We Have Met the Enemy
And He is Us – The Danger Model and Neonatal Disease

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Disclosures
- There are no financial or other relationships between the speaker and any commercial enterprise or product mentioned in this lecture
- There are no discussions of clinical testing or therapies in this lecture

Objectives
- Overview of the Immune System
- Explanations and Hypotheses for Tolerance
- The Danger Model
- PAMPs, DAMPs and PRRs
- The Danger Model and BPD
- The Danger Model and CNS Injury
- The Danger Model and NEC

Real Objective
- Introduce (or refresh) a new way of looking at immune response, especially innate
- Show how it might add to understanding of diseases using neonatal examples

As this is an introduction, it will be a simplified overview

The Immune Response
- “The immune system has evolved to counteract assaults on the body by non-self entities” (TK Mak, 2006)
- Critical
  - Bacteria have anti-viral enzymes
  - Defensins (cysteine-rich cationic proteins) active against bacteria, fungi, some viruses
  - Found in plants, insects, mollusks, amphibians, reptiles, mammals
  - Phagocytosis – sponges, worms, insects and higher
What Should the Immune System Do?
- The system needs to
  - Recognize the assault and the offending agent
  - Organize and effect a response
  - Control the response so that it does not injure the body
    - Modulate the response
    - Only attack what needs to be attacked

Dividing the Immune System in 2 – Different Ways of Slicing
- Non-specific vs Specific
  - General reaction (like forming pus in an abscess) vs tailored response (unique antibody production)
- Extracellular vs Intracellular
  - Sending out chemokine signals vs releasing defensins into phagosome
- Innate vs Adaptive

Adaptive Immune Response
- CD4+ (T helper), CD8+ (T cytotoxic) cells, B cells, antibodies
- Specific – antigen-specific T and B cell receptors, antibody production
- Memory – Adapted response (‘or’) to repeated exposures

Innate Immune Response
- Non-inducible, non-specific like skin
- Inducible, broadly “specific” like phagocytosis, NK cells, inflammation

The Need for Tolerance
- Like artillery
  - Identify that there is a target
  - Mount an appropriate response
    - Protect the host from invasion
  - Avoid Friendly Fire
    - Auto-immune diseases
    - Indiscriminate tissue destruction
- Multiple systems developed to solve this problem
B Cells -
- B cells - Go thru 9 stages in BM. Near the end, express surface IgM that can react with marrow stromal cell antigens (self) and prevent further development or undergo apoptosis or by rearranging its light chain (receptor editing).

T-cells
- T-cells – Formed in bone marrow, sent to thymus where they differentiate
- Express cell receptors – those that either poorly express cell receptors or which bind very strongly to MHC antigens on self tissue undergo apoptosis
- Left with cells with weak MHC recognition so react well with APC cell.

Antigen Presenting Cells (APCs)
- Non-specific (usually just MHC-I expression)
  - Fibroblasts, endothelial cells, glial cells
- Professional APCs (express MHC-II)
  - Dendritic cells
  - Macrophages and their derivatives (microglia, Kupffer cells, etc.)
  - Surface Ab+ B cells with specific antigen

Importance of APCs and Innate Immunity
- Pick up and present antigens (both foreign and domestic) through MHC-II to T cell TCR
- Could result in turning on response to self antigens
- Why not?

Issues of Tolerance
- 1945 (R. Owen) – Dizygotic cattle carry their own RBCs plus antigenically different RBCs from their twin. Why no immune hemolysis?
- 1953 (P. Medawar) – Injecting mouse fetus with cells from donor rendered the adult tolerant to later skin graft.

The First Self-Nonself Model
- Burnet and Medawar Nobel Prize 1960
  - Exposure during development to foreign antigens renders the host tolerant to subsequent exposures in later life i.e. what is “seen” during development is considered Self.
  - Fetal inflammatory response
  - Immune response to TORCH
  - Tolerance induction in adults
  - Etc.
The Two-Hit Modification

- 1970 (Bretsher and Cohn) – Need a co-stimulatory signal along with antigen exposure to elicit adaptive response. T helper cells for directing B cell response
- 1975 – APCs needed for co-stimulation signaling of T cells
  - APCs are non-specific when it comes to antigen capture/presentation so how is self-recognition handled?

