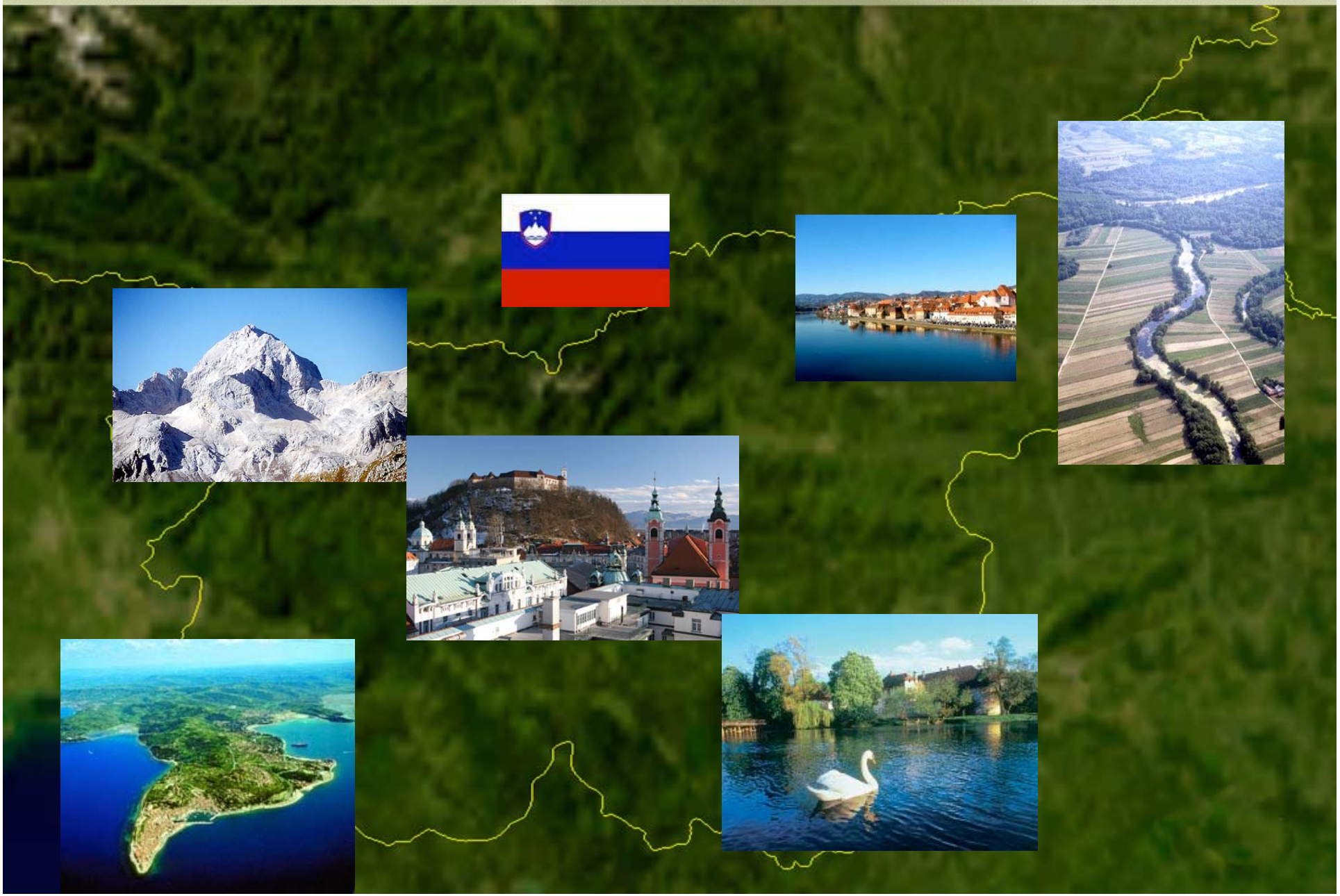
ENGINEERED HUMAN CELLS:

SAY NO STOP TO SEPSIS

iGEM 2006
Slovenija

iGEM 2006

We are from... Slovenia



The Team


Univerza v Ljubljani
National Institute
of Chemistry
Slovenia

Engineering of the logic of mammalian cells

- More similarities than differences in comparison to prokaryotic systems
- Disadvantages: more complex, slower and more expensive to work with
- Opportunities: understanding of complex systems, relevant for potential medical application

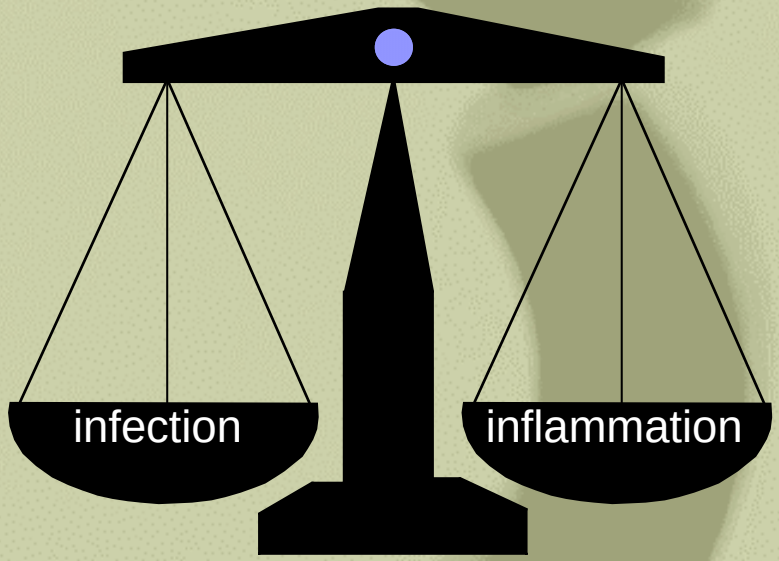
What is Sepsis?

- Strikes 750,000 people per year in the US, similar numbers for the EU
- In 1 of 5 cases it ends with death of the patient
- Among the top 10 causes of death in the US

What is Sepsis

- Excessive inflammatory response triggered by pathogens

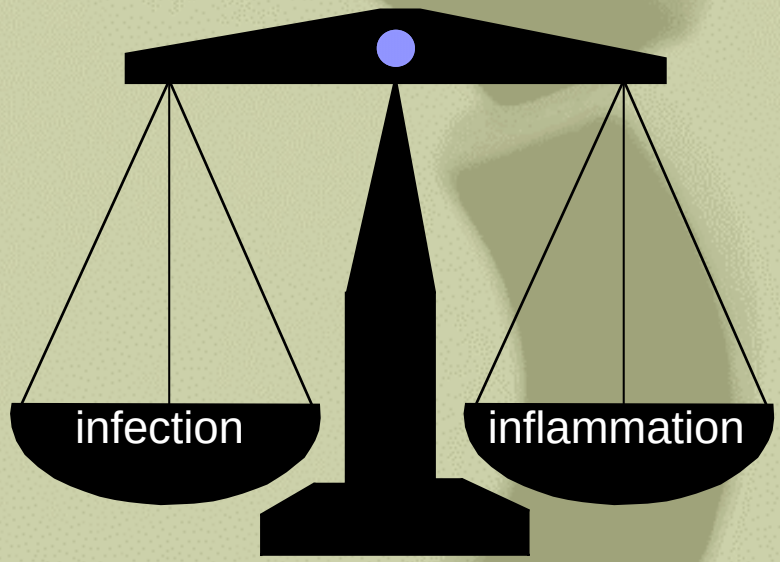
- Widespread activation of inflammation and coagulation pathways



What is Sepsis

- Results in severe organ failure

- Excessive reaction of host to the pathogen infection rather than bacteria causing pathology



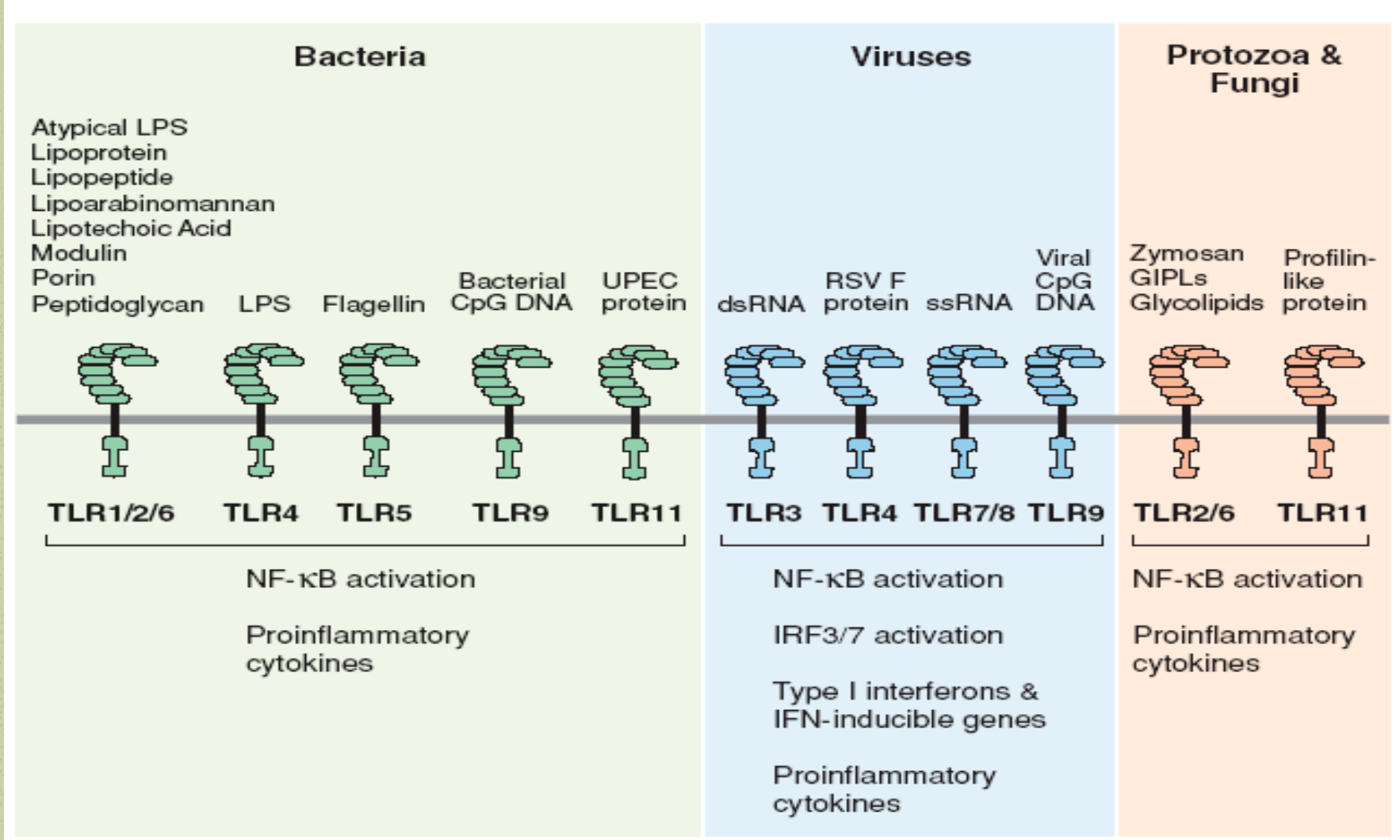
Toll-like receptors sense the presence of pathogens

