Adaptive Immune Responses to Ranaviruses and Immune Evasion Strategies of Ranaviruses

Funded By NIAID

http://www.urmc.rochester.edu/smd/mbi/xenopus

An adaptive Immune System is present in all jawed vertebrates

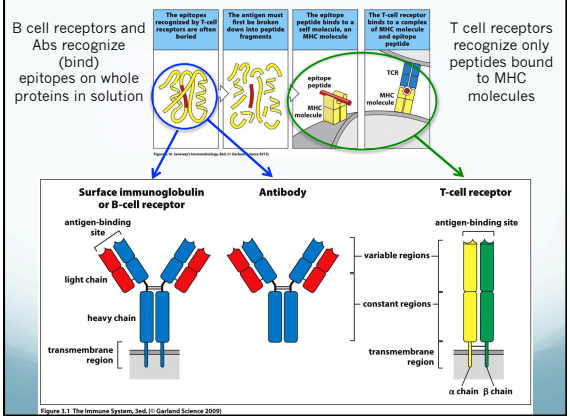
Characterized by:

- a wide somatic diversification of immune receptor repertoires
- high specificity of immune receptors for antigens,
- long term immunological memory
- and a complex cytokine- and chemokine-mediated regulatory network

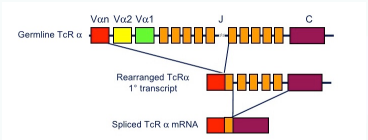
- Immunoglobulin (IgM, IgG or IgG-equivalent IgY, IgD - Fish IgZ, IgT)
- T Cell Receptor (α, β, γ, δ)
- MHC class II, classical class Ia (selection), nonclassical MHC class Ib
- RAG-1, 2 mediated gene rearrangement, TdT
- Somatic hypermutation (AID-mediated)
- Primary and secondary lymphoid tissues (e.g. thymus, spleen, bone marrow, lymph nodes)

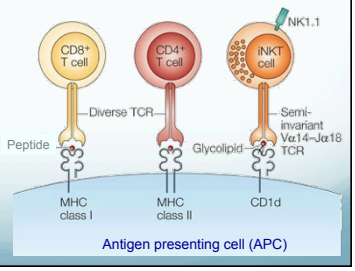
B cell receptors and Abs recognize (bind) epitopes on whole proteins in solution

T cell receptors recognize only peptides bound to MHC molecules



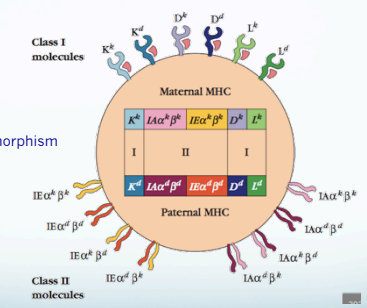
Somatic lymphocyte gene rearrangement





Nature Rev. Immunoloy

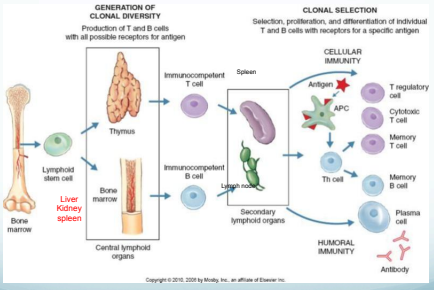
MHC haplotypes



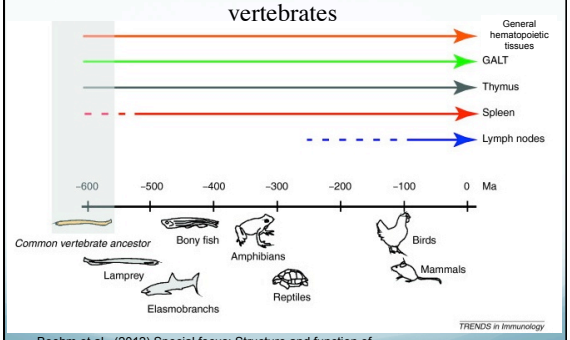
Allelic polymorphism

Some amphibian species have only 1 MHC class I gene per genome (*Xenopus*). Other have 2 or 3 genes per genomes (*Ranidae*)

