TLR agonist

Biomarkers and mechanisms of vaccine adjuvant

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In the past 20 years, 7 new adjuvants have been licensed for use in humans.

What is an adjuvant?

1) Adjuvants are the substances that are intended to enhance relevant immune response and subsequent clinical efficacy of the vaccines (WHO guidelines on nonclinical evaluation of vaccines, WHO Technical Report Series, No. 927, 2005)

2) A vaccine adjuvant is a component that potentiates the immune responses to an antigen and/or modulates it towards the desired immune responses. (EMEA guieline on adjuvants in vaccines for human use. 2005)

3) Immunologist’s dirty little secret (C Janeway 1989)
Innate control of adaptive immunity by adjuvants via signal 2 and 3

**Innate Immunity**

- Pathogens (damaged cells) to Antigen Presenting cells via Signal-1 (Antigen)
- Innate immune receptors
- "Inflammatory responses"

**Adaptive Immunity**

- Signal 1 + 2 (+3) = Immune response
- Antigen-specific B and T cell responses
- Innate control of adaptive immunity by adjuvants via signal 2 and 3

Signal 2 = adjuvants

Signal 3 = Cytokines Co-stimulation

TCR

MHC-peptide

Signal 1 only = Tolerance / Ignorance

Min - Hours - Days - Months-Years
## Innate immune receptors and their ligands

<table>
<thead>
<tr>
<th>Innate immune receptors (PRRs)</th>
<th>Ligands (PAMPs)</th>
<th>Synthetic or purified adjuvants</th>
<th>Cellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLRs</strong></td>
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<tr>
<td>TLR1/2</td>
<td>Triacyl lipopeptide</td>
<td>Pam3CSK4</td>
<td>membrane</td>
</tr>
<tr>
<td>TLR2/6</td>
<td>Diacyl lipopeptide</td>
<td>Macrophage-activating lipopeptide 2 (MALP-2)</td>
<td>membrane</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA</td>
<td>Poly I:C</td>
<td>endosome</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>Monophosphoryl lipid A (MPL)</td>
<td>membrane</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacterial flagellin</td>
<td>Flagellin-protein fusions</td>
<td>membrane</td>
</tr>
<tr>
<td>TLR7, TLR8</td>
<td>ssRNA (RNA viruses)</td>
<td>Imiquimod(R-837), Resquimod(R-848)</td>
<td>endosome</td>
</tr>
<tr>
<td>TLR9</td>
<td>unmethylated CpG DNA</td>
<td>CpG-ODNs(Type-A, -B, -C, -P)</td>
<td>endosome</td>
</tr>
<tr>
<td>TLR11</td>
<td>Profilin-like protein (T. gondii)</td>
<td>Unknown</td>
<td>membrane</td>
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<tr>
<td><strong>RLRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIG-I</td>
<td>5’-PPP ssRNA or Short (~1 kb) dsRNA</td>
<td>Unknown</td>
<td>cytosol</td>
</tr>
<tr>
<td>MDA5</td>
<td>Long (&gt; 2 kb) dsRNA</td>
<td>Poly I:C</td>
<td>cytosol</td>
</tr>
<tr>
<td><strong>NLRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD1</td>
<td>Peptidoglycans, Diaminopimelic acid(iE-DAP)</td>
<td>FK156, FK565</td>
<td>cytosol</td>
</tr>
<tr>
<td>NOD2</td>
<td>Peptidoglycans, Muramyl dipeptides (MDP)</td>
<td>Muramyl dipeptides (MDP)</td>
<td>cytosol</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Cellular stress, lysosomal damage</td>
<td>Aluminum salts, MSU, Silica</td>
<td>cytosol</td>
</tr>
<tr>
<td>NAIP5</td>
<td>Bacterial flagellin</td>
<td>Flagellin-protein fusions</td>
<td>cytosol</td>
</tr>
<tr>
<td><strong>CLRs</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Dectin-1</td>
<td>β1,3-glucan</td>
<td>Curdlan, lentinan, schizophyllan</td>
<td>membrane</td>
</tr>
<tr>
<td>Dectin-2</td>
<td>High mannose structures</td>
<td>Man9GlcNAc2</td>
<td>membrane</td>
</tr>
<tr>
<td>Mincle</td>
<td>Trehalose-6,6-dimycolate(TDM)</td>
<td>Trehalose-6,6-dibehenate(TDB)</td>
<td>membrane</td>
</tr>
<tr>
<td><strong>ALRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIM-2</td>
<td>dsDNA</td>
<td>Unknown</td>
<td>cytosol</td>
</tr>
<tr>
<td>IFI16</td>
<td>dsDNA</td>
<td>Unknown</td>
<td>cytosol</td>
</tr>
</tbody>
</table>
Vaccine signaling pathways by nucleic acid-adjuvants

