Talk Outline

• Innate Immunity
• Adaptive Immunity
  – Cellular and humoral responses
  – Assays and detection of responses
  – Mechanisms of antibody control
• Immunity and antigenic variation
• Immunity in aging
**INNATE IMMUNITY**

Ancient
Activated In Hours
Receptors Do Not Rearrange
Receptors Nonclonal
Recognizes conserved molecular patterns (LPS, carbohydrates etc)
Self-Nonself discrimination is perfect
Epithelial Cells, Monocytes
Neutrophils, NK Cells, complement,

**ADAPTIVE IMMUNITY**

Recent
Takes Longer (Days)
Receptors Rearrange
Receptors Are Clonally Distributed
Complicated structures, proteins, peptides
Imperfect
T and B Cells
1. Block the entry of microbes into host tissue (first line of defense)

2. Elimination or early control or of microbes that succeed in entering the host tissue

3. Instructs cells of the adaptive immune system (T and B cells) to eliminate the microbe if innate immunity is unsuccessful

KEY ROLES of innate immunity
Principal components of innate immunity

- Anatomical
  - Ciliated epithelium

- Secreted molecules
  - Immediate or induced

- Cells
  - Neutrophils
  - Macrophages
  - NK cells
Innate Immune System Recognizes Pathogens through Pathogen-Associated Molecular Patterns (PAMPs)

• The body detects the presence of microorganisms by recognizing molecules unique to microorganisms that are not associated with human cells
• PAMPs are structural or genetic components that stimulate pathogen sensors
  – Cell wall components
    • Proteins
    • Lipids
    • Carbohydrates
  – Nucleic acids
• PAMPs are recognized by Pathogen Recognition Receptors (PRRs)
Pathogen Recognition Receptors (PRRs)

**Extracellular-soluble**
- Collectins and ficolins
  - SP A and D
  - MBL
  - Ficolins L, M, and H
- Pentraxins
  - PTX3, PTX4, NPTX1, NPTX2
  - CRP, SAP
- PLUNC
  - BPI
  - a protein
- Complement

**Membrane-bound**
- Toll-like receptors
  - TLRs 1, 2; 2, 6; 4, 5
- Lectin receptors
  - Mannose receptor
  - DC-SIGN
  - SIGNR1
  - Langerin
  - Dectin-1 and -2
- Scavenger receptors
  - SR-AI, SR-AII, SR-AI, SR-AII
  - MARCO
- Integrins
  - Mac-1

**Vesicular**
- Toll-like receptors
  - TLR-3
  - TLR-7/8
  - TLR-9

**Cytoplasmic**
- RNA sensors
  - PKR
- RIG-I-like receptors
  - RIG-I
  - MDA5
- Peptidoglycan sensors
  - NLRX1
- NOD-like receptors
  - NOD1 and 2
  - Ipaf
cryopyrin
- DNA sensors
  - DAI (DLM-1/ZBP1)
  - AIM2
Immediate Innate defences in the respiratory tract

• Non-specific inhibitors in mucins

• Collectins are soluble PRRs
  • carbohydrate binding proteins with globular domains
  • Lung surfactant proteins SP-A and SP-D
  • Mannose binding protein (MBL)

• Bind to virus via the carbohydrate side chains on HA and NA, inhibiting the virus engaging its receptor

• Also bind to HA and NA on the infected cell surface

• Trigger the lectin pathway of the complement cascade

• → uptake of virus by macrophages, lysis of infected cells
Recognition of PAMPs in Influenza Virus Infected Cells

- Retinoic acid inducible gene-I (RIG-I)
  - Found in most cell types in cytosol
  - Recognizes ssRNA bearing 5’ triphosphate
  - Results in induction of type I interferons
  - Influenza A NS1 suppresses RIG-I signaling

- Toll-like Receptor (TLR)-7
  - Recognize genomic RNA in endosomes of dendritic cells
  - Plays important role in development of adaptive immunity
  - Both live and killed virus can induce type I IFN through TLR-7
Induction of type I Interferons and proinflammatory cytokines