Organization of the immune system



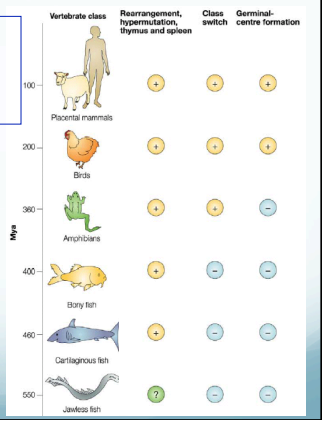
Evolutionary trajectory of lymphoid tissues in vertebrates



Boehm et al., (2012) Special focus: Structure and function of lymphoid tissues. Trends Immunol. 33:315

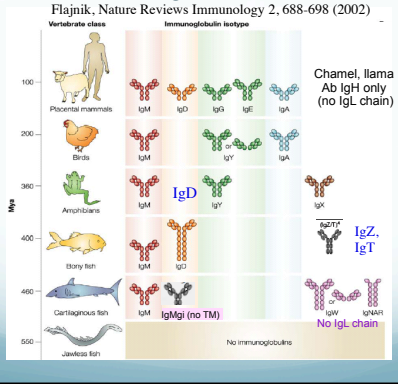
Features of an Adaptive Immune System

- Ig, TCR, MHC
- RAG 1, 2 expression
- Lymphoid Compartments

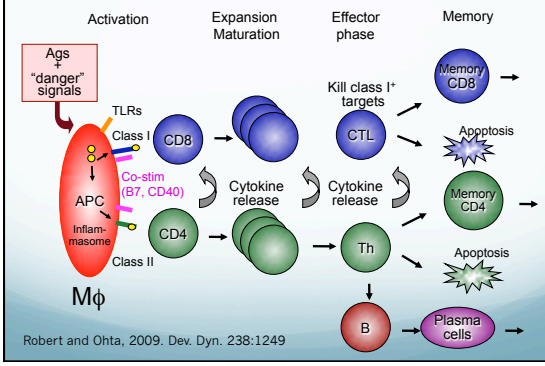


Flajnik, Nature Rev. Immunology 2, 688-698 (2002)

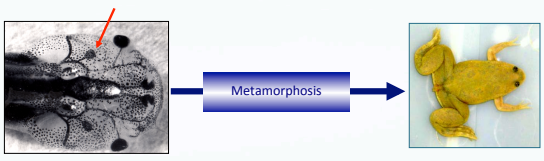
Immunoglobulin Evolution



Antiviral immune responses



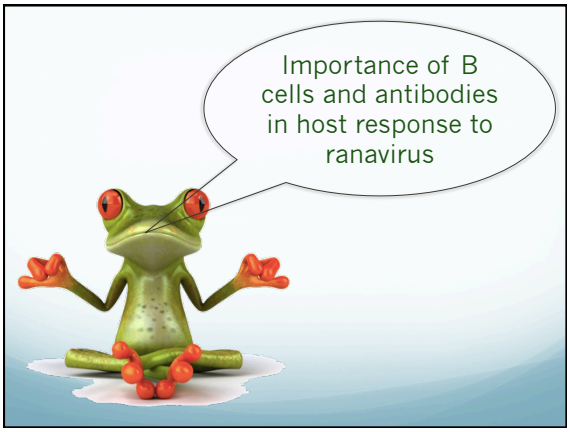
Robert and Ohta, 2009. Dev. Dyn. 238:1249



- External development , absence of maternal influences on embryos
- Tadpoles are immunocompetent but immature
- Immune system develop early (10 days of age)
- Only about 20,000 T cells, mainly innate T cells, in tadpoles
- No classical MHC class I protein expression until metamorphosis
- No NK cells, weaker T cell responses than adults
- Drastic remodeling of the immune system during metamorphosis
- Thymocytes degenerate, new thymic education from new progenitors

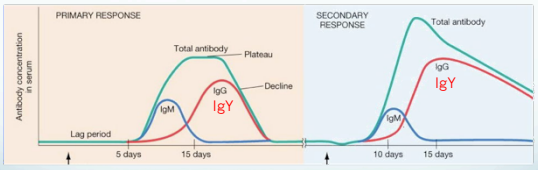
Urodelean adaptive immunity

- Relatively poor adaptive immunity compared to anurans
- Low IgM antibody heterogeneity (no specific IgY is produced
- Expanded MHC class I repertoire (~100 genes) that may include classical and nonclassical MHC class I as well as a non-polymorphic MHC class II
- Based on chronic rejection of allografts and xenografts, weak immune responses appear to characterize most species of salamanders
- High susceptibility to ranavirus infection
- But still able to survive in pathogen-rich environments

