

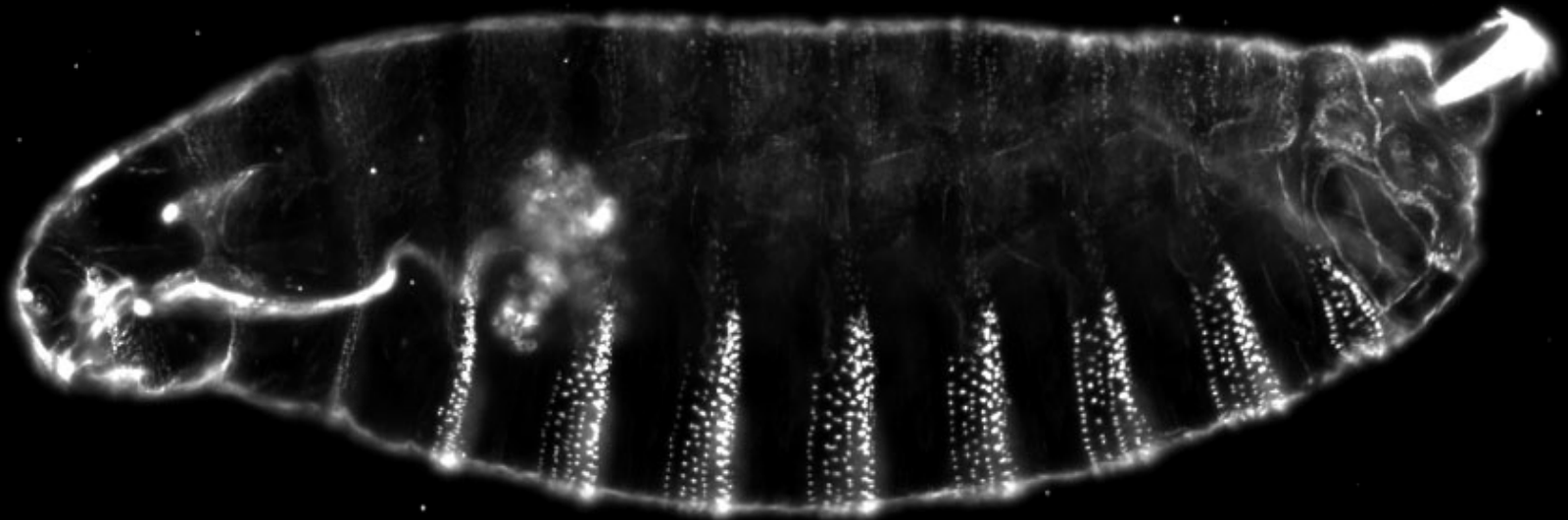
# Lessons from the field: parasite DNA drives the innate immune response to malaria (it's not just the caiparinhas)



University of  
Massachusetts  
Medical School

Douglas Golenbock, MD  
Chief, Infectious Diseases

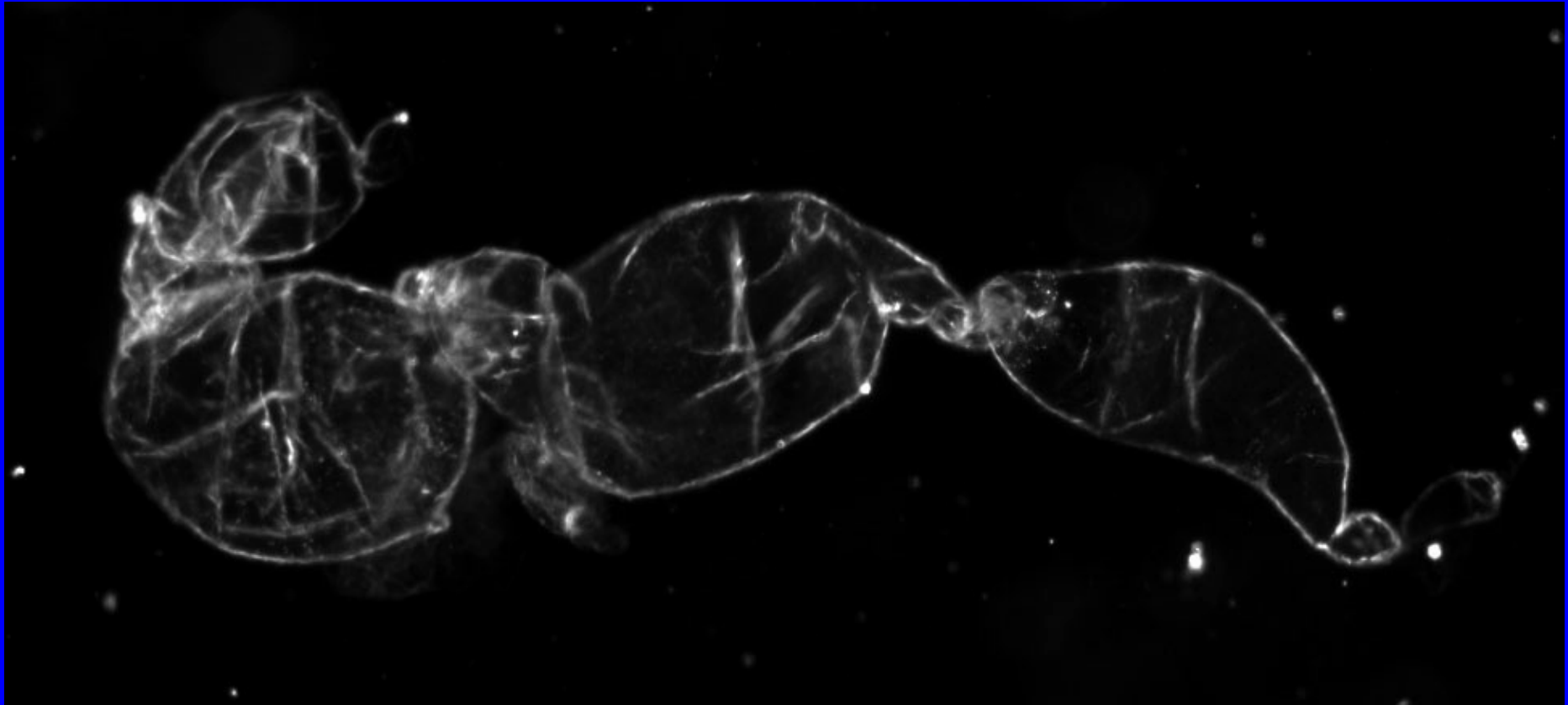
# ***Drosophila melanogaster* larvae**



# Christiane Nüsslein-Volhard, Nobel Laureate (Medicine, 1995)



# mutant *Drosophila melanogaster* larvae



Anderson *et al.* *Cell* 1985; 42: 791-98

**Christiane Nüsslein-Volhard,  
Nobel Laureate (Medicine, 1995)**



**Toll**

# Christiane Nüsslein-Volhard, Nobel Laureate (Medicine, 1995)



**Toll**

## **TOLL**

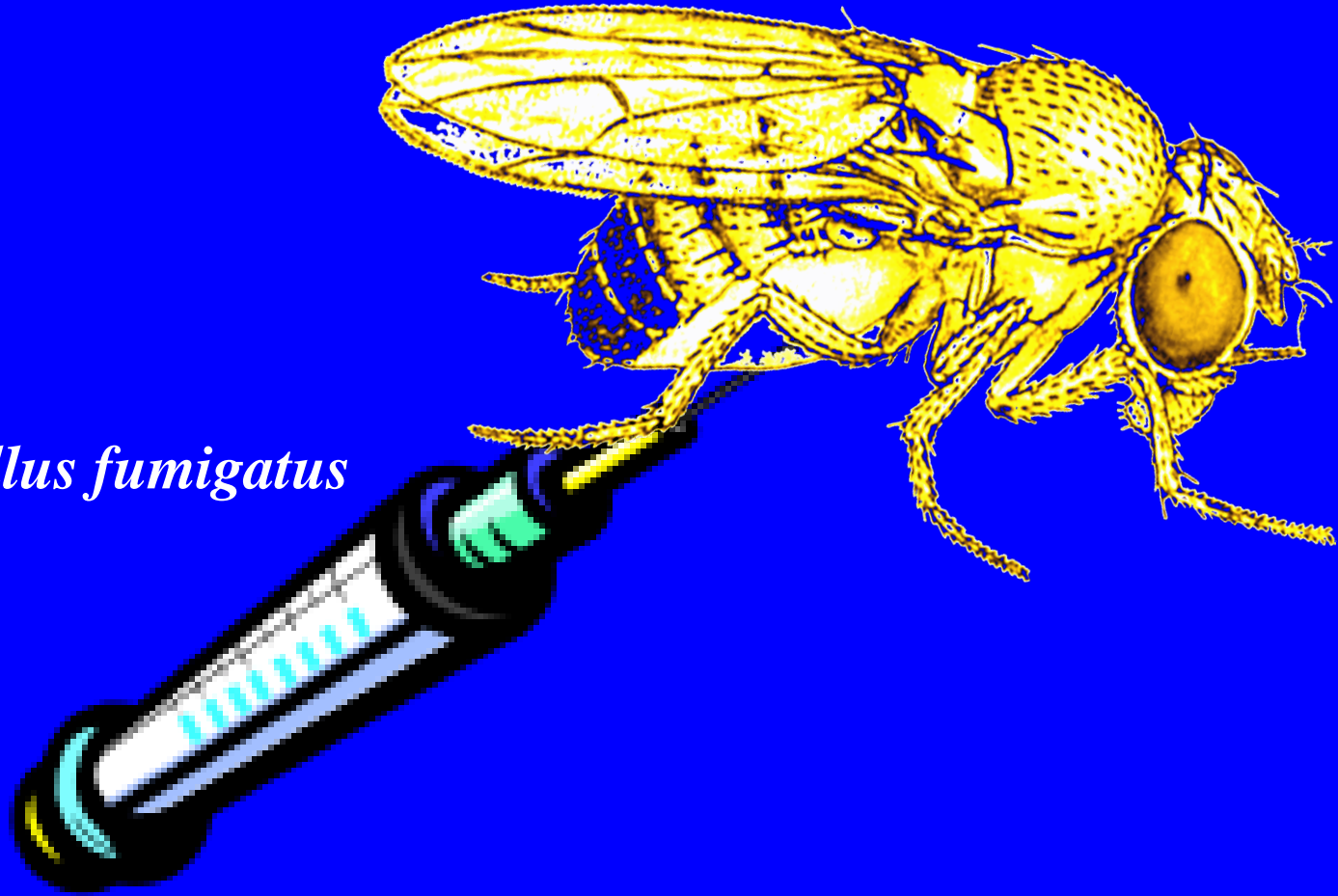
Amazing  
Bodacious  
Cool  
Corky  
Crazy  
Frantic  
Furious  
Great  
Like blazes  
Mad  
Madcap  
Screaming  
Super  
Wild  
Wicked  
Wow

Cloning of *Toll*:

homology to the human IL-1  
receptor

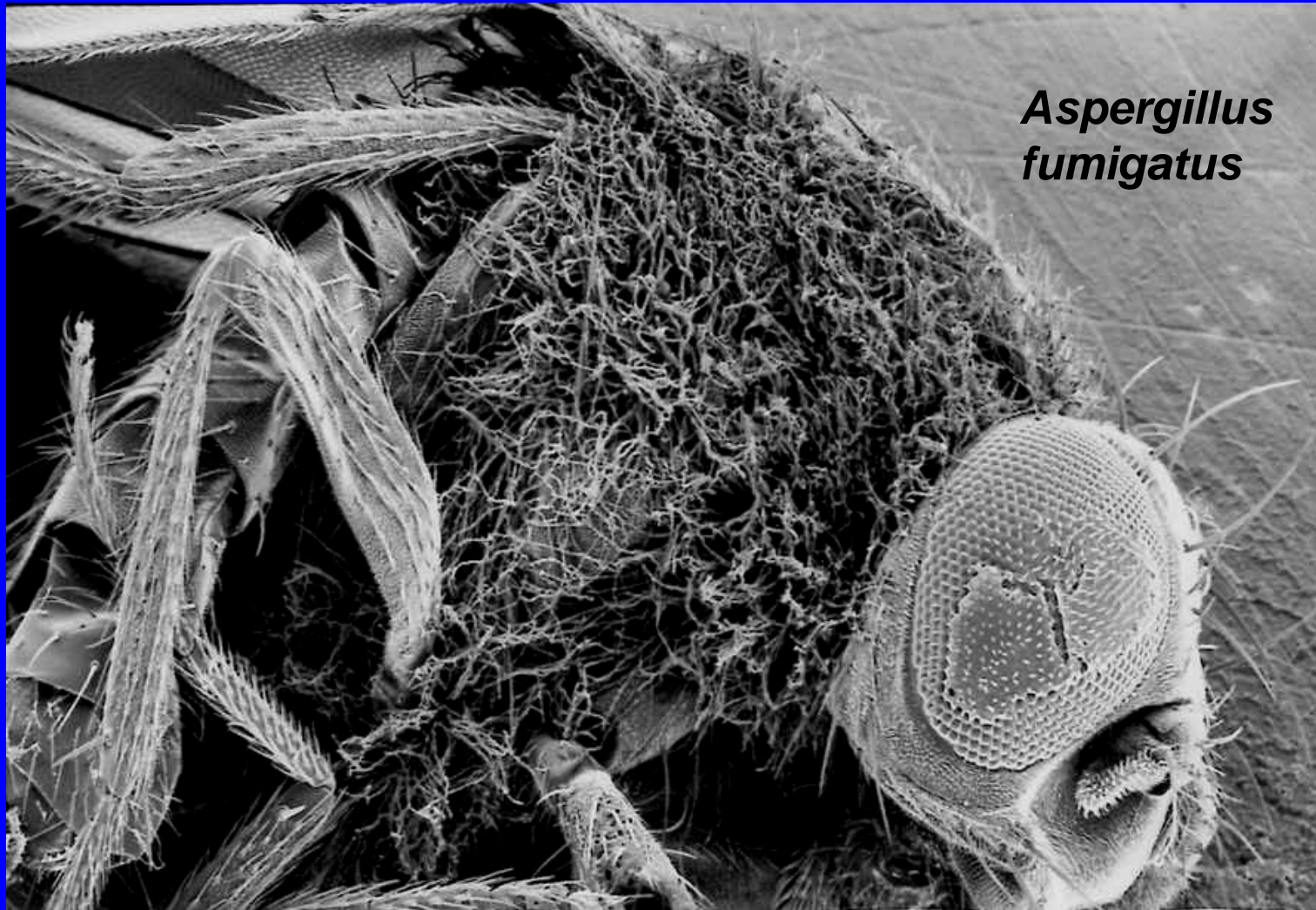
Lemaitre, Hoffmann *et al. Cell* 1996; 86: 973-83

