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INFLUENZA SPECIFIC T- AND B-CELL RESPONSES IN IMMUNOSUPPRESSED PATIENTS

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Cover page – Depiction of pandemic influenza H1N1 virus, a single virion (hemagglutinin in blue, neuraminidase in pink and M2 protein in purple) and the epitope VEPGDKITFEATGNL (251-265 aa) on the hemagglutinin (influenza H1N1 images courtesy of CDC, USA and concept/design by Thomas Poiret and Aditya Ambati)
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Influenza specific T- and B-cell responses in immunosuppressed patients
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedicated to my family

ABSTRACT

Influenza, known as the 'flu', is a recurrent acute viral infection that might cause severe inflammation, particularly in vulnerable individuals, i.e. young children, the elderly, and immune-suppressed patients, such as stem cell transplant recipients. Prevention strategies, primarily vaccination, and possibly the use of anti-viral drugs, are recommended with the aim to reduce mortality and morbidity. Influenza vaccination responses are often sub-optimal in immune-compromised patients. There is therefore a need to evaluate other vaccination systems and schedules to improve vaccine efficacy.

We mapped the humoral and cellular anti-flu directed immune responses and studied in a first set of experiments the immune responses in immune competent individuals prior to, and following a natural pandemic influenza infection, as well as after adjuvanted Pandemrix® influenza vaccination. This was performed prospectively during the H1N1 pandemic influenza of 2009. 'High content' influenza proteome peptide arrays were used to gauge serum IgG epitope signatures prior to and after Pandemrix® vaccination/ or H1N1 pandemic infection described in **paper I**. A novel epitope residing in the sialic acid receptor-binding domain of VEPGDKITFEATGNL (251-265) of the pandemic flu hemagglutinin was identified. This epitope was found to be exclusively recognized in serum from previously vaccinated individuals and never in serum from individuals with H1N1 infection. The natural H1N1 infection induced a different footprint of IgG epitope recognition patterns as compared to the Pandemrix® H1N1 vaccination.

Pre-transplant influenza vaccination of the donor or allogeneic hematopoietic stem cell (HSCT) candidate was evaluated in a randomized study of 122 HSCT patients reported in **paper II**. The antibody titers against H1 ($p=0.028$) and H3 ($p<0.001$) were highest in the pre-transplant recipient vaccination group until d.180 after transplantation. A significant difference was found concerning the specific Ig levels against pandemic H1N1 at 6 months after HSCT ($p=0.02$). The mean IgG levels against pandemic H1N1, generic H1N1 and H3N2 were highest in the pre-transplant recipient vaccination group. Pre-transplant influenza vaccination of the donor or the HSCT candidate was found to be beneficial in eliciting seroprotective titers.

The immunogenicity after a single dose of adjuvanted trivalent virosomal vaccination was evaluated in a cohort of 21 HSCT recipients and compared to a control cohort of 30 HSCT recipients who received a single dose of non-adjuvanted seasonal trivalent subunit vaccination, reported in **Paper III**. The delta change of IFNγ production in response to pandemic influenza H1N1 ($p=0.005$) and influenza B antigens ($p=0.01$) were significantly increased in blood from individuals who received the virosomal, as compared to the non-adjuvanted vaccine. Virosomal vaccination was found to be beneficial in eliciting robust cellular immune responses to influenza pandemic H1N1.

Pandemic influenza hemagglutinin MHC class 1 peptide restricted CD8 T-cells were enumerated over the course of a natural pandemic influenza infection and Pandemrix®

vaccination in a prospective study reported in **Paper IV**. PBMCs from vaccinated control individuals exhibited a significantly increased percentage of (p=0.003) hemagglutinin specific CD8 T-cells that resided in the terminally differentiated effector memory compartment, as compared to PBMCs from individuals that contracted H1N1 infection. The cellular immune signatures were found to be different elicited by a natural flu infection as compared to vaccination concerning the phenotype/maturation of antigen-specific CD8 T-cells.

LIST OF SCIENTIFIC PAPERS

I. **Ambati A**, Valentini D, Montomoli E, Lapini G, Buiso F, Magalhaes I, Maeurer M
H1N1 viral proteome peptide microarray predicts individuals at risk for H1N1 infection and segregates infection versus pandemrix® -vaccination. Immunology. 2015 Feb 1. doi: 10.1111/imm.12448. [Epub ahead of print].

II. **Ambati A**, Vilas Boas L S, P. Ljungman, L. Testa, Aoun M, J.F.de Oliveira, V. Colturato, M. Maeurer, C.M. Machado
Evaluation of pre-transplant influenza vaccination in hematopoietic stem cell transplantation: a randomized prospective study.
Bone Marrow Transplant. 2015 March 1. doi:10.1038/bmt.2015.47. [Epub ahead of print].

III. **Ambati A**, Einarsdottir S, Magalhaes I, Poiret T, Bodenstein R, LeBlanc K, Brune M, Maeurer M, Ljungman P
Immunogenicity of virosomal adjuvanted trivalent influenza vaccination in allogeneic stem cell transplant recipients.
Transplant Infectious Disease. 2015 March 28. doi:10.1111/tid.12382. [Epub ahead of print].

IV. **Ambati A**, Magalhaes I, Rane L, Axelsson Robertson R, Maeurer M
Influenza specific CD3+ CD8+ cytotoxic lymphocytes reside in precursor CD45RA+CCR7+ T-cell populations in individuals after pandemic influenza infection in contrast to Pandemrix® vaccination.
(2015 Manuscript).

SCIENTIFIC PAPERS NOT IN THE SCOPE OF THIS THESIS

I. Magalhaes I, Eriksson M, Linde C, Muhammad R, Rane L, **Ambati A**, Axelsson-Robertson R, Khalaj B, Alvarez-Corrales N, Lapini G, Montomoli E, Linde A, Pedersen NL, Maeurer M.
Difference in immune response in vaccinated and unvaccinated Swedish individuals after the 2009 Influenza pandemic.
BMC Infectious Disease. 2014 Jun 11;14(1):319.

II. Ahmed RK, Poiret T, **Ambati A**, Rane L, Remberger M, Omazic B, Vudattu NK, Winiarski J, Ernberg I, Axelsson-Robertson R, Magalhaes I, Castelli C, Ringden O, Maeurer M.
TCR+CD4-CD8- T cells in Antigen-specific MHC Class I-restricted T-cell Responses After Allogeneic Hematopoietic Stem Cell Transplantation.
Journal of Immunotherapy. 2014 Oct;37(8):416-25.

III. **Ambati A,** Poiret T, Svahn BM, Valentini D, Khademi M, Kockum I, Lima I, Arnheim-Dahlström L, Lamb F, Fink K, Qingda M, Olsson T, Maeurer M,
Increased β-hemolytic Group A Streptococcal M6 serotype and streptodornase B-specific cellular immune responses in Swedish narcolepsy cases.
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LIST OF ABBREVIATIONS

PRR	Pattern Recognition Receptor
PAMPs	Pathogen Associated Molecular Patterns
MBL	Mannan-Binding Lectin
dsRNA	Double Stranded Ribonucleic Acid
LPS	Lipopolysaccharides
LTA	Lipoteichoic Acid
PKR	Protein Kinase RNA activated
OAS/RNaseL	2′,5′-oligoadenylate synthetase/Ribonuclease L
NOD-like	Nucleotide oligomerization domain
NLRP3	NOD-like receptor family, pyrin domain containing 3
IL	Interleukin family
TLR	Toll-like receptors
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IFN	Interferon
PMN	Polymorphonuclear leukocytes
CNS	Central nervous system
TGF-β	Transforming growth factor-β
ROS	Reactive oxygen species
iNOS	Inducible nitric oxide species
MHC	Major histocompatability class
NK	Natural killer cells
MDSC	Myeloid-derived suppressor cell
TAM	Tumor associated macrophages
TNF	Tumor necrosis factor
NKG2	Natural Killer group 2
KIR	Killer cell immunoglobulin-like receptors
ITAMs	Immunoreceptor tyrosine-based activation motifs
ITIMs	Immunoreceptor tyrosine-based inhibition motifs
Ig	Immunoglobulin

ADCC	Antibody dependant cellular cytotoxicity
MAIT	Mucosal associated invariant T-cells
MR1	MHC related class I-like molecule
γδ	Gamma-Delta T-cells
NKT	Natural killer T-cells
ILC	Innate lymphoid cells
MAC	Membrane attack complex
TCR	T-cell receptor
APC	Antigen presenting cell
CLIP	Class II-associated invariant chain peptide
Th	T helper cell
STAT	Signal Transducer and Activator of Transcription
T-bet	T box transcription factor
GATA3	GATA binding transcription factor
RORγt	RAR-related orphan receptor gamma
Treg	T-regulatory cells
FOXP3	Forkhead box P3
TAP	Transport associated with antigen processing
HA	Influenza hemagglutinin protein
NA	Influenza neuraminidase protein
M	Influenza matrix 1 and 2 proteins
NS	Non-structural proteins
RT-PCR	Reverse transcriptase-polymerase chain reaction
MDCK	Madin-Darby canine kidney cells
PMK	Primary rhesus monkey kidney cells
DFA	Direct fluorscent staining
CTL	Cytotoxic CD8 T-cells
GVHD	Graft-versus-host disease
aHSCT	Allogeneic hematopoietic stem cell transplantation
HI	Hemagglutinin inhibition

1 INTRODUCTION

1.1 The immune response

The consolidated effort procured by molecules, organelles, cells and tissues, in preventing infection and eliminating transformed cells, represents the immune response, which is crucial for survival. Severely immundeficient individuals are highly susceptible to infections and thereby more likely succumb to a range of viral, bacterial and fungal diseases, if no therapeutic intervention is provided. The cells and tissues of the human immune system vary in complexity and function, starting from the very basic protection, elaborated by skin epithelial and mucosal surfaces, to highly complex and specialized adaptable immune cells, capable of immunological memory. Each of these components contributes with specialized roles involved in fighting off infections, prevention of tumors, recognizing and mediating allograft and foreign tissue rejection. The immune response can be divided into two arms; one arm that is quick to respond to pathogens is referred to as innate immunity. The second arm represents the adaptive immune response that usually develops slowly and evolves upon subsequent encounters with the nominal pathogen(s).

1.2 Innate immune response

The innate immune response is characterized by a defined set of reactions to invading microbes and pathogens. These can be broadly classified into inflammation and anti-viral defense mechanisms on the basis of germ-line coded receptors that are 'non-specific' concerning recognition of microbial pathogens [1]. Receptors sensing invading pathogens are referred to as pattern recognition receptors (PRR) and are widely distributed on different cell types [2]. A broad array of pathogen derived molecules are recognized by these receptors; these patterns are referred to as pathogen associated molecular patterns (PAMPS) [2]. PRRs can for example be secreted as mannan-binding lectin (MBL), C-reactive protein and serum amyloid proteins. They can also be found on the cell surface, such as the macrophage scavenger receptor with a broad specificity to many ligands including dsRNA, LPS, LTA [3] or may be located intracellularly, such as the protein kinase PKR, OAS/RNaseL systems and the family of NOD-like receptor proteins [4-6]. The NOD like receptors, especially the NLRP-3, are important components of the inflammasome that releases the mature form of IL-1β after its engagement with the nominal target, resulting in acute inflammation [7].

Apart from these PRRs, ten biologically relevant toll-like receptors (TLRs) have been identified, for instance the extracellular receptors, e.g. TLR4, which is a critical LPS sensing receptor [8]. TLR2 recognizes peptidoglycan and bacterial lipoproteins [9], TLR5 recognizes bacterial flagellin [10]. Intracellular receptors, e.g. TLR9 recognizes unmethylated CPG motifs in DNA [11] and the TLRs 3, 7, 8 that recognize viral nucleic acids (dsRNA) [12]. The recognition of their cognate ligands sets off a downstream signaling pathway leading to the activation of the NF-κB transcription factor which consequently leads to cytokine production, particularly IFNα/β and to increased expression of co-stimulatory molecules [13].

Once activated by the corresponding PRR receptors, phagocytes, including neutrophils and monocytes/macrophages, enter the site of infection, where they ingest microbial pathogens. The polymorphonuclear (PMNs) leukocytes in blood (neutrophils) are exceptionally efficient in phagocytosis of bacterial and fungal pathogens. The blood monocytes enter into extravascular spaces and differentiate into resident macrophages for e.g microglia (CNS), into Kupffer cells (liver), alveolar macrophages (lungs), osteoclasts (bone); these cells are programmed to survive in their tissue environments, unlike neutrophils that succumb after ingestion of their targets [14].

Macrophages may differentiate into subsets with distinct functions associated with the nature of the stimulation. For instance, activation by either PRRs or effector cytokines such as IFNγ promotes "classically activated" M1 macrophages. These M1 macrophages are pro-inflammatory and mediate host defence against bacteria, viruses, protozoa and to some extent antitumor responses. M2 macrophages are anti-inflammatory and promote wound repair. Regulatory macrophages secrete copious amounts of IL-10 in response to Fc receptor-γ ligation. Myeloid-derived suppressor cell (MDSCs) maybe precursors to tumor associated macrophages (TAM) that suppress antitumor immune responses. The M2, regulatory, TAM and MDSC subsets of macrophages promote immune suppressive activity [15].

However, macrophages adopt context dependent phenotypes that can either promote or inhibit immune responses and may represent a spectrum of activated phenotypes, rather than discrete stable fates. Many studies describe plasticity in macrophages switching from one phenotype to another in response to the local cytokine milieu. Some macrophage effector functions may combine activities that have classically been labelled as an M1 or M2 response [14, 16].

M1 macrophages (i) produce cytokines such as TNF, IL-1, IL-6 and IL-12 that increase inflammation and aid adaptive immune responses, they also (ii) secrete reactive oxygen species (ROS) and nitric oxide (iNOS) (iii) upregulate the MHC class I and II machinery. M2 macrophages have pronounced anti-inflammatory effects including wound repair and fibrosis, predominantly through production of TGF-β and IL-10 [16].

Dendritic cells are cell subsets that bridge the innate and adaptive immune responses; they recognize microbes and subsequently produce cytokines and display microbial peptide antigens on their cell surface that may activate the adaptive cellular immune response [17]. Mast cells, derived from the myeloid lineage, have abundant cytoplasmic granules that contain histamines and heparin. These cells play a major role in allergy, anaphylaxis and defense against helminthes [18].

Natural killer (NK) cells are critical players in an innate immune response; they contain abundant cytoplasmic granules effective in killing infected cells. Their killing mechanisms are very similar to those seen in cytotoxic CD8 T-lymphocytes. Their functions are evident in anti-viral responses, where they synergistically act with macrophages to eliminate intracellular viral reservoirs. Activated NK cells produce IFNγ that acts on macrophages by

increasing antigen presentation pathways. Production of IL-12, IL-15 and type 1 interferons by macrophages play a pivotal role in NK cell development and proliferation. NK cells display a host of activating and inhibitory receptors.

The activating receptors consist of NKG2D and KIRs (killer cell immunoglobulin-like receptors) (KIR2DL4, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DS1) which recognize stressed cells in conjunction with MHC class I, these receptors have signaling subunits called ITAMs (immune receptor tyrosine-based activation motifs) which get phosphorylated and activate cytoplasmic tyrosine kinases leading to cytotoxic granule and IFNγ release [19, 20]. Another activating receptor is the CD16 (FcγRIII) that is specific for IgG antibodies, its activation causes the discharge of cytotoxic granules and is instrumental in ADCC (antibody dependent cell cytotoxicity)[21].

The inhibitory receptors consist of KIRs (KIR2DL1, KIR2DL2/3, KIR2DL5, and KIR3DL2) which may be crucial to the "missing-self hypothesis" which states that NK cells preferentially kill cells with missing or compromised self MHC class I expression [22-24]. The other well characterized receptor is the CD94-NKG2 receptor that recognizes the non-classical MHC molecule such as HLA-E [25]. The inhibitory receptors, when activated by MHC class I, contain in their cytoplasmic domains signaling units known as ITIMs (immune receptor tyrosine-based inhibition motifs) that are phosphorylated and activate cytoplasmic tyrosine phosphatases that remove phosphate groups from ITAMS preventing activation cascade [20].

Non-conventional T-lymphocytes represent a subset of cells already capable of cytokine production and cytolytic function as they emerge from the thymus; they exhibit limited diversity in their antigen recognition receptors. These correspond to three major cell types MAIT cells, γδ T-cells and NKT cells, which are at the interface of innate and adaptive immune response. MAIT cells (mucosal associated invariant T-cells) are present in mucosal surfaces and restricted to MR1 (MHC related class I-like molecule) that presents vitamin B metabolites. These cells may be important in response to pathogenic and commensal bacteria [26, 27]. γδ T-cells are mostly CD1 restricted and recognize phospoantigens, they may have anti-viral cytolytic functions [28]. In particular, Vγ9Vδ2 T-cells were described to exhibit anti-influenza reactivity [29]. Natural killer T-cells (NKT) recognize endogenous and exogenous lipid antigens presented by CD1d molecules on APCs [30], the best characterized are the type 1 NKT cells that express a restricted TCR with an invariant α chain (Vα24-Jα18) paired with a limited array of TCR Vβ chains. These cells have been described to contain inflammation - which could lead to lung injury by selectively lysing inflammatory monocytes during a severe influenza infection in a CD1d dependent manner [31].

Innate lymphoid cells (ILCs) are a heterogeneous cell populations playing a critical role in intestinal and mucosal immunity with some subsets even producing cytokines such as IL-5, IL-13, IL-22 and/or IL-17A [32]. These cells have been described to drive Th2 associated immune response against helminth infections [33] and more recently in the context of acute

influenza infection, where they were shown to restore tissue homeostasis and airway epithelial integrity after flu infection [34].

The complement system is an evolutionary ancient form of host defence that is organized into proteolytic cascades leading to inflammation, lysis and opsonization of pathogens. The complement system can be activated by three pathways i.e. (i) the classical pathway – is initiated when the C1q complex along with C1r and C1s binds to the Fc region of IgG1 and IgM antibodies that are bound to pathogens, (ii) alternate pathway – is initiated by the pathogenic surface itself (iii) lectin pathway- is initiated by MBL receptor when it recognizes PAMPs. All of these pathways lead to production of C3a and further C3b which activates the late complement cascade and culminates in the formation of membrane attack complex (MAC), this complex is efficient in lysis of thin walled microbial pathogens [35].

1.3 Adaptive immune response

The innate immune responses set the stage for the adaptive or acquired immune response by upregulating the expression of co-stimulatory molecules and by increased cytokine production. Adaptive immune responses are specific, directed to the microbe and often long lived. They can be recalled faster during second and subsequent encounters with the cognate pathogen. The two main components of an adaptive immune response are the (i) cellular immune response characterized by thymus educated T-lymphocytes and their effector cytokines that specifically act on intracellular and phagocytized pathogens. (ii) Humoral responses, elaborated by B-lymphocytes that produce antibodies, targeting pathogens in extracellular spaces.

1.3.1 T-cell mediated immune responses

These responses are defined by T-lymphocytes and are critical in the elimination of intracellular pathogens. There are two ways in which the intracellular pathogens may survive (i) pathogens, e.g. intracellular bacteria are phagocytosed by macrophages and continue to multiply in the cytosol by evading the phagolysosome complex. (ii) Viral pathogens that infect non-phagocytic cells such as epithelial cells. In addition to elimination of intracellular pathogens, classes of T-lymphocytes expressing the CD4 co-receptor are called helper T-cells; orchestrate both cellular and humoral responses by producing cytokines (see below in section 1.3.1.1).

1.3.1.1 CD4 T-Lymphocytes

The CD4 T-lymphocytes are central to immune protection from pathogens; they provide help to B cells (for maturation and differentiation and to secrete antibodies), activate macrophages and direct PMNs to sites of infection. The naïve CD4 T-lymphocytes patrolling the lymph nodes are activated by their T-cell receptor (TCR) complex via the major histocompatibility class (MHC) II molecules on the surface of dendritic cells that present microbial peptide antigens, usually 10-30 amino acids. The peripheral lymph nodes sample protein antigens from epithelial and connective tissues, whereas the blood borne antigens are concentrated by

the spleen. These antigens are processed via the MHC class I or II pathways by professional antigen presenting cells (APCs) e.g. dendritic cells, macrophages and B-cells. When dendritic cell encounter antigens - and are sufficiently stimulated by PRRs - they express a chemokine receptor CCR7 and start migrating towards peripheral lymph nodes. In this process, they mature into APCs that can very effectively stimulate T-cells. In brief, the MHC II pathway is initiated by the active uptake of extracellular protein into the endocytic vesicles of the APCs; these internalized proteins are processed into many unique small peptide fragments by the fusion of the endosomal and lysosomal vesicles. Following this processing, the newly synthesized MHC II αβ dimer, along with an invariant chain containing the CLIP peptide that is strongly bound to the peptide binding cleft of the MHC II, are transported to the late endosomal vesicle containing the processed peptide fragments. The late endosomal vesicle contains another MHC II like complex, called HLA-DM, which facilitates the exchange of the CLIP peptide for higher affinity antigenic peptides. The antigenic peptide is then bound to the groove of the MHC II molecule which stabilizes the complex and this is further transported to the cell surface in wait for potential CD4 TCR engagement in the lymph nodes [36].

Once the T-cell receptor complex, along with the CD4 co-receptor, is engaged to its cognate peptide MHC class II on APCs, a second co-stimulatory CD28 receptor on T-cells may bind to the B7.1/7.2 ligands on the APC to complete activation. This activation signals leads to the maturation and differentiation of naïve CD4 T-cells into four different possible fates i.e Th1, Th2, Th17 and Tregs. Each fate is specialized and committed to produce a certain set of cytokines and transcription factors as indicated below. More different subsets have been reported recently like Tfh (follicular helper T-cells that provide specialized help to B-cells and help in the maintenance and formation of germinal centers [37]), Th9 (polarized in the presence of TGF-β/IL-4, producing IL-9 that contributes to anti-parasite responses [38]) and Th22 (skin homing cells polarized in the presence of IL-6/TNF-α,producing IL-22 [39]).