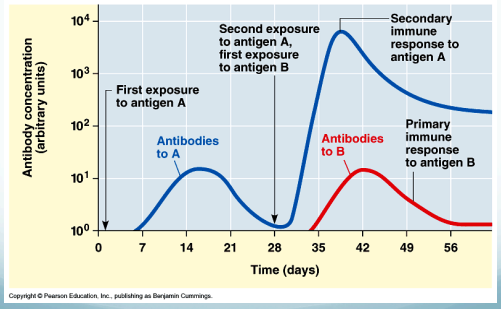


Humoral (antibody) response

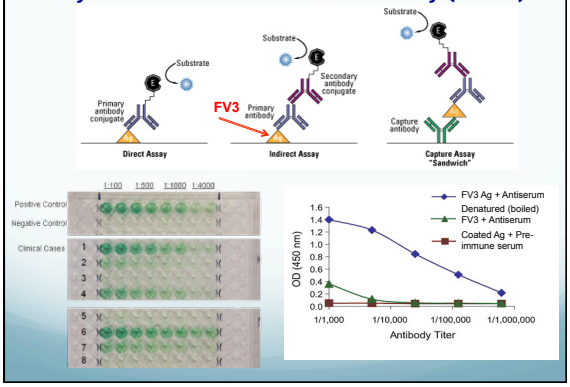
Primary *vs* secondary antibody responses



Humoral (antibody) response



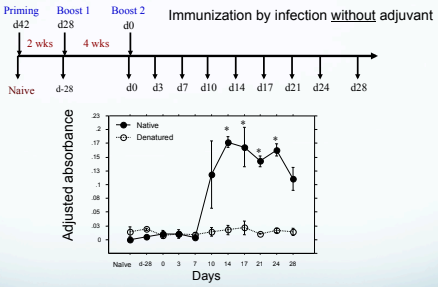
Enzyme-Linked Immunosorbant Assay (ELISA)



Humoral response

- *Xenopus* and mammals have similar organization and usage of their Ig genes (RAG-dependent VDJ rearrangements)
- Thymus-dependent switch IgM to IgY (IgG functional equivalent), T-B collaboration
- But *Xenopus* antibodies are limited in heterogeneity, mature poorly in affinity (less than 10 fold) and their serum titer increase only slightly during a secondary response
- How important is the humoral response in the resistance against natural pathogens such as FV3 infection?

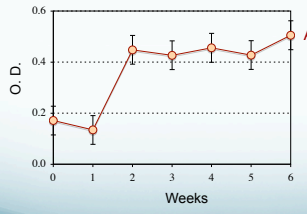
Anti-FV3 IgY antibody response



Xenopus anti-FV3 IgY (**1:200** dilution, O.D. = 0.4)
Rabbit anti-FV3 IgG (**1:20,000** dil, Oution.D. = 1.1)

Maniero et al., Dev Comp Immunol 2006, 30:649

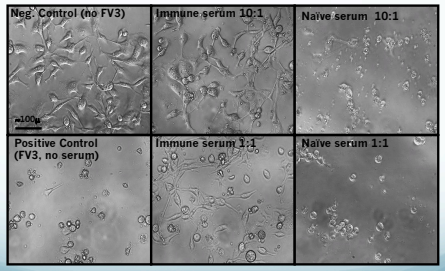
Long lasting B cell memory
(Re-infection 15 months after primary infection)



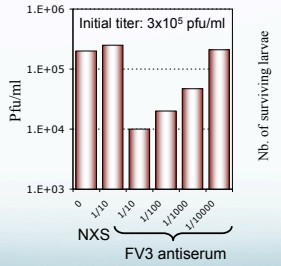
Anti-FV3 IgY (1:100 dil.)