*Aspergillus fumigatus*



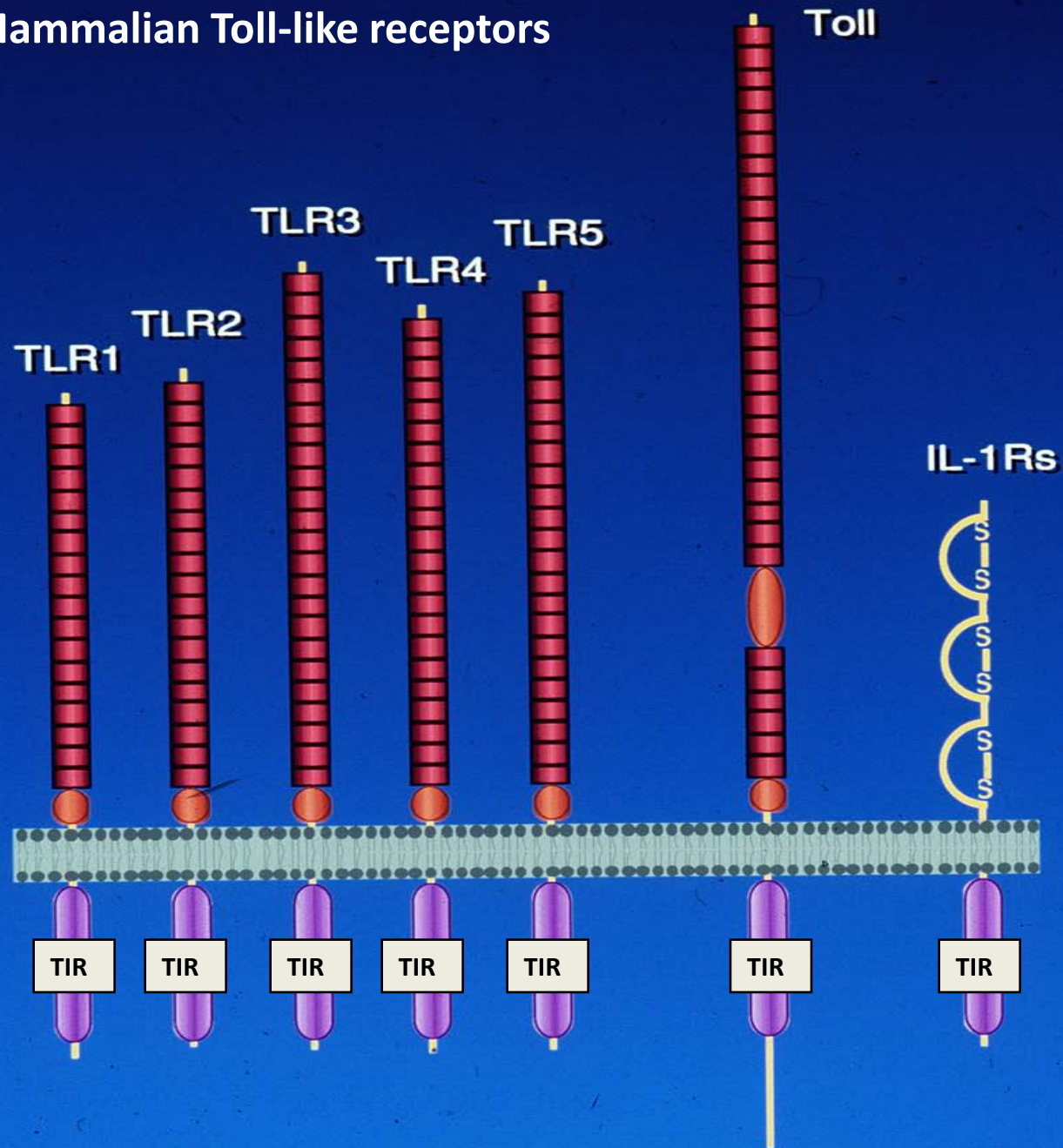


# Mutations in *Toll* predispose to fungal infections



Lemaitre et al. *Cell* 1996; 86: 973-83

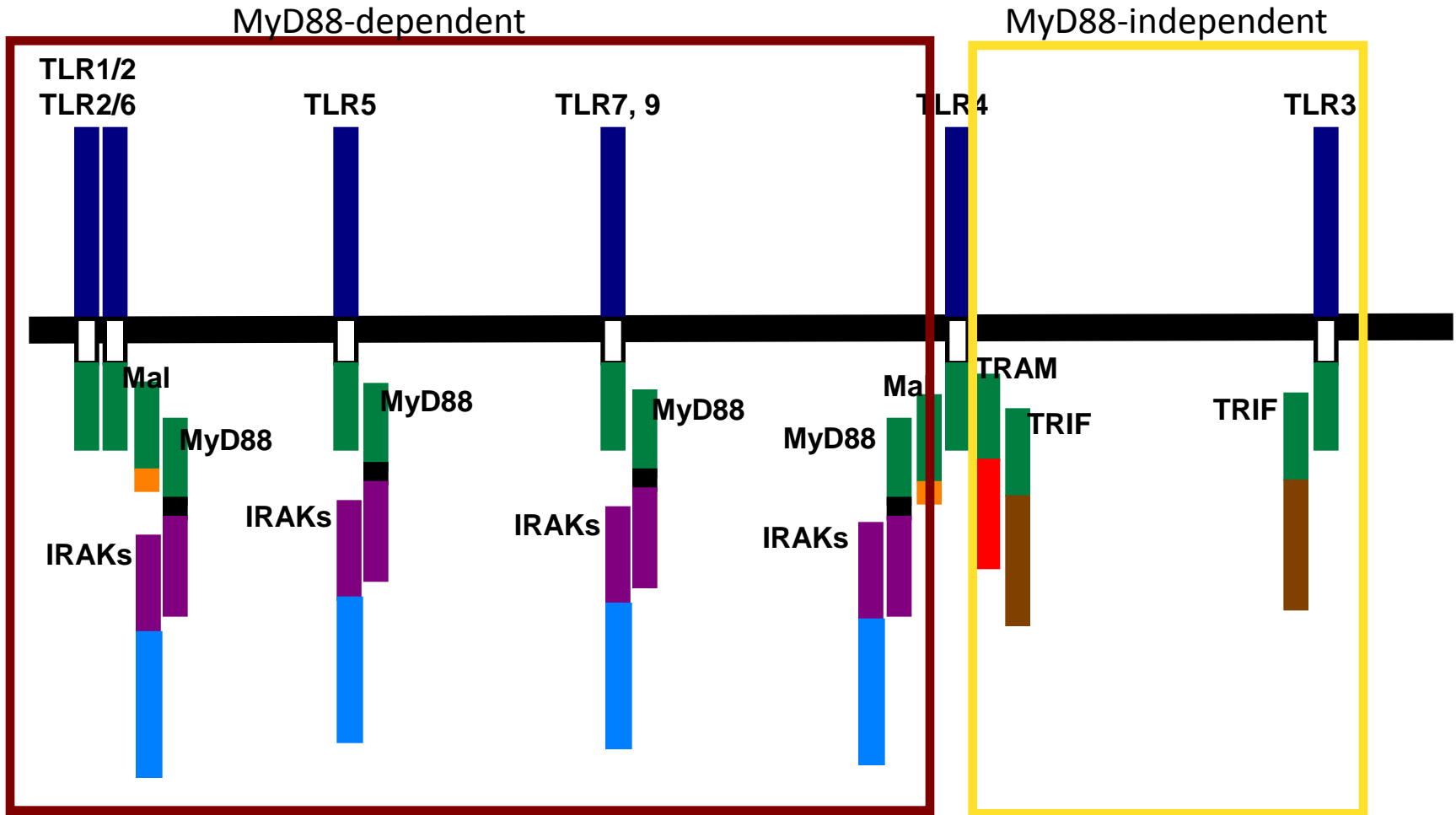
# Mammalian Toll-like receptors



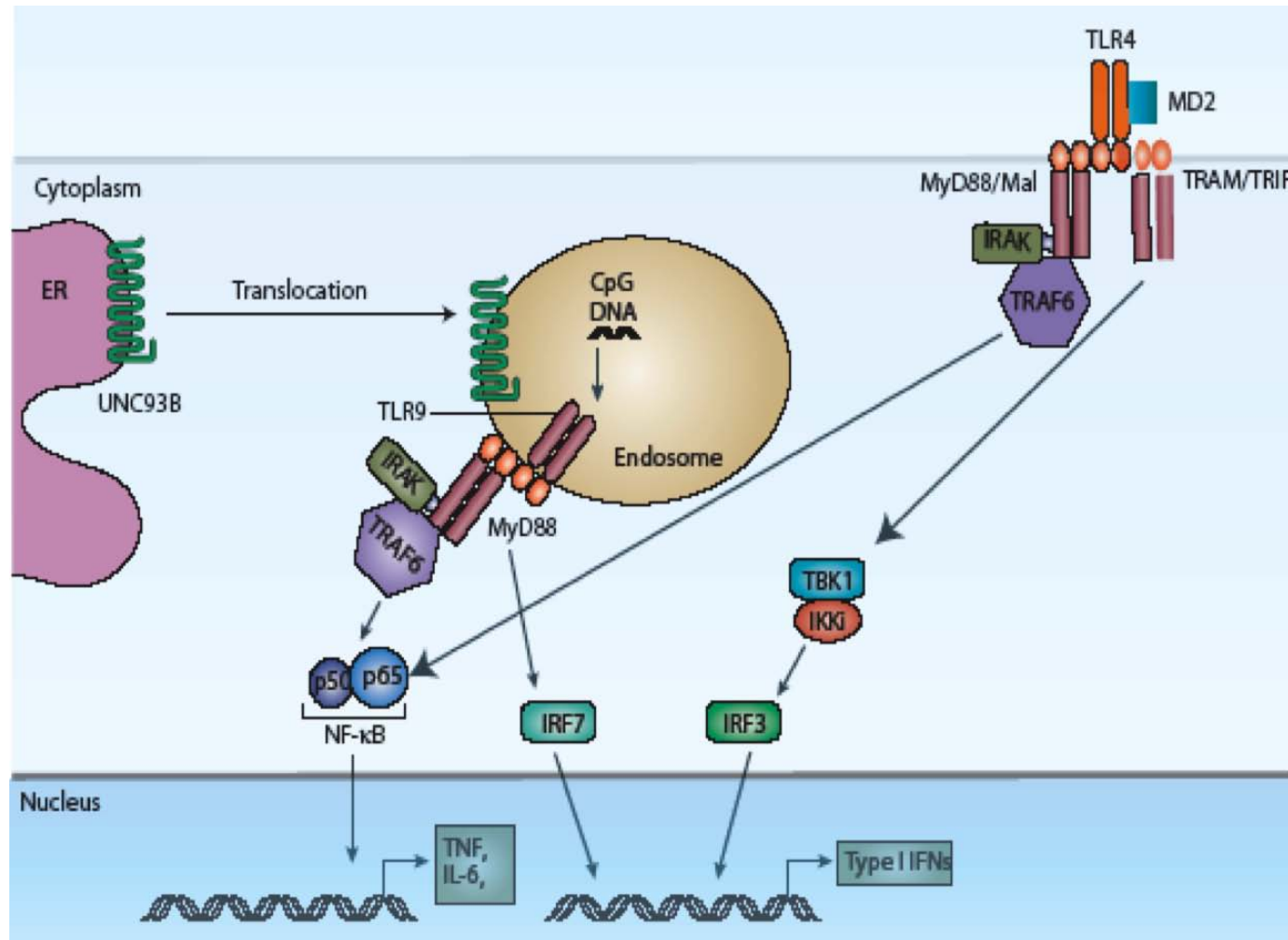
10 human TLRs  
4 adapter TLRs



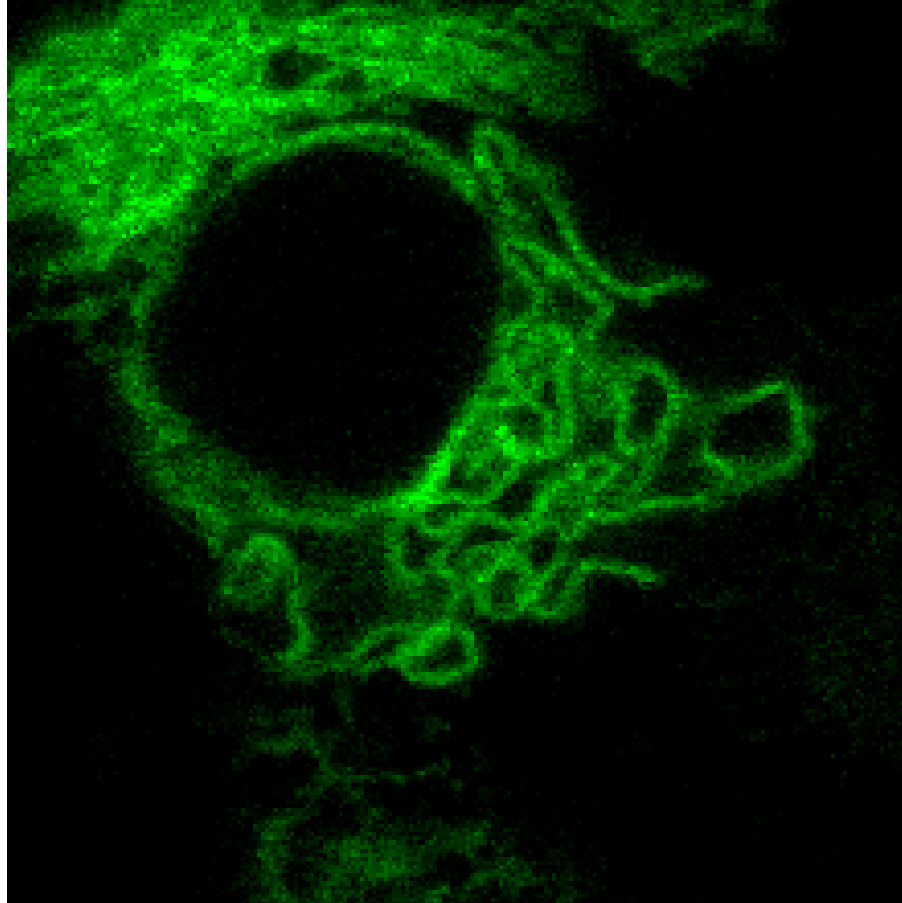
# There are 4 TLR adapter molecules



There are two broad classes of TLRs:  
endosomal and surface TLRs.



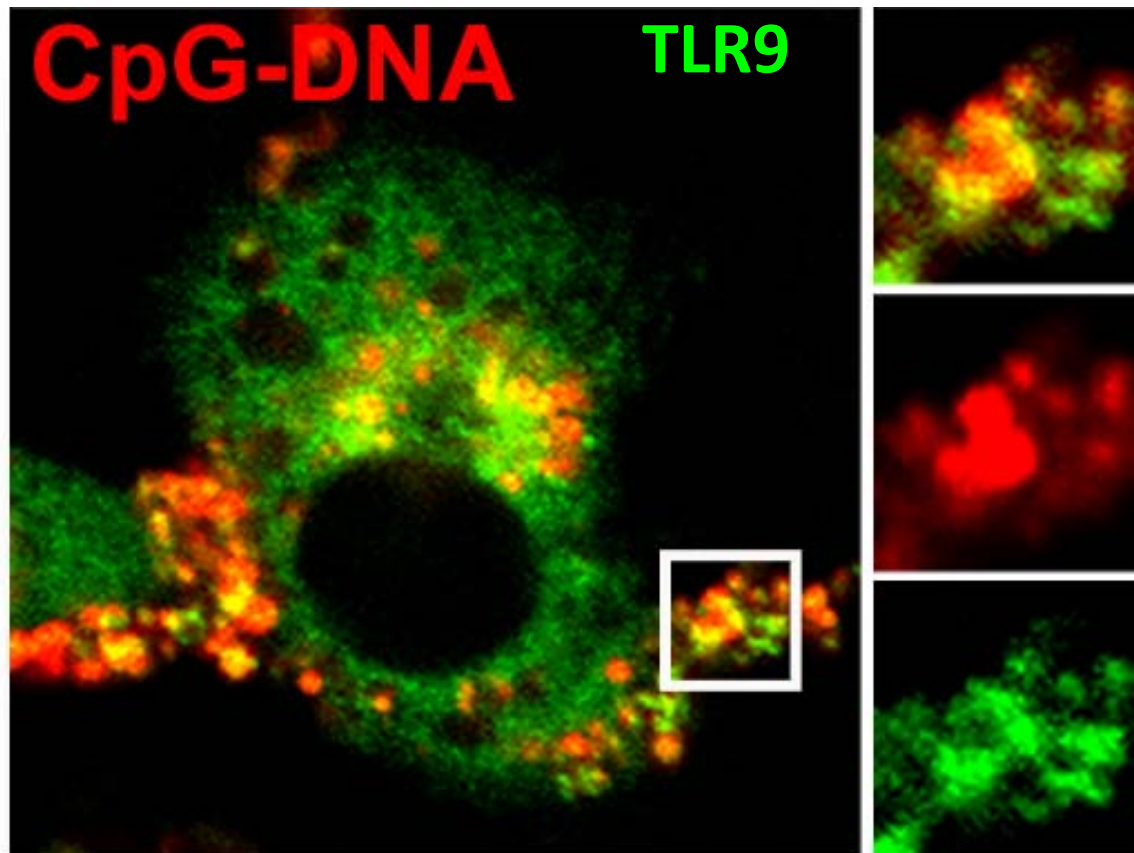
TLR9 is the prototypical endosomal TLR.



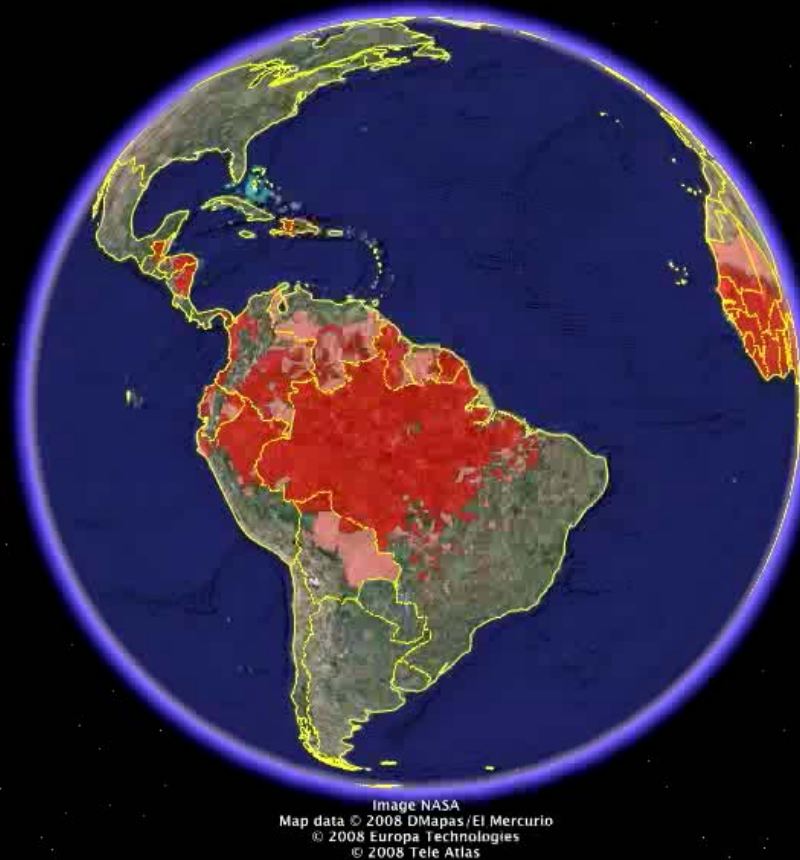
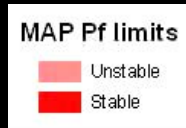


TLR9 must move from the ER to the endosome to encounter its ligand (CpG DNA).