APCs and Pattern Recognition Receptors

- 1989 (Janeway) – APCs are quiescent until activated via germ-line encoded receptors that recognize highly conserved molecular patterns
  - Receptors call Pattern Recognition Receptors
  - Ligands are Pathogen-Associated Molecular Patterns (PAMPs) – ancient, conserved
  - Activation produces co-stimulatory signal plus antigen presentation (For Infectious – Non-Self)
  - If Noninfectious – Self Ag – no activation or co-stimulatory signal

Issues with the Infectious-Nonself vs Noninfectious Self Theory

- Why do viruses stimulate immunity if they do not have PAMPs?
- Why transplant/graft rejection?
- Why are there autoimmune diseases?
- Why are some tumors rejected?
- [Why haven’t pathogen patterns evolved?]

Matzinger’s Danger Model - 1994

- It doesn’t matter what the antigen is, it only matters if the APC presents an antigen in conjunction with a “Danger signal”
- Danger signals also called alarm signals, alarmins, etc.
- Danger signals can be PAMPs like LPS
- Danger signals can be DAMPs like intracellular products released in response to stress or due to damage
- Must have a receptor on cell to respond

Who is Polly Matzinger?

- Born 1947
- By the time she was 27 she had been a Playboy bunny, jazz musician, dog trainer and waitress
- One of her customers persuaded her to finish college at UC-Davis in the sciences
- She got her Ph.D. at UCSD, did post-doc at Cambridge and Basel and established the Ghost Lab at NIH
- “Ghost” because it was empty for almost a year while she thought about how to apply Chaos Theory to immunology
- Banned from J Exp Medicine for 15 years
The Pattern Recognition Receptor

- Theory before fact
  - Theory in 1989, Danger receptor 1994
- Primary receptor family identified in 1994 – function unknown
- Toll receptor from Drosophila important for fighting fungal infections – 1996
- Human analog had role in initiating adaptive immune response – 1997
- LPS identified as ligand – The prototype PRR - 1998

The Receptor (s)

- TLRs – A family of at least 13 mammalian proteins
  - The intracellular portion looks like the IL-1 receptor and is needed for transmembrane signaling to initiate inflammatory responses
  - The extracellular portion is variable and confers the ability to bind to lots of important things
  - Phylogenetically old (plants, amphibians, birds, mammals)

The TLR Family & Their PAMPs

- TLR1 – mycobacteria
- TLR2 – bacteria, fungi
- TLR4 – LPS
- TLR5 – bacteria
- TLR6 – bacteria
- TLR9 – viruses
- TLR10 – ?
- TLR7 – viruses
- TLR8 – viruses
- TLR9 – viruses, bacteria, protozoa

But The TLRs Have Other Ligands!

- Heat Shock Proteins
  - 22, 60, 70
  - Bind to and activate TLR2/4
- DNA-Immune complex
  - Binds to and activates TLR9
- Surfactant Protein A
  - Binds to and activates TLR2
- Surfactant Protein D
  - Binds to and activates TLR2/4
- ECM components
  - Biglycan
  - Fibronectin
  - Hyaluronan
  - To TLR 2/4
- HMGB -1
  - Binds and activates TLR4

Types of Cell Death & Immune Response

**Apoptosis**

- Apoptosis = Programmed Cell Death
- Series of proteolytic enzyme-mediated organelle destructions
- Eventual apoptotic body phagocytosed without eliciting much inflammatory reaction

**Necrosis**

- Necrosis = traumatic cell death
- Series of internal steps usually mediated by Ca”
- Eventual loss of membrane integrity, spillage of intracellular contents
- Provokes inflammatory response
HMGB1

- Abundant (1 x 10^6/cell)
- Role is to open up spots on DNA
- Usually held closely in nucleus
- In apoptosis remains bound to condensed chromatin
- In necrosis it is released and becomes a potent Chemoattractant

How Does HMGB1 Promote Inflammation?

