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# Creating and Use of an New Experimental Preclinical HLA Transgenic Mice Model to Mapping HLA-restricted T Cells Epitopes for Polyepitopes Vaccine Design

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Ecole doctorale de signalisation et réseaux intégratifs en biologie

Doctorat

Immunologie

Présenté et soutenu par

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Exploitation d'un modèle expérimentale préclinique de souris HLA  
transgénique pour l'identification des épitopes T HLA-restreint afin de  
concevoir des vaccins poly-épitopiques

Thèse dirigée par Dr Yu-Chun LONE

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## Abbreviation

Abbreviation	Full Name
Aa	Amino acid
APC	Antigen presenting cell
bp	Paire de bases
BSA	Bovine serum albumin
CTL	Cytotoxic T Lymphocyte
CPM	Counts per minute
DNA	Deoxyribonucleic Acid
DC	Dendritic cell
D-MEM	dulbecco's minimum essential medium
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
EBV	Epstein-Barr Virus
FBS	fetal bovine serum
g	gram
h	hour
HA	Hemagglutinin
HBc	HBV Capsule
HBs	HBV envelope Protein
hCD4	Human CD4 molecule
HLA	Human leucocyte antigen
HRP	horseradish peroxidase
IC <sub>50</sub>	half maximal inhibitory concentration
IPTG	isopropyl $\beta$ -D-thiogalactoside
IgG	immunoglobulin G
IFN- $\gamma$	Interferon $\gamma$
IL	Interleukine
Kb	Kilobase
kDa	Kilodalton
LB	Luria-Benrtani medium
min	Minute
mg	milligram
ml	milliliter

mM	millimole per liter
M1	Matrix protein 1
NP	Nucleoprotein
NA	Neuraminidase
OD <sub>450</sub>	Absorption value at 450nm
OVA	Albumin from chicken egg white
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBMCs	Peripheral blood mononuclear cells
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
PMA	phorbol myristate acetate
rpm	rotate per minute
SDS	sodium dodecyl sulfate
SI	Stimulation index
T CD4+	CD4+ T lymphocyte
T CD8+	CD8+ T lymphocyte
TBS	tris buffered saline
TAP	Transporter associated with antigen processing
TCR	T cell receptor
WB	western-blot
μg	microgram
μl	microliter
μm	micrometer
μM	micromole per liter

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# *Introduction*

## I. Major Histocompatibility Complex (MHC)

Major histocompatibility complex (MHC) is a complicated antigen system which is widely present in mammalian cells and it was encoded by a large gene family in all vertebrate. Currently, the function of MHC antigens has been basically stated: it poses an important role in the differentiation and maturation of peripheral T-cell, initiation and regulation of T immune response and it closely related to the autoimmunity. Therefore, it is one of the most important molecules in the immune system and it's valuable to further exploit its potential function.

MHC gene families varies from different species, for example, chickens MHC (B antigen) having among the smallest known MHC regions (19 genes)<sup>[1]</sup>; the murine MHC gene region spans approximately 4 Mb of chromosome 17 (23.0 cM, cytoband B–C) and contains 3 major classes of highly polymorphic gene sets: class I (H-2-K, H-2-D, Q, H-2-T18 genes), class II (H-2-I genes), and class III (H-2-S genes)<sup>[2]</sup>; in humans the MHC region located on the chromosome 6, between the flanking genetic markers MOG and COL11A2, and contains 140 genes spanning 3.6 mega base pairs(3.6 Mb)<sup>[3]</sup>.

The MHC gene family consists of three subgroups: class I, class II and class III, which are shown in *Table 1*, take charge of encoding corresponding antigen to mediate immunity.

Class	Antigen	Molecular structure
I	<ul style="list-style-type: none"> <li>● Peptide-binding proteins, which bind short amino acids sequences for antigen presentation</li> <li>● Molecules aiding antigen-processing e.g. TAP and Tapasin</li> </ul>	<p>The diagram shows the MHC Class I structure. It consists of a heavy chain (α) with three domains: α<sub>1</sub>, α<sub>2</sub>, and α<sub>3</sub>. The α<sub>1</sub> and α<sub>2</sub> domains form a peptide-binding groove. A light chain, β-2-microglobulin (β<sub>2m</sub>), is associated with the α<sub>1</sub> domain. The α<sub>3</sub> domain is transmembrane and anchors the molecule into the cell membrane.</p>
II	<ul style="list-style-type: none"> <li>● Peptide-binding proteins, which bind longer amino acid sequences for antigen presentation</li> <li>● Proteins assisting antigen loading onto MHC class II's peptide-binding proteins</li> </ul>	<p>The diagram shows the MHC Class II structure. It consists of two heavy chains (α and β) with two domains each: α<sub>1</sub>, α<sub>2</sub> and β<sub>1</sub>, β<sub>2</sub>. The α<sub>1</sub> and β<sub>1</sub> domains form a peptide-binding groove. The α<sub>2</sub> and β<sub>2</sub> domains are transmembrane and anchor the molecule into the cell membrane.</p>
III	<ul style="list-style-type: none"> <li>● Other immune protein e.g. Complement cascade</li> <li>● Cytokines of immune signaling</li> </ul>	Various

Table 1. Three MHC gene subgroups and their functions.

## A. Structure of MHC antigen

In three kinds of MHC antigens, MHC class I and class II were proved to be more significant in specific cellular immunity and both of antigens are the most studied antigen in MHC antigen.

MHC class I antigen presents on the surface of all nucleated cells –in essence all cells but red blood cells. Actually, there are two kinds of MHC class I molecules, including classical MHC molecules and nonclassical molecules; however, we mainly focused on the classical MHC molecules. MHC class I occurs as an  $\alpha$  chain composed of three domains- $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ . The  $\alpha_1$  subunit stands with a unit of the non-MHC molecule  $\beta_2m$  which encoded on human chromosome 15. The  $\alpha_2$  subunit form a

peptide-binding groove with  $\alpha 1$  ( $\alpha 1/\alpha 2$  heterodimer) to present epitope to CTL. The  $\alpha 3$  subunit is transmembrane, anchoring the MHC class I molecule to the cell membrane. What's more,  $\alpha 3$  subunit allow CD8 molecule of CTLs dock on its surface and initiate cellular immunity.

MHC class II antigen can be conditionally expressed by all cell types, but normally occurs only on professional antigen-presenting cells (APC), such as macrophages, B cells and especially dendritic cells (DCs). MHC class II molecule is constructed by two chains,  $\alpha$  and  $\beta$ , and each chain contain two domains- $\alpha 1$  and  $\alpha 2$  and  $\beta 1$  and  $\beta 2$ . Among them,  $\alpha 1$  and  $\beta 1$  form a heterodimer and the peptide being presented is held by the floor of the peptide-binding groove, in the central region of the  $\alpha 1/\beta 1$  heterodimer;  $\alpha 2$  and  $\beta 2$  is transmembrane domain that anchoring the MHC class II molecule to the cell membrane.

## **B. Immunological function of MHC molecule**

### **a. MHC restriction**

During the development of T cells in the thymus, immature T lymphocytes have to go through a positive selection, which make sure T lymphocytes can get self MHC restriction and subsequently develop into CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes, and a negative selection, which avoid T lymphocytes recognize the self-antigen and MHC complex. In this way, T cell receptor (TCR) of all mature T lymphocytes possesses dual

specificity: recognizes self MHC as well as nonself antigens.

### **b. Antigen processing and presentation**

For MHC class I pathway, proteins in the cytosol are cleaved into fragments by proteasome, liberating peptides internalized by TAP (transporter associated antigen presenting) channel in the Endoplasmic reticulum(ER), then assembles with freshly synthesized MHC-I. These peptide-MHC-I complexes are following glycosylated, secreted out of cell membrane and finally recognized by TCR of CD8<sup>+</sup> T lymphocytes. Once CD8 molecule in CTLs dock to the MHC-I accompany with the recognition of TCR to the matching epitope, the CTLs transduce signals prompting the target cell's apoptosis. In this way, MHC-I antigen participates to mediate cellular immunity (Figure 1).

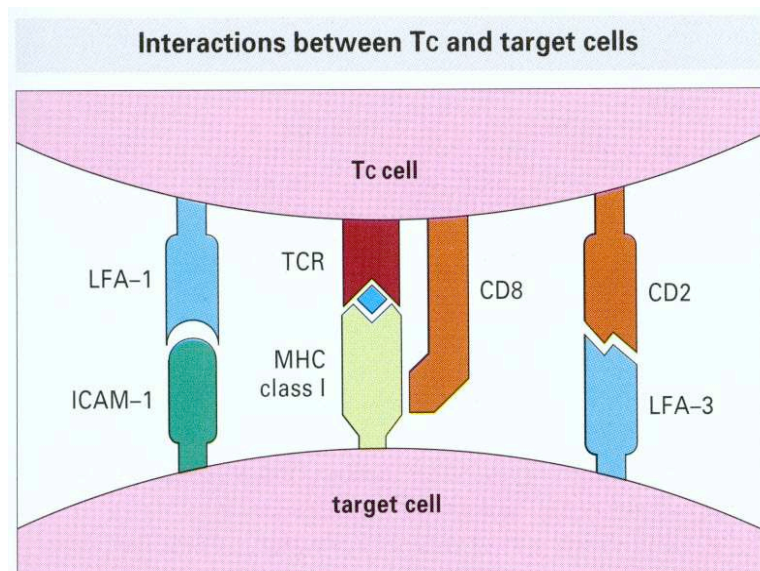


Figure 1. Mechanism of MHC class I-mediated CTL response.

Several experiments demonstrated that peptides that can be bound by MHC-I molecule with a bias: they are mostly a linear structure; from 9 to

10 amino acids for the length, because it was reported that HLA binding affinity of 9-amino acids peptide is 100-1000 times greater than that of more or less 9 amino acids; the anchor residues of these peptides are mainly located on the first N-terminal 2 and 9 to bind HLA molecule through hydrogen bonds; their hydrophobic carboxyl-terminal is constituted by aliphatic amino acids.

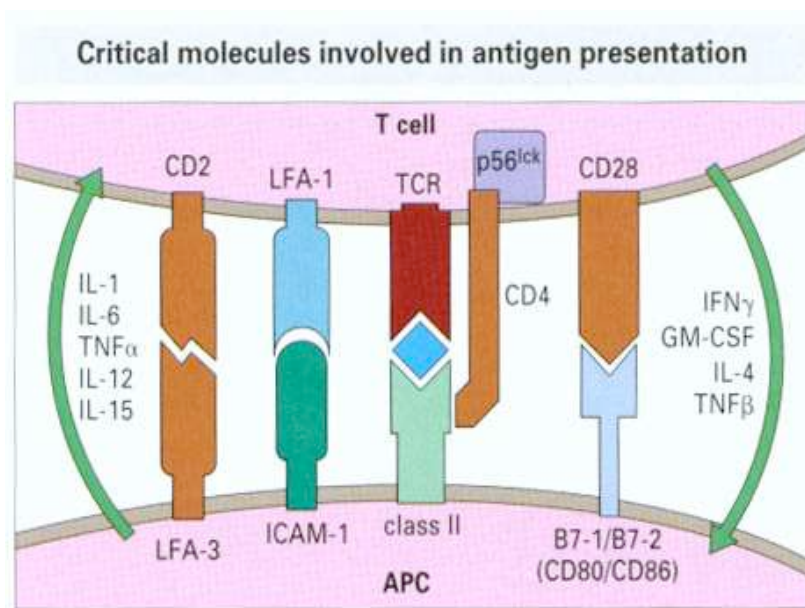


Figure 2. Mechanism of MHC class II-mediated CTL response.

For MHC class II pathway, antigen processing is mainly performed by phagocytes, such as macrophage, immature dendritic cells, mononuclear phagocytes, B lymphocytes, endothelial cells and epithelium of thymus. These phagocytes uptake entities into phagosomes and the uptaken proteins are cleaved into many different short peptides. Then the peptides assemble with MHC-II in endoplasmic reticulum (ER) and further present on the surface of phagocytes to be recognized by T cell receptors (TCR)

of CD4+ T lymphocytes. In this way, CD4+ T lymphocytes are stimulated and differentiate into effector T cells to mediate specific cellular response (shown in Figure 2).

As distinct from MHC class I epitopes, peptides that can be bound by MHC-II molecules display peptides of variable length(usually 12-26 mers), often encompassing a common binding core sequence, which is variably extended in both the N- and C-terminal directions<sup>[4]</sup>. Crystal structure of complexes between human MHC class II molecules and peptides have indicated that the peptide binding grooves of the different MHC class II isotype are superimposable and that the backbone of peptides bound to the binding grooves is highly conserved. The binding grooves are mainly characterized by properties of the so-called P1, P4, P6 and P9 pockets (as shown in Figure 3), which confer the specificity to the anchor residues of the peptides bound to the groove<sup>[5]</sup>. This 9 peptide binding core, or nonamer, is sufficient to bind MHC class II molecule<sup>[6]</sup>.

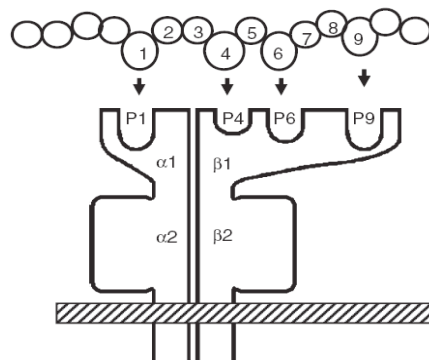


Figure 3. Schematic picture of an MHC class II molecule and a peptide.

### c. MHC and diseases susceptibility

It is reported that some MHC alleles, such as HLA alleles was often

found at a much higher frequency in those suffering from certain diseases than in the general population. Most of these diseases associated with particular MHC alleles, for instance, as autoimmune disorders, certain viral diseases, disorders of the complement system, some neurologic disorders, and several different allergies, more detail was shown in *Table 2*.

Disease	Associated HLA allele	Relative Risk*
Ankylosing spondylitis	B27	90
Hereditary hemochromatosis	A3	9.3
	B14	2.3
	A3/B14	90
Psoriasis	B13	
	B17	
Diabetes	B8	
	Bw15	
	B18	
Reactive arthritis (Yersinia, Salmonella, Gonococcus)	B27	18
Reiter's syndrome	B27	37
Goodpasture's syndrome	DR2	16
Gluten-sensitive enteropathy	DR3	12
Insulin-dependent diabetes mellitus	DR4/DR3	20
Multiple sclerosis	DR2	5
Myasthenia gravis	DR3	10
Narcolepsy	DR2	130
Rheumatoid arthritis	DR4	10
Sjogren's syndrome	Dw3	6
Systemic lupus erythematosus	DR3	5

**\*Relative risk is calculated by dividing the frequency of the HLA allele in the patient population by the frequency in the general populations:**

$$RR = \frac{\frac{Ag^+}{Ag^-} \text{ (Disease)}}{\frac{Ag^+}{Ag^-} \text{ (Control)}}$$

*Table 2.* HLA and disease susceptibility.

From the discovery of human HLA antigen, it is discovered that more than 50 human diseases are related to one or more human HLA antigens.



For example, the HLA-B27 antigen arises from about 90% ankylosing spondylitis disease <sup>[7]</sup>; crowd carrying HLA-A29 monomer tends to suffer from birdshot chorioretinopathy<sup>[8]</sup>; IDDM is related to HLA-B8, HLA-Bw15 and HLA-B18 and so on <sup>[9]</sup>; the patients with psoriasis carries HLA-B13 or HLA-B17 alleles and so on <sup>[10]</sup>, therefore, specific HLA clinically becomes the genetic markers for some diseases. Now, the correlation of HLA alleles and a given disease can be predicted by determining the frequency of the HLA alleles in patients afflicted with the disease, then comparing these data with the frequency of the same alleles in the general population. In this way, the susceptibility can be predicted and at present, HLA typing in autoimmunity is being increasingly used as a tool in diagnosis.

The built HLA transgenic mouse model can be used for the researches on the relevance between specific HLA and diseases. For example, Breban et al built the HLA-B27 transgenic mouse model to study the relevance between this haploid and spondylarthritis, and determined the protection and susceptibility of specific HLA for diseases through understanding of the disorder mechanism of the immune system, so as to provide references for clinical prevention and therapeutic schedule and so on.

#### **d. MHC in transplant rejection**

It is well known that MHC also could be considered as an antigen in a

transplant procedure and it can stimulate immune response in the recipient. Normally, even though high MHC polymorphism at the population level, an individual bears at most 18 MHC I or II alleles. For each person, around six HLA class I alleles and six to eight HLA class II alleles simultaneously occur in human haplotype. Thus, serology-based HLA typing is the most important determinant factor in transplantation.

In summary, MHC antigen is one of the most crucial molecules in the immunity. It is necessary to further exploit its biological function as well as mechanism in initiation and regulation of immunity.

## II. Murine MHC (H-2)

Mouse MHC antigen is also called H-2 antigen. Its gene locates on mouse chromosome 17. Gene structure of mouse MHC (H-2) is shown in Figure 4. Most studied H-2 antigen is two major classes: class I and class II.

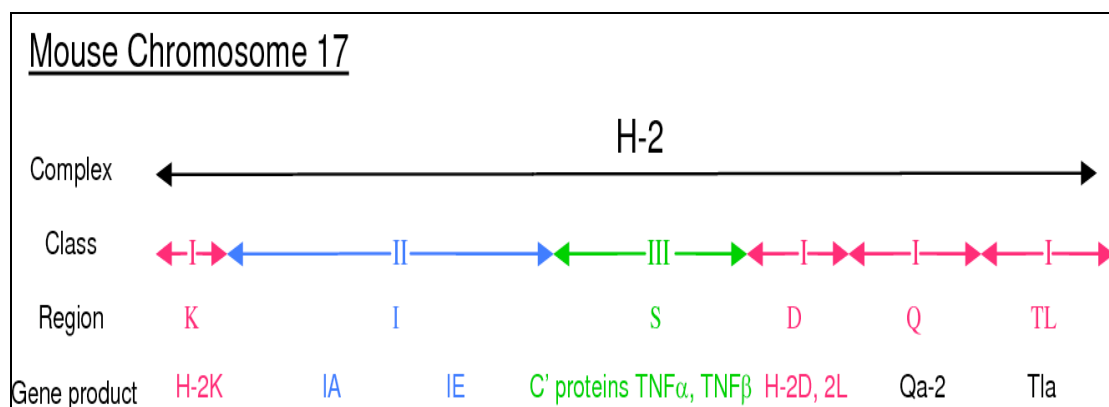


Figure 4 .Gene structure of mouse MHC (H-2)

Mouse MHC is composed of 11 subclasses. It includes the “classical

MHC class I” (also called MHC-Ia) that comprises H-2D, H-2K and H-2L subclasses; the “non-classical MHC class I” (MHC-Ib) that comprises H-2Q, H-2M and H-2T subclasses; the “classical MHC class II”(MHC-II a) that includes H-2A(I-A) and H-2E(I-E) subclasses, and the “non-classical MHC class II” (MHC-II b) comprises H-2M and H-2O. H-2 molecules are highly polymorphic. Each laboratory mouse strain is homozygous and has a unique MHC haplotype. The MHC haplotype in these strains is designated by a small letter (a, b, d, k, q, s, etc.). Take the two most useful animal models BALB/ and C57BL/ for example, the mouse leukocyte alloantigen of BALB strains is d, it has H-2K<sup>d</sup>, H-2D<sup>d</sup>, H-2L<sup>d</sup>, IA<sup>d</sup>, I-E<sup>d</sup>, while for C57BL strains is b, it only has H-2K<sup>b</sup>, H-2D<sup>b</sup> for class I and I-A<sup>b</sup> for class II. Mouse Leukocyte Alloantigens Chart is shown in *Table 3*. Thus; the MHC background of C57BL strains is less complicated than BALB.

H-2 class I molecules consist of a 45 kD highly glycosylated heavy chain non-covalently associated with a 12kD  $\beta$ 2-microglobulin, a polypeptide that is also found free in serum. H-2 class I antigens are expressed on almost all nucleated murine cells and are reported in many animal trials that they play an important role in presentation of altered self cell antigens (virally infected or tumor cells) to CD8+ cytotoxicity T cells.

MOUSE STRAINS	MHC haplotype									
	H-2K	I-A <sub>g</sub>	I-A <sub>b</sub>	I-E	H-2D	H-2L	Qa-2	Qa-1		
101/-	k	k	k	k	k	k	-			
129/-	b	b	b	b	-	b	-	a (+)		
A/J	a	k	k	k	k	d	d	a (lo)	a	
A2G	a	k	k	k	k	d	d			
AKR/J	k	k	k	k	k	k	-	b (-)	b	
AL/N	a	k	k	k	k	d	d	a (lo)		
AU/SsJ	q	q	q	q	-	q	q			
BALB/cAnN	d	d	d	d	d	d	d	b (-)		
BALB/cJ	d	d	d	d	d	d	d	a (lo)	b	
BDP/J	p	p	p	p	p	p	-	b (-)		
BUB/BnJ	q	q	q	q	-	q	q			
BXSB/Mp	b	b	b	b	-	b	-			
C.B-17	d	d	d	d	d	d	d	a (lo)	b	
C3H/Bi	k	k	k	k	k	k	-	b (-)		
C3H/He	k	k	k	k	k	k	-	b (-)	b	
C57BL/-	b	b	b	b	-	b	-	a (hi)	b	
C57BR/cd	k	k	k	k	k	k	-	b (-)	a	
C57L/-	b	b	b	b	-	b	-	a (+)	b	
C58/-	k	k	k	k	k	k	-	b (-)		
CBA/Ca	k	k	k	k	k	k	-	b (-)		
CBA/J	k	k	k	k	k	k	-	b (-)	b	
CBA/N	k	k	k	k	k	k	-	b (-)		
CE/-	k	k	k	k	k	k	-	b (-)		
DA/HuSn	qp1	q	q	q	-	s				
DBA/1	q	q	q	q	-	q	q	a (lo)		
DBA/2	d	d	d	d	d	d	d	a (lo)	b	

Table 3. Mouse Leukocyte Alloantigens Chart

Murine MHC class II molecules are composed of a 33 kD  $\alpha$  chain and a 28 kD  $\beta$  chain. H-2 class II antigens are expressed on antigen presenting cells (B cells, monocytes/ macrophages, dendritic cells, and Langerhans cells, etc.) and they involve in presentation of processed peptide antigens to CD4+ cells.

### III. Human MHC (Human leukocyte antigen, HLA)

The discovery of MHC in 20th century, especially the discovery of the first human HLA antigen in 1958, was the important milestone in the immunological development course. At present, function of the HLA

antigen is practically illustrated as follows: it not only participates in the maturation of peripheral T cell and starts and regulates the function of the immune state together with TCR, but also is related to many autoimmune diseases, and is one of the most important links in the immune system.

## **A. HLA structure**

The MHC antigen in humans was firstly found on the surface of human leukocyte, so it was called Human Leukocyte Antigen (HLA). The encoding super locus contains a large number of genes related to immune system function in humans and it locates in the short arm of human chromosome 6, encoding cell-surface antigen-presenting MHC proteins and other proteins.

MHC class I allele exists of three loci: HLA-A, -B and -C and presents peptides on all nucleated cells in the body. MHC class II allele also exists of three loci: HLA-DP, -DQ and -DR and presents peptides on special antigen presenting cells (APC) like macrophages, dendritic cells and B-lymphocytes. Other proteins, such as TAP (Transporter associated with Antigen Presentation) and proteasome are also encoded in the MHC class II region. MHC class III is not involved in antigen-presenting but it closely related to immune functions: complements of the complement system (such as C2, C4), cytokines (such as TNF- $\alpha$ , LTA, LTB), and heat shock proteins (hsp). Therefore, HLA studies mainly focus on the HLA

class I and class II. Gene map of HLA is shown in Figure 3.

In the figure 5, it is illustrated that HLA class I contains HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G; HLA class II contains HLA-DR, HLA-DQ, and HLA-DP. According to the structure of HLA class II, each of HLA class II molecules is constituted by two Chains, such as HLA-DRA1 and HLA-DRB1, HLA-DQA1 and HLA-DQB1, HLA-DPA1 and HLA-DPB1. As other MHC class I molecules, HLA-A, HLA-B, and HLA-C present peptides from inside the cell, while HLA-DR, HLA-DM, HLA-DQ, and HLA-DP present peptides from outside of the cell to T lymphocytes, leading to stimulate the differentiation of T-helper cells, which in turn stimulate B-cells to produce antibodies, to that specific antigen.

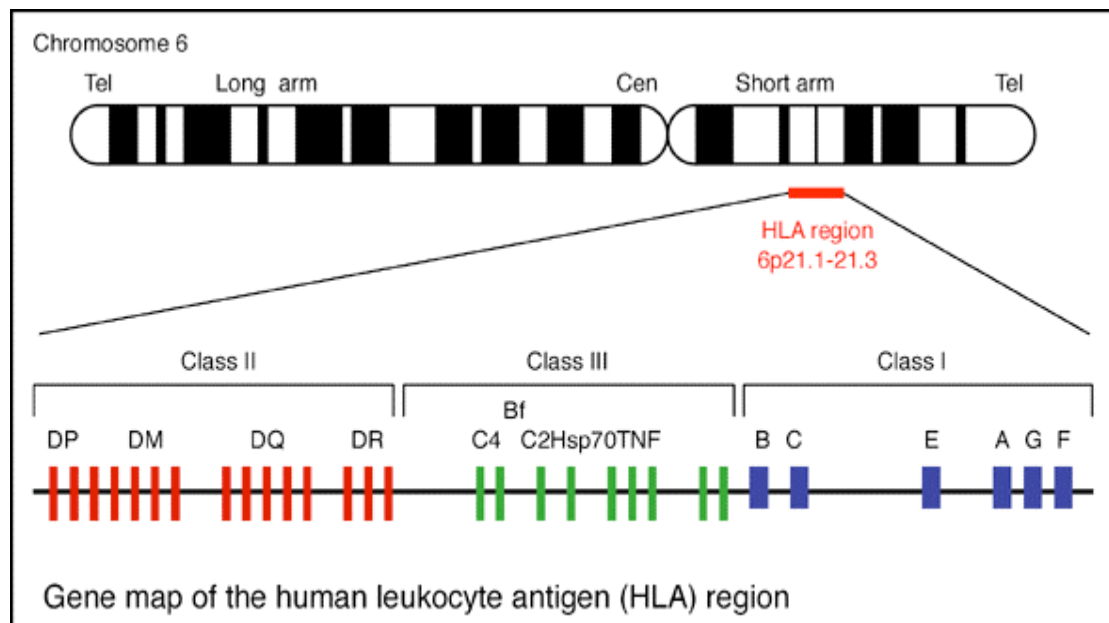


Figure 5. Gene map of the human leukocyte antigen (HLA) region.

