}$ mice following WSN-SIY infection was defective, as shown by the low

expression of DC activation markers CD40, CD80 and CD86. The inefficient maturation of DCs in B7-H1$^{-/-}$ mice provided a possible explanation for the decreased activation and proliferation of endogenous CD8 T cells and B7-H1$^{-/-}$ 2C CD8 T cells in these mice, respectively. More important, this finding illustrated a new and so far unknown function of B7-H1 during immune response. Our data suggested that B7-H1 may deliver essential signals that are beneficial for DC maturation. Without this stimulatory signaling in B7-H1$^{-/-}$ mice, generation of active and mature DCs is defective and as a result of this, antigen-presentation and stimulation of CD8 T cells response is impaired.


## 5.2.2 B7-H1 expressing T cells restore dendritic cell maturation

Our studies have suggested that B7-H1 has an important stimulatory function in DC maturation after infection with WSN-SIY virus. However, despite the fact that DCs in B7-H1$^{-/-}$ mice are defective, activation and proliferation of adoptively transferred 2C CD8 T cells (B7-H1$^{+}$) in these mice occurred normal when compared to WT mice. One study demonstrated that signals derived from T cells may play a critical role in the generation of mature DCs that are capable of effectively polarizing naïve antigen-specific T cells to produce Th1 cytokines (Leslie et al., 2002). Although, there are likely to be normal interactions between endogenous T cells and DCs in B7-H1$^{-/-}$ mice, it is possible that signals induced through key effector molecules required for DC maturation may be missing from this interaction. The CD40-ligand (CD40L), expressed on CD4 T cells, has been described as an effector molecule and its ligation to CD40 on DCs has been shown to increase DC viability and induce DC maturation (Banchereau et al., 1994; Sallusto and Lanzaveccia, 1994, Mackey et al., 1998; Grewal and Flavell, 1995). But in contrast, various other studies have also shown that maturation of DCs can be mediated by CD8 T cells in a CD4 T cell-independent manner (Ruedl et al., 1999; Lu et al., 2000; Tripp et al., 1995; Bachman ad Zinkernagel, 1998). This and other studies provided a direct evidence for T cell mediated DC activation but did not show the exact manner in which T cells influence DC maturation.

In our study, the reason for normal 2C CD8 T cell activation and proliferation in B7-H1$^{-/-}$ mice can be explained due to restored DC maturation by B7-H1 signals that are most likely derived from the 2C CD8 T cells. In return, functional mature DCs then efficiently induced 2C CD8 T cell priming, resulting in normal activation and proliferation of 2C CD8 T cells in B7-H1$^{-/-}$ mice. This was further affirmed by the transfer of B7-H1-

expressing CD8 T cells into B7-H1$^{-/-}$ mice and the analysis of DC maturation in these mice after WSN-SIY infection. Maturation of DCs was completely restored in B7-H1$^{-/-}$ mice that had received B7-H1-expressing CD8 T cells. However, this experiment was not very conclusive, as recovery of DC maturation was examined by comparison of DC status in B7-H1$^{-/-}$ mice that received B7-H1$^{+}$ T cells and B7-H1$^{-/-}$ mice without T cell transfer. But it is possible that the process of cell transfer into mice itself may trigger signals that could influence DC maturation independent of B7-H1-signaling. To this end, B7-H1$^{+}$ and B7-H1$^{-/-}$ T cells were adoptively transferred into B7-H1$^{-/-}$ recipients and DC maturation in both mice was assayed after infection. This approach allowed a fair comparison between both mice. The upregulation of all three markers for DC maturation (CD40, CD80 and CD86) in mice that had received B7-H1$^{+}$ T cells but not B7-H1$^{-/-}$ T cells provided direct evidence that DC maturation is indeed restored by B7-H1-expressing T cell.