Rabbit serum
anti-FV3
1:20,000 dilution
O.D. = 1.1

Xenopus adult produce neutralizing anti-FV3 antibodies



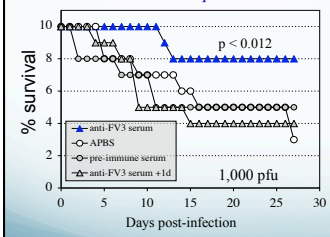
Neutralization capacity of *Xenopus* anti-FV3 serum by TCID50



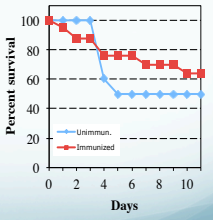
Maniero et al., Dev Comp Immunol 2006, 30:649

Tadpole exhibit poor anti-ranavirus antibody responses

Passive protection of anti-FV3 antiserum in susceptible larvae



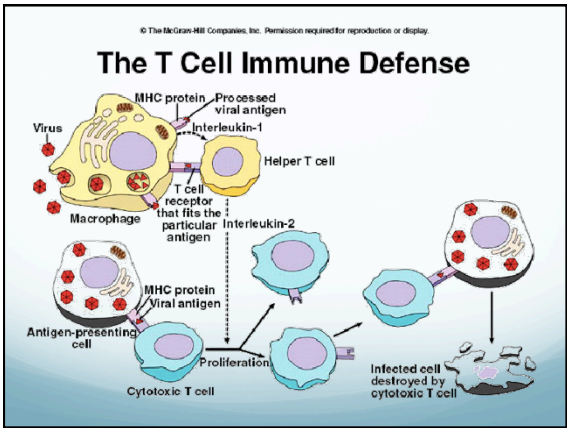
Immunization FV3 Heat inactivated + alum



1,000 pfu/animal
~ 10 ug of protein

Summary I

- Anuran amphibians like *Xenopus* are capable to generate effective antibodies (IgM and IgY) against ranaviruses
- More efficient, IgY, antibody response is elicited during a secondary infection (No anti-FV3 Ab detected in adult sera during a primary infection in absence of adjuvant in *Xenopus*)
- FV3-specific IgY antibodies (thymus-dependent IgG equivalent) detected from 10 up to 24 days after re-infection (no adjuvent)
- B cell memory lasting at least 15 months after a first infection
- Serum of immunized frogs contain antibodies that can neutralize ranavirus (*Xenopus* adults can generate potent neutralizing anti-FV3 antibodies, that are able to provide passive protection to susceptible tadpoles
- Compared to adult frogs, tadpoles exhibit poor anti-ranavirus antibody response



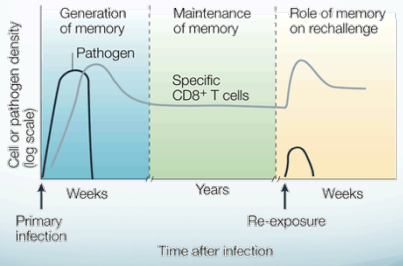
Assessing T function by sublethal γ-irradiation

- T cell differentiation in the thymus is dependent on cell division, which is very sensitive to γ-irradiation
- Whole body γ-irradiation 5 to 10 Gray depletes mostly thymocytes and T cells
- This impairs adaptive immunity for 1 to 2 week (e.g., Skin graft rejection)
- Resistant adult *Xenopus* become susceptible and die from FV3 infection following sublethal γ-irradiation
- Infected γ-irradiated frogs also release more virus into the environment

More specific assessment of CD8 T cells by Ab treatment

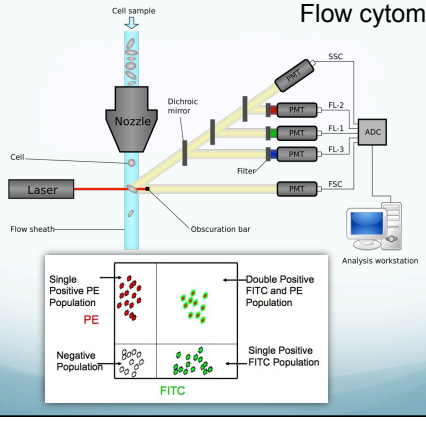
- *In vivo* CD8 depletion by anti-CD8 mAb-treatment increases susceptibility to FV3 in adults

T cell memory



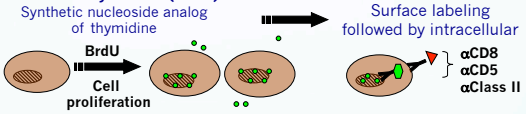
Nature Reviews | Immunology

Flow cytometry



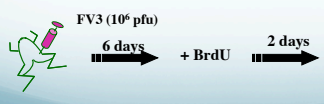
Detecting *in vivo* cell proliferation upon FV3 infection, primary response