## **B. HLA Nomenclature**

At present, there are two parallel systems of nomenclature that are widely applied to HLA. The first nomenclature is based on serology recognition. In this system, antigens were eventually assigned letters and numbers (e.g., HLA-A02 or, shortened, A2). The other nomenclature will provide more information. In this system, most designations begin with HLA- in conjunction with a letter “\*”, following four-or-more number of digits specifying the allele, such as HLA-A\*02:01, HLA-DP\*04:01 and HLA-DP\*04:02. However, antigen serotype is a crude measure of identify of cells. For instance, HLA-A9 serotype recognizes cells of HLA-A23- and A24- bearing individuals<sup>[11]</sup>.

Actually, many other kinds of nomenclature are also developing. Cellular assay based on the mixed lymphocyte culture (MLC) was used to determine the HLA class II types<sup>[12]</sup>. This kind of typing used “Dw” to designate and it is more sensitive than serotype due to minor differences unrecognized by alloantisera can also stimulate T cells. Together with difficulty of cellular assay in generating and maintaining cellular typing reagents, cellular assay is being replaced by DNA-based typing method.

Another method is called sequence specific primer (SSP-PCR)<sup>[13]</sup>. Sequence specific primers are designed to a variant region of HLA region and these overlapping primers will amplify the specific sequence from the different core sequence. Finally, the production of PCR will be separated

and observed by electrophoresis.

### **C. Polymorphism**

The specificity of HLA haplotype varies from race, such as in the Middle Easterners, Africans and other countries. In Caucasians, three polymorphism types, HLA-DR01, HLA-DR03 and HLA-DR04, account approximately 80% of the population; for Middle Easterners, HLA-DR03, HLA-DR04 and HLA-DR07 are dominated, while for Chinese, the predominant genotypes are HLA-DR09, HLA-DR15 and HLA-DR12, accounting for about 70-80% of the total population. In Asia, the Japanese population shares a similar HLA haplotype distribution with Chinese population, while Asian Muslim population and Indian population differ significantly from the Chinese and Japanese population in MHC polymorphism. Therefore, there is a great HLA polymorphism in different races in the world.

## **IV. MHC mediated immunology**

Cellular immunity mainly includes specific cytotoxicity T lymphocytes responses, helper T lymphocytes response as well as unspecific NK killing response. For specific immunity, it was widely accepted that MHC molecule not only participate in antigen presentation to directly mediate



cellular immunology, but also indirectly regulate humoral response by secreting cytokines. Several studies reported that lack of CD8<sup>+</sup> CTL delays viral clearance and increases mortality after infection with a virulent strain of influenza virus <sup>[14]</sup>, some CD4<sup>+</sup> T cells can also kill target cells as CD8<sup>+</sup> T cells <sup>[15,16]</sup>. However, mice lacking both CD4<sup>+</sup> and CD8<sup>+</sup> CTL do not clear virus or survive <sup>[17]</sup>. Therefore, MHC mediated immunity play an important role in antigen specific immunity.

### **A. Cytotoxicity T Lymphocytes response (CTL)**

Cytotoxicity T Lymphocytes is not a single population of lymphocytes but a group of lymphocytes with the specific killing activity. It contains CD8<sup>+</sup> T lymphocytes and some CD4<sup>+</sup> T lymphocytes. All of these lymphocytes can specifically and rapidly kill target cells.

The procedure of CTLs cytolysis is divided into 4 phases. 1. Recognition and initiation: TCR of precursor CTL recognize the peptide-MHC-I complex together with other ligands combination is the first step of cytotoxicity. 2. Proliferation: after the recognition between precursor CTLs and p-MHC, much IL-2R will be expressed on the surface of precursor CTLs. Meanwhile, Th cells are activated and many kinds of cytokines, such as IL-2, IL-4, IL-7, IL-10, IL-12 and IL-15 are secreted by Th cells will help precursor CTLs develop into effective CTLs to enhance the cytotoxicity. 3. Initiation and dissociation: After TCR

recognize p-MHC, CD11a/CD18 (LFA-1) combine the ICAM-1 or ICAM-2 that expressed on the surface of T lymphocytes, some cytoskeleton composition coupling with TCR, such as tubule and actin, will make organelles in effective T cells rearrange and move to the conjunction between CTLs and target cells. Further, cytoplasmic granules that contain the proteins perforin and granzymes from effective CTLs will attack membranes of target cells to form pores in the target cell membrane and then CTLs dissociate from target cells to search other target cells. 4. Apoptosis: target cells will be killed or initiate apoptosis. The whole procedure is illustrated in Figure 6.

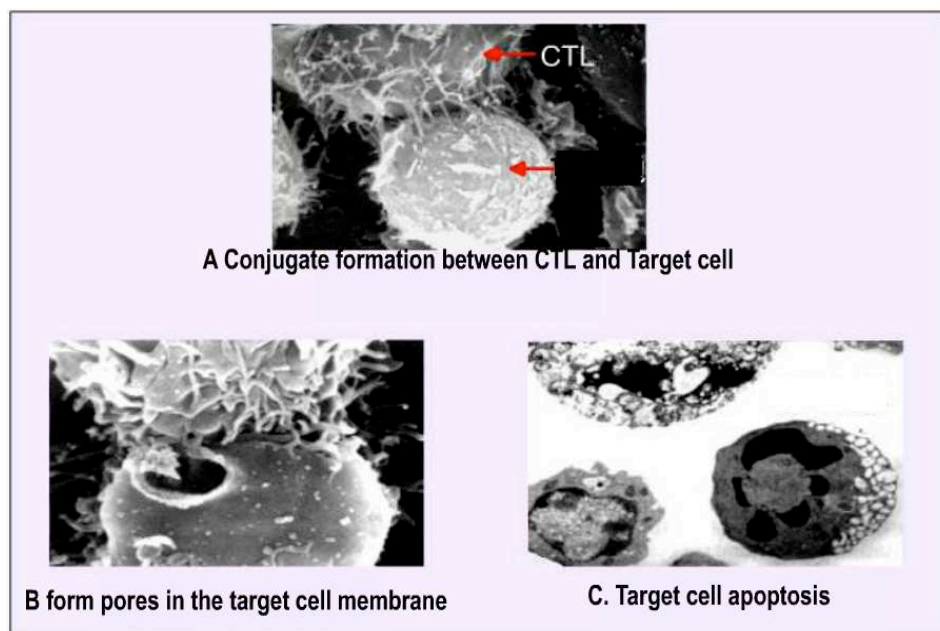


Figure 6. Perforin/Granzyme Killing mechanism of cytotoxic T lymphocytes to kill target cells

There are two kinds mechanism for killing the target cells. Apart form Perforin/Granzyme Killing, the other way is FasL/Fas killing pathway. It

is known that most potential CTL target cells express a receptor for FasL designated Fas. FAS/APO-1(CD95) is a transmembrane protein that widely expressed on the activated T, B cells, NK cells, monocytes and fibroblast; while CTLs express a transmembrane protein that called FasL on their surface, which is widely expressed on the lymphocytes, macrophage, dendritic cells and monocytes. When cytotoxic T cells recognize (bind to) their target, they produce more FasL at their surface to binds with the Fas on the surface of target cell leading to its death by apoptosis, as shown in Figure 7.

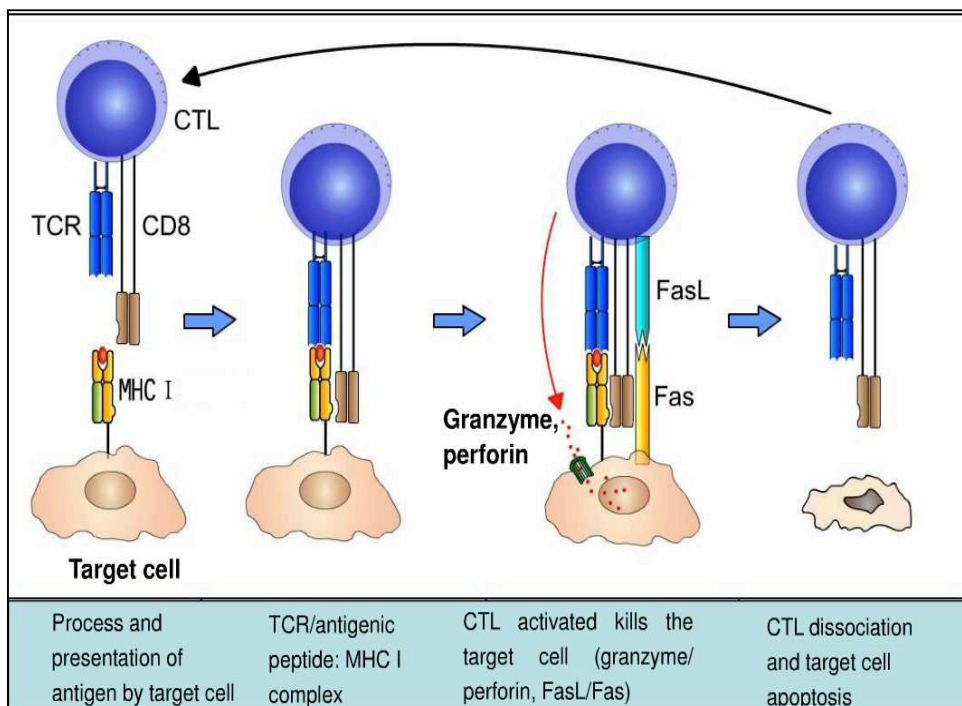


Figure 7.

7. The mechanism of CTL killing target cells.

## **B. Helper T lymphocytes response (Th)**

Helper T Lymphocytes are a cell subset which regulates the body's immune functions. This cell subset generally has no cytotoxic or phagocytic activity, and therefore could not kill infected cells or pathogens directly, but these cells play key regulatory roles through other types of lymphocytes. For example, they stimulate B lymphocytes to perform humoral immunity and stimulate CD8 lymphocytes to perform cellular immunity, and enhance the killing activity of macrophages. With the depth of helper T cells studies, it has become an important direction of study in immune prevention in better utilizing Helper T cells' immune regulatory role in vaccine development.

Helper T cells play important roles in many aspects such as infection immunity, tumor immunity, autoimmunity and immune regulation. In pathogen infections, in particular viral infections, Helper T cells play a vital role in recognizing the Th epitopes of pathogen proteins and regulating CTL and humoral immune response.

Through complicated regulatory mechanisms, an organism's immune system performs two basic biological functions: protecting itself from invasion by various exogenous pathogens and inhibiting immune responses toward its own antigens. When the immune system is certain of an immune response, it has to produce appropriate functional response to each and every different pathogen. One key cell type performing this

function of mediation and regulation is CD4<sup>+</sup> T cells, and it has been demonstrated that cytokines secreted by CD4 T cells play important roles in the process of fine tuning its functions <sup>[18]</sup>. Based on the cytokines they produced, CD4<sup>+</sup>T cells are categorized into two types: Helper cell type 1 (Th1) and type 2 (Th2). When the T cell receptor (TCR) molecules on the surface of CD4<sup>+</sup> T cells bind with antigen-MHC-II complex presented by antigen presenting cells (APC), a first signal is released which results in abundant expression on CD4<sup>+</sup> T cell surfaces multiple cytokine receptors such as IL-1R and IL-2R; meanwhile, CD4<sup>+</sup> T cells recognize the co-stimulatory molecule on APC surface and form the second signal which is the secretion of multiple cytokines, mainly IL-2 but also including some IL-4, IL-10, IL-12, interferon- $\gamma$  and others. Subsequently, the IL-2R on CD4<sup>+</sup> T cell surface binds to IL-2 secreted in the autocrine or paracrine fashion, which activates the cell to produce many progeny clone Th0 cells. Th0 cells differentiate into Th1 or Th2 cells under the actions of different cytokines. IFN- $\gamma$  promotes Th0 cells to differentiate into Th1 cells which, through IL-2 and IFN- $\gamma$  they secreted, enhance the cellular immune response of CD8 T cells. IL-4 promotes Th0 cells to differentiate into Th2 cells which, through cytokines such as IL-4 and IL-5 they secreted, enhance the humoral immune response of B cells <sup>[19]</sup>. Activated Th1 cells secrete IFN- $\gamma$ , which activates macrophages, TNF- $\beta$ , which kills chronically infected macrophages; IL-2, which induces T cell

proliferation; IL-3 and GM-CSF, which act synergistically to induce bone marrow monocytes to differentiate; LT and MCT, which synergistically activate endothelial cells and induce infiltration of macrophages; MCF and MIF, which synergistically attract macrophages to move toward infected foci. Under these physiological responses, Th1 cells upregulate immune functions to resist infection of intracellular pathogens. In contrast, activated Th2 cells have a main function which is to promote B cells to produce antibodies to play the role of humoral immunity <sup>[20]</sup>. Therefore, systematic and in-depth study of Th epitopes of pathogen proteins is of theoretical significance for understanding the immunity to infection, vaccine reaction characteristics and immune protection mechanisms.

### **C. CD4+ T cells epitope**

Helper T cell epitope is the molecular basis for Th cells to perform immune regulatory functions, and plays important roles in preventing pathogen infection. Systematically studying the Th epitopes of major viruses such as HIV and HBV is of scientific significance for elucidating immune reactions to viral infections and immune protection mechanism. There are two types of T cell epitopes: MHC-I restricted and MHC-II restricted. Studies on MHC-I restricted epitopes mediated CD8+ T cell response have been quite systematic while it is less so for MHC-II epitopes, both in relevance of MHC restricted epitopes to CTL or humoral

response mechanism, and in epitope screening and application. However, as more studies gradually reveal more about Th cell functions and biological significances, the study of Th has attracted wide attention, and in particular, the field has been moving fast in identifying new MHC-II molecules and MHC-II restricted epitopes.

MHC-II restricted epitopes are helper T cell epitopes formed when antigens are cleaved inside the host organism and are 8-25 amino acids in length. The epitopes and MHC-II molecules on APC surface form antigen epitope peptide-MHC-II complex, which binds to TCR-CD3 on CD4+ T cell surface; at the action of the second signal (CD8 or CD86 molecules on APC surface binds to CD28+ molecules on CD4+ cell surface), they promote cells to differentiate into effector cells, and stimulate cells to highly express a series of cytokines such as IL-2, IL-12, IFN- $\gamma$ , IL-4, IL-10 and other immune regulatory factors. The microenvironment formed by these cytokines is the messenger which mediates the activation of Th cells and ultimate realization of the immune regulatory function of Th cells.

Many studies have demonstrated Th epitopes derive from the HBV are essential for the immune response against HBV infection. In chronic HBV infected individuals, inefficient binding between hepatocyte surface MHC-II molecules with HBsAg antigen epitopes might account for the dysfunction or weak specific CTL of HBV infected patients<sup>[21]</sup>, which

further cause persistent HBV infection. Similar studies on the NS3 region of HCV revealed that specific Th epitopes on NS3 can contribute to the clearance of HCV infection <sup>[22]</sup>, Th epitopes of HIV viral proteins could enhance CTL cell toxicity toward infected cells and are associated with long-term non-progression of HIV infected individuals <sup>[23]</sup>. Therefore, Helper T cell epitopes could effectively enhance protective cellular and humoral immune response, and have important applications in immune preventives and therapies such as polypeptide vaccines, multiple-epitope recombinant vaccines and therapeutic vaccines.

Developing safer, more effective and more economic vaccines is the trend in vaccine R&D. Novel vaccines have advantages in safety, cost and stability and are the main directions of vaccines in the twenty-first century. Traditional vaccines mainly refer to those biological products which are produced from attenuation or killing of pathogenic microorganisms and can stimulate the body to produce microorganism -specific antibody or cellular immunity. Novel vaccines refer to subunit vaccines, polypeptide vaccines, nucleic acid vaccines, live vector vaccines and therapeutic vaccines. It is a trend for the future to modify and develop novel vaccines based on traditional ones. As studies go into depth, the role of Th epitopes in vaccines, in particular in novel vaccines, has received increasingly more attention. Appropriate Th epitopes will not only enhance cellular immune response such as CTL, but also could raise the level of humoral



immune response.

## **V. Humanized mouse Model (HLA transgenic mice)**

At present, animal models used for preclinical studies mainly include the strongly repetitive low-cost small animal models, such as inbreeding mice or birds, and large animal model with closer genetic background to the human, such as monkey, orangutan and other nonhuman primates. However, many small animal models are unable to accurately forecast the human response because their genetic background is greatly different from that of the human; the nonhuman primate and other large animal model suffers from high cost, limited sample number and ethical dispute. Moreover, both models are unable to eliminate the influence of species specificity on preclinical studies, so that many preclinical animal experiment results are greatly different from the human clinical response, and therefore neither of the above two kinds of animal models are the ideal animal models for predicting the human immune response.

Several studies indicated that the hereditary difference of interspecies MHC molecules is one of the important factors resulting in the difference. Therefore the accuracy for the animal model to forecast the human immune response can be effectively enhanced by replacing the animal MHC molecules with the human HLA molecules in the experimental animal body.

Mouse shares as high as 95% genes and 80% genetic products with human. The genetic background of mice were well decrypted and selecting the inbreeding mouse as the human HLA carrier can effectively reduce the influence of other genetic backgrounds when we are observing the regulation of the cellular immune response with HLA molecules. In the 1980s, the rapid development of molecular biology and maturation of transgenic technology <sup>[24]</sup> provides technical support for inserting the human specific HLA genes cloned *in vitro* into the mouse genome <sup>[25]</sup>. What's more important, each individual inherited several different HLA haplotype due to the polymorphism and linkage of MHC; it results in the difficulty to observe the single HLA restricted response in human body. Thus, transferring of certain HLA molecule into mouse body can separately inspect the immunological characteristics of this HLA molecule *in vivo* to avoid the competition from other HLA molecules in antigen presenting.

HLA transgenic mouse models are created by introducing typical human MHC gene into the mouse and knocking out murine H-2 gene. Therefore, the immune response in these transgenic mouse models is only restricted by HLA molecules and the cellular immune responses should be consistent with those of humans. Doubtlessly, HLA transgenic mouse model is an effective tool to enhance the accuracy of preclinical studies. At present, the reported humanized MHC transgenic animal model

includes HLA-I and II transgenic mouse models, which respectively focuses on inspecting the function of cytotoxic T lymphocyte (CTLs) and helper T cell (Th).

### **A. HLA- class I transgenic mouse models**

Kievits F et al reported the first humanized MHC class I transgenic mouse model HLA-B27 <sup>[26]</sup> in 1987 and proved that the cytotoxic T lymphocyte (CTLs) of this model can specifically recognize virus proteins using the humanized HLA-B27 restricted molecules. Later, despite researches demonstrated that the epitope library presented by the mouse H-2-I molecules is not exactly the same as that presented by the human HLA-I molecules <sup>[27,28]</sup>, Sesma L et al proved through in vitro experiments that among the 1551 epitopes presented by mouse-based cells and 1372 epitopes presented by humanized cells, 1161 epitopes are the same <sup>[29]</sup>. Therefore, the micro environment in mouse body can satisfy the normal replication, modification and transportation of the human HLA class I molecules, and perform the antigen presentation process similar to the human body.

### **B. Optimization of the HLA-I transgenic mouse model**

HLA-I molecules are alloantigen, which present high polymorphism

and are composed of a glycosylated  $\alpha$  heavy chain and a  $\beta$  light chain through non-covalent bonding. With deep understanding of the structure and immunological function of HLA-I molecule, HLA class I transgenic mice were unceasingly optimized to solve the problems that encounter in application of HLA class I transgenic mice. This procedure can be divided into three stages.

**Stage 1:**

The independent  $\alpha$  heavy chain or  $\alpha$  heavy chain and light chain  $\beta 2m$  of human HLA molecules are directly transferred to the wild mouse genome through transgene, so as to realize the expression of human HLA molecules under the mouse-based background, and enable the mouse T cell to recognize the extraneous HLA molecules and the HLA restricted epitopes presented thereof. Where, the representative mouse model includes HLA-B27, HLA-B7, HLA-A2, HLA-Cw3 and HLA-B35 and so on [30-35]. In these models, TCR on the rearranged mature mouse-based T cell surface can recognize the extraneous peptide presented by the human HLA-I molecules, and give rise to the HLA-I restricted T cell response [36-38]. But the HLA heavy chain genes ( $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$ ) are completely originated from the human, the interaction between functional area of humanized  $\alpha 3$  and mCD8 is not as strong as that of the mouse-based  $\alpha 3$ , and the majority of HLA restricted T cell response is competitively inhibited by the mouse H-2-I restricted T cell response [39-40], therefore,

the mouse CTL still mainly produces the mouse-based H-2-I restricted CTL response.

**Stage 2:**

The secondary lymphatic organ of the first generation of HLA-I transgenic mouse model has limited quantity of HLA restricted T cells, and the interspecies hereditary difference affects the interaction between mouse-based co-receptor CD8 and the human I-type molecules, therefore enhancing the utilization of HLA-I molecules in producing the CTL response is the key to improve the humanized HLA-I transgenic mouse model. The improved model includes HLA-A2, HLA-B27, HLA-B7, HLA-A24 and HLA-A11 and so on <sup>[41-46]</sup>. One strategy is to transfer human hCD8+ gene into the mouse genome, so that its T lymphocyte simultaneously expresses mouse-based and humanized CD8+ molecules. Under the circumstances, extraneous HLA-I molecules have more opportunities to combine with CD8+ on mouse CTL surface, and efficiently start the second signal. Another strategy is to optimize the molecular structure of extraneous HLA, replace the humanized intracellular  $\alpha 3$  functional area in the transmembrane area with mouse-based one, and build chimeric HLA molecules, namely the chimerism between humanized  $\alpha 1$  and  $\alpha 2$  functional areas and the mouse-based  $\alpha 3$  functional area, HHM for short. This structure can enhance the bonding of mouse CD8 molecules and human MHC class I

molecules<sup>[41-43]</sup>. The mouse model optimized with the above method can produce non-specific and HLA restricted specific response, but the majority of CTL response is still the mouse-based H2 restricted response [42, 43].

### **Stage 3:**

In view of the fact that the first two generations of mouse models mainly generate the mouse-based H-2-I restricted CTL response, it is necessary to eliminate the competitive inhibition of endogenous H-2-I molecules on HLA-I molecules in the HLA-I transgenic mouse model. In the 1990s, with the maturation and application of the gene knockout technology, the mouse H-2-I system was knocked out. Barra C et al reported<sup>[47]</sup> that after the mouse H-2-I gene is knocked out, the HLA-I restricted CTL response is still very strong. In the event that no H-2-I molecule exists, the HLA-I transgenic mouse model can form a huge TCR cell library using the normal differentiation of TCRV $\beta$ <sup>[48]</sup>. Therefore, the Lemonnier team proposed to build the HLA-I transgenic mouse model with the H-2-I gene knockout, so that the CTL maturation and activation only relies on the human HLA-I molecules. At present, the mouse H-2-I genes can be knocked out with two methods: the first method is to knock out the heavy chain gene encoding the mouse H-2-I molecules, namely first respectively knock out the mouse-based H-2Kb and H-2Db, then mate with HHM mouse, and finally inbred to

homozygous mouse model <sup>[49, 50]</sup>. But this method suffers from a time-consuming complex process, and will still produce the antigen presentation participated by non-classical H-2-I molecules <sup>[51]</sup> and CD1 restricted NKT cell response (CD1 expression depends on  $\beta 2m$ ) as a result of the existence of mouse-based  $\beta 2m$  molecules. Therefore, at present, the third generation of HLA-I transgenic mouse model created by using another simpler method, namely first knock out the mouse-based  $\beta 2m$ , so that the H-2-I molecules are unable to form the functional unit due to lack of light chain, thereby achieving the purpose of silencing H-2-I molecule functions; meanwhile construct an HHD structure with heavy  $\alpha$  chain of HHM combine with human light chain  $\beta 2m$  through a linker, so as to prevent the binding of human  $\beta 2m$  and endogenous H-2-I heavy chain. Even though the mouse model built with this method could still conduct background expression of H-2-I antigen <sup>[52, 53]</sup>, it does not participate in the classical and non-classical antigen presentation of endogenous H-2-I molecules, therefore the HHD mouse model mainly produces the HLA-I restricted CTL response. It was demonstrated that both the third generation of mouse models possess not only CD8+T cell library with diversified TCR  $V\beta$  and  $V\alpha$  on their T cell surface, but also stronger antigen specific HLA-I restricted response than the second generation of mouse model <sup>[54,55]</sup>. Therefore, such transgenic mouse models are widely applied in related fields of preclinical studies.

### **C. HLA class II transgenic mouse models**

Experimental therapy in humans is limited by technological and ethical considerations; however, no effective wide animal models can instead human cases to meet this requirement. In this case, HLA-II transgenic mouse models were originally developed in order to study the susceptibility between HLA and some diseases, and further used to observe the CD4+ T cells response against virus, parasites, tumour as well as autoimmunity. So far, a range of transgenic mouse models humanized for MHC II molecules, such as HLA-DR01, HLA-DR03, HLA-DP4, HLA-DR15, DQ6 and DQ8<sup>[56]</sup>, were created to study rheumatoid arthritis, multiple sclerosis, insulin-dependent diabetes mellitus or celiac disease.

Similar as the HLA-class I transgenic mice, the development of molecular biology and transgenic technology lay a foundation for the feasibility of creation of HLA-class II transgenic mice. First HLA-class II transgenic mice were created by introducing HLA -DR gene into murine cells in 1983<sup>[57]</sup>. After 2 years, HLA DP transgenic mice were reported and their HLA-DP restricted immunological function of CD4+ T cells was observed as expected<sup>[58]</sup>. However, these humanized mouse models expressed both human class II and murine MHC class II antigens. It brought great difficulty to distinguish that the immune response were



initiated by the murine MHC class II molecules or that were mediated by the human MHC class II molecules. In this situation, many strategies were used to optimize these mouse models in following aspects.

#### **D. Optimization of the HLA-II transgenic mouse model**

As other MHC class II molecules, both HLA class II and H-2 class II molecules are heterodimer which are composed of a  $\alpha$  chain and a  $\beta$  chain, and each chain contain two domains- $\alpha 1$ ,  $-\alpha 2$  and  $-\beta 1$ ,  $-\beta 2$ . In antigen presentation,  $\alpha 1$  and  $\beta 1$  form a groove to bind epitopes and take charge of antigen presenting;  $\alpha 2$  and  $\beta 2$  form transmembrane domain that anchoring the MHC class II molecule to the cell membrane. Therefore, the optimization of HLA-II transgenic mouse model was designed on basis of MHC-II molecule structure.