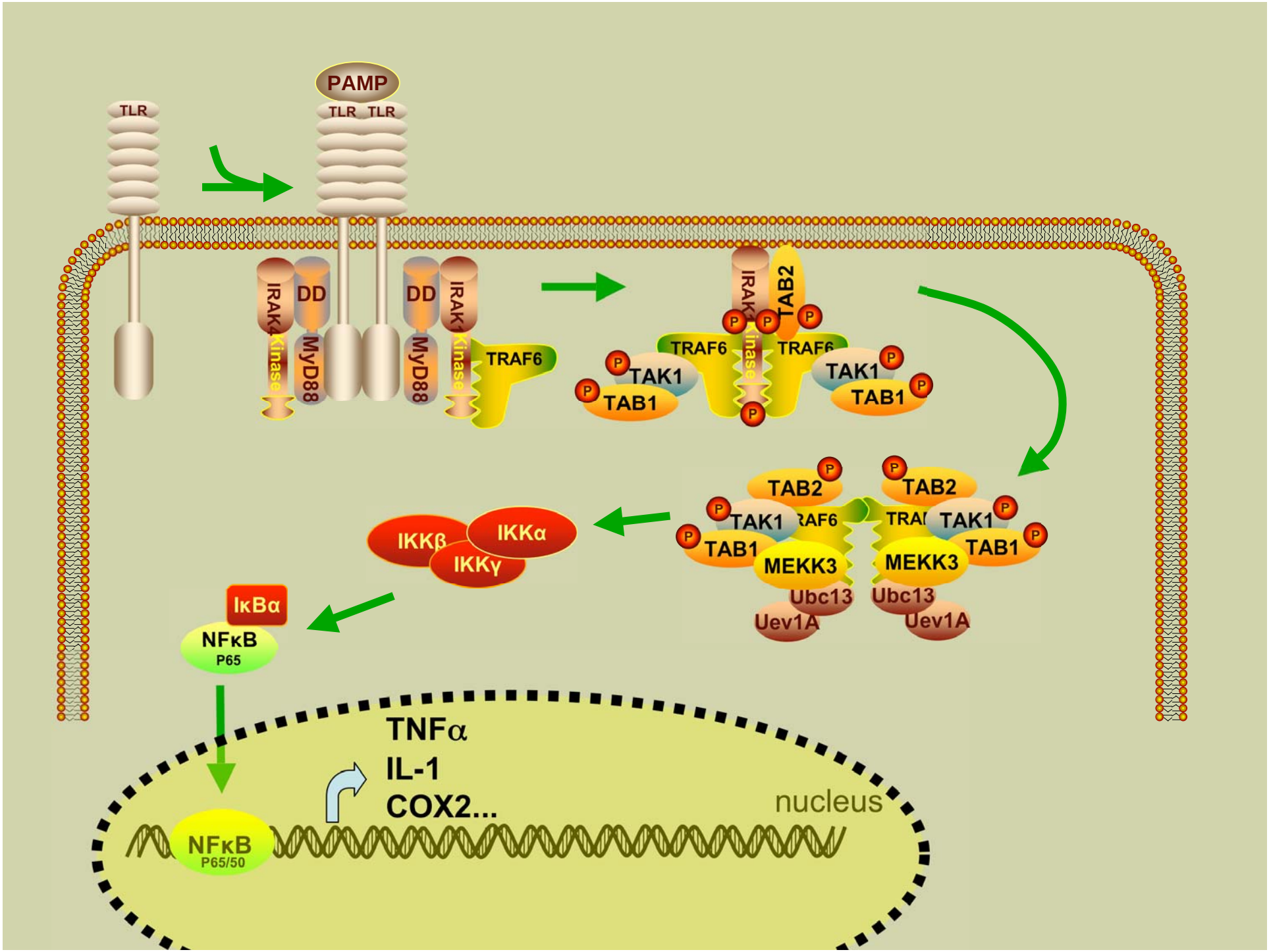
- main sensors of the innate immune response

Toll-like receptor molecules:

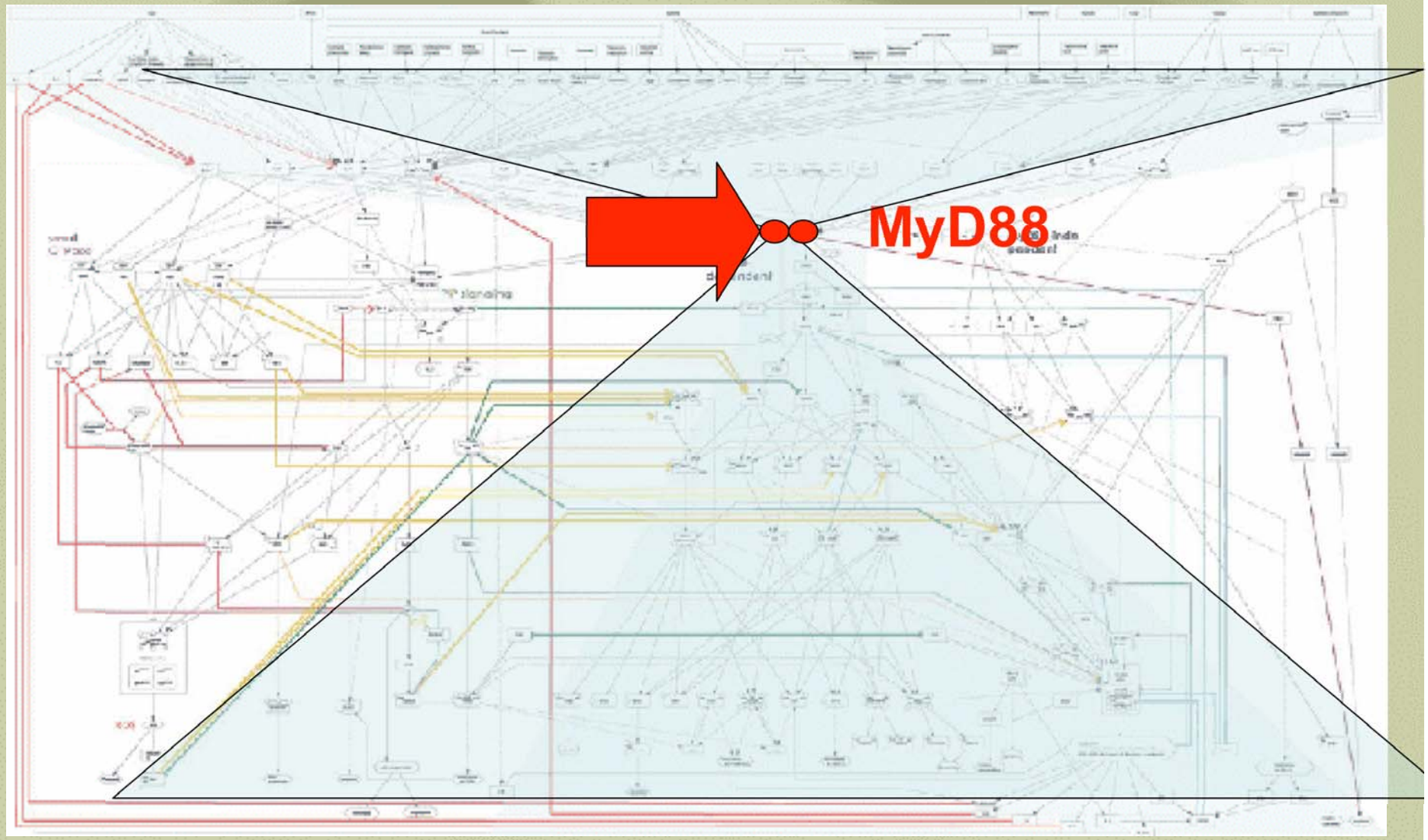
- 11 different human membrane receptores
- recognize different molecules distinctive for pathogens

TLRs and their agonists




PAMP
TLR
TLR TLR
IRAK4
Kinase
DD
MyD88
DD
MyD88
IRAK1
Kinase
TRAF6
IRAK1
Kinase
TAB2
TRAF6
TRAF6
TAK1
TAB1
TAK1
TAB1
TAB2
TAB2
TAK1
TAB1
TAK1
TAB1
MEKK3
MEKK3
Ubc13
Uev1A
Ubc13
Uev1A
IKKβ
IKKα
IKKγ
IκBα
NFκB
P65
TNFα
IL-1
COX2...
nucleus
NFκB
P65/50