Naïve CD4 T-cells are polarized into a Th1 subset in the presence of IL-12, IL-18 or IFNγ when activated by a TCR stimulus in conjunction with peptide MHC class II. Th1 cells have been described to be effective in responses against intracellular pathogens; their signature cytokines are IFNγ, IL-2 and lymphotoxin α. The transcription factors that defines this subset is T-bet and STAT5. IFNγ is a potent activator of macrophages and increases the antimicrobial activity of macrophages (described above) and IL-2 is critical for T-cell memory formation.

Th2 cells are subsets that have effective response to extracellular pathogens and helminthic infections; CD4 T-cells are often polarized into Th2 cells in the presence of IL-2 and IL-4. The signature cytokine(s) produced by this subset are IL-4, IL-5 and IL-13. Th2 cells have GATA3 and STAT6 as their transcription factors. IL-4 is a crucial cytokine in inducing IgE class switching in B-cells. Particularly IL-5 is involved in the development and activation of eosinophils and mast cells. IgE binds to the FcεR1 on basophils and mast cells leading to

secretion of histamines setting up an inflammatory milieu. IL-13 is described to be crucial in clearing helminthic infections [40].

Th17 cells are polarized cells in the presence of IL-6 and TGF-β and play a major role in responses against extracellular bacteria and fungi. Their effector signature cytokines are IL-17a, IL-17f and IL-21 and their transcription factors are RORγt and STAT3. IL-17a and IL-17f are both critical in recruiting and activating neutrophils to sites of infection; IL-21 acts on Th17 cells further promoting the survival of this subset in an autocrine manner. IL-21 also has profound effects on CD8 T-cells, B-cells and NK cells e.g. by rescuing cells from activation-induced cell death [41].

Treg cells are generated in the presence of TGF-β and IL-2 and play a major role in promoting self-tolerance and regulating immune responses. The signature cytokines produced by these cells are IL-10 and TGF-β, their respective transcription factors are FOXP3 and STAT5 [42].

1.3.1.2 CD8 T-Lymphocytes

CD8 T-cells are lymphocytes derived from the bone marrow and are educated in the thymus bearing a TCR and CD8 co-receptor. These cells primarily recognize pathogen derived peptide MHC class I complexes on the surface of nucleated cells. A requirement for the activation of CD8 T-cells is the cross presentation of cytoplasmic antigens by dendritic cells. Dendritic cells generally ingest virus-infected cells and process and present peptide antigens in the cytosol via the MHC class I to CD8 T-cells [43].

The MHC class I complex is a dimer of a heavy α chain noncovalently linked to a protein (β_2-microglobulin) generally expressed on all nucleated cells. The heavy α chain is composed of α1 and α2 subunits that form the peptide binding cleft which usually holds an 8-10 mer peptide fragment, the α3 subunit that interacts with the CD8 co-receptor during the TCR engagement. The MHC class I pathway is initiated by the antigenic proteins in the cytosol, e.g. by viral proteins in the infected cell, in addition to the cell's own misfolded proteins that are targeted for destruction by a ubiquitin-proteasome pathway. These proteins are unfolded and tagged with ubiquitin and then processed through a proteasome complex that cleaves the parent protein into small peptide fragments (8-10aa) in the cytosol. Since the MHC class I machinery is synthesized in the endoplasmic reticulum (ER), the peptide fragments are transported by a specialized molecules called transport associated with antigen processing (TAP), located in the ER. The TAP protein actively pumps peptide fragments from the cytosolic side into the ER, where the newly synthesized pre-mature MHC class I heavy α chain linked to tapasin is present. The tapasin links the MHC class I α chain to the TAP complex and if there are peptides with high affinity, the whole complex is stabilized along with β_2-microglobulin; the mature MHC class I/peptide/beta-2 microglobulin complex is unlinked from TAP and released to the cell surface [36] .

Upon activation, CD8 T-cells express a broad repertoire of effector molecules in defense against microbial pathogens; of importance is the direct cytotoxic effect of target cells due to

release of perforin and granzymes. Perforin is an effective disruptor of target cell membranes facilitating the entry of granzymes in particular granzyme B that activates caspases which induce apoptosis there in. Another killing mechanism is via the fas ligand that binds to death inducing receptor CD95 activating the caspase system resulting in subsequent apoptosis. The CD8 T-cells also secrete IFNγ and TNFα that are crucial in creating an inflammatory state enabling effective clearance of the respective viral pathogens [43].

The naïve/ precursor CD8 T-cells are defined by expression of CCR7, CD62L and CD45RA CD8 T-cells are constantly circulating between blood and lymph nodes surveying APCs to find their cognate antigen peptide MHC class I. Upon sufficient activation and CD4 T-cell help, CD8 T-cells are able to generate long-lived memory responses [44] via clonal expansion. Central memory T-cells, characterized by expression of CCR7+ and the loss of CD45RA- are generated, which have increased sensitivity to antigen stimulation and are often independent of co-stimulation. These cells produce IL-2 and proliferate into effector CD8 T-cells (CCR7-CD45RA-) that migrate out of the secondary lymph nodes in search of their cognate antigens. Upon encounter with their target(s), they produce IFNγ and TNFα in addition to perforin and granzyme production. This expansion/effector phase is followed by phase of contraction wherein 95% of these effector CD8s undergo cell death barring a few resulting in early memory formation. The terminal effector cells (CCR7-CD45RA+) have abundant cytoplasmic granzyme and perforin for immediate deployment upon secondary antigen encounter. This heterogeneity seems to differ according to the source of antigenic stimulus and the pathogen encountered, for instance in some studies CMV specific CD8 T-cells were entirely defined to reside in the terminally differentiated effector compartment, whereas in an human immunodeficiency virus (HIV) setting, antigen-specific T-cells were found in the effector memory T-cell subset probably due to interaction of CD27 on T-cells with CD70 on APCs promoting an effector phenotype to compensate for limited CD4 T-cell help [45]. In contrast, antigen – specific T-cells have been found to reside in the naïve/precursor compartment in melanoma patients, yet these T-cells could not sufficiently produce cytokines [46].

1.3.2 Humoral immune responses

The hallmarks of humoral responses are antibody mediated and orchestrated by B-lymphocytes. The humoral response serves to neutralize extracellular pathogens and their toxins. Antibodies may also target non-protein antigens such as polysaccharides and lipids. The B-lymphocytes produce antibodies of 4 major classes upon activation with their cognate targets i.e. IgD, IgM, IgA and IgG. Each class of antibodies performs highly specialized functions to neutralize extracellular pathogens as discussed further.

1.3.2.1 B-Lymphocytes

Naïve B-cells are generated in the fetal liver, bone marrow, and adult bone marrow [47, 48]. These B-cells express membrane-bound immunoglobulins IgM and IgD, they are often activated in the lymph nodes, spleen and mucosal surfaces where antigens are concentrated.

In lymph nodes, macrophages take the captured antigens to the B-cell rich follicles and marginal zones wherein the antigens are displayed. Membrane bound Ig of the B-cells engages with displayed antigens subsequently triggering the B-cell receptor signaling. The activating antigens can be divided into two groups on the basis of T-cell help requirement i.e. T-cell dependent antigens (TD) or T-cell independent (TI). TD antigens are those that after binding to surface Ig of the B-cells are internalized and processed through the MHC class II pathway. The MHC class II-peptide antigen complex is recognized by activated CD4 T helper cells via its TCR along with the engagement of co-stimulatory receptors CD40 on B-cells with CD40L on T-cells. This is called the linked recognition, the epitope recognized by CD4 T-cells is linked to the epitope recognized by the B-cell surface Ig [49]. This stimulates and provides IL-4 cytokine signals to B-cells for isotype switching and affinity maturation. These B-cells are referred to as follicular B-cells and make the bulk of antibodies to TD antigens and give rise to long-lived plasma cells capable of producing IgG, IgA and IgE. The TI antigens are generally non-protein structures such as polysaccharides and lipids, often stimulate B-cells by cross-linking the Ig receptors; they are independent of T -cell help. The marginal zones B-cells in the splenic white pulp are the main responders to the blood derived polysaccharide antigens whereas the B-1 cells respond to other non-protein and lipid antigens. Both these subsets produce IgM class of immunoglobulin and often do not yield long-lived immune memory [50].

1.3.2.2 Immunoglobulins

An antibody is composed of four polypeptide chains; 2 heavy (H) and 2 light chains (L), each containing a variable and constant region highly homologous to antigen receptors in T-lymphocytes. The two mechanisms by which immunoglobulins enhance their response are (i) isotype switching –production of antibodies with same specificities but different isotype leading to varied effector functions (ii) affinity maturation –repeated stimulation with protein antigens leads to production of antibodies with increased affinity for that antigen. As described the four different classes of immunoglobulins are discussed below.

IgM Immunoglobulins –These are the first immunoglobulins expressed on B-cells in monomeric form, on activation and maturation by antigenic stimulus, these B-cells secrete multimeric IgM antibodies (generally pentameric). Secreted IgM constitutes about 10% of all serum immunoglobulins. The IgM antibodies are associated with primary response and tend to be more polyreactive as compared to other isotypes, due to reduced affinity maturation; IgM antibodies have also been described to facilitate the removal of apoptotic cells [51].

IgD Immunoglobulins – Secreted IgD is a monomer and constitutes just 0.25 % of all the serum immunoglobulins with a half-life of 2.8 days, the secreted IgD has been described to bind unspecifically to many bacteria through the Fc region; its role in the humoral response is not yet clear. The IgD immunoglobulin is also present on the surface of B-cells where it is co-expressed with IgM, IgD has been described to be involved in B-cell receptor signaling and in regulation of B-cell activation states [52].

IgG Immunoglobulins- these are the predominant isotypes upto 75 % of all the immunoglobulins found in the body with longest serum half-life of upto 23 days. The monomeric IgG are subdivided into four different classes based on their structural and functional differences in the constant regions of the heavy chain (figure 1). The serum concentrations are in the order of IgG1 > IgG2 > IgG3 > IgG4 in healthy individuals [53]. IgG1, IgG2 and IgG3 can fix complement in the order of IgG3 >IgG1 >IgG2 and can mark pathogens for phagocytosis known as opsonization. IgG1 is crucial in secondary response; IgG2 and IgG3 neutralize virus and toxins. IgG4 is the only sub-class that does not fix complement. Their affinities to FcγR (I, II and III) also differ; IgG1 and IgG3 bind to all the three classes, IgG4 binds to II and III, IgG2 binds only to II. Taken together IgG antibodies have the following functions (i) neutralization of microbes and toxins (ii) opsonization (iii) activation of the classical complement pathway (iv) ADCC mediated by NK cells (v) neonatal immunity (vi) feedback inhibition of B-cell activation.

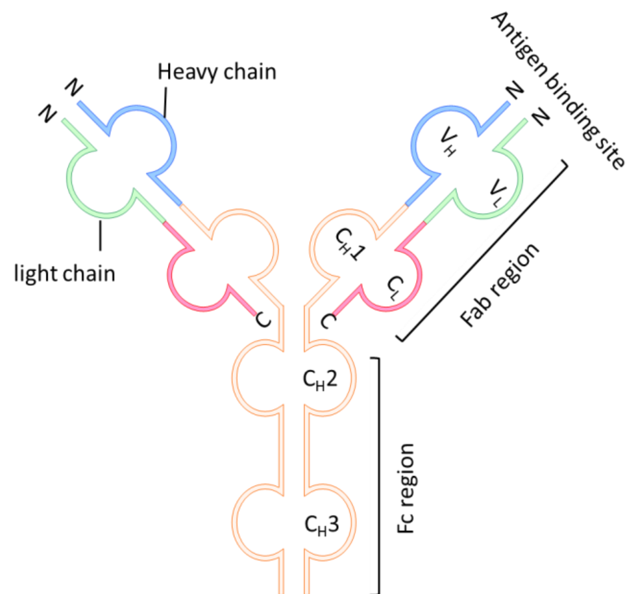

Figure 1: A secreted Immunoglobulin g (IgG) molecule illustrating the heavy and light chains and the antigen binding site on the fab region (adapted from Basic Immunology, 4th edition by Abul K Abbas, Andrew H Lichtman and Shiv Pillai).

IgA Immunoglobulins- IgA antibodies exist in either dimeric or monomeric forms and are predominantly present on mucosal surfaces (sIgA), in secretions such as saliva and in breast milk. They are also present as monomers in serum constituting 15% of total immunoglobulins. The IgA are subdivided into two classes IgA1 and IgA2 based on their structural differences, IgA1 makes up 90% of the serum IgA and IgA2 is present at mucosal surfaces. The sIgA are critical in responses against viruses and bacteria at mucosal surfaces by means of direct neutralization. Furthermore, intracellular IgA has been described and could reduce microbial pathogenesis. Neutrophils express IgA receptor and maybe activated to perform ADCC, IgA may act as an immunopotentiator e.g. by limiting the activation of dendritic cells and promoting the local homeostasis in the gastrointestinal tract [53].

IgE Immunoglobulins- IgE is a monomeric antibody that constitutes less than > 0.01 % of serum immunoglobulins, It has the shortest half-life. IgE is characterized by its potency to induce type I hypersensitivity and allergic reactions. Furthermore it’s a critical player in the response against parasitic helminths. IgE binds to FcεRI that is expressed on mast cells, basophils and esoinophils with very high affinity leading to activation of these cell subsets [54].

1.4 Influenza virus

1.4.1 Influenza biology and pathogenesis

Influenza viruses are enveloped negative strand RNA viruses that belong to the family *Orthomyxoviridae.* The characteristic features are the segmented genome containing seven to eight segments [55]. The influenza viruses are spherical particles with a host-derived lipid bi layer embedded with the surface immunogenicity defining proteins hemagglutinin neuraminidase and M2 protein, followed by inner Matrix 1 layer in which the eight strands of negative RNA are present held together by ribonucleoprotein RNP (containing the PB1, PB2 and PA)(figure 2) [56].

The hemagglutinin (HA) homotrimer is the major antigenic surface protein that is cleaved into HA1 and HA2 subunits by the host derived proteases and is mandatory for fusion of the virion and the host cell sialic acid receptors [57, 58]. The RNA segment 4 encodes the HA protein, due to faulty RNA polymerase activity the HA protein is subject to high number of mutations and is driven also in part by immune selection pressures, resulting in many different subtypes (at least 18 have been described)[59].

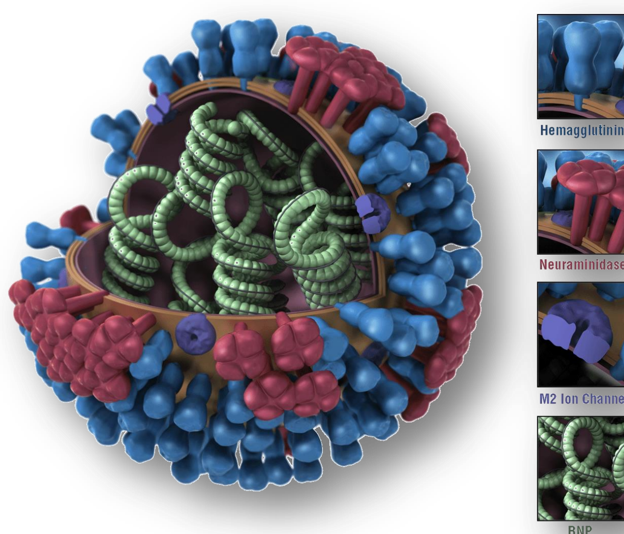

Figure 2: A graphical representation in 3D showing the surface and internal proteins of influenza A virion (adapted from http://www.cdc.gov/h1n1flu/images.htm).

The neuraminidase (NA) is encoded by the RNA segment 6 and is another major antigenic surface protein, the NA is crucial in cleaving the terminal sialic acid from glycoprotein and glycolipids to permit the exit of newly synthesized viral particles from host cells [60]. Likewise NA is subject to high number of mutations and many subtypes ranging from N1 through N11 have been described [59].

The matrix 1 (M1) and matrix 2 (M2) proteins are encoded by RNA segment 7, M1 protein forms a sheath around the nucleoprotein complex and is present in abundance in cytoplasm and nuclei of the infected host cells [61]. The M2 protein is a membrane protein and signals for transport to cell surface of the host cell [62].

PB1 and PB2 polymerases are encoded by RNA segment 2 and 1 respectively. The PB2 polymerase function is in the initiation of viral mRNA transcription and it recognizes the 5' capI structures of host cell mRNAs to be used as primers. The PB1 polymerase functions by elongating the primed nascent mRNA. The polymerase PA is the product of RNA segment 3, it functions by unwinding the helix. The non-structural proteins NS1 and NS2 are encoded by

RNA segment 8 and functions in the export of viral RNPs to the nucleus of the host cell [63] and in some reports the NS1 can also function as Interferon antagonist [64].

1.4.2 History and epidemiology

Influenza viruses, often referred to as the flu, are the causative agents of respiratory infections. Influenza virus has caused many pandemics with regular intervals and has been mentioned historically as early as 412 BC [65]. Richard E. Shope at the Rockefeller institute first identified the influenza virus in early 1930s, much later than the devastating pandemic of 1918 [66, 67]. Influenza virus was first isolated from humans in 1933 by Christopher Andrewes, Wilson Smith and Patrick Laidlaw [68]. Shope also showed that sera from individuals who experienced the 1918 H1N1 infection could neutralize the swine virus [69]. The well-characterized influenza virus types are the A, B and C viruses that can be readily distinguished from each other by genetic testing but appear similar in manifestation of symptoms. The human influenza A and B viruses are responsible for yearly seasonal epidemics unlike the influenza C virus that causes sporadic cases [70].The influenza A viruses are defined into subtypes based on their hemagglutinin and neuraminidase variants e.g. H1N1, H3N2 and H5N1, and most of these subtypes circulate in birds and swine. There are no subtypes of influenza B circulating in animals, as humans are the only hosts [56, 59]. Influenza A viruses are very susceptible to mutations resulting in antigen drifts, and after accumulation of these mutations, reassortments between subtypes an antigenic shift can occur, where in a previously immune individuals become susceptible to a symptomatic disease. [24]. A wide range of influenza subtypes are endemic to pigs and birds, occasionally causing severe zoonotic infections in humans and consequently, adapting to new host species [25]. Reassortment is process by which an influenza subtype can adapt to a new host due to

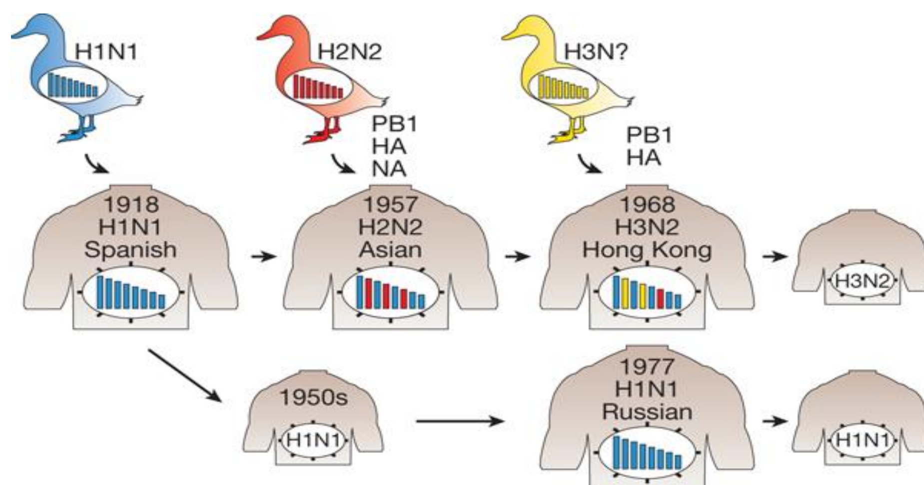

Figure 3: Emergence of Spanish pandemic 1918 H1N1 influenza viruses by the transmission of avian influenza virus to humans, the 1957 H2N2 was resultant of introduction of avian influenza RNA segments into human population, similarly the 1968 H3N2 pandemic was a result of avian H3 HA and PB into human population. (adapted from Neumann et al. 2009).

exchange of RNA segments; for e.g. It is described to occur when an avian influenza subtype such as H5N1, human influenza subtype such as H3N2 and swine influenza H1N1 infect a

common host i.e. pigs that have the receptors to bind all the three subtypes. Thereby, triple reassortments can occur with the three subtypes, while packaging their RNA segments. This is linked to pandemic spread of flu infections[71].

Perhaps the 1918-19 H1N1 pandemic was the worst in terms of mortality with some reports putting the death toll at 50 million around the world [72].The two other pandemic outbreaks in the 20th century were Asian influenza H2N2 of 1957 with a mortality of 70,000 and the Hong Kong influenza H3N2 outbreak of 1968 resulting in a lower disease burden since influenza vaccination had been introduced [73-75]. There were smaller contained outbreaks of 1943 and 1947 among US military personnel and the failure of vaccine that was supposed to prevent the 1943 H1N1 [76] (figure 3). This was followed by a milder Russian outbreak of 1977 of an H1N1 subtype [77]. In 1997 the highly pathogenic H5N1 subtype struck with six fatalities and 18 infections, but it was controlled rapidly by extensive culling of live birds [75].

The first large outbreak of the 21st century started in the Mexican town of La Gloria, Veracruz in mid-February of 2009 [78]. This strain was characterized as swine-origin influenza H1N1 and by May of 2009, 41 countries reported fatalities. Subsequently, the WHO declared this wave a pandemic. In general, the infections were mild and did not require hospitalization [79] but severe cases were reported in pregnant women and immunocompromised individuals. This novel pandemic H1N1 shared a very limited repertoire of cytotoxic and humoral epitopes with its generic seasonal counterparts [80] resulting in substantial illness among children and young adults [81]. The estimated global mortality was 201,200 respiratory deaths and 83,300 cardiovascular deaths, 80% of the respiratory deaths occurred in younger people and predominantly in Southeast Asia and Africa [82].

However, also in the non-pandemic years influenza viruses circulate widely in the population with changes in the antigenic composition called “antigenic drift”. The accumulation of mutations in the hemagglutinin epitopes recognized by neutralizing antibodies results in this phenomenon. Influenza virus adjusts its receptor binding avidity in response to immune pressures by altering simultaneously many amino acids on its globular head [83]. Therefore yearly, influenza viruses are responsible for more than 30,000 deaths and 200,000 hospitalizations yearly in the United States alone [84].