##### **Stage 1:**

In this stage, HLA-class II transgenic mouse models only transgene class II single  $\alpha$  chain or  $\beta$  chain of human MHC, and coupled with murine complementary chain *in vivo*. For example, initial HLA-DR transgenic mouse models were only DR $\alpha$  chain transgenic and paired with murine E $\beta$ , with absence of DR  $\beta$  chain<sup>[59]</sup>, or only DR  $\beta$  chain transgenic and paired with murine E $\alpha$ , with absence of DR  $\alpha$  chain<sup>[60]</sup>. In this case, murine T cells can be partially educated under human HLA class II molecule.

Subsequent improvement was fulfilled by simultaneously introducing both human  $\alpha$  chain and  $\beta$  chain genes into murine genome so as to allow the integrated HLA antigen can take action in murine background, such as HLA-DR1 and HLA-DR2 transgenic mice. Except the structure of HLA genes, the promoter of HLA was also considered. Some mouse models, such as HLA-DQw6, DR3, DRw17 and DR1, used the promoter from human MHC region <sup>[61-63]</sup>, while some other mouse models used the murine MHC region promoter for expressing DR4 <sup>[64, 65]</sup>. All of these mouse models were still reported to be low efficient in HLA restricted response.

### **Stage 2:**

In addition to the optimization of human MHC class II molecule expression, the interaction between CD4 molecule of T lymphocytes and MHC class II molecules of target cells was considered. Some groups reported that the interaction between mCD4 and exogenous HLA molecule was relatively weak <sup>[66-68]</sup>, compared with that between hCD4 and exogenous HLA molecule <sup>[69]</sup>. Therefore, there are two alternatives, one is to modify MHC class II molecule as a chimeric structure, Woods team took this strategy and replaced human  $\alpha 2$  and  $\beta 2$  domain by murine  $\alpha 2$  and  $\beta 2$  domain in HLA-DR transgenic mice, they reported that the chimeric structure (DR- $\alpha 1$ ,  $\beta 1$  and I-E $\alpha 2$ ,  $\beta 2$ ) help to stimulate a stronger CD4<sup>+</sup> T cells response in mice <sup>[64, 65]</sup>. The other method is to replace

mCD4 by hCD4. Even though Altmann et al reported that the mobilization of CD4<sup>+</sup> T cells repertoire of HLA-DR1/hCD4 transgenic mice was the same as that of HLA-DR1 transgenic mice<sup>[63]</sup>, more groups reported that the interaction between HLA and hCD4 was better than that between HLA and mCD4. What's more, the promoters for hCD4 were from murine genome, such as murine CD3 $\delta$  promoter<sup>[70]</sup> in HLA-DR4 mice and murine CD2 promoter<sup>[71]</sup> in HLA-DQ6 mice.

### **Stage 3:**

As mentioned above, much effort was done to improve the similarity of HLA class II transgenic mice with human. However, many studies demonstrated that the co-expression of H-2 with HLA molecule would lead to the murine immunity preference. Thus, in 1991, Cosgrove et al<sup>[72]</sup> developed a mouse model that was deficient in H-2 class II, and in 1999, Madsen et al<sup>[73]</sup> generated another knockout mouse that lacked all four of the classic murine MHC class II gene via a large (80-Kb) deletion of the entire class II region that was engineered by homologous recombination and Cre recombinase-mediated excision. These mouse models subsequently were widely used for developing mouse models that expressed only human MHC class II molecules with absence of all murine H-2 class II molecules to highlight the HLA class II restricted immunity. The human MHC class II molecules in these new mouse models have been shown to be functionally activated and participated in

shaping the CD4<sup>+</sup> T cell repertoire in thymus and mediate CD4<sup>+</sup> T cell responses in the periphery. It was reported that, mature CD4<sup>+</sup> T cells repertoire in HLA transgenic and H-2 class II knock out mouse model is different from that in C57BL/B6 mice [74-76]. Importantly, peptides presented to CD4<sup>+</sup> T cells in these mouse models were shown to be similar to peptides presented to CD4<sup>+</sup> cells in patients who carry the same MHC class II haplotype [77].

In summary, the currently available HLA class II transgenic mice have high capacity to mimic human cellular response. However, there are still some limitations to be solved. HLA class II molecule in these mice is only one of the HLA class II antigens; whereas CD4<sup>+</sup> T cells response in human is the mutual result of different HLA class II molecules. Therefore, more different types of mouse models should be developed for experimental therapy.

## **E. HLA-I/II double transgenic mouse models**

Compared with HLA-I or II single transgenic mice, HLA-I and II double-transgenic mice would allow the observation of synergy effects of MHC-I and -II molecules in immune response, which is uniquely advantageous either studying viral pathogenesis or screening for anti-viral vaccines. In 2004, Anthony et al successfully established a human MHC HLA-A2/DR1 transgenic mouse model by introducing human HLA-A2

and HLA-DR1 gene and knocking out the mouse H-2 class I and class II molecules <sup>[78]</sup>. For these mice, their cellular immune responses are restricted completely by human HLA molecules and extremely consistent with human cellular immune responses. Especially, HLA-DR1 molecule is capable of regulating mouse B cells mediated humoral immune response and further influence the CTL response. Even though the percentage of CD8<sup>+</sup> T cells population is lower than wild-type mice, expression of functional transgenic HLA-A2.1 molecules led to an increase in the size of the peripheral CD8<sup>+</sup> T cell population, which reached 2-3% of the total splenocytes in HLA-A2/DR1 mice, compared to 0.6-1% in  $\beta$ 2m-KO MHC-I deficient mice. Furthermore, the size of functional TCR repertoire from peripheral CD8<sup>+</sup> T cell is highly diverse as comparing to wild type B6 mice, this suggest that CD8 T cell activity is highly competent. Similarly, HLA-DR1 molecule leads to an equivalent expression in HLA-A2/DR1 transgenic mice, which CD4<sup>+</sup> T cells represented 13–14% of the splenocytes population. In contrast, only 2–3% of the cells were CD4<sup>+</sup> T cells population in H-2 class II-KO mice; in agreement with the initial report on mice lacking MHC class II molecules <sup>[72]</sup>. Therefore, when this mouse model are used to study T cell epitopes and to evaluate cellular immunity based protective vaccines, it could reflect the human natural immune response, and represent a rather ideal technology and model for vaccine research and evaluation.

Moreover, this double transgenic and double knockout mouse model is a moderate small animal model for transplanting and reconstructing human hemolymphoid cells and tissues.

## **VI. Highly pathogenic Influenza A virus (H5N1)**

HPAIV is a highly pathogenic subtype of the avian influenza A virus. Its mortality rates in infected flocks often approach 100%. Even though many reports indicated that H5N1 presented a highly poultry-specific infectivity and seldom infect humans, there are an increasing number of human cases infected by H5N1. According to the statistics provided by World Health Organization (WHO) (shown in *Table 4*), H5N1 has totally caused 573 human infected cases worldwide, including 336 deaths since 2003<sup>[79]</sup>. Moreover, the influenza H1N1 pandemic in 2009, which partial segment derive from H5N1 virus and arose worldwide panic and ever be speculated to generate the third human pandemic disease like 1918 Spanish pandemic, alerted people to draw more attention on the potential risk of H5N1. Once H5N1 virus spread through species barrier to infect humans and carries its 59% mortality, there will be a disaster. Therefore, for the high lethality and virulence of HPAIV (H5N1), huge potential host repertoire, and its significant ongoing mutations, the H5N1 is doubtlessly the world's largest current pandemic threat.

Country	2003		2004		2005		2006		2007		2008		2009		2010		2011		Total	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	0	0	8	5
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	3	0
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	1	1	8	8	19	16
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	2	1	0	0	40	28
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	29	13	36	13	155	53
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	9	7	11	9	182	150
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	3	2
Lao People's Democratic Republic	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	3	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	0	0	12	4
Viet Nam	3	3	29	20	61	19	0	0	8	5	6	5	5	5	7	2	0	0	119	59
Total	4	4	46	32	98	43	115	79	88	59	44	33	73	32	48	24	57	30	573	336

*Table 4.* Cumulative number of confirmed human cases for avian influenza A (H5N1) reported to WHO, 2003-2011

## A. Structure of HPAIV H5N1 virus

H5N1 is a subtype of the species Influenza A virus and belonging to Influenza A genus of the orthomyxoviridae family. Like other subtypes of influenza A virus, H5N1 is a single stranded, negative-sense RNA virus that composed of 8 negative segments which encoding 10 proteins, as shown in Figure 8, including hemagglutinin (HA), neuraminidase (NA), matrix protein 1(M1) and matrix protein 2(M2), nucleoprotein (NP), polymerase basic 1(PB1) and PB2, nonstructural protein NS1 and NS2 and polymerase acidic (PA)<sup>[80]</sup>. There are comprehensive studies on every viral antigen up to date. In our studies, we mainly focus on the HA, NA, M1, M2 and NP related immunity and thus, other proteins we will not make a further introduction.

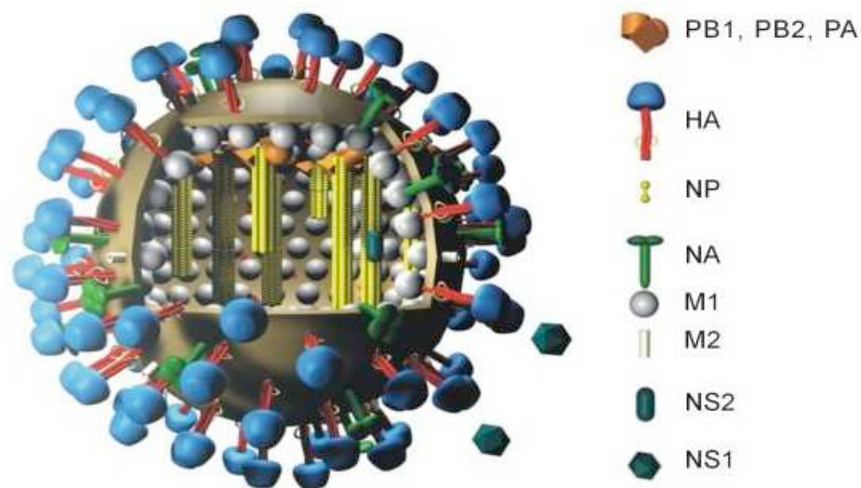


Figure 8. Molecular structure of H5N1 virus.

Hemagglutinin (HA) and the neuraminidase (NA) proteins are the two surface antigens of H5N1; they play key roles in interactions between the virus and host cells. Structure analysis has shown that HAs are trimeric glycoproteins while NAs present on viral cell surface as tetramers<sup>[81, 82]</sup>. Both HAs and NAs can bind to sialic acids in cell surface receptors. After protease cleaves HAs into two chains, HA1 and HA2, HA2 can mediate the entry of viruses into host cells<sup>[83]</sup>. After the HA is cleaved by a protease, the cell imports the virus by endocytosis. NAs enhance the cleavage of sialic acids from virus and host cell glyco-junctions and facilitate the release of mature virions<sup>[84]</sup>. It is demonstrated that HA cleavage is the premise of H5N1 infection<sup>[85]</sup>.

Matrix protein is encoded by RNA segment 7 and has two forms, including M1 and M2. M1 protein is the most abundant viral protein and it takes up nearly 40% articles in the whole viral protein .Thus, its



antigenic specificity in different influenza strains could be used for diagnosis and virus typing<sup>[86]</sup>. Therefore, M1 protein poses a vital role in maintaining virus structure and integrity. Moreover, M1 protein binds to RNP cores and therefore takes significant functions in the virus life cycle, such as inhibits RNA synthesis by the RNP-associated transcriptase of influenza virus and participates in assembly and budding of progeny virions<sup>[87]</sup>. The other form of matrix protein, M2-protein, is a tetrameric, type III transmembrane protein scarcely present on virus particles, but abundant on virus-infected cells<sup>[88, 89]</sup>. It can form a proton channel during infection which is essential for infection. The extracellular domain of M2 protein contains a 24 amino acid residues that called M2e-sequence. Significantly, this short sequence has remained nearly unchanged since the first human influenza strain was isolated in 1933, despite numerous epidemics and two major pandemics since then<sup>[90]</sup>.

Nucleoprotein is encoded by RNA segment 7 and make up of 498 amino acids. It is one of the most important structural proteins, according to the NP antigenicity; influenza virus can be divided into three subtypes A, B and C. Hence, it is the basic protein that used as classification and diagnosis. In the infected cell newly synthesized NPs are imported into the nucleus where they take part in a RNA elongation/RNP assembly process. According to a favoured assembly model one or a few NPs bind to newly synthesized viral RNA, which will then stimulate further NP

associations by cooperative NP-NP and NP-RNA interactions<sup>[91]</sup>. In this way, NP participate in the virus replication.

## **B. Pathogenesis**

The transmission of Human influenza A virus infection can be easily fulfilled result from the enrichment of hemagglutinin proteins locating in the upper part of the respiratory tract to infect epithelial cells, typically in the mouth, nose as well as throat and it can be easily transmitted by people coughing and sneezing. However, the symptom and characteristic of HPAIV infection is different from that of human influenza A virus. Because the avian influenza hemagglutinin binds to avian cell alpha 2-3 sialic acid receptors(SA  $\alpha$ -2,3), while human influenza hemagglutinin bind to human cell alpha 2-6 sialic acid receptors(SA  $\alpha$ -2,6)<sup>[92]</sup>. Once hemagglutinin fuses the viral envelope with the vacuole's membrane, then the M2 ion channel allows protons to move through the viral envelope and acidify the core of the virus, which lead the core to disassemble and release the viral RNA and core proteins. Owing to more alpha 2-6 sialic acid receptors (SA  $\alpha$ -2, 6) exist in the deeper respiratory tract of human body, the severe tissue damage even death occurs more often in patients after H5N1 virus infection due to inflammatory cascade that triggered by H5N1 virus<sup>[93]</sup>. This so called a “cytokines storm” might be aroused by what seems to be a positive feedback process of damage to

the body resulting from immune system stimulation. It also account for the reason that H5N1 virus can replicate in the lower respiratory tract and further cause viral pneumonia<sup>[94, 95]</sup> in the lungs.

Much concerns about the H5N1 pandemic due to the high mutation of HA. It was widely demonstrated that the ability of various influenza strains to show species-selectivity is largely due to variation in the hemagglutinin genes. Even a single amino acid substitution might dramatically change the ability of viral HA to bind to receptors on the surface of host cells, which further make virus strains being inefficient at infecting human cells change into a more contagious virus strains as the seasonal human influenza viruses. Another apprehension about the H5N1 pandemic is attributing to the ability of H5N1 genetic recombination by segments reassortment in the host who had been infected by more than two kinds of subtype, which alter H5N1 virus from not pathogenic in humans to become pathogenic in humans. Take A/2009/H1N1 for example, the special structure of influenza virus allows the segments from swine flu, bird flu and human flu reconstitute a new H1N1 influenza virus which emerged in Mexico, the United States, and several other nations and caused a public panic<sup>[96]</sup>.

### **C. Prophylaxis**

Currently, four antivirals against H5N1 have been proved effective in

the treatment and prophylaxis of influenza A infections: two M2 inhibitors (amantadine and rimantadine) and two neuraminidase inhibitors (zanamivir and oseltamivir)<sup>[97, 98]</sup>. However, there are many factors should be considered, such as drug resistance and costs. Mass vaccination is the best form protection to prevent and reduce the chance of severe illness or death in people during a H5N1 pandemic.

The development and potentially imminent availability of approved human vaccines against H5N1 marks are of great interest to address this threat. Although, the history of human H5N1 vaccines is a relatively young in many aspects, such as immunogenicity, safety, dosage, degree and duration of their protective effects, at least 16 different manufacturers have an H5N1 vaccine in relatively advanced development based on a range of approaches (including egg and cell culture grown viruses, live virus and inactivated vaccines, whole and split antigen and vaccines with and without different adjuvants). Additional novel vaccines are also under consideration and development<sup>[99]</sup>.

Current two licensed H5N1 vaccines by Food and Drug Administration (FDA) of USA are the conventional inactivated virus vaccine (CIV) and the live-attenuated vaccine (LAV). Both of them have been demonstrated to be effective for protection. However, whether these traditional vaccines against H5N1 can provide protection against new emerging H5N1 virus is hard to be speculated because of the high mutation of H5N1 surface

antigens Hemagglutinin (HA) and Neuraminidase (NA). Therefore, many novel vaccines were developed to deal with the disadvantages of current licensed vaccines, including split vaccine, subunit vaccine <sup>[100]</sup>, VLPs vaccine <sup>[101]</sup> and DNA vaccine <sup>[102]</sup>, however, most of these vaccines still focus on stimulating the neutralizing antibodies against external glycoproteins HA and NA and related reports largely published. Several researches reported that the vaccines based on the H5N1 Hemagglutinin has lower efficiency than seasonal human influenza vaccines and unadjuvanted inactivated whole virus H5N1 vaccines appear to be more immunogenic than unadjuvanted split or subunit vaccines. In more recent years, “universal flu vaccines” <sup>[103]</sup>, which based on the conserved viral antigens or epitopes, are developing to potentially provide longer-lasting protection against different subtypes <sup>[104,105]</sup>. For this reason, less mutated proteins nucleoprotein, matrix protein, polymerase base as well as many conserved CTL epitopes, B-cell epitopes and Th epitopes are essential targets for next generation vaccine design.

## **VII. Hepatitis B Virus (HBV)**

Hepatitis B virus is a member of the hepadnavirus family. It was reported that 2 billion people had been infected by hepatitis B virus (HBV), more than 350 millions of people worldwide are chronic carrier of the virus and 600,000 people die each year due to the acute or chronic

consequences of hepatitis B <sup>[106]</sup>. Therefore, HBV is one of the most contagious viruses to threaten human health.

### **A. Structure of hepatitis B virus**

Hepatitis B surface antigen (HBsAg) particles carry the common determinant a, d or y and w or r subtype determinants, and are classified into the four major subtypes, i.e., adw, adr, ayw and ayr, which depend on antigenic epitopes present on its envelope protein <sup>[107]</sup>. It is also divided into 8 genotypes based on overall nucleotide sequence variation of the genome. Hepatitis B virus particle is constituted by an outer lipid envelope and an icosahedral nucleocapsid core (as shown in Figure 9). The component of the outer lipid envelope is surface protein which composed surface protein, middle protein and large protein. This envelope protein, which is also called HBsAg, often present in the sera of patients with viral hepatitis B with or without clinical symptoms. Thus, it can indicate the current HBV infection in clinical diagnosis. The nucleocapsid core is a complex of viral DNA and a DNA polymerase that has reverse transcriptase activity similar to retroviruses. Apart from HBsAg and Polymerase, HBcAg and HBeAg antigens occur in the HBV particle. HBcAg is a structural protein in HBV and wrapped up by HBsAg. It is an indicator of active viral replication; which means the person infected with Hepatitis B can likely transmit the virus on to another person. HBcAg

soluble form antigen is HBeAg and it is also the marker of active viral replication. When anti-HBcAg and anti-HBeAg present can be detected in the sera of patients, it reveals a decline in viral replication and the recovery from HBV infection.

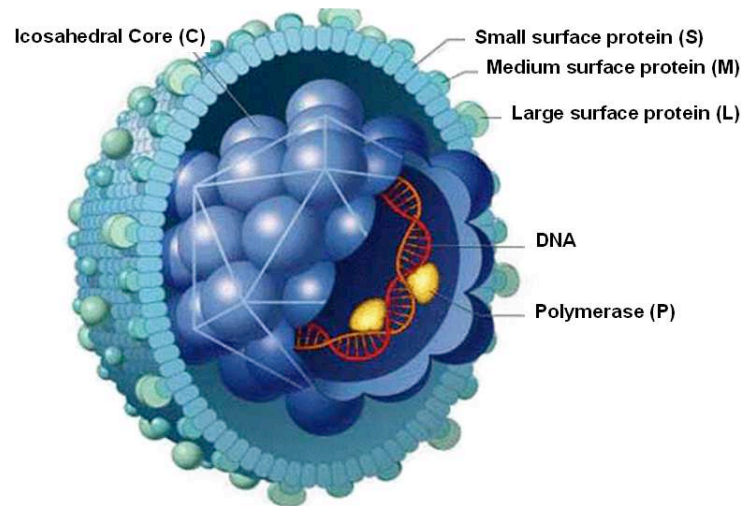


Figure 9. Molecular structure of hepatitis B virus.

The genome of HBV is made of the smallest circular DNA, a not fully double-stranded 3.2k bases DNA, which composed of a full length anti-sense strand and a variant length of the sense strand. The anti-sense strand has 4 ORF, including S, C, P and X segments and they encode all the known HBV proteins, whereas sense strand does not encode any protein(as shown in Figure 10). S gene can be divided into two genes; pre S and S gene: there are 678 bases for S gene, Pre S gene locates at the upstream of S gene, it encodes a protein with 163 amino acids, including Pre S1 and Pre S2 proteins.C gene is also divided into two parts, Pre C and C genes, which encode HBeAg and HBcAg, respectively; P gene

mainly encodes the polymerases for viral replication; X gene encodes a 154 aa basic polypeptide.

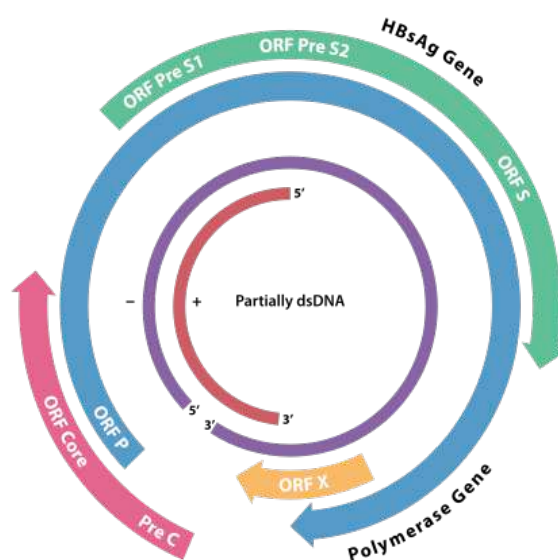


Figure 10. Genome structure of Hepatitis B virus.

## B. Pathogenesis

Hepatitis B virus (HBV) is a non-cytopathic DNA virus that causes acute and chronic hepatitis<sup>[108,109]</sup>. Both the adaptive and innate immune responses are known to be involved in viral clearance during HBV infection<sup>[110]</sup>. It was reported that the host immune response against HBV is the key factor to determine whether patients naturally resolve viral infection or develop chronic infection<sup>[111]</sup>. In acute HBV virus infection, multi-specific cellular response in patients could be observed, with the adequate production of anti-HBV antibodies and antiviral cytokines. On the contrary, patients suffering from chronic infection have very weak or functionally impaired immune responses. When persistent inflammation occurs during HBV infection, many liver diseases would be caused, such



as fulminant hepatitis, liver cirrhosis (LC) or hepatocellular carcinoma (HCC) <sup>[112,113]</sup>.

As other pathogenic infection, the initial immune response against HBV infection is innate immunity that fulfills by type 1 interferon (IFN) production and activation of natural killer (NK) cells <sup>[114]</sup> to control the virus replication in a low level. 6-8 weeks after HBV post infection, HBV replication will enter into a rapid exponential phase and viral loads get dramatically raised. In this procedure, antigen specific CTLs plays a crucial role in virus clearance. It was indicated that, in acute HBV infection, a comprehensive CTLs response against envelop, HBcAg, and polymerase proteins were observed in patients who successfully clear the virus <sup>[115-117]</sup> and the CTL response can last for decades after recovery from acute infection or/and after treatment-mediated resolution of chronicity. In addition, the Th response against HBcAg- and HBeAg- also contributed to enhance the cellular response. On the contrary, a weaker cellular response against HBsAg, HBcAg and HBeAg were reported in HBV chronic infected patients. However, hypersensitive T cell immune response against HBV in turns account for the liver injuries in the liver <sup>[114]</sup>.

### **C. Prophylaxis**

The first available HBV vaccine were composed of hepatitis B surface

antigen(HBsAg) purified from the blood of HBV carrier in 1982 and it made great contribution to decrease the incidence of HBV infection in past decades <sup>[118]</sup>. Current prophylactic HBV vaccine was manufactured by HBsAg antigen expression in recombinant yeast. Once anti-HBs antibody titer can reach to 10IU/ml, the vaccine could be considered as protective. Usually, the primary series consisted of two or three doses given at intervals of 1-2 months.

However, this kind of vaccine is only effective to protect the uninfected people rather than HBV infected patients. The clinical use drugs to abbreviate the chronic HBV infection mainly target on the HBV polymerase to control viral replication and no HBV therapeutic vaccines is available up to now. Although, the present available anti-HBV drugs efficiently decrease serum viral load to undetectable levels, they fail to eradicate infection due to the persistence of HBV covalently closed circular DNA (cccDNA) in hepatocytes and the emergence of resistant viruses <sup>[119,120]</sup>. Moreover, these drugs are not proper for long-term treatment due to its immunological limitation, great expense and sometimes toxicity. For this reason, the strategy for developing therapeutic vaccine against chronic HBV infection is likely to be a good alternative to enhance or awake the impairment of T cells immune response. This theory may help us to optimize current HBV vaccine and to solve the low effective problem for 10% of the population, who can not

be protected or requires multiple shots. It is worth noting that the design of therapeutic HBV vaccines followed the idea that using the facilitating function of helper T cells to induce stronger CTL cytotoxicity response, i.e., introducing Th epitopes in traditional vaccines and using them to raise the recognition and clearance of CTL cells for HBV infected patients. For example, a multi-epitope HBV DNA vaccine, which embeds 30 CTL epitopes and 16 Th epitopes was developed by Innogenics Company in Belgium; an epitope based HBV vaccine that combines CTL specific epitope HBc 18-27, pan tetanus toxoid Th epitope as well as N-2-palmitic acid was designed by Epimmune Company in USA; a therapeutic vaccine developed in China, which contains HBcAg18-27 CTL epitope, Tetanus toxoid helper T cell epitopes TT830-843 and B cell epitope PreS218-24 was developed. All of therapeutic vaccine candidate mentioned above were proved effective and have no safety problem yet. Some of them have already implemented into phase II clinical trials. Therefore, the systematic identification of viral epitopes in HBV virus will be of great interest for the future vaccine design.

# *Issues*

Currently, several HLA transgenic mice were developed and their immunological characteristic is becoming closer and closer to human immunity. Most of these human MHC transgenic animal models are HLA class I or HLA class II single transgenic mice which were used to study cytotoxic T cells (CTLs) and helper T cell (Th) function, respectively. It leads to they could only examine one type of responses and fail to observe cooperation of B cells, Th cells and CTL cells in a natural condition. In 2004, a novel HLA class I / class II double transgenic with murine H-2 class I /class II double knockout mouse model was created in our lab and its capacity to completely mimic human cellular immunity was verified in many researches on Hepatitis B, HIV and HCV.

