How mammals sense infection: from endotoxin to the Toll-like receptors

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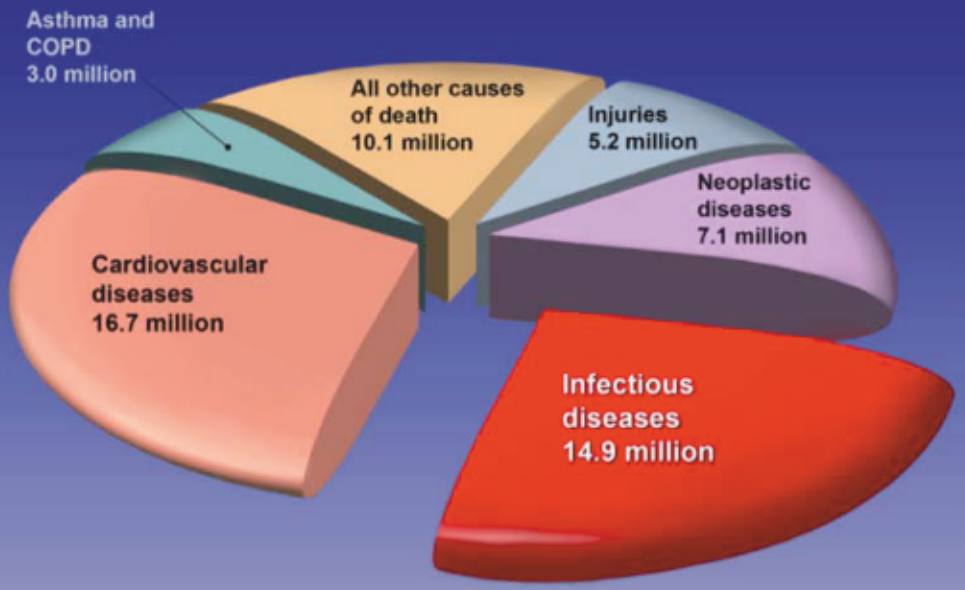
Ernest Beutler, M.D., 1928-2008



Infectious Diseases	Annual deaths (millions)
Respiratory infections	3.96
HIV/AIDS	2.77
Diarrhoeal diseases	1.80
Tuberculosis	1.56
Vaccine-preventable childhood diseases	1.12
Malaria	1.27
STD's (other than HIV)	0.18
Meningitis	0.17
Hepatitis B and C	0.16
Tropical parasitic diseases	0.13
Dengue	0.02
Other infectious disease	1.76

Based on: The challenge of emerging and re-emerging infectious diseases, D.M. Morens, G.K. Folkers, and A. S. Fauci,*Nature* **463**, 122(7 January 2010)

Infections and their transmissible character were known in antiquity…

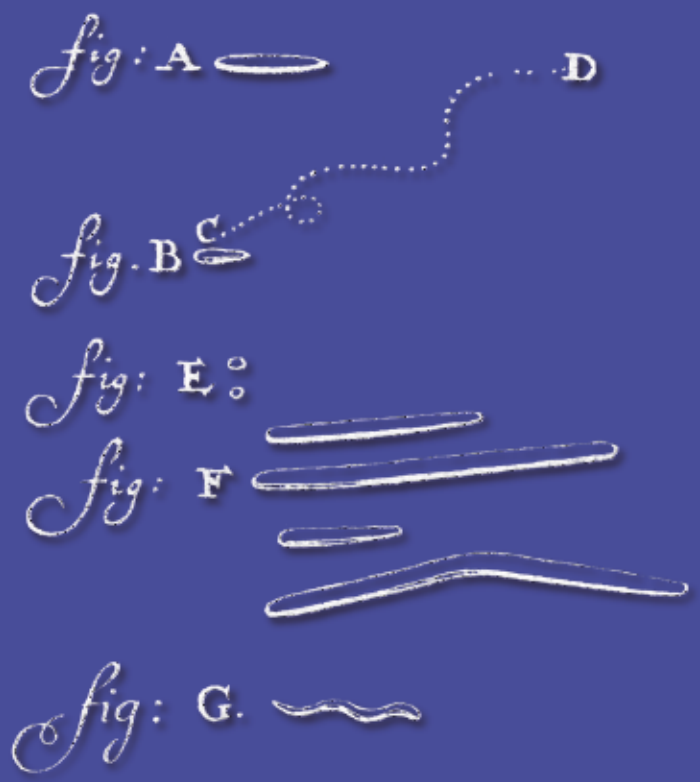
460 - 370, BCE

129 – 199, AD

1135-1204, AD

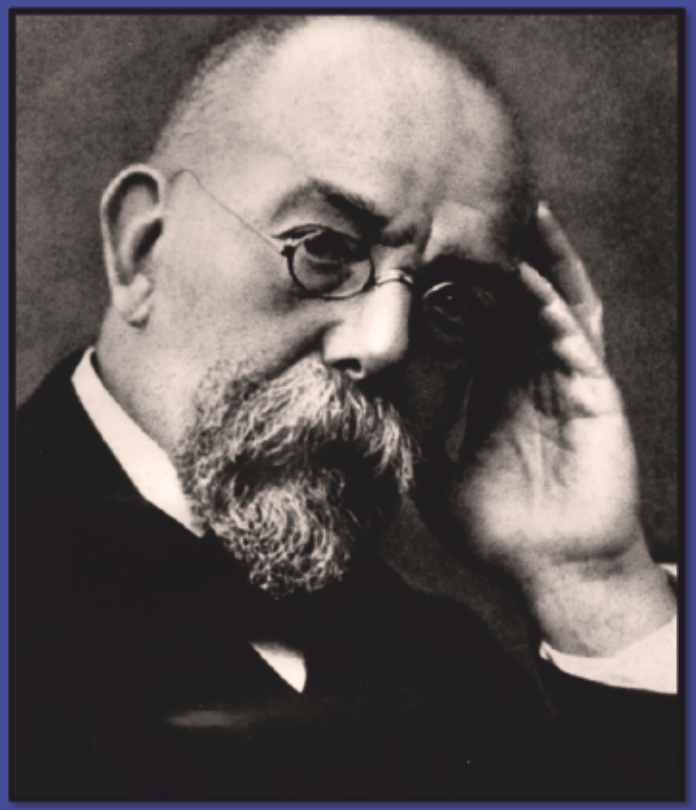
…while microbes were only discovered in the 17th century…