Bromo deoxyUridine (BrdU)
Synthetic nucleoside analog of thymidine

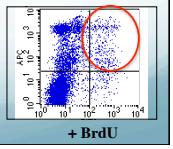


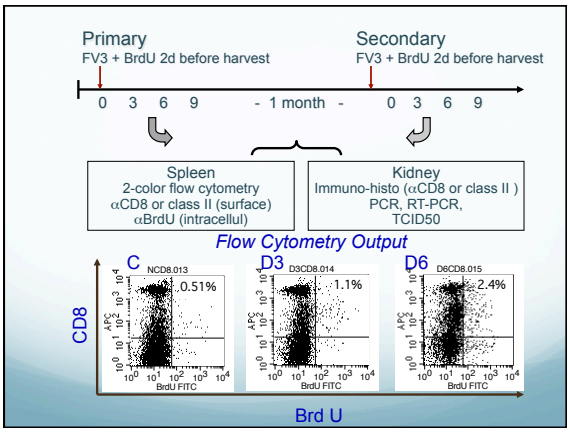
Surface labeling followed by intracellular

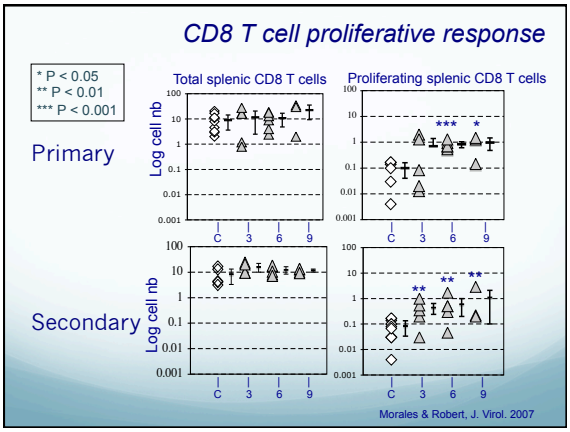
FV3 infection and BrdU incubation (added in water in obscurity)