- Infected epithelial cells produce type I interferons (IFN-α/β)
- PAMP - PRR interactions (TLR7) induce macrophages and immature DCs at site of infection to produce type I interferons and proinflammatory cytokines (IL-1, IL-6, IL-12, TNF-α and chemokines (MIP-1α/β, MCP-1, IL-8)
- Leads to amplification of the inflammatory response
Role of Type I interferons (IFNα/β)

- Protect uninfected cells from virus infection. Their binding to IFNRI triggers antiviral functions in the cell
- Induce the influx and activation of NK (natural killer) cells to kill infected epithelial cells before virus release
- Upregulate MHC class I expression to make cells better targets for lysis
Systemic effects of proinflammatory cytokines

- IL-1, IL-6 and TNF act on the brain to produce symptoms of illness
  - Fever, lethargy, muscle aches

- Also act in liver to cause release of acute-phase proteins
  - Activation of Complement proteins
  - Mannose binding proteins

- Colony stimulating factors activated by inflammatory responses can stimulate lymphocyte production

Adapted from A. S. Hamblin, *Cytokines and Cytokine Receptors* (IRL Press, Oxford, United Kingdom, 1993), with permission.

*From Principles of Virology, SJ Flint et al., (eds)*
ASM Press, 2004
The Inflammatory Response in Fatal Human Avian H5N1 Virus Infection
(deJong et al., Nat Med 2006;12:1203)

- Fatal human H5N1 virus infection characterized by:
  - High viral load
  - vRNA detection in blood or GI tract
  - Low peripheral blood lymphocytes
  - High levels of some chemokines and cytokines
Possible Mechanisms of H5N1 Virus Disease

- Differences in host responses to H5N1 viruses versus seasonal viruses
  - Heightened cytokine/chemokine responses

- H5N1 virus has tropism for alveolar epithelium and macrophages

- Cytokine induced inflammatory responses thought to play major role in Acute Respiratory Disease Syndrome (ARDS)

Peiris et al., Trends Immunol 2009; 30:574-84
Natural Killer (NK) Cells and Influenza

- Major lymphocyte effector cells of the innate response
- Regulated by inhibitory and activation receptors
  - Inhibitory receptors recognize MHC class I molecule expression
  - Recognise and bind virus-infected cells through receptors that recognise eg. cell “stress”
  - Natural cytotoxicity receptors (NKp46) recognizes HA
- Activation leads to release of granules which damage target cells and induce apoptosis
- More severe influenza disease in mice depleted of NK cells
- Activated NK cells produce a variety of cytokines (IFN-gamma) chemokines and are an important bridge between innate and adaptive immunity
- NK cells are also effectors for adaptive responses mediated by antibodies (ADCC)
The innate response to primary influenza provides early control, slowing replication, but adaptive immunity ultimately clears the infection and allows recovery.

From Principles of Virology, SJ Flint et al., (eds)  
ASM Press, 2004
T cell-mediated immunity
Dendritic cells are central to the initiation and regulation of the adaptive immunity.

DC and microenvironment influence polarization of T cells.

*Pulendran B, Nat Immunol, 2010*
Dendritic Cell Subsets

Two subsets of DCs: plasmacytoid and conventional or myloid DC
Major Histocompatibility Class (MHC) I and II Antigen Structures and Interaction with CD8+ and CD4+ T cells, Respectively
Peptides are 8-10 aa long
Anchor residues at each end

Peptides are at least 13 aa long
Anchor residues at each end
Same core sequence for same MHC molecule
T cell Receptor Binding to MHC-peptide Complex

T cell receptor (TCR)

MHC-peptide complex

MHC-peptide complex showing TCR contact area

Figure 3-22 Immunobiology, 7ed. (© Garland Science 2008)
APCs deliver three kinds of signals to naive T cells