### 5.2.3 DC maturation in T cell-deficient RAG-1$^{-/-}$ mice is defective

The interplay of T lymphocytes and DCs has been analyzed mainly with respect to the stimulation of naive T cells by DCs. However, various studies in the past suggested a more complex reciprocal relationship between these two. Murine DC lines have been shown to undergo maturation upon interacting with T cells in vitro in a phenomenon that has been appropriately referred to as "T cell-mediated terminal maturation of DCs" (Volkmann et al., 1996; Kitajima et al., 1996). For instance, RAG-1$^{-/-}$ and RAG-2$^{-/-}$ mice (Recombination activating gene-1/2) are T cell and B cell-deficient mice and also known to have defective DC maturation after stimulation with antigen (Muraille et al., 2002). Furthermore, mice with a mutation in the RAG-1 gene have phenotypes similar to that of SCID (severe combined immune deficiency) mice (Chen et al, 1993; Shinkai et al., 1992; Mombaerts et al., 1992). However, the impaired ability of DCs to present antigen was corrected by reconstituting T cell deficient RAG-2$^{-/-}$ mice with normal T lymphocytes (Shreedhar et al., 1999). In accordance with previously published data, our study demonstrated that regulatory signals derived from T cells during interaction with DCs that promote DC activation. However to date, the detailed mechanisms of how this interaction restored DC maturation as well as the nature of the responsible T cell molecule(s) are not completely understood, but it is assumed that stimulatory signals mediated by effector molecules, such as CD40L or B7-1/2, might be involved.

In this study, we have shown that B7-H1 expression on T cells positively influenced DC maturation. But so far it was not clear whether B7-H1-mediated DC maturation was induced during direct interaction with T cells. Therefore, introducing B7-H1$^{-/-}$ CD8 T cells and B7-H1$^{+}$ CD8 T cells, respectively, into RAG-deficient mice allowed us a more detailed examination of the mechanism in how B7-H1 induces DC maturation. The lack of B cells in this case is negligible since DC maturation seems to be B cell-independent. It is maybe worth mentioning that DC maturation in all the previous experiments was assayed by intranasal infection with a high dose of the WSN-SIY virus (1000 pfu) and isolation of DCs from the mediastinal lymphnode (MLN) that drains the lung. Since T and B cells are absent in RAG-1$^{-/-}$ mice, the MLNs are very small and almost impossible to detect. Therefore, several attempts to isolate and analyze DCs from the MLN of RAG-1$^{-/-}$ mice failed, even after pooling multiple mice, due to very low DC numbers. As a solution, RAG-1$^{-/-}$ mice were infected intraperitoneally (i.p.) (into the body cavity) with a very high dose (1 x 10$^6$ pfu) of virus and DCs were isolated from the mesenteric lymphnodes that drain the intestines. While i.p. infections with influenza are not productive and the virus cannot replicate in the peritoneal cavity, it still causes activation, migration and antigen presentation of peritoneal resident APCs. To be able to infect multiple mice with such a high dose of virus, the concentration of WSN-SIY virus was too low (8 x 10$^4$ pfu/ml), and therefore mice were infected with the high titered WSN virus (5 x 10$^6$ pfu/ml).

As expected, DC maturation in RAG-1$^{-/-}$ mice without any T cell transfer was defective. Furthermore, only DCs in RAG-1$^{-/-}$ mice that had received B7-H1$^{+}$ T cells had restored maturation, as illustrated by the upregulation of maturation markers CD40, CD80 and CD86. Maturation of DCs in RAG-1$^{-/-}$ mice that received B7-H1$^{-/-}$ T cells remained defective. This observation did not only confirm one more time that B7-H1-mediated signals are necessary to generate efficient DC maturation, more important, it suggested that T cell-DC interaction is necessary to promote DC maturation. It is very likely that T cells provide an environment during interaction with DCs that favors B7-H1-mediated positive signals to DCs. If this is the case, DCs in RAG-1$^{-/-}$ mice are missing this signaling which then explains their defective maturation. However, at this point we are unable to rule out possible additional factors that could promote defective DC maturation in RAG-1$^{-/-}$ mice as well.

## 5.2.4 DC conditioning during T cell-DC interaction

T cells are capable to interact with immature DCs long before infection (Hugues et al., 2006). Although this interaction is not very distinctive and of short-duration, it is possible that during this short period of time T cells may induce signals through B7-H1 and set DCs in a modified state ("conditioned") where they uptake more readily antigens and efficiently mature (Altmann and Boyton, 2003; Filatenkov et al., 2005). If this is the case, DCs from WT mice are likely to be in such a "conditioned state", since they reside in an environment that would allow them to interact with B7-H1$^+$ T cells, and upon infection they are capable to successfully stimulate T cell activation.