Mouse dendritic cells after 5 min



# Malaria: the facts in 2010



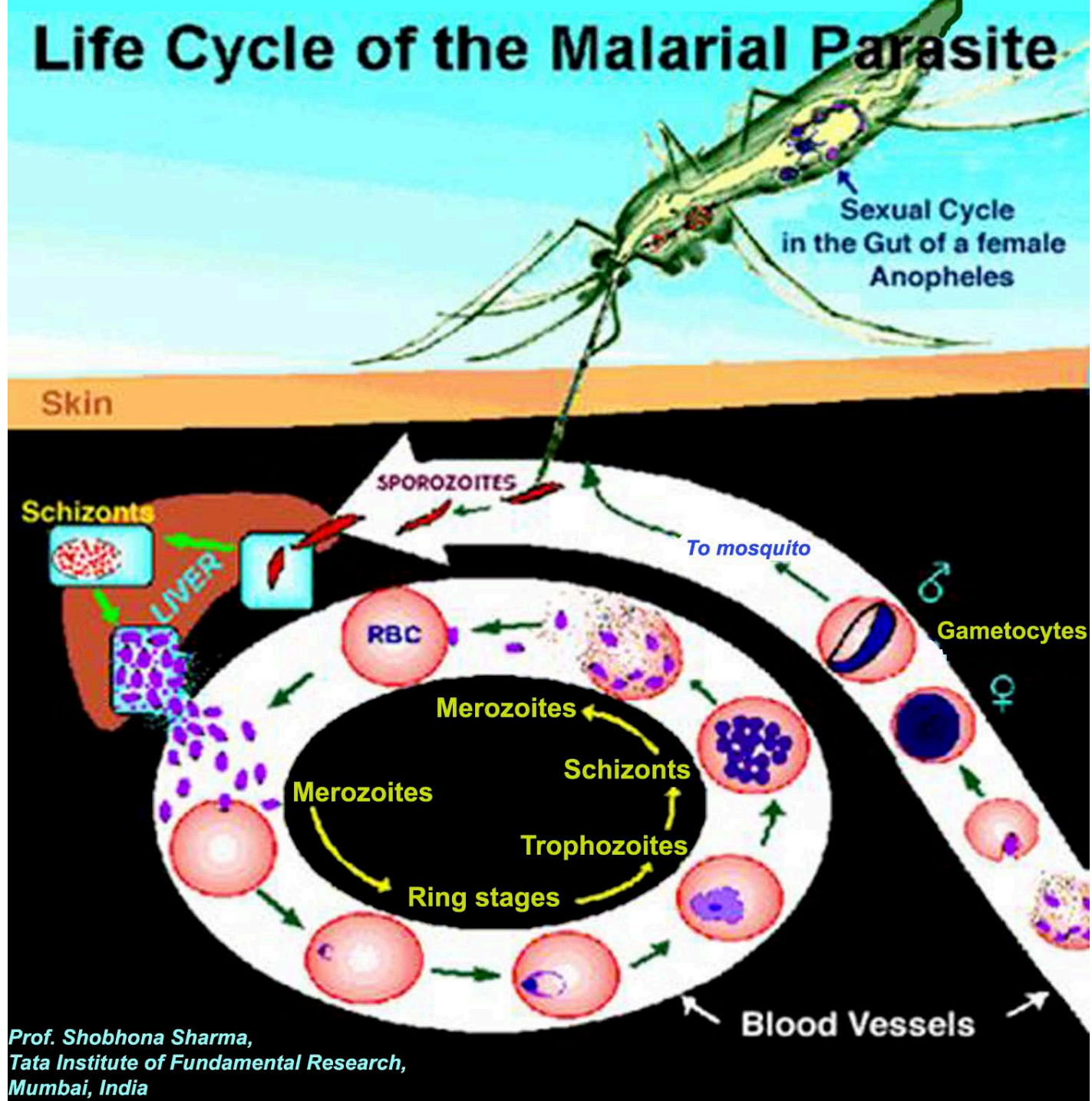
35% of the worlds population is at risk

# Epidemiology

- Malaria is generally considered to be a tropical disease.
- Four major species of plasmodium, but *P. falciparum* and *P. vivax* are by far the most important.
- *P. falciparum* is worldwide; *P. vivax* is not frequent in Africa but frequent in South America and Asia.

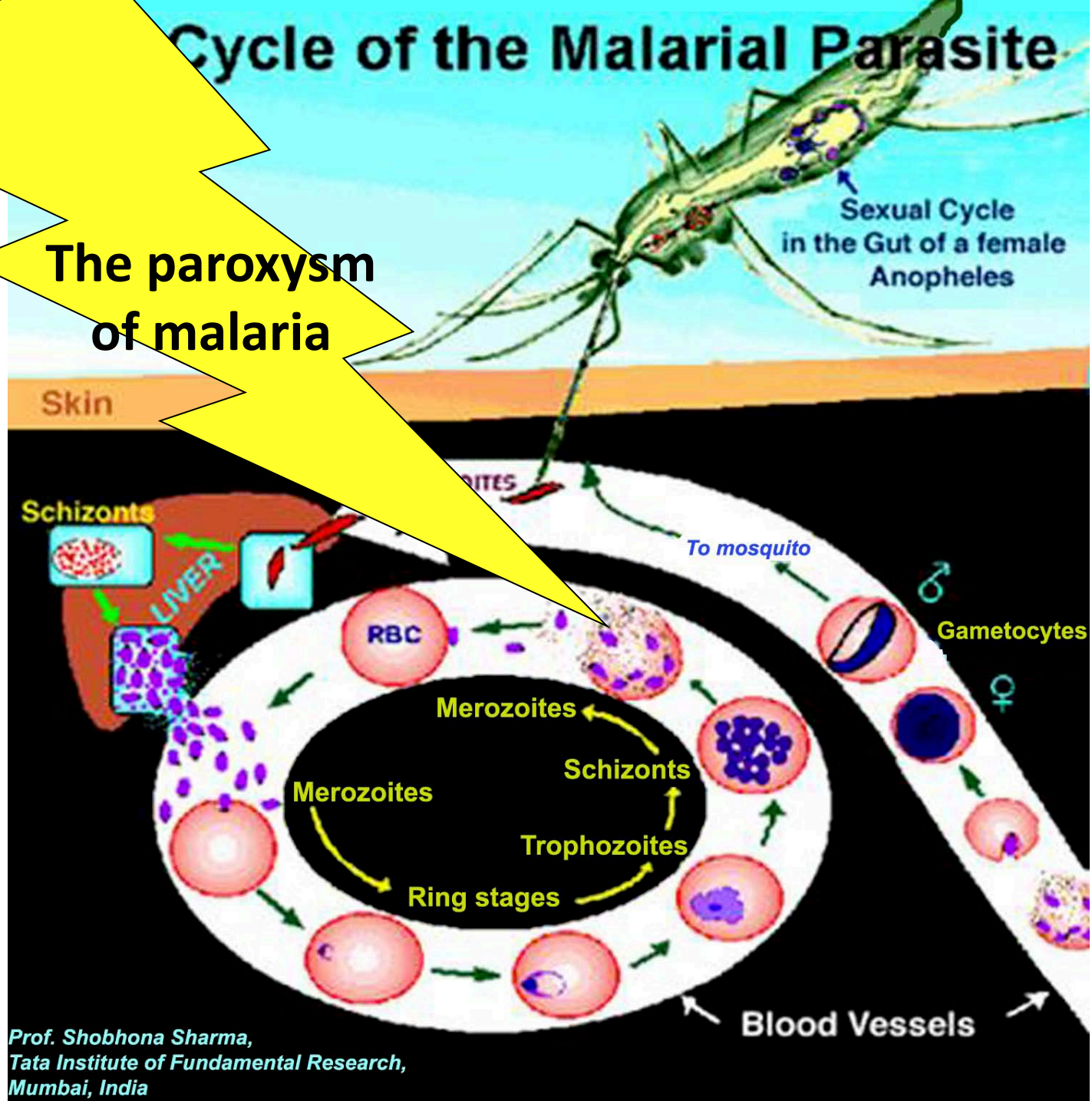


# Life Cycle of the Malarial Parasite



# Cycle of the Malarial Parasite

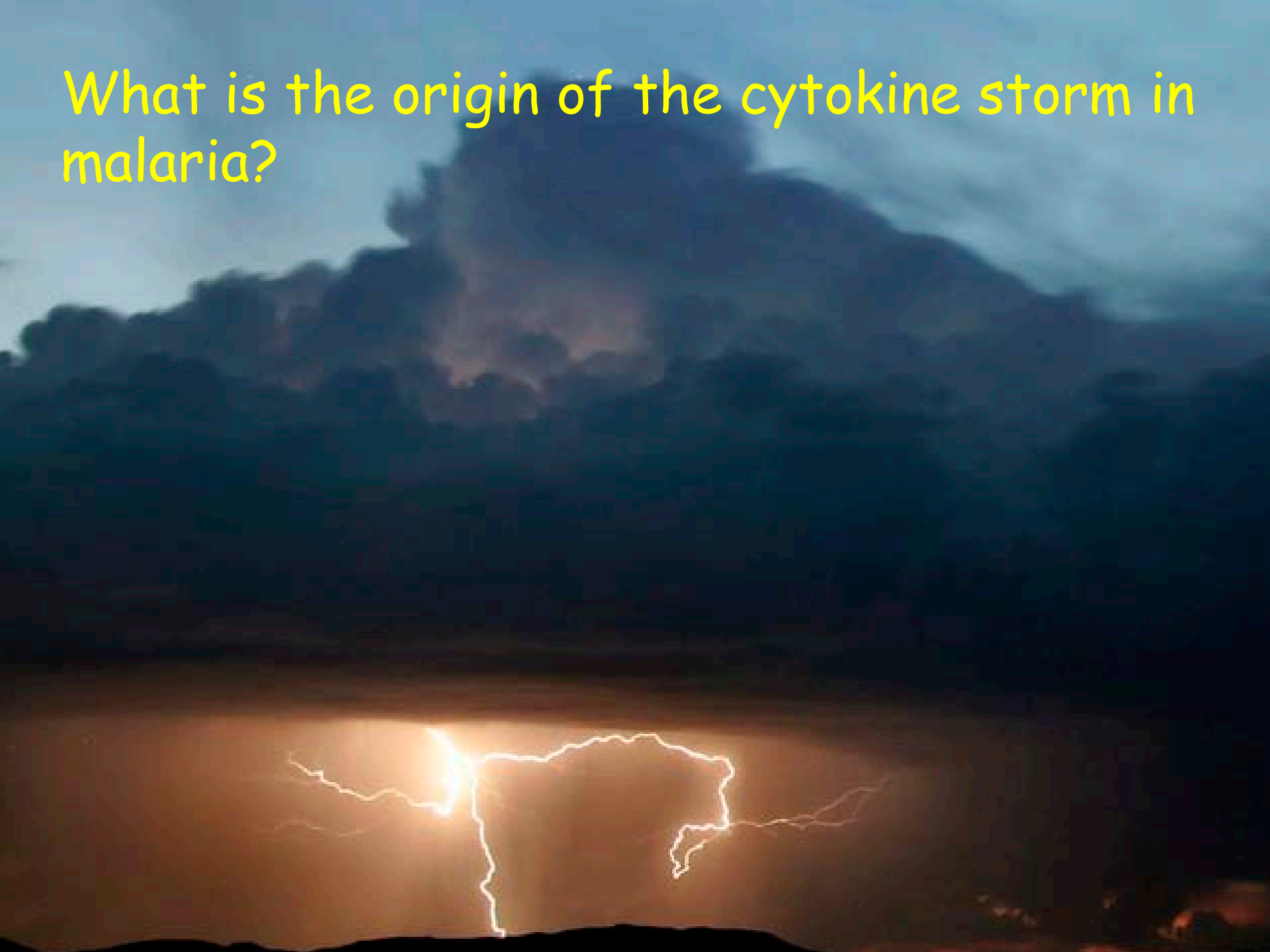
The paroxysm  
of malaria



# The paroxysm of malaria represents an inflammatory cytokine storm

- Fever (up to 104°F or 40°C)
- Rigor
- Headache
- Myalgias
  - During the paroxysm, extremely high levels of TNF $\alpha$  and IL1 $\beta$  have been measured
- The paroxysm is often followed by a period of extreme fatigue

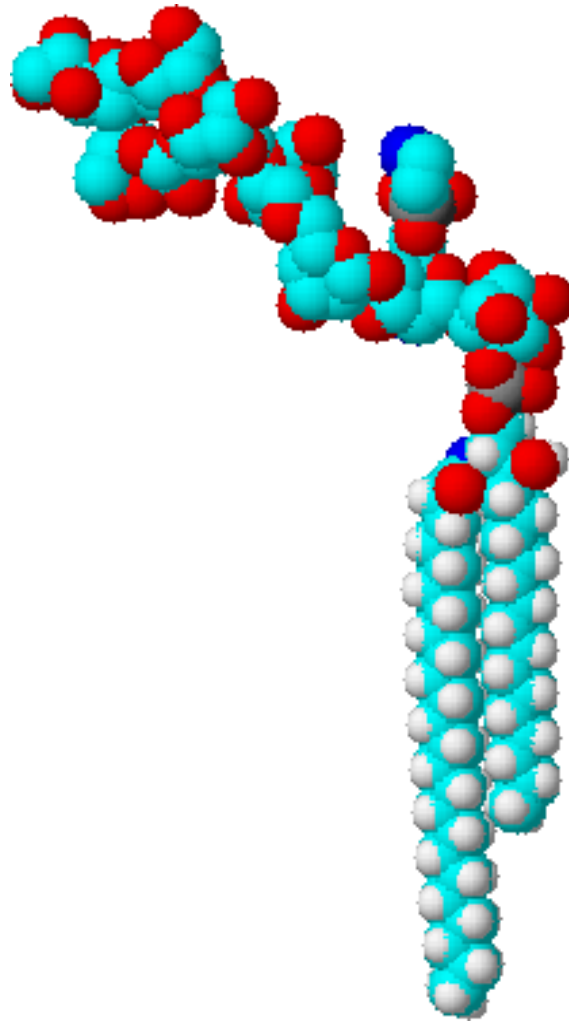
What is the origin of the cytokine storm in malaria?



# Our initial hypothesis

- The fever in malaria is caused by the activation of a TLR.

The Malarial Parasite is Coated with a  
Glycosylphosphatidyl Inositol Anchor (GPI)

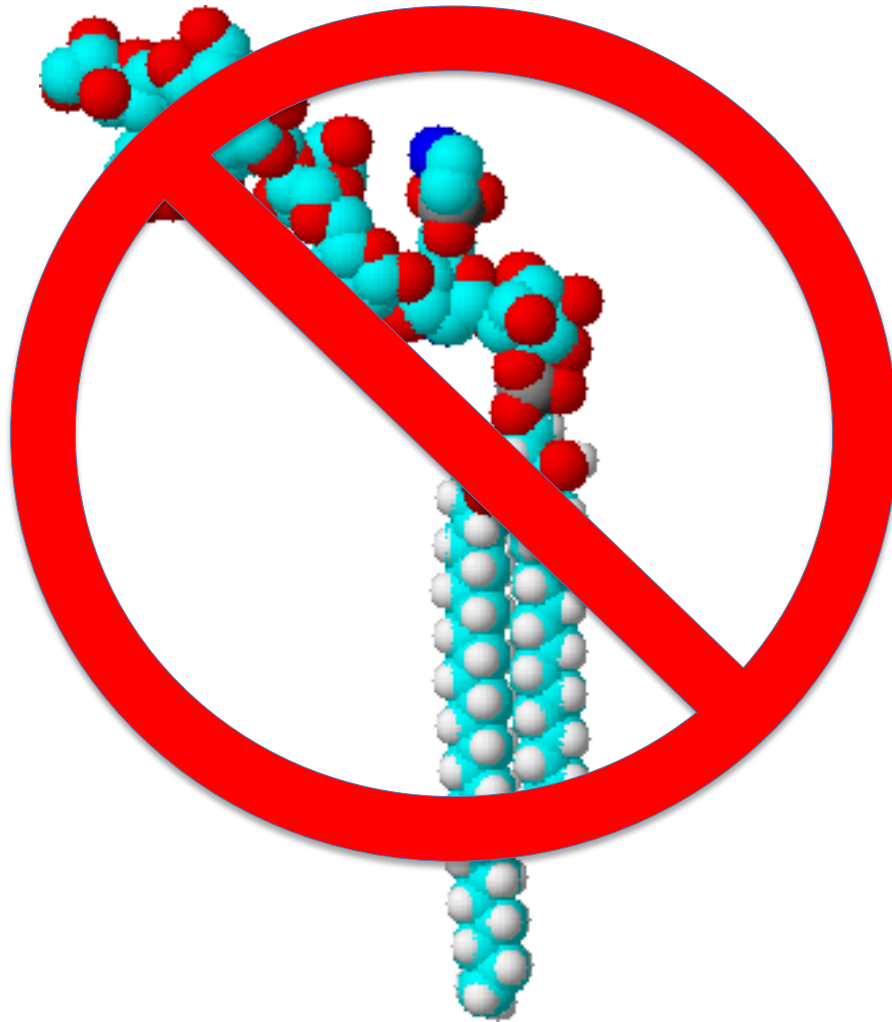


Gowda *et al.*, 2010. J Immunol.