1. CD4
2. B7.1
3. IL-6
4. IL-12
5. TGF-β

MHC class II

TCR

CD28

cytokines

Activation

Survival

Differentiation

Figure 8-19 Immunobiology, 7ed. (© Garland Science 2008)
Th1 Response

TNF-α

MHC Class I

TC

MHC Class II

TNF-β

T_{H1}

IL-2

IFN-γ

NK

IFN-γ

IL-12

TNF-α

TNF-α

MHC Class I

MHC Class II
CD8+ T cell Effectors Mediate Lysis of Virus Infected Cells By Release of Cytotoxic Granules
### Human influenza A virus CTL epitopes

Numerous CTL epitopes have been identified on most influenza viral proteins.*

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<th>HLA restriction</th>
<th>Protein (amino acids)</th>
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<td>A*01</td>
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* Data are from the Influenza Sequence Database (http://www.flu.lanl.gov) (26).

*Berkhoff et al., J Virol 2004;78:5216-5222*
Inverse Correlation between Virus Shedding and CD8+ Cytotoxic T cell Responses in Seronegative Adults

McMichael et al., NEJM 1983 and Epstein et al., Exp Rev Vac 2010
Intracellular Cytokine Staining

1. Stimulate cells with antigen of interest to generate cytokine production.

2. Add golgi blocker to accumulate cytokines in the cells.

3. Stain surface markers with fluorescently labeled monoclonal antibodies (CD4, CD8, CD25, CD69, etc.)

4. Stain intracellular cytokines with fluorescently labeled monoclonal antibodies (IFN-γ, TNF-α, IL-2).

5. Analyze surface marker and cytokine expression by flow cytometry.
CD4+ Helper T cell Response to Influenza Infection

• The B cell response to influenza virus HA is dependent on help from CD4+ T cells
• CD4+ T cells provide help for B cell activation and affinity maturation
• Epitopes recognized by CD4+ T cells are found on majority of influenza virus proteins
  – Recognition depends on HLA (MHC antigens) profile of an individual
• T cell memory is essentially life long
• In general, CD4+ T cells exert their effect by proving help for B cells and CD8+ T cells, and do not act directly to clear virus
  – Mice that lack B cells and CD8+ T cells succumb to infection
Interferon-gamma Producing CD4+ T cell Responses in Children and Adults following Vaccination with LAIV or TIV

(He et al., J Virol 2006; 80:11756)

In children, 5-9 yrs, LAIV stimulated stronger CD4+ and CD8+ T cell responses compared with TIV. Compared with adults, children made more robust responses to LAIV. TIV did not stimulate CD4+ or CD8+ T cell responses to a significant level.
B cell-mediated immunity
B cell differentiation pathway

Naïve B cell

$T_{dep} \text{ Ag}$ (influenza virus)

Foci of short-lived plasma cells (low affinity IgM)

Germinal center B cell

Upregulated activation markers and lower threshold for activation

Memory B cells

Somatic hypermutation Selection

Long-lived plasma cells in bone marrow

High affinity Class switched Antibodies (IgG, IgA)

Germinal center B cell
Kinetics of Serum Antibody Response to Primary Influenza Infection

Antibody titer

Weeks post-infection

Serum IgG
Serum IgM
Serum IgA

1 2 4 12 26
Kinetics of Neutralizing Antibody Response
Humans infected with Avian Influenza

Estimated days post symptom onset

Neutralizing Ab GMT (Log$_2$)
Secretory IgA responses are an important defense against mucosal pathogens

- Secretory or polymeric IgA is produced by B cells at mucosal surfaces of upper respiratory tract
- IgA binds to receptor on epithelial cells and is endocytosed
- Internal IgA can bind viral proteins in internal compartments
- Free IgA binds virus in lumen
- IgA is produced at mucosal surfaces following influenza infection
  - Can be detected for at least 3 months
  - Neutralizing activity
  - Memory B cells detected in nasal mucosa

*From Principles of Virology, SJ Flint et al., (eds)*
ASM Press, 2004
Serologic Assays for Detection of Human anti-HA Antibodies