Antonie van Leeuwenhoek
1632 - 1723



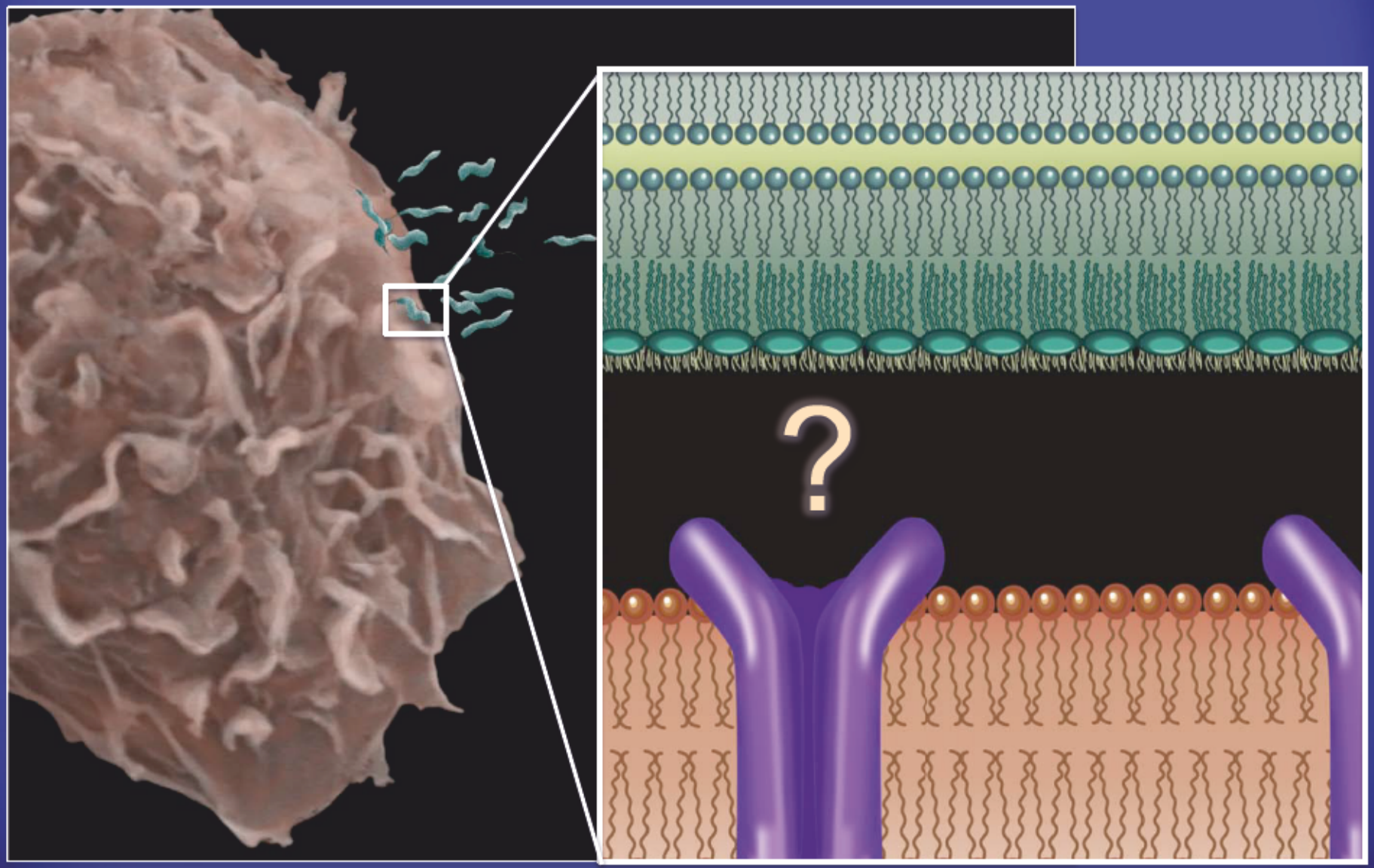
…and the association between microbes and infection was only discovered in the 19th century.

Louis Pasteur
1822-1895

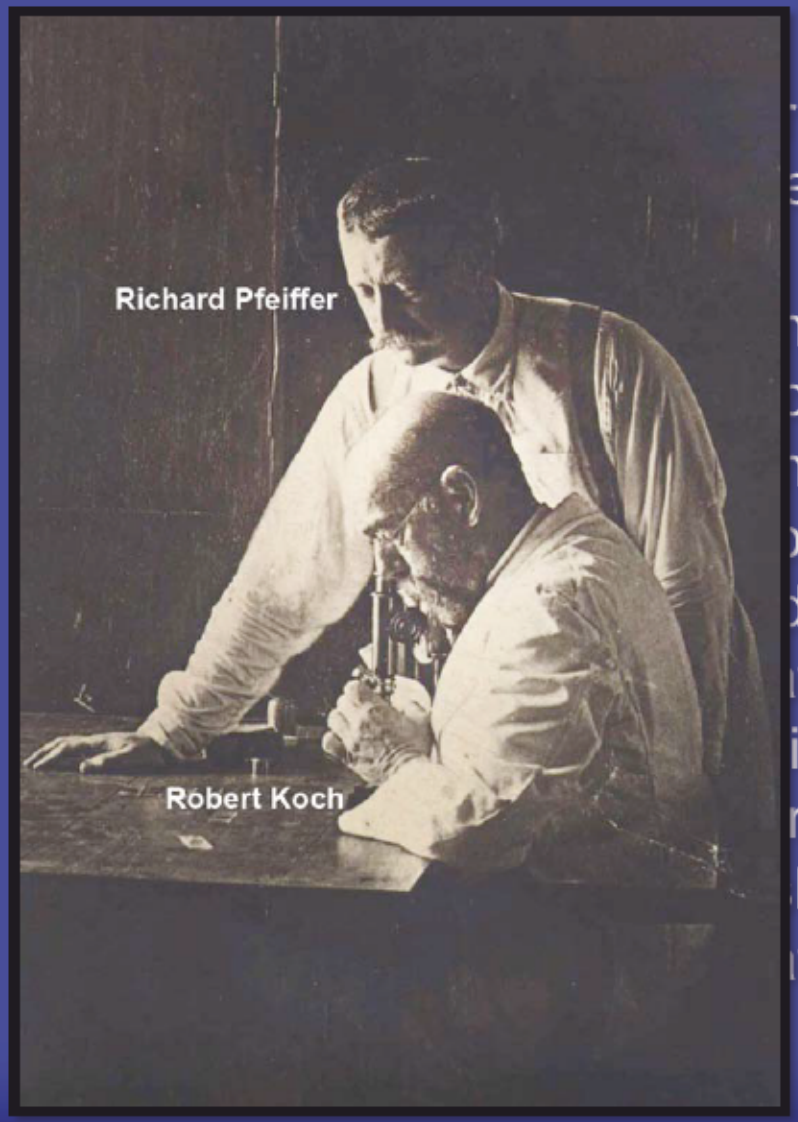


Robert Koch
1843-1910

What might be the nature of contact between microbe and host?



Soon after microbes were discovered, it was appreciated that mammals recognize them as foreign and mount an intense inflammatory response