According to statistics from human HLA allele frequency database, we found that HLA-A\*0201 and HLA-DP4 alleles are the most frequent HLA class I and II haplotype, which represent 30-50% and 15-76 % of the global population. However, there is only HLA-A\*0201 transgenic mouse model is available at present and no systematic study was reported on HLA-DP4 transgenic mouse model. Therefore, our objective of this thesis is to create a HLA-A2/DP4 double transgenic mouse model and analyze its HLA -A2 restricted and HLA-DP4 restricted immunities. In addition, HLA-A2 restricted CTL epitope mapping for Matrix protein 1 of a highly pathogenic avian influenza H5N1 strain (A/Shenzhen/406H

/06) and HLA-DP4 restricted Th epitope mapping for HBsAg of Hepatitis B virus will be done by using new established HLA-A2/DP4 transgenic mouse model.

The main tasks of thesis contain following parts:

- 1) Development of a humanized HLA-A2.1/DP4 transgenic mouse model and the use of this model to map HLA-DP4-restricted epitopes of HBV envelope protein.
- 2) Identification of novel HLA-A\*0201 restricted epitopes from H5N1 Matrix protein 1 by immunization of DNA vaccine.
- 3) An H5N1 M2e-based multiple antigenic peptide vaccine confers heterosubtypic protection from lethal infection with pandemic 2009 H1N1 virus.

## **Article 1**

*Development of a humanized HLA-A2.1/DP4 transgenic mouse model and the use of this model to map HLA-DP4-restricted epitopes of HBV envelope protein*

PLoS ONE(Accepted, PONE-D-11-17039, 10.1371/journal.pone.0032247)

HLA class I and class II double transgenic mouse model was reported as an optimized mouse model that possess the capability to reflect CTL response and Th response simultaneously and allow researchers to observe the mobilization of the B cell response, CTL response and Th response. For this goal, we obtained a novel homozygous HLA-transgenic mouse model, HLA-A2.1<sup>+/+</sup>HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup>β2m<sup>-/-</sup> (HLA-A2/DP4), by crossing the previously characterized HLA-A2<sup>+/+</sup>β2m<sup>-/-</sup> (A2) mouse strain and an HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup> (DP4) mouse strain that both established in our laboratory.

In order to ensure the immunological characteristics of this mouse model, 3 different methods, including specific PCR amplification for genotyping, cell surface expression for cytometry as well as antigen specific response for *in vivo* immunological assay, were implemented. All of the results showed that new developed HLA-A2/DP4 mouse model could be used to predict human cellular immunity.

In HLA-A2/DP4 mice, both the HLA-A2 and HLA-DP4 molecules took function in shaping T cell repertoire and well educated the development of CD8<sup>+</sup> T cells, CD4<sup>+</sup> T cells and Treg cells. In present study, we predicted HLA-DP4-restricted epitopes derived from HBV envelop protein by bioinformatics, which developed by Professor Bernard Maillere for searching for the high affinity peptides with HLA-DP4 molecule, and observed DP4 binding affinity of each epitope by vitro



refolding assay.

Meanwhile, HLA-A2/DP4 mice were intramuscularly immunized 3 times with the recombinant plasmid pCMV-S2S that encoding both HBV S protein and pre-S2 protein. 10 days after the last immunization, S protein and pre-S2 protein antibodies could be detected in sera of the immunized mice. Subsequently, the splenocytes derived from the primed mice were stimulated *in vitro* with the 12 candidate epitopes. We could observe the significant lymphocyte proliferation after stimulated by peptides S109-121, S256-268, S326-338, S347-358 and S352-364.

On purpose of confirming whether these 5 selected peptides could stimulate the proliferation of human lymphocytes, we stimulated the PBMC from human PBMC from 4 HBV vaccinees and 2 non immunized donors to observe their proliferation. The result showed that 4 of 5 peptides (S256-268, S326-338, S347-358, and S352-364) could stimulate obvious human PBMC proliferation in HBV vaccinees. Therefore, 4 new identified HLA-DP4-restricted epitopes (S256-268, S326-338, S347-358 and S352-364) that could trigger T helper cell responses in vaccinated HLA-A2/DP4 mice is similar to those observed in HBV-vaccinated DP4-positive human donors.

Based on the results we obtained in this research, we demonstrated that the HLA-A2/DP4 mice represent a promising preclinical research animal model that shares immunological traits with approximately one-quarter of

the human population (30-50% for HLA-A2 and 20-80% for HLA-DP4). Thus, this animal model should facilitate the identification of novel HLA-A2- and/or HLA-DP4-restricted epitopes that could be further developed as biomarkers and vaccine components.

## **Article 2**

*Identification of novel HLA-A\*0201 restricted epitopes from H5N1 Matrix protein 1 by immunization of DNA vaccine (Manuscript in preparation)*

Analysis of structure and conservation of H5N1 virus showed that matrix protein 1(M1) is one of major structural proteins and it is highly conserved. Several studies reported that cytotoxic T cells (CTLs) response induced by M1 plays an important role in clearance of virus and recovery; therefore, developing a new cross-protective vaccine which contains conserved CTLs epitopes of M1 is of great value. Part of our research is to do epitope mapping for HLA-A\*0201 restricted CTL epitopes derive from M1 by using our established HLA-A2/DP4 animal model that is capable to produce the HLA-A\*0201restricted cellular responses.

After we obtained the M1 gene of H5N1 virus, prokaryotic and eukaryotic expression plasmids with target M1 gene were constructed to express M1 proteins. Specific primers of matrix protein M1 were synthesized for amplification M1 gene (750bp) by using cDNA from A/Shenzhen/406H/06 H5N1. After sequencing, both M1 gene were subcloned into prokaryotic expression vectors PQE30 as well as the eukaryotic expression vector pJW4303.

The recombinant M1 protein was expressed in M15 E.coli in prokaryotic way after IPTG induction, results of SDS-PAGE showed that the full-length M1 gene could be expressed in the vectors PQE30. As Parallel, M1 gene was also cloned into eukaryotic vector PJW4303 and verified its expression in 293T cells.

In order to simplified our CTL epitope mapping for M1 protein, we predicted potential HLA-A\*0201 restricted epitopes in M1 full length amino acid sequence by using the bioinformatics software BIMAS, SYFPEITHI and MAPPP. 10 potential peptides with top scores were selected for further verification. Meanwhile, HLA-A\*0201/DP4 transgenic mice were intramuscularly immunized 3 times with the pJW4303-M1 recombinant plasmid. 10 days after last immunization, humoral and cellular responses were observed by ELISA and ELISPOT, respectively. Results showed that specific M1 antibody could be detected in the sera and the HLA-A\*0201 specific CTL response could be observed by M1<sub>3-10</sub> LLTEVETYV. It demonstrated that our established HLA-A\*0201/DP4 transgenic mouse model has the capacity to apply for HLA-A\*0201 epitope.

**Article 3**

*An H5N1 M2e-based multiple antigenic peptide vaccine confers heterosubtypic protection from lethal infection with pandemic 2009 H1N1 virus*

Virology Journal 2010, 7:151

The extracellular domain of influenza Matrix protein 2 (M2e) is a highly conserved polypeptide across different influenza A subtypes and it is also the antiviral target of current treatment and prophylaxis of influenza A infections, such as two M2 inhibitors (amantadine and rimantadine). However, the poor immunogenicity of M2e alone compels us to find a better way to stimulate a strong immunity. Apart from virus-like particles, recombinant proteins, DNA and synthesized peptides, multiple antigenic peptides (MAPs) was proved to be a effective way to present M2e peptides.

In our previous study in tetra-branched multiple antigenic peptide carrying four copies of M2e peptide of H5N1 virus (H5N1-M2e-MAP), we reported that this H5N1-M2e-MAP vaccine elicited high titers of H5N1-M2e-specific serum antibodies and conferred efficacious protection against different clades of H5N1 virus. However, whether the H5N1-M2e-MAP vaccine could stimulate a strong immunity to provide the heterosubtypic protection against pandemic 2009 H1N1 strain still need to be verified.

In this study, mice were vaccinated subcutaneously (s. c) with H5N1-M2e-MAP in the presence of Freund's complete adjuvant or intramuscularly (i. m) plus aluminum adjuvant. After immunization, mice were intranasally (i. n) challenged with a lethal dose (10LD<sub>50</sub>) of A/Beijing/501/09 to observe their weights and immunological response

against the challenge. It was shown that vaccination with H5N1-M2e-MAP induced high titers of cross-creative antibodies against H1N1-M2e, which provided complete protection against H1N1 virus challenge, with the survival rate reaching 100%.

In brief, this study demonstrated that the conserved H5N1-M2e can provide a cross-protection against pandemic H1N1 infection; therefore, M2e is a promising target to be selected as an ingredient of “universal flu vaccine” which will greatly help to decrease the risk of future influenza epidemic and pandemic.



# *Discussion*

There are several species animal models were used in our preclinical and experimental research. However, several species animals were constrained to be used due to the high cost, limited resources, and low production, sometimes with ethnical problems. What's more important is that paucity of genetic models for human disease and unsatisfied accordance between human and animal models in immunological response. Let alone, the genetically inbred animal models suitable for stem cell or tissue transplantation. In this situation, even though much limitation involves on the application of HLA humanized mice, there might be no alternative that could be better mimic human immunological diseases *in vivo*. Therefore, our established HLA transgenic mouse model is a more ideal animal model no matter in fundamental researches or in application researches.

According to current research, different races differ markedly in MHC restriction. For HLA class I alleles, many studies of HLA polymorphism distribution demonstrated that the genotype for HLA-A02, is around 45-50% in West Europeans and North Americans, which is similar to that in Chinese (50%), and it is 30-40% in Middle Easterners and Indian populations. The genotypes A11 and A24 are predominant in Chinese population, each approximately 20-30%, while their distribution in West Europeans, North Americans, Middle Easterners and Indian populations are much lower(10-15%). For HLA class II alleles, in Caucasians, the

allele frequencies for HLA-DR01 is 20-25%, HLA-DR03 is 20-30%, HLA-DR04 is about 30%, and the three types add to about 80%. In Middle Eastern populations, HLA-DR03 (25-35%), HLA-DR04 (20-25%), HLA-DR07 (25-30%) and HLA-DR15 (20%) are dominating alleles. While in Chinese the dominant genotypes are LA-DR09 (20-35%), HLA-DR15 (25-30%), HLA-DR12 (20%), HLA-DR04 (~20%), which add to about 80% of total. It differs clearly from that in different races<sup>[121]</sup>. However, HLA-DP4 locus was proved later as one of the most abundant HLA alleles worldwide (20-80% of the population), being even more abundant than HLA-A2 (30-50%). It consists of two subtypes, DP0401 and DP0402, which differ from each other by 3 amino acids. Preliminary reports have shown that the presence of both DP0401 and DP0402 occurs at a frequency of 50% in Europe, 60% in South America, 80% in North America, 60% in India, 40% in the Xinjiang district in China, 25% in Africa and 15% in Japan<sup>[122,123]</sup>. Therefore, it is of great value and interest to study DP4 allele.

### **DP4 is the most frequent allele worldwide**

Despite the currently known HLA-DP alleles is comprise of 17  $\alpha$ -chain alleles and  $\beta$ -chain alleles, the HLA DP4 allele is still the most abundant in the general population. HLA DP4 include two molecules, DP401 (DP\*A0103/DP\*0401) expressed by 64% of Caucasians and the

less common DP402 (DP\*A0103/DP\*0402) found in 21% of Caucasians with 3 amino acid variants from the former <sup>[122]</sup>. Together, both molecules carried by 76% of individuals which is more frequent as the well known HLA-A2 molecules. However, approximately six molecules HLA-DR which are most studied can cover the same percentage of population.

There are only three amino acid substitutions between HLA-DP401 and DP402 molecule. These substitutions reside in the P9 pocket and has been reported that do not provoke major changes in the amino acid preferences of this pocket. Therefore, it is not surprising that they share very similar binding motifs <sup>[122]</sup>. Moreover; it was reported that two peptides derives from the two tumoral antigens, NY-ESO1 and Mage 3, restricted by HLA-DP4 have been shown to also bind to multiple HLA-DR molecules<sup>[124,125]</sup>. It may be hypothesized that the two HLA-DP4 molecules can also share the same motif with some other HLA class II restricted molecules <sup>[126]</sup>.

Apart from the binding motif, the peptides restricted by HLA-DP4 molecule were identified to be immunodominant. Two NY-ESO-1 peptides, either NY-ESO-1 87-111 or NY-ESO-1 157-170 specific CD4 T cells response could be observed in nearly 67% of the HLA-DP4 positive patients(8 in 12) with NY-ESO-1 expressing melanomas and even 25% of patients (3 of 12) responded the specific CD4+ T cells response simultaneously against both peptides<sup>[124]</sup>, a strong Th1 response against

one DP4 restricted peptide derived from Mage 3 could be observed in a majority of vaccinated patients with dendritic cells pulsed with this peptide<sup>[127]</sup>. Similar phenomenon could be also observed in viral peptide, memory effector response to an hexon T cell epitope derived from adenovirus and restricted by HLA-DP4 were detected in 78% healthy adults<sup>[128]</sup>.

Currently, several vaccine strategies were attempted to apply for preclinical immunotherapy, such as peptide pulsed dendritic cells, or recombinant proteins combined or no with adjuvant led to the induction of HLA-DP4 restricted CD4+ T cell responses against peptides derived from malignant tumour. These responses were often of high intensity and a 3000 fold amplification of anti-Mage 3 DP4 restricted CD4+ T cells has been recorded after immunization<sup>[129]</sup>. What's more important, HLA-DP4 tumour peptides were demonstrated to be naturally processed by tumour cells in some models. Curiously, only a limited number of HLA-DP4 restricted epitopes have been documented for viral infection <sup>[130-135]</sup> and tumours <sup>[136-137]</sup>.

### **Significance of DP4 restricted epitope identification**

CD8+ T cells are generally considered as potent mediators of antigen specific immunity. During these last years, identification of many CD8+ T cell epitopes has allowed a comprehensive analysis of the role and

function anti-viral CD8+ T cells <sup>[138,139]</sup>. In the contrary, the role of CD4+ T cells is more complex and characterization of tumor peptides recognized by CD4+ T cells progress slower than that for CD8+ T cells. CD4+ T cells are often considered to be a double-edged sword. CD4+ T cells play a central role in initiating and maintaining antigen specific immune responses <sup>[140, 141]</sup>. In the absence of CD4+ T cells help, specific CD8+ T cells can become lethargic <sup>[142]</sup> or to be deleted <sup>[143]</sup>. CD4+ T cell help is needed during the primary antigen specific response to imprint CD8+ T cells with the ability to develop into long-lived functional memory cells <sup>[144]</sup>. A number of studies have indicated that antigen-specific CD8+ T cell response were significantly enhanced and maintained over extended period of time in the presence of CD4+ T helper cells. Helpless CD8+ T cells that are primed in the absence of CD4+ T cells, can mediate effector functions such as cytotoxicity and cytokine secretion upon restimulation, but do not undergo a second round of clonal expansion <sup>[145]</sup>. The CD4+ T cells help CD8+ T cells via specific DC, by providing general growth factors or by inhibiting the death by activation-induced cell death upon secondary stimulation of CD8+ T cells <sup>[146]</sup>.

In cancer research, CD4+ T cells have also been demonstrated to be important to sustain the functions of adoptively transferred anti-tumor CD8+ T cells. However, transfer of CD4+ T cells in the absence of CD8+

T cells can also protect and cure mice against tumor. Indeed, CD4<sup>+</sup> T cells could inhibit tumor growth in the absence of CD8<sup>+</sup> T cells by directly lysing MHC class II positive tumor cells or by prompting the recruitment of other effector cells, such as macrophages and eosinophils [147, 148]. Furthermore, IFN- $\gamma$  secreted by tumor-specific CD4<sup>+</sup> T cells around the tumor has been reported to be very essential for tumor regression involving inhibition of tumor-induced angiogenesis [148].

On the other hand, a CD4<sup>+</sup> T cell subsets that called regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells, has emerged as the dominant T cell population governing peripheral self- tolerance by inhibiting effector T cells [149]. Since the CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> phenotype also identifies activated T cells , new markers such as FoxP3 have been described which appear to discriminate activated and regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells. In animal models, removal of CD4<sup>+</sup>CD25<sup>+</sup> T cells improved immune mediated tumor clearance and enhanced the response to immunotherapy [150, 151]. The frequency of regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells has been demonstrated to be significantly elevated in the peripheral blood and tumor site of cancer patients [152, 153]. It has been proposed that tumor cells convert DCs into regulatory cells that secrete bioactive TGF- $\beta$  and stimulate CD4<sup>+</sup> T reg cell proliferation during the development of Cancer [154]. Due to this dual activity, a better characterization of specific anti-tumor CD4<sup>+</sup> T cells will allow a better understanding of their role during the development of

cancer.

Moreover, most CD4 peptides have been reported to be presented by HLA-DR or DQ molecules. However, the identification of CD4+ restricted by other MHC class II molecules is also attractive because of the frequency of their HLA class II alleles, the immunodominance of these restricted peptides in different disease animal models and the fact that the peptides which bind to these unpopular MHC class II molecules are often promiscuous. These new CD4 targets will allow a better dissection of the role and function of these CD4+ T cells in human disease. They will also represent valuable tools for the immunomonitoring of immunotherapy protocols and for the design of vaccines.

New strategy of therapeutic vaccine is to introduce the Th epitope into traditional vaccine so as to enhance the recognition of CTL cells for HBV infected cells. As mention above, many DR and DQ restricted Th epitopes were largely identified, however, there is few DP4 restricted Th epitope reported, even though the allele frequency of HLA-DP4 is higher than DR or DQ haplotype. Therefore, screening the DP4 restricted Th epitope is valuable for further vaccine design.

### **DP4 restricted epitope mapping in vitro and in vivo**

In general, the immunogenicity of both T helper and CTL epitopes are



related to their MHC-binding capacities, with good T helper inducers generally having a high affinity for MHC class II molecules. Based on this theory, we used two methods to identify the DP4 restricted epitope mapping in vitro; one is the bioinformatics prediction, the other is a DP4 peptide specific binding assay.

The bioinformatics to predict the potential epitope in antigen is based on the peptide binding motif of HLA-DP4, of which the main anchors residues are accommodated by the P1 and P6 pocket. In our study, the sequences of HBsAg was inputted in a excel files and submitted to the bioinformatics of binding prediction to HLA-DP401 and DP402 <sup>[124]</sup>. Peptides will be ranked based on their predicted IC50. 12 peptides were selected, provided their predicted IC50 is not superior to 1000nM. They were synthesized as 15 mer peptides.

To further verify the synthesized peptides, a binding assay was implemented. To identify the CD4 peptide, a series of the most popular HLA class II molecules in the European and North-American population have been constituted and produced, including HLA-DR1, DR3, DR4, DR7, DR11, DR13, DR15, DRB3, DRB4, and DRB5 and they have been used to characterize CD4<sup>+</sup> T cell epitopes for various tumor antigens as EphA3, Mage3, and CAMEL <sup>[155-157]</sup>. For each molecule, a peptide specific binding assay has been developed relying upon a competitive ELISA principle. This kind of assay allows us to evaluate the binding

capacity of 20-30 peptides in the same run. At present, these operations have been recently adapted to an automated workstation called “HLA express” in order to increase the rate and quality of the measurement. In order to identify the DP4 restricted epitope, the most preponderant HLA class II molecules worldwide, a similar DP4 peptide specific binding assay, as mentioned in our research, was developed to further analyze the DP4 restricted peptide binding capacity. Details were shown as follow, immunopurified HLA DP4 molecules will be incubated with a dose range of peptides, an appropriate dilution of a biotinylated peptide. After 24h incubation, HLA class II complexes will be transferred in ELISA plates on which a HLA class II specific monoclonal antibody has been absorbed. Presence of the complexes will be revealed by addition of streptavidin-phosphatase and a fluorescent substrate. This assay proved to be as sensitive and reproducible as the HLA-DR specific binding assays in our study.

Preliminary work on the binding capacity of DP4 molecules greatly facilitates the procedure of DP4 epitope mapping. However, which of the good binders in candidate peptides are immunological should be further verified *in vivo* and in the clinical validation.

Taking account of much limitation in using human sample, we developed a more accessible and convenient assay, HLA-A2/DP4 transgenic mice for observation of DP4 restricted CD4 T cells response.

Later, all the results, which obtained from mouse model, repeated and observed in the HLA-DP4 positive donors.

### **Immunological characteristics of HLA-A2/DP4 transgenic mice**

The final objective to develop HLA transgenic mice is to solve the problems in therapeutic or diagnosis purpose. In our study, HLA-A2/DP4 transgenic mouse model was created by replacing murine H2 molecules with the most abundant human HLA molecules, HLA-A2 and HLA-DP4, which can take corresponding human biological function in C57BL/B6 murine background. In this case, new established mouse model can be used to mimic human cellular immune response in the largest population to predict both HLA-A2 and HLA-DP4 mediated T cell responses against exogenous and endogenous antigen in human immunity. As known, only MHC class I and class II mainly regulate the antigen specific cellular immunity, herein, the HLA class I and HLA class II in the murine cells can directly or indirectly involves in the all cellular immunity. Therefore, building this HLA-I and HLA-II double transgenic mouse model has essential significance for preclinical study.

In order to improve Immunological characteristics of HLA-A2/DP4 (HLA-A2.1<sup>+/+</sup>HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IA $\beta$ <sup>-/-</sup> $\beta$ 2m<sup>-/-</sup>) transgenic mouse model, the parental HLA-A2-transgenic (HLA-A2.1<sup>+/+</sup> $\beta$ 2m<sup>-/-</sup>)

mouse model <sup>[158]</sup> and HLA-DP4/hCD4 transgenic H-2 class II (IA $\beta$ )/mCD4-KO (HLA-DP4+/+hCD4+/+mCD4-/-IA $\beta$ -/-) mouse model <sup>[159]</sup> were well designed. HLA-A2-transgenic mouse model <sup>[158]</sup> expressing a chimeric monochain named HHD molecule:  $\alpha$ 1- $\alpha$ 2 domains of HLA-A2,  $\alpha$ 3 to the cytoplasmic domains of H-2 Db, linked at its N-terminus to the C-terminus of human  $\beta$ 2m by a 15-amino-acid peptide linker, this kind of structure was proved to be very effective in many HLA class I mediated immunology. Expression of this functional transgenic HLA-A2.1 molecules led to an increase in the size of the peripheral CD8<sup>+</sup> T cell population, which reached 2-3% of the total splenocytes in HLA-A2/DR1 mice, compared to 0.6-1% in  $\beta$ 2m-KO MHC-I deficient mice <sup>[78]</sup>. Furthermore, the size of functional TCR repertoire from peripheral CD8<sup>+</sup> T cell is highly diverse as comparing to wild type B6 mice, this suggest that CD8 T cell activity is highly competent and supported by several studies on HBV and HIV <sup>[160, 161]</sup>

For HLA-DP4/hCD4 transgenic H-2 class II (IA $\beta$ )/mCD4-KO mouse model, more optimization focused on the interaction between the HLA DP4 molecule and the CD4<sup>+</sup> T lymphocytes. This work focused on the optimization of the HLA-DP4/ IA $\beta$  (HLA-DP4+/+IA $\beta$ -/-) transgenic mice and derivatives. Previously, we have created and used HLA-DP4/ IA $\beta$  (HLA-DP4+/+IA $\beta$ -/-) transgenic mouse model to identify new HLA-class II restricted epitopes from several antigens. However, these mice have

only 3% of CD4<sup>+</sup> T lymphocytes compared to 0.8% of residual CD4<sup>+</sup> T lymphocytes in H-2 class II KO mice (H-2 class II KO). To optimize their ability and sensibility for epitope mapping, we generated the new HLA-DP4/IAβ/hCD4/mCD4 (HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup>) mice. In this mouse models, we observed that there are 12% of CD4<sup>+</sup> T lymphocytes compared to 3% of CD4<sup>+</sup> T lymphocytes in HLA-DP4/IAβ (HLA-DP4<sup>+/+</sup>IAβ<sup>-/-</sup>) transgenic mice. These data indicate that correct interaction between hCD4 and DP4 are very important during thymus education process. Therefore, the new developed HLA-A2/DP4/β2m/IAβ (HLA-A2.1<sup>+/+</sup>HLA-DP4<sup>+/+</sup>IAβ<sup>-/-</sup>β2m<sup>-/-</sup>) transgenic mouse model were further developed into HLA-A2/DP4/β2m/IAβ/hCD4/mCD4 (HLA-A2.1<sup>+/+</sup>HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup>β2m<sup>-/-</sup>) transgenic mice.

In the HLA-A2/DP4 mouse model, HLA-A2 and HLA-DP4 molecules expression could be detected, it allows for the selection of peripheral CD8<sup>+</sup> and CD4<sup>+</sup> T cells and shaping the CD8<sup>+</sup> and CD4<sup>+</sup> T cells repertoire. Around 2.3% CD8<sup>+</sup> T cells could be observed in CD3<sup>+</sup> T lymphocytes of HLA-A2/DP4 transgenic mice, compared with 0.6–1 % CD8<sup>+</sup> T cells in β2-microglobulin (β2m)-deficient MHC class I-deficient mice. Around 89–90% CD4<sup>+</sup> T cells could be observed in CD3<sup>+</sup> T lymphocytes of HLA-A2/DP4 transgenic mice, while only 2–3% CD4<sup>+</sup> T cells in the H-2 class II-KO mice. We further analyzed the hCD4 expression in CD4<sup>+</sup> T lymphocytes because the differentiation of

immature CD4<sup>+</sup>CD8<sup>+</sup> double-positive T cells into CD4<sup>+</sup> T cells in the thymus is highly dependent on the expression of human CD4 (hCD4)<sup>[162]</sup>, around 89–90% of the CD4<sup>+</sup> T cells express hCD4 rather than mCD4. Doubtlessly, the cellular immunity in these mouse models are under HLA molecules regulation. On the other hand, we confirmed that no specific IA $\beta$  and  $\beta$ 2m DNA amplification were detected by using HLA-A2/DP4 mouse model's genome. This indicated that H-2 antigen system was “silenced” by human HLA molecules and the murine endogeneous H2 loss the chance to compete for the immune response with the HLA class I and class II transgenes, which contribute to improve the reliability of transgenic mouse model. Significantly, this new model could be used to observe CD4<sup>+</sup> T cells response, CD8<sup>+</sup> T cell response as well as the collaboration of CD4<sup>+</sup> and CD8<sup>+</sup> T cell response.