- Hemagglutination-Inhibition (HI)
  - Detects Ab that bind around receptor-binding site in globular head and block agglutination
  - Titer $\geq 40$ correlated with protection

- Microneutralization (MN) or Virus Neutralization (VN)
  - Detects Ab that bind around globular head and block virus attachment/entry
  - Detects cross-reactive Ab that bind to stem region
  - No correlate of protection established

- Single radial hemolysis (SRH) Assay
  - Area of $\geq 25\text{mm}^2$ correlated with protection

- Indirect ELISA using rHA or purified virus
  - Detects any Ab that binds entire HA including Ab against linear epitopes if HA is partially denatured
  - Not suitable for detection of strain-specific responses
  - Suitable to detect IgG, IgM, IgA in serum and nasal washes
Hemagglutination inhibition assay

1. Treat serum, 2-fold serial dilutions
2. Add 4 HA units of virus, 30 min @ RT
3. Add 0.5% turkey RBC, 30 min @ RT
4. Record HI titer

Photos courtesy of A. Balish
Mioneutralization Assay

1. Add heat inactivated sera

2. Add virus
   100 or 200 TCID/well

3. Add MDCK cells
   1.5 x 10^4 cells/well

4. Wash/Fix

5. ELISA
   α-NP Ab

1 hr @ 37°C

18-20 hr @ 37°C
Influenza A virus Proteins as Antibody Targets

Anti-HA Abs block attachment or fusion of virus to inhibit replication

Anti-NA Abs prevent virus release and spread

Abs to M2 external domain block virus budding/release and are target for ADCC

Abs to NP reduce viral shedding in mice
Antibodies to the HA Globular Head Block Binding to Receptor on Host Cell

Subbarao and Joseph,
Nat Rev Immunol 2007: 7:267
HA Stem Region Antibodies

- Based on recent identification of human MAbs that recognize conserved stalk region of HA that encompasses fusion peptide (*Throsby et al., PLoS One 2008; Ekiert et al., Science 2009; Sui et al., Nat Struct Mol Biol 2009*)

- 2 structural groups for 16 HA subtypes

- Antibodies inhibit membrane fusion
  - Neutralization in vitro?

- Low levels of these heterosubtypic Abs detected in human sera (*Sui et al., CID 2011*)

- Vaccination may boost these responses (*Corti et al., JCI 2010;120:1663*)
Non-neutralizing antibodies can inhibit release of virus from infected cell

MDCK cells infected with H3N2 virus
In the presence and absence of anti-M2 Ab

Subbarao and Joseph,
Nat Rev Immunol 2007: 7:267

Hughey et al., Virology 1995;212:411-21
Antibody-dependent Cell-mediated Cytotoxicity (ADCC)

Effector NK cell → Virus-infected cell

Perforin, Granzymes

Fc receptor (CD16), Fc domain

(Anti-M2 antibodies)

M2 antigen

Cell lysis
B cell ELISPOT assay

1. Coat plate with antigen
2. Add PBMC or plasma blasts
3. Add enzyme-linked antibody to human Ig and develop plates
4. Culture cells with activators to produce plasma blasts
5. Memory B cells
Serum Antibody Responses to Influenza Virus Infection is Long-lived

- In 1977-78, H1N1 reemerged after absence of more than 20 years
  - Only children and young adults susceptible

- Memory B cells specific for 1918 virus isolated from elderly persons exposed to 1918 virus
  - Pre-existing serum antibody cross-reactive with 2009 H1N1pdm virus found in older adults
Circulating and Memory B cell Responses Following Immunization with seasonal LAIV or TIV (H3N2 response)  
(Sasaki et al., J Virol 2007;81:215)

- ~80% of adults and older children made circulating IgG ASC responses to LAIV or TIV on days 7-12 post infection
- Elevated IgG ASC were detected more frequently in adult LAIV recipients compared with serum Abs
- TIV, but not LAIV resulted in increased levels of memory B cells in blood
- Memory IgG B cell responses increase with age in young children
# Protective Role of Antibody and CMI Responses to Influenza