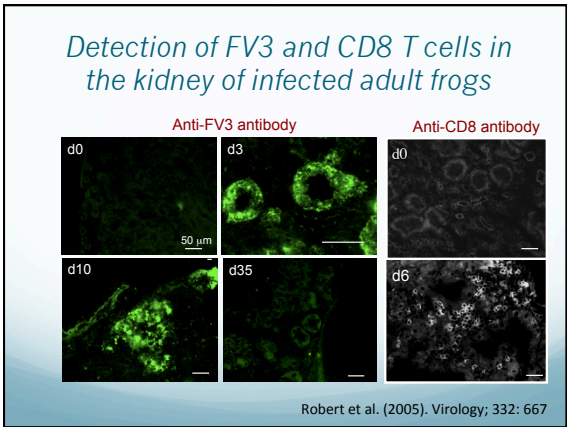


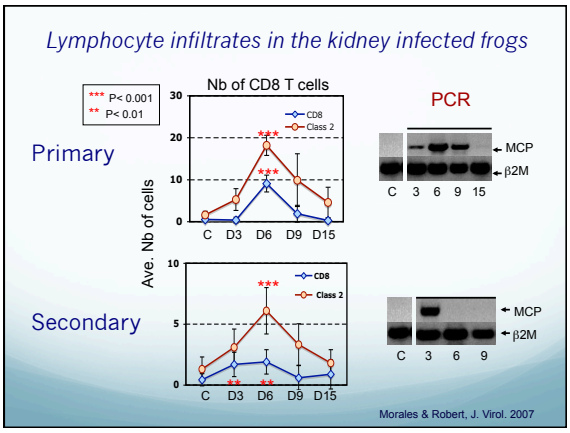
Flow cytometry

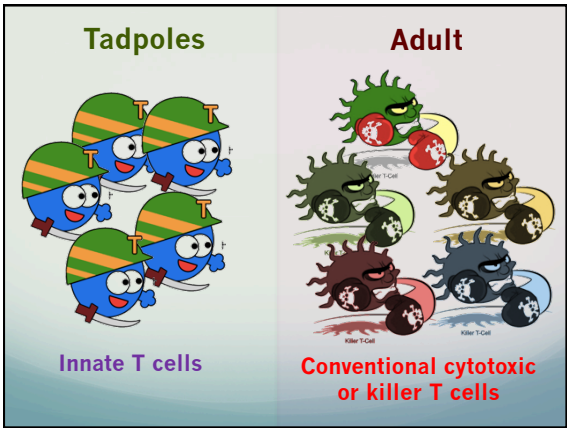


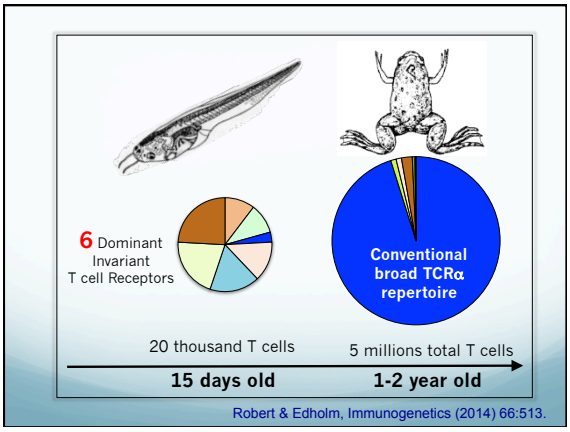

Primary
FV3 + BrdU 2d before harvest
Secondary
FV3 + BrdU 2d before harvest
0 3 6 9
- 1 month -
0 3 6 9
Spleen
2-color flow cytometry
αCD8 or class II (surface)
αBrdU (intracellul)
Kidney
Immuno-histo (αCD8 or class II)
PCR, RT-PCR,
TCID50
Flow Cytometry Output
C
D3
D6
NCD8.013
0.51%
D3CD8.014
1.1%
D6CD8.015
2.4%
CD8
APC
BrdU FITC
Brd U


CD8 T cell proliferative response
* P < 0.05
** P < 0.01
*** P < 0.001
Total splenic CD8 T cells
Proliferating splenic CD8 T cells
Primary
Secondary
Log cell nb
C 3 6 9
Morales & Robert, J. Virol. 2007

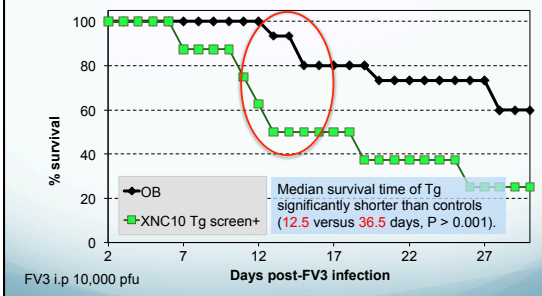

Detection of FV3 and CD8 T cells in the kidney of infected adult frogs
Anti-FV3 antibody
Anti-CD8 antibody
d0
d3
d0
50 µm
d10
d35
d6
Robert et al. (2005). Virology; 332: 667


Lymphocyte infiltrates in the kidney infected frogs
*** P< 0.001
** P< 0.01
Nb of CD8 T cells
Primary
Secondary
Ave. Nb of cells
CD8
Class 2
C D3 D6 D9 D15
PCR
MCP
β2M
C 3 6 9 15
Morales & Robert, J. Virol. 2007


Tadpoles
Adult
Innate T cells
Conventional cytotoxic or killer T cells


6 Dominant Invariant T cell Receptors
Conventional broad TCRα repertoire
20 thousand T cells
5 millions total T cells
15 days old
1-2 year old
Robert & Edholm, Immunogenetics (2014) 66:513.

*Increased susceptibility to FV3 infection of **XNC10 - deficient** Tg tadpoles lacking XNC10- iT cells*