Our observations in this study supported the theory of conditioned DCs in B7-H1$^+$ (WT) mice, as response of endogenous SIY-specific CD8 T cell to WSN-SIY was completely restored in B7-H1$^{-/-}$ mice that were adoptively transferred with DCs (conditioned) from WT mice. Moreover, endogenous CD8 T cell response remained impaired in B7-H1$^{-/-}$ recipients that received DCs from B7-H1$^{-/-}$ mice (non-conditioned DCs), demonstrating a B7-H1-dependent conditioning of DCs. Interestingly, although "conditioned" DCs from WT mice were immature when transferred into B7-H1$^{-/-}$ mice and could not receive any further B7-H1-stimulus, endogenous T cells response were still restored by these DCs. This suggested that immature DCs that are conditioned by B7-H1 are capable to efficiently mature and activate T cells in the absence of further B7-H1 signaling.

The experiment in which WT DCs were adoptively transferred into B7-H1$^{-/-}$ recipients revealed that B7-H1-mediated signals set DCs into a "conditioned state" which seems to be a prerequisite for efficient DC activation. This was further shown by the recovery of antigen-specific CD8 T cell response in B7-H1$^{-/-}$ mice that were injected with conditioned RAG-1$^{-/-}$ DCs. The fact that endogenous CD8 T cell response in B7-H1$^{-/-}$ mice was only restored by DCs, isolated from RAG-1$^{-/-}$ mice that had been transferred with B7-H1$^+$ T cells but not DCs from RAG-1$^{-/-}$ mice, injected with B7-H1$^{-/-}$ T cells, provided evidence that DC-conditioning is B7-H1-dependent. Moreover, since RAG-1$^{-/-}$ mice were not infected after T cells were transferred demonstrates that short interactions between DCs and T cells in the absence of inflammatory stimuli are sufficient to condition DCs.

Taken together, this part of the thesis described a novel function for the B7-H1 molecules that are expressed on T cells. The analysis of dendritic cell maturation in B7-H1$^{-/-}$ and RAG-1$^{-/-}$ mice following antigenic stimulation revealed that DC maturation

requires a preceding conditioning that is provided by B7-H1 signals during T cell-DC interaction. Furthermore, DCs in both mice were conditioned in the absence of inflammation, suggesting that short-lived T cell-DC interaction in naïve mice is sufficient to induce DC conditioning.

## 5.3 Potential interaction partners of B7-H1

### 5.3.1 PD-1, a receptor for B7-H1, is not involved in B7-H1-mediated DC conditioning

Most of the previous experiments, shown and discussed in this study, served to demonstrated the stimulatory function of B7-H1. It was clearly shown that B7-H1-mediated signals during T cell-DC interaction induce "conditioning" of DCs. However, the functional binding partner to B7-H1 that is expressed on DCs has not been identified.

In the past few years, an increasing number of scientists have proposed that B7-H1 can bind to an unknown molecule, mediating stimulatory signals, but until recently, the only known receptor or binding partner of B7-H1 has been the PD-1 receptor (Dong et al. 2002; Yamazaki et al. 2005). As mentioned earlier, the glycoprotein PD-1 is only expressed on activated T cells, B cells and Monocytes. Although there is no evidence of PD-1 expression on immature murine DCs, we have sought to confirm this by staining for PD-1 (Agata et al., 1996). Flow cytometric analysis demonstrated no detectable PD-1 expression on murine dendritic cells and therefore, we can confidently exclude PD-1 as a binding partner that induces DC conditioning. In addition to this, it is well established that PD-1 signaling generates inhibitory effects on the immune response (Nishimura et al., 1999; Salama et al., 2003; Freeman et al., 2000), and a possible interaction between B7-H1 and PD-1 would most likely inhibit initiation of DC maturation and subsequent T cell activation.