d Pfeiffer, a
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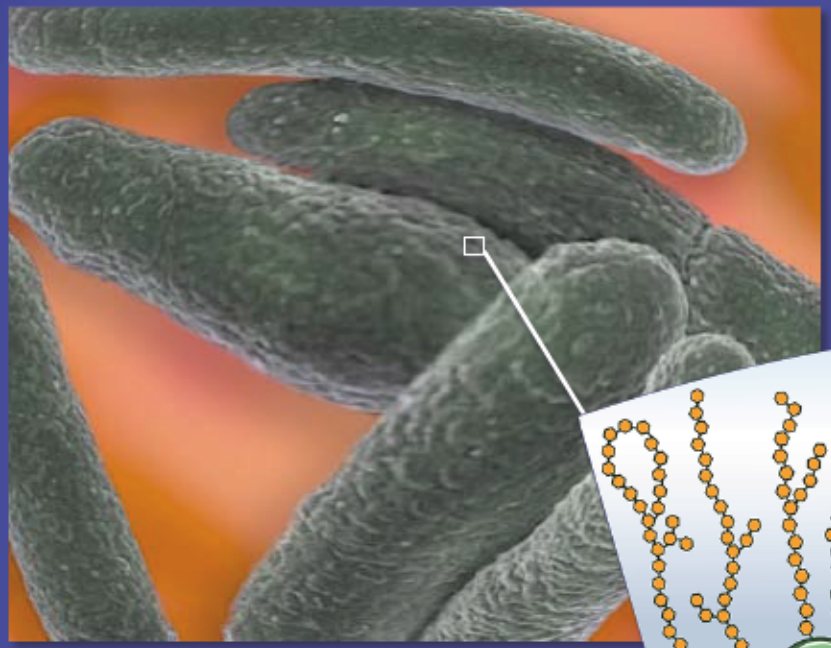
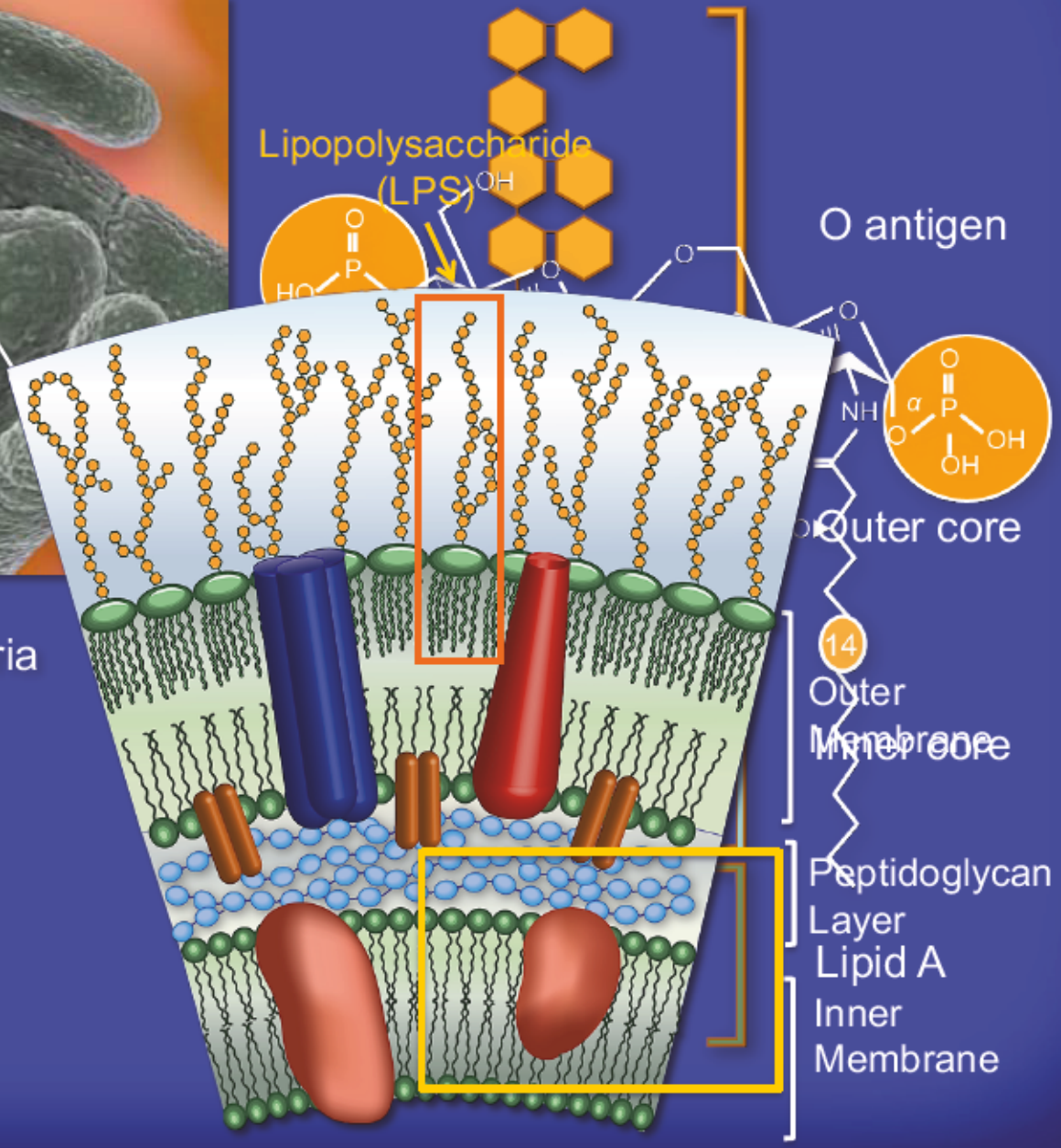
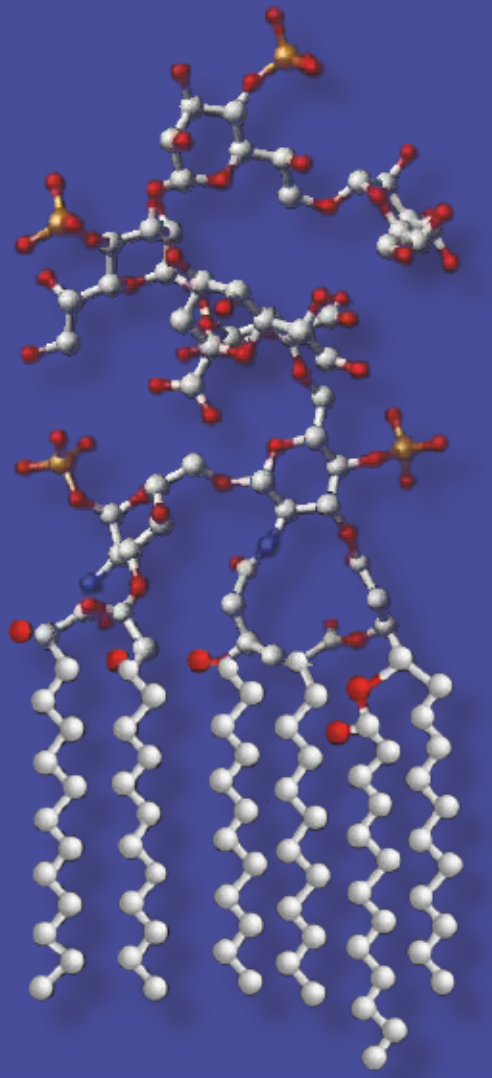