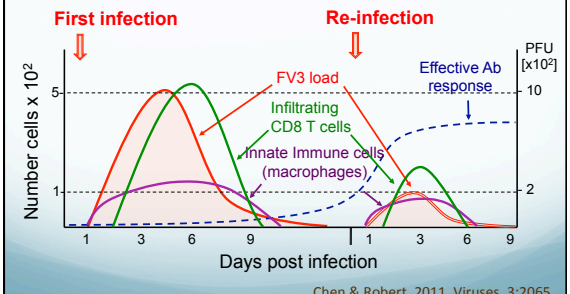


Edholm et al., PNAS. 2013, 110:14342

Summary II

- **CD8 T cells play a major role during a primary ranaviral infection**
  - γ-irradiated adults are more susceptible to FV3 infection
  - *In vivo* CD8 depletion with anti-CD8 mAb-treatment increases susceptibility to FV3 in adults
  - CD8 T cell infiltrate infected tissues then contract during viral clearance
- **Critical involvement of CD8 T cells during a ranaviral secondary infection and immunological memory**
  - Faster recovery of Infected adults
  - Faster infiltration of CD8 T cells and class II^+ cell in kidneys
  - Faster viral clearance
- **Critical involvement of XNC10-restricted innate T cells**

Xenopus adult immune response kinetics in infected kidneys



Chen & Robert, 2011, Viruses, 3:2065

How ranavirus can overcome host immune defenses?

Virulence

Ability of a virus to cause disease in the infected host animal

Virulence genes encode molecules that contribute to the pathogenicity of the organism and enable them to achieve the following:

- Viral replication
- Invasiveness (colonization of a niche in the host, attachment to cells)
- Tropism
- Enable the virus to spread in the host
- Intrinsic cell killing effects
- Obtain nutrition from the host
- **Immune evasion**, immune suppression (avoiding immune recognition, modification and inhibition of immune response)

Immune modulators:

- Apoptosis
- Cytokine or immune receptor mimics (*Virokines*, *viroreceptors*)
- Complement binding proteins
- Modifiers of MHC class I and class II pathways

Immune evasion strategies of ranaviruses

Ranaviruses can:

- Cross species barriers of many ectothermic vertebrates, suggesting potent immune evasion strategies
- Persist quiescently in resistant host species, which may serve as asymptomatic carriers for viral dissemination
- Disseminate to immune privileged and distal end-organs and tissues and immune
- Persist quiescent in cells such as macrophages
- Likely to use an arsenal of virulence and immune evasion viral genes (function of only 1/3 of the 98-105 ORFs known or inferred based on sequence homology)

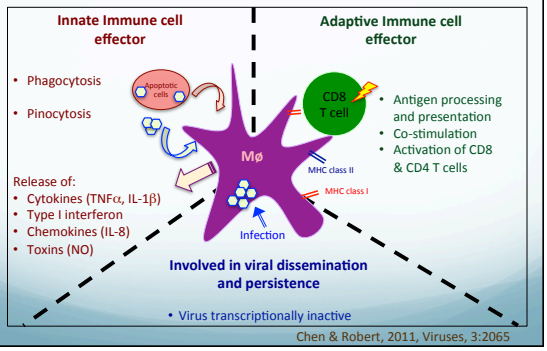
Putative ranavirus virulence and immune evasion genes

- ❑ Some virulence genes identified by sequence homology
- ❑ Characterization of immune evasion genes by site-specific viral gene deletion or knockout

1. **vIF2α homologue**: Antagonist of protein kinase R (PKR)
2. **Caspase activation and recruitment domain-containing (CARD) protein**: Interfere with CARD domains containing pro-apoptotic, pro-inflammatory and/or interferon responsive
3. **β-hydroxysteroid dehydrogenase homolog**: may play a role in dampening host immune responses
4. **18K immediate-early protein**: unknown function but conserved among ranaviruses

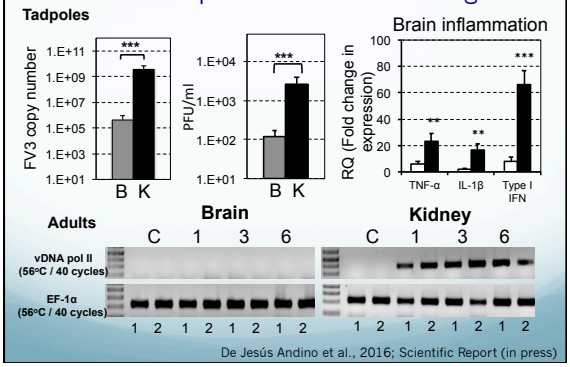
De Jesús Andino et al., 2015; Virology 485:162