- ssRNA
  - Influenza RNA
  - TLR7
  - RIG-I
  - MyD88
  - IKKαβγ
  - TBK1
  - Th1
  - B
  - CTL

- dsRNA
  - poly IC
  - TLR3
  - MDA5
  - IPS-1
  - TRIF
  - TBK1
  - Th1
  - B
  - ?
  - CTL

- ssDNA
  - CpG ODN
  - TLR9
  - MyD88
  - IKKαβγ
  - STING, IPS-1
  - TBK1
  - Th1
  - B
  - CTL

- dsDNA
  - B-form DNA within DNA vaccine
  - AIM2
  - endosome
  - DAI? Pol-III, H2B, lrrfip1, ifi16
  - cytosol
  - STING, IPS-1
  - TBK1
  - Th1
  - B
  - CTL

- Stromal cell (e.g. muscle)

Influenza A virus infection

TLR7/MyD88

RIG-I/IPS-1

cDC

Fibroblast

pDC

Type-I IFNs

Th0

Th1

B

IgG2a.2c

IFN γ

Killing IFNγ

CTL

Th1 type adaptive immune response to live virus infection is dominantly controlled by TLR7-dependent pathway.

Innate control of various influenza vaccine immunogenicity

**Live virus**
- Flu vaccine subtypes
- mDCs, TLR7, pDCs, RIG-I, Uncertain NLR, Epithelial cells, Macrophages
- Type-I IFNs, Proinflammatory cytokines
- Naïve host

**Whole virus vaccine**
- Formalin inactivated
- Viability
- Virus RNA
- pDCs
- TLR7
- No innate response
- No immunity

**Split vaccine**
- Surface antigen enriched
- No innate response
- Exposed host

**Key cells and innate immune receptors**
- Key effectors

**Type-I IFNs**
- Cytotoxicity
- CD8+Tcell
- CD4+Tcell
- Bcell
- Th1-type antibody
- IFNγ

**Resultant adaptive immune activation**
- Memory CD4+Tcell
- IFNγ

**Summary**

Innate response is not necessarily required
Inactivated influenza vaccines

- **Killed whole virion vaccine**
  - Treat with Formalin

- **Inactivated influenza vaccines**
  - Treat with Ether & gradient centrifugation

- **Commercially available Split HA vaccine**
  - HA
  - NA

- **Novel adjuvant combined split vaccine**
  - HA
  - NA

SSRNA

SPG = CpG DNA forming a triple helix with β-glucan
Vaccine signaling pathways by nucleic acid-adjuvants

**ssRNA**
- *Influenza RNA*
  - TLR7
  - RIG-I
  - MyD88
  - IKKαβγ
  - TBK1
  - Th1
  - B

**dsRNA**
- *poly IC*
  - TLR3
  - MDA5
  - TRIF
  - IPS-1
  - TBK1
  - CTL

**ssDNA**
- *CpG ODN*
  - TLR9
  - MyD88
  - IKKαβγ
  - TBK1
  - Th1
  - B

**dsDNA**
- *B-form DNA within DNA vaccine*
  - DAI? Pol-III,H2B,lrrfip1,ifi16
  - STING,IPS-1
  - TBK1
  - Th1
  - CTL
  - B

*Stromal cell (e.g. muscle)*

**References**
Molecular Basis of TLR-Ligand Interaction
Humanization of CpG DNA (ODN): Distinct types of CpG

- **K(B) -Type CpG ODN (PS)**: NNNNTCGT/ANNN
- **D(A)-type CpG ODN (mix)**: GGnnnntcgatnnngggGG

Mouse CpG
NN-Pu-Pu-CpG-Py-PyNN

- **B cell**
- **mDC**
- **pDC**

**B cell**

- **Proliferation**
- **Polyclonal activation**
- **IL-6, IgM, chemokines**

**mDC**

- **APC function**
- **CD8 T cell activation**

**pDC**

- **MHC, CD40, CD86**
- **IFNα, TNFα, chemokines**

**NK**

- **NK activity**
- **IFNγ**

Development of 2$^{nd}$ generation ‘DDS-CpG adjuvant’; CpG DNA-$\beta$-1,3-1,6-glucan (SPG) complex.
Schizophyllan (SPG), a b-1,3 glucan, is derived from a mushroom “Suehirotake”

SPG has not only been used as a Eastern medicine for years, but also been used as an approved anti-cancer drug in Japan for treating cervical cancer in conjunction with conventional chemotherapy.
One way to achieve enhancing potency and reducing toxicity at the same time for the TLR-ligands is to target them to specific antigen specific cells in lymphoid organ with certain delivery system.