The concentration of GPI on the surface of merozoites is too low to account for the ability of parasite extracts to stimulate cytokine production.



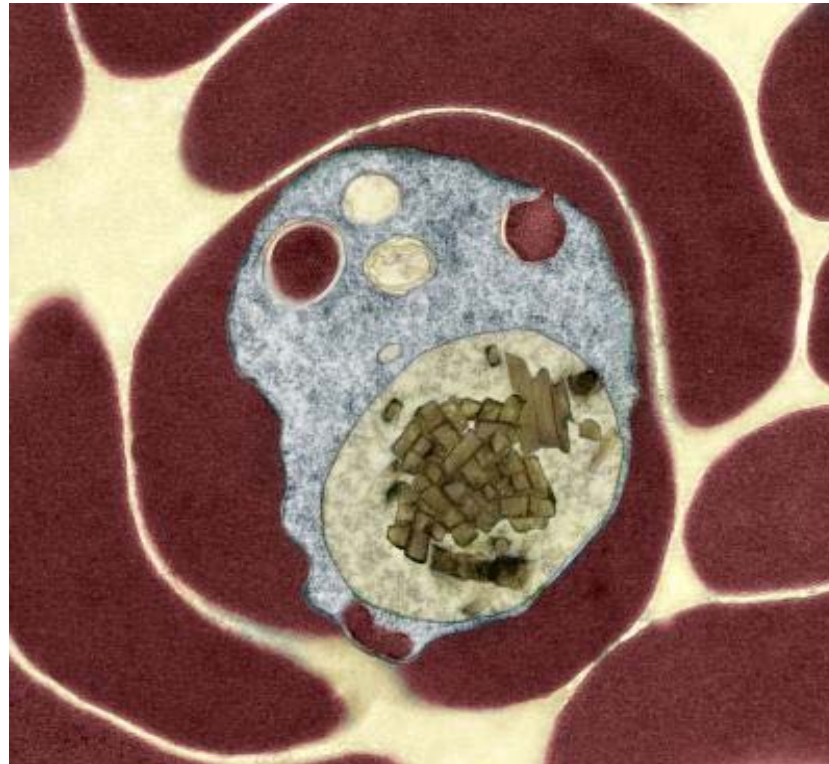
GPI is not likely to be an important cause of cytokine stimulation in malaria.





# Hemozoin

- *P. falciparum* metabolizes hemoglobin into hemin, which is subsequently detoxified by forming the inert crystal, hemozoin.



Hemozoin in parasitophorous  
vacuole

# Shizuo Akira



# Akira *et al.*

Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin.

*J Exp Med.* 2005;201(1):19-25.

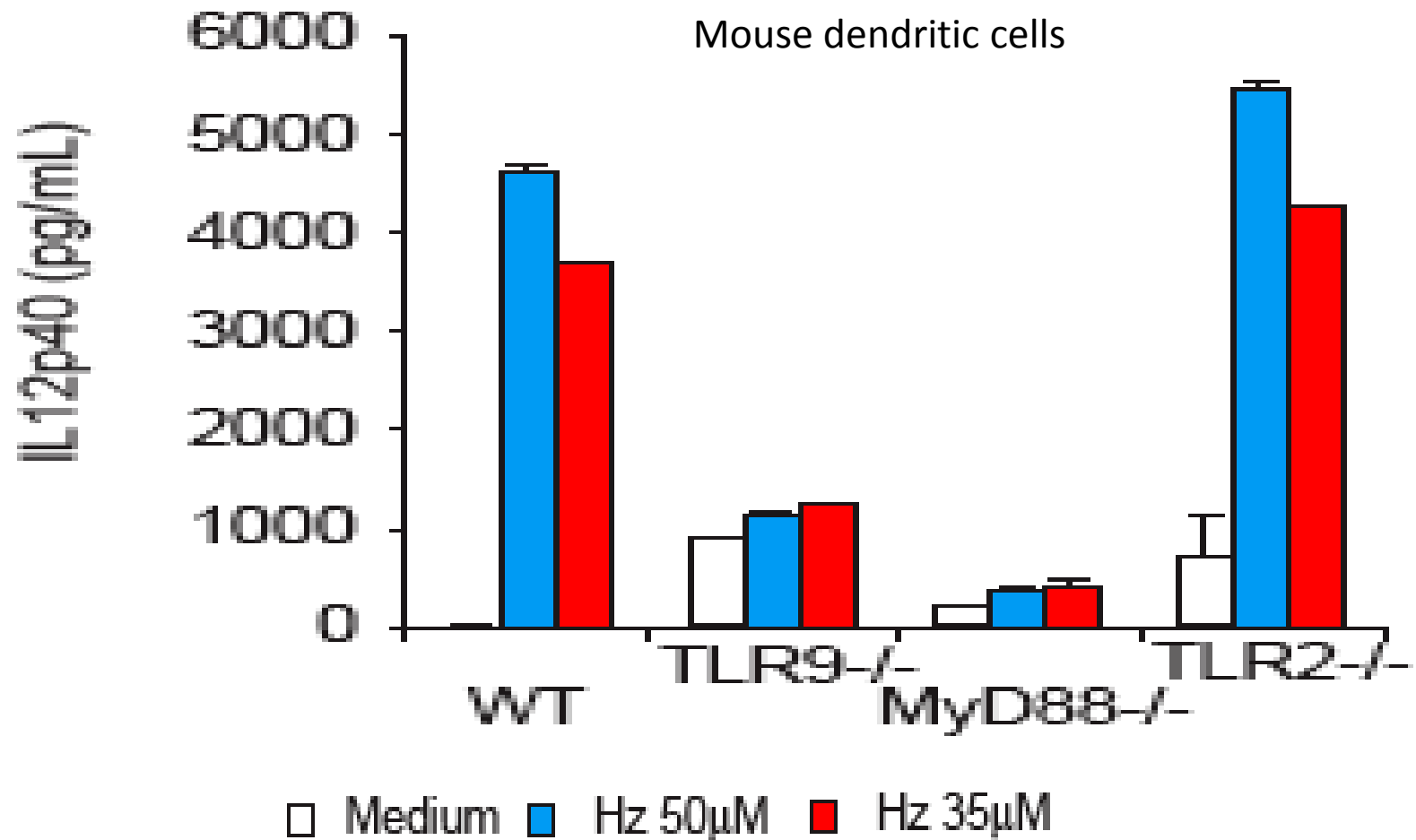


Scooped by Akira...again!

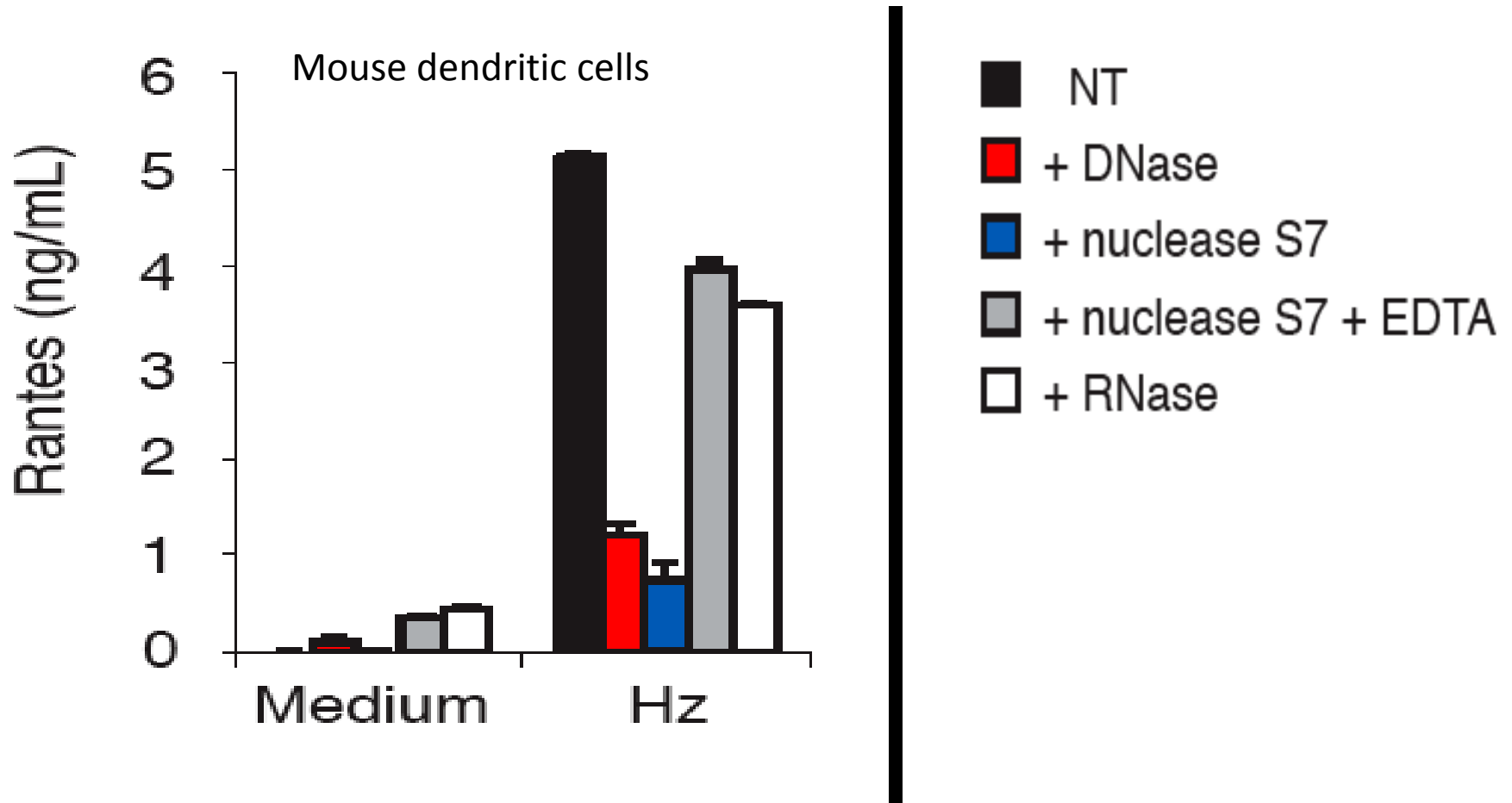


DTG

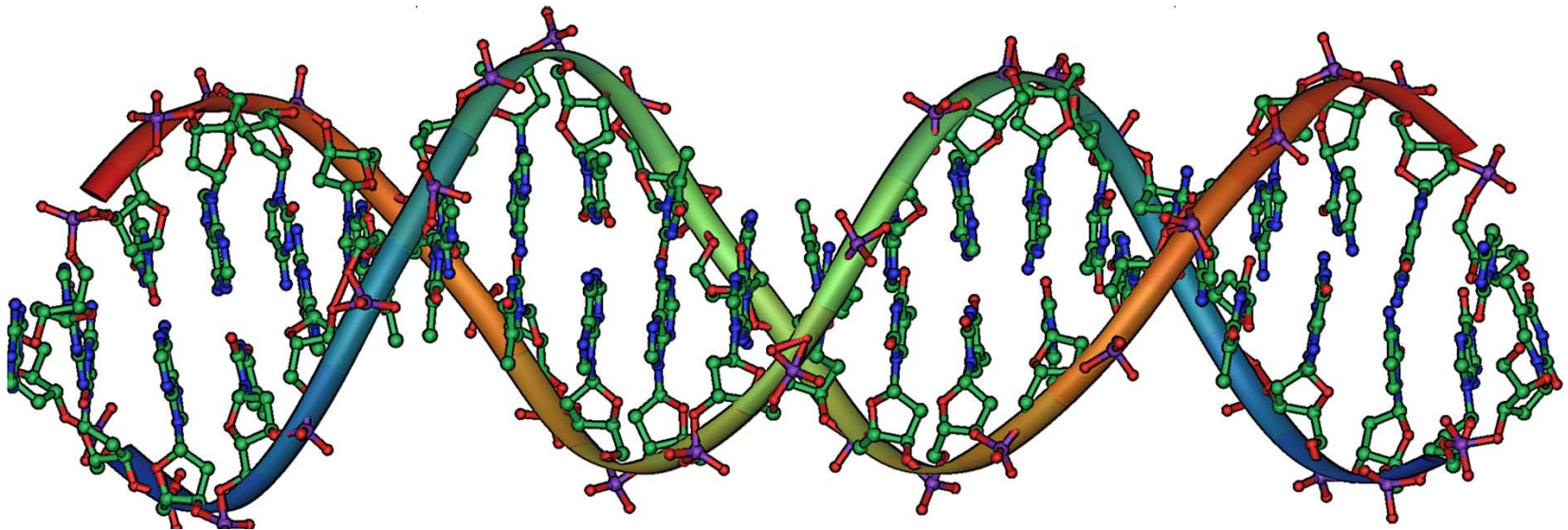
# Natural Hemozoin Activates Cytokine Production via TLR9 and its adapter protein, MyD88



# The stimulatory activity of hemozoin was destroyed by Dnase.

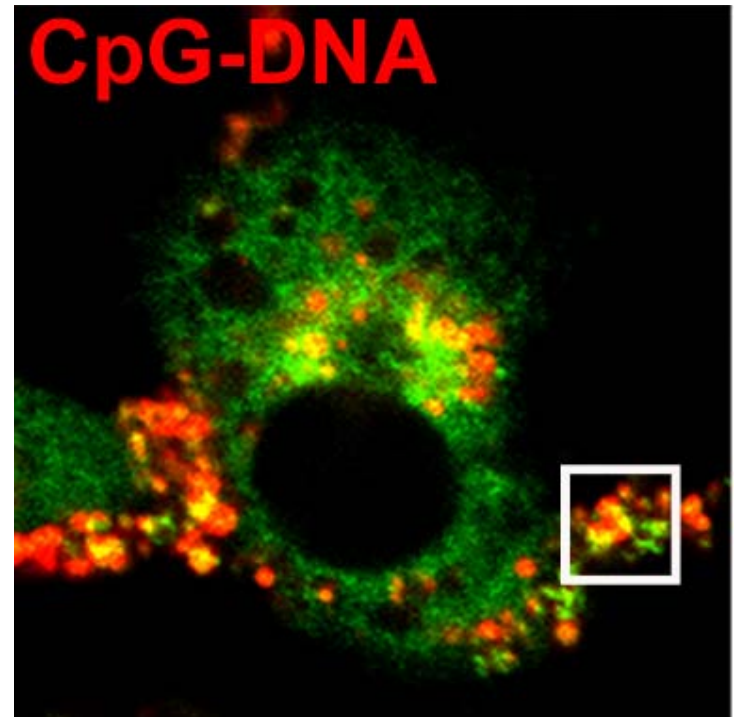
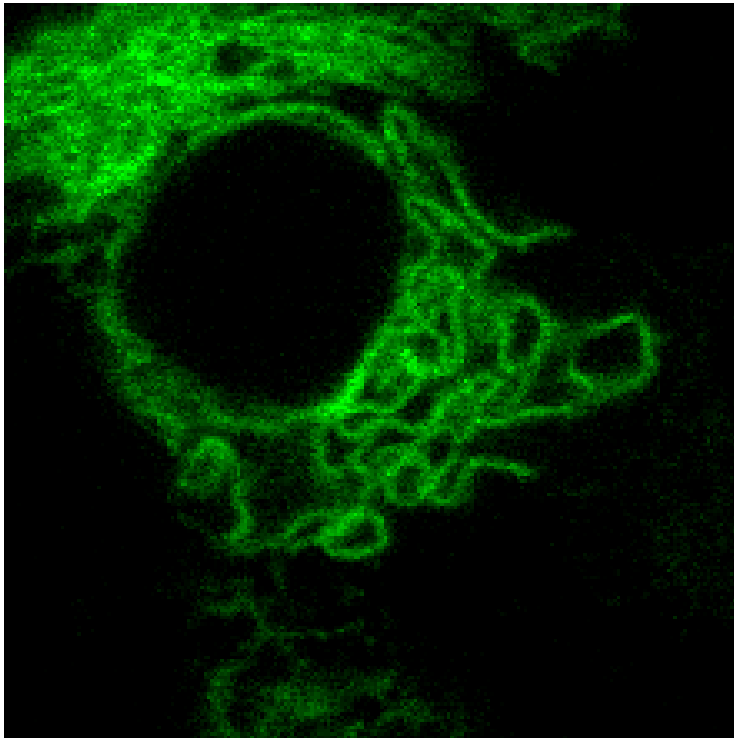


Thus, the cytokine inducing component  
of hemozoin is (plasmodial) DNA



## Current hypothesis:

*Hemozoin functions to traffic DNA into an intracellular compartment to which TLR9 can be recruited*



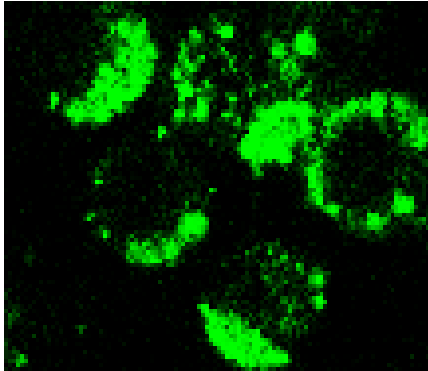


Parisa Kalantari, Ph.D.