In order to observe whether the immunological characteristic of HLA-A2/DP4 mouse model is similar to that of human response, we compared the humoral response and HLA-A2-restricted response in the HLA-A2/DP4 mice with the responses that have been previously reported for naturally infected patients or immunized humans. As expected, we observed the HLA-A2 restricted CD8<sup>+</sup> T cells response and the HLA-DP4 restricted CD4<sup>+</sup> T cells response in HLA-A2/DP4 mouse model respectively. Both the T cells response against reported HLA-A2 and HLA-DP4 restricted epitopes are accordance with that of other HLA

transgenic mice and human.

## **HLA-A2/DP4 mice application in DP4 restricted epitope identification**

For HBV proteins MHC-I-restricted epitopes, there are abundant data on HLA-A2 restricted CTL epitopes in related databases, which provides a solid basis for vaccine design and experimentation based on CTL epitopes. However, MHC-II restricted epitopes are relatively rare and they are mostly Caucasian dominated HLA-DR01 restricted epitopes and they can not present by DP4 molecule. Therefore, these epitopes probably won't work well in the large DP4 positive population, let alone directly be used in designing polypeptide vaccines or multiple epitope vaccines. Furthermore, establishment of suitable animal models that reflect human immune status for carrying out epitope mapping and vaccine evaluation is a practical approach for vaccine research.

Considering the great value of DP4 epitope in therapeutic and experimental research, an HBV DNA vaccine that encodes two HBV envelope proteins (preS2 and S proteins) was implemented for searching for the potential target in HBsAg. The referred two proteins can self-assemble into particles carrying HBsAg, and they are the two protein components of currently used vaccine against hepatitis B. In one part of our study, immunological characteristics of HLA-A2/DP4 transgenic

mice were well confirmed and the results indicated that HLA-A2/DP4 mice could respond to whole HBs antigen and to a previously described HLA-DP4-restricted peptide, S181-S192. We inferred that novel HLA-DP4-restricted epitopes in the HBs envelope protein can be identified by this new model. The whole length of hepatitis B envelope protein was scanned with the help of HLA-DP4 epitope prediction bioinformatics, 12 candidate peptides were selected and the affinities for each of them were evaluated. Meanwhile, HLA-A2/DP4 mice were immunized intramuscularly with the pCMV-S2.S DNA vaccine to observe the DP4 CD4<sup>+</sup> T cells specific proliferation *in vivo*, including previously reported epitope HBs181-192. The peptide-specific proliferation aroused by peptides S109-121, S256-268, S326-338, S347-358 and S352-364 was detected in HLA-A2/DP4 mice, as we expected. Subsequently, we were curious about whether the epitopes that presented in the HLA-A2/DP4 mice could also be presented and stimulate human immunity. We obtained some PBMCs from 6 HLA-DP4<sup>+</sup> donors, including 4 donors who had been immunized against HBV and 2 donors who were never immunized, were utilized to analyze their HLA-DP4-restricted CD4<sup>+</sup> T cell responses.

Results of the human PBMCs proliferation assays showed that three out of four immunized subjects responded to the reported Celis (S181-S192) peptide. The responses of two donors were directed against



the S326-338 and S256-268 peptides. One response was observed against the S352-364 and S347-358 peptides. No significant responses against the S109-121 peptide (which was derived from the S2 part of the antigen) were observed in the four donors who had received the HBV vaccine. All together, four out of five newly identified DP4-restricted HBs epitopes were functional to detect specific T cell responses in vaccinated humans. All of the results demonstrated that the HLA-A2/DP4 mouse model can be used in novel epitope mapping as the complementary method *in vivo* with their 80% accordance with human cases.

### **The application of HLA-A2/DP4 mouse model**

The objective of this study is to create a HLA-A2/DP4 double transgenic mouse model with HLA-A2 restricted CTL response and HLA-DP4 restricted Th response. With the application of this model, we confirmed one novel HLA-A2 restricted CTL epitope and four novel HLA-DP4 restricted Th epitope in HBsAg. Thus, this mouse model represents a promiscuous animal model in following domains.

1. Screening and classification of both HLA-A2 and HLA-DP4 restricted immunodominant epitope of tumor and pathogen

The cytotoxic T lymphocyte mediated specific cellular immunity plays an important role in eliminating tumor and removing virus infection. At present, effective stimulation of the specific CTL response is an important

objective of the researches on therapeutic anti-tumor and anti-pathogen vaccine. Many immunodominant epitope from many pathogens, for instance, the human highly pathogenic bird flu <sup>[163]</sup>, human cytomegalovirus <sup>[164]</sup> and hepatitis D virus <sup>[165]</sup>, and malignant tumors such as the melanoma <sup>[166]</sup>, breast cancer<sup>[167]</sup> were obtained by using the single HLA-A2 mouse model and so on. Some of them are already used to design the clinical test markers and polypeptide vaccines and so on.

Traditional method for screening CTL epitope includes the bioinformatics prediction, biochemical method, T2 cell binding assay and other in vitro screening methods based on the combining capacity between MHC-I molecule and antigenic peptide. But the experiment proved that the combining capacity between MHC-I molecule and antigenic peptide is non-absolutely related with the immunogenicity of this antigenic peptide, which is also directly affected by other factors in the intracellular antigen presentation process. Up to now, our team has developed several strains of mouse models for human HLA class I molecules with absence of H-2 class I molecule, such as HLA-A2<sup>+</sup>/β2m<sup>-</sup>, HLA-B7<sup>+</sup>/β2m<sup>-</sup> and HLA-A3<sup>+</sup>/β2m<sup>-</sup>. All of them are murine β microglobulin knock-out to avoid competition with the HLA-class I molecules. These transgenic mice have been shown to be robust and versatile for the identification of the HLA class I T cell epitopes which can induce CD8<sup>+</sup> T cell response *in vivo* and they have been successfully

used by many laboratories to identify new HLA-class I epitopes from several viral and tumor antigens<sup>[168-170]</sup>. Therefore, the new created HLA-A2/DP4 mouse model is new member to screen more immunodominant epitopes of the antigen from tumors and pathogenic proteins as the vaccine targets through ELISPOT, *in vivo* CTL killing experiment (CTL toxicity test), tetramer binding and other test methods.

As studies go into depth, the role of helper T cell epitopes receives more and more attention. However, for Th epitopes identified for major proteins of important pathogens, such as HIV, HBV and MTB, are mainly HLA-DR1 epitopes, which do not match the HLA-DP4 restriction dominance in the population, and these epitopes won't function well in regulating cell-mediated immunity and humoral immunity in DP4 positive population, and can not be directly applied to the design of polypeptide or multi-epitope vaccines. As Parallel with HLA class I transgenic mice, several strains human HLA class II strains mouse models with deletion of H-2 class II molecule, notably the HLA-DR1<sup>+</sup>/IAβ<sup>-</sup>, HLA-DR3<sup>+</sup>/IAβ<sup>-</sup> and HLA-DQ8<sup>+</sup>/IAβ<sup>-</sup> were also established. These transgenic mice have been shown to be robust and versatile for the identification of the HLA class I and II restricted T cell epitopes which can induce CD4<sup>+</sup> and CD8<sup>+</sup> T cell response *in vivo* and they have been used to identify new HLA class II restricted epitopes from several viral and tumor antigens<sup>[170,171]</sup>. However, the alleles in these mice

is not most abundant, therefore, the establishment of HLA-A2/DP4 can greatly expand the human coverage of HLA transgenic mice. In addition, most of former mouse models are single HLA class I or class II transgenic mice and fail to evaluate the synergistic effect between the CTL and Th immunity.

It is convincing that the identification of HLA-DP4 restricted peptides could be very useful for the improvement of CTL, one goal of vaccine, by two ways: It has been shown that polypeptide that contain both MHC class I and class II restricted epitopes is more efficient for CTL induction *in vivo*<sup>[172]</sup>. Vaccines that simultaneously induce helper T cells and CTLs are more effective than those that induces CTLs only<sup>[173,174]</sup>. Therefore, one way to improve vaccines efficiency would be to associate DP4 restricted peptide to known CD8 tumor peptide within a long polypeptide.

Another concern for us is the universal epitope. There are two non specific T-helper epitopes are use in some therapeutic cancer vaccination for the activation of the CD4+ T lymphocytes and the enhancement of CTL induction. One is a pan HLA-DR-binding epitope (PADRE) that has been reported to be immunogenic when used as adjuvant<sup>[175,176]</sup>. It has been shown to improve the induction of an effective CTL response *in vivo* <sup>[177-178]</sup>. Furthermore, recent data from clinical trial using peptide-pulsed dendritic cells vaccination in metastatic renal cancer patients, also demonstrate that the addition of PADRE peptide during T

cell priming might increase the cytotoxic activity of antigen-specific T lymphocytes supporting the immunogenicity of this peptide. Another CD4<sup>+</sup> T helper epitope derived from tetanus toxoid has also been proposed as adjuvant because of its promiscuous binding to MHC class II molecules. Tetanus derived helper peptide has also been shown to increase specific CTL response in vivo and in vitro<sup>[179,180]</sup>. In this case, it's very attractive to identify some DP restricted universal epitopes and to verify the immunological efficiency of known universal epitope under the DP4 regulation.

## 2. Safety and efficacy validation of new vaccines

It generally takes 10-15 years to research and develop a new medicine. Furthermore, the new medicine is less likely to succeed: only a few medicines can be finally approved through strict screening and validation in various stages such as the in vitro experiment, animal experiment, preclinical experiment and so on, 1/5000 in the US and 1/4000 in Japan for instance. HLA transgenic mouse model has a closer vaccine reaction to the human body than traditional mouse models, therefore enhancing the accuracy for the animal model evaluation helps to shorten the research and development cycle of drugs and vaccines. What is worth mentioning is that the HLA transgenic mouse model has unique superiority in evaluating the immunoprophylaxis and treatment of new vaccines for human such as the polypeptide vaccine, recombinant multi-epitope

vaccine, therapeutic vaccine and so on based on epitope development, including evaluating the dosage form, vaccination way, dosage and medication times of polypeptide, and comparing the influence of the mutational site of epitope on the epitope immunogenicity and so on.

### 3. Reconstitution of immunodeficient mice with human hematopoietic stem cells

All reported HLA transgenic mouse models, including HLA class I transgenic mice, HLA class II transgenic mice and HLA class I and class II double transgenic mice, can regulate the T cell maturation and antigen presentation by replaced human HLA antigen system instead of mouse H-2 antigen system. It allows the human stem cells can be transplanted and grow in these mouse models without the species specific MHC rejection. Further, when the human stem cells developed into different kinds of lymphocytes, both the antigen specific humoral response and cellular response would be observed more likely same to that in human. At present, such mouse models are still at the exploration stage, and are mainly intended to transfer the human HLA gene into SCID, NOD-SCID or Rag2<sup>-/-</sup>γc<sup>-/-</sup> severe combined immunodeficiency mouse model [181,182], inject human hemopoietic stem cell into the mouse body after killing the mouse lymphocyte through radiation exposure, so as to complete the growth and maturation of lymphocyte in vivo, and establish a more humanized animal model based on the mouse model. Recently, Shultz LD

et al reported that ,after the human hemopoietic stem cell was injected into HLA-A2-NOD-SCID- $\gamma$  c<sup>-/-</sup> severe combined immunodeficiency mouse model, it can develop in the marrow and spleen to TCR with double positive  $\alpha\beta$  CD4+CD8+, TCR with double negative  $\gamma\delta$  CD4-CD8-, T cell with positive CD8+, or Th17, Th1 and Th2. Recombined immune mouse model infected with EBV can have the lymphadenosis and corresponding CTL response, which proves the feasibility of this method [183]. However, CD4+ T cells in this animal models was still developed under the murine H-2 class II molecule, thus HLA-A2-NOD-SCID- $\gamma$  c<sup>-/-</sup> severe combined immunodeficiency mouse model still need to be improved as a more humanized mice. Our previously developed HLA-A2/DR1 mouse model and the present HLA-A2/DP4 mouse model might be a more rational proxy as the recipient of human hemopoietic stem cell.

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# *Annexes*

# *Article 1*

**Development of a Humanized HLA-A2.1/DP4 Transgenic Mouse Model and the Use of this Model to Map HLA-DP4-Restricted Epitopes of HBV Envelope Protein**

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## **Abstract**

A new homozygous humanized transgenic mouse strain, HLA-A2.1<sup>+/+</sup>HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup>β2m<sup>-/-</sup> (HLA-A2/DP4), was obtained by crossing the previously characterized HLA-A2<sup>+/+</sup>β2m<sup>-/-</sup> (A2) mouse and our previously created HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup> (DP4) mouse. We confirmed that the transgenes (HLA-A2, HLA-DP4, hCD4) inherited from the parental A2 and DP4 mice are functional in the HLA-A2/DP4 mice. After immunizing HLA-A2/DP4 mice with a hepatitis B DNA vaccine, hepatitis B virus-specific antibodies, HLA-A2-restricted and HLA-DP4-restricted responses were observed to be similar to those in naturally infected humans. Therefore, the present study demonstrated that HLA-A2/DP4 transgenic mice can faithfully mimic human cellular responses. Furthermore, we reported four new HLA-DP4-restricted epitopes derived from HBsAg that were identified in both vaccinated HLA-A2/DP4 mice and HLA-DP4-positive human individuals. The HLA-A2/DP4 mouse model is a promising preclinical animal model carrying alleles present to more than a quarter of the human population. This model should facilitate the identification of novel HLA-A2- and HLA-DP4-restricted epitopes and vaccine development as well as the characterization of HLA-DP4-restricted responses against infection in humans.

**Keywords:** HLA-A2/DP4, epitopes, hepatitis B virus, humanized mice



## ***Introduction:***

Protective immunity requires the effective mobilization of B cells, cytotoxic T cells and helper T cells <sup>[1]</sup>. Major Histocompatibility Complex (MHC) molecules play a pivotal role in shaping both the specificity and the functional outcome of adaptive immune responses. The repertoire of functional T cell receptors on CD4<sup>+</sup> and CD8<sup>+</sup> T cells is dependent upon the presence of MHC molecules implicated in the positive and negative selection of thymocytes in the thymus <sup>[2]</sup>. Moreover, the MHC controls the activity of T cells through peripheral tolerance mechanisms and the ability to select and present MHC-restricted epitopes <sup>[3]</sup>.

HLA-restricted epitopes was shown to be important for boosting the effectiveness of HLA-restricted epitope-based vaccine candidates. There is considerable interest in inducing specific CTLs to prevent or control viral infections or to cure autoimmune diseases. Promising protective antiviral immunity using CTL epitope-based vaccines has been demonstrated in several experimental models of infection <sup>[4]</sup>. Similarly, CD4<sup>+</sup> T cell epitope-based vaccines have been designed for several experimental autoimmune and pathogenic diseases <sup>[5-8]</sup>. However, these vaccines did not induce significant or sufficient protection in human preclinical trials. The insufficient efficacy could be due to the lack of simultaneous activation of specific B cells, cytotoxic T cells and helper T cells, which is required for protective immunity <sup>[9]</sup>. Given the practical difficulty of studying immune responses in humans, several transgenic mouse models expressing human HLA class I or class II molecules have been developed to map potential pathogenic and tumoral epitopes as well as to predict human immunity <sup>[10,11]</sup>.

To facilitate the development of a new generation of candidate vaccines and to evaluate their efficacy *in vivo*, a predictive preclinical “humanized” transgenic mouse model that expresses both HLA-A2 and HLA-DR1 while lacking H2-IA $\beta$  and H2- $\beta$ 2m was developed <sup>[12]</sup>. These mice allow for the simultaneous evaluation of specific B cells, HLA class I-restricted CD8<sup>+</sup> T cells and HLA class II-restricted CD4<sup>+</sup> T cells of human interest. However, this humanized mouse model represents the

immunity of approximately 3-9% of the human population (30-50% for HLA-A2.1, 6-18% for HLA-DR1)<sup>[13]</sup>, thus limiting the usefulness of this model for preclinical research.

The HLA-DP4 locus is one of the most abundant HLA alleles worldwide (20-80% of the population), being even more abundant than HLA-A2 (30-50%). It consists of two subtypes, DP0401 and DP0402, which differ from each other by 3 amino acids. Preliminary reports have shown that the presence of both DP0401 and DP0402 occurs at a frequency of 50% in Europe, 60% in South America, 80% in North America, 60% in India, 40% in the Xinjiang district in China, 25% in Africa and 15% in Japan<sup>[14, 15]</sup>. Interestingly, some reports have suggested that antigen presentation by HLA-DP4 molecules may be critical for viral elimination and that such antigen presentation plays an important role in the pathogenesis of chronic hepatitis B infection<sup>[16, 17]</sup>. These studies highlight the importance of HLA-DP4 in the immune response to infection and in some autoimmunity diseases<sup>[18, 19]</sup>. Increasing evidence of the importance of HLA-DP4 and its high frequency in the human population suggests that HLA-DP4 epitope-based vaccines could induce immune protection in a large proportion of the population. However, a limited number of HLA-DP4-restricted epitopes have been documented for pathogenic infection<sup>[20-25]</sup> and tumors<sup>[26-28]</sup>.

In this study, we describe a homozygous humanized HLA-transgenic mouse strain, HLA-A2.1<sup>+/+</sup>HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup>β2m<sup>-/-</sup> (HLA-A2/DP4), which was produced by crossing the previously characterized HLA-A2<sup>+/+</sup>β2m<sup>-/-</sup> (A2) mouse<sup>[29]</sup> and an HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup> (DP4) mouse strain<sup>[30]</sup> that we established in our laboratory. We then confirmed the phenotypes and immunological activities characteristic of the transgenes (HLA-A2 and HLA-DP4) inherited from the parental HLA-A2 and HLA-DP4 mice. Finally, we screened for HLA-DP4-restricted epitopes derived from HBV envelop protein using the HLA-A2/DP4 mouse, and we identified 4 new HLA-DP4-restricted epitopes (S<sub>256-268</sub>, S<sub>326-338</sub>, S<sub>347-358</sub> and S<sub>352-364</sub>) that could trigger T helper cell responses similar to those observed in vaccinated HLA-A2/DP4 mice and HBV-vaccinated DP4-positive human donors. Our results demonstrate that the HLA-A2/DP4 mouse represent a promising preclinical research animal model that

shares immunological traits with approximately one-quarter of the human population (30-50% for HLA-A2 and 20-80% for HLA-DP4). Thus, this animal model should facilitate the identification of novel HLA-A2- and/or HLA-DP4-restricted epitopes that could be further developed as biomarkers and vaccine components.

## ***Materials and Methods***

### **Generation of transgenic mice:**

HLA-DP4/hCD4 transgenic H-2 class II (IA $\beta$ )/mCD4-KO mice <sup>[30]</sup> were obtained at the INSERM by crossing our established HLA-DP4-transgenic mice (HLA-DP4<sup>+/+</sup> hCD4<sup>+/+</sup> mCD4<sup>-/-</sup>) with H-2 class II (IA $\beta$ )-KO (IA $\beta$ <sup>-/-</sup>) mice. The HLA-A2.1-transgenic mice, expressing a chimeric monochain (HHD molecule:  $\alpha$ 1– $\alpha$ 2 domains of HLA-A2.1,  $\alpha$ 3 to the cytoplasmic domains of H-2 D<sup>b</sup>, linked at its N-terminus to the C-terminus of human  $\beta$ 2m by a 15-amino-acid peptide linker), were created in Pasteur Institute <sup>[29]</sup>. HLA-A2.1 (HHD)-transgenic H-2 class I ( $\beta$ 2m)-KO and HLA-DP4/hCD4-transgenic H-2 class II (IA $\beta$ )/mCD4-KO mice were intercrossed. After six generation, the heterozygote progeny with HLA-A2.1-HLA-DP4- hCD4 were selected to continue intercrossing till mCD4<sup>-/-</sup>IA $\beta$ <sup>-/-</sup> $\beta$ 2m<sup>-/-</sup> mice were obtained. Finally, HLA-A2.1<sup>+/+</sup>HLA-DP4<sup>+/+</sup> hCD4<sup>+/+</sup> mice with the background of mCD4<sup>-/-</sup>IA $\beta$ <sup>-/-</sup> $\beta$ 2m<sup>-/-</sup> mice were obtained and used for the experiments described in this report. Mice were bred in the animal facilities at INSERM U1014 (Paris, France) under barrier conditions and provided with commercial mouse chow and water ad libitum. All experiments involving mice were performed according to approved protocols and the guidelines of the animal facility of Hospital Paul Brousse (Villejuif, France) under agreement number 94-076-32 and permit number 94–241 from the Directorate of Veterinary Services.

### **Genotype identification:**

The HLA-A2.1 (HHD)-transgenic H-2 class I-KO and DP4-transgenic H-2 class transgenic II-KO (IA $\beta$ ) mice were identified by PCR. Mice genomic DNA was extracted using genomic DNA isolation protocols. Briefly, tails were digested by incubation with 100 mM NaCl, 50 mM Tris-HCl, pH 7.2, 100 mM EDTA, 1% SDS and 0.5 mg/mL proteinase K (Merck, Darmstadt, Germany) overnight at 56°C, followed by the addition of 250  $\mu$ L of saturated NaCl solution and isopropanol precipitation. Pellets were washed 2 times with 70% ethanol and resuspended in 100

μL deionized water. After homogenization of the DNA concentration, PCR was used to amplify transgenes with different pairs of forward and reverse primers, as follows: HDD: 5'CATTGAGACAGAGCGCTTGGCACAGAAGCAG3', 5'GGATGACGTGAGTAAACCTGAATCTTTGGAGTACGC; DP04α: 5'TAATACAAAGTCTGCAGCTGGC3', 5'AGCAATGTTAGCCAGCC3'; DP04β: 5'GGGATTGGAAAGAGGCTC3', 5'GCACTGCCCCGCTTCTCC3'; hCD4: 5'TCAGTGCAATGTAGGAGTCCAA G 3', 5'CACGATGTCTATTTTGAAGTCCAC3'; mCD4: 5'GGAGTTGTGGGTGTTCAAAGTG3', 5'AGAGTTGCTATCCAAGGTCAGGG3'; 5'GCTTCCTCGTGCTTTACGGTATC3'; β2m: 5'CTGAGCTCTGTTTTTCGTCTG3', 5'CTAACTCTGCA GCGTATG3'; 5'CCTGCCGAGAAAGTATCCA3'; and IAB: 5'TTCGTGTACCAGTTCATGGG3', 5'TAGTTGTGTCTGCACACCGT3', 5'CCTGCCGAGAAAGTATCCA 3'.

### **FACS analysis**

Splenic cells were separated by Ficoll gradients (GE Lifesciences, Uppsala, Sweden). Cytofluorometry studies were performed on splenocytes using PE-conjugated W6/32 (anti-HLA-ABC; eBioscience, San Diego, CA). Analysis of MHC class II molecule expression was conducted after saturation of Fc receptors with the 2.4G2 mAb using FITC-labeled anti-HLA-DP (B7/21) and PE-labeled anti-CD19. CD4<sup>+</sup>/CD8<sup>+</sup> positive lymphocytes that were first labeled with APC anti-CD3 (BD Biosciences, San Diego, CA, USA). Portions of the single mCD8<sup>+</sup>, mCD4<sup>+</sup> and hCD4<sup>+</sup> lymphocytes were labeled using PE-labeled anti-mouse CD8, FITC-labeled anti-mouse CD4, and PECy7-labeled anti-human CD4. Wild-type C57BL/B6 mice were chosen as a control.

### **DNA immunization**

The hepatitis DNA vaccine pCMV-S2.S<sup>[31]</sup> was used to verify the consistency in the *in vivo* cellular responses between the transgenic mice and humans. At the age of 8 weeks, transgenic mice were pre-immunized with cardiotoxin. After 5 days, the mice were immunized 3 times intramuscularly at 10-day intervals, each time with a 100-μg

DNA vaccine injection. Ten days after the last immunization, the mice were used for further analyses.

### **ELISA assay**

Sera from immunized mice were individually assayed by ELISA<sup>[31]</sup> on either purified HBsAg or preS2 synthetic HBs109–134 peptide. After blocking with PBS supplemented with 0.1% Tween-20, 10% FCS and washings three times, bound antibodies were detected with horseradish peroxidase-labeled anti-mouse IgG (Serotec, Cergy-Saint-Christophe, France). Antibody titers (means of at least three determinations) were determined by the serial end-point dilution method.

### **ELISPOT assay**

An ELISPOT assay was implemented to detect IFN- $\gamma$  secreted by CD8<sup>+</sup> T lymphocytes. Briefly, membrane-backed 96-well ELISPOT plates (Millipore, Bedford, MA) were coated with anti-IFN- $\gamma$  mAb (Diaclone, Besancon, France) overnight at 4°C and then blocked with 1% skim milk. CD4-lymphocyte-depleted cells ( $2 \times 10^5$ /well) were added to each well, cultured with 20  $\mu$ g/mL synthetic peptides and incubated for 20 h at 37°C under 5% CO<sub>2</sub>. The IFN- $\gamma$ -secreting cells were captured by coating with anti-IFN- $\gamma$  mAb and detected by incubation with biotinylated anti-mouse IFN- $\gamma$ Ab (Diaclone) for 90 min at 37°C, followed by incubation with streptavidin-HRP for 1 h. Finally, the plates were developed using substrate BEC (Diaclone, ready to use), washed, and dried. Spots were counted using an ELISPOT reader (CTL, Germany).

### **Proliferation assay:**

Ten days after the final immunization, splenocytes were RBC-depleted, submitted to a Ficoll gradient, and adjusted to  $10 \times 10^6$  cells/mL ( $5 \times 10^5$  cells/well)<sup>[12]</sup>. Splenocytes were co-cultured with peptide-pulsed (20  $\mu$ g/mL) in HL1 serum-free medium supplemented with 10 mM HEPES, 1 mM sodium pyruvate,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 100 IU/mL penicillin and 100  $\mu$ g streptomycin for 72 h at 37°C in

5% CO<sub>2</sub>. One microcurie of [<sup>3</sup>H]thymidine was added to each well 16 hours before the cells were harvested using a TOMTEC collector (Perkin Elmer Applied Biosystems), and the incorporated radioactivity was measured using a micro-β-counter (Perkin Elmer Applied Biosystems). The results are given as the stimulation index (SI) = cpm with specific peptide/cpm with control peptide (Mag-3<sub>243-258</sub> KKLLTQHFVQENYLEY)<sup>[32]</sup>.