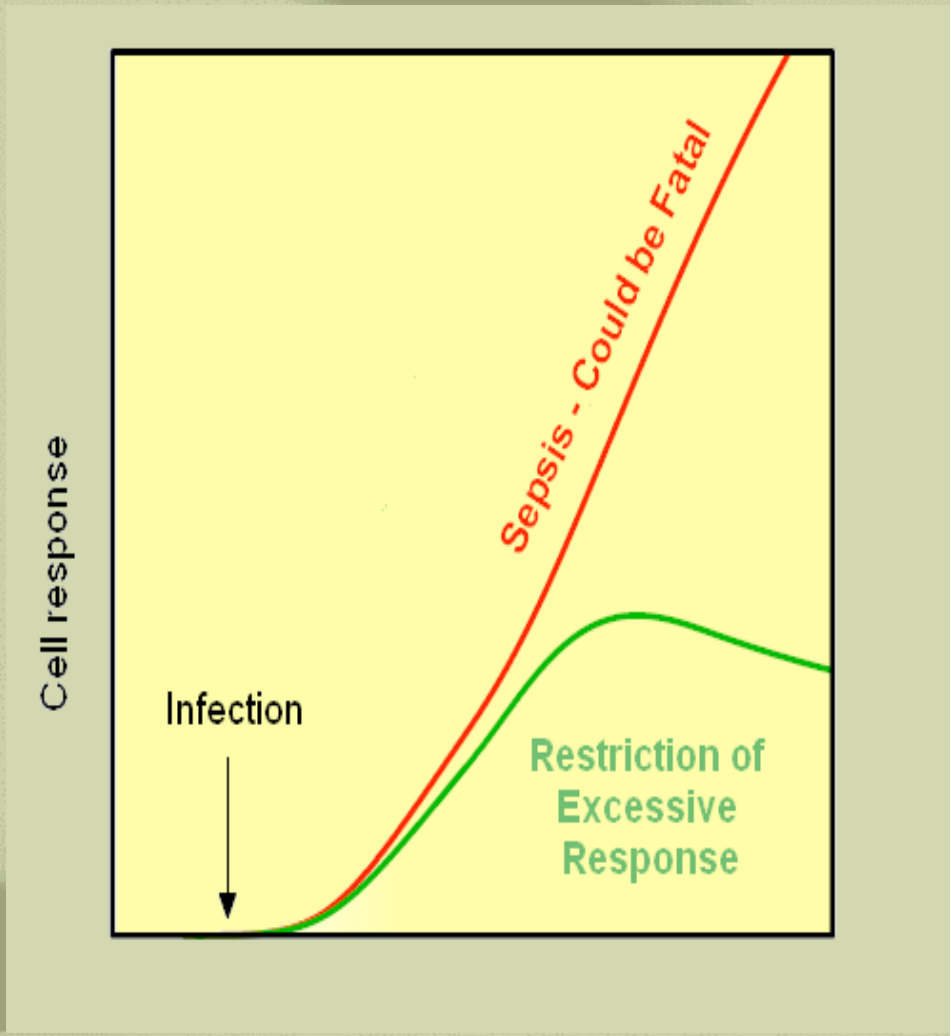
“All Paths lead through MyD88”



Our Project

Basic concept:

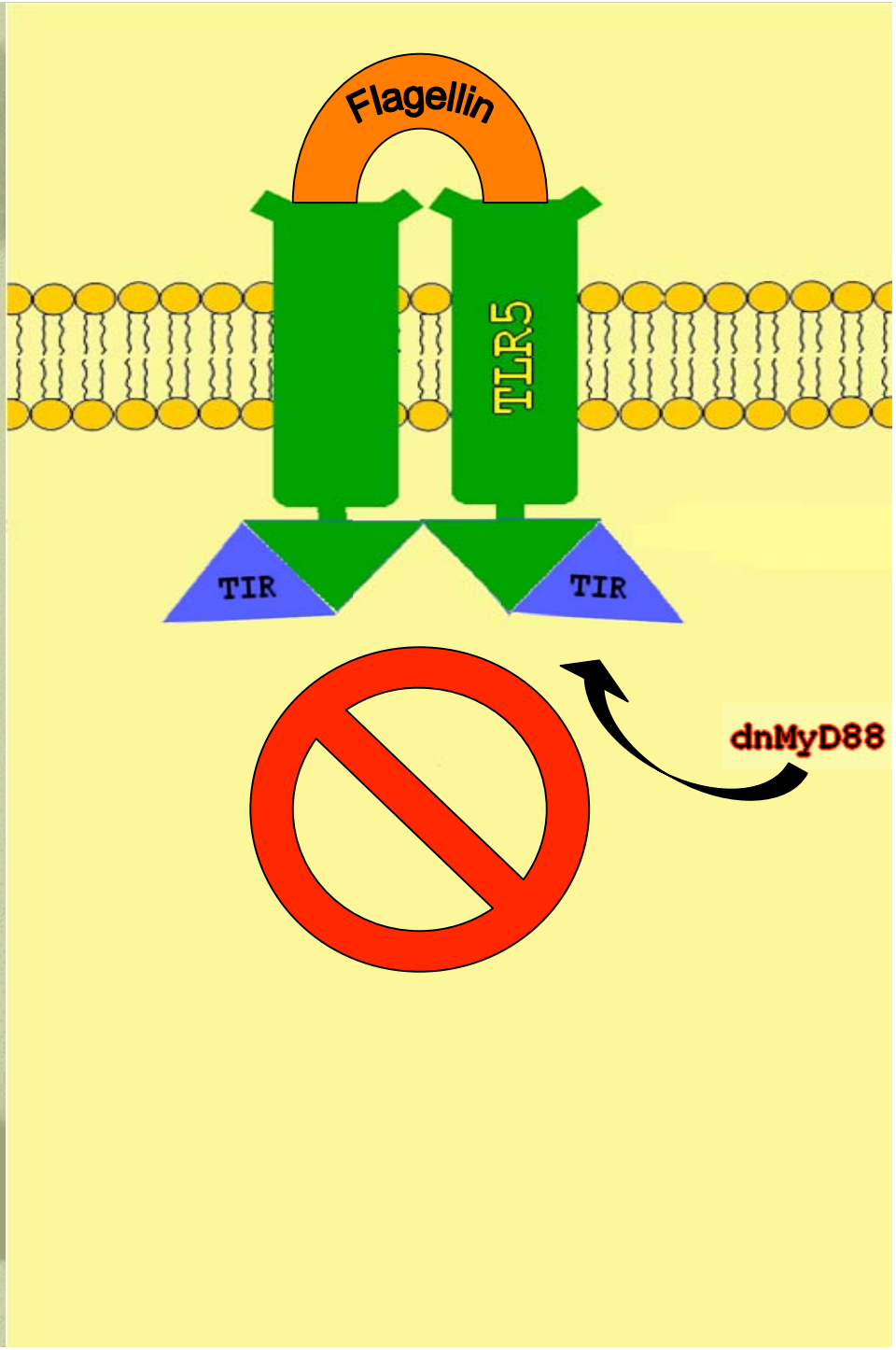
Inhibit the excessive cellular activation without of completely abolishing the cellular responsiveness



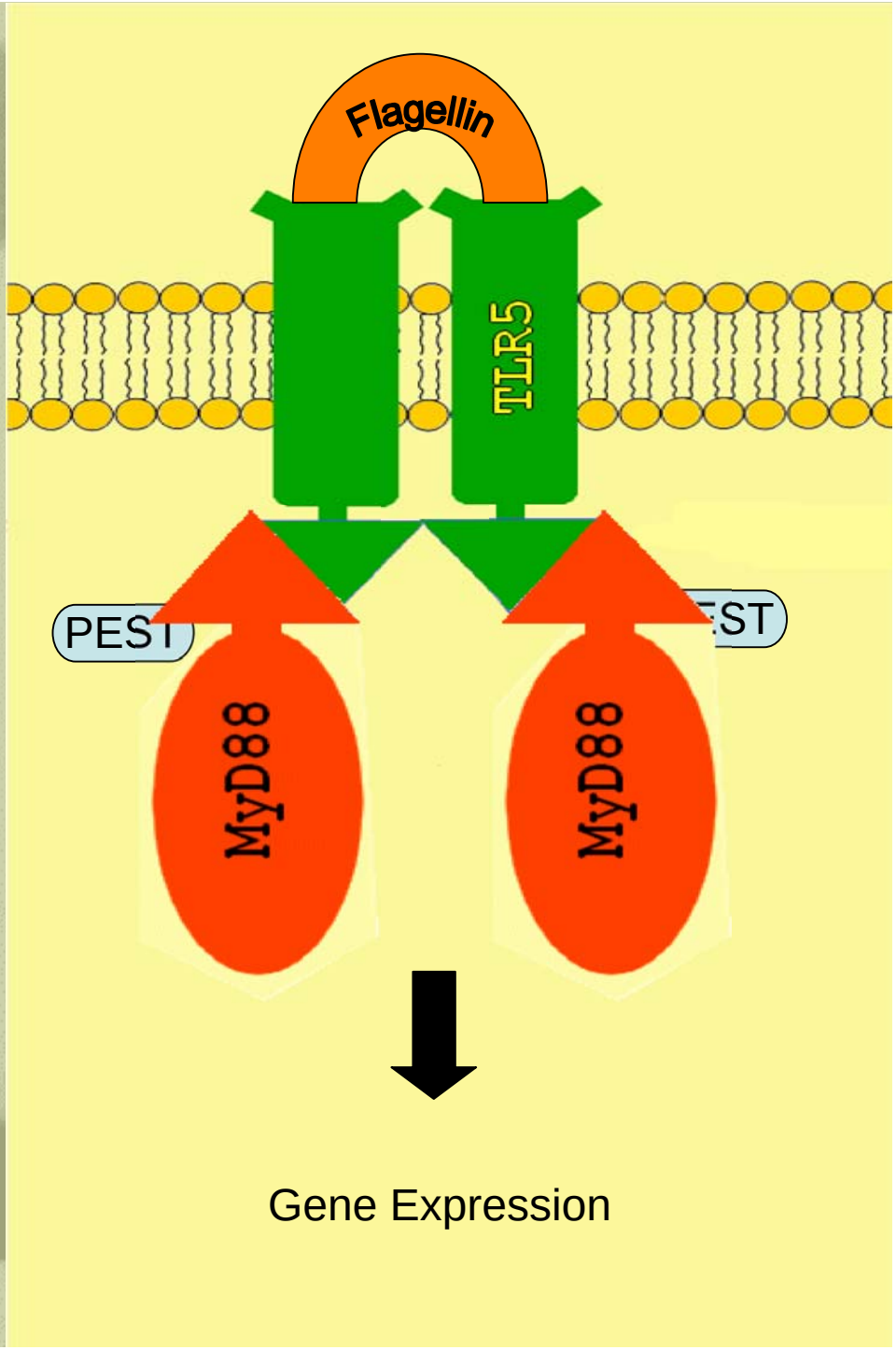
Implementation:

Insert into mammalian cells a feedback device with inhibitor

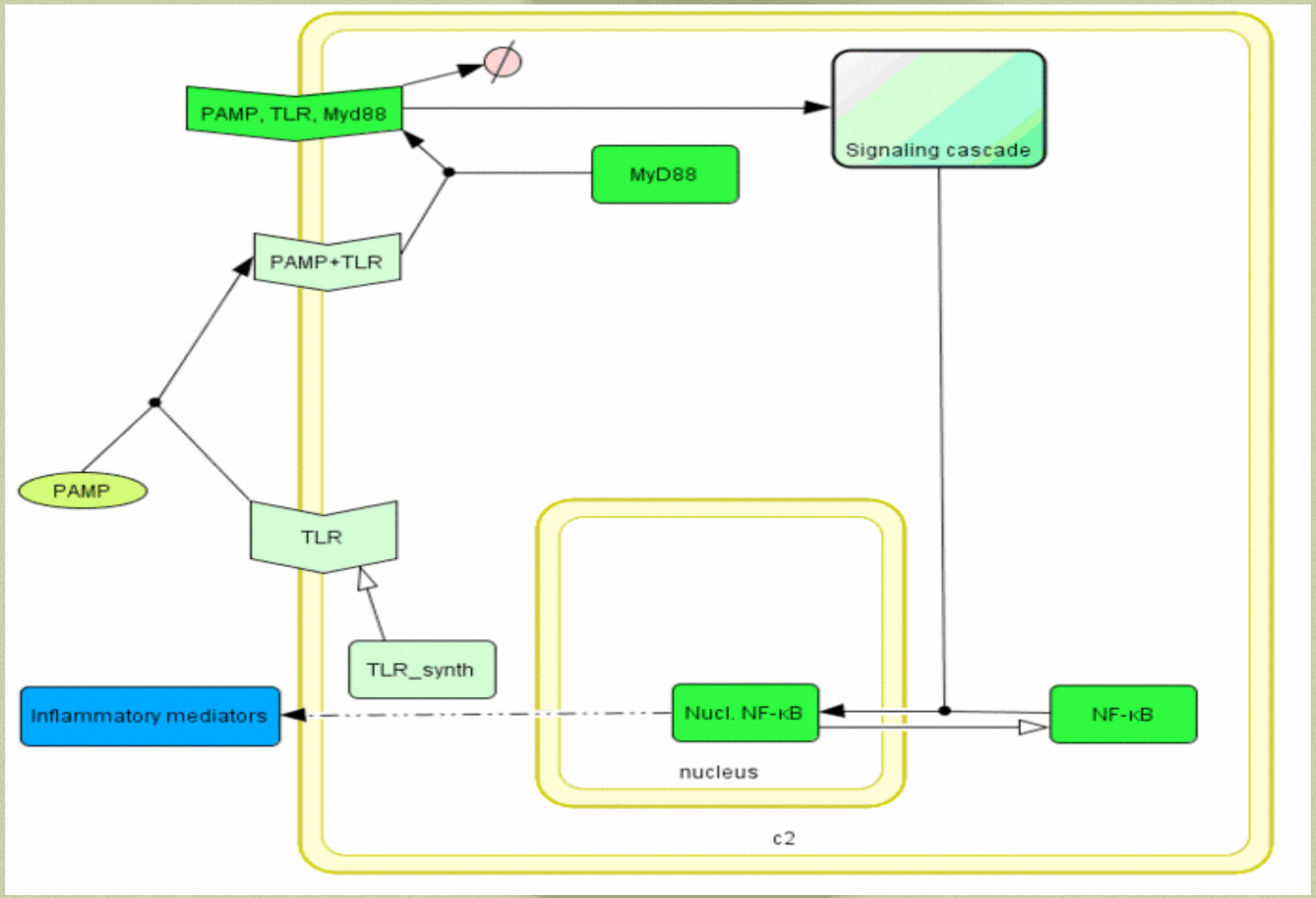
(dnMyD88) that would repress the signaling of TLR pathway for a limited period of time.