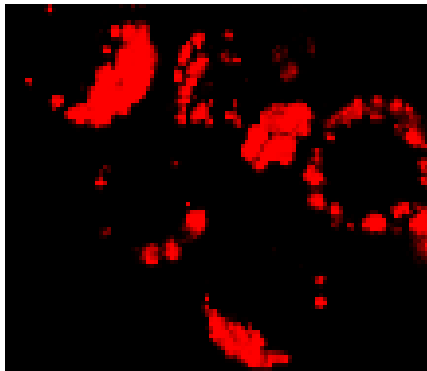


# Hemozoin/DNA complexes are rapidly internalized into a lysosomal compartment in macrophages

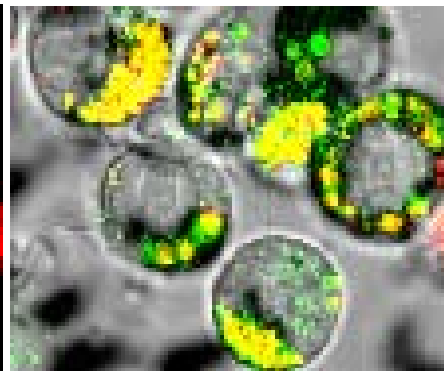
Reflection (sHz)



CpG-Alexa642



Overlay



100 ug of sHz and 30ug of CpG-Alexa 642-



Adding CpG-Alexa 642 and sHz in a microcentrifuge tube



Rocked for 2 hours at RT



Washed 4 times with PBS



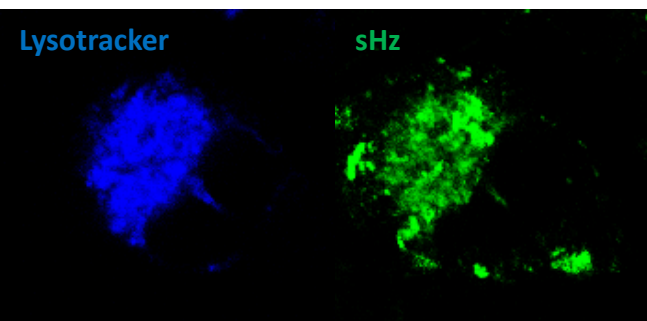
Added to the cells



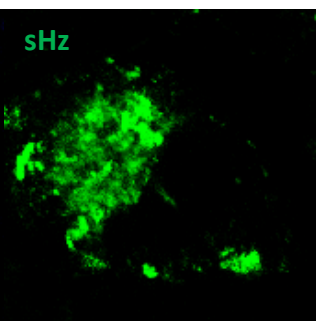
Confocal Microscopy

CpG DNA is internalized into **lysosomes** when complexed to Hemozoin

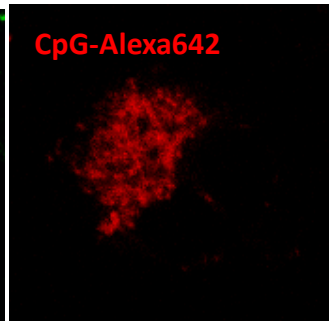
Lysotracker



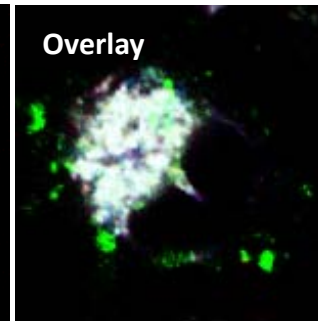
sHz



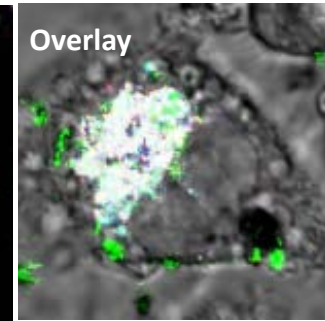
CpG-Alexa642



Overlay

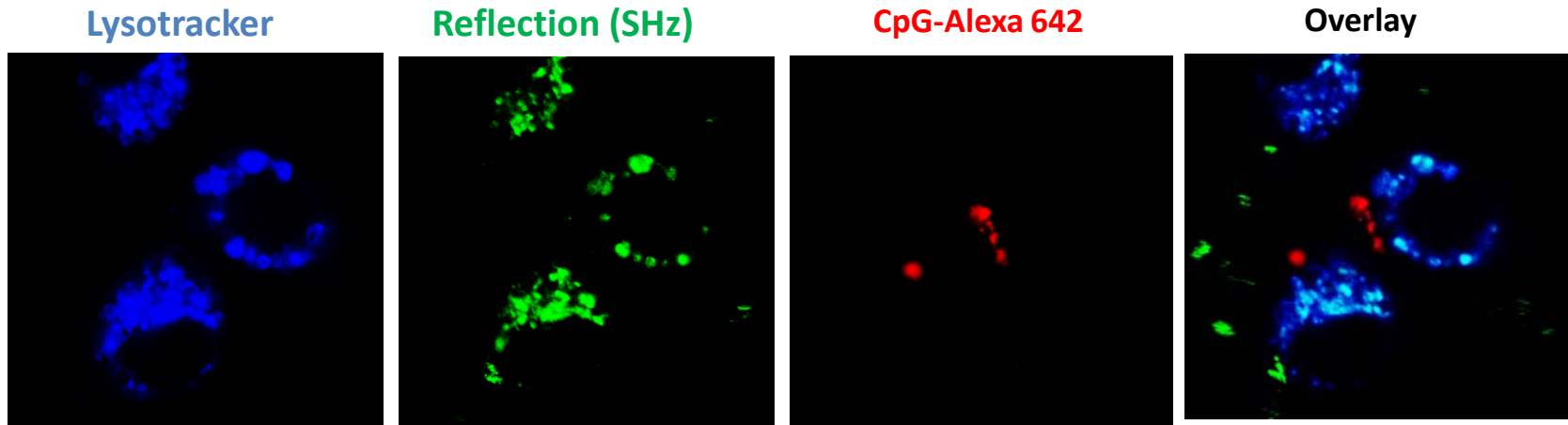


Overlay

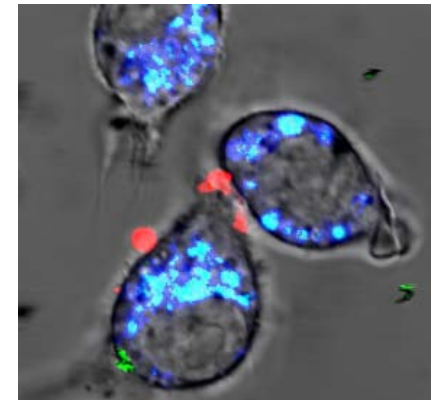


30 min

**Free CpG DNA (unlike hemozoin-bound DNA) has delayed internalization into a lysosomal compartment.**



Hz and CpG added to macrophages- No prebinding



30 minutes

Lysotracker:  
Ex:380

The concept that plasmodial DNA is introduced into cells by hemozoin to activate TLR9 via CpG DNA motifs was compelling, except for the fact that plasmodial DNA is highly AT-rich!

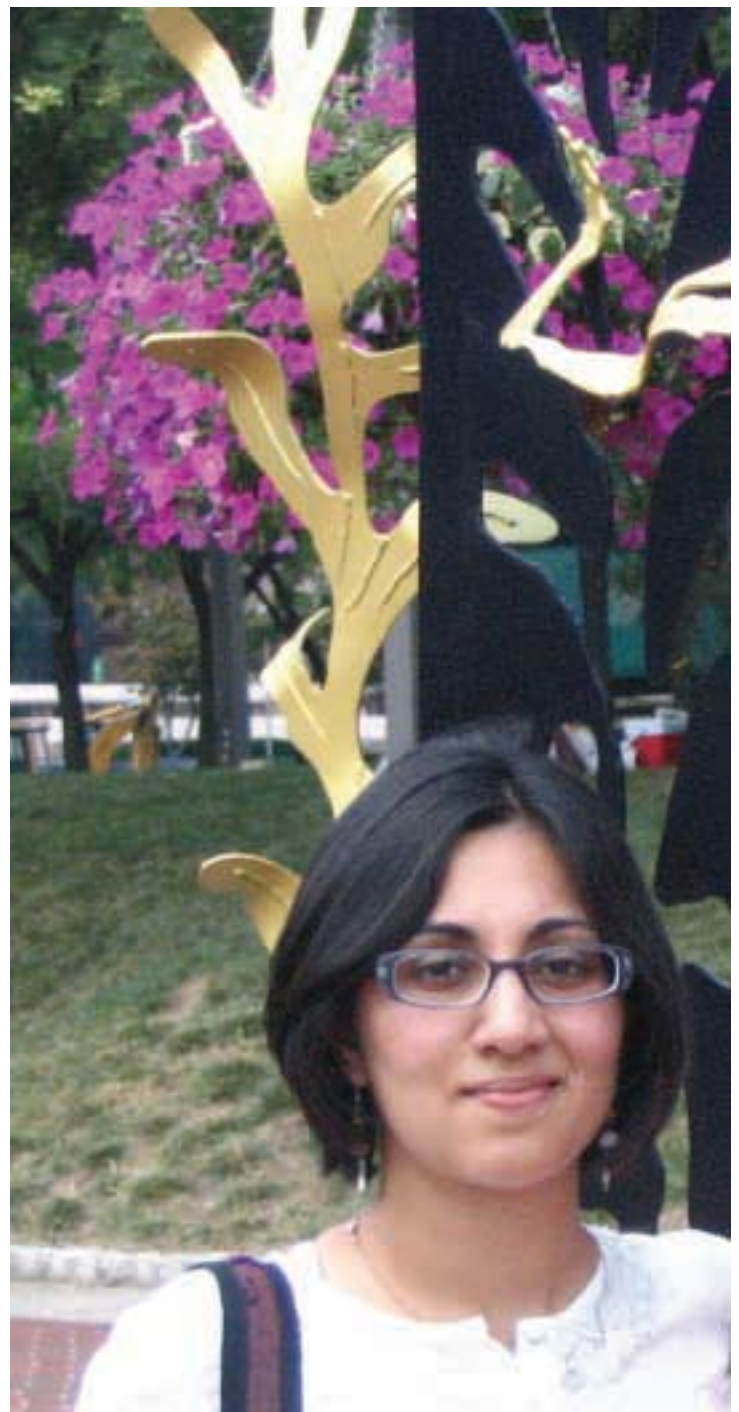
The concept that plasmodial DNA is introduced into cells by hemozoin to activate TLR9 via CpG DNA motifs was compelling, except for the fact that plasmodial DNA is highly AT-rich!

- The *P. falciparum* genome contains a stem loop motif: ATTTTAC over 6000 times!

## The AT-r stem-loop motif is found in a variety of other organisms

Genome (size)	AT content (%)	Number of putative AT-ODN motifs
<i>Plasmodium falciparum</i> (22.8Mb)	82%	6130
<i>Variola</i> (371.1kb)	68%	24
<i>Vaccinia</i> (389.4kb)	67%	25
<i>Listeria monocytogenes</i> (5.8Mb)	61%	480
<i>Homo sapiens</i> (3.08 Gb)	56%	268828
<i>Human adenovirus 5</i> (35.9kb)	45%	5
<i>Leishmania major</i> (32.8Mb)	38%	184
<i>Human herpesvirus 2</i> (154.7kb)	30%	2

Shruti Sharma

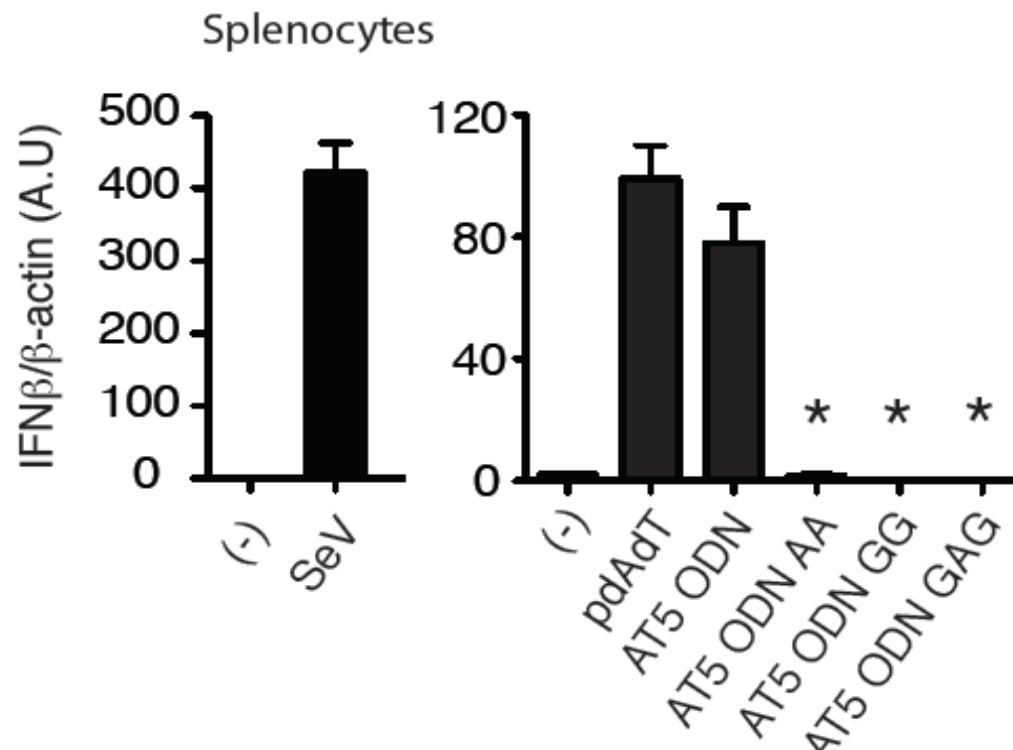
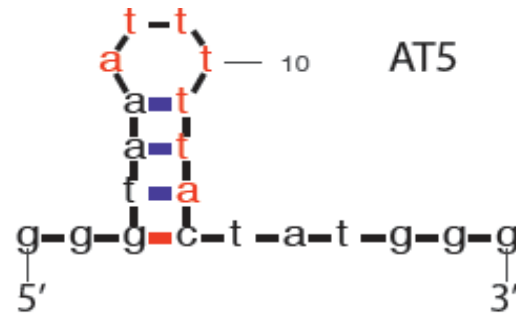


One can study the AT-r stem-loop motif  
using synthetic ODN

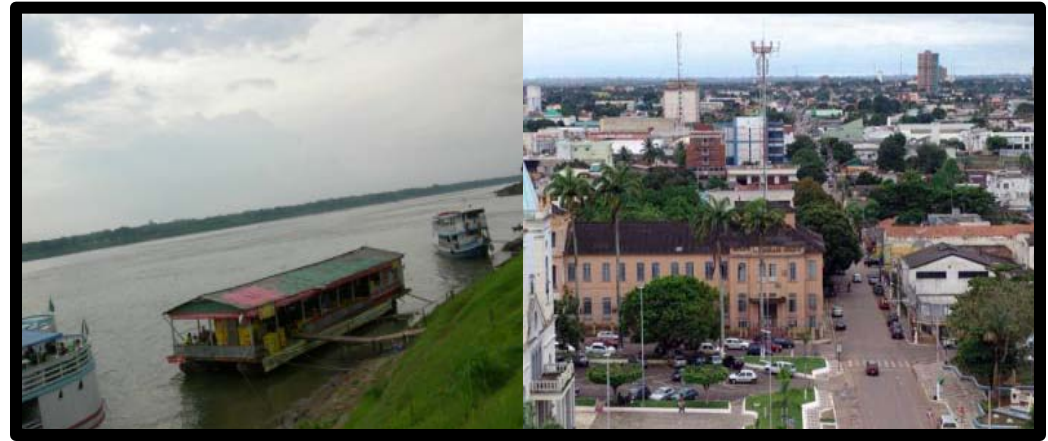
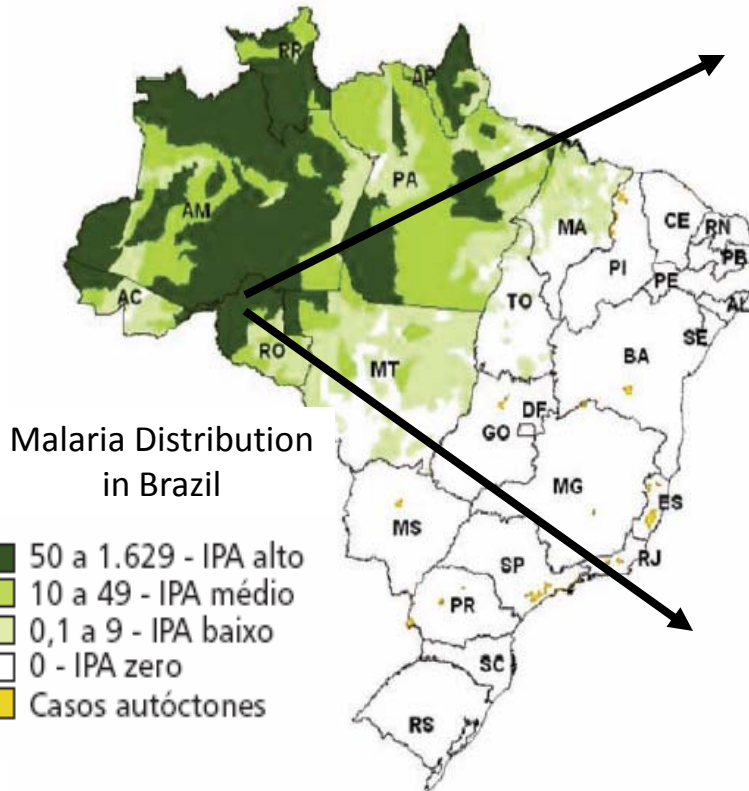
- AT-2: GCACAC**ATTTTAC**TAAAAC