1.4.3 Clinical symptoms

Influenza transmissions may occur through respiratory droplets and often requires close contact or when in contact with contaminated surfaces, the average incubation period of influenza is 2 days following which symptoms develop within 5 to 7 days. Transmission can also happen through hand-to-hand contact between individuals. Common symptoms are fever, myalgia (muscle pain), headache, cough, rhinitis, sore throat, and gastro-intestinal symptoms in some cases. An uncomplicated influenza usually resolves within 7 days, but secondary infections such bacterial pneumonia can occur. In immunosuppressed individuals

such as those with HIV infection and in stem cell transplant recipients, the clinical manifestations can be severe and result in mortality. Influenza can be fatal in young children, pregnant women mainly in the third trimester and the elderly (>64 years)[85].In some very rare cases neurological manifestations may occur such as Guillain-Barre syndrome [86]. In the recent pandemic H1N1 influenza wave the most common clinical manifestations among 426 patients in China were fever (67.4) and cough (69.5%), lymphopenia also occurred in 68.1 % of adults and 92.3% of children, the fever duration was generally 3 days [87]. In another report among 642 patients in the United States the most common symptoms were fever (94%) cough (92%) sore throat (66%), diarrhea and/or vomiting (25%) [79].

1.4.4 Laboratory diagnosis

The clinical diagnosis commonly called “influenza like illness” is generally accurate during the influenza season if the subjects appear with cough and/or fever with a predictive value of 75- 80% as reported in several studies [88-91].

The laboratory diagnosis can be established by four different assays; virus culture, serological diagnosis, detection of influenza antigens, and viral nucleic acid detection by polymerase chain reaction (PCR). Virus culture using Madin-Darby canine kidney (MDCK) cells or primary rhesus monkey kidney cells (PMK) are considered the gold standard [92, 93] with the main readout being the cytopathic effect and additional confirmation by monoclonal antibodies to surface viral antigens if available, this is particularly important for characterizing new subtypes at the start of an influenza epidemic. Although considered to be very high sensitive the disadvantage is that results are available in 4-5 days. Therefore, this technique is no longer used in routine patient care and has been replaced by more rapid tests.

Direct fluorescent staining assay of influenza surface antigen (DFA) in the nasopharyngeal aspirate is reliable and generally the results are offered on the same day, though sensitivities are variable ranging from 40% to 100% depending on the handling of specimens, the experience of the technician, and the staining procedures [94-97]. Rapid enzyme optical immunoassays or NA (neuraminidase) enzymatic assays have also been used. Implementing rapid influenza diagnostic tests (RIDTs) based on antigen detection is controversial due to the lack of sensitivity [98, 99]. Negative results of RIDTs must be independently established by a PCR based laboratory diagnosis in the immunocompromised patient group.

The polymerase chain reaction has simplified diagnostic testing in many viral diseases, and influenza testing has benefited immensely. Through the use of a multiplex panel of primers and RT-PCR (reverse transcriptase PCR), many influenza subtypes as well as other respiratory viruses can be diagnosed in a single run [100]. These tests have proven to be very sensitive and were employed during the recent H1N1 pandemic of 2009 [101, 102] with a turnaround time in hours. Serological diagnoses are generally retrospectively performed, with detection of influenza hemagglutinin specific IgG antibodies. A four-fold rise in serum titer between acute and convalescent sera is considered positive. There are different techniques for serological diagnosis such as hemagglutinin inhibition, virus neutralization followed by

enzyme immunoassay to detect internal viral nucleoproteins, pseudotype viral particle neutralization in lieu of highly pathogenic viruses such as H5N1, single radial hemolysis to detect complement-mediated lysis, and ELISA (enzyme linked immunosorbent assay) to purified influenza antigenic proteins [103]. Serological methods are not recommended in the diagnosis of acute infection, since serum antibodies are induced 4-5 days post exposure or occurrence of symptoms and requirement of paired serum samples is a necessity to make a conclusive diagnosis [103].

1.5 Innate immune responses to influenza

The initial host response to influenza is the formation of physical barriers such as mucins and collectins [104]. The innate immune response is triggered by PRRs, like TLR7/3, RIG-1 and NOD like (NLRP3) receptors, which promote the production of IFNα/β and IL-1β by respiratory epithelial cells [105-107]. However, the influenza virus has developed a mechanism of evasion by NS1 protein that acts as an antagonist of IFNα/β and is likely to delay an effective response [64, 108]. Nevertheless, the flu virus activates neutrophils and natural killer cells during the early phase of the infection.

Influenza infected cells are destroyed if NK cells recognize reduced expression of MHC class I, in conjunction with the NK cell NKp46 receptor that has been described to interact with influenza hemagglutinin [109, 110]. In addition, NK cells with their CD16 (FcγRIII) can bind to the Fc portion of influenza bound IgG and effectively induce ADCC of infected cells [111]. The innate immune responses at this stage serve to limit influenza viral replication and promote adaptive immune responses by upregulation of MHC machinery and co-stimulatory molecules on accessory cells.

Alveolar macrophages are recruited to the site of infection by epithelial cells via the production of CCL2 [112], upon activation, alveolar macrophages produce IL-6, IL-12 and TNFα creating a pro-inflammatory cytokine milieu [113, 114]. These alveolar macrophages have a very critical role in limiting the spread of new infections by phagocytosing the infected cells [115-117]. The depletion of alveolar macrophages also results in impaired cytotoxic CD8 T-cells and reduced antibody titers [117].

The DCs during an influenza infection acquire antigens via two pathways: (i) DCs themselves can be infected with the influenza virus [118, 119] and subsequently the viral antigens are processed by a MHC class I pathway and presented to cytotoxic lymphocytes, (ii) the second route of influenza antigen accrual is by active phagocytosis of virus particles and infected epithelial cells, followed by degradation and processing of viral protein antigens via the MHC II pathway and subsequent antigen presentation to CD4 T helper cells, A mechanism described previously as cross presentation can occur activating CD8 T-cells in parallel [120, 121].

1.6 T-cell mediated immune responses to influenza

Influenza virus infection generates a range of effector CD4 and CD8 T-cell responses. These have been found to be critical in regulating an effective anti-flu response, reducing symptomatic infections and are crucial in protection in the course of a second encounter with influenza. Flu-induced activation of CD4 and CD8 T-cells is responsible in generation of heterosubtypic immunity i.e. protection against infection with new subtype of influenza due to encounters with previous pathogens showing related subtype [122].

1.6.1 CD4 T-Lymphocytes in response to influenza

CD4 T-cells are activated after recognizing influenza antigenic peptides in conjunction with MHC class II on the surface of dendritic cells. Activation of CD4 T-cells is often based on the strength of the stimulation and the cytokine micro-environment that lead them to differentiate into T-helper subsets [123]. Th2 differentiated cells are responsible for activation and production of antibodies from B-cells, mainly through production of IL-4, IL-5 and IL-13 [40, 124]; yet other reports differed in the beneficial role of Th2 type cells [125] suggesting that Th1 responses inhibit a Th2 response during an influenza infection [126]. Th2 cells provide signals for antibody isotype switching and affinity maturation of influenza specific antibodies, T-cell clones specific for internal flu proteins were equally efficient in inducing hemagglutinin specific B-cell responses as compared to hemagglutinin specific T-cell clones [127].

Th1 responses to influenza are characterized by the production of IFNγ and IL-2 that results in the activation of macrophages and CD8 T-cells [127, 128]. In addition, long-lived memory CD8 T-cells can be generated with help from Th1 subsets [129]. Memory CD4 Th1 type cells in the lungs can enhance the quality of innate immune inflammatory cytokines and chemokines independent of IFNγ and TNFα [130]. Lung resident memory CD4 T-cells have been described, that can quickly respond to the second encounter with the nominal pathogen [131]. Pre-existing CD4 T-cells specific for pandemic influenza 2009 showed cytotoxic potential and were responsible for decreased illness and disease severity in the absence of neutralizing antibodies [132]. The CD4 T-cells that migrate to lung during an influenza infection are characterized by an effector phenotype having reduced expression of CCR7 and CD62L, stable expression of CD44 and CD49d and low expression of CCR5 and CD25; these cells produced copious amounts of IFNγ [133]. Heterosubtypic immunity is a very important mechanism by which memory CD4 T-cells can mediate protection: healthy volunteers could also generate potent anti-pandemic influenza responses when vaccinated with the generic seasonal influenza vaccine [134]. Memory T-cells specific for H3N2 influenza were also able to cross-react with H5N1 subtype in healthy individuals [135].

1.6.2 CD8 T-Lymphocytes in response to influenza

CD8 T-cells are activated by influenza antigenic peptides on MHC class I molecules on APCs in the lymph nodes, followed by their differentiation into cytotoxic CD8 T-cells (CTLs) and migration to the sites of infection. CD8 T-cells may subsequently eliminate

infected cells and prevent further production of viral progeny [136]. These cytotoxic CD8 T-cells show a very high specificity to the internal conserved proteins of Influenza such as M1, NP including PB1 and PA, that maybe central to the heterosubtypic protection against influenza A viruses [137-139]. Heterosubtypic CTL response to the newly identified influenza A virus H7N9 subtype was also described, induced from exposure to seasonal H3N2, H1N1 and pandemic H1N1 2009 [140, 141]. In addition, large numbers of cross-reactive CTL epitopes were conserved in the pandemic 2009 strain blunting disease severity [80].

The specific CTLs lytic activity is mediated via two pathways i.e. either by releasing perforin and granzymes that cleave essential components of infected cellular machinery necessary for viral replication [142, 143] or by the induction of apoptosis in influenza infected cells via the fas ligand interaction with the death inducing receptor CD95 (activating the caspase system in the infected cells) [144]. The cytokine production is skewed towards pro-inflammatory molecules, characterized by abundant production of TNFα and IFNγ [145]. An influenza infection or vaccination creates a pool of long-lived influenza specific CTLs that are mainly located in the central and effector memory T-cell pool. These immune cells can be quickly recruited upon re-encounter with the nominal targets without the need of dividing into daughter cells [146-148]. A Prospective study, conducted during the pandemic influenza has also emphasized the importance of terminal effector memory CTLs specific to the conserved M1 peptide epitope, these CTLs were the most significant correlate of immune protection against pandemic influenza [149].

1.7 Humoral immune responses to influenza

Influenza infection or vaccination generates influenza directed antibodies produced by activated B-cells [150]. The antibodies induced by the influenza hemagglutinin primarily correlate with immune protection, in particular they can prevent viral attachment to host cells and block receptor mediated endocytosis [151]. The sterilizing immunity resultant of antibodies targeting the trimeric hemagglutinin complex is strain-dependent and often fails to neutralize intrasubtypic drift strains or other subtypes [152]. This is particularly evident following the H1N1 pandemic influenza 2009 wave, where individuals, born before 1950, were relatively sparred from symptomatic infection [153-155].

Antibodies have been described to neuraminidase (NA) protein, which is pivotal to the release of newly formed virions. Antibodies directed to NA do not neutralize the virus but limit the spread of infection [156, 157]. In addition, antibodies to the transmembrane M2 protein have also been characterized. M2 protein unpacks the virus after the receptor-mediated endocytosis. Since the M2 protein is relatively well conserved, it may also contribute to heterosubtypic immune responses [158-160]. Antibodies against the viral nucleoproteins (RNP) that are highly conserved have also been described and could also be important in contributing to heterosubtypic immunity [161]. The non-neutralizing antibodies induced against NA, M2 and viral nucleoproteins could further contribute to protective immunity by the mechanism of ADCC [162]. However other reports suggest that high titers

of low avidity non-neutralizing antibodies maybe detrimental and are associated with poorer outcome [163]. Other studies have described the importance of the classically activated complement pathway in individuals with C1q genetic polymorphisms, these individuals had poorer clinical outcomes and developed pneumonia after the recent pandemic influenza infection [164].

IgM, IgA and IgG isotype of antibodies can be generated by the primary infection. IgM is not produced upon a secondary infection [165]. Other studies have suggested the importance of IgM and its role in activation of complement C3, which is crucial in maintenance of long-lived memory B-cell responses [166]. The presence of Influenza nucleoprotein specific serum IgA has also been correlated with a recent onset of influenza infection [167]. Mucosal sIgA (secretory), produced by resident B-cells that accumulate in lung following infection, were described to be important for reduced disease severity and protection [168-170].

1.8 Influenza vaccination systems and prevention

The major causative agents of epidemics and pandemics resulting often in severe cases are influenza A virus subtypes followed by the influenza B. while the influenza C subtype is of limited concern. Therefore, all vaccine formulations contain Influenza A and Influenza B antigens. The antigenic drift to which both influenza A and B subtypes are susceptible mandates the change in antigen formulations every year based on epidemiological surveillance. To successfully induce protection, the vaccine antigen strain must have at least 85% consensus with those strains that are currently in circulation [171]. Since different influenza A subtypes have resulted in epidemics and pandemics, the current vaccine formulations are often trivalent inactivated vaccines (TIV) i.e. two different subtypes generally a H1N1 and H3N2 from influenza A and an influenza B strain are included or quadrivalent where two antigenically (B/Yamagata and B/Victoria) different B strains are included [172-174].

The currently licensed influenza vaccines fall into two broad categories inactivated and live virus vaccines. Inactivated vaccines are composed of whole inactivated virus or split virus containing purified surface antigens. The inactivated vaccines are available in three formats i.e inactivated whole virus, split and sub-unit vaccines. The live viruses are grown in chicken eggs or cells, and thereafter formaldehyde inactivated, purified, and concentrated to 15µg doses of Hemagglutinin [175], the split vaccines are additionally detergent treated to dissociate the viral envelope proteins [176], the subunit vaccines are put through further purification steps [177], further the split and subunit vaccines have comparable immunogenicity relative to the whole virus inactivated vaccine.

The TIV vaccines are generally administered intramuscularly and induce predominantly serum IgG responses against strain specific hemagglutinins and neuraminidases [178]. Since the TIV are relatively little immunogenic, they might be coupled with adjuvant systems to enhance the influenza specific immune response. In times of pandemics, when the demand for protective vaccines is high, the adjuvants can greatly decrease the concentration of the

purified antigen. The licensed adjuvants in use are alum salts, oil in water emulsions such as MF59 (Novartis), ASO3 (GSK Biologicals), and virosomes. The oil in water emulsion adjuvanted vaccines were superior in immunogenicity when compared to non-adjuvanted vaccines tested during the pandemic 2009 [179, 180]. However, following the AS03 adjuvanted vaccination campaign several European countries reported increased incidence of narcolepsy cases [181]. The virosomal adjuvanted vaccine or the virus like particles (VLP) are composed of phospholipids and phosphatidylcholine, complexed with influenza envelope glycoproteins hemagglutinin and neuraminidase [182]. The virosomal vaccine has been tested in adults, the elderly, and in immunocompromised patients with good immunogenicity [183].

The live attenuated virus vaccines (LAIV) were based on the concept of mimicking a course of natural flu infection, and were hypothesized to induce both humoral and cellular immune responses; they were developed by passaging the virus in suboptimal conditions resulting in attenuation. These attenuated virus strains are temperature sensitive and adapted to grow at 25°C, which is the temperature of the nasal passage, but not adapted to grow at 35°C, the temperature of the lower respiratory tract [184]. The LAIV vaccines are administered intranasally and are described to induce a longer lasting humoral response and are especially effective in children [185]. These LAIV are not recommended in immunosuppressed patient groups such as aHSCT recipients and HIV infected individuals.

Antiviral agents with effect against influenza include amantadine/rimantadine, oseltamivir, and zanamivir [186]. The amantadines were used before the development of neuraminidase inhibitors; these inhibit viral uncoating by blocking the proton channel activity. The limitations include limited effectiveness against influenza B viruses and rapid development of resistance [187] and these drugs are today used very rarely. The second class of drugs blocks the active site of neuraminidase on infected host cells thereby preventing the release of virus and includes sialic acid analogues such as oseltamivir (oral) and zanamivir (inhalation) [188].

1.9 Allogeneic hematopoietic stem cell transplantation (aHSCT)

Allogeneic HSCT is used primarily for treatment of hematologic malignancies, non-malignant and congenital diseases. For non-malignant diseases the aim is to replace the abnormal hematopoietic compartment with healthy donor stem cells or to provide factors lacking in patients with enzyme defects. [189]. For treatment of malignancies, the recipient is conditioned using two broadly classified types of regimens (i) myeloablative regimens that eliminate malignant cancer cells and induce general immunosuppression allowing engraftment (ii) reduced intensity regimens that is mainly immunosuppressive and relies on the graft to perform eradication of malignant cancer cells called the graft-versus-leukemia effect (GVL) [190]. The donor hematopoietic stem cells are characterized by expression of the CD34 antigen and can be from different sources i.e. bone marrow cells, cord blood cells, or peripheral blood stem cells; the latter mobilized from the donors using granulocyte colony stimulating factor (G-CSF) and sometimes depleted of T -lymphocytes. The donors are usually HLA-identical siblings or unrelated registry donors. Early on mostly well HLA matched donors were used but in recent years, better prevention strategies against graft-vs-host disease (GVHD) allow the use of, mismatched, or haploidentical donors [190].

GVHD results from immunological attack by the allogenic graft primarily mediated by donor T-cells on the tissues and organs of the recipient. The severity and outcome of GVHD are dependent on recipient age, stem cell source and conditioning regimens. GVHD within the first 100 days post transplantation is commonly referred to as acute GVHD (aGVHD) and is predominantly mediated by T-cells in response to donor cell-surfaces and secreted factors due to the conditioning regimens. The aGVHD pathogenesis consists of three stages (i) initiation phase – the MHC disparities and danger signals (PAMPs and DAMPs) are the triggers which activate host innate immune cells and APCs to present antigens activating T-cells, (ii) effector phase – T-lymphocytes activated by APCs home to secondary lymphoid organs and proliferate creating an inflammatory cytokine milieu, (iii) treatment phase – where the main prophylaxis is topical steroids and methylprednisone. The primary target organs are the skin, gastrointestinal tract and liver, with aGVHD being divided into 4 grades based on the severity of symptoms [191, 192].

Chronic GVHD (cGVHD) has a different clinical presentation and usually develops after the first 100 days post transplantation; its symptoms are frequently similar to those caused by autoimmune diseases. It is predominantly mediated by CD4 T-cells polarized into Th2 cells that produce IL-4, IL-5 and IL-11. These Th2 cells are described to be the resultant of faulty negative selection due to thymic damage caused by conditioning regimens. The Th2 cytokines activate the production of fibrogenic cytokines (IL-2, IL-10, TGFβ1), which further activates macrophages to produce platelet-derived growth factor (PDGF) and TGFβ1. These factors promote proliferation of tissue fibroblasts. Further there is dysregulation of B cell activation due to presence of BAFF (B-cell activating factor), all these mechanisms contribute to a systemic autoimmune-like syndrome and predominantly present as symptoms in oral, mucosal, skin, kidneys, lungs, liver and gut tissues [192]. Consequently, chronic

GVHD is major complication limiting the success of HSCT with a 13% fatality rate among recipients [193].

1.10 Immune reconstitution following HSCT

Early after transplantation the susceptibility to infections is pronounced due to strongly suppressed marrow function with very low circulating granulocytes (figure 4). The granulocyte recovery is within 12-14 days with peripheral blood stem cells and a few days

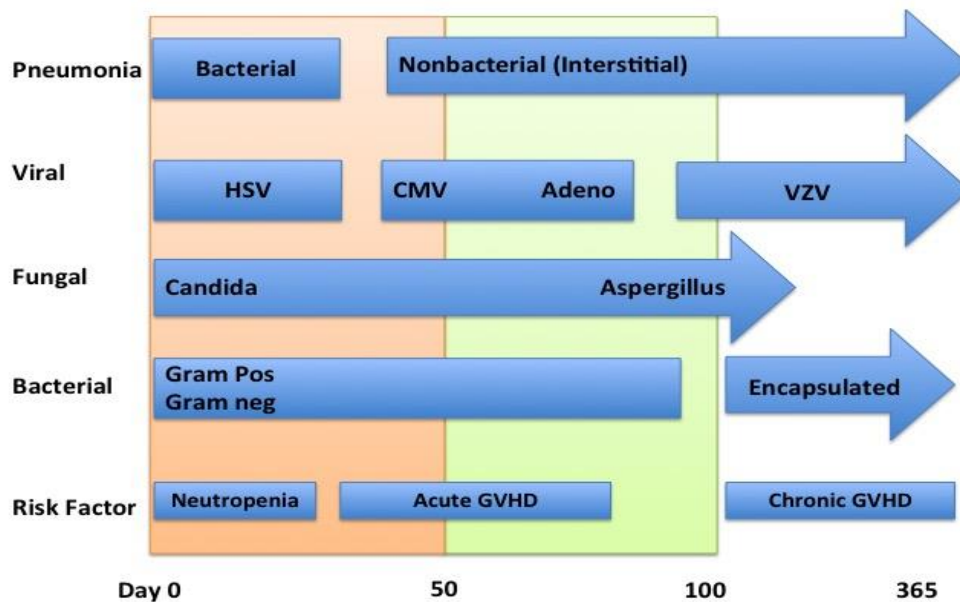

Figure 4 – Types of infections that occur relative to time after marrow infusion or transplantation before the implementation of prophylaxis. Adeno-Adenovirus, CMV-cytomegalovirus, HSV-herpes simplex virus, VZV-varicella zoster virus and GVHD. (Adapted from 2nd edition Transplant infections / editors, Raleigh A. Bowden, Per Ljungman, Carlos V.Paya).

later if bone marrow is used. Monocytes recover within a month but may not be functionally competent for over a year [194], dendritic precursors recover within 6 months and in particular the follicular dendritic cells recover at a relatively slow pace resulting in delayed function of germinal centers and memory B-cells [195]. The NK cells recover rapidly post transplantation reaching optimal levels within a month [196].

The B cell recovery is slow over a period of 1-2 years, though relatively faster rates have been observed after PBSC grafts In addition the B cell recovery is dependent on GVHD and/or its treatment [197-199]. In the process of recovery, the predominant phenotype of B-cells was CD9c, CD38, IgM, and IgD [200]. The immunoglobulin gene usage was skewed towards regions that are seen in neonatal B-cells. The serum immunoglobulins recover in the order of IgM, IgG1 and IgG3, followed much later by IgG2, IgG4 and IgA. These early isotypes are characterized by irrelevant reactivity and consist of autoantibodies; but also may be specific as Wahren et al describe that anti-viral IgG was present early and persisted in some cases up to 12 months after transplantation [201, 202]. The specific reactivity against protein antigens recovers faster than reactivity against polysaccharide antigens. Other studies have indicated that blood stem cell grafts delayed the serum IgG reconstitution as compared

to bone marrow grafts [203]. Vaccination induced antibody responses are frequently sub-optimal especially the first year after transplantation and repeated boosters are almost always needed to achieve similar protection rates as in immune competent individuals.