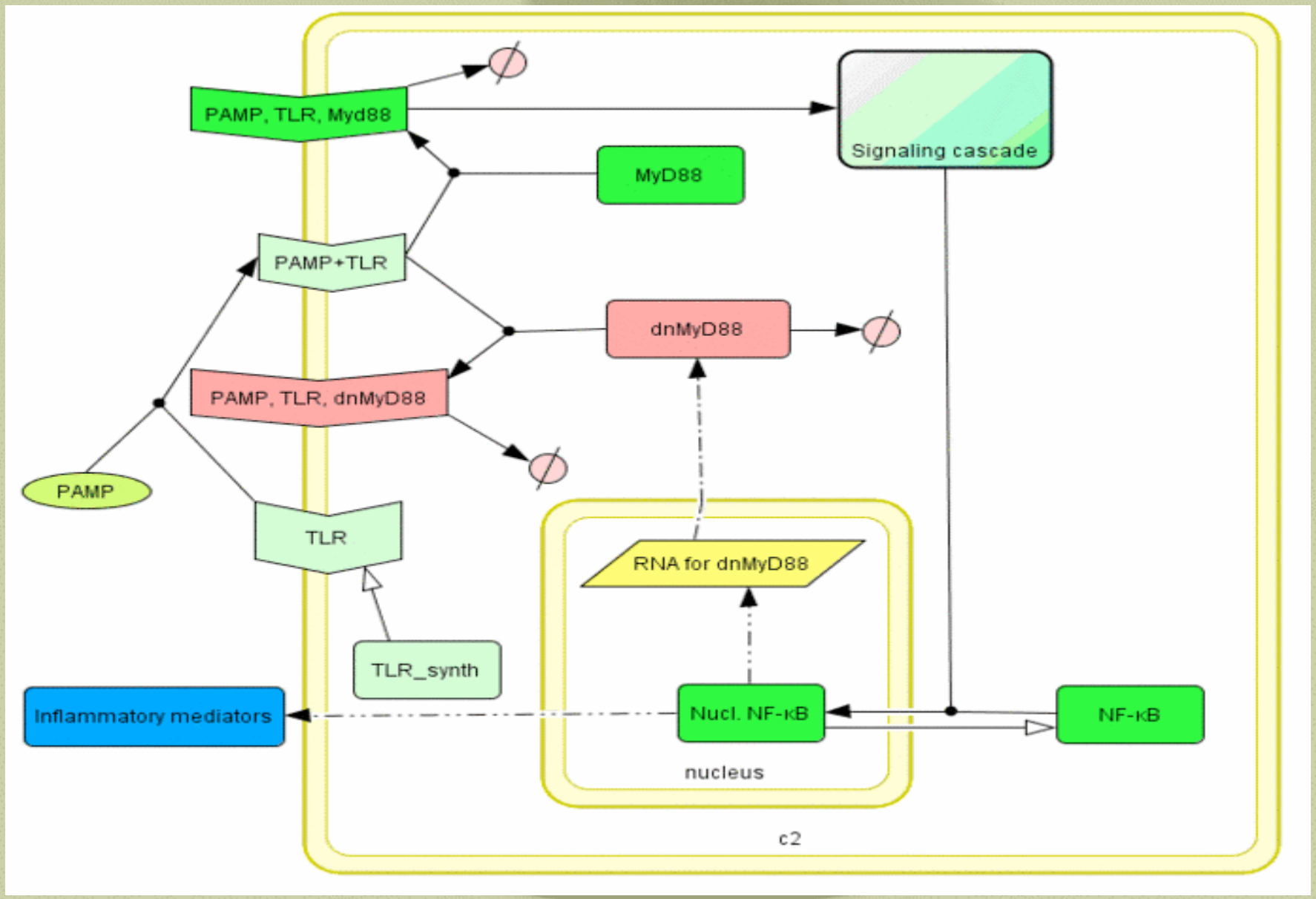
PEST sequence



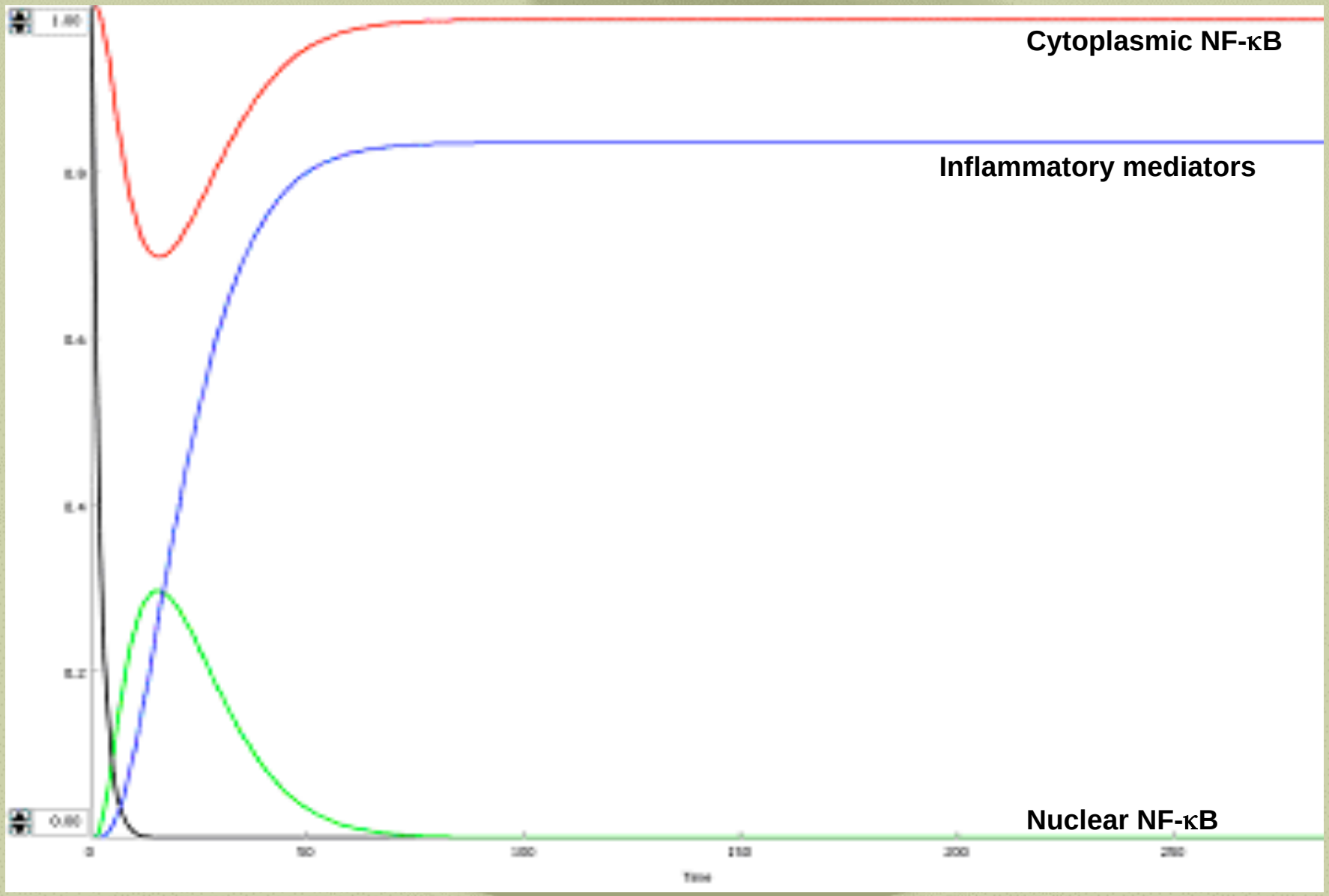
Mathematical Model



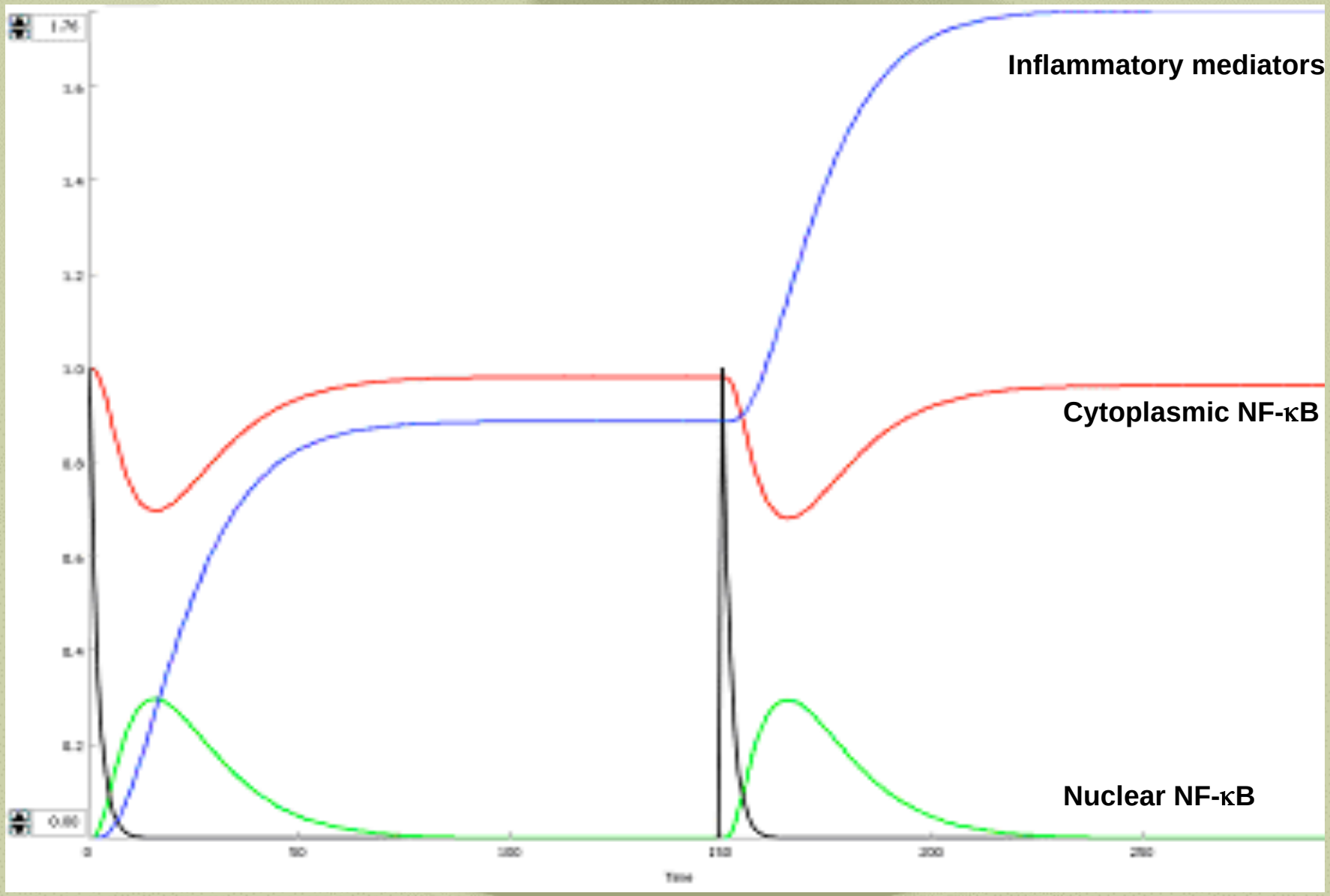
Simplified model of TLR signaling



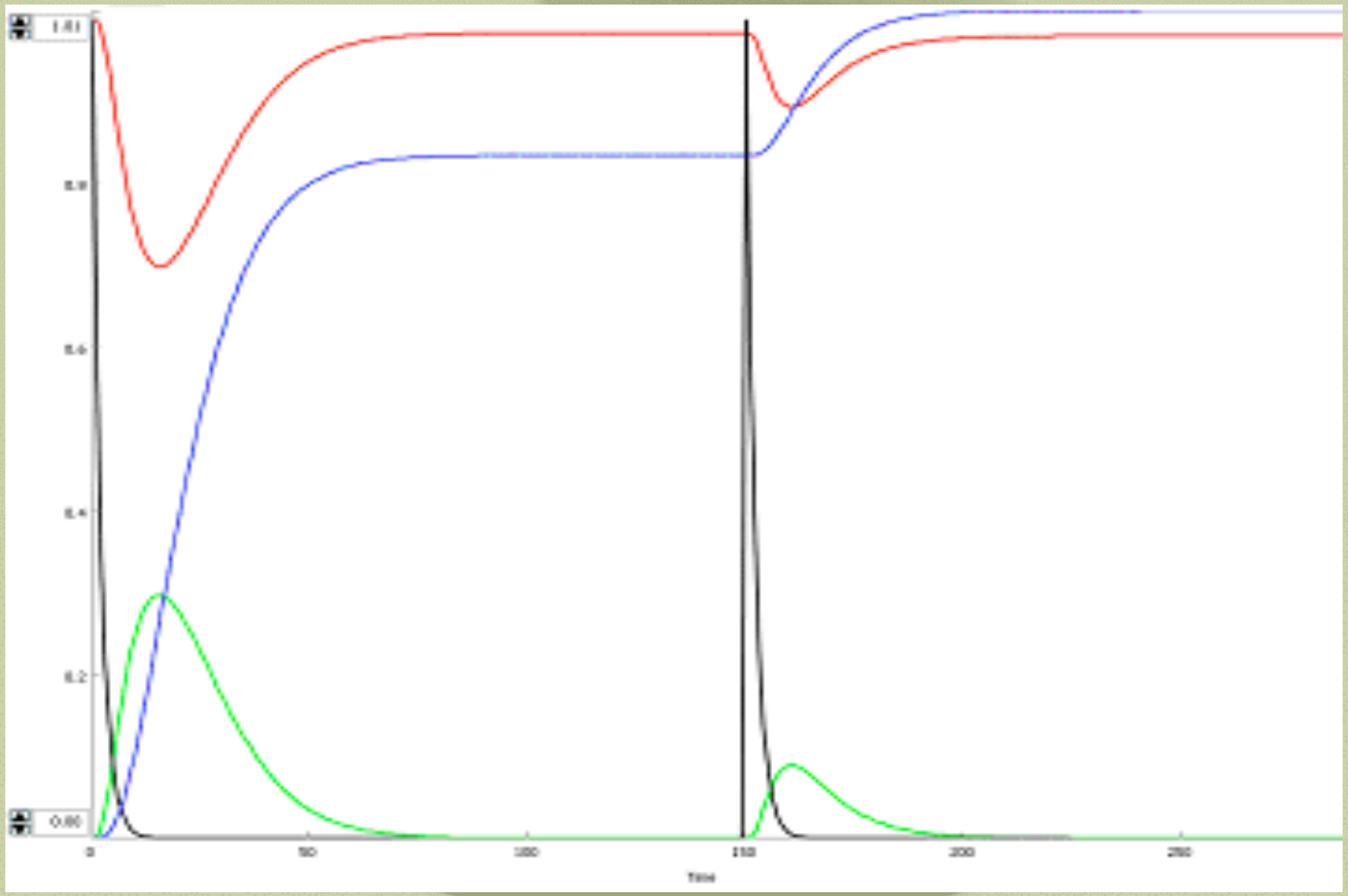
Model with additional dnMyD88 feedback


Cytoplasmic NF-κB
Inflammatory mediators
Nuclear NF-κB
Time

Normal cellular response to repeated stimulus



Response to repeated stimulus in cells with inserted feedback device



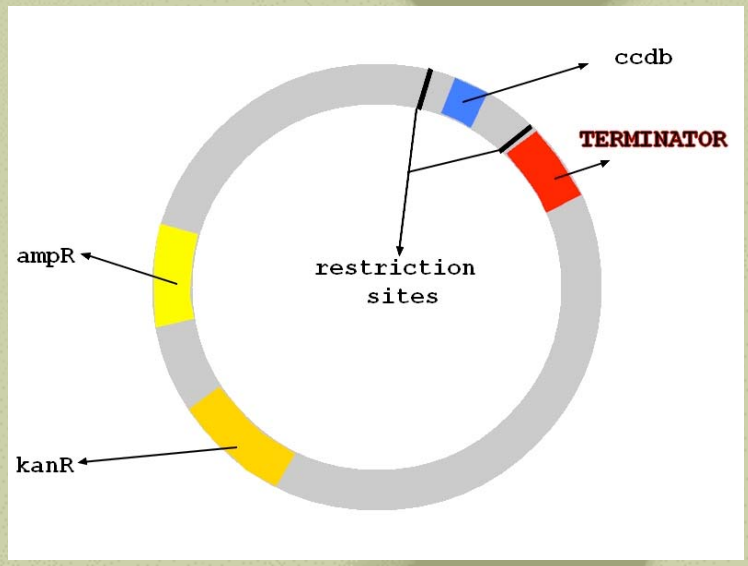
Our Parts

Registration number	Part's Name
BBa_J52008	rluc
BBa_J52010	NFκB
BBa_J52011	dnMyD88-linker-rLuc
BBa_J52012	rluc-linker-PEST191
BBa_J52013	dnMyD88-linker-rluc-link-pest191
BBa_J52014	NFκB+dnMyD88-linker-rLuc
BBa_J52016	eukaryotic terminator
BBa_J52017	eukaryotic terminator vector
BBa_J52018	NFκB+rLuc
BBa_J52019	dnTRAF6
BBa_J52021	dnTRAF6-linker-GFP
BBa_J52022	NFκB+dnTRAF6-linker-GFP
BBa_J52023	NFκB+rLuc-linker-PEST191