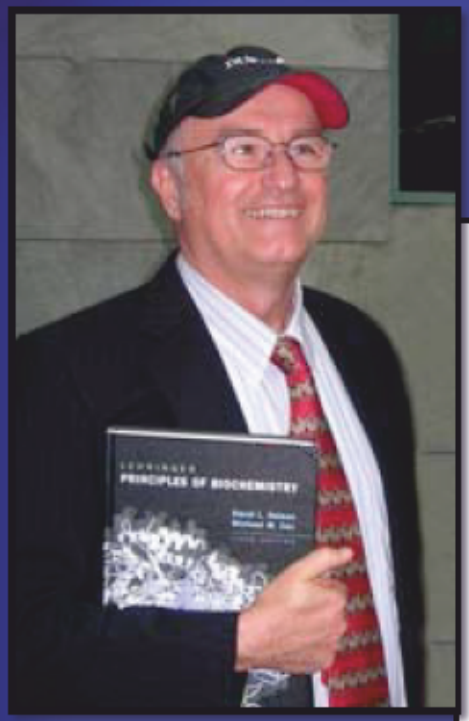
Gram negative bacteria
Eschericia coli



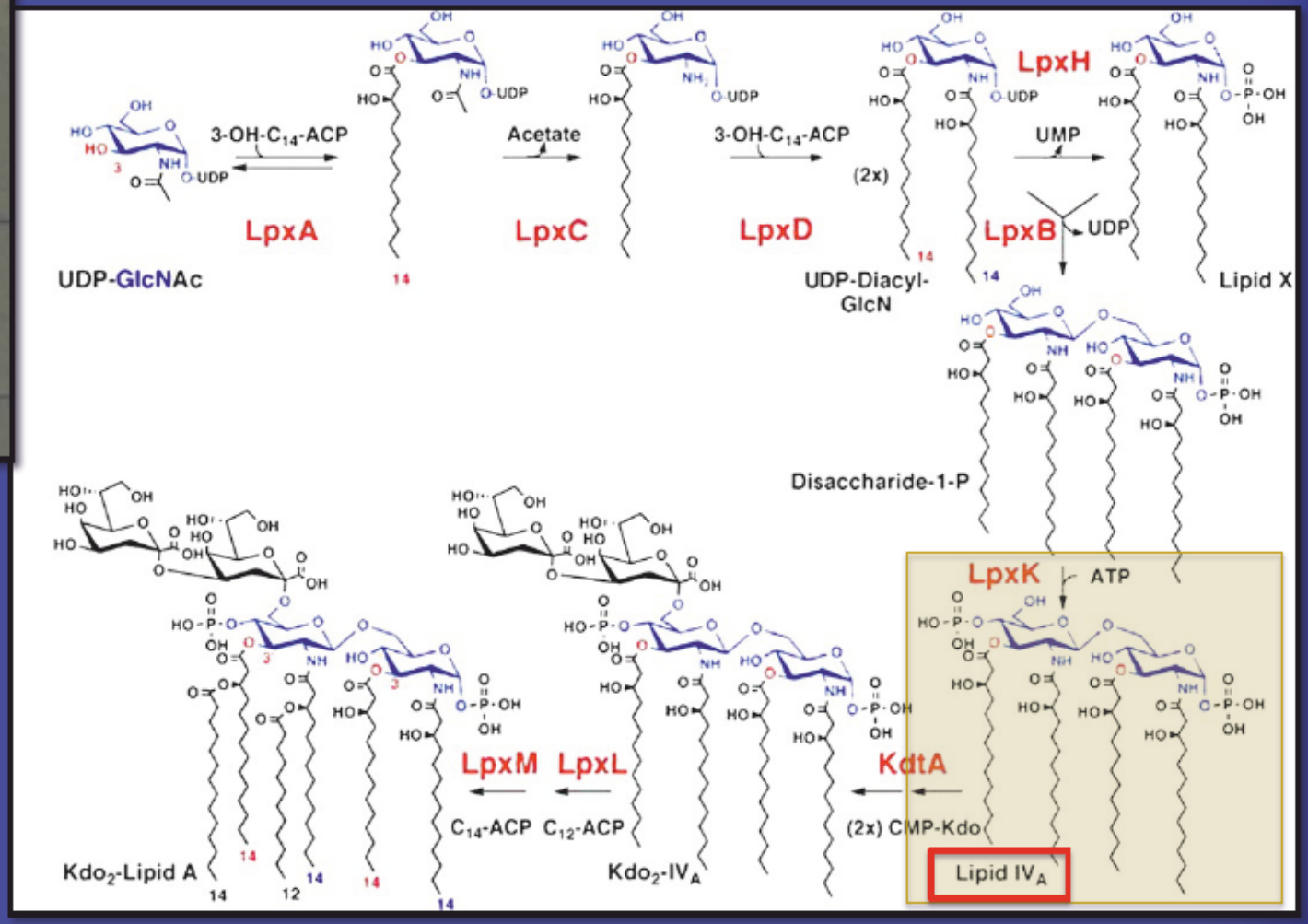
LPS



Biosynthesis of Lipid A



Christian R.H. Raetz
1946 - 2011



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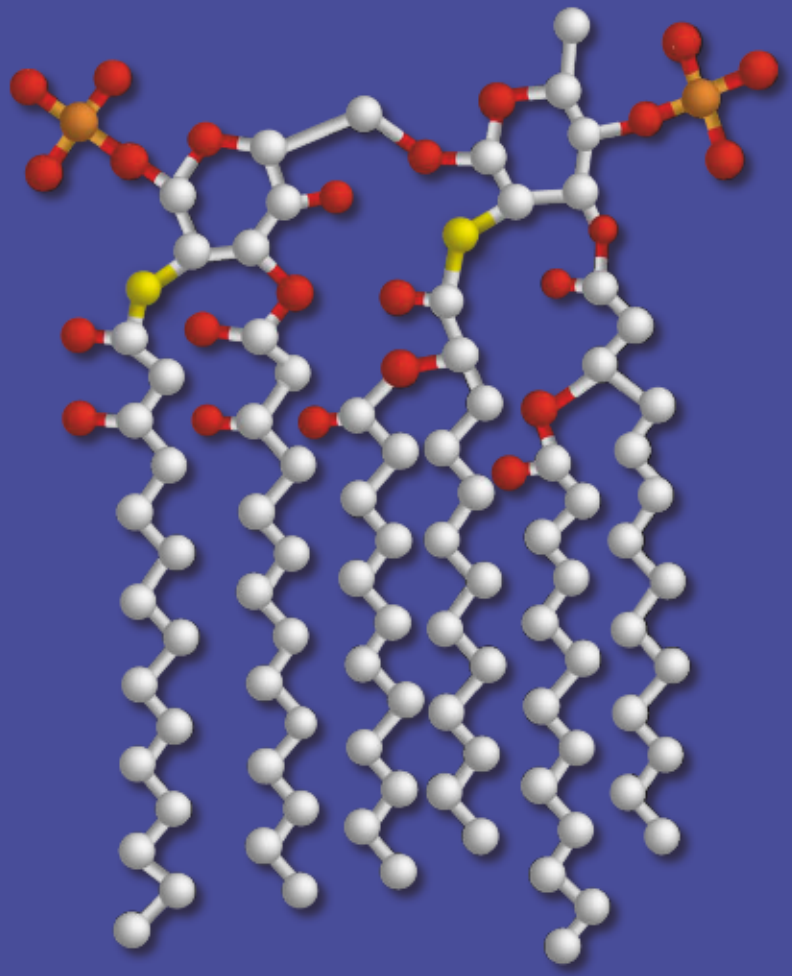
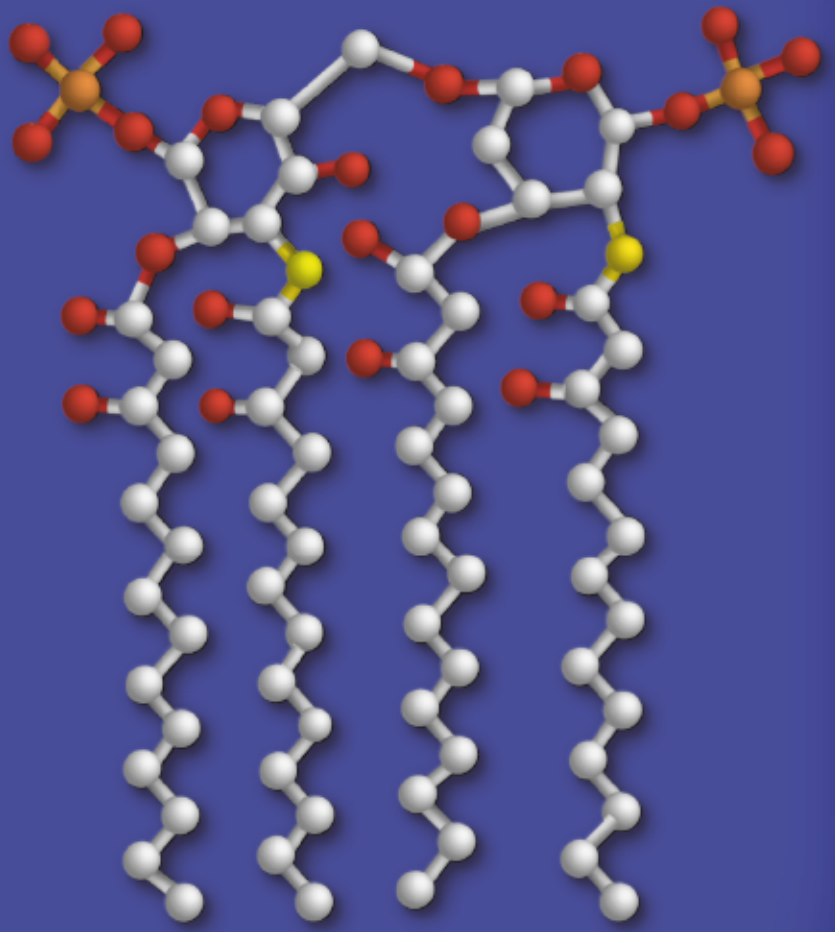
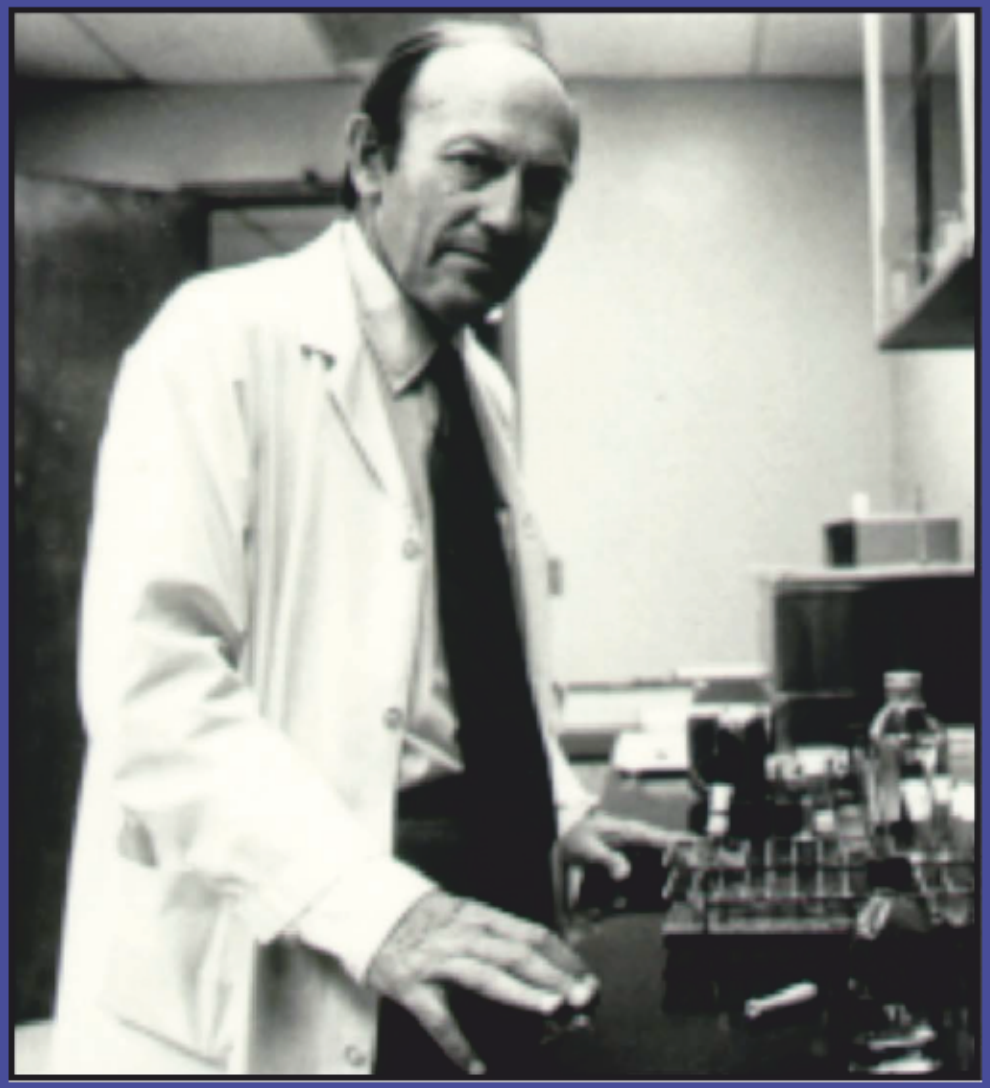
Lipid A