Complex role of macrophages in Xenopus host defenses against RV

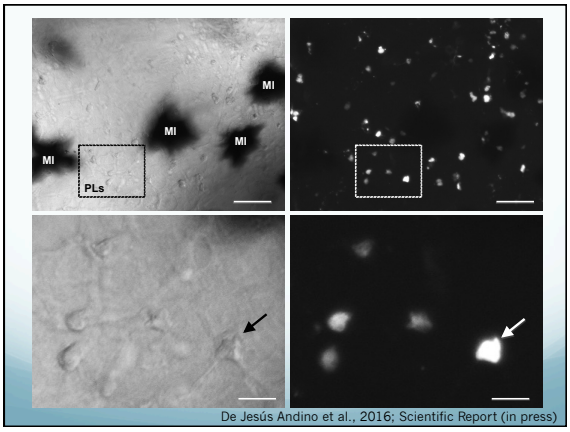


Chen & Robert, 2011, Viruses, 3:2065

FV3 disseminate into the brain of tadpoles but not adult frogs

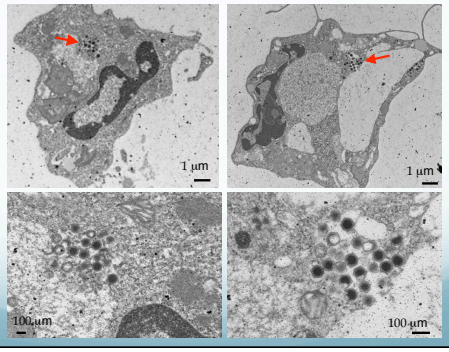


De Jesús Andino et al., 2016; Scientific Report (in press)



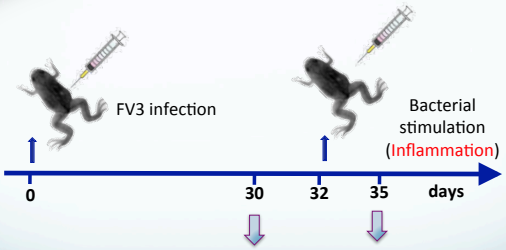
De Jesús Andino et al., 2016; Scientific Report (in press)

Mø infected in vitro for 2 days with FV3

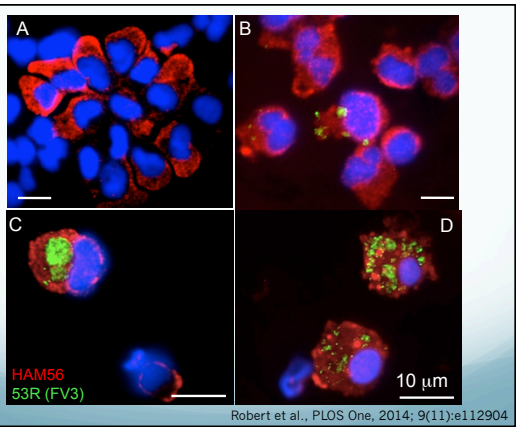


Morales et al., 2010, J. Virol. 84:4912

Experimental Method

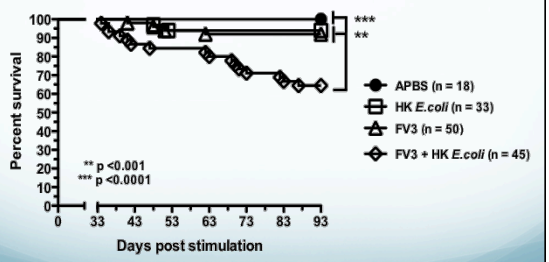


Collect macrophages
RNA and DNA isolation
Virus load, viral transcription



Robert et al., PLOS One, 2014; 9(11):e112904

Survival curve in adult frogs after FV3 infection, followed by bacterial stimulation



Robert et al., PLOS One, 2014; 9(11):e112904

Host immunity to ranavirus

- **Adults: Resistant, clear FV3 within 2 weeks**
  - Early innate immune response
  - Critical involvement of cytotoxic T cells and antibodies
  - FV3 persists quiescent in some asymptomatic adults
  - Immunological memory. Upon secondary infection: faster recovery, viral clearance & T cell response; and protective antibodies
- **Tadpoles: More susceptible, most succumb infection**
  - Less efficient B and T cell responses (mainly innate T cells)
  - delayed and/or inadequate innate anti-FV3 response
  - Inefficient viral clearance & wider tissue dissemination
  - Ranaviruses may be more pathogenic in tadpoles