#### **HLA-DP4 specific binding assay**

HLA-DP4 binding assays were performed as described elsewhere<sup>[14]</sup>. In brief, they were performed in 10mM phosphate, 150mM NaCl, 1mM n-dodecyl β-D-maltoside, 10mM citrate, and 0.003% thimerosal (PH 5) buffer with 10 mM of bOxy271-287 peptide, an appropriate dilution of HLA-DP4 molecules (~0.1μg/mL), and serial mid-dilutions of competitor peptides. After 24h incubation at 37°C, samples were neutralized and applied to B7/21-coated plates for 2h. Bound biotinylated peptide was detected by means of streptavidin-alkaline phosphatase conjugate (Amersham, Little Chalfont, UK) and 4-methylumbelliferyl phosphate substrate (Sigma-Aldrich). Emitted fluorescence was measured at 450nm upon excitation at 365 nm in a Victor II spectrofluorometer (PerkinElmer Instruments, Les Ulis, France). Data were expressed as the peptide concentration that prevented binding of 50% of the labeled peptide (IC<sub>50</sub>). IC<sub>50</sub> values of the Oxy<sub>271-287</sub> peptides served as reference in each experiment.

#### **Human T cell proliferation assays**

Human peripheral blood mononuclear cells (PBMCs) were incubated with the HBsAg peptides and the positive control peptide at 20 μg/mL in T cell medium containing 7.5% AB serum for 5 days and pulsing with one microcurie of [<sup>3</sup>H]thymidine for 16 hours before harvesting. Wells were harvested using the TOMTEC collector (Perkin Elmer Applied Biosystems), and the incorporated radioactivity was measured using a micro-β counter (Perkin Elmer Applied

Biosystems). The results are given as the stimulation index (SI) = (cpm with specific peptide)/ (cpm with control peptide).

## ***Results***

### **Cell surface expression of MHC molecules in HLA-A2/DP4 mice.**

HLA-A2/DP4 mice were obtained by crossing the parental HLA-A2<sup>+/+</sup>β2m<sup>-/-</sup> (A2) mice and HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup> (DP4) mice. The genotype and cell surface expression of HLA-A2.1 and HLA-DP4 were confirmed on splenocytes from the HLA-A2/DP4 mice by PCR (data not shown) and flow cytometry. As illustrated in Figure 1A, HLA-A2.1 expression was observed in HLA-A2/DP4 mice, whereas no expression was detected in wild-type C57BL/B6 mice. As shown in Figure 1B and 1C, HLA-DP4 expression was observed only in the HLA-A2/DP4 mice (Figure 1C), however, no HLA-DP4 expression was detected as expected (Figure 1B). In addition, DP4<sup>+</sup>CD19<sup>-</sup> T lymphocytes were further analyzed by staining PEcy7-labeled anti-CD11b and FITC-labeled anti-CD11c in Figure S1.

### **Peripheral CD8<sup>+</sup> T and CD4<sup>+</sup> T lymphocytes in HLA-A2/DP4 mice.**

Splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers were determined by immunostaining and flow cytometry analysis. T lymphocytes were labeled with APC-conjugated anti-CD3<sup>+</sup> antibody and then with PE-conjugated anti-mCD8<sup>+</sup>, FITC-conjugated anti-mCD4<sup>+</sup> and PECy7-conjugated anti-hCD4<sup>+</sup> antibodies, as illustrated in Figures 2-3.

The expression of HLA-A2.1 and HLA-DP4 molecules allowed for the selection of peripheral CD8<sup>+</sup> and CD4<sup>+</sup> T cells in HLA-A2/DP4 mice. As shown in Figure 2, around 2.3% CD8<sup>+</sup> T cells (Figure 2A and 2C), compared with 0.6–1 % CD8<sup>+</sup> T cells in β2-microglobulin (β2m)-deficient MHC class I-deficient mice<sup>[29]</sup>; there were 89–90% CD4<sup>+</sup> T cells (Figure 3D and 3E) compared with 2–3% CD4<sup>+</sup> T cells in the H-2 class II-KO mice<sup>[33]</sup>. In comparison, wild-type C57BL/B6 mice possess around 17.2% CD8<sup>+</sup> T cells (Figure 2B and 2C) and 77% CD4<sup>+</sup> T cells (Figure 3C) in CD3<sup>+</sup> T cells.

It has been reported that the differentiation of immature CD4<sup>+</sup>CD8<sup>+</sup> double-positive



T cells into CD4<sup>+</sup> T cells in the thymus is highly dependent on the expression of human CD4 (hCD4)<sup>[34]</sup>. As shown in Figures 3D and 3E, 89–90% of CD4<sup>+</sup> T cell expressed hCD4, and 0–0.3% of the remaining cells exhibited nonspecific staining with FITC-labeled anti-murine CD4 (mCD4) in HLA-A2/DP4 mice (Figure 3A and 3B). In contrast, 77.3% of the CD4<sup>+</sup> T cells in wild-type C57BL/B6 mice C57BL/B6 mice express mCD4 (Figure 3C) rather than hCD4 (0.03%) (Figure 3F). In addition, flow cytometric analysis was also used to observe the percentage of Treg cells in CD4<sup>+</sup> T cells in Figure S2.

### **Humoral responses and HLA-A2-restricted responses in HLA-A2/DP4 mice**

To evaluate the immunological potential of HLA-A2/DP4 mice and their ability to predict human responses, we compared the humoral response and the immunodominant HLA-A2-restricted response in the HLA-A2/DP4 mice with the responses that have been previously reported for naturally infected patients or immunized humans. We immunized the HLA-A2/DP4 mice with an HBV DNA vaccine that encodes two HBV envelope proteins (preS2 and S proteins). These two proteins can self-assemble into particles carrying HBsAg, and they are the two protein components of currently used vaccine against hepatitis B.

To show that the CD8<sup>+</sup> T cells in the periphery of HLA-A2/DP4 mice are functionally restricted by transgenic human class I molecules (as reported for the transgenic HLA-A2 mice<sup>[29]</sup>), we examined the HBsAg-specific CD8<sup>+</sup> CTL responses by monitoring the immunodominant HLA-A2.1-restricted epitope responses directed against HBsAg<sub>348–357</sub><sup>[35]</sup> and HBsAg<sub>335–343</sub><sup>[36]</sup> epitopes. The results showed that after immunization with the pCMV-S2.S HBV DNA vaccine, the transgenic mice exhibited HBsAg-specific humoral responses (Figure 4A) and HLA-A2-restricted CD8<sup>+</sup> T cell responses (Figure 4B). The immunodominant HLA-A2.1-restricted response is directed against the HBsAg<sub>348–357</sub> and HBsAg<sub>335–343</sub> epitopes, whereas in wild-type C57BL/B6 mice, the H-2 K<sup>b</sup>-restricted HBsAg-specific CTL response is directed against the HBsAg<sub>371–378</sub> epitope<sup>[37]</sup>. To determine whether HLA-A2/DP4 mice mount the same HLA-A2-restricted CTL response as HLA-A2/DR1 mice and humans,

splenic T cells were stimulated with the relevant HLA-A2.1-restricted peptides, HBsAg<sub>348-357</sub>, HBsAg<sub>335-343</sub> and a control (HBsAg<sub>371-378</sub>, H-2 K<sup>b</sup>-restricted), and the secretion of IFN- $\gamma$  was measured as a read-out. As expected, HBsAg DNA immunization elicited a significant CTL response against HBsAg<sub>348-357</sub> and HBsAg<sub>335-343</sub>, and there was no response against HBsAg<sub>371-378</sub> (Figure 4B). The results demonstrate that CD8<sup>+</sup> T cell responses in HLA-A2/DP4 mice are HLA-A2-restricted and are similar to the CD8<sup>+</sup> CTL response after immunization of HLA-A2/DR1 transgenic mice with the same vaccine <sup>[12]</sup>.

### **HLA-DP4-restricted responses in HLA-A2/DP4 mice**

To evaluate the behavior of CD4<sup>+</sup> T cells in HLA-A2/DP4 mice, we immunized the mice with a hepatitis B virus DNA vaccine, pCMV-S2.S, by intramuscular injection. H2-class II-deficient mice were used as a control <sup>[33]</sup>. As shown in Figure 5A, HBs protein- and PreS2 antigen-specific antibodies were induced in HLA-A2/DP4 transgenic mice (solid column) at 10 days after the third immunization. Conversely, no antigen-specific antibodies were detected in H2-class II-deficient mice. This result demonstrates that potent humoral responses require the help of CD4<sup>+</sup> T cells.

In the *in vitro* proliferation assays (Figure 5B), HBsAg DP4-restricted CD4<sup>+</sup> T cells responded to whole HBs antigen and to the previously described HLA-DP4-restricted peptide, S<sub>181</sub>-S<sub>192</sub> <sup>[20]</sup>. No responses were observed in the H2-class II-deficient mice (hollow column) or to the control HLA-DP4-restricted peptide, Mage-3<sub>243-258</sub> <sup>[32]</sup>. The results demonstrate that HBsAg could induce the HLA-DP4-restricted proliferation of CD4<sup>+</sup> T cells in HLA-A2/DP4 transgenic mice.

### **Identification of novel HLA-DP4 epitopes that respond to the HBsAg DNA vaccine**

Having documented that the HLA-A2/DP4 mice could respond to HBsAg, a previously described DP4-restricted epitope, we next sought to identify novel HLA-DP4-restricted epitopes in the HBs envelope protein. The immunogenicity of both T helper and CTL epitopes are related to their MHC-binding capacities, with

good T helper inducers generally having a high affinity for MHC class II molecules. We therefore scanned the hepatitis B envelope protein based on the HLA-DP4 peptide-binding motif<sup>[38]</sup> and selected 11 candidate peptides. The affinities for each of the candidate peptides were evaluated (Table 1). We also immunized HLA-A2/DP4 transgenic mice intramuscularly with the pCMV-S2.S DNA vaccine. The splenocytes derived from the primed mice were separated and then stimulated *in vitro* with 12 HLA-DP4-restricted peptides, including the HLA-DP4-restricted epitope HBs<sub>181-192</sub>. The peptides S109-121, S256-268, S326-338, S347-358 and S352-364 induced proliferative responses in HLA-A2/DP4 mice, whereas no responses were observed in the control H-2 class II-deficient mice. Thus, the HLA-A2.1<sup>+/+</sup>HLA-DP4<sup>+/+</sup>hCD4<sup>+/+</sup>mCD4<sup>-/-</sup>IAβ<sup>-/-</sup>β2m<sup>-/-</sup> mice allowed us to identify 5 novel HLA-DP4-restricted epitopes from the hepatitis B envelope protein.

### **HLA-DP4-restricted CD4<sup>+</sup> T cell responses in vaccinated humans**

To determine whether the epitopes that we identified in the HLA-A2/DP4 mice were relevant for human immune responses, PBMCs from 6 HLA-DP4<sup>+</sup> donors, including 4 donors who had been immunized against HBV and 2 donors who were never immunized, were utilized to analyze their HLA-DP4-restricted CD4<sup>+</sup> T cell responses. The results of the proliferation assays (Table 2) showed that three out of four immunized subjects responded to the reported Celis (S181-S192) peptide. The responses of two donors were directed against the S326-338 and S256-268 peptides. One response was observed against the S352-364 and S347-358 peptides. No significant responses against the S109-121 peptide (which was derived from the S2 part of the antigen) were observed in the four donors who had received the HBV vaccine. All together, four out of five newly identified DP4-restricted HBs epitopes were functional to detect specific T cell responses in vaccinated humans.

### ***Discussion:***

In the present study, we developed an HLA transgenic mouse model that lacked an

H-2 antigen system. As we mentioned above, this newly created HLA-A2/DP4 transgenic mouse model combines the most frequent HLA class I allele, A\*0201, with the most frequent class II allele, HLA-DP4. At least a quarter of the Caucasian population (which is ~50% positive for HLA-A\*0201 and ~76% for HLA-DP4<sup>[13]</sup>), for example, carries the HLA-A2/DP4 genotype. Thus, this mouse model could predict human cellular responses for a larger proportion of the human population than our previously established HLA-A2/DR1 mouse model.

According to human allele frequency statistics, DP4 is the most abundant allele in the world, and it shares a motif with other subtypes DPB1\*0101, DPB1\*0501 and DPB1\*0201<sup>[39]</sup> as DR and DQ superfamilies. Furthermore, the HLA-DP4 allele has become increasingly valuable in preclinical research. Accumulating evidence has proven that DP4 is important in immunity, and it is a key allele in preventing viral infection, autoimmunity and transplant rejection<sup>[40]</sup>. A recent study indicated that DP4 is a protective allele in preventing chronic infection with HBV. Other studies have shown that the presence of DP4 is correlated with diabetes and multiple sclerosis<sup>[18,19]</sup>. These studies were based on statistical analyses, and further explanation of this mechanism is required. However, polymorphisms among different species and individuals as well as a lack of human samples have hampered HLA studies leading to DP-related research, and there are surprisingly few mouse models for DP.

In the HLA-A2/DP4 mouse model, the development of a repertoire of CTLs and T helper cells and the mobilization of effector cells in the periphery should be restricted to HLA molecules rather than murine H-2 molecules. We confirmed HLA-A2 and DP4 molecules were expressed on the cell surface in HLA-A2/DP4 mouse model. With the regulation of HLA molecules, there is a normal population of T lymphocytes in the periphery. Even though the percentage of CD8 T cells is relatively low in HLA-A2/DP4 mice, this observation is in accordance with our previously reported HLA-A2/DR1 mice in 2004. Even in this case, expression of transgenic HLA-A2.1 molecule led to an increase in the size of the peripheral CD8<sup>+</sup> T cell population, which reached 2-3% of the total splenocytes in HLA-A2/DR1 mice, compared to 0.6-1% in  $\beta$ 2m-KO MHC-I deficient mice<sup>[12]</sup>. Furthermore, this phenomenon had

been discussed and several studies on HBV and HIV proved that the low percentage for CD8+ T cell doesn't significantly affect the usage of HLA-A2/DR1 mice in immunological analysis.<sup>[41, 42]</sup>

Then, we observed the humoral and HLA-restricted immune responses in this mouse model by studying HBV DNA immunization. After DNA immunization, we could detect a significant humoral response against target proteins. Meanwhile, we confirmed that both the CTL response and the Th response were HLA-restricted responses rather than H-2 restricted responses. Our results demonstrated that these mice exhibit HLA-DP4 responses similar to those observed in humans, and the mice should be useful, not only for the identification of new HLA-DP4-restricted epitopes and assessing the efficiency and safety of novel vaccines but also for analysis of the cooperation between HLA-A2 and HLA-DP4 and their contribution to immunity.

Compared with HLA-A2, DP4 restricted epitopes of HBV are rarely reported. Therefore, mapping immunodominant DP4 epitopes will be of great value for vaccine applications. Given that one epitope identified in HLA-A2/DP4 transgenic mice did not induce a significant response in vaccinated humans, it is possible that competition among different HLA-class II molecules in human body results in the selection of immunodominant epitopes that would be concentrated on the human MHC molecules in the humanized mice. In a natural situation, several HLA genes are codominantly expressed in the ER, and they can compete for overlapping epitopes<sup>[43-45]</sup>. Thus, the set of peptides associated with an HLA molecule can be influenced by the presence of other HLA molecules. In addition, we speculate that other factors may have influenced the results, such as Treg cells; it is reported that Tregs epitopes can suppress the proliferation of other T cells after peptide stimulation *in vitro*<sup>[46]</sup> but this speculation need further verification.

In conclusion, we reported the creation of a new HLA-A2/DP4 mouse model that can be reliably used to identify immunodominant epitopes in humans and to rank their ability to prime CTLs and T helper cells. This mouse model thus represents a promising surrogate model for studying the immune responses before human preclinical trials.

## ***Acknowledgments***

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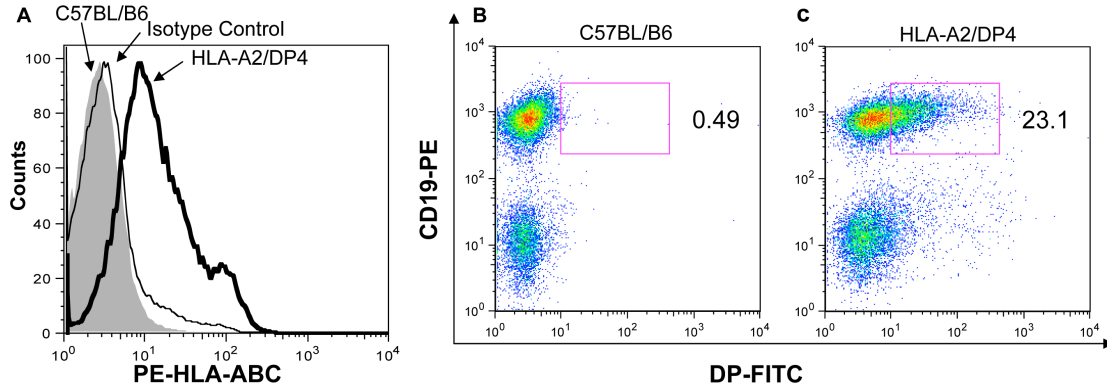
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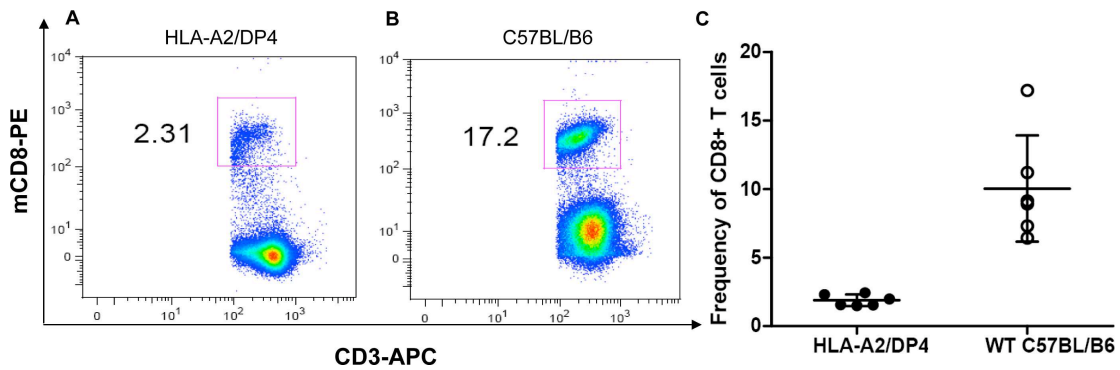
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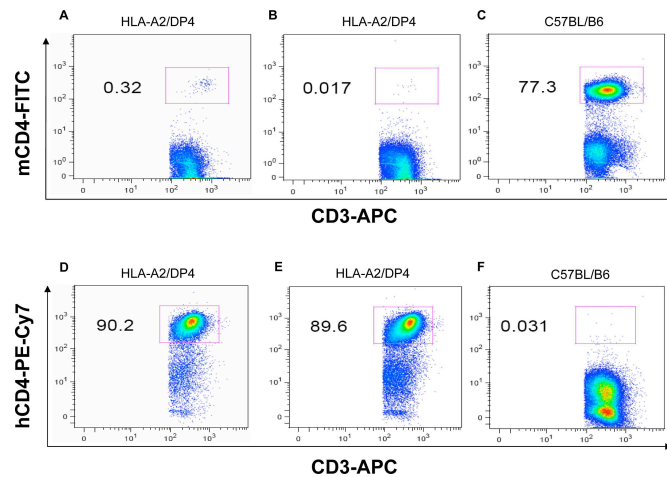
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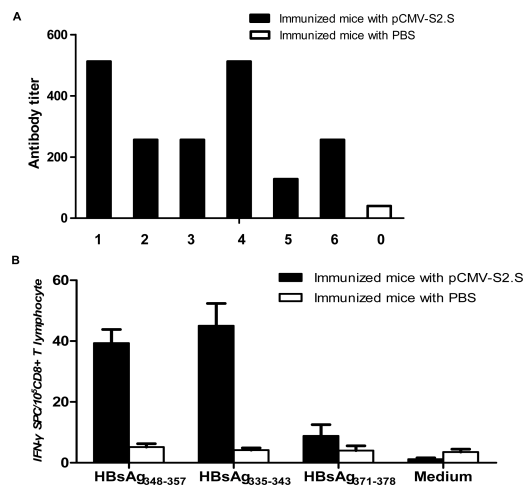
**Figure 1. Flow cytometric analysis of HLA-A2 and HLA-DP4 expression of transgenic molecules.** (A) Splenocytes from HLA-A2/DP4 (bold histogram) and wild-type C57BL/B6 (shadowed histogram) mice were isolated and stained with PE-labeled anti-HLA-ABC mAb to observe the HLA-A2 expression. PE-labeled mouse IgG2a, k mAb was used as isotype control (*thinned histogram*) for staining HLA-A2/DP4 mouse. (B) Splenocytes from wild-type C57BL/B6 mice (Figure 1B) and HLA-A2/DP4 (Figure 1C) were isolated and stained with PE-labeled anti-CD19 and FITC-labeled anti-HLA-DP mAb to observe the HLA-DP4 expression.



**Figure 2. Flow cytometric analysis of peripheral CD8<sup>+</sup> T lymphocytes.** Splenocytes from HLA-A2/DP4 (Figure 2A) and wild-type C57BL/B6 mice (Figure 2B) were isolated and CD3<sup>+</sup> T cells were gated by staining APC-conjugated anti-CD3 mAb and CD8<sup>+</sup> T cells were gated by staining PE-conjugated anti-mCD8 mAb. Frequencies of CD8<sup>+</sup> T cells among total CD3<sup>+</sup> T cells from 6 mice of each group were shown in Figure 2C.

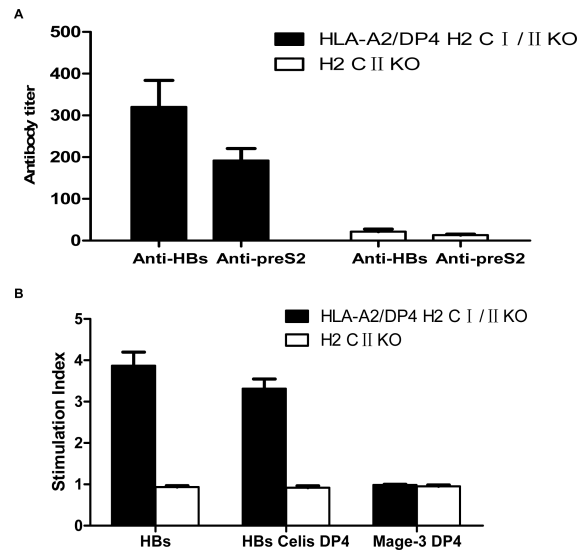


**Figure 3. Flow cytometric analysis of peripheral mCD4<sup>+</sup> and hCD4<sup>+</sup> T lymphocytes.** Splenocytes from HLA-A2/DP4 (Figure 3A, 3B, 3D and 3E) and wild-type C57BL/6 (Figure 3C and 3F) mice were isolated and CD3<sup>+</sup> T cells were gated by staining with APC-conjugated anti-CD3 mAb. Meanwhile, FITC-conjugated anti-mCD4 and PE-Cy7-conjugated anti-hCD4 antibodies were simultaneously used to observe the mCD4 and hCD4 expression in HLA-A2/DP4 and WT C57BL/6 mice.

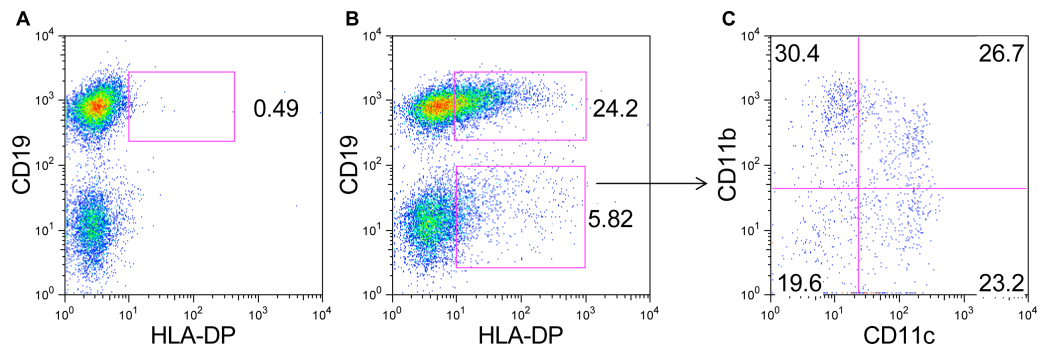


**Figure 4. HBs-specific antibody and cytotoxic responses.** (A) Sera were collected 10 days after the third immunization, and the titers of the antibody (IgG) against HBs particles in the immunized mice (solid bar) were determined and compared with the mean responses of six PBS-immunized mice (hollow bar) in an ELISA assay. (B) HBs epitope-specific IFN- $\gamma$  production by CTLs was determined by measuring response to the HLA-A2-restricted epitopes HBsAg348–357 and HBsAg335–343 and

to the H-2 K<sup>b</sup>-restricted epitope HBsAg371–378 in immunized mice (solid bar) and PBS-immunized mice (hollow bar).

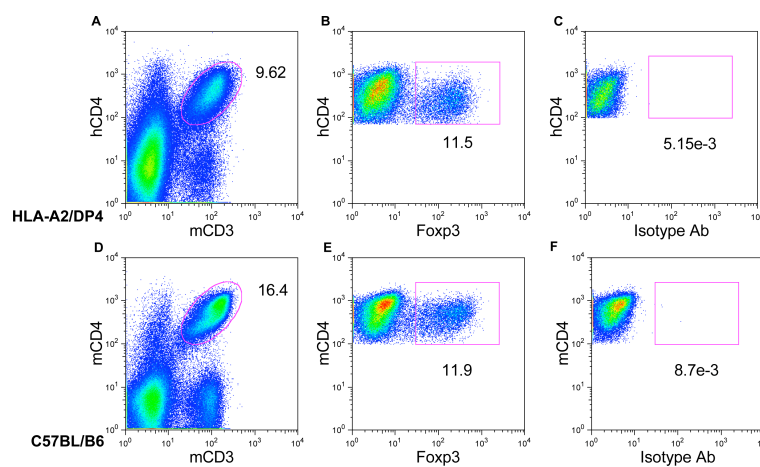


**Figure 5. HBs-specific antibody and proliferative response.** (A) The titers of antibody (IgG) from HLA-A2/DP4 transgenic mice (solid bar) and H-2 class II-deficient mice (hollow bar) against HBs particles and the preS2<sub>109-134</sub> peptide were determined using an ELISA assay (Figure 5A). (B) The HLA-DP4-restricted proliferation of CD4<sup>+</sup> T cells from HLA-A2/DP4 transgenic mice (solid bar) and H-2 class II-deficient mice (hollow bar) after stimulation with HBs particles, the previously described HBV DP4 restricted peptide, S181-S192, or the control HLA-DP4-restricted epitope, Mag-3<sub>243-258</sub> (Figure 5B).