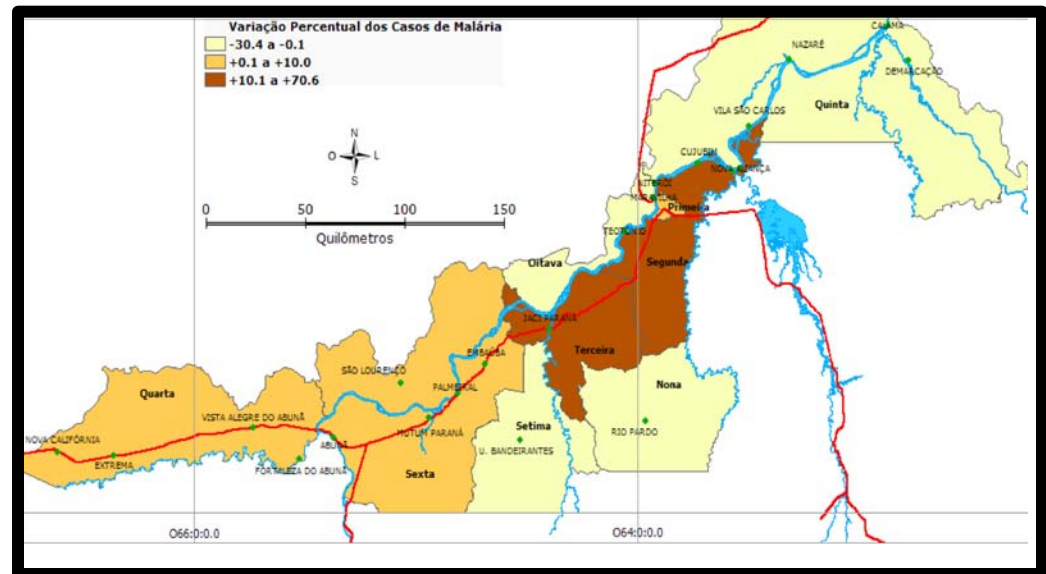
# The stem-loop structure is *essential* for innate immune activity



# Malaria Clinical Research Site in Porto Velho, Rondônia, Brazil



## Malaria Distribution in Porto Velho - 2010



**Porto Velho** is the capital city of **Rondonia State**; it is the most rapidly growing major city in Brazil and currently has a population of about 400,000 persons.



# The ideal caiparinha comes from caipira (“hillbilly”)- an example of T3 research



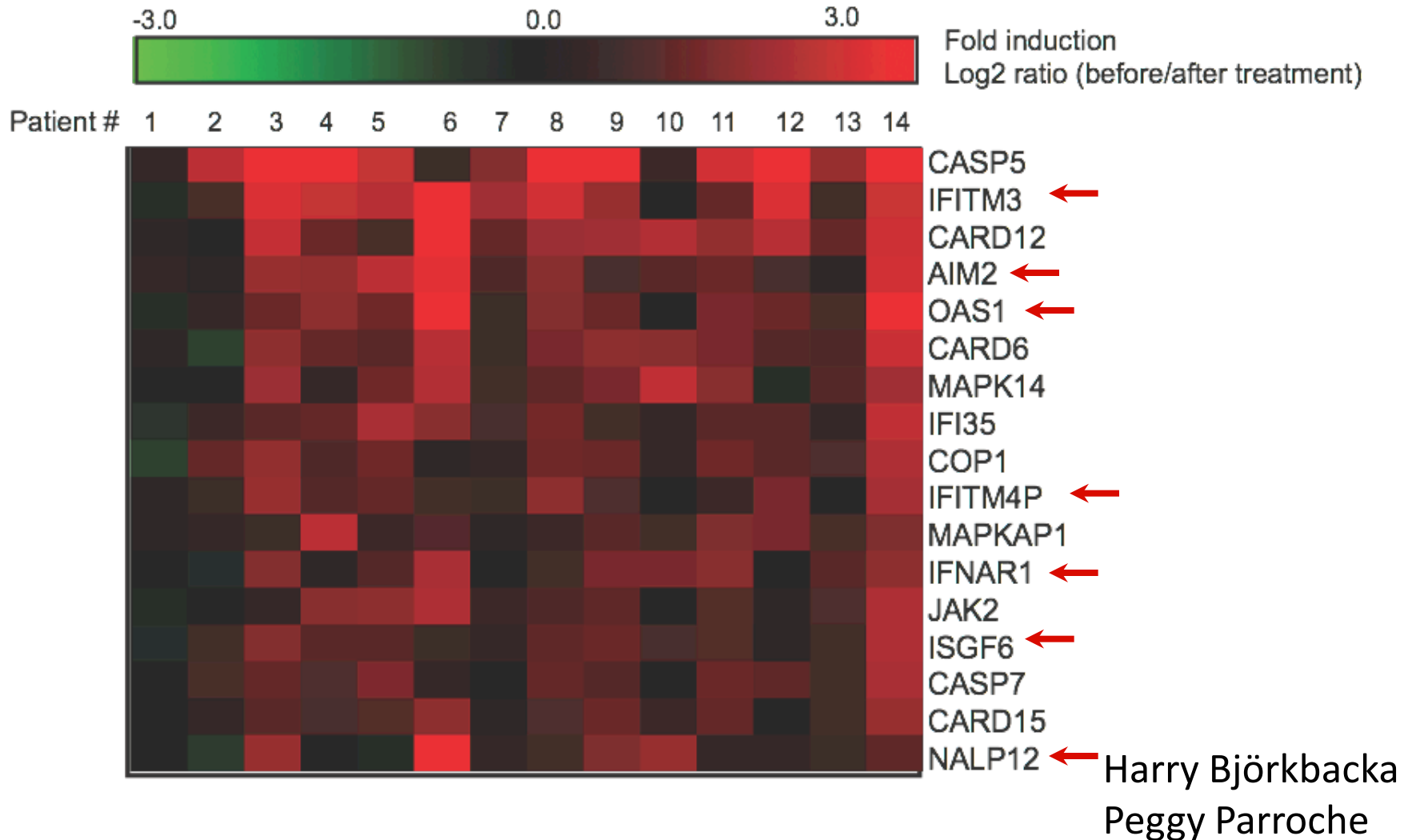
## Ingredients

- \* 1/2 lime, quartered
- \* 1 teaspoon white sugar
- \* 2 1/2 fluid ounces cachaca
- \* 1 cup ice cubes

## Directions

1. In a large rocks glass squeeze and drop in 2 eighths of lime. Add sugar, crush and mix with a spoon. Pour in the cachaca and plenty of ice. Stir well.

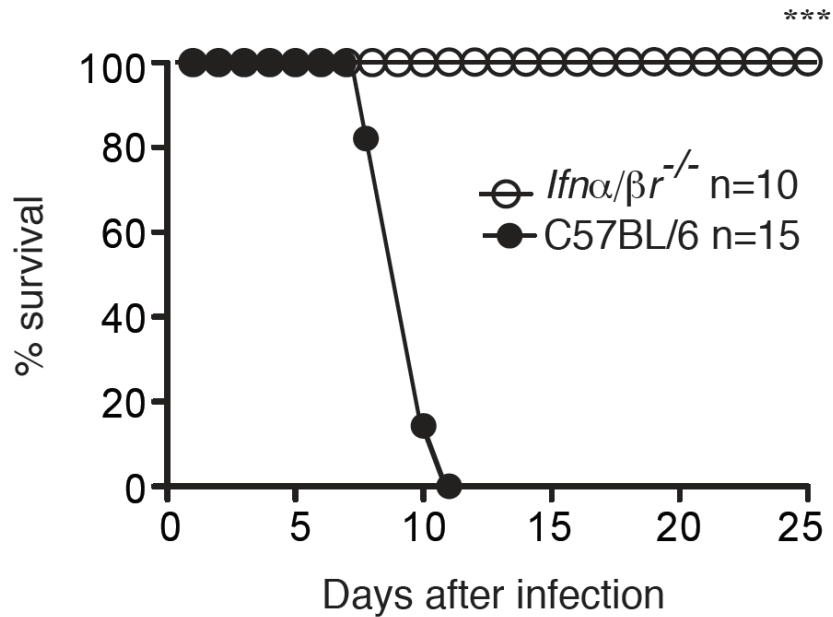
Studies of febrile malaria patients (*P. falciparum*) demonstrated that IFN-stimulated genes (ISG) are up-regulated during malaria



# Rosane de Oliveira

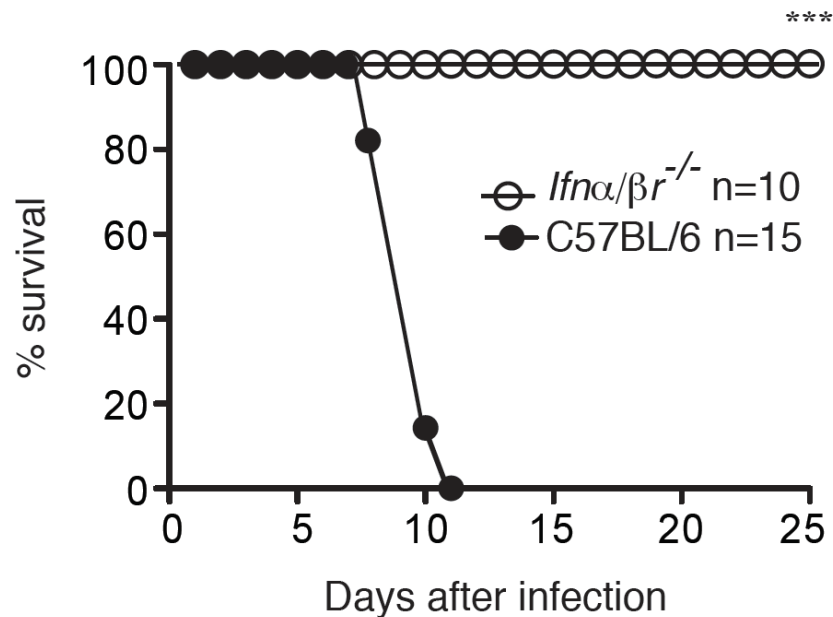


# IFN alpha/beta receptor (IFNAR) knockouts are highly resistant to mouse cerebral malaria





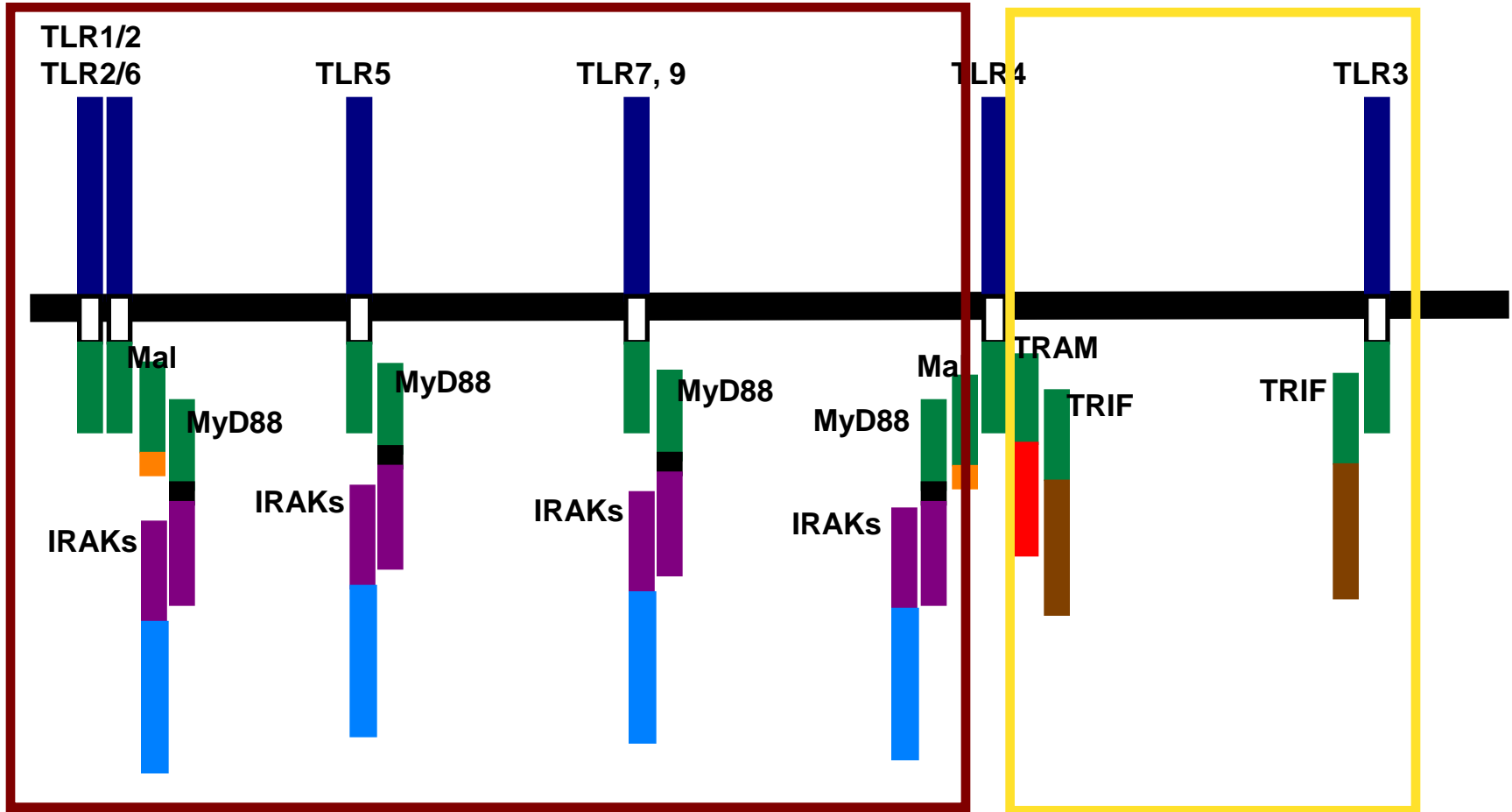
Hence, type I IFNs appear to be an essential part of the inflammation seen in malaria



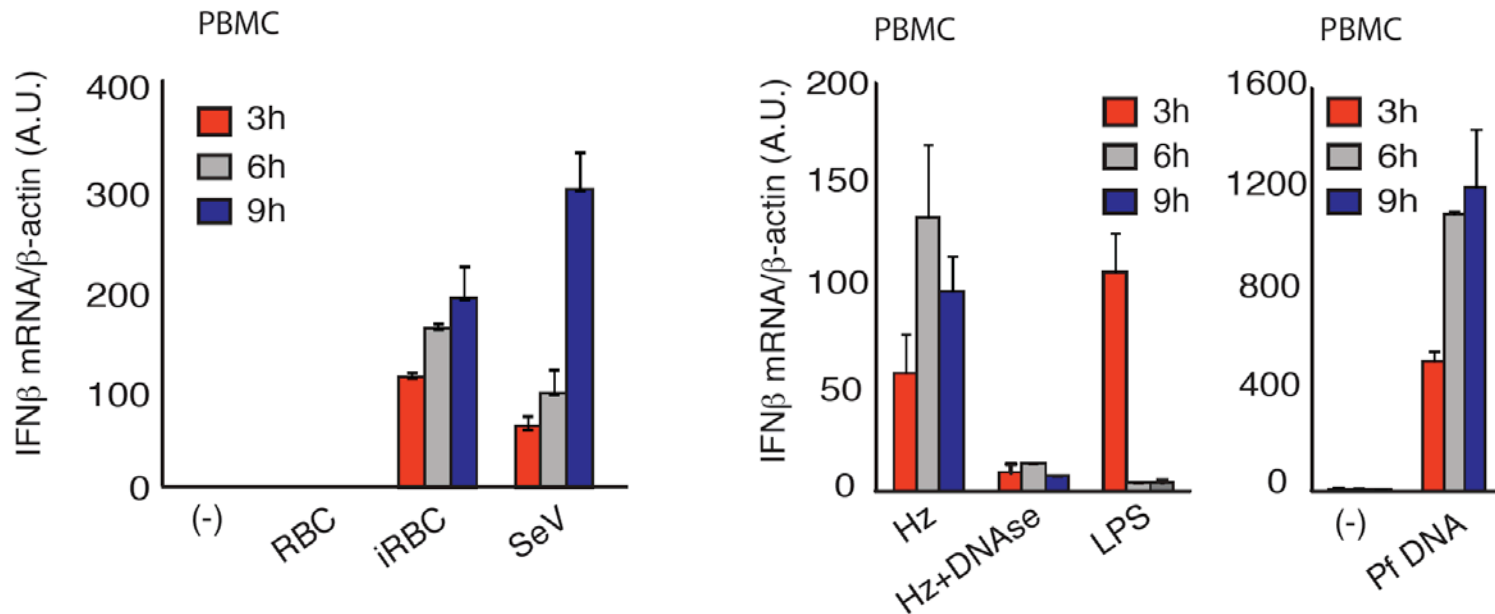
# There are two major TLR signaling pathways