BBa_J52024	NFκB+dnMyD88-linker-rLuc-link-PEST191
BBa_J52026	dnMyD88-linker-GFP
BBa_J52027	NFκB+dnMyD88-linker-GFP
BBa_J52028	GFP-PEST191
BBa_J52029	NFκB+GFP-PEST191
BBa_J52034	CMV
BBa_J52035	dnMyD88
BBa_J52036	NFκB+dnMyD88
BBa_J52038	CMV-rLuc
BBa_J52039	CMV+rLuc-linker-PEST191
BBa_J52040	CMV+GFP-PEST191
BBa_J52642	GFP
BBa_J52648	CMV+GFP

Building of BioBricks:

- Preparation of special fusion protein constructs with use of PCR Overlap Extension method
- Cloning in BioBrick plasmids with ccdB domain
- Construction of a modified vector with incorporated terminator
- Construction of final Composites using of BioBrick assembly technique



Transfection:

Cell line: HEK293

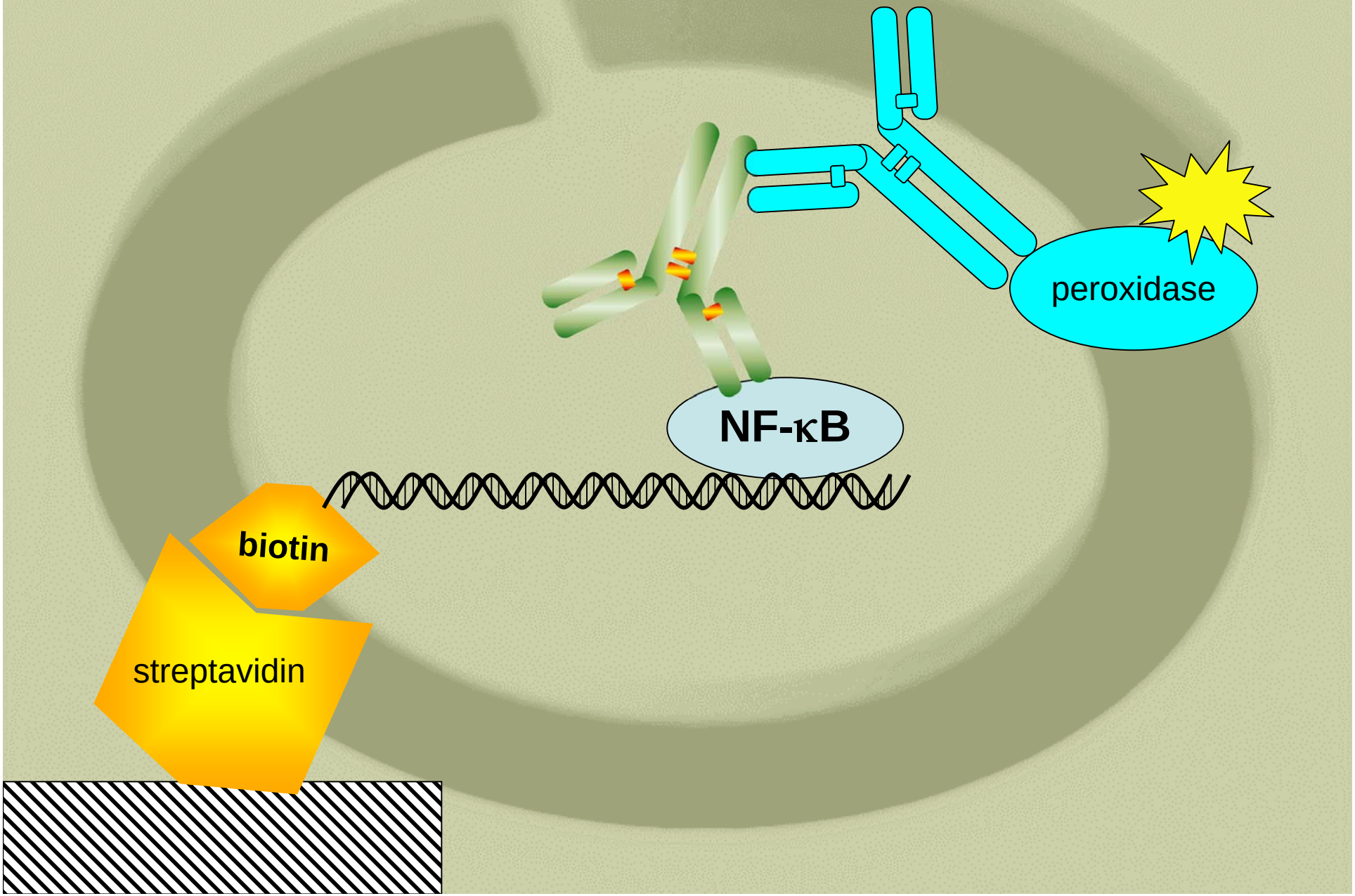
- **don’t express TLRs**
- **have conserved signaling pathway**

Methods

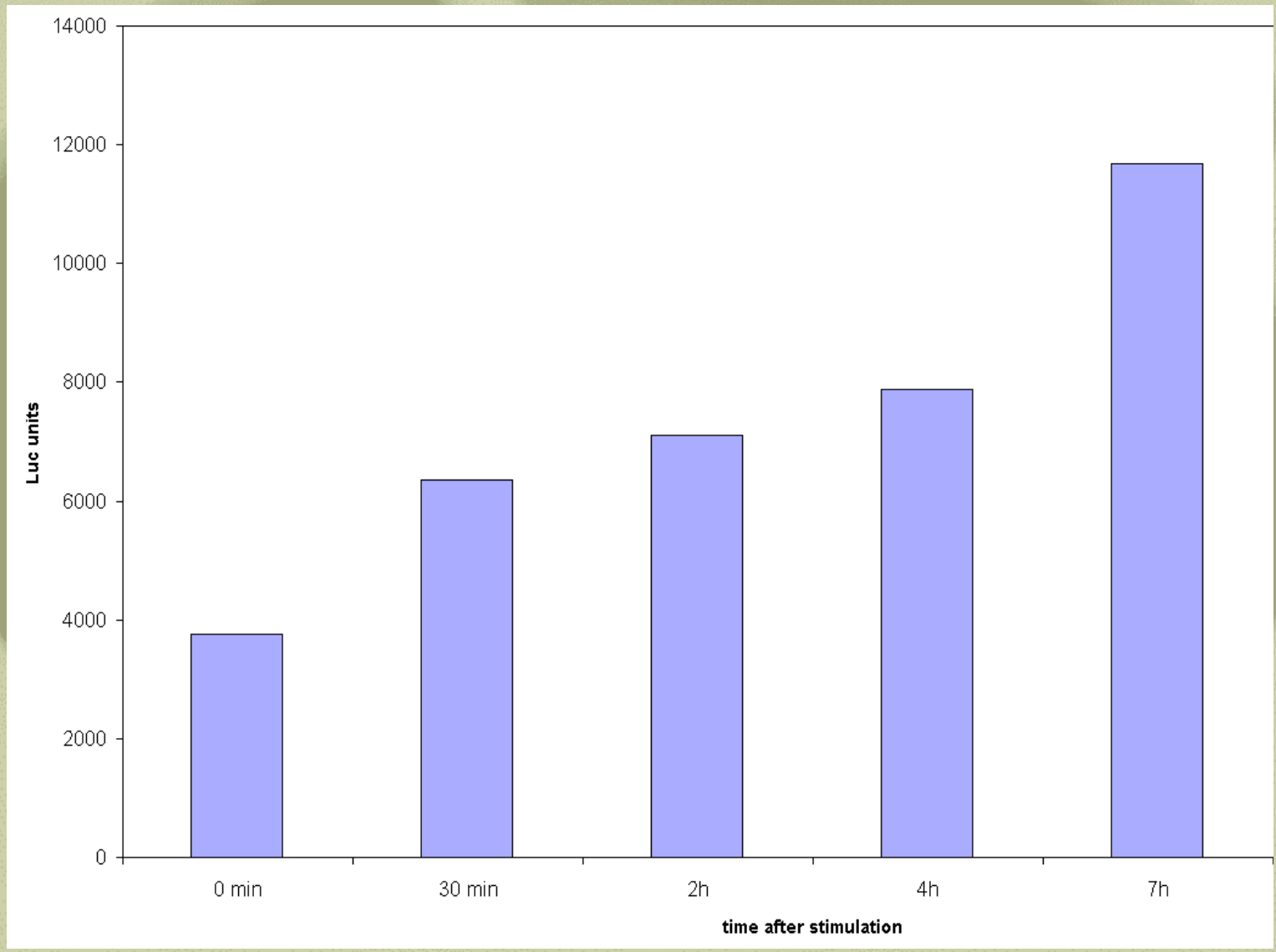
Detection systems to monitor the time course of the cellular response:

- **ELISA**
- **Flow Cytometry**
- **Luciferase Assay**
- **Microscopy**

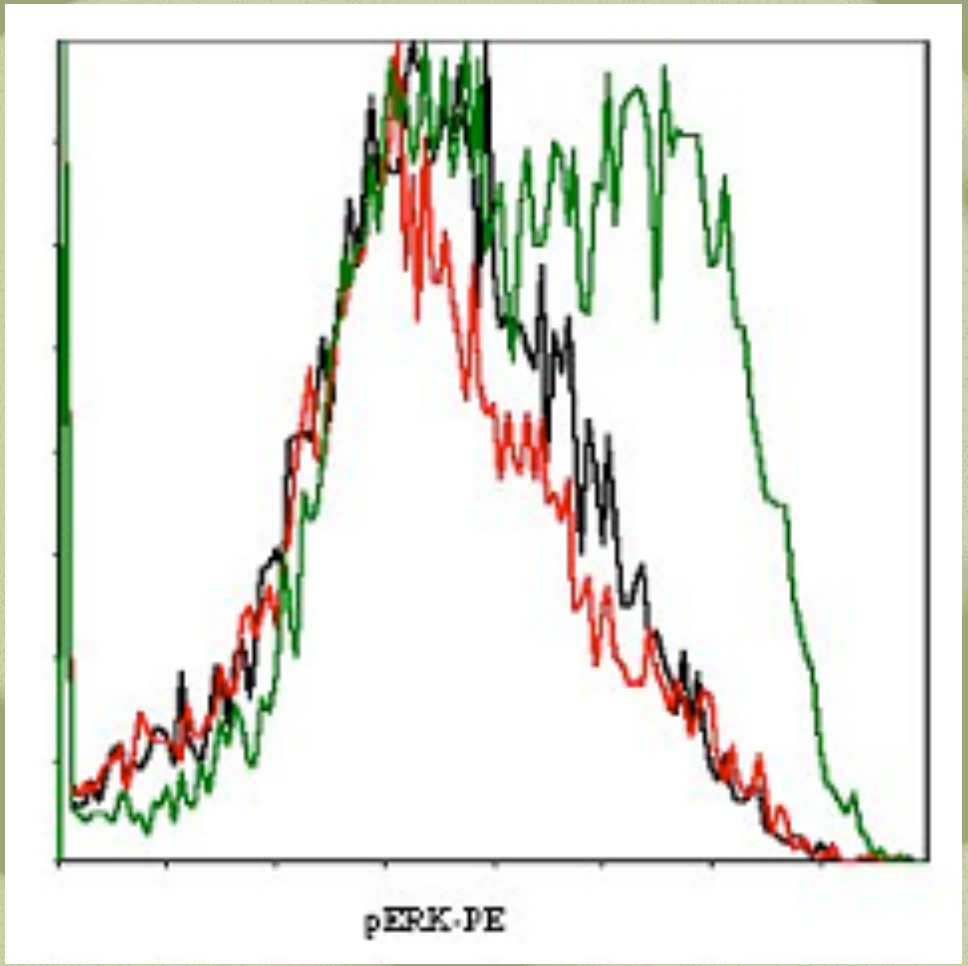
ELISA for the detection of free NF-κB



ELISA - Results



Flow Cytometry for the detection of phosphorylated ERK

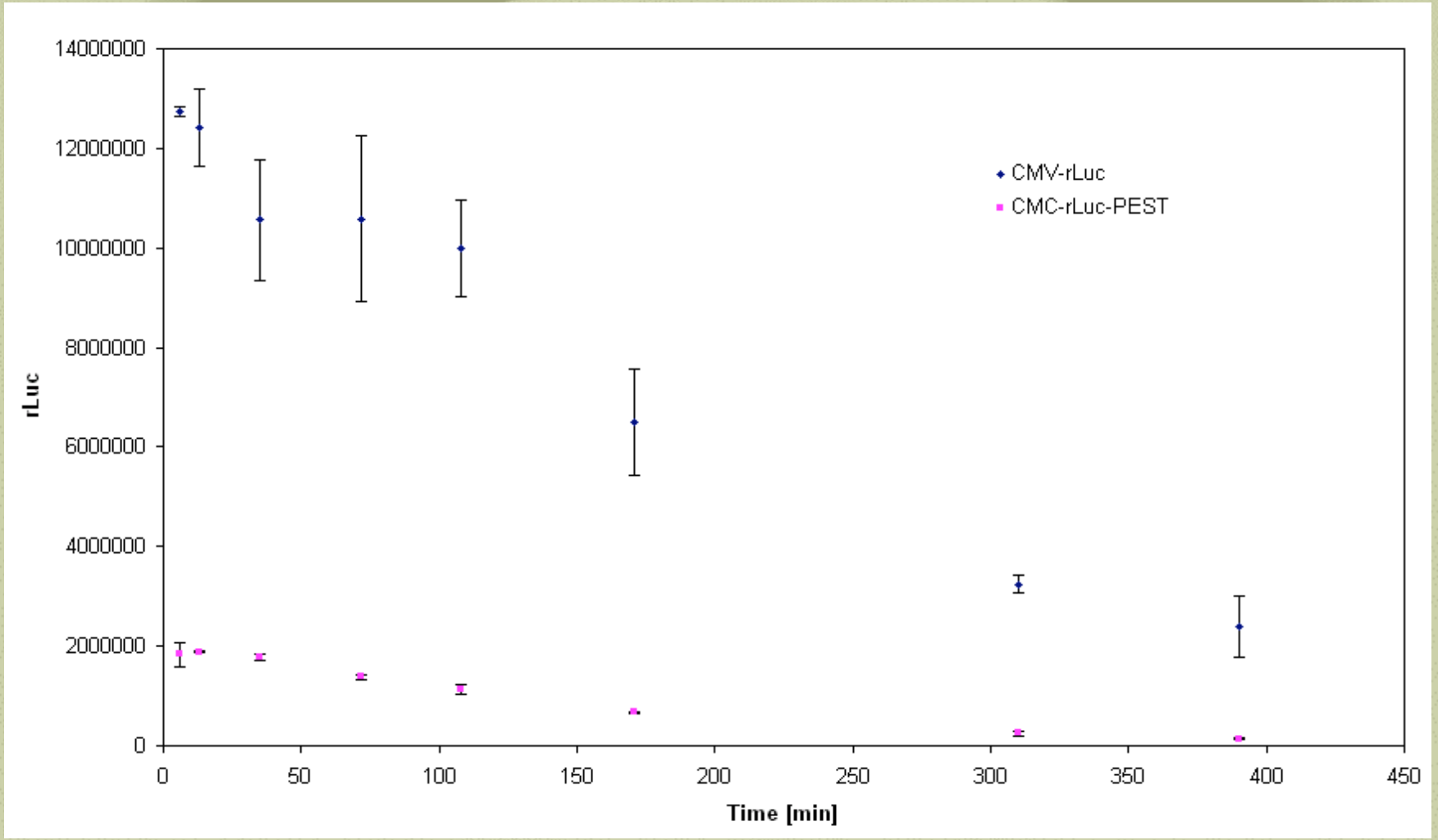


Detection of transcriptional activation by luciferase assay

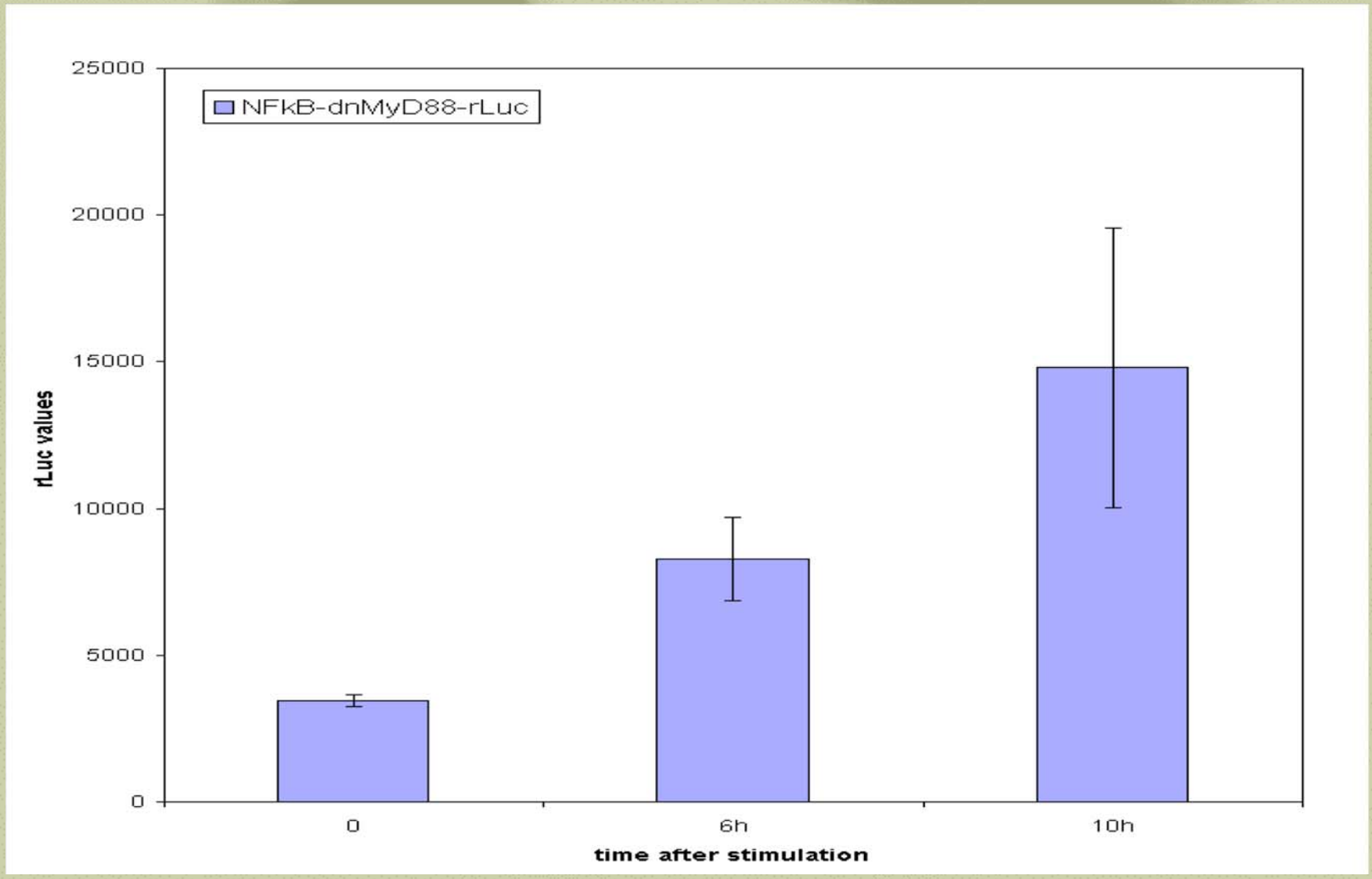
Dual luciferase assay

- **Two different luciferase reporter enzymes**
  - **The experimental reporter (NF-κB-fLuc)**
  - **The constitutive reporter gene (CMV-rLuc)**

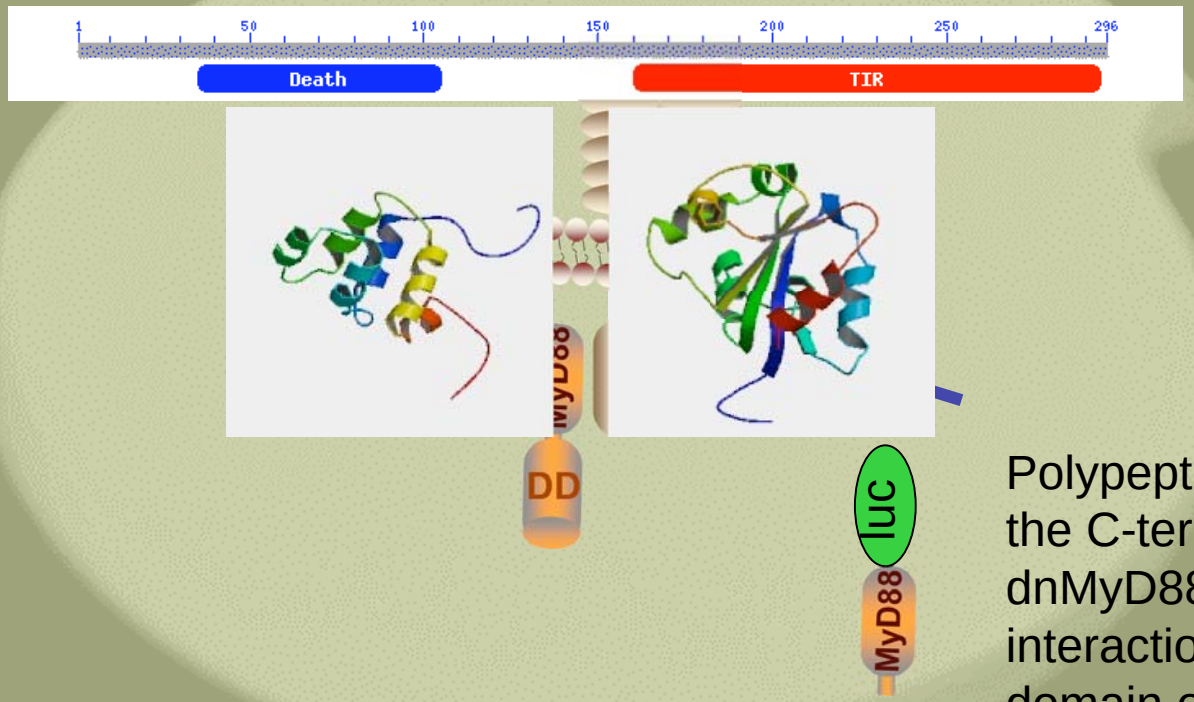
Decreased steady state level and increased degradation rate of proteins with attached PEST tag



Inducible expression of protein under the control of NF-κB promoter



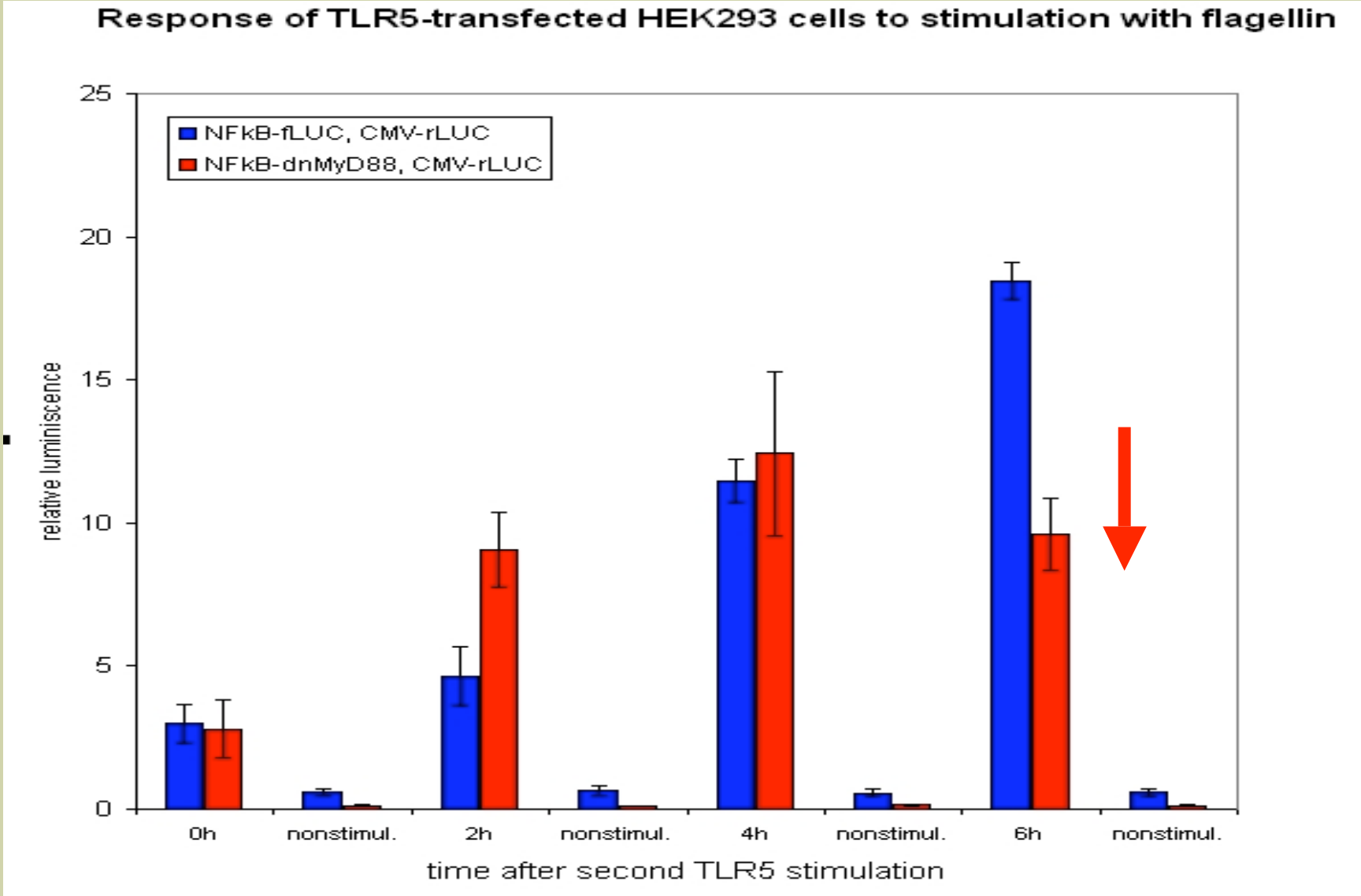
Why is there no inhibition ?



Polypeptide domain at the C-terminus of dnMyD88 prevents its interaction with TIR domain of TLR

If true, it should work without of the C-terminal addition, does it ?

Yes, the feedback device works!



Conclusions

- **We have transferred the BioBrick principle into the mammalian cells using transient transfection.**
- **We have succesfully implemented the feedback device that restricts the cellular activation in inflammation.**
- **Our constructed device mimicks the natural mechanism of tolerance only that it is activated faster.**
- **Simplified model of the TLR signaling qualitatively captures the main features of the signaling kinetics.**

Prospects for the future

- **Modulation of the lifetime of the inhibitor (and signal repression) based on the different rate of degradation by the addition of N-terminal PEST tag.**

- **Construction of BioBrick vectors for stable transfection (additional resistance for cell culture lines).**


Hvala lepa