The CD4 T-lymphocyte recovery is slow; the numbers are typically low over 1 to 3 month period, followed by gradual rise over several years [204]. There is predominantly a memory phenotype of CD4 T-cells characterized by expression of CD45RO, CD11a, HLA-DR and CD29 and to lesser extent CD45RA, CD28 and CD62L. In the absence of chronic GVHD the naïve CD4 T-cell numbers after a year were comparable to normal individuals [205]. The naïve CD4 T-cells recover at a slower pace as compared to the memory CD4 T-cells, due to reduced thymic function and often correlate with age, with younger patients having a faster recovery. PBSC grafts are relatively abundant with both naïve and memory CD4 T-cells and recovery is enhanced as compared to bone marrow grafts [206]. The ability to generate antigen specific responses when stimulated with tetanus toxoid correlated coincided with generation of naïve CD4 T-cells [207]. The T-cell receptor repertoire recovered faster if a patient received cord blood graft as compared to T-cell depleted PBSC graft [208].

The CD8 T-cells are suppressed for 2-3 months, Thereafter, their numbers rise rapidly resulting in a CD4:CD8 inversion ratio, These cells are memory CD8 T-cells characterized by CD45RO, CD11a, CD11b, CD29, CD57 and HLA-DR expression [209]. Some studies described this particular phenotype of CD8 T-cells as suppressor or anergic cells that inhibit cell mediated cytolysis. These CD8 T-cells have been reported to be increased in chronic GVHD [210, 211].

1.11 Influenza infections and risk factors after HSCT

Influenza infections have over the years caused significant morbidity in this particular patient group. During the influenza epidemic of 1991-92 in Houston, Texas, influenza A virus was isolated from 8/28 patients (29%) and among 6 of these HSCT patients with pneumonia, the mortality was 17% [212]. The incidence of influenza was 3.5% in allo-HSCT patients with direct influenza related mortality of 15.3 % in 37 European centers over three seasons 1997-2000. In another retrospective study the documented influenza incidence rate over 12 seasons (1989-2002) in 4797 HSCT patients was 62 (1.3%), 18/62 cases developed pneumonia (29%) and the mortality among these was 5/18 (28%) [213]. During the first season of the pandemic H1N1 influenza, Ljungman et al. conducted the largest published study involving 64 HSCT centers with 286 patients (222 allo-HSCT and 66 auto-HSCT) of whom 125/286 (44%) were hospitalized, 93/286 developed lower respiratory tract infection (LRTI), 11.5% required ventilation support, and the mortality was 6.3% (18/286) [214]. Choi et al reported that pandemic influenza infection in HSCT patients manifested as severe respiratory disease with prolonged viral shedding relative to the seasonal influenza infections [215]. In another evaluation study of 37 HSCT patients, 18 were hospitalized and 6 required ventilation support; the mortality was 7/37 (18.9%) within 30 days of symptom onset and 11/37 (29.7%) within 60 days of symptom onset [216]. Similar mortality rates were observed in many different centers during the pandemic influenza season [217-220].

The main risk factor for severe outcome (LRTI and mortality) has in several studies been reported to be lymphopenia as reviewed by Engelhard et al [219]. Machado et al report that seasonal exposure and intensive conditioning were risk factors for acquiring influenza [221]. In other studies use of a myeloablative conditioning regimen and autologous HSCT were associated with higher LRTI compared to non-myeloablative regimen [222]. Furthermore, chronic steroid use amounting to ≥20mg/day at onset of symptoms was associated with LRTI and mortality [220]. In the multicenter study by Ljungman et al., it was found that older age and lymphopenia were risk factors for LRTI, while influenza onset early after transplant during neutropenia, older age and infection with oseltamivir resistant strains were risk factors associated with mortality [214].

1.12 Influenza vaccination in HSCT recipients

Influenza vaccination is the mainstay for the prevention of influenza in HSCT patients. Most studies have assessed only the antibody responses that generally are poorer than in immune competent individuals. In a study of 48 HSCT patients vaccinated with a seasonal trivalent influenza vaccination, the longer time to vaccination from transplant was a significant variable in eliciting protective antibody responses [223]. In a randomized study including 117 patients, the response rates were 25-34% and the administration of GM-CSF only marginally improved the response [224]. Seasonal influenza vaccination early after transplant within 6 months was ineffective as measured by HI and in some studies before 12 months after HSCT [223, 224]. The only study assessing clinical efficacy although not randomized, showed a protection of 80% in a cohort of 177 HSCT patients [221].

During and after the pandemic influenza 2009, many centers conducted vaccination efficacy studies. Humoral responses were evaluated in 22 adult HSCT patients after ASO3 adjuvanted pandemic influenza vaccination, with seroprotection rates of 45.5 % (10/22) and after a second dose the rate of 73 % (16/22) [225]. Two doses of adjuvanted pandemic vaccine resulted in better rates of seroprotection than a single dose [225-231]. In another study the responses were dependent on chronic GVHD with modest 53% seroprotection as measured by HI. Issa et al observed that after a single dose of non-adjuvanted pandemic influenza vaccine in 82 HSCT patients the seroprotection rate was 51% and dependent on the timing of vaccination from transplant, with patients vaccinated further away from transplant having improved seroprotective rates [232].

Cellular immune responses may be better indicators of immunogenicity by the vaccines and possibly mediate protection against severe influenza especially in the absence of protective HI titers. Haining et al describe a significant increase in influenza specific CD4 T-cell proliferation after vaccination in five pediatric allo-HSCT patients, while there were no increases in influenza specific IgG [233]. Avetisyan et al. show that an influenza specific cellular response could be induced early within 3 months after transplantation when vaccinated with seroprotection rate of 29% among 14 HSCT patients [234]. Increases in IFNγ production and influenza specific CD8 T-cells measured by soluble MHC class I/peptide multimers after seasonal vaccination could be measured, although decreased as compared to

healthy controls in another study [234]. In a study with the pH1N1 vaccine, significantly improved cellular responses ($p=0.008$) were also seen [235]. In another study with the use of both TIV and ASO3 adjuvanted pH1N1 vaccine, CD4 T-cell cross-reactivity to both H1 and H3 antigens could be characterized in allo-HSCT patients [236]. Mariotti et al observed minimal increases in influenza specific CD137+ T-cells as measured at day 50 post vaccination among 9 allo-HSCT patients [237]. Garland et al observed in a prospective study that among 4 allo-HSCT patients with influenza H1N1 infection, all of them exhibited impaired cellular immune responses [238].

2 AIMS

The aim of this thesis was to study and improve influenza vaccination responses in aHSCT recipients since influenza vaccination responses are sub-optimal in aHSCT recipients. In order to first understand correlates of vaccination mediated immune response, a prospective study of over 2000 immune competent individuals was conducted during the H1N1 pandemic influenza season of 2009 in Sweden, following which these specific aims were formulated.

Specific aims

To define humoral immune profiles after a pandemic influenza infection or adjuvanted influenza vaccination in immune competent individuals using a high content influenza proteome peptide microarrays. (Paper I)

To evaluate the benefit of pre-transplant influenza vaccination of aHSCT candidates or their donors on the influenza seroprotection rate in the first months after transplant. (Paper II)

To characterize the antibody and T-cell responses to an adjuvanted virosomal influenza vaccine as compared to the responses to the seasonal non-adjuvanted vaccine in aHSCT recipients. (Paper III)

To determine if pandemic influenza hemagglutinin specific CD8 T-cells generated during the course of natural pandemic influenza infections are phenotypically different as compared to vaccination with the adjuvanted influenza vaccine. (Paper IV)

3 RESULTS AND DISCUSSION

3.1 Pandemic H1N1 viral proteome peptide microarray (Paper I)

Due to the paucity in pandemic influenza epitope data clearly linked to clinical endpoints i.e. epitopes linked to natural pandemic influenza infection and/or pandemrix® vaccination. We designed a high content peptide microarray (figure 5) containing the entire proteome of pandemic Influenza A virus (A/California/08/2009(H1N1)) proteome and hemagglutinin proteins from 12 other influenza A subtypes (2304 unique features) to answer these questions i) what is the immune recognition pattern that segregates individuals at high risk to be infected with influenza, ii) what are the differences in IgG recognition patterns induced by vaccination versus infection, iii) and if there exist shared epitope recognition patterns comparing different hemagglutinin proteins from influenza A strains, including the hemagglutinin from the pandemic strain Influenza A virus (A/South Carolina/1/1918(H1N1). This was possible in the current study using the serum aliquots from a prospective study [239-241], where 2000 individuals were followed before and after vaccination, or H1N1 infection, respectively. We identified 19 individuals with a positive pandemic H1N1 RNA Swab with a pre-infection and post-infection serum samples. These were used to assay the detailed Flu epitope recognition pattern. Serum samples from 19 (age and sex-matched) individuals vaccinated with the AS03 adjuvanted vaccine against pandemic H1N1 influenza (Pandemrix®) were used as controls.

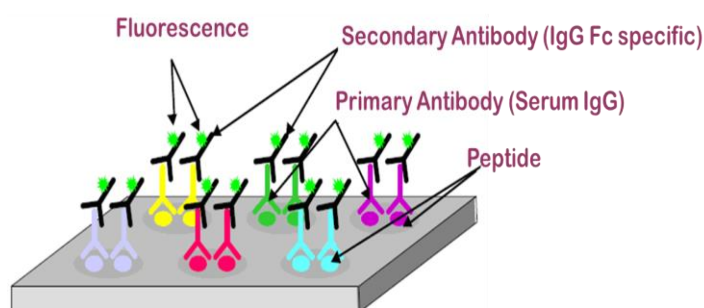

Figure 5 – Principle of peptide microarray (courtesy- Davide Valentini)

The prospective design of the study allowed characterizing serum-epitope recognition patterns before and after pandemic influenza infection and/ or pandemrix® vaccination. Clusters of 262 epitopes were differentially recognized before the pandemic season i.e. before pandemic flu infection/vaccination. Following either pandemic flu infection or pandemrix® vaccination a cluster of 250 epitopes were differentially recognized.

Pre-existing pandemic influenza specific IgG before the flu season in individuals with previous vaccinations was observed, 10 epitopes from the pandemic strain (6 from polymerase PA, 2 from matrix1, 1 from polymerase PB1 and 1 epitope from hemagglutinin VEPGDKITFEATGNL). In contrast Only 2 epitopes from the 2009 pandemic flu strain were recognized in the serum from individuals who later developed a pandemic flu infection, i.e. polymerase PA (GRDRIMAWTVVNSIC) and neuraminidase (NFSIKQDIVGINEWS). Pre-existing serum IgG to pandemic hemagglutinin in vaccinated individuals before the onset of the flu season probably due to past exposures [242-245] and previous vaccinations (the interviews of the study participants showed that individuals who chose to be vaccinated did also previously prior to 2009/2010 and *vice versa* [241]). Seven epitopes from flu internal

proteins in serum from individuals who chose to be vaccinated were recognized. These non-neutralizing antibodies might decrease morbidity and lead to increased viral clearance through binding to FcRs and subsequent activation of CD8 T-cells [246-250]. Conserved influenza internal proteins may therefore aid to elicit memory B-cells that are long-lived [251, 252] and contribute to heterosubtypic immunity during antigenic drifts.

Pandemic Influenza infection generated new neuraminidase specific IgG reactivity leading to the recognition of the epitopes QGALLNDKHSNGTIK, NDKHSNGTIKDRSPY from neuraminidase [Influenza A virus (A/California/08/2009(H1N1)]. Moreover, in the absence of neutralizing HA antibodies, neuraminidase (NA) specific antibodies would be instrumental to mount protective immune responses against the pandemic virus [253]. Of note, we observed in serum from the current cohort an increase in NA epitope reactivity, while no pandemic H1 epitopes were commonly recognized.

Differential serum IgG recognition was highly focused on hemagglutinin (H1) and was restricted to classical H1 antigenic sites in the serum from individuals both before and after pandemic infection and vaccination. The peptide epitope SRYSKKFKPEIAARP from the HA [Influenza A virus (A/Swine/Indiana/P12439/00 (H1N2)] was strongly recognized in serum from individuals both prior to and after flu infection (2.50 and 2.60 fold change respectively), this epitope belongs to the Ca antigenic site on the H1 and is highly homologous to the SRYSKKFKPEIAIRP epitope from HA [Influenza A virus (A/California/08/2009(H1N1)] on the HA receptor binding domain. Antibodies against swine influenza virus were found to neutralize pandemic flu in experimental models [69, 254], at present there is insufficient evidence for the beneficial role of these antibodies in humans. However non-neutralizing antibodies produced against the swine origin H1 - due to past exposures - could enhance virus fusion and promote infection [255]. Most significantly the subjects in the current study who experienced H1N1 infection exhibited increased levels of IgG against swine origin SRYSKKFKPEIAARP.

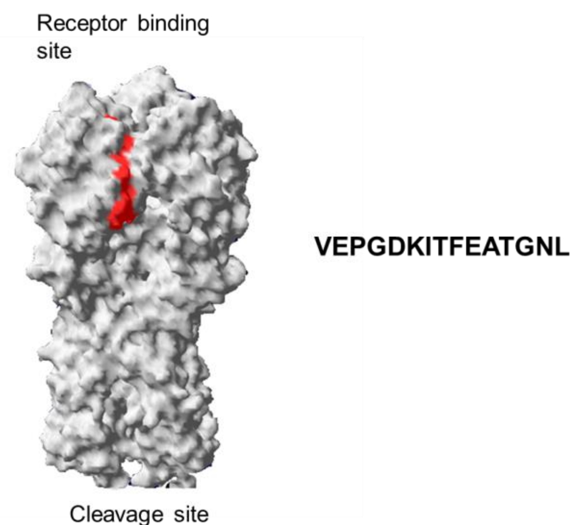

Figure 6 - The epitope VEPGDKITFEATGNL (highlighted in red) exclusively recognized in serum from individuals (n=17) who were vaccinated mapped on the crystal structure of the 2009 H1N1 influenza virus hemagglutinin receptor-binding domain (PDB ID - 3LZG)

A novel epitope VEPGDKITFEATGNL on the antigenic site of the pandemic flu hemagglutinin was exclusively recognized in serum from individuals prior to flu season (16/19 individuals). This was also found to be true for the post-flu season period

(serum from 17/19 individuals). Consequently, we further mapped the epitope VEPGDKITFEATGNL (251-265aa) from hemagglutinin [Influenza A virus (A/California/08/2009(H1N1))] using the PDB entry 3LZG (figure 6) and 3MLH of the Crystal structure of the 2009 H1N1 influenza virus hemagglutinin receptor-binding domain [256]. This reactivity was completely absent from the IgG-epitope recognition repertoire of the flu-infected individuals prior to and after the flu season. Most likely, this epitope represents a dominant response associated with previous Flu vaccination. A rabbit mono-specific antibody directed against this epitope was prepared but did not neutralize the pandemic flu in-vitro; functional significance of this epitope could not be established.

Although the current study may be limited due to the fact that only IgG directed against linear epitopes could be characterized with conformational epitopes being missed, however a distinct IgG recognition pattern was identified. These patterns segregated based on H1N1 infection and vaccination and could be clearly linked to a clinically relevant endpoint: pandemic influenza infection leads to a different array of epitope recognition as compared to the Pandemrix® vaccine.

3.2 Evaluation of pre-transplant influenza vaccination in aHSCT (Paper II)

Pre-transplant vaccination of the donor, the recipient, or both has been evaluated previously in the context of hepatitis, tetanus, polio, and pneumococcal antigens and found to positively influence antigen specific IgG levels in the first year after transplant [257-260]. However, this has not been studied previously for vaccination against influenza. Accordingly, a randomized prospective study was conducted from Oct 2007 to January 2010 in two Brazilian HSCT centers to evaluate alternative schedules of pre-transplant influenza vaccination in an aHSCT setting.

122 HSCT recipients and their donors were assigned to three randomization groups: No pre-transplant vaccination (n=38) (group1), donor pre-transplant vaccination (n=44) (group 2), or recipient pre-transplant vaccination (n=40) (group 3).

Donor serum samples were taken at study admission (baseline), and around the day of HSCT. Recipient serum samples were taken at baseline and on days zero (dzero), +30 (d30), +60 (d60), +100 (d100), +180 (d180) and after d180 vaccination. Specific serum IgG was assessed by both hemagglutinin inhibition (HI) and, in 57 patients; by an indirect influenza-specific ELISA at specified times after HSCT. Seroprotection was defined by the presence of HI Ab ≥ 1:40. Seroresponse was defined by ≥ 4-fold rise in HI Ab titers after vaccination.

Donors belonging to the randomization group 2 had seroprotective HI Ab titers against H1 and H3 antigens compared to donors belonging to groups 1 and 3 (p<0.001). The recipient seroprotection rates at baseline for H1(18.5%), H3 (33.6%) and B (64.7%) antigens. 40 HSCT candidates assigned to group 3 received pre-transplant vaccinations at a median of 10.5 days before transplantation. Seroresponse rates evaluated in 39 patients upto day 30 were H1 (17.9%), H3 (25.6%) and B (30.8%) antigens.

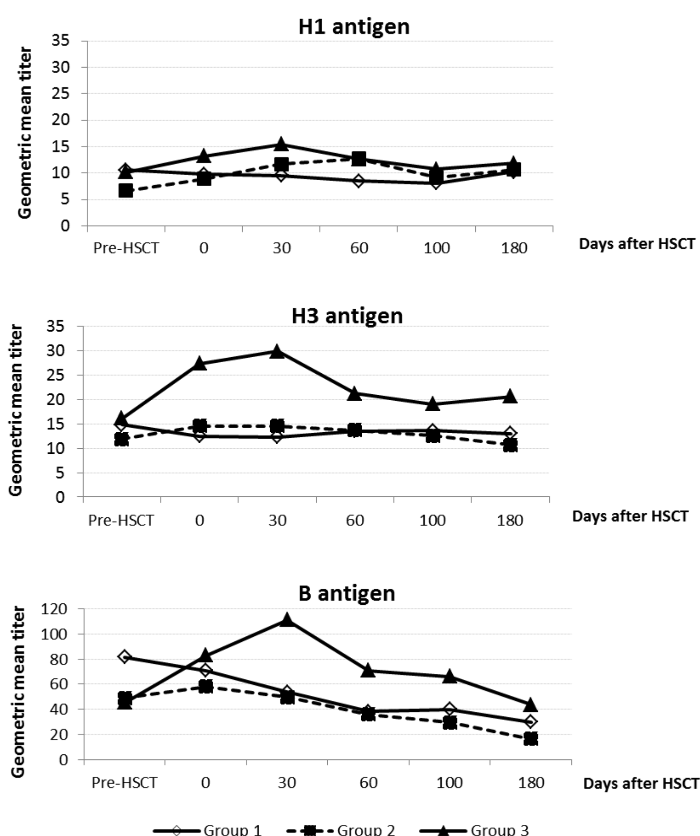

Figure 7 - Dynamics of HI IgG titers up to day 180 according to randomization and different influenza antigens

During the follow-up the geometric titers (GMT) against H1 (p=0.028) and H3 (p<0.001) were highest in the pre-transplant recipient vaccination group (group 3) until d.180 after transplantation (figure 7).

Influenza specific IgG was analyzed using an indirect ELISA assay in a subgroup of 57 patients; 17 from the group 1, 20 from the group 2, and 20 from group 3. There was a significant difference in the antigen specific serum IgG level against A/H1N1/California/2009 between the 3 study groups at 6 months (day 180) after HSCT with the strongest increase seen in serum from recipients vaccinated pre-transplant (group3) (p=0.02) (figure 8). In addition to HI, we assessed specific IgG using an indirect ELISA to broaden the assessment of the humoral responses, since the ELISA measures IgG against epitopes from the hemagglutinin, the neuraminidase, and trace amounts of the matrix 1 protein [261-264], apart from the neutralization epitopes of HA; these other epitopes may be beneficial in ADCC and complement fixation.

The influenza vaccine was administered to 63 of the 122 recipients (51.6%), at a median of 193 (145 to 281d) days after transplantation. The seroresponse was evaluated in 59 patients; we could not identify any significant variables associated with seroprotective titers on day 180.After d180 vaccination, recipient seroresponse rates were also poor when assessed by HI, varying from 8% (antigen B) to 20% (H1) in concordance with previous studies [225, 236, 265, 266].

The primary study objective was to improve recipient seroprotection during the first months after transplantation; a period in which influenza vaccine is frequently ineffective [223]. The titer at baseline was significantly associated with protective titers to all three influenza antigens at d60 in accordance with previous observations that if either donor or recipient is immunized pre-transplant, the likelihood of transfer or persistence of immunity is increased [260, 267-269]. Although we did not see a significant effect of donor pre-transplant vaccination, the pre-transplant influenza vaccination of the HSCT candidate significantly improved the antibody titers to H1 and H3 antigens until day 180. An earlier post-transplant vaccination would have been beneficial to prolong the immunity as seen in other studies assessing vaccination responses to other target antigens [270-273]. Despite the poor immunogenicity of the vaccine reflected by the low seroresponse and seroprotective rates during follow-up, the pre-transplant vaccine efficacy was higher than 65%. Future vaccination strategy might be to vaccinate the recipient pre-transplant and boost this response early within 3 months after transplantation in the recipient. Efforts should also therefore be made to limit the risk of influenza exposure in HSCT recipients.

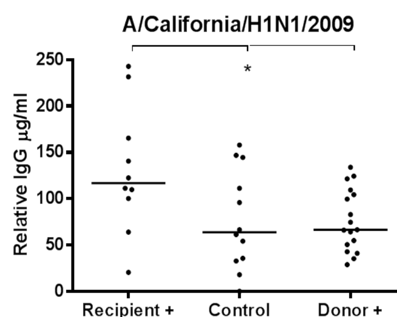

Figure 8 - Influenza specific relative serum IgG assayed by an indirect ELISA in a sub-group (n= 57) at day 180. Medians are indicated (p<0.05*). (Recipient + group 3, Control- group 1, Donor + group 2).