MyD88-dependent

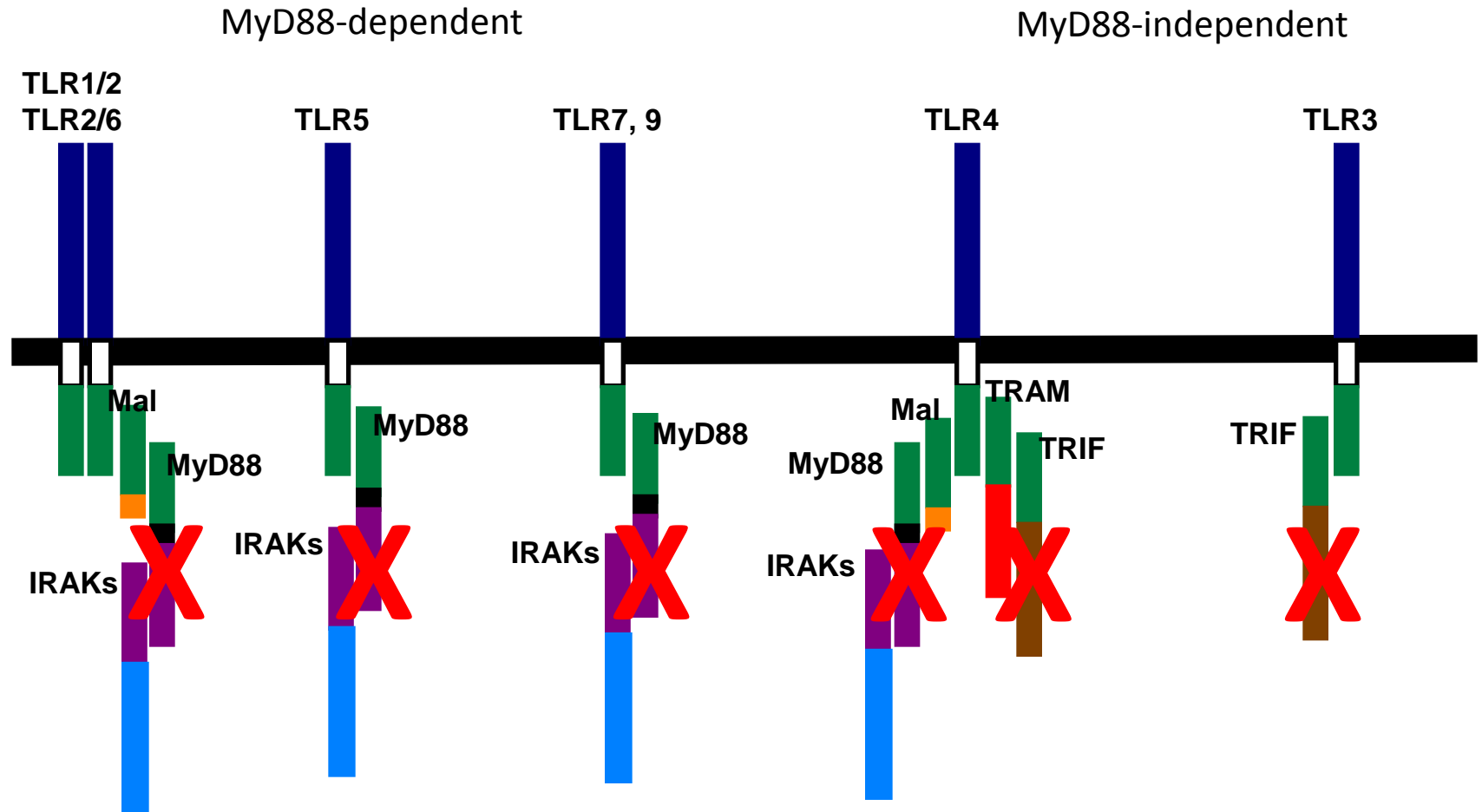
MyD88-independent  
/TRIF dependent



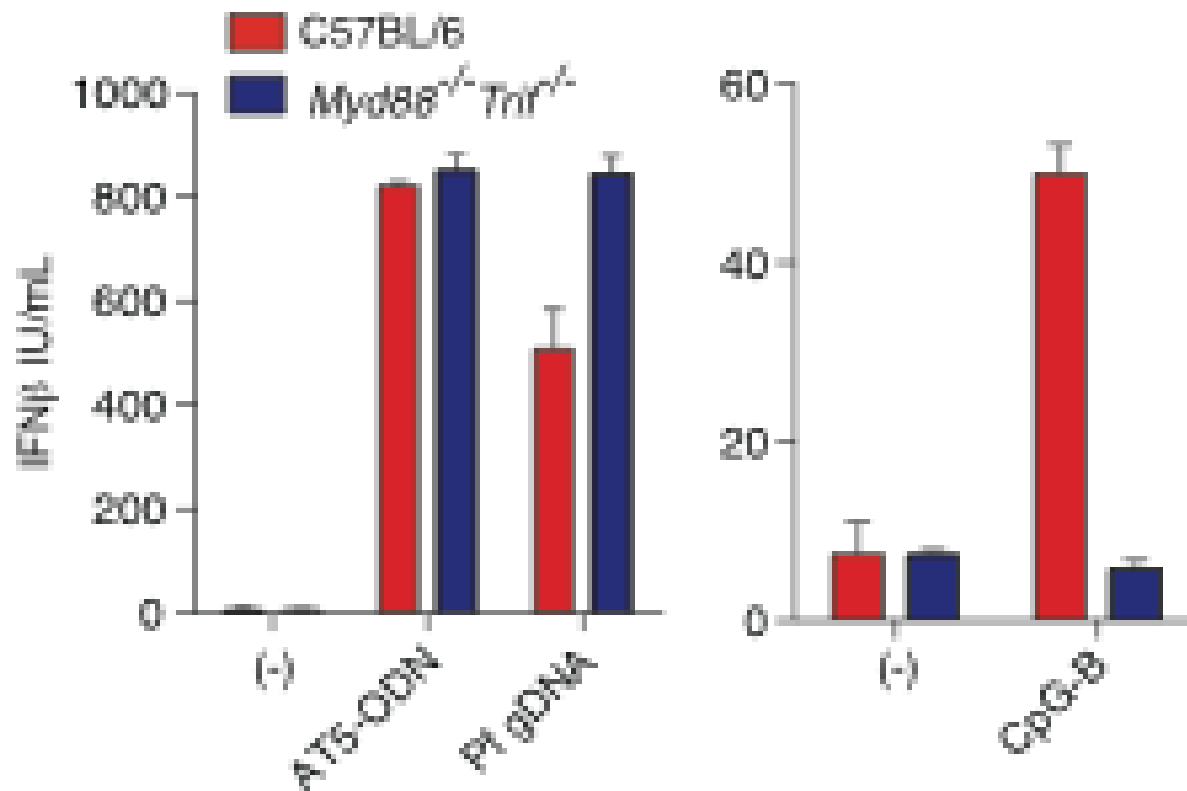
# *P. falciparum* infected rbc, hemozoin/DNA and purified malaria DNA activate a type I interferon response



# TRIF/MyD88 DKO have no TLR function

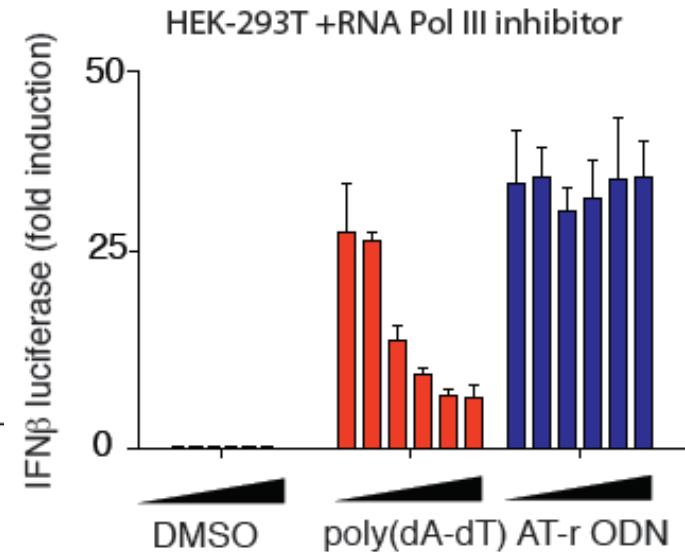
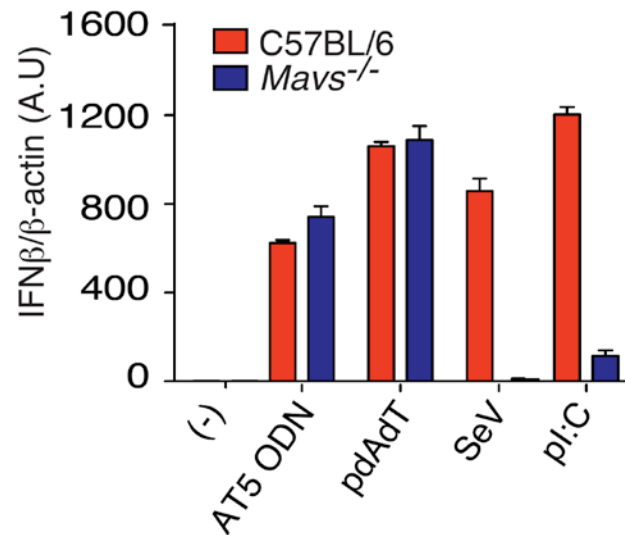
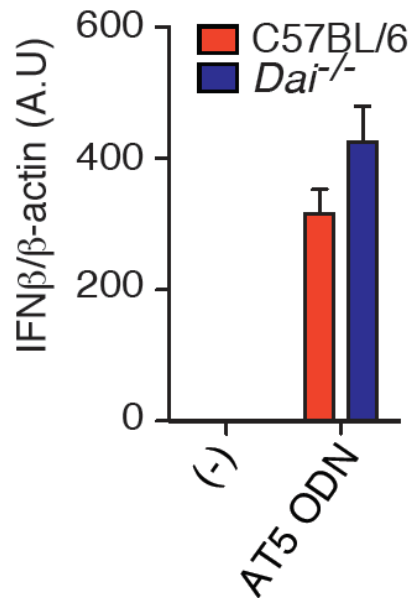


Neither AT-r ODN nor plasmidial genomic DNA induce IFN production via TLRs!

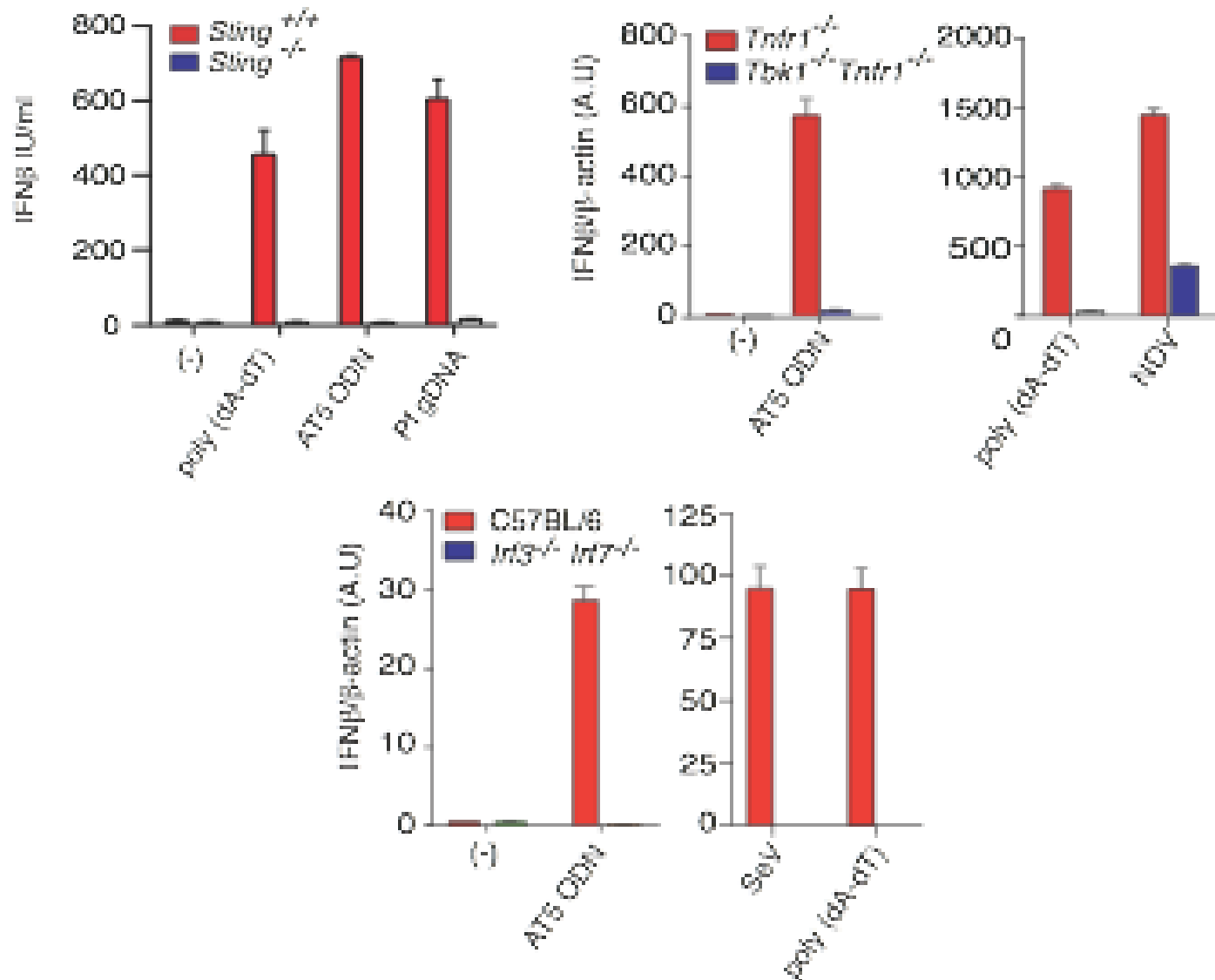


# AT-r activation of type I interferons is not due to activation of known nucleotide sensors

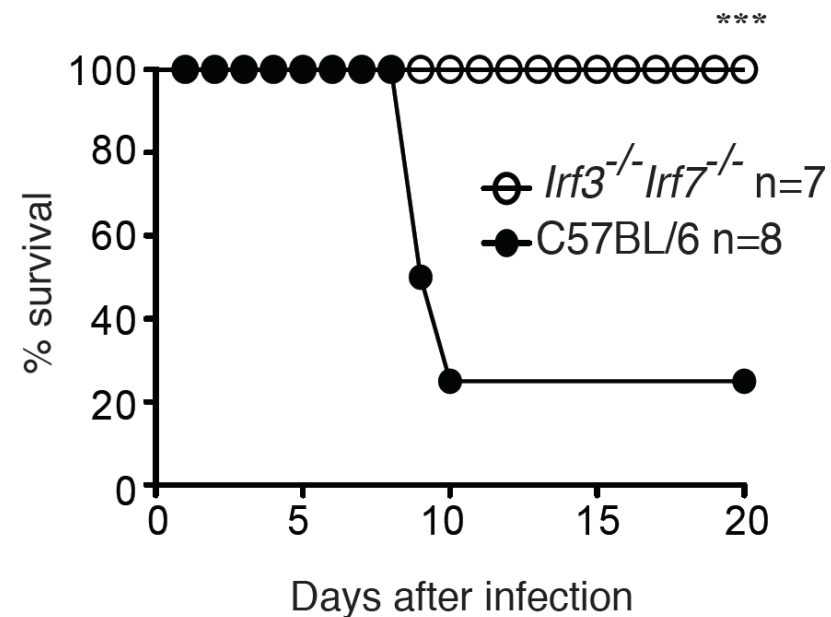
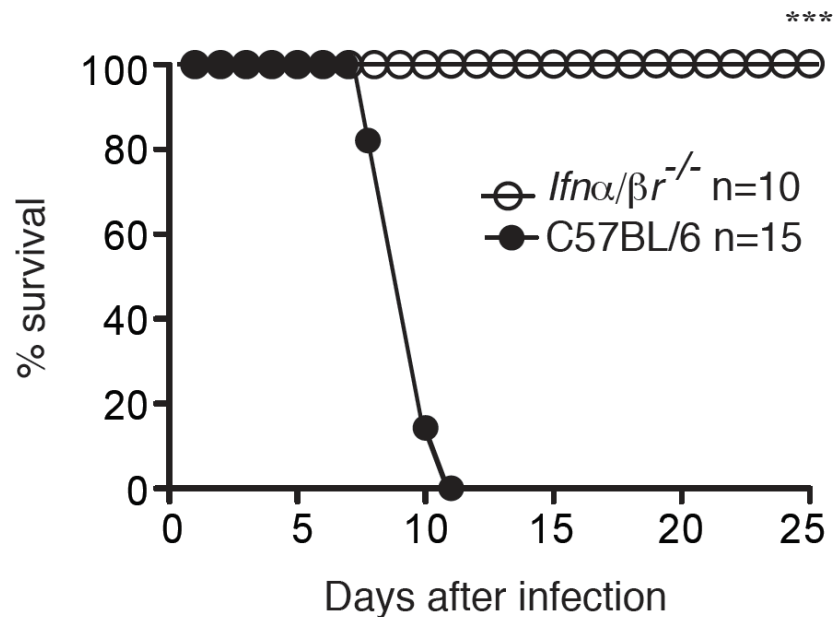
- DAI
- RNA helicases
- RNA Polymerase III