<table>
<thead>
<tr>
<th></th>
<th>Specificity</th>
<th>Protection from infection</th>
<th>Reduction of virus shedding</th>
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<tbody>
<tr>
<td><strong>Antibody</strong></td>
<td>HA (strain specific)</td>
<td>Yes</td>
<td>Yes</td>
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<td></td>
<td>HA (cross-reactive)</td>
<td>Yes/No?</td>
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<td>NA</td>
<td>No</td>
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<td>M2</td>
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<tr>
<td><strong>CTL</strong></td>
<td>Various</td>
<td>No</td>
<td>Yes</td>
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</table>
Immunity and Antigenic Variation of Influenza Viruses
Antigenic Drift

- Leads to the creation of new strains within a subtype
- Amino acid changes in viral proteins arise because of errors of replication of the viral RNA-dependent RNA polymerase (= drift)
- Those that occur in the sites on HA (or NA) where antibodies bind may be advantageous if the antibody can no longer bind the mutated protein sequence.
- Viruses with such a mutation will be selected in the presence of antibodies to that site (= antigenic drift)
- So even though antibodies induced by natural infection are very long lived, they become ineffective in neutralizing variant strains
Specificity of HA Antibody Response

- Following natural infection of children, the majority of antibody induced is highly specific for infecting strain.

- Likewise, following vaccination with inactivated virus, children make a “strain-specific” antibody response.

- In contrast, infection or vaccination of adults induces anti-HA antibody that is predominantly cross-reactive for antigenically related viruses within the subtype.
Original Antigenic Sin

- Concept based on patterns of serum antibody responses among persons with varying histories of influenza virus infection or vaccination (Francis)
- Exposure to initial influenza virus leaves a lasting “imprint” on the immune response
- Subsequent re-exposure to variant of same subtype results in immune response dominated by antibodies that cross-react with earlier strain
- Pre-existing memory B cells use available CD4+ T cell help to expand
  - Reduced expansion of naïve B cells recognizing new epitopes
Antigenic Shift of Influenza A Viruses Infecting Humans

- H1N1
- H2N2
- H3N2
- H5
- H9
- H7

- Spanish Influenza
- Asian Influenza
- Hong Kong Influenza

Timeline:
- 1918: Spanish Influenza
- 1957: Asian Influenza
- 1968: H2N2 Hong Kong Influenza
- 1977: H1N1
- 1997: H5
- 1999: H9
- 2003: H7
- 2005: H1N1 pdm
- 2009:
Cross-Protective Immunity to Influenza A Viruses

• **Heterosubtypic immunity (Het-I)**
  – Protection by virus or antigen of one influenza A strain against a virus of a different subtype

• **Heterotypic immunity**
  – Protection within a subtype
  – E.g. across H5N1 clades

• Het-I induction is the goal of “Universal Vaccines”
Heterosubtypic Immunity in Animal Models

- Mild infection of animals with one strain protects against subsequent severe challenge with virus bearing different HA and NA
  - Infection is not prevented
  - Reduction of virus shedding and less severe disease

- Both T and B cell-mediated immunity involved
  - CD8+ CTL recognize epitopes on conserved internal proteins
  - Anti-M2 antibody and HA stem region antibody
  - Role for CD4+ T cells may be to support and enhance CTL responses and provide help for B cell responses
Evidence for Heterosubtypic Immunity in Human During 1957 H2N2 Pandemic

*Epstein and Price, Expert Rev Vaccines 2010; 9(11):1325-41*

**Subject reported ILI**

**Laboratory confirmed**
Cross-recognition of avian H5N1 viruses by human cytotoxic CD8+ T cells induced by human viruses (Kreijtz et al., *J Virol* 2008; 82:5161)

- Epitopes in PB1, M1, NS1, NP were >95% conserved
- Majority of individuals with different HLA types CD8+ T cell responses that cross-reacted with H5N1 viruses