**Figure S1. Flow cytometric analysis of HLA-DP4 expression of transgenic molecules.**

Splenocytes from wild-type C57BL/B6 mice (Figure S1A) and HLA-A2/DP4 (Figure S1B) were isolated and stained with APC-labeled anti-CD19 and PE-labeled anti-HLA-DP mAb to observe the HLA-DP4 expression. In addition, DP4<sup>+</sup> CD19<sup>-</sup> T lymphocytes were further analyzed by staining PEcy7-labeled anti-CD11b and FITC-labeled anti-CD11c(Figure S1C).



**Figure S2. Flow cytometric analysis of the percentage of Treg cells.**

Splenocytes from HLA-A2/DP4 and wild-type C57BL/B6 mice were isolated and CD3<sup>+</sup> T cells were gated by staining with FITC-labeled anti-CD3 mAb. Meanwhile, PEcy7-labeled anti-hCD4 mAb and APC-labeled anti-Foxp3 mAb were simultaneously used to observe the Treg frequency in hCD4<sup>+</sup> T cells of HLA-A2/DP4 mice(Figure S2A, S2B, and S2C), while PEcy7-conjugated anti-mCD4 mAb and APC-labeled anti-Foxp3 mAb were simultaneously used to observe the Treg frequency in mCD4<sup>+</sup> T cells of WT C57BL/B6 mice(Figure S2D, S2E, and S2F).

**Table 1. DP4 epitopes binding ability and proliferative response in HLA-A2/DP4 mice.**

Positions	Anchors			Exp	Obs	SI	
	1	6	9	IC50(nM)	IC50(nM)	HLA-A2/DP4	H2 CII KO
S109-121	MQWNSTT <b>F</b> HQ <b>T</b> LQ			40	144	2	1
S165-177	ENITSG <b>F</b> LG <b>P</b> LLV			180	>10000	1	1
S181-192	GF <b>F</b> LLTRIL <b>T</b> IPQ			70	2	2-3	1
S197-209	SW <b>W</b> TSLN <b>F</b> LG <b>G</b> TT			300	737	1	1
S256-268	FL <b>L</b> VLLD <b>Y</b> Q <b>G</b> MLP			120	>10000	2-3	1
S318-331	SS <b>W</b> AFG <b>K</b> FL <b>W</b> EWA			110	46	1	1
S319-331	W <b>A</b> FG <b>K</b> FL <b>W</b> E <b>A</b> SA			160	221	1	1
S326-338	W <b>E</b> W <b>A</b> SAR <b>F</b> SWLSL			10	77	2	1
S347-358	V <b>G</b> LSPT <b>V</b> WLS <b>V</b> I			120	30	2-3	1
S352-364	TV <b>W</b> LS <b>V</b> I <b>W</b> MM <b>W</b> YW			28	>1000	2-3	1
S362-374	W <b>Y</b> W <b>G</b> PS <b>L</b> Y <b>S</b> IL <b>S</b> P			40	952	1	1
S376-388	L <b>P</b> LL <b>P</b> IF <b>F</b> CL <b>W</b> VY			60	626	1	1

Twelve HLA-DP4-restricted epitopes were predicted by scanning the entire HBs protein and were synthesized. Exp IC50 (nM) represents the predicted affinities according to algorithms, while Obs (nM) represents the affinities obtained from MHC class II binding assays. [<sup>3</sup>H]thymidine incorporation assays were used to measure the proliferation of CD4<sup>+</sup> T cells from DNA-vaccine-immunized HLA-A2/DP4 and H-2 class II-deficient mice stimulated with the twelve synthesized peptides, including the previously reported epitope, S181-192. Exp=Expected, Obs=Observed, SI, stimulation index.

**Table 2. Proliferative response in DP4-positive donors.**

<b>Positions</b>	<b>DP4+ HBV vaccinated (SI)</b>				<b>DP4+ unvaccinated (SI)</b>	
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
S109-121	1.1	0.9	0.8	1.0	0.8	0.9
S181-192*	7.2	3.6	0.8	3.1	0.8	1.1
S256-268	2.4	1.4	0.4	3.1	0.7	1
S326-338	1.4	2.3	0.5	3.5	1	1
S347-358	1.2	2.2	0.6	1.1	1	0.9
S352-364	1.2	3.1	0.7	1.6	1	0.7

\* S181-S192 peptide was used as positive control.

The proliferation of CD4<sup>+</sup> T cells in 4 HBV vaccinated and 2 unvaccinated HLA-DP4<sup>+</sup> donors after *in vitro* stimulation with 5 newly identified HLA-DP4-restricted epitopes and one positive control peptide S181-S192. SI, stimulation index.

## *Article 2*



# Identification of two novel HLA-A\*0201 restricted epitopes from H5N1 Matrix protein 1 by immunization of HLA-A2/DP4 transgenic mice

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## Abstract

General consensus on universal H5N1 influenza virus vaccine is that conserved CTL epitopes in the internal proteins of H5N1 would contribute to improve future universal vaccine with more immunogenicity and broader-spectrum. In present study, we confirmed that, in recent 13 years, 88% M1 amino acid sequences from H5N1 human infected strains are conserved and mapped M1 protein to look for more potential CTLs epitopes. In this study, we isolated a strain (A/ Shenzhen strain/2003) from one patient that infected by H5N1 and cloned one of its segments M1. Following, Bioinformatics online prediction tools BIMAS, SYFPEITHI and MAPPP were used to predict CTLs epitopes in M1 protein and then candidate epitopes were synthesized and further identified by immunizing HLA-A2/DR1/β2m<sup>-/-</sup>/IAβ<sup>-/-</sup> transgenic mice with DNA vaccine pJW4303-M1. The results of ELISA, RMA-S/HHD cell binding assay, ELISPOT and vivo cytotoxicity indicated that two novel 9-mer HLA-A2-restricted CTL epitopes M1<sub>3-11</sub>(LLTEVETYV) and M1<sub>134-142</sub>(RMGTVTTEV) were identified. These two novel epitopes would possibly contribute to future vaccine design and production.

*Key words:* H5N1, M1, CTL, HHD transgenic mice, DNA vaccine

## Introduction

H5N1 influenza virus, as a highly contagious virus, constituted a potential great threat to both human and poultry. Even though many reports indicated that H5N1 presented a highly poultry-specific infectivity and seldom infect humans, there are an increasing number of patients infected by H5N1. According to the statistics provided by World Health Organization (WHO) since 2003, H5N1 has totally caused 499 human infected cases, including 295 deaths in the world<sup>[1]</sup>. Moreover, the influenza H1N1 pandemic in 2009, which arose worldwide panic and ever be speculated to generate the third human pandemic disease like 1918 Spanish pandemic, alerted people to draw more attention on the potential risk of H5N1. Once H5N1 virus spread through species barrier to infect humans and carries its 59% mortality, there will become a disaster.

In present, vaccines against H5N1 have been manufactured to prevent H5N1 virus prevalence in human population and the two licensed H5N1 vaccines are the conventional inactivated virus vaccine (CIV) and the live-attenuated vaccine (LAV). Both of them have been proved to be effective—safe and stable against targeted strains, however, their efficiency against new emerging H5N1 virus is hard to be speculated due to the high mutation of H5N1 surface proteins Hemagglutinin (HA) and Neuraminidase (NA)<sup>[2]</sup>. Therefore, many vaccine candidates were designed to remedy the disadvantages of current licensed vaccines, such as split vaccine, subunit vaccine<sup>[3,4]</sup>, VLPs vaccine<sup>[5,6]</sup> and DNA vaccine<sup>[7]</sup>, however, most of these vaccines still tried to stimulate the neutralizing antibodies against external glycoproteins HA and NA with related reports largely published.

In fact, in addition to HA and NA humoral responses, cellular immune response especially CTLs response also contribute to provide protection against H5N1 infection and their response against conserved proteins or conserved peptides could provide heterologous protective against influenza viruses of different subtypes. This conclusion was supported by many animal models experiments<sup>[10, 11]</sup>. Moreover, two recent researches reported that most human subjects have CD4<sup>+</sup> and CD8<sup>+</sup> T cells directed to human influenza virus, including some conserved CTL epitopes on protein NP and M1<sup>[12, 13]</sup>. Consequently, Vaccine embedding effective conserved CTLs epitopes might be a promising “cocktail” strategy for development of universal vaccines against H5N1. However, documented CTLs epitopes are still not sufficient

for further usage and screening conserved proteins to search for more CTLs epitopes is necessary.

CTLs response is restricted by HLA allele and HLA-A\*0201 is the most abundant allele; it takes up 50% in the world population. At the same time, M1 protein as the main internal viral proteins which has many structural and biological function, such as inhibits RNA synthesis by the RNP-associated transcriptase of influenza virus and participates poses in assembly and budding of progeny virions [14-16]. Thus, identification of potential HLA-A\*0201 restricted M1 protein epitopes would be much utile in future applications. In present study, our team worked on mapping novel HLA-A\*0201 restricted CTL epitopes with the help of bioinformatics and immunological technology. At the beginning of this study, we made alignment of M1 amino acid sequences in human infected H5N1 cases during 1997-2009 to look for their conserved segment and utilized Silico prediction online databases to predict all possible epitopes. Finally, top 10 peptides were synthesized and further identified was implemented by immunizing HHD (HLA-A2/DR1/ $\beta$ 2m<sup>-/-</sup>/IA $\beta$ <sup>-/-</sup>) transgenic mice with DNA vaccine M1-pJW4303. Finally, two novel 9-mer HLA-A\*0201 restricted CTL epitopes M1<sub>3-11</sub>(LLTEVETYV) and M1<sub>134-142</sub>(RMGTVTTEV) were identified and they would possibly contribute to future vaccine design and production.

## **Materials and methods**

### *1. Virus and peptides*

The whole RNA (A/Shenzhen strain/2006) was extracted from patient and then rever-transcribed into cDNA. Specified primers synthesized by ShanghaiShengong company. Peptides (purity>85%) were predicted by Online-database: BIMAS [2] SYFPEITHI and MAPPP and then manufactured, high-performance-liquid chromatography purified and analyzed by mass spectrometry(Genescript, New Jersey, USA).Peptides were dissolved in dimethyl sulfoxide (5.0mg/ml), diluted in deionized H<sub>2</sub>O to 1mg/ml, and stored in -20°C.

### *2 Constructions of DNA vaccines encoding M1 protein*

The gene encoding the long length M1 protein was amplified by RT-PCR by using primer 5' (GGA TCC ATG AGT CTT CTA ACC GAG GTG CAA), which contains an BamHI site and primer 3' (GAA TTC CTT GAA TCG CTG CAT CTG), which contains an EcoRI site and subcloned into pJW4303 eukaryotic expression vector (It

was kindly provided by a collaborated lab in USA). After enzyme-digestion verification, the endotoxin-free plasmids were largely prepared by using the Tiangen EndoFree Plasmid Maxi kit (Qiagen, Beijing, China.). Meanwhile, the M1 gene was cloned into PQE30 prokaryotic vector to express recombinant M1 protein by using same primer 5' and primer 3' (GAA TTC CTT GAA TCG CTG CAT CTG), which replace an EcoRI site into HindIII.

### *3 Procedure of DNA immunization for HLA-A2/DR1 mice*

HLA-A2<sup>+/+</sup> / DR1<sup>+/+</sup>/β2m<sup>-/-</sup>/IAβ<sup>-/-</sup> transgenic mice were developed by our lab in 2004<sup>[17]</sup> and maintained in INSERM (Paris, France) U1014 under barrier conditions and fed commercial mouse chow and water ad libitum. All operations followed the corresponding rule in INSERM. For evaluation of mice humoral responses and cellular response after DNA immunization, at the age of 8 weeks, 14 transgenic mice were pre- immunized by cardiotoxin. After 5 days, mice were separated into 2 groups and immunized four times intramuscularly at 10-days intervals with pJW4303-M1 and pJW4303 vector, respectively. Each time with 100μg DNA vaccine injection per mouse. 10 days after the last immunization, mice were used for further analyses.

### *4 ELISA*

ELISA was used to measure the production of specific antibody against M1 in the sera. Sera samples were obtained every 10 days by centrifugation (4°C ,3000rpm/min for 15 minutes) for assessment of the production of M1 specific antibody .The sera were stored at -20°C for later use in serologic tests. M1 recombinant protein was diluted in coating buffer (0.1M Na2CO3, PH9.6) and dispensed 50μL of diluted solution (10μg/ml) to each well of a 96-well plate (Nunc, Roskilde, Denmark.), 4□ overnight. The Plate were washed three times with phosphate buffered saline (PBS), blocked with 1%BSA–PBS for 1 h at 37□, and washed twice. Added 50μL serum serial dilutions from 1:25 to 1:51200 in wells for 1h at 37□。 The plate was washed three times with 0.05% Tween 20–phosphate buffered saline (PBS), and after 45 min incubation with 50μL goat anti-mouse IgG conjugated to HRP (Serotec, Cergy-Saint-Christophe, France) diluted solution (1:1000) in every well, washed three times with 0.05% Tween 20– PBS. After washing, 50μL ready-to-use ELISA substrate (Roche, Mannheim, Germany) was dispensed in every well .15minutes later, the reaction βwas stopped by addition of 50 ml of 3 M HCl and measured at 405nm by using a Bioserv(BIOSERV, Rostock, Germany).

### *5 ELISPOT assay*

ELISPOT assay was implemented to detect IFN- $\gamma$  secreted by CD8+ T lymphocyte as described previously [18]. Membrane-backed 96-well ELISPOT plates (Millipore, Bedford, MA) were coated with anti-IFN- $\gamma$  mAb (Diacclone, Besancone, France) overnight at 4°C and then blocked with 1% skimmed milk. Lymphocyte (2 x 10<sup>5</sup>/well) were added to every well and cultured with 10  $\mu$ g/ml synthetic peptides and incubated for 20 h at 37°C, 5%CO<sub>2</sub>. The IFN- $\gamma$ -secreting cells were captured by coating anti-IFN- $\gamma$  mAb and detected by incubation with biotinylated anti-mouse IFN- $\gamma$ Ab (Diacclone) for 1h30 at 37°C, followed by incubation with Streptavidin-HRP for 1h). Finally, the plates are developed using substrate BEC (Diacclone, ready to use), washed, and dried. Spots are counted using the ELISPOT reader (CTL, Germany)

### *6 Cytotoxicity in Vivo*

CFSE/PI staining cytotoxicity in vivo was operated to verify the affinity of peptides. Lymphocytes were collected from 3 non-immunized HLA-A2/DR1 mice and separated by Ficolls (*GE Lifesciences, Uppsala, Sweden*) and finally divided into three parts, each part was in turn pulsed with 10 $\mu$ g/ml single peptide M1 58-66, predicted peptides and NP10 at 37°C for 1h, M1 58-66 and NP10 as positive and negative peptides respectively. Followed CFSE was used to stain each part as the sequence above with graded concentrations 0.4 $\mu$ M, 2 $\mu$ M and 10 $\mu$ M for 10min, at room temperature. After washing 2 times with PBS, the three parts of lymphocytes were mixed and injected into mice through orbital plexiform venous. Within 24h, mice were sacrificed to observe the stained target cells by FACS analysis.

## **Results**

### **The high conserved characteristic of M1 protein.**

We analyzed the M1 amino acid sequences derive from all reported human infected H5N1 influenza virus strains which were prevalent during 1997-2009. 65 amino acid sequences could represent that from the total 241 strains. M1 sequences from 27 strains were the most popular and 16 of 27 could appear in 80.9% strains. Especially, A/Vietnam/UT3028/2003(H5N1) shares identical M1 amino acid sequence with 47 strains; A/Indonesia/CDC287E/2005(H5N1) shares identical M1 amino acid sequence with 41 strains; A/HK/212/03 (H5N1) strain shares identical M1 amino acid sequence with 35 strains; A/Anhui/1/2005(H5N1) shares identical M1 amino acid sequence with 19 strains; A/Indonesia/5/2005(H5N1) shares identical M1 amino acid sequence with 16 strains. Moreover, most of mutation confine to some certain positions and with the same substitutions, such as 28K 107I, 144F as well as 157S, their counterparts are 28R, 107M, 144L and 157A, respectively.

### **The bioinformatics prediction for M1 derived epitopes**

Based on Silico examination of the M1 protein amino acid sequence by using BIMAS, SYFPEITHI and MAPPP prediction methods, the HLA-A\*0201 restricted epitopes listed in Table 1 were selected to be synthesized. All of the epitopes are linear and consistent with many influenza strains. Each epitope was indicated its sequence and the amino acids location within it. The bioinformatics tools of SYFPEITHI and MAPPP have the same results.

Most of epitopes in the Table 2 are the top 20 scores of candidates in three prediction methods, except M159-67(LGFVFTLTV) and M1138-146(VTTEVAFGL). However, we selected the two epitopes got high scores in BIMAS method in order to increase the impartiality of prediction.

### **Humoral responses responses in HLA-A2/DR1 mice**

In order to identify the novel epitopes in the M1 proteins, we constructed a DNA vaccine with the full length of M1 antigen, pJW4303-M1, for immunization. 10 days after the third immunization. M1 specific antibody could be detected by ELISA. It indicated that the new constructed DNA vaccine could significantly stimulate the humoral response in the HLA-A2/DR1 mice. While, there was no response could be observed in pJW4303 (Vector) group.

### **HLA-A2-restricted responses in HLA-A2/DP4 mice**

In order to observe the immunogenicity of each synthesized peptides, we implemented an ELISPOT essay to determine. After 10 days of third immunization, the splenocytes of HLA-A2/DP4 mice were isolated by Ficolls and diluted into 1 million/ml suspending cells. Every peptide stimulated the CD4 deleted lymphocytes respectively. Finally, only M1<sub>58-66</sub>, M1<sub>2-10</sub> and M1<sub>134-142</sub> could stimulate the lymphocytes to secrete IFN- $\gamma$  in pJW4303-M1 group, whereas other peptides and the negative control group had no IFN- $\gamma$  secretion. It revealed that peptides M1<sub>2-10</sub> and M1<sub>134-142</sub> are the potential HLA-A\*0201 restricted CTL epitopes.

### **Discussion**

For a long time, HA and NA glycoprotein were used to develop vaccines against influenza virus due to their antigenicity can provide significant protection against influenza virus even though their efficiency was greatly influenced by virus mutation. However, humoral response against HA and NA is only one part of human immunity

and it is supported by the fact that influenza virus exists for a long time while actually large pandemics in humans occur only in some cases regardless the rapid mutation of HA and NA. Other immunity mechanisms must possess equivalent importance in host immunity.

At the moment, it was widely accepted that future vaccines for influenza virus should be universal and with broader-spectrum for different subtypes. In other words, it should combine high neutralizing epitopes, conserved CTLs epitopes and pan-T helper epitopes. Among the three “ingredient”, the screening of conserved CTLs epitopes could be reached easier than others.

Primarily, conserved segments can be easily targeted by alignment of different strains, plus, many strains share the identical sequences as the Table 1 showed; 65 amino acid sequences of M1 protein cover that from total 241 human infected H5N1 strains and notably, 16 sequences appear in 80.9% strains, this characteristic greatly reduce the complexity of vaccine design. Meanwhile, problems on CTL epitopes HLA-specificities were abbreviated by a report that 12 HLA-I supertypes (A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, B62) could present in all ethnic groups<sup>[5,19,8]</sup> and among these alleles, some ones like HLA-A2, it present about 50% populations. It's helpful to expansion the Last but not the least, most of CTL epitopes are linear structures rather than conformation structures, it means that some key amino acid mutation in the epitopes will not only change the structure but also the immunogenicity. Normally, position 2 and 9 are anchor points for the MHC-I epitope, however, in our study, the epitope M1 58-66 GILGFVFTL could be also be recognized by CTLs directed to M1 58-66 GMLGFVFTL. On the contrary, if there is certain mutated amino acid happens in the neutralizing epitope, it might change its structure and escape from human immunity. This inference may account for humoral immunity often lose the surveillance for HA and NA mutation.

Another point we mentioned is the application of humanized MHC transgenic mice on adaptive immunity. In our previously study, several human CTL and Th epitopes from HIV and HBV viruses had been identified by HLA-A2/DR1/ $\beta 2m^{-/-}$ /IA $\beta^{-/-}$  mice and results consistent with the ones from patients. Doubtlessly, this mice is also valuable in H5N1 study. Two reasons are the valuable tools to Health human PBMC were often used to evaluate the CTL response against peptides, but in most of case, we don't know whether the donors have been exposed to virus.

The result of challenge experiment in humanized mice should more authentic, since after infection, human HLA instead animal MHC to initiate the adaptive immunity and directly regulate cellular response and partial humoral response.

There is interesting results is that, when HLA-A2/DP4 was used to map epitopes in M1 protein from influenza virus H5N1 by using ELISPOT ,a epitope M1134-142 could be identified in HLA-A2/DR1 (results don't show here) ,while in HLA-A2/DP4, there was hardly no response. The possible reason is that in M1 protein, there exist HLA-DR restricted Th epitopes and they could boost CTLs response whereas no definite HLA-DP4 specific Th epitopes was reported could enhance CTLs responses, if it is the right explanation is that mean Caucasian people are more vulnerable to be infected by H5N1.

To sum up, we have identified two novel H5N1 -derived CTL epitopes, both of them are conserved in the human infected H5N1 cases, in addition, in term of human CTL response against H5N1 is largely across with other epidemic strains, they could be applied in rational design of CTL epitope-based vaccines to fight against H5N1 as well as new pandemic influenza virus strains.

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**Table 1. 27 strains of human infected H5N1 influenza viruses during 1997-2009.**

Year	District	H5N1 Strains	Number share Identical aa sequence*
1997	China/Hong Kong	<b>A/Hong Kong/156/97(H5N1)</b>	4
1997	China/Hong Kong	<b>A/Hong Kong/482/97(H5N1)</b>	3
1997	China/Hong Kong	<b>A/Hong Kong/483/1997(H5N1)</b>	3
1998	China/Hong Kong	<b>A/HongKong/97/98(H5N1)</b>	2
2001	China/Hong Kong	<b>A/HongKong/378.1/2001(H5N1)</b>	1
2003	China/Hong Kong	<b>A/HK/212/03 (H5N1)</b>	35
2003	Vietnam	<b>A/Vietnam/UT3028/2003(H5N1)</b>	47
2004	Vietnam	<b>A/Vietnam/1196/2004(H5N1)</b>	1
2004	Thailand	<b>A/Thailand/1(KAN-1)/2004(H5N1)</b>	4
2005	Indonesia	<b>A/Indonesia/5/2005(H5N1)</b>	16
2005	China	<b>A/Anhui/1/2005(H5N1)</b>	19
2005	Indonesia	<b>A/Indonesia/CDC287E/2005(H5N1)</b>	41
2006	Turkey	<b>A/Turkey/651242/2006(H5N1)</b>	8
2006	Azerbaijan	A/Azerbaijan/001-161/2006(H5N1)	0
2006	Iraq	A/Iraq/1/2006(H5N1)	0
2006	China	A/Anhui/T2/2006(H5N1)	0
2006	Indonesia	<b>A/Indonesia/CDC669/2006(H5N1)</b>	5
2007	Laos	A/Laos/Nong Khai 1/2007(H5N1)	0
2007	Indonesia	<b>A/Indonesia/CDC1032/2007(H5N1)</b>	3
2007	China	A/Fujian/1/2007(H5N1)	0
2007	Viet Nam	A/Vietnam/UT31244III/2007(H5N1)	0
2007	Laos	A/Laos/Nong Khai 1/2007(H5N1)	0
2008	China	A/Hunan/1/2008(H5N1)	0
2008	Viet Nam	A/Vietnam/UT31394II/2008(H5N1)	0
2008	Bangladesh	A/Bangladesh/207095/2008(H5N1)	0
2008	China	<b>A/Guangdong/1/2008(H5N1)</b>	3
2009	China	A/Hunan/2/2009(H5N1)	0

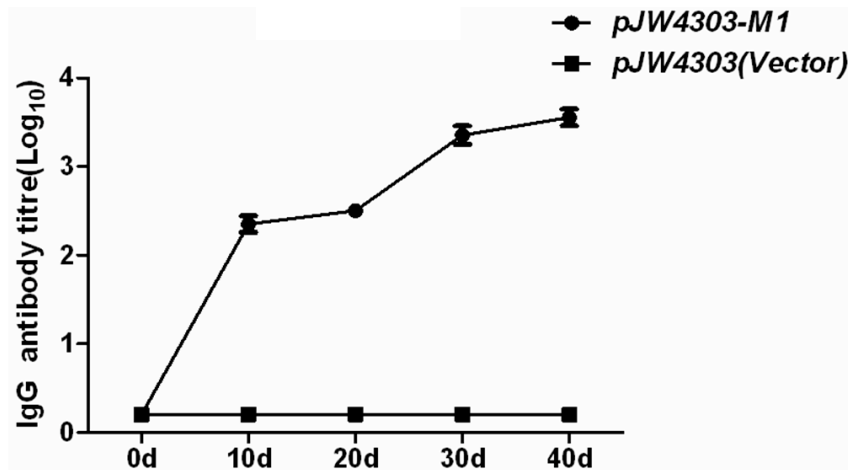
27 strains of human infected H5N1 influenza viruses that are most prevalent during 1997-2009, including all years and countries. 65 amino acid sequences of M1 protein represent total 241 human infected H5N1 strains, 16 sequences appear in 80.9% strains. Most of mutation confine to some certain positions and with the same substitutions, such as 28K 107I→144F as well as 157S ,their counterparts are 28R→107M→144L and 157A respectively.