Amongst variety of methodology and approaches, we chose SPG-CpG, a second generation humanized TLR-ligand-based adjuvant developed in our lab complexed with GMP lot of commercial soluble beta-1.3-1.6 glucan, namely SPG, recently shown to form specific complex with nucleic acids such as CpG ODN.

SPG-CpG targets dectin-1 expressing antigen presenting cells including certain subsets of dendritic cells and macrophages in the draining lymph node.

SPG-CpG enhances both humoral (IgG and IgA (in the case of intra-nasal administration) and cellular immune responses (CD4 Th1 and CD8 CTL) to co-administered antigens, including split HA antigen, resulting in potent immunogenicity and protective efficacy in both murine and NHP models.
Why so much alarm for vaccines?

- Despite humanity’s daily encounters with microbial antigens, vaccines still seem unnatural and frightening to some people.

- Perception of risk is amplified in today’s setting.

- The public’s understanding of immunization is superficial.
Adjuvant research and development: promising, but dangerous

Vaccine adjuvant

Gene therapy with viral vector
Good or bad; Rationales for innovating novel Agonistic and/or antagonistic adjuvants

Adjuvants

Antagonistic adjuvant

BAD

GOOD

↑Innate immune activation

Pathogen

↑IFNs, IL-12, NO, chemokines
↑NK activity, phagocytosis

Tumor

↑Th1, ↓Th2

Vaccine

↑IgG2a, IFNγ, CTL

Th1 Adjuvant

Anti-infection

Anti-Cancer

Anti-Allergen

INNATE IMMUNITY ↔ ADAPTIVE IMMUNITY

Tissue damage

Chronic inflammation

Autoimmunity

Activation of Mϕ, endothelial cells

Endogenous or exogenous adjuvant factor(s)

DC maturation

B cell activation

IFNs, IL-12, NO, chemokines
NK activity, phagocytosis

GOOD

BAD
Pre-pandemic vaccines consisted of whole virion + Alum caused high fever in children, but not adult after the first, but not the second immunization.

<table>
<thead>
<tr>
<th></th>
<th>After 1\textsuperscript{st} imm</th>
<th></th>
<th>After 2\textsuperscript{nd} imm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># of fever+</td>
<td>Ratio (%)</td>
<td># of fever+</td>
<td>Ratio (%)</td>
</tr>
<tr>
<td><strong>BK-PIFA</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Children (6M-20y)</td>
<td>109/187</td>
<td>58.3</td>
<td>20/184</td>
<td>10.9</td>
</tr>
<tr>
<td>Adults (20-65y)</td>
<td>3/150</td>
<td>2.0</td>
<td>1/148</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>KIB-PIA</strong></td>
<td></td>
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</tr>
<tr>
<td>Children (6M-20y)</td>
<td>83/187</td>
<td>44.4</td>
<td>13/183</td>
<td>7.1</td>
</tr>
<tr>
<td>Adults (20-65y)</td>
<td>0/150</td>
<td>0.0</td>
<td>1/149</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Relation ship between fever event and antibody titer

## IgG subclass antibody responses

<table>
<thead>
<tr>
<th></th>
<th>NT</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
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</thead>
<tbody>
<tr>
<td><strong>2009 Pdm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Natural infection</td>
<td>50</td>
<td>42</td>
<td>2</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>(n=53)</td>
<td>(94.3%)</td>
<td>(79.2%)</td>
<td>(3.8%)</td>
<td>(30.2%)</td>
<td>(45.3%)</td>
</tr>
<tr>
<td><strong>2009 Pdm</strong></td>
<td>55</td>
<td>23</td>
<td>2</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Vac. split</td>
<td>(74.3%)</td>
<td>(41.8%)</td>
<td>(2.7%)</td>
<td>(16.2%)</td>
<td>(0%)</td>
</tr>
<tr>
<td>(n=74)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>H5 WIV Alum</strong></td>
<td>193</td>
<td>67</td>
<td>12</td>
<td>4</td>
<td>42</td>
</tr>
<tr>
<td>Vac. children</td>
<td>(100%)</td>
<td>(34.7%)</td>
<td>(6.2%)</td>
<td>(2.1%)</td>
<td>(21.8%)</td>
</tr>
<tr>
<td>(n=193)</td>
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More than 4-fold increase