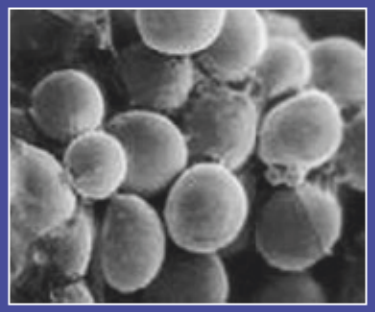
Lipid IVa





Abraham Braude, 1917-1984

Wasting disease (cachexia) in a cow with African trypanosomiasis



3T3-L1
pre-adipocytes

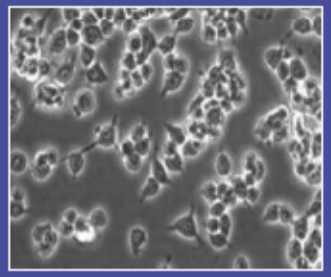
↑ Plasma triglyceride

↓ LPL, AcCoAC, FAS

Adipocyte

Tumors ? Cachectin

Other tissue ?



RAW 264.7
macrophages

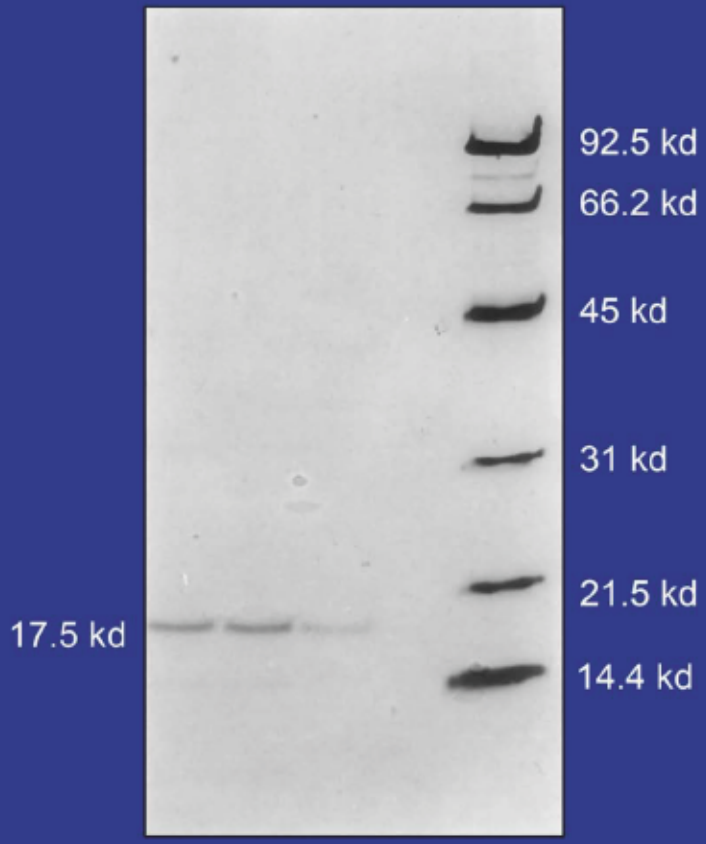
Endotoxin Tryoanosome factors Tumor factors

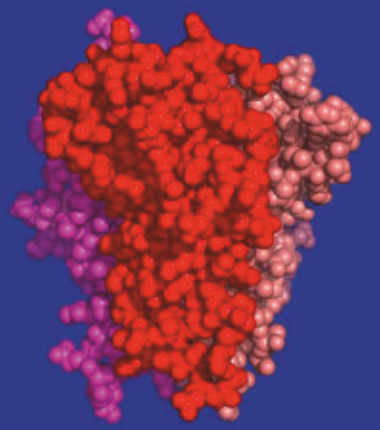
Infection Malignancy

1983

Isolation of mouse cachectin

- Pressure dialysis of medium from ~500 10 cm plates of LPS-activated RAW 264.7 cells (early harvest)
- ConA sepharose chromatography
- Isoelectric focusing in a glycerol gradient
- Preparative native gel electrophoresis
- Preparative SDS gel electrophoresis
- Yielded microgram quantities of an apparently pure 17.5 kD protein with approximately 2% yield of initial biological activity (prior to denaturing gel electrophoresis).
- Cachectin comprised 1-2% of the protein secreted by RAW 264.7 cells during the first two hours following LPS activation.





TNF

Cachectin = Mouse tumor necrosis factor

(mouse CACH)

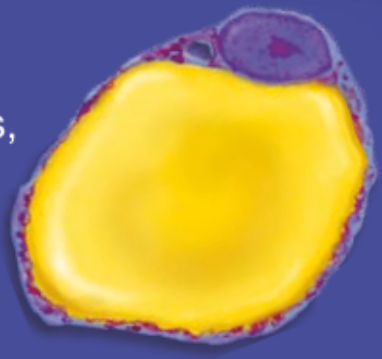
H_2N LEU-ARG-SER-SER-SER-GLU-ASN-SER-SER-ASP-PRO-PRO-VAL-ALA- ? -VAL-VAL-ALA-ASN…

H_2N VAL-ARG-SER-SER-SER-ARG-THR-PRO-SER-ASP-LYS-PRO-VAL-ALA-HIS-VAL-VAL-ALA-ASN…

(human TNF)

1 µg of cachectin had 10^8 U of TNF activity

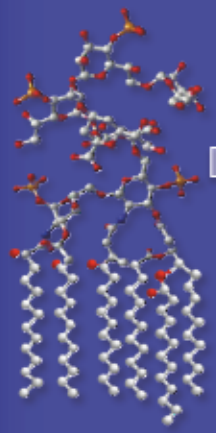
This raised the question: might TNF mediate *all* effects of LPS, including the lethal effect?