**Table 2. HLA-A\*0201 restricted epitope prediction by Bioinformatics for M1 protein.**

Position	Epitope	Rank		
		BIMAS	SYFPEITHI	MAPP
M1 <sub>2-10</sub>	LLTEVETYV	1	4	4
M1 <sub>58-66</sub>	GILGFVFTL	2	1	1
M1 <sub>134-142</sub>	RMGTVTTEV	3	10	10
<b>MI<sub>59-67</sub></b>	<b>LG FVFTLV</b>	-----	<b>6</b>	<b>6</b>
M1 <sub>51-59</sub>	ILSPLTKGI	5	2	2
M1 <sub>130-138</sub>	LIYNRMGTV	6	5	5
M1 <sub>164-172</sub>	QMATITNPL	7	6	6
<b>MI<sub>138-146</sub></b>	<b>VTTEVAFGL</b>	<b>8</b>	-----	-----
M1 <sub>60-68</sub>	LG FVFTLV	9	19	19
M1 <sub>180-188</sub>	VLASTTAKA	10	7	7
M1 <sub>123-131</sub>	ALASCMGLI	17	9	9
M <sub>116-124</sub>	ALSYSTGAL	20	3	3

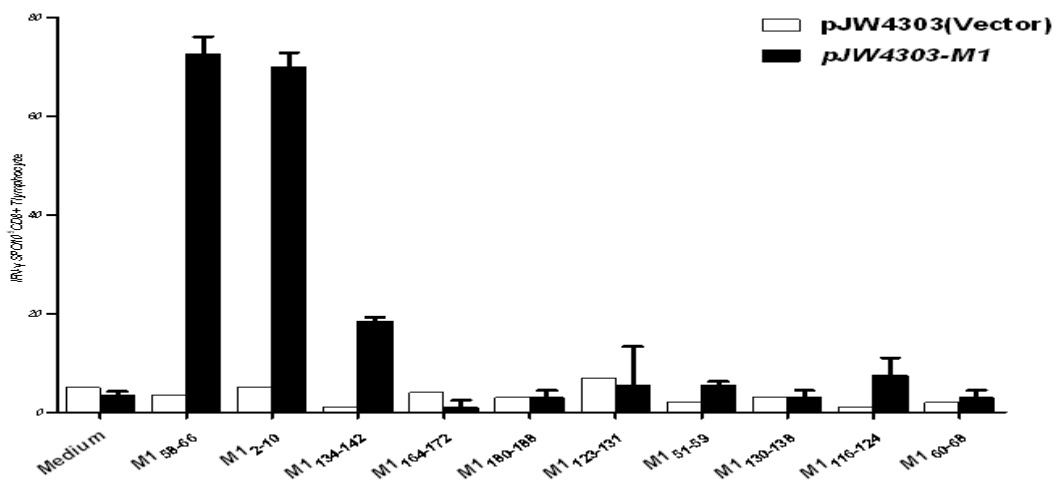
\*Shaded epitopes represent the peptides only be selected in one method, however it was in the top 10 for each method.

Full length of M1 amino acid sequence were analyzed by three kinds of bioinformatics tools to predict the potential HLA-A\*0201 peptides. 10 peptides were selected to synthesize, which got ranks in the 20 highest scores.



**Figure 1.** M1-specific antibody induced by pJW4303-M1 and pJW4303 DNA vaccines.

**Figure 1.** M1 specific antibodies were detected in every 10 days after the immunization. The pJW4303-M1 DNA vaccine (circle points) and pJW4303 (vector) plasmid (Square points) were used to stimulate the murine immunity.



**Figure 2.** M1-specific cytotoxic response induced by synthesized peptides.

**Figure 2.** 10 days after third immunization, 10 synthesized peptides were used to stimulate the IFN- $\gamma$  secretion in an ELISPOT assay. Peptides M1<sub>58-66</sub>, M1<sub>2-10</sub> and M1<sub>134-142</sub> could stimulate the specific IFN- $\gamma$  secretion, no response after other peptides stimulation (Solid). Medium and pJW4303 immunized mice (hollow) as the negative control.

# *Article 3*

RESEARCH

Open Access

# An H5N1 M2e-based multiple antigenic peptide vaccine confers heterosubtypic protection from lethal infection with pandemic 2009 H1N1 virus

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## Abstract

**Background:** A 2009 global influenza pandemic caused by a novel swine-origin H1N1 influenza A virus has posed an increasing threat of a potential pandemic by the highly pathogenic avian influenza (HPAI) H5N1 virus, driving us to develop an influenza vaccine which confers cross-protection against both H5N1 and H1N1 viruses. Previously, we have shown that a tetra-branched multiple antigenic peptide (MAP) vaccine based on the extracellular domain of M2 protein (M2e) from H5N1 virus (H5N1-M2e-MAP) induced strong immune responses and cross-protection against different clades of HPAI H5N1 viruses. In this report, we investigated whether such M2e-MAP presenting the H5N1-M2e consensus sequence can afford heterosubtypic protection from lethal challenge with the pandemic 2009 H1N1 virus.

**Results:** Our results demonstrated that H5N1-M2e-MAP plus Freund's or aluminum adjuvant induced strong cross-reactive IgG antibody responses against M2e of the pandemic H1N1 virus which contains one amino acid variation with M2e of H5N1 at position 13. These cross-reactive antibodies may maintain for 6 months and bounced back quickly to the previous high level after the 2nd boost administered 2 weeks before virus challenge. H5N1-M2e-MAP could afford heterosubtypic protection against lethal challenge with pandemic H1N1 virus, showing significant decrease of viral replications and obvious alleviation of histopathological damages in the challenged mouse lungs. 100% and 80% of the H5N1-M2e-MAP-vaccinated mice with Freund's and aluminum adjuvant, respectively, survived the lethal challenge with pandemic H1N1 virus.

**Conclusions:** Our results suggest that H5N1-M2e-MAP has a great potential to prevent the threat from re-emergence of pandemic H1N1 influenza and possible novel influenza pandemic due to the reassortment of HPAI H5N1 virus with the 2009 swine-origin H1N1 influenza virus.

## Background

At the same time concern was raised about a possible pandemic resulting from the highly pathogenic avian influenza (HPAI) H5N1 virus, the world confronted the first influenza pandemic of the 21st century caused by a novel influenza A H1N1 virus [1]. This pandemic H1N1 virus was first identified in April 2009 and demonstrated a rapid rate of spread. As of 9 May, 2010, WHO has reported at least 18,036 fatal cases in more than 214

countries [2]. Although H5N1 virus has not yet evolved to become transmissible among humans, it has still presented a high mortality rate of approximately 60% [3]. Consequently, WHO considers the H5N1 virus to be a potential human pandemic [4]. Antigenic and genetic analysis has suggested that the current pandemic 2009 H1N1 virus is a product of reassortment between genes in the human, avian and swine influenza strains [5]. There is a concern that one more human pandemic influenza virus could be derived in the future from animal reservoirs, such as avian, possessing both rapid interpersonal transmissibility and high lethality. The potential shortage of pandemic influenza vaccines and

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the absence of specific-immunity in the human population make the development of a cross-protective influenza vaccine, which is based on conserved antigens, a promising prophylactic strategy.

The extracellular domain of influenza M2 protein (M2e) is highly conserved across influenza A subtypes and has become an attractive antigen target for producing a cross-protective influenza vaccine with broad-spectrum prevention [6]. To date, many groups have reported M2e-based vaccine candidates in different forms such as virus-like particles [7-9], recombinant proteins [10-12], DNA [13], and synthetic peptides [14,15]. However, most of these M2e vaccines require either chemical or genetic strategies to form M2e-carrier fusion constructs in order to overcome the poor immunogenicity of M2e alone. Therefore, multiple antigenic peptides (MAPs) would provide an ideal platform for the application of a vaccine based on a short peptide antigen, such as M2e. This type of construct would result in a large macromolecule with a high molar ratio of target peptide antigen to a small immunologically inert core molecule without requiring further conjugation to a carrier protein [16,17]. In our previous study, we designed and synthesized a MAP with radially branching lysine dendrites onto which four copies of conserved M2e of H5N1 virus were attached. Such H5N1-M2e-MAP vaccine elicited high titers of H5N1-M2e-specific serum antibodies and conferred efficacious protection against different clades of H5N1 virus [18]. Here, we further proved that the H5N1-M2e-MAP candidate vaccine can provide heterosubtypic protection against lethal infection of pandemic 2009 H1N1 virus.

## Materials and methods

### Mice

Six- to eight-week-old female BALB/c mice were purchased from the Beijing Animal Center (Beijing, China). All mice were maintained in a specific pathogen-free facility and housed in cages containing sterilized feed, autoclaved bedding and water. The animal study was approved by the Institutional Animal Care and Use Committee (IACUC).

### Virus

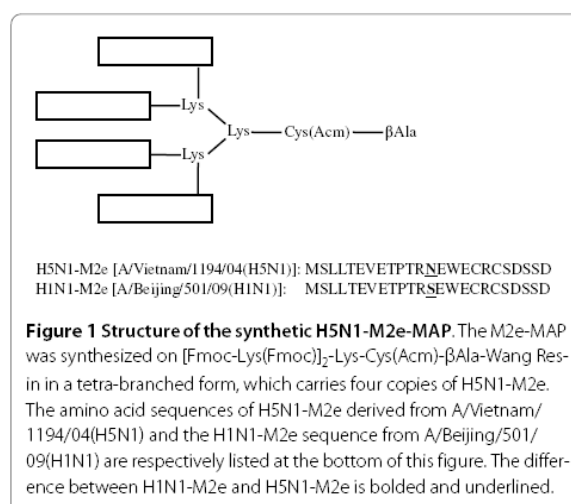
The pandemic 2009 H1N1 virus used in this study is A/Beijing/501/09 isolated from a confirmed H1N1 case in China. Virus was grown in the allantoic cavities of 10-day-old embryonated chicken eggs. Virus-containing allantoic fluid was harvested and stored in aliquots at -80°C until use. The 50% lethal dose (LD<sub>50</sub>) was determined in mice after serial dilutions of the virus stock. All infectious experiments were performed in an approved biosafety level 3 (BSL-3) facility.

### H5N1-M2e-MAP

As described previously [18], tetra-branched multiple antigenic peptide carrying four copies of M2e peptide of H5N1 virus (H5N1-M2e-MAP) was synthesized on [Fmoc-Lys(Fmoc)]<sub>2</sub>-Lys-Cys(Acm)-βAla-Wang Resin (Advanced ChemTech, Louisville, Kentucky, USA) on a 0.02 mM scale using an Applied Biosystems model 433A peptide synthesizer. Cleavage of the peptide from the resin was performed by treatment with trifluoroacetic acid (TFA), DTT, water, and triisopropylsilane (TIPS) in the ratio 88:5:5:2 (TFA/DTT/H<sub>2</sub>O/TIPS). Crude peptide was purified by reversed phase high-performance liquid chromatography (RP-HPLC). The structure of H5N1-M2e-MAP is shown in Fig. 1.

### Immunization and virus challenge

Mice were vaccinated subcutaneously (s.c.) with 10 μg of H5N1-M2e-MAP in the presence of Freund's complete adjuvant (Sigma, MO) or intramuscularly (i.m.) plus aluminum adjuvant (Sigma). The 1<sup>st</sup> boost was given with the same amount of H5N1-M2e-MAP in Freund's incomplete adjuvant (Sigma) or aluminum adjuvant three weeks later. The mice received the 2<sup>nd</sup> boost six months later. Mice injected with Freund's or aluminum adjuvant alone were used as the respective control. Sera were collected at 1 week and 3 and 6 months after 1<sup>st</sup> boost and 1 week after 2<sup>nd</sup> boost to detect cross-reactive antibodies against H1N1-M2e. Sera collected before immunization were used as negative controls. Two weeks after 2<sup>nd</sup> boost, mice were intraperitoneally (i.p.) anesthetized with ketamine (75 mg/kg) and intranasally (i.n.) challenged with a lethal dose (10LD<sub>50</sub>) of A/Beijing/501/09. Infected mice were observed and weighed daily for 2 weeks. Lung tissues were collected from euthanized mice 3 days post-challenge for further virological testing and histopathological analysis.





## ELISA

The cross-reactivity of H5N1-M2e-MAP-induced antibody against H1N1-M2e was detected by ELISA. Briefly, 96-well microtiter plates were pre-coated with 10 µg/ml of H1N1-M2e peptide (SBS Genetech, Beijing, China) overnight at 4°C. After blocking with 5% BSA containing 0.05% Tween-20 in PBS, serial diluted mouse sera were added to the plates, followed by addition of HRP-conjugated rabbit anti-mouse IgG (1:2,000, Invitrogen, Carlsbad, CA) at 37°C for 0.5 h. Assay was developed using 3,3',5,5'-tetramethylbenzidine (TMB) (Zymed, Carlsbad, CA), and the reaction was stopped by adding 1N H<sub>2</sub>SO<sub>4</sub>. The absorbance at 450 nm was measured by an ELISA plate reader (Sunrise™ microplate reader, TECAN, NC).

## Viral titers in lung tissues

Viral titers in lungs were determined by 50% Tissue culture infective dose (TCID<sub>50</sub>) as described [18], except that TPCK-trypsin (Sigma) was added into the medium (2 µg/ml), and 0.5% chicken erythrocytes were used in hemagglutination confirming cytopathic effect (CPE) endpoint.

## Histopathological analysis

The lung tissues of challenged mice were immediately fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were made at 4 - 6 µm thickness and mounted on slides. Histopathological changes were examined by H & E staining and observed under light microscopy.

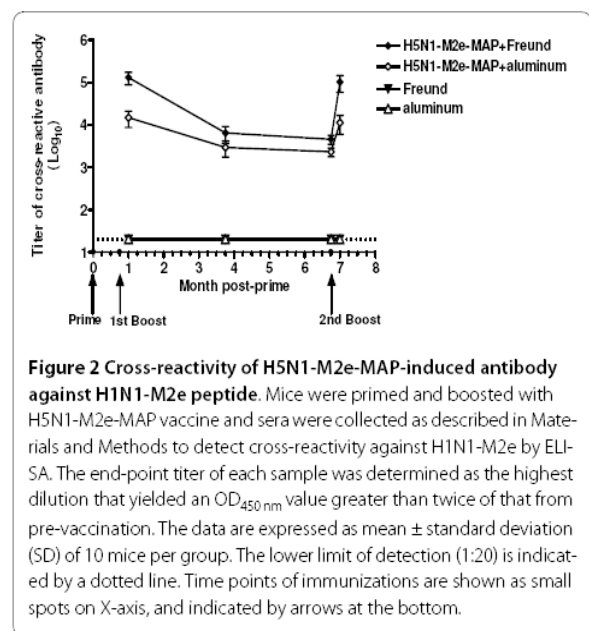
## Statistical analysis

The significance between survival curves was analyzed by Kaplan-Meier survival analysis with log-rank test. Other data were analyzed using the 2-tailed Student's t test.  $P < 0.001$  was considered significant. All analyses were performed in Graphpad Prism software.

## Results

### H5N1-M2e-MAP vaccination induced high titers of cross-reactive antibody against H1N1-M2e peptide

The M2e of human influenza virus is significantly different from that of avian influenza virus [19]. However, the pandemic 2009 H1N1 virus and H5N1 virus share a similar M2e sequence, except for one amino acid, as indicated in Fig. 1 with representative virus stains. The serological examination illustrated the cross-reactivity of H5N1-M2e-MAP-induced antibodies that recognized H1N1-M2e. As shown in Fig. 2, one week after the 1<sup>st</sup> boost, H5N1-M2e-MAP in Freund's adjuvant or aluminum adjuvant elicited strong cross-reactive IgG antibody against H1N1-M2e with the titer reaching 1:10<sup>5</sup> or 1:10<sup>4</sup>, respectively. Despite a slight decrease in the following six months, the titers of cross-reactive antibody bounced back quickly to the previous high level after the 2<sup>nd</sup> boost.



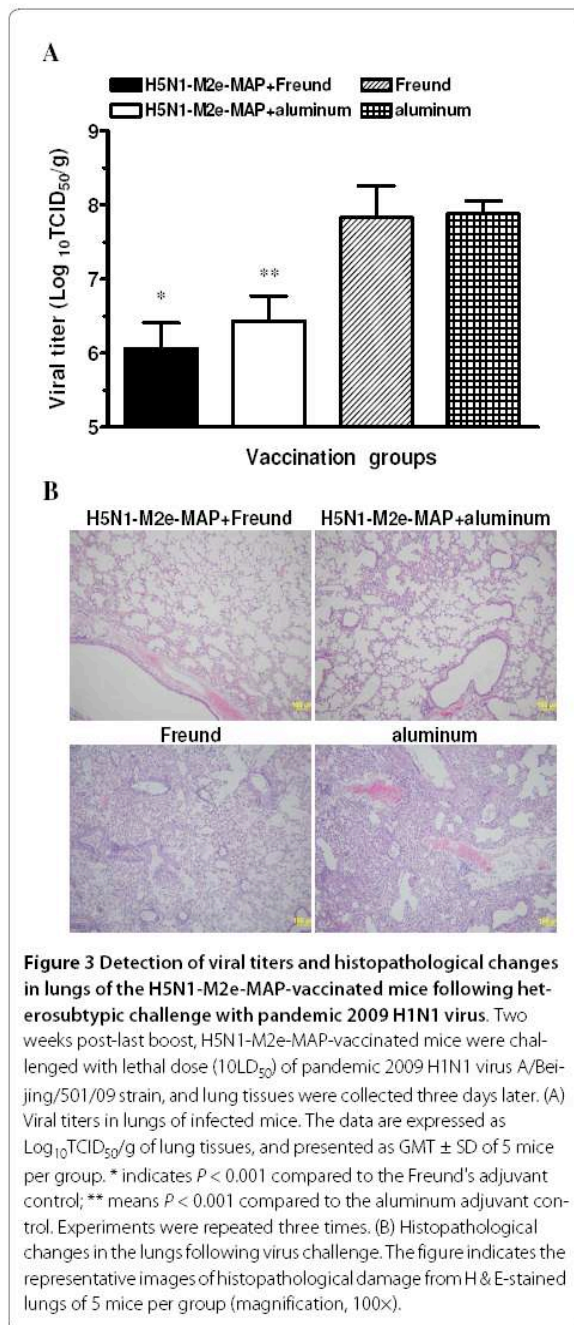
**Figure 2 Cross-reactivity of H5N1-M2e-MAP-induced antibody against H1N1-M2e peptide.** Mice were primed and boosted with H5N1-M2e-MAP vaccine and sera were collected as described in Materials and Methods to detect cross-reactivity against H1N1-M2e by ELISA. The end-point titer of each sample was determined as the highest dilution that yielded an OD<sub>450 nm</sub> value greater than twice of that from pre-vaccination. The data are expressed as mean ± standard deviation (SD) of 10 mice per group. The lower limit of detection (1:20) is indicated by a dotted line. Time points of immunizations are shown as small spots on X-axis, and indicated by arrows at the bottom.

In contrast, only background level of anti-H1N1-M2e antibody response was detected in Freund's or aluminum adjuvant controls. These results suggest that H5N1-M2e-MAP can induce long-term and potent antibody responses that may be effective against both homologous HPAI H5N1 virus and heterologous pandemic 2009 H1N1 virus.

### H5N1-M2e-MAP vaccination limited replication of pandemic 2009 H1N1 and attenuated virus-induced lung pathology

In our established mouse model, the virus replicated in the lung tissue to the peak, reaching the highest titer three days post-lethal infection of pandemic 2009 H1N1 virus (data not shown). To determine whether the H5N1-M2e-MAP-induced immune responses confer heterosubtypic protection against infection with pandemic 2009 H1N1 virus, the vaccinated mice were sacrificed three days after lethal virus challenge (10LD<sub>50</sub>) of A/Beijing/501/09 and lung tissues were collected for detection of viral titers and histopathological examination. Compared with the matched adjuvant control group, the average viral titer in lungs of H5N1-M2e-MAP-vaccinated mice was significantly lower ( $P < 0.001$ ) after lethal challenge with the pandemic 2009 H1N1 virus (Fig. 3A), suggesting that the H5N1-M2e-MAP can induce potent protective immunity against viral replication following pandemic 2009 H1N1 virus infection.

Further examination of the lung tissues of virus-challenged mice revealed that dramatic lung damages mainly occurred in the control mice, which were characterized by diffused alveolar lesion with pneumocytes denatured, alveolar macrophages and cellular debris mixed with

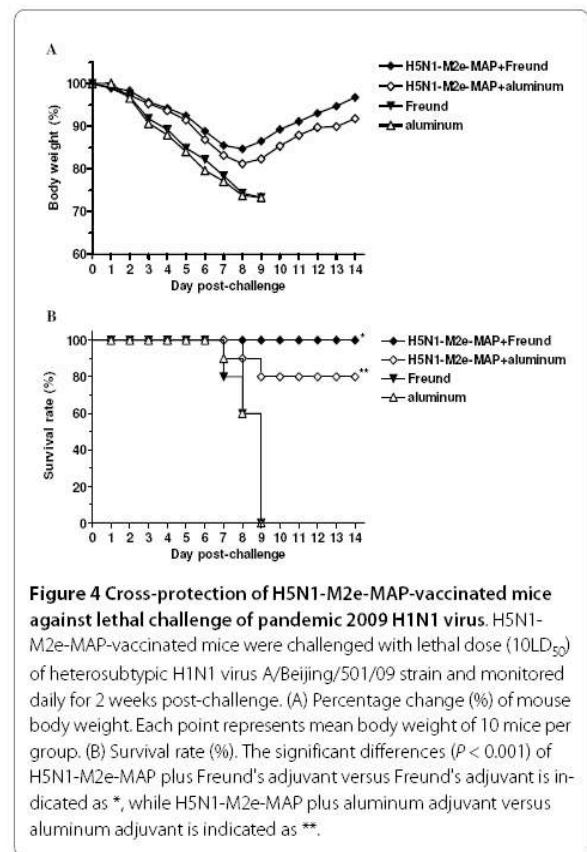


fibrin in alveolar lumina, and widened lung septa with the infiltration of lymphocytes and a few neutrophils. The bronchiolar epithelium was degenerated and collapsed with multifocal peribronchiolar infiltration of lymphocytes and a few neutrophils. The blood vessel endothelium was damaged with moderate edema and infiltration of lymphocytes and neutrophils in the periendothelium. However, lungs of H5N1-M2e-MAP-vaccinated mice exhibited fewer histopathological changes, with only mild pulmonary interstitial pneumonia and moderate lympho-

cytic infiltration (Fig. 3B). The above data implied that H5N1-M2e-MAP vaccination protects mice against lethal infection with the pandemic 2009 H1N1 virus through a combination of limiting viral replication in the lungs and attenuating virus-induced lung pathology.

#### H5N1-M2e-MAP vaccination provided effective heterosubtypic protection from lethal challenge with pandemic 2009 H1N1 virus

To further confirm cross-protection conferred by H5N1-M2e-MAP against heterologous infection with the pandemic 2009 H1N1 virus, mice received lethal challenge (10LD<sub>50</sub>) of A/Beijing/501/09 strain were monitored for weight loss and death for the subsequent two weeks post-challenge. As shown in Fig. 4A, mice vaccinated with H5N1-M2e-MAP in Freund's or aluminum adjuvant rallied from body weight loss eight days after virus infection. In contrast, body weight of the mice in the adjuvant control group dramatically decreased, even more than 25%, in some cases. All mice received adjuvant alone died within 10 days after lethal virus challenge. In comparison, 100% and 80% of the mice survived in the groups vaccinated with H5N1-M2e-MAP in Freund's and aluminum adjuvants, respectively, with the survival rate significantly different from the matched adjuvant control (*P* < 0.001)



(Fig. 4B). These data illustrated that H5N1-M2e-MAP can afford heterosubtypic protection against lethal challenge of pandemic 2009 H1N1 virus.

## Discussion

To develop safe and effective prophylactic strategies to combat human infections by both pandemic 2009 H1N1 virus and H5N1 virus, the highly conserved M2e of influenza A virus has proven to be a promising target antigen to produce cross-protective influenza vaccines. In this study, H5N1-M2e-MAP, whose cross-protection against different clades of H5N1 virus has already been established in our previous report [18], was shown to confer heterosubtypic protection from lethal infection with the pandemic 2009 H1N1 virus. Remarkably, all mice vaccinated with H5N1-M2e-MAP plus Freund's adjuvant survived the lethal heterologous virus challenge. Although there is partial protection (80%) in the H5N1-M2e-MAP plus aluminum adjuvant group, H5N1-M2e-MAP vaccination in both Freund's and aluminum adjuvants demonstrated similar efficacy in limiting viral replication and attenuating virus-producing histopathological damage in lung tissues, thus showing the ability to control disease transmission.

Notably, it seems that higher titers of cross-reactive antibodies against H1N1-M2e result in better heterosubtypic protection from lethal virus infection (Figs. 2 and 4), implying that M2e-induced cross-reactive antibody is a crucial component in heterosubtypic protection in M2e-based vaccines. Other groups have reported that the antiviral effect of M2e-based vaccines was mediated by antibodies to M2e antigen and that its mechanism was antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-mediated cytotoxicity (CDC) [20,21]. Therefore, the cross-protection of the M2e vaccine was based on the premise that antibody responses with high levels of cross-reactivity were induced following vaccination. However, the difference of M2e amino acid sequences between human-type and avian-type viruses would affect their mutual recognition in varying degrees. Specifically, Fan et al. [14] reported that antisera against human-type M2e sequence failed to react with avian-type M2e peptide. Also, Liu et al. [22] indicated that mAb specific to a region (aa 6 - 13) of human-type M2e sequence can only weakly recognize, or not recognize at all, avian-type M2e sequence with variations in the same range. Nevertheless, in our studies, H5N1-M2e-MAP not only induced high titers of specific antibody against H5N1-M2e [18] but also elicited potent and prolonged cross-reactive antibody recognizing H1N1-M2e (Fig. 2). Although it is not certain whether the only amino acid difference between the H1N1-M2e and H5N1-M2e (as indicated in Fig. 1) is located outside the region containing B-cell epitopes, the induction of cross-reactive

antibody by H5N1-M2e-MAP against H1N1-M2e is obvious, and it is confirmed that H5N1-M2e-MAP can afford heterosubtypic protection against pandemic 2009 H1N1 virus (Figs. 3 and 4), regardless of the difference in M2e sequence between H1N1 and H5N1 by one amino acid.

Given that the immunization regimen consisting of H5N1-M2e-MAP and aluminum, the only adjuvant approved for use in humans, induced weaker immune responses (Fig. 2) and protective immunity (Fig. 4) than that is comprised of H5N1-M2e-MAP and Freund's adjuvant, it is urgently needed to develop more effective and safe adjuvant than aluminum for clinical use of human vaccines.

## Conclusions

The highly conserved M2e is an appropriate antigen target for the development of a cross-protective influenza vaccine. The present study revealed that the H5N1-M2e-MAP vaccine, whose cross-clade protection against divergent H5N1 viruses was confirmed in our previous report, can afford heterosubtypic protection against pandemic 2009 H1N1 virus, supporting the concept of an M2e-based vaccine with broad-spectrum protection against both the existing influenza virus and the emergent variant.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

GZ and YZ designed research. GZ, SS, WX, YG, and HY performed research. GZ, SS, ZR and ZK analyzed data. GZ, SS, LD, SJ, YL, BZ and YZ wrote and modified the paper. GZ and SS have equal contributions to this paper. All authors read and approved the final manuscript.

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