3.3 Immunogenicity of virosomal adjuvanted trivalent influenza vaccination in aHSCT (Paper III)

Since the seasonal subunit vaccination response is frequently sub-optimal, it is important to evaluate other possibly more efficient vaccination systems. The immunogenicity after a single dose (0.5 mL) of adjuvanted trivalent virosomal vaccination was therefore evaluated in a cohort of 21 HSCT recipients and compared to a control cohort of 30 HSCT recipients who received a single dose (0.5 mL) of non-adjuvanted seasonal trivalent subunit vaccination over four seasons from 2010-2014.

Serum and blood samples were collected before and at 4 weeks after respective vaccination Whole blood IFNγ (gamma-interferon) release assays were tested both prior to and 30 days after vaccination in response to influenza pandemic (pdm) H1N1, H3N2 and B antigens. HLA-A*02 dextramers (GILGFVFTL Matrix 1 protein), (RLATGLRNV hemagglutinin (pdm 2009)) to gauge for the absolute number of antigen-specific CD8 T-cells, and pandemic 2009 hemagglutinin inhibition (HI) assays, to test for neutralizing antibodies, were used as immunological readouts.

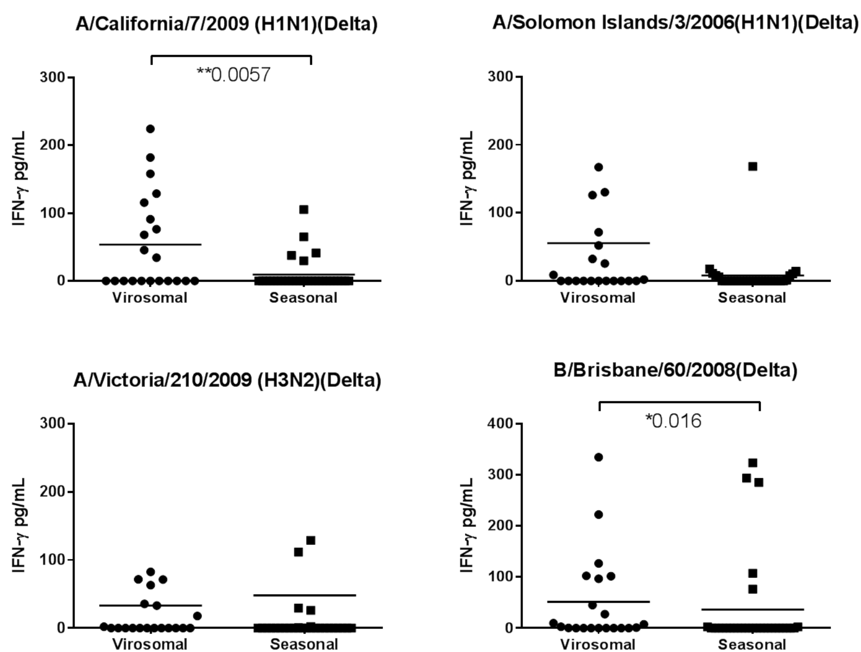

Figure 9 -δ production of IFN-γ in response to influenza monovalent antigens in the two cohorts

The hemagglutinin inhibition (HI) titer responses to pandemic H1 were poor in both cohorts, with only 13.3 % (4/30) after the seasonal vaccination and 23% (5/21) after virosomal vaccination exhibiting protective titers. There were no differences in HI titers if the recipient was vaccinated earlier or later than 6 months post transplantation in both cohorts.

After virosomal vaccination, the median antigen-specific IFNγ production increased 5-fold from pre-vaccination to post vaccination (8.7pg/mL to 45.5pg/mL) in response to the. A/California/H1N1/2009 antigen (p=0.05). This response is in contrast to the response after

seasonal non-adjuvanted vaccination, where there was no increase in median IFNγ production after vaccination in the response to the A/California/H1N1/2009 antigen (p=0.19).

The increase in cytokine production from pre to post vaccination response, i.e the δ–production response was calculated. The virosomal vaccine induced significantly higher δ-IFNγ responses (p<0.01) to A/California/7/2009(H1N1) with a trend to (p=0.07) increased δ IFNγ production in response to the generic H1N1 strain (A/Solomon Islands/3/2006(H1N1) and a significantly (p=0.02) increased δ IFNγ response to B/Brisbane/60/2008 as compared to the seasonal non-adjuvanted vaccine. Patients following virosomal vaccination later than 6 months after HSCT had significantly higher IFNγ production (p<0.001) in response to A/California/7/2009(H1N1) and significantly higher δ (delta) (p=0.006) values as compared to the control cohort that received the non-adjuvanted vaccine (figure 9).

The matrix 1 protein specific HLA A*02- GILGFVFTL restricted CD8 T-cells were enumerated in a subgroup of patients in the two vaccination cohorts by flow cytometric analysis. Analysis of PBMCs from patients vaccinated with the virosomal vaccine exhibited increased frequencies of matrix 1 specific CD8 T-cells (p=0.01) measured after vaccination while there was no difference in the control cohort (p=0.1) after non-adjuvanted vaccination (figure 10). No significant differences were observed in the frequency of pandemic influenza hemagglutinin specific HLA A*02- RLATGLRNV restricted CD8 T cells in the peripheral blood between the cohorts.

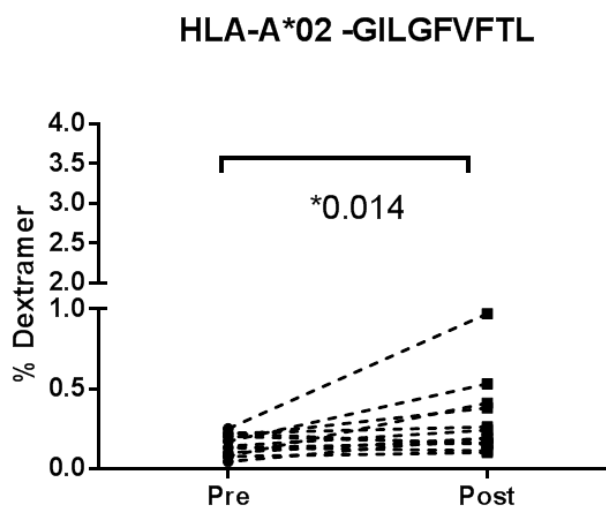

Figure 10 - Influenza matrix 1 GILGFVFTL specific and HLA-A*02 restricted CD8 T cells were analysed in the study cohort (n=13) after

The virosomal vaccine did not improve the serological responses compared to the standard vaccine. This reflect most likely the dysfunctional humoral immune response during the first year after stem cell transplantation [274], it is possible that a two-dose adjuvanted vaccine might have had a better response as observed using the AS03 adjuvanted vaccine [225, 228].

In contrast to the poor serological responses, we found that the adjuvanted virosomal trivalent vaccine was capable to elicit robust IFNγ production in aHSCT patients. And there could be several reasons for this improved response: i) the virosome resembles the native viral structure and has similar cell entry and functional receptor binding characteristics [275] ii) the virosome sufficiently induces the maturation of dendritic cells as measured by up regulation of MHC class I, MHC class II, ICAM-1, B7.1, B7.2 and CD40 [276] (iii) the T helper cell

and CTL compartment is potently activated by virosome as reflected by increased IFNγ production [277, 278].

We also observed significant increases in Matrix 1 HLA-A*02 restricted CD8 T-cells in blood from patients who received the virosomal vaccine. This could be attributed to the fact that both the virosomal and seasonal subunit vaccines are manufactured from purified monovalent bulks that could have traces of matrix 1 protein [261-264, 279] that might promote matrix 1 specific cellular immune responses. Influenza specific MHC class I restricted CD8 T-cells studied previously following non-adjuvanted influenza vaccination elicited minimal increases in antigen specific CD8 T-cell frequencies [234].

The time to vaccination was a crucial variable with poorer responses in both cohorts prior to 6 months after HSCT while the virosomal vaccine elicited better responses in the group vaccinated >6M after HSCT. This maybe in part due to an improved immunostimulatory effect on the PRRs of dendritic cells and macrophages; reflecting a native virus, in the absence of B cells after HSCT [275]. The influenza specific cellular immune responses may modify the course of the influenza infection, since it has been shown in elderly individuals that the risk for influenza disease was comparable in individuals demonstrating a cell-mediated response alone, an antibody response alone, or both types of responses [235].

3.4 Influenza specific CD3+CD8+ cytotoxic lymphocytes reside in precursor CD45RA+CCR7+ T-cells in individuals after pandemic influenza (Paper IV)

CD8 T-cells have been implicated in direct killing of influenza-infected cells reducing greatly disease severity; a cellular immune response is often correlated with vaccine-induced protection [280, 281]. The majority of CD8 T-cell epitopes that have been reported are present in the internal conserved proteins of the pandemic influenza [282].We showed previously in paper 1 that the AS03 adjuvanted influenza vaccine induced a different humoral response profile as compared to the natural pandemic flu infection in the current cohort [283], further we asked if the same phenomenon applies to cellular immune responses i.e. if pandemic hemagglutinin specific CD8 T-cells generated during a flu infection and after the vaccination have different immune memory profiles.

Nine HLA-A*02 individuals with a positive pandemic 2009 H1N1 RNA Swab and blood draws at onset of infection (September-November 2009), 2 weeks after onset of symptoms, as well as 6 months post-infection (April-May 2010) were studied. Blood from 8 (age and sex-matched) HLA-A*02 individuals before (September-November 2009) and 6 months (April-May 2010) after adjuvanted vaccination against pandemic H1N1 influenza (Pandemrix®) were used as controls. MHC class I restricted T-cells directed against the newly identified pandemic Influenza hemagglutinin peptide RLATGLRNV and the Matrix 1 peptide GILGFVFTL. HLA-A*02 restricted CD8 T-cells and their memory phenotype was characterized by flow cytometry, defined by CD45RA and CCR7 expression. Whole blood influenza antigen specific IFNγ release assays were performed to quantify the effector IFNγ production in PBMCs.

At the onset of pandemic influenza season, the median frequencies of A*02 RLATGLRNV hemagglutinin (HA) restricted CD8 T-cells were higher (6.28%, with a trend to significance (p=0.05) in the PBMCs from the control group as compared to PBMCs from individuals (2.7%) who later contracted a pandemic flu infection. HA specific terminal effector memory CD8 T-cells (CD45RA+CCR7-) in the PBMCs from control individuals was higher (45.8%) as compared to the PBMCs (21.3 %) in susceptible individuals who later contracted influenza infection. These individuals also exhibited higher median frequencies (59.3%) of T-cells exhibiting the precursor phenotype characterized by co-expression of CD45RA+ and CCR7+ as compared to T-cells from individuals in the control group (28.25%).

Influenza hemagglutinin specific CD8 T-cells during the pandemic infection were predominantly in the precursor phenotype with co-expression of CD45RA+ CCR7+ (median 55.5%, i.e. significantly higher, p<0.05) than other T-cell compartments (central memory- 3.1 %, effector memory – 13.1% and terminal effectors – 27.9%).

Following the pandemic influenza infection, central memory hemagglutinin specific CD8 T–cells (CD45RA-CCR7+) had a trend to increased frequencies (p=0.05) and a significant decrease (p=0.02) in terminal effector (CD45RA+CCR7-) in relation to PBMCs tested prior to pandemic influenza infection. Following the pandemrix® vaccination, a significant increase

(p=0.03) in the effector hemagglutinin specific CD8 T-cells (CD45RA-CCR7-) and trend to decreased precursor hemagglutinin specific CD8 T-cells (CD45RA+CCR7+) in relation to PBMCs tested prior to pandemrix® vaccination was observed. Influenza hemagglutinin (pdm 2009) restricted CD8 T-cells after vaccination are concentrated in the terminally differentiated effector pool and significantly different (p=0.003) in relation to PBMCs tested from individuals after pandemic influenza infection.

We did not find any significant differences in the frequencies of Matrix 1 GILGFVFTL. HLA-A*02 restricted CD8 T-cells in PBMCs prior to or following either pandemic influenza infection or vaccination. Of importance was that M1 specific CD8 T-cells in PBMCs from individuals after pandemic influenza exhibited higher frequencies of central memory CD8 T-cells (p=0.06) compared to the group that was vaccinated in contrast to the PBMCs following flu vaccination where the M1 specific CD8 T-cells resided in the terminal effector memory pool after Flu vaccination: and significantly higher (p=0.003).

We observed higher frequencies of influenza hemagglutinin precursor CD8 T-cells in PBMCs before pandemic flu infection and a significant increase following pandemic influenza infection as compared to PBMCs from individuals after the flu vaccination. This could have two implications (i) The presence of these antigen specific cells in this phenotype is detrimental to the individual and reportedly can correlate with a symptomatic influenza infection as observed in murine models [284] (ii) These are beneficial antigen-experienced T-cells with a precursor like phenotype that have been observed in other infectious disease contexts [285-288]. These antigen-experienced T-cells are able to differentiate into other T-cell memory and effector CD117+ (c-kit) CD45RA+CCR7+ T-cell subsets with capacity to produce IFNγ [289-291].

The clinical relevance of such subset has to be further studied and the cytokine patterns have to be characterized. Identification of 'multipotent' precursor-like memory-cells in the course of Flu infection could be clinically relevant, particularly in the development of improved Flu vaccines. In conclusion, influenza vaccination with AS03 adjuvanted vaccine induced a different memory profile of Influenza specific CD8 T-cells as compared to the natural pandemic flu infection.

4 CONCLUSIONS

Influenza infections can represent important complications after aHSCT resulting in lower respiratory tract infections and mortality. Preventive strategies such as influenza vaccination are therefore important. Since the current immune responses to vaccination are frequently suboptimal, we evaluated alternative vaccination systems and vaccination schedules to improve vaccination efficacy. In addition we identified correlates of influenza vaccine mediated immune responses in immunocompetent individuals.

In our **paper I**, we asked if the pandemic influenza infection generated a different humoral immune profile as compared to influenza vaccination.

Our paper I resulted in new insights into the influenza epitope serum IgG recognition patterns and these could be clearly linked to clinical endpoints i.e. before and after pandemic influenza infection or vaccination. The individuals with symptomatic influenza infection completely lacked any serum IgG response to pandemic hemagglutinin linear epitopes studied by our technique, which may have contributed to this outcome. Further these individuals had a very dominant IgG response to hemagglutinin epitopes from swine H1N1 strains in contrast to the individuals who did not have the symptomatic influenza infection. We also found that absence of reactivity to pandemic hemagglutinin epitope $_{(251\text{-}265)}$ could be a predictor for susceptibility to symptomatic pandemic influenza infection. These findings should be further characterized retrospectively in serum aliquots from larger cohorts that are available from the pandemic H1N1 season of 2009-2010.

In our **paper II**, we asked if there is any beneficial effect of pre-transplant influenza vaccination on seroprotection early after transplant.

Pre-transplant recipient vaccination in particular was significantly associated with protective responses to H1 and H3 antigens up to day 180 after transplantation. Retention of vaccine antigens or antigen-antibody immune complexes in the recipient's follicular dendritic cells [292, 293] post HSCT may drive the proliferation of donor lymphocytes. Another finding was that response to vaccination before HSCT was very poor and most patients who had seroprotective titers on HSCT day were able to maintain them until d60. We did not notice any effect of pre-transplant vaccination on the day 180 vaccination sero-responses, which were poor (8 % to antigen B and 20% to antigen H1). An earlier vaccination within three months post-transplant would have been beneficial similar to what have been seen in studies with other antigens such as tetanus toxoid.

In our **paper III**, we asked if an influenza adjuvanted virosomal vaccination would result in improved immune responses compared to non-adjuvanted seasonal vaccination in aHSCT recipients

An enhanced cellular immune response to influenza pandemic (pdm) H1N1 antigen following a virosomal vaccination as compared to the seasonal non-adjuvanted vaccination was found. This can be attributed to the virosomal formulation that reflects closely the native

virus in having potent immunostimulatory effects leading to enhanced cellular immune responses. We did not see any beneficial effect on the seroresponse rates after virosomal vaccination as compared to non-adjuvanted vaccination maybe due to impaired B-cell compartments. Further, we observed poor IFNγ and serum HI titers in recipients early after transplant (≤6 months), where they are most vulnerable, Additional studies are necessary to improve the response rates in this period.

In our **paper IV**, we asked if the presence and/or memory phenotype of HLA-A*02 influenza pandemic hemagglutinin peptide restricted CD8 T-cells correlate with either symptomatic influenza or adjuvanted AS03 vaccination

Although there were no significant differences in the frequency of HLA-A*02 influenza pandemic hemagglutinin peptide restricted CD8 T-cells after pandemic influenza infection and vaccination, the δ frequency of antigen specific T-cells was significantly higher after adjuvanted vaccination. We also observed that these antigen-specific T-cells belonged to a terminally differentiated effector memory phenotype characterized by CD45RA+ CCR7- in individuals after vaccination, in contrast to PBMCS obtained from individuals after symptomatic influenza infection. Here, the antigen specific T-cells resided in the precursor/naïve phenotype characterized by CD45RA+ CCR7+. We conclude that the cellular immune signatures are different in a natural influenza infection and vaccination.

5 FUTURE PERSPECTIVES

Influenza infections are a constant threat to immunocompetent and immunosuppressed individuals. Improved understanding of protective immune responses will greatly benefit in rational vaccine designs. Future work should unravel the functional significance of IgG reactivity to VEPGDKITFEATGNL, the epitope on the antigenic site of the pandemic flu hemagglutinin. The antigen specific plasma cells from these vaccinated individuals could be further characterized using deep sequencing of their antigen receptors and compared to plasma cells from individuals after influenza infection.

Further influenza vaccination studies should address a 2-dose strategy by priming the patient early after HSCT (<6M) with an adjuvanted vaccine followed by a yearly boosting immunization with a non-adjuvanted vaccine matched for antigens during the influenza season. Another strategy could be the vaccination of related donors pre-transplantation with an adjuvanted influenza vaccine that may have beneficial seroprotective effect early after transplant. A higher dose of influenza vaccine (60µg HA) administered intramuscularly or intradermal TIV with regular dose (15µg HA) could also be tested in HSCT patients, which may promote superior response as seen in previous studies in older adults [294, 295].

Since we identified different memory profiles of influenza of HLA-A*02 influenza pandemic hemagglutinin peptide restricted CD8 T-cells in natural influenza infection and vaccination, the effector cytokine profiles of these specific cells will have to be addressed, i.e. if a symptomatic influenza infection is due to polyfunctionality or *vice versa*. Deep sequencing of the TCR Vβ repertoire would allow visualization of dynamics of TCR usage prior to and following pandemic influenza infection/vaccination: it would demonstrate whether the existing antigen specific T-cell pool is expanded or rather new clonotypes are being recruited upon influenza infection/vaccination The detailed functional analysis of the anti-Flu T-cell responses will provide interesting cues whether protective and long-lasting immune responses are rather focused (defined by the limited use TCRs directed against the nominal target), or rather oligoclonal/polyclonal with several TCRs targeting MHC class I-peptide complexes.

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7 REFERENCES

1. Medzhitov R, Janeway Jr CA: **Innate Immunity: The Virtues of a Nonclonal System of Recognition**. *Cell* 1997, **91**(3):295-298.

2. Janeway CA, Jr.: **Approaching the asymptote? Evolution and revolution in immunology**. *Cold Spring Harbor symposia on quantitative biology* 1989, **54 Pt 1**:1-13.

3. Janeway CA, Jr., Medzhitov R: **Innate immune recognition**. *Annual review of immunology* 2002, **20**:197-216.

4. Williams BR: **PKR; a sentinel kinase for cellular stress**. *Oncogene* 1999, **18**(45):6112-6120.

5. Kumar M, Carmichael GG: **Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes**. *Microbiology and molecular biology reviews : MMBR* 1998, **62**(4):1415-1434.

6. Bertin J, Nir W-J, Fischer CM, Tayber OV, Errada PR, Grant JR, Keilty JJ, Gosselin ML, Robison KE, Wong GHW *et al*: **Human CARD4 Protein Is a Novel CED-4/Apaf-1 Cell Death Family Member That Activates NF-κB**. *Journal of Biological Chemistry* 1999, **274**(19):12955-12958.

7. Latz E, Xiao TS, Stutz A: **Activation and regulation of the inflammasomes**. *Nat Rev Immunol* 2013, **13**(6):397-411.

8. Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, Fenton MJ, Oikawa M, Qureshi N, Monks B *et al*: **Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide**. *The Journal of clinical investigation* 2000, **105**(4):497-504.

9. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S: **Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components**. *Immunity* 1999, **11**(4):443-451.

10. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A: **The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5**. *Nature* 2001, **410**(6832):1099-1103.

11. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K *et al*: **A Toll-like receptor recognizes bacterial DNA**. *Nature* 2000, **408**(6813):740-745.

12. Blasius AL, Beutler B: **Intracellular Toll-like Receptors**. *Immunity* 2010, **32**(3):305-315.

13. Akira S, Takeda K: **Toll-like receptor signalling**. *Nat Rev Immunol* 2004, **4**(7):499-511.

14. Murray PJ, Wynn TA: **Protective and pathogenic functions of macrophage subsets**. *Nat Rev Immunol* 2011, **11**(11):723-737.

15. Murray PJ, Wynn TA: **Obstacles and opportunities for understanding macrophage polarization**. *Journal of leukocyte biology* 2011, **89**(4):557-563.

16. Murray Peter J, Allen Judith E, Biswas Subhra K, Fisher Edward A, Gilroy Derek W, Goerdt S, Gordon S, Hamilton John A, Ivashkiv Lionel B, Lawrence T *et al*:

Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* 2014, **41**(1):14-20.

17. Merad M, Sathe P, Helft J, Miller J, Mortha A: **The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting**. *Annual review of immunology* 2013, **31**:563-604.

18. Abraham SN, St. John AL: **Mast cell-orchestrated immunity to pathogens**. *Nat Rev Immunol* 2010, **10**(6):440-452.

19. Bryceson YT, Chiang SC, Darmanin S, Fauriat C, Schlums H, Theorell J, Wood SM: **Molecular mechanisms of natural killer cell activation**. *Journal of innate immunity* 2011, **3**(3):216-226.

20. Lanier LL: **Up on the tightrope: natural killer cell activation and inhibition**. *Nature immunology* 2008, **9**(5):495-502.