Two major receptor pathways
- RAGE = Receptor for advanced glycation end products
- TLRs 2 and 4
Net result is NF-κB activation and inflammatory gene expression

Interim Summary

Reaction to external threat or to endogenous damage or danger can cause similar responses.

Not perfect model. Doesn’t explain it all and tries to squeeze in some observations that don’t really fit. More likely additive to INS model. Is it useful in helping unravel disease processes?

Not Everyone Likes the Danger Model

- Purity of DAMPs
  - Can anything be completely LPS-free?
- Why doesn’t cell necrosis releasing DAMPs which help stimulate/direct the immune response not promote more autoimmune disease?
- Collateral or uncontrolled damage from sterile inflammation due to a small amount of necrosis may be a large price to pay

A New Approach?

- Look at some neonatal conditions
- What has been the common approach?
- What evidence is there that points toward a Danger Model influence?
- Goal is to provoke new approaches, hypotheses and experiments

BPD Issues

- Disease of prematurity characterized by increased inflammation and with inhibition or arrest of lung development

Normal Lung in 3 mo old Surfactant-treated Infant with BPD
BPD – Risk Factors

Alistair Philip’s Formula (1975)

$$\text{BPD} = \frac{\text{Oxygen} \times \text{Pressure} \times \text{Time}}{\text{Degree of Prematurity}}$$

Infection -
- Prenatal
- Maternal chorioamnionitis
- Amniotic LPS = less RDS, more BPD
- Postnatal
- Sepsis, Ureaplasma

Current Studies of Oxygen and BPD

- Premature infants who develop BPD have lower lung glutathione concentrations and higher malondialdehyde (peroxidation marker) – Collard et al., Arch Dis Child 2004; 89: F412
- Multiple studies of lungs and hyperoxia have found ↑ in: IL-1β, IL-6, IL-8, TNF-α, MIP-2, MCP-1, IFN-γ – Ryan et al., Clin Rev Allergy Immunol 2008; 34: 174
- Exposing newborn animals to hyperoxia causes lung simplification and altered growth factors/receptors – e.g. Voelkel et al. Am J Physiol 2006; 290: L209

Oxygen and the Danger Model

- Expose lung epithelial cells to hyperoxia in vitro causes necrosis and NF-κB activation
- In adults, prolonged ventilation or VAP led to higher HMGB-1 levels in lung lavage
  - Van Zoelen et al. Shock 2008; 29: 441
- Mice with mutant TLR4 exposed to hyperoxia for 4 days had far less inflammation

Current Studies of Trauma and BPD

- Newborn rats ventilated with 25 ml/kg TV vs 10 ml/kg - ↑CTFG levels, ↑Smad
  - Wu et al., Pediatr Res 2008; 63: 245
- Atelectrauma – 15 preterms on SIPPV VT=5 ml/kg vs 15 on SIPPV VT = 3 ml/kg
  - Lista et al. Pediatr Pulmonol 2006; 41: 357

Trauma and the Danger Model

- Increased stretch leads to increased cell death Release of danger signals?

Current Studies of Infection and BPD

- Ureaplasma urealyticum increases risk of BPD
- Fetal intramniotic LPS in rats inhibits postnatal alveolarization – Ueda et al., Pediatr Res 2006; 59: 396

- CONS as risk factor:
  - BPD if no sepsis = 24%
  - BPD if non-CONS sepsis = 41%
  - BPD if VONS sepsis = 64%
Infection and the Danger Model

- Intra-amniotic LPS at E15 caused decreased lung branching and increased luminal spaces (A and B)
- This effect was absent in mutant TLR4 mice (F and G)

Prince et al. Dev Dyn 2005; 233: 553

BPD and Danger Model Questions

- Increased lung ECM products in BPD lungs – are these DAMPs?
- HMGB-1 elevated in adults on prolonged vent – Is this true in babies esp. preterms?
- What is the effect of development on lung APC function?
- What are the downstream relationships between TLR4 and lung development factors?