Evaluation of vaccine adjuvant; Data base seeking biomarkers for safety and efficacy

Innate Immunity

Adaptive Immunity

Min Hour Day Month-Year

Current

In vivo animal model

Empirical attenuation

In vitro assays

Humoral responses (Ab)

Immunological understanding at cellular level

Transcriptome, proteome, metabolome, micro RNAs

Identification of surrogate markers

Efforts to detect any biological responses at molecular level

Intra-cellular signaling pathways

Comprehensive analysis by Omics (Systems immunology)

In vitro-in vivo

On going

Vaccine vehicle

Signal 1 = Ag

Signal 2

Signal 3

Cytokines etc

APC

DC, MΦ, B

TCR

Cellular responses

Vaccine adjuvant
miRNA: potential and stable biomarker in serum

<table>
<thead>
<tr>
<th>Group</th>
<th>Abtiter</th>
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<tbody>
<tr>
<td></td>
<td>+++</td>
<td>-/+</td>
</tr>
<tr>
<td>Fever 38℃～</td>
<td>+++</td>
<td>G1</td>
</tr>
<tr>
<td></td>
<td>-/+</td>
<td>G3</td>
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<tr>
<td></td>
<td></td>
<td>G4</td>
</tr>
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Biomarker for fever = side effect

Biomarker for Ab = immunogenicity
Nibio Initiates Japanese Vaccine Adjuvant Research Consortium

Japan's National Institute of Biomedical Research (NIBIO) has established a research team to tackle research and development of next-generation adjuvants for vaccines. The team is consisted of relevant researchers from academia, industries and regulatory agencies to vaccines and adjuvants.

Board member;
Koishi Yamanishi (NIBIO)
Shizuo Akira (IFREC Osaka U)
Hiroshi Kiyono (Tokyo U)
Kenji Nakanishi (Hyogo Med College)
Tsukasa Seya (Hokkaido U)
Ken Ishii (NIBIO / IFREC Osaka U)

Official members
PMDA
Novartis
Pfizer
MSD
GSK
Sanofi Pasteur
Astellas Pharma
Otsuka Pharmaceutical
Shionogi & Co
Zeria Pharmaceutical
Daiichi Sankyo
Dainippon Sumitomo Pharma
Takeda Pharmaceutical
Mitsubishi Tanabe Pharma
Chugai Pharma
Ono Pharma
Biken,
Denka Seiken,
Kitasato-Daiichi-Sankyo
Kaketsuken
and several biotech companies

A book published:
“Advanced Technologies for adjuvant research and development”
by CMC press Japan
Vaccine Science and Technology Consortium

- Human Immunology
- Drug Delivery System (DDS)
- Mucosal Immune system
- Bio-3D, 4Dimaging
- Vaccine Technology
- Bioinformatics (vaccinome, systems immunology)
- Field & Molecular Epidemiology
- HTP screening system
- Basic and clinical researches on vaccine target diseases
- Protective antigen
- Delivery vehicle
- Adjuvant
- Production & Regulation
- Toxico-genomics/metabolomics
- Innate immune system
- Organic chemistry

HTP screening system

Vaccine Science and Technology Consortium Production & Regulation
It takes time, costs, but deserves to develop vaccines!

“The French historian Yves-Marie Bercé, is the author of a book entitled Le Chaudron et la Lancette (right) which tells how the Englishman Edward Jenner’s discovery of a treatment for smallpox in the summer of 1798 then spread to the rest of Europe.

To quote: "The news spread through Europe like wildfire. However, this was a time when the continent was in a state of war; the seas were in the hands of pirates and roads were cut off by armies. Despite all these obstacles, the vaccine against smallpox found ways of getting through. Within a few years, and in all countries, not only were the professors in the universities aware of the discovery, but so too were ordinary doctors in private practices, despite not being in the main run of intellectual ideas, and they were able to make the vaccine available in their localities, and, furthermore, with a high degree of efficiency."

More by Kenzaburo Oe
Acknowledgement

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Osaka U.

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Daiichi Sankyo
Tanabe Mitsubishi
Shionogi
Roles of PAMPs and DAMPs during vaccination