# STING/TBK1 and IRF3/7 are critical components of AT-r DNA sensing pathway.

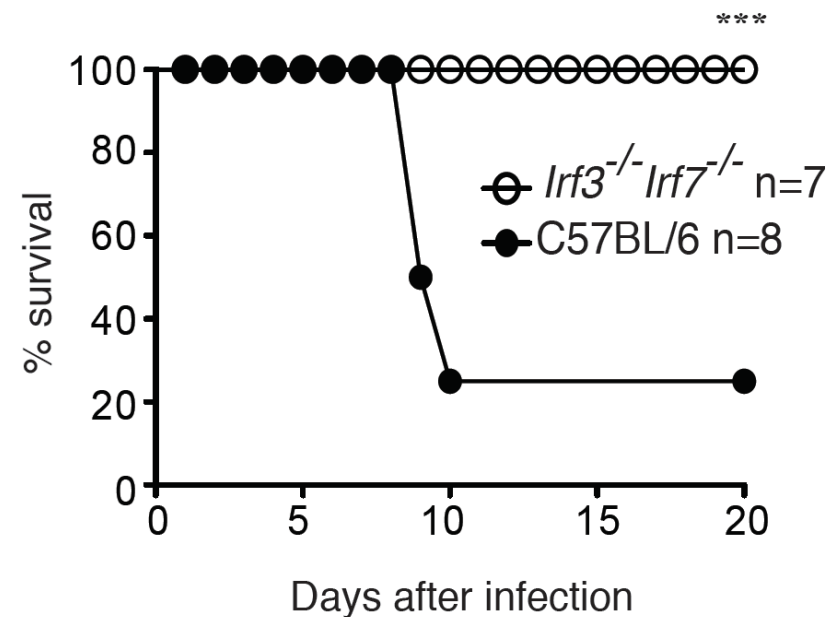
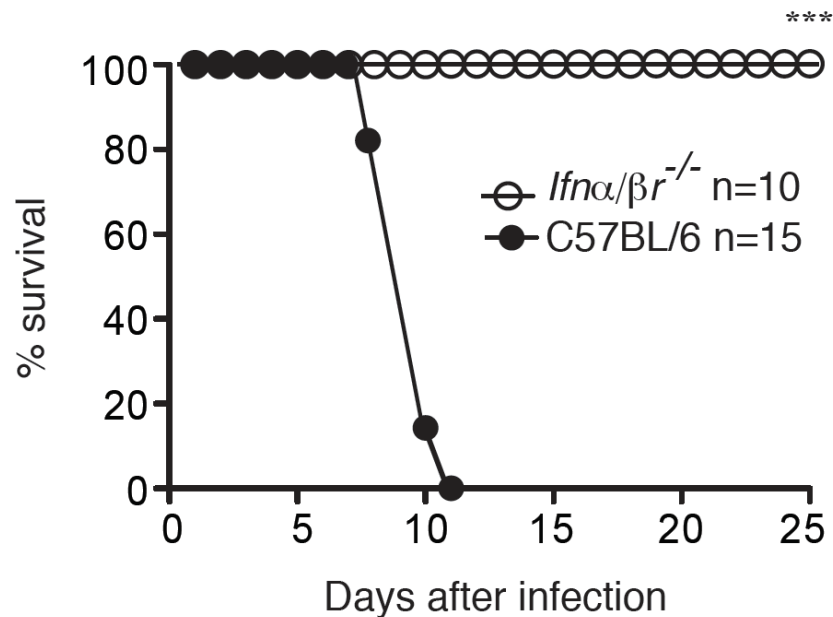


Like the IFNAR KOs, IRF3/7 double knockouts are similarly resistant to cerebral malaria





# Both STING KOs and TBK1 hypomorphic mice appear to be resistant to cerebral malaria



AT-rich DNA must be in the cytosol of cells to activate IFN or pro-inflammatory cytokines.

How does the DNA on the surface of hemozoin move from the phagosome to the cytosol?

# Hemozoin traffics into the phagolysosome and then into the cytosol

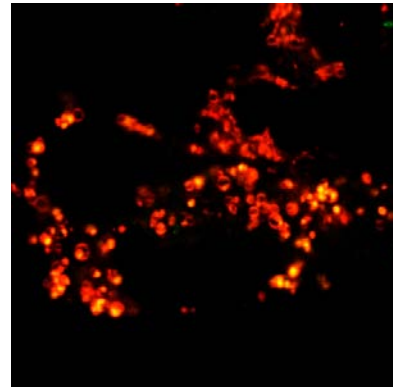
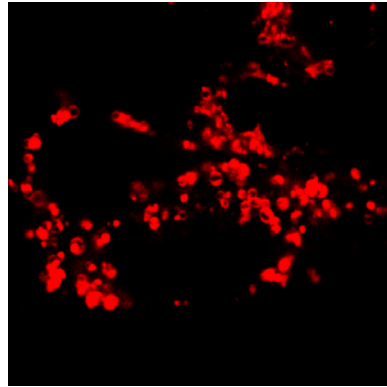
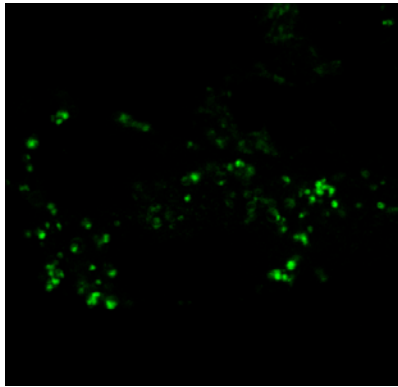
Hemozoin

Lysosome

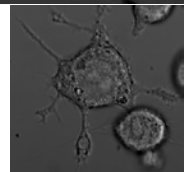
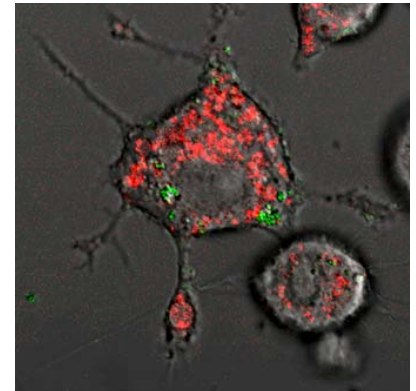
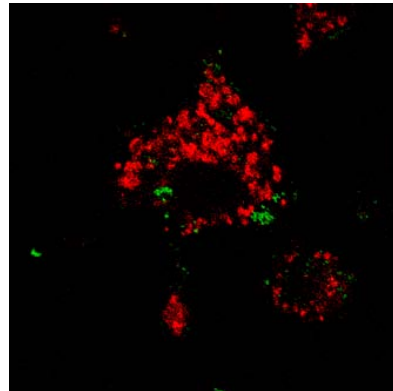
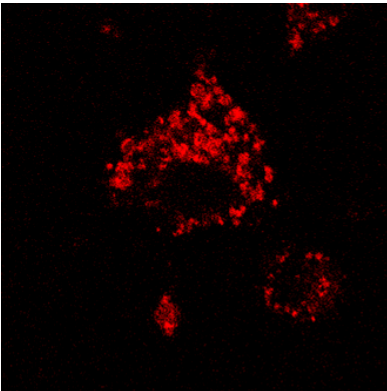
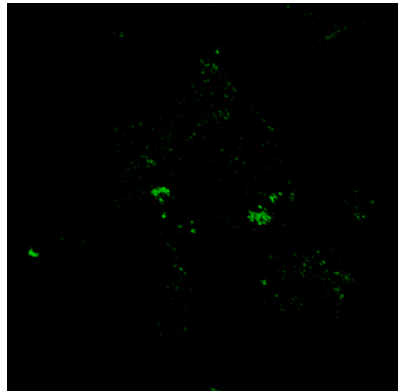
Overlay

Overlay

1 hr



6hrs



The phagocytosis of inert particles results in phagolysosomal leakage.

- Silicic acid
- Urate
- Asbestos

The phagocytosis of inert particles results in phagolysosomal leakage.

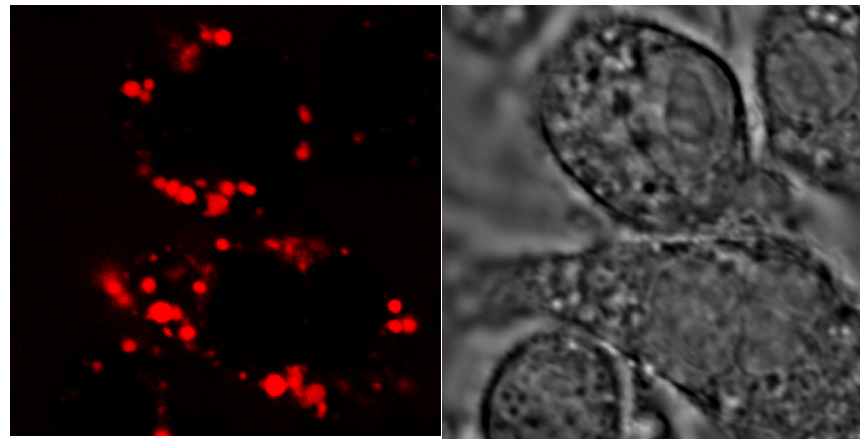
- Silicic acid
- Urate
- Asbestos
- Hemozoin

Hemozoin crystals lead to lysosomal rupture and leakage of lysosomal content into the cytosol

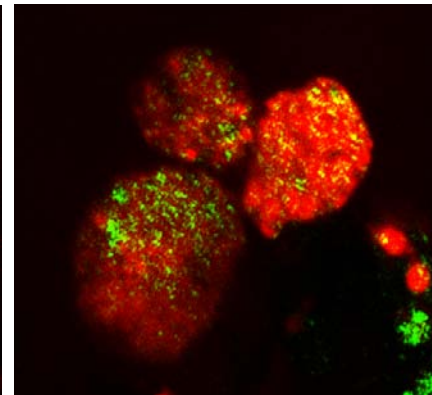
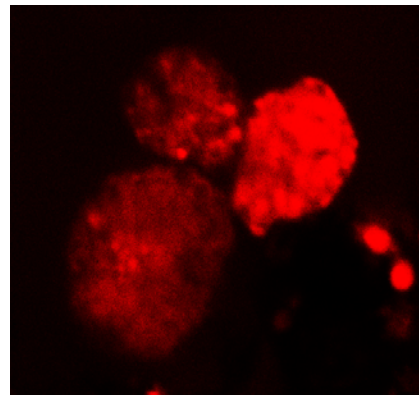
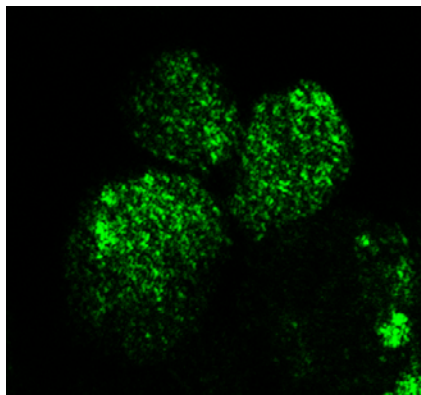
**Hemozoin  
(reflection)**

**Dextran 10kDa**

**Overlay**

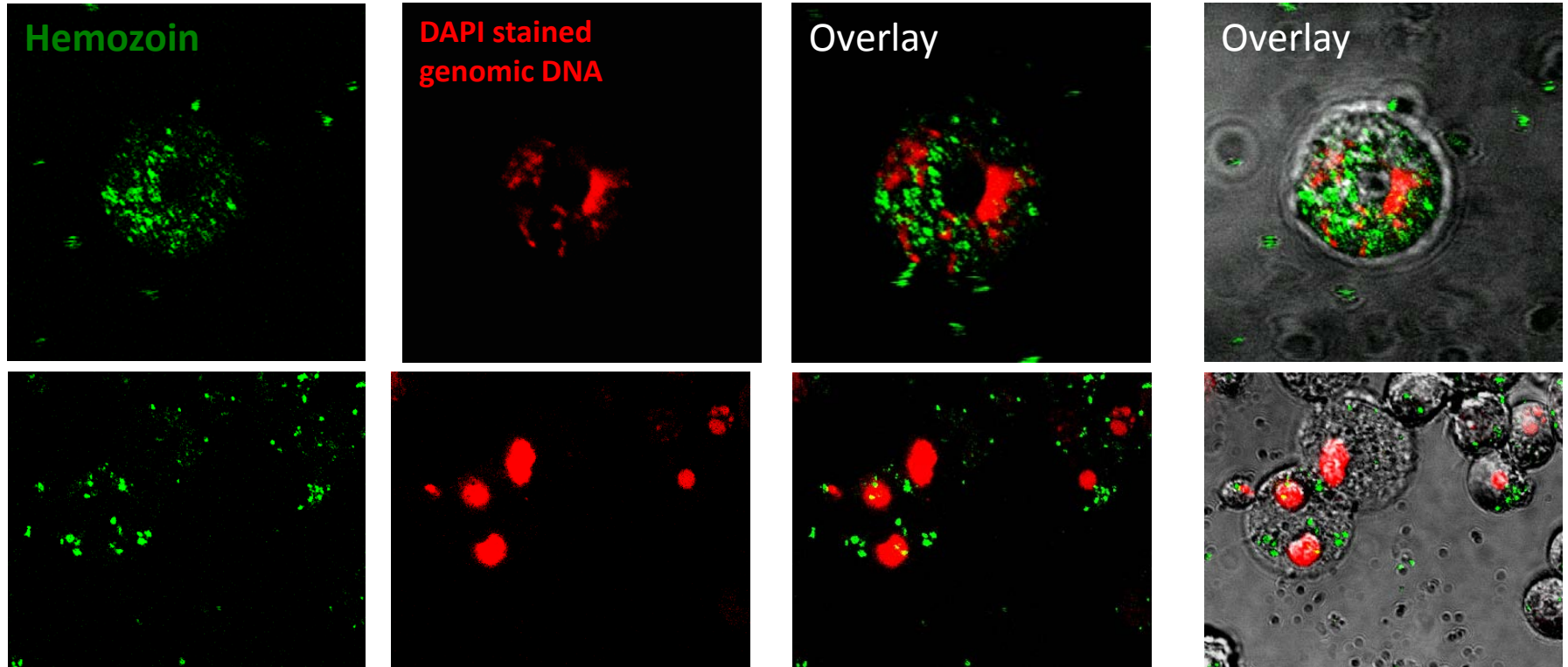


**No Hemozoin**



**Hemozoin**

# Genomic DNA dissociates from the surface of hemozoin and is released into the cytosol





# Recap

- ◆ **DNA recognition appears to be a major cause of inflammation in malaria.**
- ◆ **There appear to be several ways that the human host responds to plasmodial DNA:**
  - ◆ As DNA traffics through the lysosomal compartment, it engages TLR9 via CpG motifs.
  - ◆ When DNA gains access to the cytosol, an AT-rich motif activates an as yet unknown receptor via a TBK-1, IRF3/7 and STING dependent mechanism to produce type I interferons.
  - ◆ (Direct activation of inflammasomes)
- ◆ **Hemozoin promotes innate immune activation.**
  - ◆ By carrying DNA into the phagolysosomal compartment, where it engages TLR9, and then by allowing the DNA to escape to the cytosol.
  - ◆ (By activating the NLRP3 inflammasome activation)

# Acknowledgements

University of Massachusetts Medical School

**Fitzgerald/Golenbock/Gazinelli Malaria Group**

**Kate Fitzgerald**  
**Douglas Golenbock**  
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**Parisa Kalantari**  
**Daniella C. Bartholomeu**  
**Brian Monks**  
**Jennie Chan**  
**Vijay Rathinam**  
**Zhaozhao Jiang**  
**Donghai Wang**

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**Mikayla Thompson**  
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**John Kaminski**  
**Joseph Conlon**  
**Anna Cerny**

## **Other lab members**

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Rahul Gupta  
Kristen Halmen  
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