CNS Injury Issues

- 50% of VLBW infants have some degree of white matter injury on brain MRI –
- Major antecedent for 5-10% CP rate in preterms
- Two major risk factors/pathological pathways
  - Ischemia
  - Infection/Inflammation

White Matter Injury and Ischemia

- Long and short penetrating arteries create watershed in white matter so one common cause is ischemia – Risk zone
- Impaired cerebral blood flow auto regulation so more premature or sicker, more pressure-passive system
- Focal or diffuse ischemia causing focal/diffuse necrosis

CNS Necrosis, HSP60 and TLR4

- Giving HSP60 (which is produced by injured/dying cells) leads to neurotoxicity via TLR4
- Culture neurons ± HSP60 = no effect
- Culture neurons and microglia ± HSP60 = neuronal death
- Culture neurons and lps± microglia ± HSP60 = no effect


Fetal Inflammatory Response and PVL

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<th>N = 177 N = 102</th>
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*Adjusted for covariates: variables described in previous research papers or the association of clinical variables with each outcome variable.
Sheep Brain Response to Asphyxia

- Near-term fetal sheep ± asphyxia for 8 minutes then resuscitated in 21% or 100% O₂
- Asphyxia and 100% O₂ caused increased cortical/subcortical IL-1β and IL-12p40
- This group also had increased cortex/subcortex TLR2, 3, and 4

Microglia and Premature White Matter Injury

- LPS into fetal rabbits on E28, delivered E 31
- Inject labeled (1-[2-chlorophenyl]-N-methyl-N-[1-methylpropyl]-3-isoquinoline carboxamide) = activated microglial marker

Microglia – Central Role

PAMPs e.g. LPS
Cytokines e.g. IL-1β, IL-8, IL-6
Cell Death e.g. HMGB1, HSP60
TLR-4
Excitatory amino acids
Growth factors
ROS, RNS
Oligodendrocyte Death
IL-8
TNFα
IFNγ
ROS, RNS

CONS and PVL

- 204 infants < 30 wks from 2001-03
  - 55 had CONS sepsis and 78% had white matter injury on term MRI
  - Lower MDI and PDI scores at 2 yrs corrected age
- Volpe hypothesizes that PAMPs from CONS or more likely DAMPs from sepsis activates TLR4 on microglia

White Matter Injury and the Danger Model

- Is TLR4 and similar receptors on microglia part of final common pathway for ischemia and infection/inflammation causing similar what matter injury?
- Microglial invasion in third trimester. Are they functional? Do they mature?
- Would TLR4 modification stop PVL?

Necrotizing Enterocolitis

- Affects 2-5% of all premature infants with mortality rates of 10 – 50%
- Epidemiological risk factors include degree of prematurity and enteral feeding, with intra-uterine and postnatal “stress” as added factors
- Focus on gastrointestinal defense immaturity
**GI Barrier Function and NEC**
- Tight junctions disrupted by NO which is elevated in NEC – Li et al. Acta Paediatr 2005; 94: 386

**NEC and The Danger Model**
- Rats formula fed + hypoxia express more TLR2 and TLR4 – Leman-Mandat Shulze PLoS One 2007; 2: e1102
- Blocking NF-kB prevents NEC – De Plaen et al.

**TLR4 and NEC**
- Hypoxia and LPS upregulate enterocyte TLR4
- Increased TLR4 expression in animal models and resected human intestinal tissues
- Animals with mutant TLR4 protected from NEC
- Decreased repair and regeneration in TLR4 mutants
- But should have constant exposure to LPS, etc…?

**Model of TLR4 and NEC**
Stressors, contact, etc., increase TLR4 number and activity
- Many Questions – e.g.
  - What changes the message from homeostasis to Danger?
  - Why doesn’t unstressed TLR4 mutant have abnormal enteric homeostasis?
  - What does immaturity do to this model?

**Conclusions (= Questions)**
- Adding the Danger Model steps increases potential understanding of mechanisms behind many disease processes and opens new lines of inquiry
- How does the innate immune system develop?
- Is APC function affected by gestational age?
- Do the Danger responses differ in newborns? In preterms?
  - In general? By organ/tissue type? By stressor?
- Are the Danger receptors affected by gestational age?
- Stay tuned