### 5.3.2 Binding of B7-H1 to the B7.1-ligand does not induce DC conditioning

In a recent study, it has been demonstrated that B7.1 (CD80), a co-stimulatory member of the B7-family, is capable to interact to B7-H1 with intermediate affinity. Further, it has

been shown that this interaction was functionally significant and induced inhibitory signals (Sharpe et al., 2007). Since B7.1 is constitutively expressed on DCs, it is tempting to assume that B7.1 may be the putative binding partner to B7-H1 that induces DC conditioning. To test this possibility, we utilized mice that are B7.1-deficient. If conditioning of DCs is indeed triggered by the interaction between of B7.1 and B7-H1, then the assumption is that B7.1-deficient mice should exhibit defective DC maturation. However, DC maturation, such as induction of CD86 and CD40 expression, occurred normally in B7-H1$^{-/-}$ mice in response to influenza infection. More importantly, transfer of B7.1-deficient DCs into B7-H1$^{-/-}$ mice enhanced endogenous CD8 T cell response to influenza virus, suggesting normal DC maturation in the absence of B7.1. Therefore, B7-H1 likely interacts with a new partner to mediate the T cell-mediated DC maturation. Numerous investigators have proposed that B7-H1 may bind to an unknown molecule, which appears to play a stimulatory role in T cell response. Findings presented here are consistent with this proposal. However, whether the yet to be identified molecule is the interacting partner of B7-H1 in T cell-mediated DC maturation remains to be determined.

## 5.4 Conclusions

B7-H1 is widely expressed on both hematopoietic and non-hematopoietic lineage cells and interacts with multiple ligands. Results presented here and published previously suggest that B7-H1's diverse expression and interacting with multiple partners underlie its diverse functions in regulating immune responses. Following influenza infection, the impaired CD8 T cell activation and proliferation in the draining lymphnodes (DLN) and reduced number of antigen-specific CD8 T cells in B7-H1$^{-/-}$ mice clearly illustrates a stimulatory function of B7-H1 in the initiation of T cell immune response. As shown in this study, the stimulatory function is mediated by B7-H1-dependent T cell conditioning of DCs. Thus, following influenza virus infection, DC cells in the respiratory tract take up antigen and migrate to DLN, where they then interact with T cells in B7-H1-dependent manner. The interaction and recognition of viral components results in efficient DC maturation, including expression of CD40, CD80 and CD86. The mature DCs then interact with antigen-specific CD8 T cells via TCR-peptide/MHC and CD28-CD80/CD86, resulting in activation and proliferation of antigen-specific T cells. The activated CD8 T cells acquire effector function and ability to migrate to the site of infection, where they

help to eliminate infecting virus. The extensive interactions between T cells and DCs revealed here are consistent with results from live cell imaging analysis between antigen-specific T cells and DC cells in lymph nodes, where their interactions can last for several hours (Stoll et al., 2002; Miller et al., 2002). It is possible that all the step-wise interactions revealed from this study occur without T cells and DCs ever separating from each other.

Interestingly, the impaired T cell responses in B7-H1$^{-/-}$ mice are restricted to the early phase of the responses. By 14 and 30 dpi the percentage and number of antigen-specific CD8 T cells in the spleen and the lung of B7-H1$^{-/-}$ mice are similar to those in WT mice, suggesting an inhibitory role of B7-H1 in late phase of T cell responses. The inhibitory effect is likely mediated by peripheral tissue, such as the lung, expressed B7-H1 that interacts with PD-1 expressed by effector T cells, resulting in an inhibition of activated T cells (Sharpe A, 2003; Barber et al, 2006; Koup et al, 2006). The inhibitory role of tissue-expressed B7-H1 exhibits its effect only when effector T cells reach the tissue site at the late phase of immune response, most likely to avoid excessive accumulation of effector T cells after the virus is cleared. Thus, while T cell number in WT mice rapidly decreases 14 dpi, the decline of antigen-specific T cell number in B7-H1$^{-/-}$ mice is slowed due to the lack of this inhibition.

In summary, our findings demonstrate a novel function for B7-H1 on T cells in DC maturation and early immune response. Immature DCs must enter a 'conditioned state' in order to undergo rapid maturation and effective T cell activation following microbial challenge. This conditioning is mediated by B7-H1 during interaction between DCs and B7-H1 expressing naïve T cells, even in a non-inflammatory environment.

# 6. References

# 6. References

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