### Table 1. Variant sequences of known CTL epitopes in H5N1 viruses

<table>
<thead>
<tr>
<th>Epitope Variant</th>
<th>HLA-A1 PHB (591–599)</th>
<th>HLA-A3 M1 (15–21)</th>
<th>HLA-A*0201 M1 (58–66)</th>
<th>HLA-B*0201 M1 (128–135)</th>
<th>HLA-A*0201 NS1 (122–132)</th>
<th>HLA-A*0201 NS1 (158–166)</th>
<th>HLA-B*44 NP (44–52)</th>
<th>HLA-A*6801 NP (91–99)</th>
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<tr>
<td>1</td>
<td>VDFQGOFVX</td>
<td>S111PSQDPX</td>
<td>QIGIPVPLS</td>
<td>AEGHLYX</td>
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</table>

* Variant sequences of known CTL epitopes were ranked according to their relative prevalence. Anchor residues of the epitopes are underlined. Amino acid residue positions are given in parentheses.
Variable CTL Epitopes identified in H3N2 Influenza Viruses
(*Rimmelzwaan et al., Vaccine 2009;27:6363*)

<table>
<thead>
<tr>
<th>Epitope</th>
<th>HLA restriction</th>
<th>Amino acid sequence</th>
<th>Years of isolation</th>
<th>reference</th>
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<td>-K- - - - - - - - -</td>
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</table>

*During years of co-circulation of two different variants, the most dominant variants are indicated in bold and underlined.

162 viruses
Spanning 10 years
Impact of Mutations within CD8+ T cell Epitopes

- Mutations in amino acids that anchor peptide binding to MHC class I molecule or engage T cell receptor can reduce/eliminate CD8+ CTL response in vitro
- If mutation in immunodominant epitope, may lose major CTL response to virus
  - Responses to other epitopes may not “fill-in”
- In mice, loss of immunodominant epitope response results in delayed virus clearance and increased morbidity
- Impact in humans not clear

Variation in M2 Ectodomain among Influenza Virus Subtypes

Zhong et al., J Virol Meth 2010
The “Holy Grail”: An influenza Universal Vaccine:

- Conserved antibodies to M2 or HA stem region
- Conserved epitopes on NP and M1 for CD8+ T cell responses
- Need to consider variation in so-called “non-variable” targets
1 in 5 Americans is >65 years old. It will be 1 in 3.5 by 2050
1.45 billion people 65+ by 2050 in the world

- Older adults experience more severe and fatal influenza illness
- Effectiveness of vaccines is reduced
Immune Responses in Aging -I

- Immunosenescence: age-related changes in immune function
- Greater heterogeneity of responses in older adults
- Affects both innate and adaptive arms of immune response in terms of decreases in number or function of cells
  - Function of NK cells decline, in part because of increase in inhibitory receptors and decreased cytokine production
  - Macrophage numbers and cytokine secretion are reduced
  - Expression and function of PRRs is reduced
  - Effect of aging on different DC subsets not clear
    - Presenting function of aged DCs are preserved (?) but pDC subset numbers and cytokine expression decline
Immune Responses in Aging -II

• More agreement on substantial defects in adaptive responses
• Size of naïve T cell pool is reduced and memory and effector T cells is increased
• Effector T cells show reduced activation, in part because of reduced co-stimulatory signaling
• B cell responses are compromised by:
  – Decrease in naïve B cell numbers
  – Germinal center disruption leads to reduced affinity maturation
Summary and Conclusions

• Innate immunity is important to shape adaptive immune responses to influenza
  – Pathogen recognition receptors trigger inflammatory response
  – Over exuberant response may be detrimental to host

• Influenza infection induces long-lasting B and T cell immunity
  – But variant strains overcome pre-existing neutralizing antibody responses
  – TIV and LAIV stimulate circulating antibody forming cells

• Multiple antibody targets and mechanisms contribute to effective humoral immunity

• Multiple conserved targets exist for induction of Het-I-based “Universal Vaccines”
  – Even conserved epitopes can vary

• Improved vaccines for older adults will need to overcome aspects of immunosenescence
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