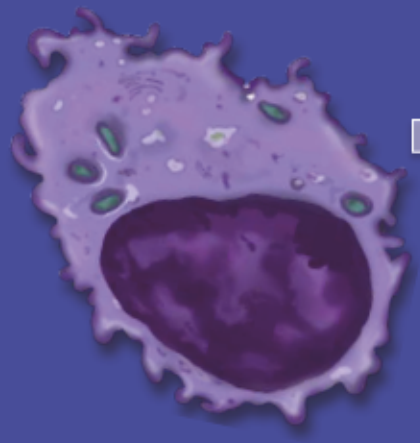
↓ Triglyceride synthesis, LPL, FAS

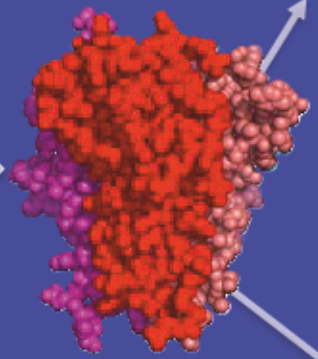
↑ AcCoA carboxylase, glycerol release

LPS



MACROPHAGE





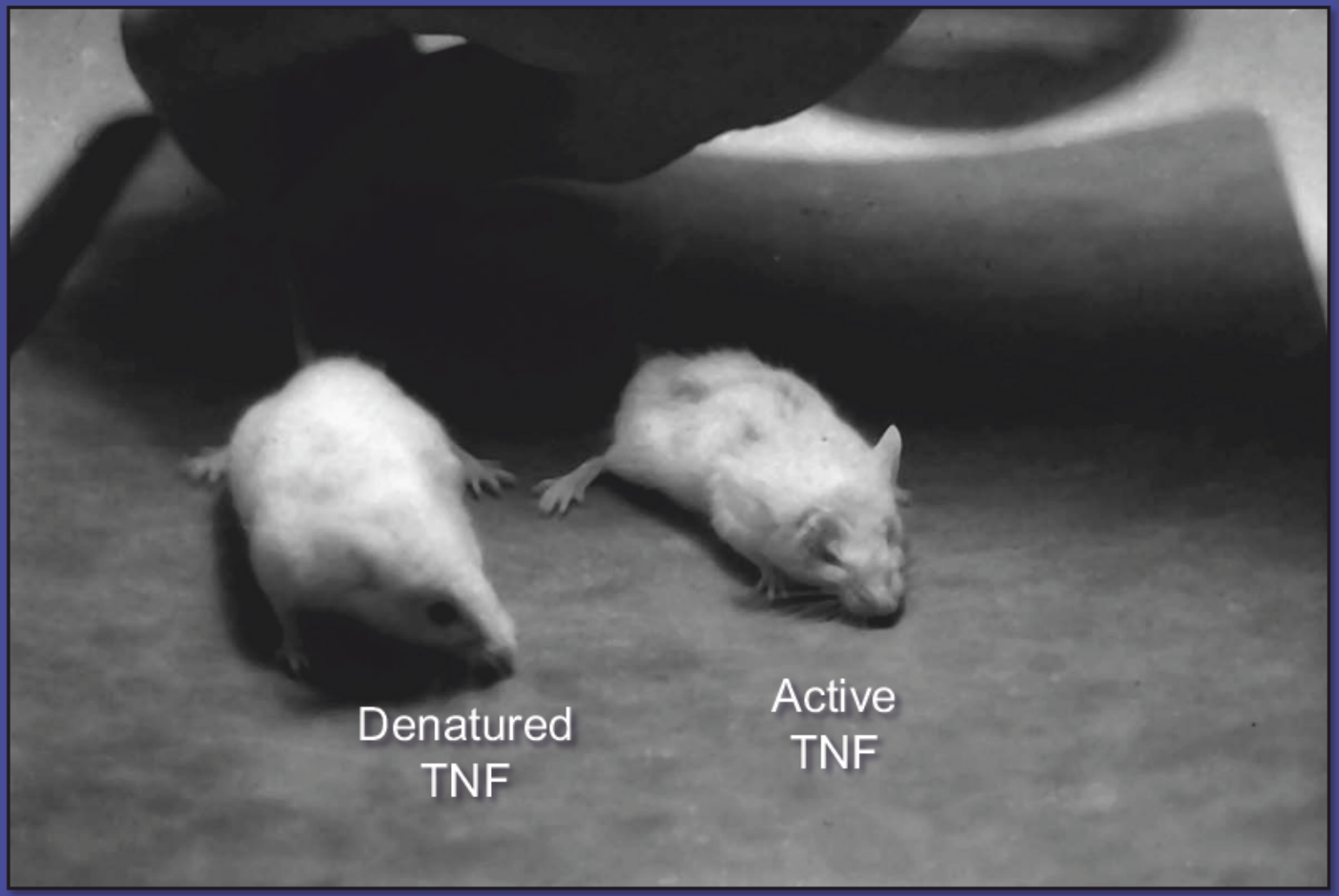
FAT

TNF

TUMOR

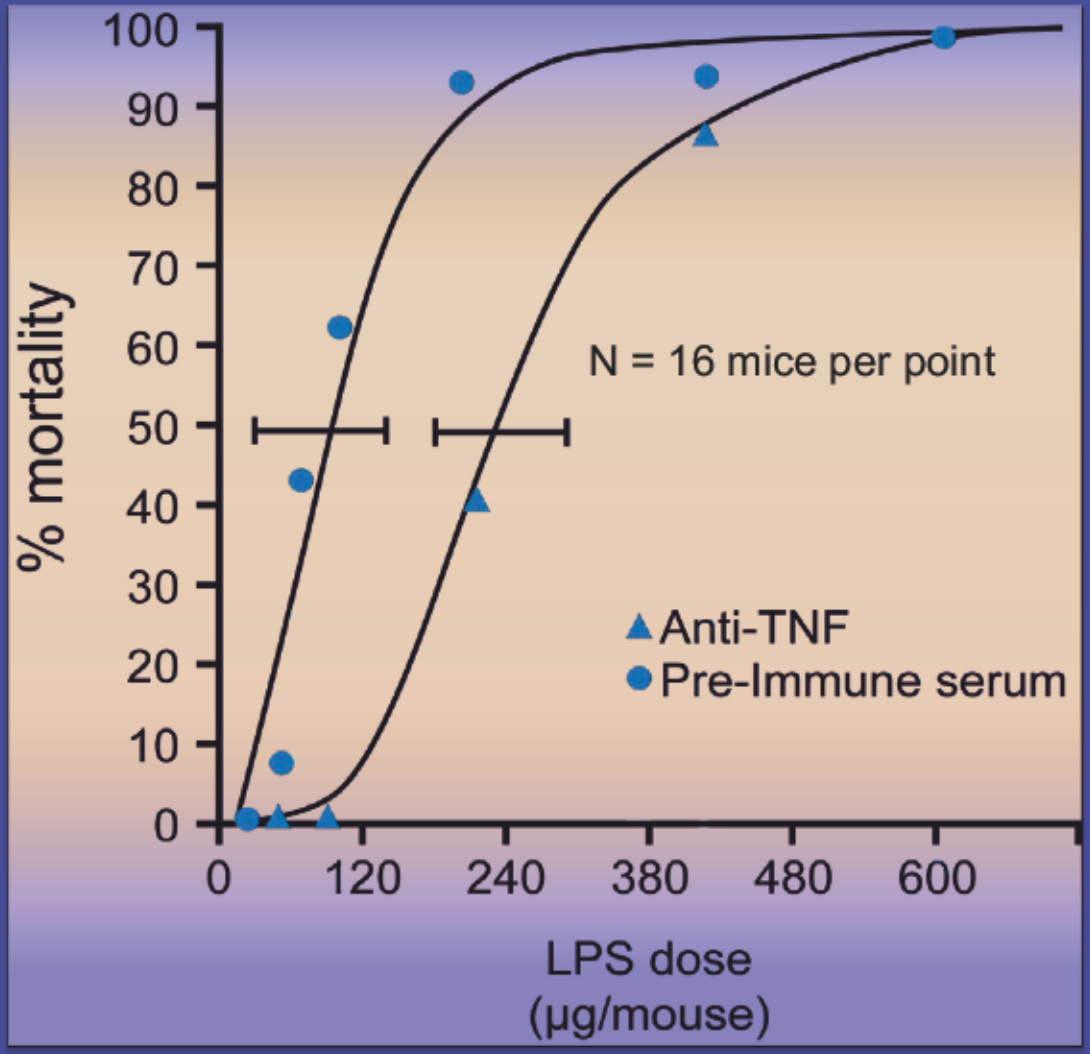
↑ Tumor necrosis and cytolysis

Purified TNF mimics LPS toxicity

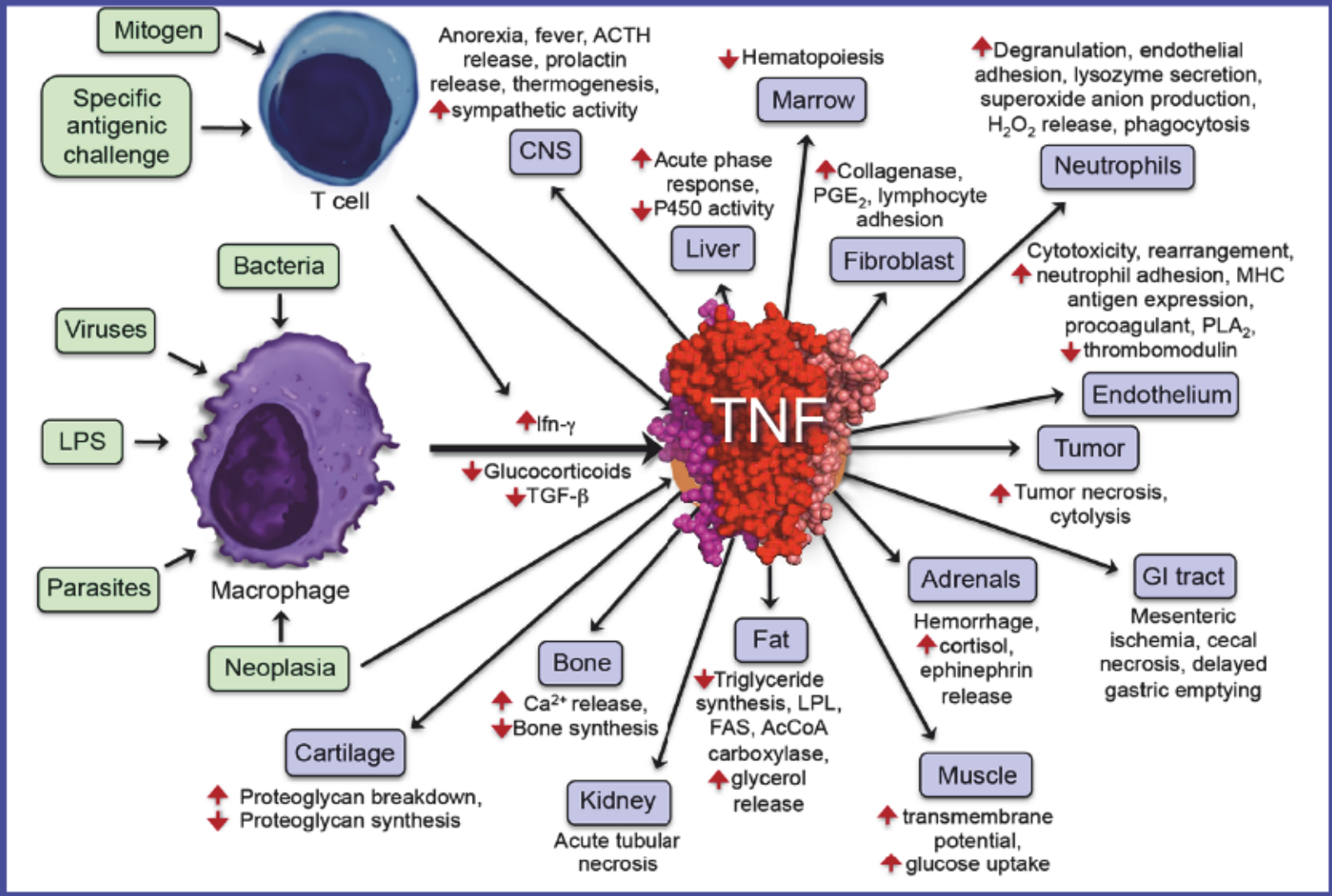


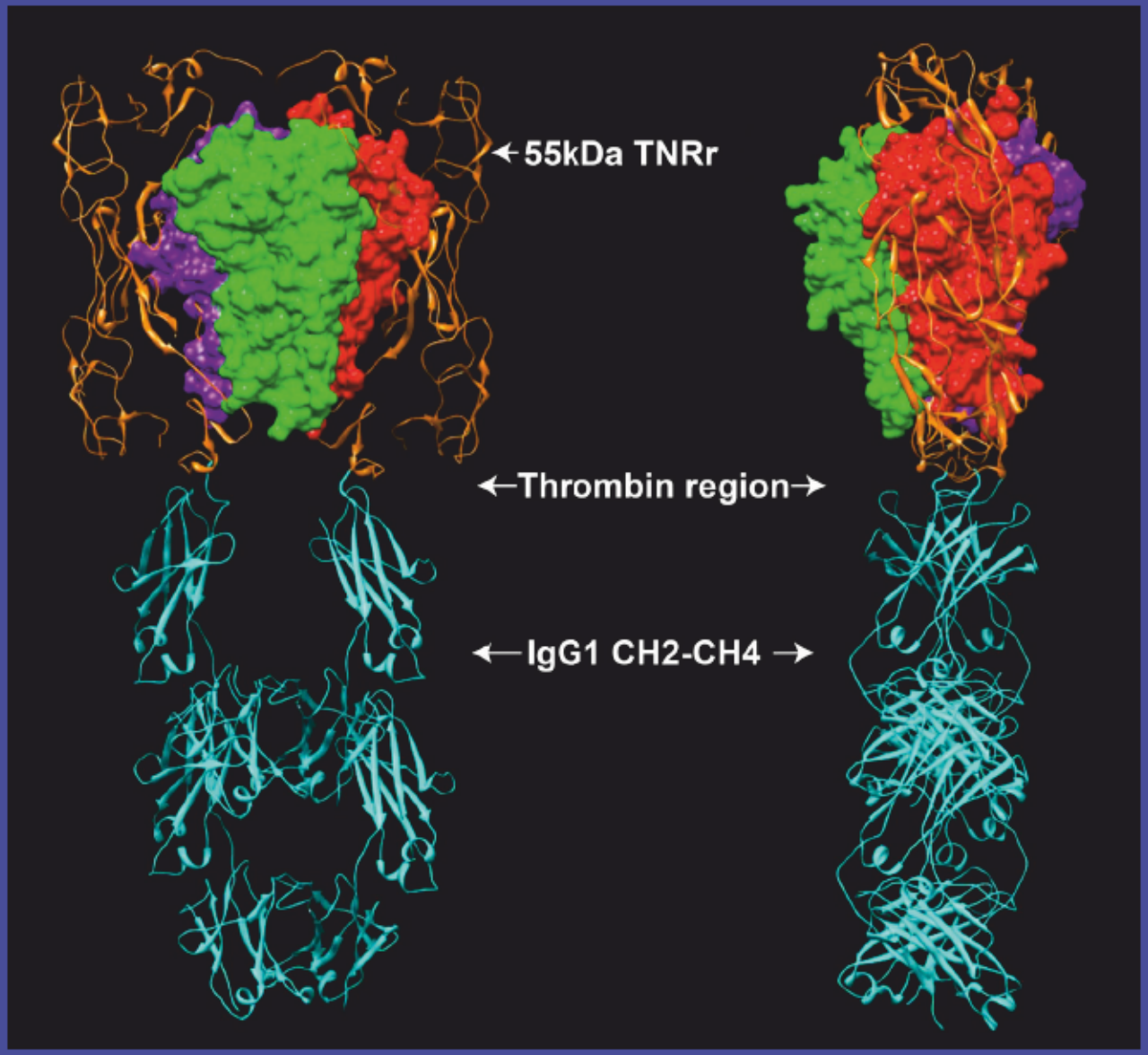
1984

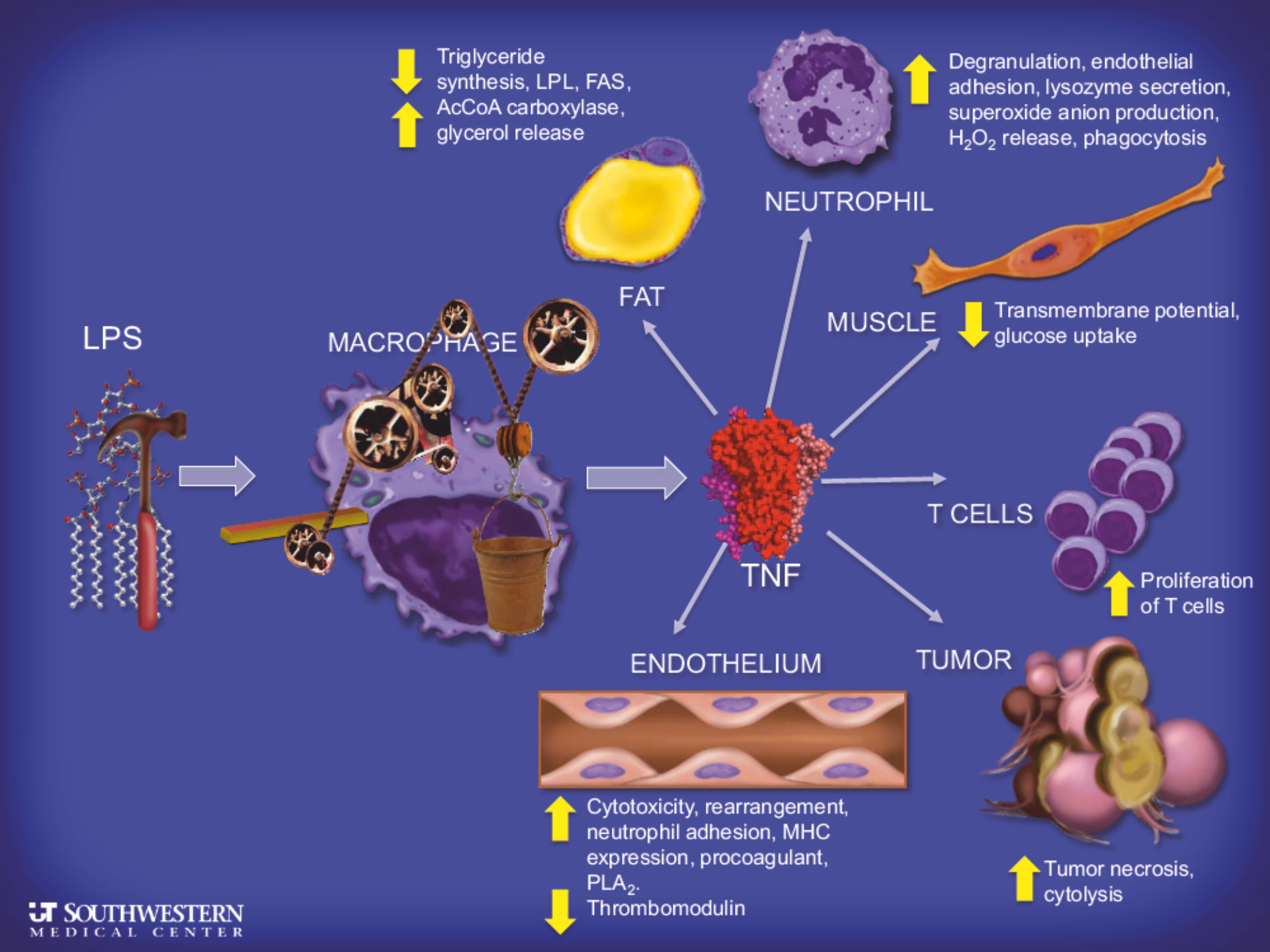
The lethal effect of LPS is attenuated by passive immunization against TNF



1985


Mitogen
Specific antigenic challenge
T cell
Anorexia, fever, ACTH release, prolactin release, thermogenesis, ↑sympathetic activity
CNS
↓Hematopoiesis
Marrow