21. Caligiuri MA: **Human natural killer cells**. *Blood* 2008, **112**(3):461-469.

22. Karre K, Ljunggren HG, Piontek G, Kiessling R: **Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy**. *Nature* 1986, **319**(6055):675-678.

23. Anfossi N, Andre P, Guia S, Falk CS, Roetynck S, Stewart CA, Breso V, Frassati C, Reviron D, Middleton D *et al*: **Human NK cell education by inhibitory receptors for MHC class I**. *Immunity* 2006, **25**(2):331-342.

24. Pegram HJ, Andrews DM, Smyth MJ, Darcy PK, Kershaw MH: **Activating and inhibitory receptors of natural killer cells**. *Immunology and cell biology* 2011, **89**(2):216-224.

25. Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH *et al*: **HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C**. *Nature* 1998, **391**(6669):795-799.

26. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, Affaticati P, Gilfillan S, Lantz O: **Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1**. *Nature* 2003, **422**(6928):164-169.

27. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, Bhati M, Chen Z, Kostenko L, Reantragoon R *et al*: **MR1 presents microbial vitamin B metabolites to MAIT cells**. *Nature* 2012, **491**(7426):717-723.

28. Bukowski JF, Morita CT, Brenner MB: **Recognition and destruction of virus-infected cells by human gamma delta CTL**. *Journal of immunology (Baltimore, Md : 1950)* 1994, **153**(11):5133-5140.

29. Qin G, Mao H, Zheng J, Sia SF, Liu Y, Chan PL, Lam KT, Peiris JS, Lau YL, Tu W: **Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses**. *The Journal of infectious diseases* 2009, **200**(6):858-865.

30. Scott-Browne JP, Matsuda JL, Mallevaey T, White J, Borg NA, McCluskey J, Rossjohn J, Kappler J, Marrack P, Gapin L: **Germline-encoded recognition of diverse glycolipids by natural killer T cells**. *Nat Immunol* 2007, **8**(10):1105-1113.

31. Kok WL, Denney L, Benam K, Cole S, Clelland C, McMichael AJ, Ho LP: **Pivotal Advance: Invariant NKT cells reduce accumulation of inflammatory monocytes**

in the lungs and decrease immune-pathology during severe influenza A virus infection. *Journal of leukocyte biology* 2012, **91**(3):357-368.

32. Spits H, Di Santo JP: **The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling**. *Nat Immunol* 2011, **12**(1):21-27.

33. Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eisley CJ, Erle DJ, Locksley RM: **Systemically dispersed innate IL-13-expressing cells in type 2 immunity**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(25):11489-11494.

34. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CGK, Doering TA, Angelosanto JM, Laidlaw BJ, Yang CY, Sathaliyawala T *et al*: **Innate lymphoid cells promote lung tissue homeostasis following acute influenza virus infection**. *Nature Immunology* 2011, **12**(11):1045-1054.

35. Dunkelberger JR, Song W-C: **Complement and its role in innate and adaptive immune responses**. *Cell Res* 2009, **20**(1):34-50.

36. Blum JS, Wearsch PA, Cresswell P: **Pathways of Antigen Processing**. *Annual review of immunology* 2013, **31**(1):443-473.

37. Crotty S: **Follicular helper CD4 T cells (TFH)**. *Annual review of immunology* 2011, **29**:621-663.

38. Tan C, Gery I: **The Unique Features of Th9 Cells and their Products**. *Critical Reviews in Immunology* 2012, **32**(1):1-10.

39. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F: **Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells**. *Nat Immunol* 2009, **10**(8):857-863.

40. Okoye IS, Wilson MS: **CD4+ T helper 2 cells--microbial triggers, differentiation requirements and effector functions**. *Immunology* 2011, **134**(4):368-377.

41. Kasaian MT, Whitters MJ, Carter LL, Lowe LD, Jussif JM, Deng B, Johnson KA, Witek JS, Senices M, Konz RF *et al*: **IL-21 Limits NK Cell Responses and Promotes Antigen-Specific T Cell Activation: A Mediator of the Transition from Innate to Adaptive Immunity**. *Immunity* 2002, **16**(4):559-569.

42. Liston A, Gray DHD: **Homeostatic control of regulatory T cell diversity**. *Nat Rev Immunol* 2014, **14**(3):154-165.

43. Harty JT, Tvinnereim AR, White DW: **CD8+ T Cell Effector Mechanisms in Resistance to Infection**. *Annual review of immunology* 2000, **18**(1):275-308.

44. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP: **CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes**. *Nature* 2003, **421**(6925):852-856.

45. Ochsenbein AF, Riddell SR, Brown M, Corey L, Baerlocher GM, Lansdorp PM, Greenberg PD: **CD27 expression promotes long-term survival of functional effector-memory CD8+ cytotoxic T lymphocytes in HIV-infected patients**. *The Journal of experimental medicine* 2004, **200**(11):1407-1417.

46. Sallusto F, Geginat J, Lanzavecchia A: **Central memory and effector memory T cell subsets: function, generation, and maintenance**. *Annual review of immunology* 2004, **22**:745-763.

47. Raff MC: **T and B lymphocytes and immune responses**. *Nature* 1973, **242**(5392):19-23.

48. LeBien TW: **Fates of human B-cell precursors**. *Blood* 2000, **96**(1):9-23.

49. Snow EC, Noelle RJ, Uhr JW, Vitetta ES: **Activation of antigen-enriched B cells. II. Role of linked recognition in B cell proliferation to thymus-dependent antigens**. *Journal of immunology (Baltimore, Md : 1950)* 1983, **130**(2):614-618.

50. Mond JJ, Vos Q, Lees A, Snapper CM: **T cell independent antigens**. *Current opinion in immunology* 1995, **7**(3):349-354.

51. Ehrenstein MR, Notley CA: **The importance of natural IgM: scavenger, protector and regulator**. *Nat Rev Immunol* 2010, **10**(11):778-786.

52. Vladutiu AO: **Immunoglobulin D: Properties, Measurement, and Clinical Relevance**. *Clinical and Diagnostic Laboratory Immunology* 2000, **7**(2):131-140.

53. Schroeder HW, Jr., Cavacini L: **Structure and function of immunoglobulins**. *Journal of Allergy and Clinical Immunology*, **125**(2):S41-S52.

54. Wu LC, Zarrin AA: **The production and regulation of IgE by the immune system**. *Nat Rev Immunol* 2014, **14**(4):247-259.

55. Taubenberger JK, Morens DM: **The Pathology of Influenza Virus Infections**. *Annual Review of Pathology: Mechanisms of Disease* 2008, **3**(1):499-522.

56. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: **Evolution and ecology of influenza A viruses**. *Microbiological reviews* 1992, **56**(1):152-179.

57. Gamblin SJ, Skehel JJ: **Influenza Hemagglutinin and Neuraminidase Membrane Glycoproteins**. *The Journal of Biological Chemistry* 2010, **285**(37):28403-28409.

58. Skehel JJ, Wiley DC: **Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin**. *Annual Review of Biochemistry* 2000, **69**(1):531-569.

59. Schrauwen EJA, Fouchier RAM: **Host adaptation and transmission of influenza A viruses in mammals**. *Emerg Microbes Infect* 2014, **3**:e9.

60. Shtyrya YA, Mochalova LV, Bovin NV: **Influenza Virus Neuraminidase: Structure and Function**. *Acta Naturae* 2009, **1**(2):26-32.

61. Rossman JS, Lamb RA: **Influenza virus assembly and budding**. *Virology* 2011, **411**(2):229-236.

62. Gómez-Puertas P, Albo C, Pérez-Pastrana E, Vivo A, Portela An: **Influenza Virus Matrix Protein Is the Major Driving Force in Virus Budding**. *Journal of Virology* 2000, **74**(24):11538-11547.

63. Enami M: **[Structure and function of influenza virus NS1 and NS2 proteins]**. *Nihon rinsho Japanese journal of clinical medicine* 1997, **55**(10):2605-2609.

64. Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T: **Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems**. *Virology* 1998, **252**(2):324-330.

65. Kuszewski K, Brydak L: **The epidemiology and history of influenza**. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2000, **54**(4):188-195.

66. Lewis PA, Shope RE: **Swine Influenza : II. A Hemophilic Bacillus From The Respiratory Tract of Infected Swine**. *The Journal of experimental medicine* 1931, **54**(3):361-371.

67. Shope RE: **Swine influenza : III. Filtration experiments and etiology**. *The Journal of experimental medicine* 1931, **54**(3):373-385.

68. Van Epps HL: **Influenza: exposing the true killer**. *The Journal of experimental medicine* 2006, **203**(4):803.

69. Shope RE: **The incidence of neutralizing antibodies for swine influenza virus in the sera of human beings of different ages**. *The Journal of experimental medicine* 1936, **63**(5):669-684.

70. Greenbaum E, Morag A, Zakay-Rones Z: **Isolation of Influenza C Virus during an Outbreak of Influenza A and B Viruses**. *Journal of Clinical Microbiology* 1998, **36**(5):1441-1442.

71. Trifonov V, Khiabanian H, Rabadan R: **Geographic Dependence, Surveillance, and Origins of the 2009 Influenza A (H1N1) Virus**. *New England Journal of Medicine* 2009, **361**(2):115-119.

72. Jeffery T, David M: **1918 Influenza: the Mother of All Pandemics**. *Emerging Infectious Disease journal* 2006, **12**(1):15.

73. Housworth J, Langmuir AD: **Excess mortality from epidemic influenza, 1957-1966**. *American journal of epidemiology* 1974, **100**(1):40-48.

74. Edwin DK: **Influenza Pandemics of the 20th Century**. *Emerging Infectious Disease journal* 2006, **12**(1):9.

75. Neumann G, Noda T, Kawaoka Y: **Emergence and pandemic potential of swine-origin H1N1 influenza virus**. *Nature* 2009, **459**(7249):931-939.

76. Kilbourne ED, Smith C, Brett I, Pokorny BA, Johansson B, Cox N: **The total influenza vaccine failure of 1947 revisited: major intrasubtypic antigenic change can explain failure of vaccine in a post-World War II epidemic**. *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**(16):10748-10752.

77. Nakajima K, Desselberger U, Palese P: **Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950**. *Nature* 1978, **274**(5669):334-339.

78. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ *et al*: **Pandemic Potential of a Strain of Influenza A (H1N1): Early Findings**. *Science* 2009, **324**(5934):1557-1561.

79. Novel Swine-Origin Influenza AVIT, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB *et al*: **Emergence of a novel swine-origin influenza A (H1N1) virus in humans**. *The New England journal of medicine* 2009, **360**(25):2605-2615.

80. Greenbaum JA, Kotturi MF, Kim Y, Oseroff C, Vaughan K, Salimi N, Vita R, Ponomarenko J, Scheuermann RH, Sette A *et al*: **Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population**.

Proceedings of the National Academy of Sciences of the United States of America 2009, **106**(48):20365-20370.

81. Pebody RG, McLean E, Zhao H, Cleary P, Bracebridge S, Foster K, Charlett A, Hardelid P, Waight P, Ellis J *et al*: **Pandemic Influenza A (H1N1) 2009 and mortality in the United Kingdom: risk factors for death, April 2009 to March 2010**. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2010, **15**(20).

82. Dawood FS, Iuliano AD, Reed C, Meltzer MI, Shay DK, Cheng PY, Bandaranayake D, Breiman RF, Brooks WA, Buchy P *et al*: **Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study**. *The Lancet Infectious diseases* 2012, **12**(9):687-695.

83. Hensley SE, Das SR, Bailey AL, Schmidt LM, Hickman HD, Jayaraman A, Viswanathan K, Raman R, Sasisekharan R, Bennink JR *et al*: **Hemagglutinin Receptor Binding Avidity Drives Influenza A Virus Antigenic Drift**. *Science* 2009, **326**(5953):734-736.

84. Clark NM, Lynch JP, 3rd: **Influenza: epidemiology, clinical features, therapy, and prevention**. *Seminars in respiratory and critical care medicine* 2011, **32**(4):373-392.

85. Van Kerkhove MD, Vandemaele KA, Shinde V, Jaramillo-Gutierrez G, Koukounari A, Donnelly CA, Carlino LO, Owen R, Paterson B, Pelletier L: **Risk factors for severe outcomes following 2009 influenza A (H1N1) infection: a global pooled analysis**. *PLoS medicine* 2011, **8**(7):e1001053.

86. Sivadon-Tardy V, Orlikowski D, Porcher R, Sharshar T, Durand MC, Enouf V, Rozenberg F, Caudie C, Annane D, van der Werf S *et al*: **Guillain-Barre syndrome and influenza virus infection**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2009, **48**(1):48-56.

87. Cao B, Li X-W, Mao Y, Wang J, Lu H-Z, Chen Y-S, Liang Z-A, Liang L, Zhang S-J, Zhang B *et al*: **Clinical Features of the Initial Cases of 2009 Pandemic Influenza A (H1N1) Virus Infection in China**. *New England Journal of Medicine* 2009, **361**(26):2507-2517.

88. Monto AS, Gravenstein S, Elliott M, Colopy M, Schweinle J: **Clinical signs and symptoms predicting influenza infection**. *Archives of internal medicine* 2000, **160**(21):3243-3247.

89. Michiels B, Thomas I, Van Royen P, Coenen S: **Clinical prediction rules combining signs, symptoms and epidemiological context to distinguish influenza from influenza-like illnesses in primary care: a cross sectional study**. *BMC Fam Pract* 2011, **12**(1):1-11.

90. Boivin G, Hardy I, Tellier G, Maziade J: **Predicting influenza infections during epidemics with use of a clinical case definition**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2000, **31**(5):1166-1169.

91. Zambon M, Hays J, Webster A, Newman R, Keene O: **Diagnosis of influenza in the community: relationship of clinical diagnosis to confirmed virological, serologic, or molecular detection of influenza**. *Archives of internal medicine* 2001, **161**(17):2116-2122.

92. Mills RD, Cain KJ, Woods GL: **Detection of influenza virus by centrifugal inoculation of MDCK cells and staining with monoclonal antibodies**. *Journal of Clinical Microbiology* 1989, **27**(11):2505-2508.

93. Hossain MJ, Perez S, Guo Z, Chen L-M, Donis RO: **Establishment and Characterization of a Madin-Darby Canine Kidney Reporter Cell Line for Influenza A Virus Assays**. *Journal of Clinical Microbiology* 2010, **48**(7):2515-2523.

94. Daisy JA, Lief FS, Friedman HM: **Rapid diagnosis of influenza A infection by direct immunofluorescence of nasopharyngeal aspirates in adults**. *J Clin Microbiol* 1979, **9**(6):688-692.

95. Kaiser L, Briones MS, Hayden FG: **Performance of virus isolation and Directigen Flu A to detect influenza A virus in experimental human infection**. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 1999, **14**(3):191-197.

96. Ray CG, Minnich LL: **Efficiency of immunofluorescence for rapid detection of common respiratory viruses**. *Journal of Clinical Microbiology* 1987, **25**(2):355-357.

97. Stokes CE, Bernstein JM, Kyger SA, Hayden FG: **Rapid diagnosis of influenza A and B by 24-h fluorescent focus assays**. *Journal of Clinical Microbiology* 1988, **26**(7):1263-1266.

98. Sintchenko V, Gilbert GL, Coiera E, Dwyer D: **Treat or test first? Decision analysis of empirical antiviral treatment of influenza virus infection versus treatment based on rapid test results**. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2002, **25**(1):15-21.

99. Tanei M, Yokokawa H, Murai K, Sakamoto R, Amari Y, Boku S, Inui A, Fujibayashi K, Uehara Y, Isonuma H *et al*: **Factors influencing the diagnostic accuracy of the rapid influenza antigen detection test (RIADT): a cross-sectional study**. *BMJ Open* 2014, **4**(1).

100. Ellis JS, Zambon MC: **Molecular diagnosis of influenza**. *Reviews in medical virology* 2002, **12**(6):375-389.

101. Poon LL, Chan KH, Smith GJ, Leung CS, Guan Y, Yuen KY, Peiris JS: **Molecular detection of a novel human influenza (H1N1) of pandemic potential by conventional and real-time quantitative RT-PCR assays**. *Clinical chemistry* 2009, **55**(8):1555-1558.

102. Faix DJ, Sherman SS, Waterman SH: **Rapid-Test Sensitivity for Novel Swine-Origin Influenza A (H1N1) Virus in Humans**. *New England Journal of Medicine* 2009, **361**(7):728-729.

103. Katz JM, Hancock K, Xu X: **Serologic assays for influenza surveillance, diagnosis and vaccine evaluation**. *Expert review of anti-infective therapy* 2011, **9**(6):669-683.

104. Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL: **Regulation of immunological homeostasis in the respiratory tract**. *Nat Rev Immunol* 2008, **8**(2):142-152.

105. Blasius AL, Beutler B: **Intracellular toll-like receptors**. *Immunity* 2010, **32**(3):305-315.

106. Pang IK, Iwasaki A: **Inflammasomes as mediators of immunity against influenza virus**. *Trends in immunology* 2011, **32**(1):34-41.

107. Takeuchi O, Akira S: **Innate immunity to virus infection**. *Immunological reviews* 2009, **227**(1):75-86.

108. Solorzano A, Webby RJ, Lager KM, Janke BH, Garcia-Sastre A, Richt JA: **Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs**. *J Virol* 2005, **79**(12):7535-7543.

109. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, Davis DM, Strominger JL, Yewdell JW, Porgador A: **Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells**. *Nature* 2001, **409**(6823):1055-1060.

110. Fujisawa H: **Inhibitory role of neutrophils on influenza virus multiplication in the lungs of mice**. *Microbiology and immunology* 2001, **45**(10):679-688.

111. Hashimoto G, Wright PF, Karzon DT: **Antibody-dependent cell-mediated cytotoxicity against influenza virus-infected cells**. *The Journal of infectious diseases* 1983, **148**(5):785-794.

112. Lin KL, Suzuki Y, Nakano H, Ramsburg E, Gunn MD: **CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality**. *Journal of immunology (Baltimore, Md : 1950)* 2008, **180**(4):2562-2572.

113. Hama Y, Kurokawa M, Imakita M, Yoshida Y, Shimizu T, Watanabe W, Shiraki K: **Interleukin 12 is a primary cytokine responding to influenza virus infection in the respiratory tract of mice**. *Acta virologica* 2009, **53**(4):233-240.

114. Becker S, Quay J, Soukup J: **Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages**. *Journal of immunology (Baltimore, Md : 1950)* 1991, **147**(12):4307-4312.

115. Hashimoto Y, Moki T, Takizawa T, Shiratsuchi A, Nakanishi Y: **Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice**. *Journal of immunology (Baltimore, Md : 1950)* 2007, **178**(4):2448-2457.

116. Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Pantin-Jackwood MJ, Schultz-Cherry S, Solorzano A, Van Rooijen N, Katz JM *et al*: **Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice**. *J Virol* 2005, **79**(23):14933-14944.

117. Kim HM, Lee YW, Lee KJ, Kim HS, Cho SW, van Rooijen N, Guan Y, Seo SH: **Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs**. *J Virol* 2008, **82**(9):4265-4274.

118. Bhardwaj N, Bender A, Gonzalez N, Bui LK, Garrett MC, Steinman RM: **Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells**. *The Journal of clinical investigation* 1994, **94**(2):797-807.

119. Hamilton-Easton A, Eichelberger M: **Virus-specific antigen presentation by different subsets of cells from lung and mediastinal lymph node tissues of influenza virus-infected mice**. *J Virol* 1995, **69**(10):6359-6366.

120. Braciale TJ, Sun J, Kim TS: **Regulating the adaptive immune response to respiratory virus infection**. *Nat Rev Immunol* 2012, **12**(4):295-305.

121. Banchereau J, Steinman RM: **Dendritic cells and the control of immunity**. *Nature* 1998, **392**(6673):245-252.

122. Schulman JL, Kilbourne ED: **Induction of partial specific heterotypic immunity in mice by a single infection with influenza a virus**. *Journal of bacteriology* 1965, **89**:170-174.

123. Zhu J, Paul WE: **Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors**. *Immunological reviews* 2010, **238**(1):247-262.

124. Lamb JR, Woody JN, Hartzman RJ, Eckels DD: **In vitro influenza virus-specific antibody production in man: antigen-specific and HLA-restricted induction of helper activity mediated by cloned human T lymphocytes**. *Journal of immunology (Baltimore, Md : 1950)* 1982, **129**(4):1465-1470.

125. Graham MB, Braciale VL, Braciale TJ: **Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection**. *The Journal of experimental medicine* 1994, **180**(4):1273-1282.

126. Wohlleben G, Müller J, Tatsch U, Hambrecht C, Herz U, Renz H, Schmitt E, Moll H, Erb KJ: **Influenza A Virus Infection Inhibits the Efficient Recruitment of Th2 Cells into the Airways and the Development of Airway Eosinophilia**. *The Journal of Immunology* 2003, **170**(9):4601-4611.

127. Scherle PA, Gerhard W: **Functional analysis of influenza-specific helper T cell clones in vivo. T cells specific for internal viral proteins provide cognate help for B cell responses to hemagglutinin**. *The Journal of experimental medicine* 1986, **164**(4):1114-1128.

128. Belz GT, Wodarz D, Diaz G, Nowak MA, Doherty PC: **Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice**. *J Virol* 2002, **76**(23):12388-12393.

129. Deliyannis G, Jackson DC, Ede NJ, Zeng W, Hourdakis I, Sakabetis E, Brown LE: **Induction of long-term memory CD8(+) T cells for recall of viral clearing responses against influenza virus**. *J Virol* 2002, **76**(9):4212-4221.

130. Strutt TM, McKinstry KK, Dibble JP, Winchell C, Kuang Y, Curtis JD, Huston G, Dutton RW, Swain SL: **Memory CD4(+) T cells induce innate responses independent of pathogen**. *Nature medicine* 2010, **16**(5):558-564.

131. Teijaro JR, Turner D, Pham Q, Wherry EJ, Lefrancois L, Farber DL: **Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection**. *Journal of immunology (Baltimore, Md : 1950)* 2011, **187**(11):5510-5514.

132. Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, Lambkin-Williams R, Gilbert A, Oxford J, Nicholas B *et al*: **Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans**. *Nat Med* 2012, **18**(2):274-280.

133. Román E, Miller E, Harmsen A, Wiley J, von Andrian UH, Huston G, Swain SL: **CD4 Effector T Cell Subsets in the Response to Influenza: Heterogeneity, Migration, and Function**. *The Journal of experimental medicine* 2002, **196**(7):957-968.

134. Richards KA, Topham D, Chaves FA, Sant AJ: **Cutting Edge: CD4 T Cells Generated from Encounter with Seasonal Influenza Viruses and Vaccines Have**

Broad Protein Specificity and Can Directly Recognize Naturally Generated Epitopes Derived from the Live Pandemic H1N1 Virus. *The Journal of Immunology* 2010, **185**(9):4998-5002.

135. Lee LY, Ha do LA, Simmons C, de Jong MD, Chau NV, Schumacher R, Peng YC, McMichael AJ, Farrar JJ, Smith GL *et al*: **Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals**. *The Journal of clinical investigation* 2008, **118**(10):3478-3490.

136. Nakanishi Y, Lu B, Gerard C, Iwasaki A: **CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help**. *Nature* 2009, **462**(7272):510-513.

137. Kreijtz JH, de Mutsert G, van Baalen CA, Fouchier RA, Osterhaus AD, Rimmelzwaan GF: **Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus**. *J Virol* 2008, **82**(11):5161-5166.

138. Jameson J, Cruz J, Ennis FA: **Human cytotoxic T-lymphocyte repertoire to influenza A viruses**. *J Virol* 1998, **72**(11):8682-8689.

139. McMichael AJ, Gotch FM, Noble GR, Beare PA: **Cytotoxic T-cell immunity to influenza**. *The New England journal of medicine* 1983, **309**(1):13-17.

140. van de Sandt CE, Kreijtz JHCM, de Mutsert G, Geelhoed-Mieras MM, Hillaire MLB, Vogelzang-van Trierum SE, Osterhaus ADME, Fouchier RAM, Rimmelzwaan GF: **Human Cytotoxic T Lymphocytes Directed to Seasonal Influenza A Viruses Cross-React with the Newly Emerging H7N9 Virus**. *Journal of Virology* 2014, **88**(3):1684-1693.

141. Quiñones-Parra S, Grant E, Loh L, Nguyen THO, Campbell K-A, Tong SYC, Miller A, Doherty PC, Vijaykrishna D, Rossjohn J *et al*: **Preexisting CD8(+) T-cell immunity to the H7N9 influenza A virus varies across ethnicities**. *Proceedings of the National Academy of Sciences of the United States of America* 2014, **111**(3):1049-1054.

142. Andrade F: **Non-cytotoxic antiviral activities of granzymes in the context of the immune antiviral state**. *Immunological reviews* 2010, **235**(1):128-146.

143. van Domselaar R, Bovenschen N: **Cell death-independent functions of granzymes: hit viruses where it hurts**. *Reviews in medical virology* 2011, **21**(5):301-314.

144. Topham DJ, Tripp RA, Doherty PC: **CD8+ T cells clear influenza virus by perforin or Fas-dependent processes**. *Journal of immunology (Baltimore, Md : 1950)* 1997, **159**(11):5197-5200.

145. La Gruta NL, Turner SJ, Doherty PC: **Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific CD8+ T cell responses: correlation of cytokine profile and TCR avidity**. *Journal of immunology (Baltimore, Md : 1950)* 2004, **172**(9):5553-5560.

146. Woodland DL, Hogan RJ, Zhong W: **Cellular immunity and memory to respiratory virus infections**. *Immunologic research* 2001, **24**(1):53-67.

147. Chang JT, Palanivel VR, Kinjyo I, Schambach F, Intlekofer AM, Banerjee A, Longworth SA, Vinup KE, Mrass P, Oliaro J *et al*: **Asymmetric T lymphocyte division in the initiation of adaptive immune responses**. *Science* 2007, **315**(5819):1687-1691.

148. Lalvani A, Brookes R, Hambleton S, Britton WJ, Hill AV, McMichael AJ: **Rapid effector function in CD8+ memory T cells**. *The Journal of experimental medicine* 1997, **186**(6):859-865.

149. Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, Bean T, Barclay W, Deeks JJ, Lalvani A: **Cellular immune correlates of protection against symptomatic pandemic influenza**. *Nat Med* 2013, **19**(10):1305-1312.

150. Waffarn EE, Baumgarth N: **Protective B cell responses to flu--no fluke!** *Journal of immunology (Baltimore, Md : 1950)* 2011, **186**(7):3823-3829.

151. Wilson IA, Cox NJ: **Structural basis of immune recognition of influenza virus hemagglutinin**. *Annual review of immunology* 1990, **8**:737-771.

152. Yu X, Tsibane T, McGraw PA, House FS, Keefer CJ, Hicar MD, Tumpey TM, Pappas C, Perrone LA, Martinez O *et al*: **Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors**. *Nature* 2008, **455**(7212):532-536.

153. Ikonen N, Strengell M, Kinnunen L, Osterlund P, Pirhonen J, Broman M, Davidkin I, Ziegler T, Julkunen I: **High frequency of cross-reacting antibodies against 2009 pandemic influenza A(H1N1) virus among the elderly in Finland**. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2010, **15**(5).

154. Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, Liu F, Dong L, DeVos JR, Gargiullo PM *et al*: **Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus**. *The New England journal of medicine* 2009, **361**(20):1945-1952.

155. Miller E, Hoschler K, Hardelid P, Stanford E, Andrews N, Zambon M: **Incidence of 2009 pandemic influenza A H1N1 infection in England: a cross-sectional serological study**. *Lancet* 2010, **375**(9720):1100-1108.

156. Bosch BJ, Bodewes R, de Vries RP, Kreijtz JH, Bartelink W, van Amerongen G, Rimmelzwaan GF, de Haan CA, Osterhaus AD, Rottier PJ: **Recombinant soluble, multimeric HA and NA exhibit distinctive types of protection against pandemic swine-origin 2009 A(H1N1) influenza virus infection in ferrets**. *J Virol* 2010, **84**(19):10366-10374.

157. Johansson BE, Bucher DJ, Kilbourne ED: **Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection**. *J Virol* 1989, **63**(3):1239-1246.

158. Zebedee SL, Lamb RA: **Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions**. *J Virol* 1988, **62**(8):2762-2772.

159. Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, Ye Z, Hogan RJ, Wu Z, Benton KA, Tumpey TM *et al*: **Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1**. *Emerging infectious diseases* 2007, **13**(3):426-435.

160. Fiers W, De Filette M, El Bakkouri K, Schepens B, Roose K, Schotsaert M, Birkett A, Saelens X: **M2e-based universal influenza A vaccine**. *Vaccine* 2009, **27**(45):6280-6283.

161. Sukeno N, Otsuki Y, Konno J, Yamane N, Odagiri T, Arikawa J, Ishida N: **Anti-nucleoprotein antibody response in influenza A infection**. *The Tohoku journal of experimental medicine* 1979, **128**(3):241-249.

162. Bodewes R, Osterhaus AD, Rimmelzwaan GF: **Targets for the induction of protective immunity against influenza a viruses**. *Viruses* 2010, **2**(1):166-188.

163. To KKW, Zhang AJX, Hung IFN, Xu T, Ip WCT, Wong RTY, Ng JCK, Chan JFW, Chan K-H, Yuen K-Y: **High Titer and Avidity of Nonneutralizing Antibodies against Influenza Vaccine Antigen Are Associated with Severe Influenza**. *Clinical and Vaccine Immunology : CVI* 2012, **19**(7):1012-1018.

164. Zúñiga J, Buendía-Roldán I, Zhao Y, Jiménez L, Torres D, Romo J, Ramírez G, Cruz A, Vargas-Alarcon G, Sheu C-C *et al*: **Genetic variants associated with severe pneumonia in A/H1N1 influenza infection**. *European Respiratory Journal* 2012, **39**(3):604-610.

165. Rimmelzwaan GF, Baars M, van Beek R, van Amerongen G, Lovgren-Bengtsson K, Claas EC, Osterhaus AD: **Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines**. *The Journal of general virology* 1997, **78 (Pt 4)**:757-765.

166. Fernandez Gonzalez S, Jayasekera JP, Carroll MC: **Complement and natural antibody are required in the long-term memory response to influenza virus**. *Vaccine* 2008, **26 Suppl 8**:I86-93.

167. Voeten JT, Groen J, van Alphen D, Claas EC, de Groot R, Osterhaus AD, Rimmelzwaan GF: **Use of recombinant nucleoproteins in enzyme-linked immunosorbent assays for detection of virus-specific immunoglobulin A (IgA) and IgG antibodies in influenza virus A- or B-infected patients**. *J Clin Microbiol* 1998, **36**(12):3527-3531.

168. Onodera T, Takahashi Y, Yokoi Y, Ato M, Kodama Y, Hachimura S, Kurosaki T, Kobayashi K: **Memory B cells in the lung participate in protective humoral immune responses to pulmonary influenza virus reinfection**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(7):2485-2490.

169. Armstrong SJ, Dimmock NJ: **Neutralization of influenza virus by low concentrations of hemagglutinin-specific polymeric immunoglobulin A inhibits viral fusion activity, but activation of the ribonucleoprotein is also inhibited**. *J Virol* 1992, **66**(6):3823-3832.

170. Mazanec MB, Coudret CL, Fletcher DR: **Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies**. *J Virol* 1995, **69**(2):1339-1343.

171. de Jong JC, Beyer WE, Palache AM, Rimmelzwaan GF, Osterhaus AD: **Mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly**. *Journal of medical virology* 2000, **61**(1):94-99.

172. Palese P, García-Sastre A: **Influenza vaccines: present and future**. *The Journal of clinical investigation* 2002, **110**(1):9-13.

173. Fiore AE, Uyeki TM, Broder K, Finelli L, Euler GL, Singleton JA, Iskander JK, Wortley PM, Shay DK, Bresee JS *et al*: **Prevention and control of influenza with**

vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. *MMWR Recomm Rep* 2010, **59**(RR-8):1-62.

174. Domachowske JB, Pankow-Culot H, Bautista M, Feng Y, Claeys C, Peeters M, Innis BL, Jain V: **A Randomized Trial of Candidate Inactivated Quadrivalent Influenza Vaccine versus Trivalent Influenza Vaccines in Children Aged 3–17 Years**. *Journal of Infectious Diseases* 2013, **207**(12):1878-1887.

175. Wood JM, Mumford J, Schild GC, Webster RG, Nicholson KG: **Single-radial-immunodiffusion potency tests of inactivated influenza vaccines for use in man and animals**. *Developments in biological standardization* 1986, **64**:169-177.

176. Duxbury AE, Hampson AW, Sievers JGM: **Antibody Response in Humans to Deoxycholate-Treated Influenza Virus Vaccine**. *The Journal of Immunology* 1968, **101**(1):62-67.

177. Laver WG, Webster RG: **Preparation and immunogenicity of a purified influenza virus haemagglutinin and neuraminidase subunit vaccine**. *Postgraduate Medical Journal* 1976, **52**(608):373-378.

178. Couch RB: **Seasonal Inactivated Influenza Virus Vaccines**. *Vaccine* 2008, **26**(Suppl 4):D5-D9.

179. Yang WH, Dionne M, Kyle M, Aggarwal N, Li P, Madariaga M, Godeaux O, Vaughn DW: **Long-term immunogenicity of an AS03-adjuvanted influenza A(H1N1)pdm09 vaccine in young and elderly adults: an observer-blind, randomized trial**. *Vaccine* 2013, **31**(40):4389-4397.

180. Tsai TF: **MF59 adjuvanted seasonal and pandemic influenza vaccines**. *Yakugaku zasshi : Journal of the Pharmaceutical Society of Japan* 2011, **131**(12):1733-1741.

181. Nohynek H, Jokinen J, Partinen M, Vaarala O, Kirjavainen T, Sundman J, Himanen SL, Hublin C, Julkunen I, Olsen P *et al*: **AS03 adjuvanted AH1N1 vaccine associated with an abrupt increase in the incidence of childhood narcolepsy in Finland**. *PloS one* 2012, **7**(3):e33536.

182. Mischler R, Metcalfe IC: **Inflexal V a trivalent virosome subunit influenza vaccine: production**. *Vaccine* 2002, **20 Suppl 5**:B17-23.

183. Herzog C, Hartmann K, Kunzi V, Kursteiner O, Mischler R, Lazar H, Gluck R: **Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine**. *Vaccine* 2009, **27**(33):4381-4387.

184. Maassab HF: **Adaptation and growth characteristics of influenza virus at 25 degrees c**. *Nature* 1967, **213**(5076):612-614.

185. Rhorer J, Ambrose CS, Dickinson S, Hamilton H, Oleka NA, Malinoski FJ, Wittes J: **Efficacy of live attenuated influenza vaccine in children: A meta-analysis of nine randomized clinical trials**. *Vaccine* 2009, **27**(7):1101-1110.

186. Tappenden P, Jackson R, Cooper K, Rees A, Simpson E, Read R, Nicholson K: **Amantadine, oseltamivir and zanamivir for the prophylaxis of influenza (including a review of existing guidance no. 67): a systematic review and economic evaluation**. *Health technology assessment (Winchester, England)* 2009, **13**(11):iii, ix-xii, 1-246.

187. Kandel R, Hartshorn KL: **Prophylaxis and treatment of influenza virus infection**. *BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy* 2001, **15**(5):303-323.

188. Palese P, Compans RW: **Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action**. *The Journal of general virology* 1976, **33**(1):159-163.

189. Jenq RR, van den Brink MRM: **Allogeneic haematopoietic stem cell transplantation: individualized stem cell and immune therapy of cancer**. *Nat Rev Cancer* 2010, **10**(3):213-221.

190. Copelan EA: **Hematopoietic Stem-Cell Transplantation**. *New England Journal of Medicine* 2006, **354**(17):1813-1826.

191. Holtan SG, Pasquini M, Weisdorf DJ: **Acute graft-versus-host disease: a bench-to-bedside update**, vol. 124; 2014.

192. Blazar BR, Murphy WJ, Abedi M: **Advances in graft-versus-host disease biology and therapy**. *Nat Rev Immunol* 2012, **12**(6):443-458.

193. Arai S, Arora M, Wang T, Spellman SR, He W, Couriel DR, Urbano-Ispizua A, Cutler CS, Bacigalupo AA, Battiwalla M *et al*: **Increasing Incidence of Chronic Graft-versus-Host Disease in Allogeneic Transplantation: A Report from the Center for International Blood and Marrow Transplant Research**. *Biology of Blood and Marrow Transplantation* 2015, **21**(2):266-274.

194. Winston DJ, Territo MC, Ho WG, Miller MJ, Gale RP, Golde DW: **Alveolar macrophage dysfunction in human bone marrow transplant recipients**. *The American journal of medicine* 1982, **73**(6):859-866.

195. Walsh LJ, Athanasas-Platsis S, Savage NW: **Reconstitution of cutaneous neural-immunological networks following bone marrow transplantation**. *Transplantation* 1996, **61**(3):413-417.

196. Niederwieser D, Gastl G, Rumpold H, Marth C, Kraft D, Huber C: **Rapid reappearance of large granular lymphocytes (LGL) with concomitant reconstitution of natural killer (NK) activity after human bone marrow transplantation (BMT)**. *British journal of haematology* 1987, **65**(3):301-305.

197. Storek J, Wells D, Dawson MA, Storer B, Maloney DG: **Factors influencing B lymphopoiesis after allogeneic hematopoietic cell transplantation**, vol. 98; 2001.

198. Storek J, Dawson MA, Storer B, Stevens-Ayers T, Maloney DG, Marr KA, Witherspoon RP, Bensinger W, Flowers MED, Martin P *et al*: **Immune reconstitution after allogeneic marrow transplantation compared with blood stem cell transplantation**, vol. 97; 2001.

199. Ottinger HD, Beelen DW, Scheulen B, Schaefer UW, Grosse-Wilde H: **Improved immune reconstitution after allotransplantation of peripheral blood stem cells instead of bone marrow**. *Blood* 1996, **88**(7):2775-2779.

200. Storek J, Ferrara S, Ku N, Giorgi JV, Champlin RE, Saxon A: **B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny?** *Bone marrow transplantation* 1993, **12**(4):387-398.

201. Gerritsen EJ, van Tol MJ, Ballieux P, Voesten A, Weiland HT, van Venrooij WJ, Daha MR, Geertzen HG, Rijkers GT, Gmelig-Meyling FH *et al*: **Search for the**

antigen-specificity of homogeneous IgG components (H-IgG) after allogeneic bone marrow transplantation. *Bone marrow transplantation* 1996, **17**(5):825-833.

202. Wahren B, Gahrton G, Linde A, Ljungman P, Lonnqvist B, Ringden O, Sundqvist VA: **Transfer and persistence of viral antibody-producing cells in bone marrow transplantation**. *The Journal of infectious diseases* 1984, **150**(3):358-365.

203. Storek J, Witherspoon RP, Maloney DG, Chauncey TR, Storb R: **Improved reconstitution of CD4 T cells and B cells but worsened reconstitution of serum IgG levels after allogeneic transplantation of blood stem cells instead of marrow**. *Blood* 1997, **89**(10):3891-3893.

204. Fujimaki K, Maruta A, Yoshida M, Kodama F, Matsuzaki M, Fujisawa S, Kanamori H, Ishigatsubo Y: **Immune reconstitution assessed during five years after allogeneic bone marrow transplantation**. *Bone marrow transplantation* 2001, **27**(12):1275-1281.

205. Weinberg K, Annett G, Kashyap A, Lenarsky C, Forman SJ, Parkman R: **The effect of thymic function on immunocompetence following bone marrow transplantation**. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 1995, **1**(1):18-23.

206. Ashihara E, Shimazaki C, Yamagata N, Hirata T, Okawa K, Oku N, Goto H, Inaba T, Fujita N, Nakagawa M: **Reconstitution of lymphocyte subsets after peripheral blood stem cell transplantation: two-color flow cytometric analysis**. *Bone marrow transplantation* 1994, **13**(4):377-381.

207. Savage WJ, Bleesing JJ, Douek D, Brown MR, Linton GM, Malech HL, Horwitz ME: **Lymphocyte reconstitution following non-myeloablative hematopoietic stem cell transplantation follows two patterns depending on age and donor/recipient chimerism**. *Bone marrow transplantation* 2001, **28**(5):463-471.

208. van Heijst JW, Ceberio I, Lipuma LB, Samilo DW, Wasilewski GD, Gonzales AM, Nieves JL, van den Brink MR, Perales MA, Pamer EG: **Quantitative assessment of T cell repertoire recovery after hematopoietic stem cell transplantation**. *Nat Med* 2013, **19**(3):372-377.

209. Atkinson K: **Clinical Bone Marrow and Blood Stem Cell Transplantation**: Cambridge University Press; 2004.

210. Autran B, Leblond V, Sadat-Sowti B, Lefranc E, Got P, Sutton L, Binet JL, Debre P: **A soluble factor released by CD8+CD57+ lymphocytes from bone marrow transplanted patients inhibits cell-mediated cytolysis**. *Blood* 1991, **77**(10):2237-2241.

211. Tsoi MS, Storb R, Dobbs S, Kopecky KJ, Santos E, Weiden PL, Thomas ED: **Nonspecific suppressor cells in patients with chronic graft-vs-host disease after marrow grafting**. *Journal of immunology (Baltimore, Md : 1950)* 1979, **123**(5):1970-1976.

212. Whimbey E, Elting LS, Couch RB, Lo W, Williams L, Champlin RE, Bodey GP: **Influenza A virus infections among hospitalized adult bone marrow transplant recipients**. *Bone marrow transplantation* 1994, **13**(4):437-440.

213. Nichols WG, Guthrie KA, Corey L, Boeckh M: **Influenza infections after hematopoietic stem cell transplantation: risk factors, mortality, and the effect of**

antiviral therapy. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2004, **39**(9):1300-1306.

214. Ljungman P, de la Camara R, Perez-Bercoff L, Abecasis M, Nieto Campuzano JB, Cannata-Ortiz MJ, Cordonnier C, Einsele H, Gonzalez-Vicent M, Espigado I *et al*: **Outcome of pandemic H1N1 infections in hematopoietic stem cell transplant recipients**. *Haematologica* 2011, **96**(8):1231-1235.

215. Choi SM, Boudreault AA, Xie H, Englund JA, Corey L, Boeckh M: **Differences in clinical outcomes after 2009 influenza A/H1N1 and seasonal influenza among hematopoietic cell transplant recipients**. *Blood* 2011, **117**(19):5050-5056.

216. Reid G, Huprikar S, Patel G, Razonable RR, Mossad S, Levi M, Gregg K, Shoham S, Humar A, Adams W *et al*: **A multicenter evaluation of pandemic influenza A/H1N1 in hematopoietic stem cell transplant recipients**. *Transplant infectious disease : an official journal of the Transplantation Society* 2013, **15**(5):487-492.

217. Dignani MC, Costantini P, Salgueira C, Jordan R, Guerrini G, Valledor A, Herrera F, Nenna A, Mora C, Roccia-Rossi I *et al*: **Pandemic 2009 Influenza A (H1N1) virus infection in cancer and hematopoietic stem cell transplant recipients; a multicenter observational study**. *F1000Research* 2014, **3**:221.

218. Pollara C, Piccinelli G, Rossi G, Cattaneo C, Perandin F, Corbellini S, Tomasi D, Bonfanti C: **Nosocomial outbreak of the pandemic Influenza A (H1N1) 2009 in critical hematologic patients during seasonal influenza 2010-2011: detection of oseltamivir resistant variant viruses**. *BMC Infect Dis* 2013, **13**(1):1-7.

219. Engelhard D, Mohty B, de la Camara R, Cordonnier C, Ljungman P: **European guidelines for prevention and management of influenza in hematopoietic stem cell transplantation and leukemia patients: summary of ECIL-4 (2011), on behalf of ECIL, a joint venture of EBMT, EORTC, ICHS, and ELN**. *Transplant Infectious Disease* 2013, **15**(3):219-232.

220. Espinosa-Aguilar L, Green JS, Forrest GN, Ball ED, Maziarz RT, Strasfeld L, Taplitz RA: **Novel H1N1 Influenza in Hematopoietic Stem Cell Transplantation Recipients: Two Centers' Experiences**. *Biology of Blood and Marrow Transplantation* 2011, **17**(4):566-573.

221. Machado CM, Cardoso MR, da Rocha IF, Boas LS, Dulley FL, Pannuti CS: **The benefit of influenza vaccination after bone marrow transplantation**. *Bone marrow transplantation* 2005, **36**(10):897-900.

222. Schiffer JT, Kirby K, Sandmaier B, Storb R, Corey L, Boeckh M: **Timing and severity of community acquired respiratory virus infections after myeloablative versus non-myeloablative hematopoietic stem cell transplantation**, vol. 94; 2009.

223. Engelhard D, Nagler A, Hardan I, Morag A, Aker M, Baciu H, Strauss N, Parag G, Naparstek E, Ravid Z *et al*: **Antibody response to a two-dose regimen of influenza vaccine in allogeneic T cell-depleted and autologous BMT recipients**. *Bone marrow transplantation* 1993, **11**(1):1-5.

224. Pauksen K, Linde A, Hammarström V, Sjölin J, Carneskog J, Jonsson G, öberga G, Engelmann H, Ljungman P: **Granulocyte-Macrophage Colony-Stimulating Factor as Immunomodulating Factor Together with Influenza Vaccination in Stem Cell Transplant Patients**. *Clinical Infectious Diseases* 2000, **30**(2):342-348.

225. de Lavallade H, Garland P, Sekine T, Hoschler K, Marin D, Stringaris K, Loucaides E, Howe K, Szydlo R, Kanfer E *et al*: **Repeated vaccination is required to optimize seroprotection against H1N1 in the immunocompromised host**. *Haematologica* 2011, **96**(2):307-314.

226. Dhedin N, Krivine A, Le Corre N, Mallet A, Lioure B, Bay JO, Rubio MT, Agape P, Thiebaut A, Le Goff J *et al*: **Comparable humoral response after two doses of adjuvanted influenza A/H1N1pdm2009 vaccine or natural infection in allogeneic stem cell transplant recipients**. *Vaccine* 2014, **32**(5):585-591.

227. Mohty B, Bel M, Vukicevic M, Nagy M, Levrat E, Meier S, Grillet S, Combescure C, Kaiser L, Chalandon Y *et al*: **Graft-versus-host disease is the major determinant of humoral responses to the AS03-adjuvanted influenza A/09/H1N1 vaccine in allogeneic hematopoietic stem cell transplant recipients**. *Haematologica* 2011, **96**(6):896-904.

228. Gueller S, Allwinn R, Mousset S, Martin H, Wieters I, Herrmann E, Serve H, Bickel M, Bug G: **Enhanced immune response after a second dose of an AS03-adjuvanted H1N1 influenza A vaccine in patients after hematopoietic stem cell transplantation**. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2011, **17**(10):1546-1550.

229. Engelhard D, Zakay-Rones Z, Shapira MY, Resnick I, Averbuch D, Grisariu S, Dray L, Djian E, Strauss-Liviatan N, Grotto I *et al*: **The humoral immune response of hematopoietic stem cell transplantation recipients to AS03-adjuvanted A/California/7/2009 (H1N1)v-like virus vaccine during the 2009 pandemic**. *Vaccine* 2011, **29**(9):1777-1782.

230. Cherif H, Höglund M, Pauksens K: **Adjuvanted influenza a (H1N1) 2009 vaccine in patients with hematological diseases: good safety and immunogenicity even in chemotherapy-treated patients**. *European Journal of Haematology* 2013, **90**(5):413-419.

231. Roll D, Ammer J, Holler B, Salzberger B, Schweiger B, Jilg W, Andreesen R, Edinger M, Wolff D, Holler E: **Vaccination against pandemic H1N1 (2009) in patients after allogeneic hematopoietic stem cell transplantation: a retrospective analysis**. *Infection* 2012, **40**(2):153-161.

232. Issa NC, Marty FM, Gagne LS, Koo S, Verrill KA, Alyea EP, Cutler CS, Koreth J, Armand P, Ho VT *et al*: **Seroprotective Titers against 2009 H1N1 Influenza A Virus after Vaccination in Allogeneic Hematopoietic Stem Cell Transplantation Recipients**. *Biology of Blood and Marrow Transplantation* 2011, **17**(3):434-438.

233. Haining WN, Evans JW, Seth NP, Callaway GD, Wucherpfennig KW, Nadler LM, Guinan EC: **Measuring T cell immunity to influenza vaccination in children after haemopoietic stem cell transplantation**. *Br J Haematol* 2004, **127**(3):322-325.

234. Avetisyan G, Aschan J, Hassan M, Ljungman P: **Evaluation of immune responses to seasonal influenza vaccination in healthy volunteers and in patients after stem cell transplantation**. *Transplantation* 2008, **86**(2):257-263.

235. de Lavallade H, Garland P, Sekine T, Hoschler K, Marin D, Stringaris K, Loucaides E, Howe K, Szydlo R, Kanfer E *et al*: **Repeated vaccination is required to optimize seroprotection against H1N1 in the immunocompromised host**, vol. 96; 2011.

236. Guérin-El Khourouj V, Duchamp M, Krivine A, Pédron B, Ouachée-Chardin M, Yakouben K, Frémond M-L, Baruchel A, Dalle J-H, Sterkers G: **Cellular and**

humoral immunity elicited by influenza vaccines in pediatric hematopoietic-stem cell transplantation. *Human Immunology* 2012, **73**(9):884-890.

237. Mariotti J, Spina F, Carniti C, Anselmi G, Lucini D, Vendramin A, Pregliasco F, Corradini P: **Long-term patterns of humoral and cellular response after vaccination against influenza A (H1N1) in patients with hematologic malignancies**. *Eur J Haematol* 2012, **89**(2):111-119.

238. Garland P, de Lavallade H, Sekine T, Hoschler K, Sriskandan S, Patel P, Brett S, Stringaris K, Loucaides E, Howe K *et al*: **Humoral and Cellular Immunity to Primary H1N1 Infection in Patients with Hematologic Malignancies following Stem Cell Transplantation**. *Biology of Blood and Marrow Transplantation* 2011, **17**(5):632-639.

239. Almqvist C, Adami HO, Franks PW, Groop L, Ingelsson E, Kere J, Lissner L, Litton JE, Maeurer M, Michaelsson K *et al*: **LifeGene--a large prospective population-based study of global relevance**. *European journal of epidemiology* 2011, **26**(1):67-77.

240. Maeurer M: **Markus Maeurer on the LifeGene project**. *Expert review of clinical immunology* 2010, **6**(5):709-712.

241. Magalhaes I, Eriksson M, Linde C, Muhammad R, Rane L, Ambati A, Axelsson-Robertson R, Khalaj B, Alvarez-Corrales N, Lapini G *et al*: **Difference in immune response in vaccinated and unvaccinated Swedish individuals after the 2009 Influenza pandemic**. *BMC Infect Dis* 2014, **14**(1):319.

242. Chi C-Y, Liu C-C, Lin C-C, Wang H-C, Cheng Y-T, Chang C-M, Wang J-R: **Preexisting Antibody Response against 2009 Pandemic Influenza H1N1 Viruses in the Taiwanese Population**. *Clinical and Vaccine Immunology* 2010, **17**(12):1958-1962.

243. Liu X, Liu Y, Zhang Y, Chen Z, Tang Z, Xu Q, Wang Y, Zhao P, Qi Z: **Pre-Existing Immunity with High Neutralizing Activity to 2009 Pandemic H1N1 Influenza Virus in Shanghai Population**. *PloS one* 2013, **8**(3):e58810.

244. Pichyangkul S, Krasaesub S, Jongkaewwattana A, Thitithanyanont A, Wiboon-Ut S, Yongvanitchit K, Limsalakpetch A, Kum-Arb U, Mongkolsirichaikul D, Khemnu N *et al*: **Pre-existing cross-reactive antibodies to avian influenza H5N1 and 2009 pandemic H1N1 in US military personnel**. *The American journal of tropical medicine and hygiene* 2014, **90**(1):149-152.

245. Xing Z, Cardona CJ: **Preexisting immunity to pandemic (H1N1) 2009**. *Emerging infectious diseases* 2009, **15**(11):1847-1849.

246. Carragher DM, Kaminski DA, Moquin A, Hartson L, Randall TD: **A Novel Role for Non-Neutralizing Antibodies against Nucleoprotein in Facilitating Resistance to Influenza Virus**. *The Journal of Immunology* 2008, **181**(6):4168-4176.

247. Pinna D, Corti D, Jarrossay D, Sallusto F, Lanzavecchia A: **Clonal dissection of the human memory B-cell repertoire following infection and vaccination**. *European journal of immunology* 2009, **39**(5):1260-1270.

248. LaMere MW, Lam H-T, Moquin A, Haynes L, Lund FE, Randall TD, Kaminski DA: **Contributions of Antinucleoprotein IgG to Heterosubtypic Immunity against Influenza Virus**. *The Journal of Immunology* 2011, **186**(7):4331-4339.

249. Wahl A, Schafer F, Bardet W, Buchli R, Air GM, Hildebrand WH: **HLA class I molecules consistently present internal influenza epitopes**. *Proceedings of the National Academy of Sciences of the United States of America* 2009, **106**(2):540-545.

250. Anderson RW, Bennink JR, Yewdell JW, Maloy WL, Coligan JE: **Influenza basic polymerase 2 peptides are recognized by influenza nucleoprotein-specific cytotoxic T lymphocytes**. *Molecular immunology* 1992, **29**(9):1089-1096.

251. Amanna IJ, Carlson NE, Slifka MK: **Duration of humoral immunity to common viral and vaccine antigens**. *The New England journal of medicine* 2007, **357**(19):1903-1915.

252. LaMere MW, Moquin A, Lee FE-H, Misra RS, Blair PJ, Haynes L, Randall TD, Lund FE, Kaminski DA: **Regulation of Antinucleoprotein IgG by Systemic Vaccination and Its Effect on Influenza Virus Clearance**. *Journal of Virology* 2011, **85**(10):5027-5035.

253. Murphy BR, Kasel JA, Chanock RM: **Association of Serum Anti-Neuraminidase Antibody with Resistance to Influenza in Man**. *New England Journal of Medicine* 1972, **286**(25):1329-1332.

254. Tsukamoto M, Hiroi S, Adachi K, Kato H, Inai M, Konishi I, Tanaka M, Yamamoto R, Sawa M, Handharyani E *et al*: **Antibodies against swine influenza virus neutralize the pandemic influenza virus A/H1N1**. *Molecular medicine reports* 2011, **4**(2):209-214.

255. Khurana S, Loving CL, Manischewitz J, King LR, Gauger PC, Henningson J, Vincent AL, Golding H: **Vaccine-Induced Anti-HA2 Antibodies Promote Virus Fusion and Enhance Influenza Virus Respiratory Disease**. *Science Translational Medicine* 2013, **5**(200):200ra114.

256. DuBois RM, Aguilar-Yañez JM, Mendoza-Ochoa GI, Oropeza-Almazán Y, Schultz-Cherry S, Alvarez MM, White SW, Russell CJ: **The Receptor-Binding Domain of Influenza Virus Hemagglutinin Produced in Escherichia coli Folds into Its Native, Immunogenic Structure**. *J Virol* 2011, **85**(2):865-872.

257. Storek J, Viganego F, Dawson MA, Herremans MMPT, Boeckh M, Flowers MED, Storer B, Bensinger WI, Witherspoon RP, Maloney DG: **Factors affecting antibody levels after allogeneic hematopoietic cell transplantation**. *Blood* 2003, **101**(8):3319-3324.

258. Ilan Y, Nagler A, Adler R, Naparstek E, Or R, Slavin S, Brautbar C, Shouval D: **Adoptive transfer of immunity to hepatitis B virus after T cell-depleted allogeneic bone marrow transplantation**. *Hepatology* 1993, **18**(2):246-252.

259. Ilan Y, Nagler A, Zeira E, Adler R, Slavin S, Shouval D: **Maintenance of immune memory to the hepatitis B envelope protein following adoptive transfer of immunity in bone marrow transplant recipients**. *Bone marrow transplantation* 2000, **26**(6):633-638.

260. Molrine DC, Antin JH, Guinan EC, Soiffer RJ, MacDonald K, Malley R, Malinoski F, Trocciola S, Wilson M, Ambrosino DM: **Donor immunization with pneumococcal conjugate vaccine and early protective antibody responses following allogeneic hematopoietic cell transplantation**. *Blood* 2003, **101**(3):831-836.

261. Terajima M, Cruz J, Leporati AM, Orphin L, Babon JAB, Co MDT, Pazoles P, Jameson J, Ennis FA: **Influenza A Virus Matrix Protein 1-Specific Human CD8+ T-Cell Response Induced in Trivalent Inactivated Vaccine Recipients**. *Journal of Virology* 2008, **82**(18):9283-9287.

262. Renfrey S, Watts A: **Morphological and biochemical characterization of influenza vaccines commercially available in the United Kingdom**. *Vaccine* 1994, **12**(8):747-752.

263. Garcia-Cañas V, Lorbetskie B, Cyr TD, Hefford MA, Smith S, Girard M: **Approach to the profiling and characterization of influenza vaccine constituents by the combined use of size-exclusion chromatography, gel electrophoresis and mass spectrometry**. *Biologicals* 2010, **38**(2):294-302.

264. Garcia-Cañas V, Lorbetskie B, Girard M: **Rapid and selective characterization of influenza virus constituents in monovalent and multivalent preparations using non-porous reversed-phase high performance liquid chromatography columns**. *Journal of Chromatography A* 2006, **1123**(2):225-232.

265. Yalcin SS, Kondolot M, Albayrak N, Altas AB, Karacan Y, Kuskonmaz B, Aksu S, Cetin M, Goker H, Yurdakok K *et al*: **Serological response to influenza vaccine after hematopoetic stem cell transplantation**. *Annals of hematology* 2010, **89**(9):913-918.

266. Engelhard D, Zakay-Rones Z, Shapira MY, Resnick I, Averbuch D, Grisariu S, Dray L, Djian E, Strauss-Liviatan N, Grotto I *et al*: **The humoral immune response of hematopoietic stem cell transplantation recipients to AS03-adjuvanted A/California/7/2009 (H1N1)v-like virus vaccine during the 2009 pandemic**. *Vaccine* 2011, **29**(9):1777-1782.

267. Wimperis JZ, Brenner MK, Prentice HG, Reittie JE, Karayiannis P, Griffiths PD, Hoffbrand AV: **Transfer of a functioning humoral immune system in transplantation of T-lymphocyte-depleted bone marrow**. *Lancet* 1986, **1**(8477):339-343.

268. Saxon A, Mitsuyasu R, Stevens R, Champlin RE, Kimata H, Gale RP: **Designed transfer of specific immune responses with bone marrow transplantation**. *The Journal of clinical investigation* 1986, **78**(4):959-967.

269. Molrine DC, Guinan EC, Antin JH, Parsons SK, Weinstein HJ, Wheeler C, McGarigle C, Blanding P, Phillips NR, Kinsella K *et al*: **Donor immunization with Haemophilus influenzae type b (HIB)-conjugate vaccine in allogeneic bone marrow transplantation**. *Blood* 1996, **87**(7):3012-3018.

270. Parkkali T, Stenvik M, Ruutu T, Hovi T, Volin L, Ruutu P: **Randomized comparison of early and late vaccination with inactivated poliovirus vaccine after allogeneic BMT**. *Bone marrow transplantation* 1997, **20**(8):663-668.

271. Parkkali T, Olander RM, Ruutu T, Vuontela K, Volin L, Eskola J, Ruutu P: **A randomized comparison between early and late vaccination with tetanus toxoid vaccine after allogeneic BMT**. *Bone marrow transplantation* 1997, **19**(9):933-938.

272. Cordonnier C, Labopin M, Chesnel V, Ribaud P, De La Camara R, Martino R, Ullmann AJ, Parkkali T, Locasciulli A, Yakouben K *et al*: **Randomized study of early versus late immunization with pneumococcal conjugate vaccine after allogeneic stem cell transplantation**. *Clin Infect Dis* 2009, **48**(10):1392-1401.

273. Vance E, George S, Guinan EC, Wheeler C, Antin JH, Ambrosino DM, Molrine DC: **Comparison of multiple immunization schedules for Haemophilus influenzae type b-conjugate and tetanus toxoid vaccines following bone marrow transplantation**. *Bone marrow transplantation* 1998, **22**(8):735-741.

274. Tomblyn M, Chiller T, Einsele H, Gress R, Sepkowitz K, Storek J, Wingard JR, Young JAH, Boeckh MA: **Guidelines for Preventing Infectious Complications among Hematopoietic Cell Transplantation Recipients: A Global Perspective**. *Biology of Blood and Marrow Transplantation* 2009, **15**(10):1143-1238.

275. Huckriede A, Bungener L, Stegmann T, Daemen T, Medema J, Palache AM, Wilschut J: **The virosome concept for influenza vaccines**. *Vaccine* 2005, **23 Suppl 1**:S26-38.

276. Huckriede A, Bungener L, ter Veer W, Holtrop M, Daemen T, Palache AM, Wilschut J: **Influenza virosomes: combining optimal presentation of hemagglutinin with immunopotentiating activity**. *Vaccine* 2003, **21**(9-10):925-931.

277. de Mare A, Bungener LB, Regts J, de Vries-Idema J, van der Zee AGJ, Wilschut J, Daemen T: **The effect of pre-existing immunity on the capacity of influenza virosomes to induce cytotoxic T lymphocyte activity**. *Vaccine* 2008, **26**(19):2314-2321.

278. Bungener L, Huckriede A, de Mare A, de Vries-Idema J, Wilschut J, Daemen T: **Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity**. *Vaccine* 2005, **23**(10):1232-1241.

279. Magalhaes I, Eriksson M, Linde C, Muhammad R, Rane L, Ambati A, Axelsson-Robertson R, Khalaj B, Alvarez-Corrales N, Lapini G *et al*: **Difference in immune response in vaccinated and unvaccinated Swedish individuals after the 2009 influenza pandemic**. *BMC infectious diseases* 2014, **14**:319.

280. Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG: **Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections**. *Immunological reviews* 1997, **159**:105-117.

281. McElhaney JE, Xie D, Hager WD, Barry MB, Wang Y, Kleppinger A, Ewen C, Kane KP, Bleackley RC: **T Cell Responses Are Better Correlates of Vaccine Protection in the Elderly**. *The Journal of Immunology* 2006, **176**(10):6333-6339.

282. Assarsson E, Bui H-H, Sidney J, Zhang Q, Glenn J, Oseroff C, Mbawuike IN, Alexander J, Newman MJ, Grey H *et al*: **Immunomic Analysis of the Repertoire of T-Cell Specificities for Influenza A Virus in Humans**. *Journal of Virology* 2008, **82**(24):12241-12251.

283. Ambati A, Valentini D, Montomoli E, Lapini G, Biuso F, Wenschuh H, Magalhaes I, Maeurer M: **H1N1 viral proteome peptide microarray predicts individuals at risk for H1N1 infection and segregates infection versus pandemrix -vaccination**. *Immunology* 2015.

284. Lawrence CW, Braciale TJ: **Activation, differentiation, and migration of naive virus-specific CD8+ T cells during pulmonary influenza virus infection**. *Journal of immunology (Baltimore, Md : 1950)* 2004, **173**(2):1209-1218.

285. Axelsson-Robertson R, Ahmed RK, Weichold FF, Ehlers MM, Kock MM, Sizemore D, Sadoff J, Maeurer M: **Human leukocyte antigens A*3001 and A*3002 show**

distinct peptide-binding patterns of the Mycobacterium tuberculosis protein TB10.4: consequences for immune recognition. *Clin Vaccine Immunol* 2011, **18**(1):125-134.

286. Tena-Coki NG, Scriba TJ, Peteni N, Eley B, Wilkinson RJ, Andersen P, Hanekom WA, Kampmann B: **CD4 and CD8 T-cell responses to mycobacterial antigens in African children**. *Am J Respir Crit Care Med* 2010, **182**(1):120-129.

287. Hohn H, Julch M, Pilch H, Kortsik C, Tully G, Neukirch C, Freitag K, Maeurer M: **Definition of the HLA-A2 restricted peptides recognized by human CD8+ effector T cells by flow-assisted sorting of the CD8+ CD45RA+ CD28- T cell subpopulation**. *Clin Exp Immunol* 2003, **131**(1):102-110.

288. Soares AP, Scriba TJ, Joseph S, Harbacheuski R, Murray RA, Gelderbloem SJ, Hawkridge A, Hussey GD, Maecker H, Kaplan G *et al*: **Bacillus Calmette-Guerin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles**. *Journal of immunology (Baltimore, Md : 1950)* 2008, **180**(5):3569-3577.

289. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C *et al*: **A human memory T cell subset with stem cell-like properties**. *Nat Med* 2011, **17**(10):1290-1297.

290. Turtle CJ, Swanson HM, Fujii N, Estey EH, Riddell SR: **A distinct subset of self-renewing human memory CD8+ T cells survives cytotoxic chemotherapy**. *Immunity* 2009, **31**(5):834-844.

291. Havenith SH, Yong SL, Henson SM, Piet B, Idu MM, Koch SD, Jonkers RE, Kragten NA, Akbar AN, van Lier RA *et al*: **Analysis of stem-cell-like properties of human CD161++IL-18Ralpha+ memory CD8+ T cells**. *Int Immunol* 2012, **24**(10):625-636.

292. Mandel TE, Phipps RP, Abbot A, Tew JG: **The follicular dendritic cell: long term antigen retention during immunity**. *Immunological reviews* 1980, **53**:29-59.

293. Tew JG, Phipps RP, Mandel TE: **The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells**. *Immunological reviews* 1980, **53**:175-201.

294. Nace DA, Lin CJ, Ross TM, Saracco S, Churilla RM, Zimmerman RK: **Randomized, Controlled Trial of High-Dose Influenza Vaccine Among Frail Residents of Long-Term Care Facilities**. *Journal of Infectious Diseases* 2014.

295. Puig-Barberà J, Natividad-Sancho A, Calabuig-Pérez J, Lluch-Rodrigo JA, Pastor-Villalba E, Martínez-Úbeda S, Díez-Domingo J: **Intradermal and virosomal influenza vaccines for preventing influenza hospitalization in the elderly during the 2011–2012 influenza season: A comparative effectiveness study using the Valencia health care information system**. *Vaccine* 2014, **32**(42):5447-5454.