↑Degranulation, endothelial adhesion, lysozyme secretion, superoxide anion production, H_2O_2 release, phagocytosis
Neutrophils
↑Acute phase response, ↓P450 activity
Liver
↑Collagenase, PGE_2, lymphocyte adhesion
Fibroblast
Cytotoxicity, rearrangement, ↑neutrophil adhesion, MHC antigen expression, procoagulant, PLA_2, ↓thrombomodulin
Endothelium
Bacteria
Viruses
LPS
Parasites
Macrophage
Neoplasia
↑Ifn-γ
↓Glucocorticoids
↓TGF-β
TNF
Tumor
↑Tumor necrosis, cytolysis
Adrenals
Hemorrhage, ↑cortisol, ephinephrin release
GI tract
Mesenteric ischemia, cecal necrosis, delayed gastric emptying
Fat
↓Triglyceride synthesis, LPL, FAS, AcCoA carboxylase, ↑glycerol release
Bone
↑ Ca^{2+} release, ↓Bone synthesis
Cartilage
↑ Proteoglycan breakdown, ↓ Proteoglycan synthesis
Kidney
Acute tubular necrosis
Muscle
↑transmembrane potential, ↑glucose uptake


55kDa TNRr
Thrombin region
IgG1 CH2-CH4


Triglyceride synthesis, LPL, FAS, AcCoA carboxylase, glycerol release
Degranulation, endothelial adhesion, lysozyme secretion, superoxide anion production, H_2O_2 release, phagocytosis
NEUTROPHIL
FAT
MUSCLE
Transmembrane potential, glucose uptake
LPS
MACROPHAGE
TNF
T CELLS
Proliferation of T cells
ENDOTHELIUM
TUMOR
Cytotoxicity, rearrangement, neutrophil adhesion, MHC expression, procoagulant, PLA_2.
Thrombomodulin
Tumor necrosis, cytolysis
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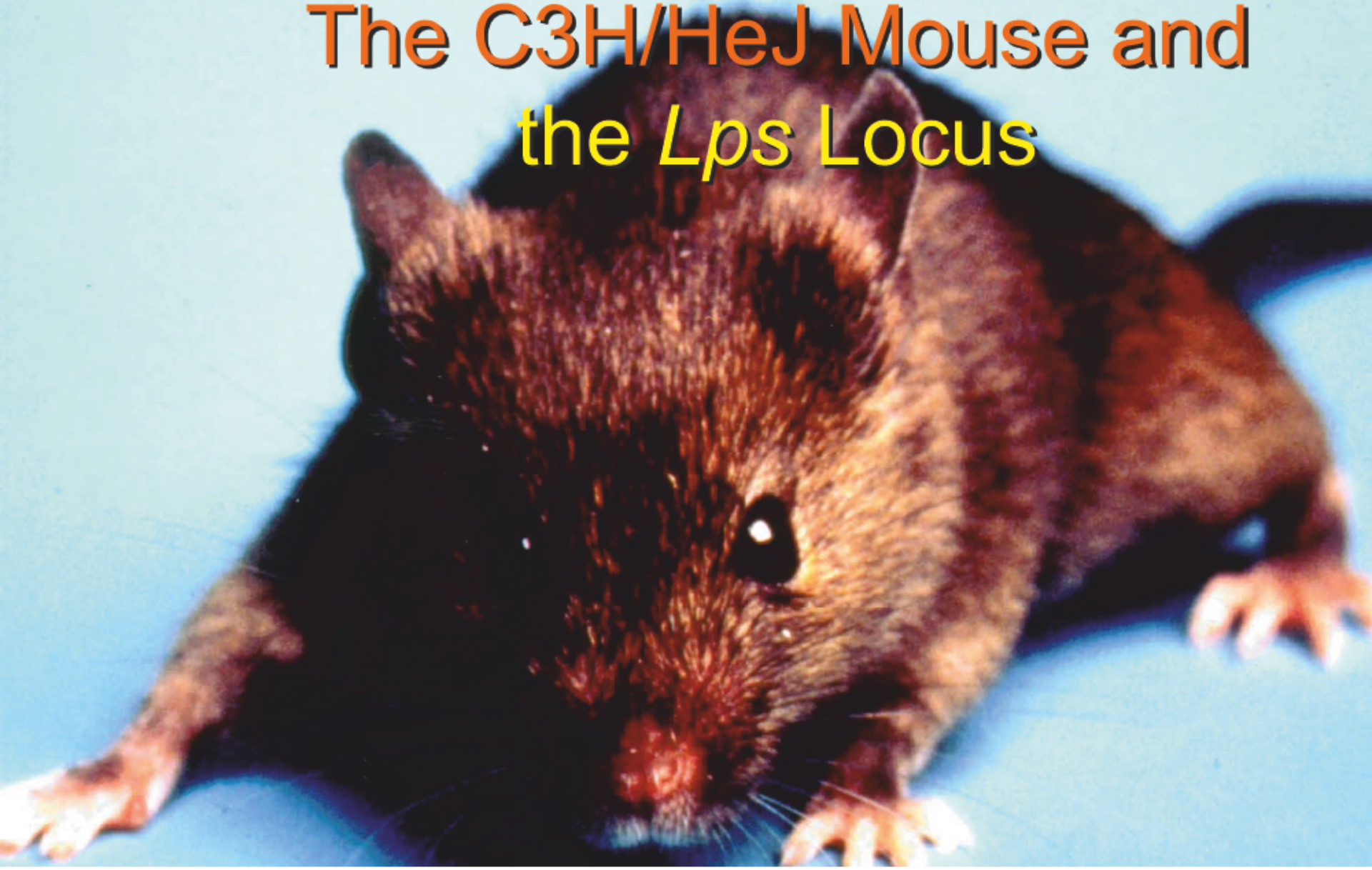
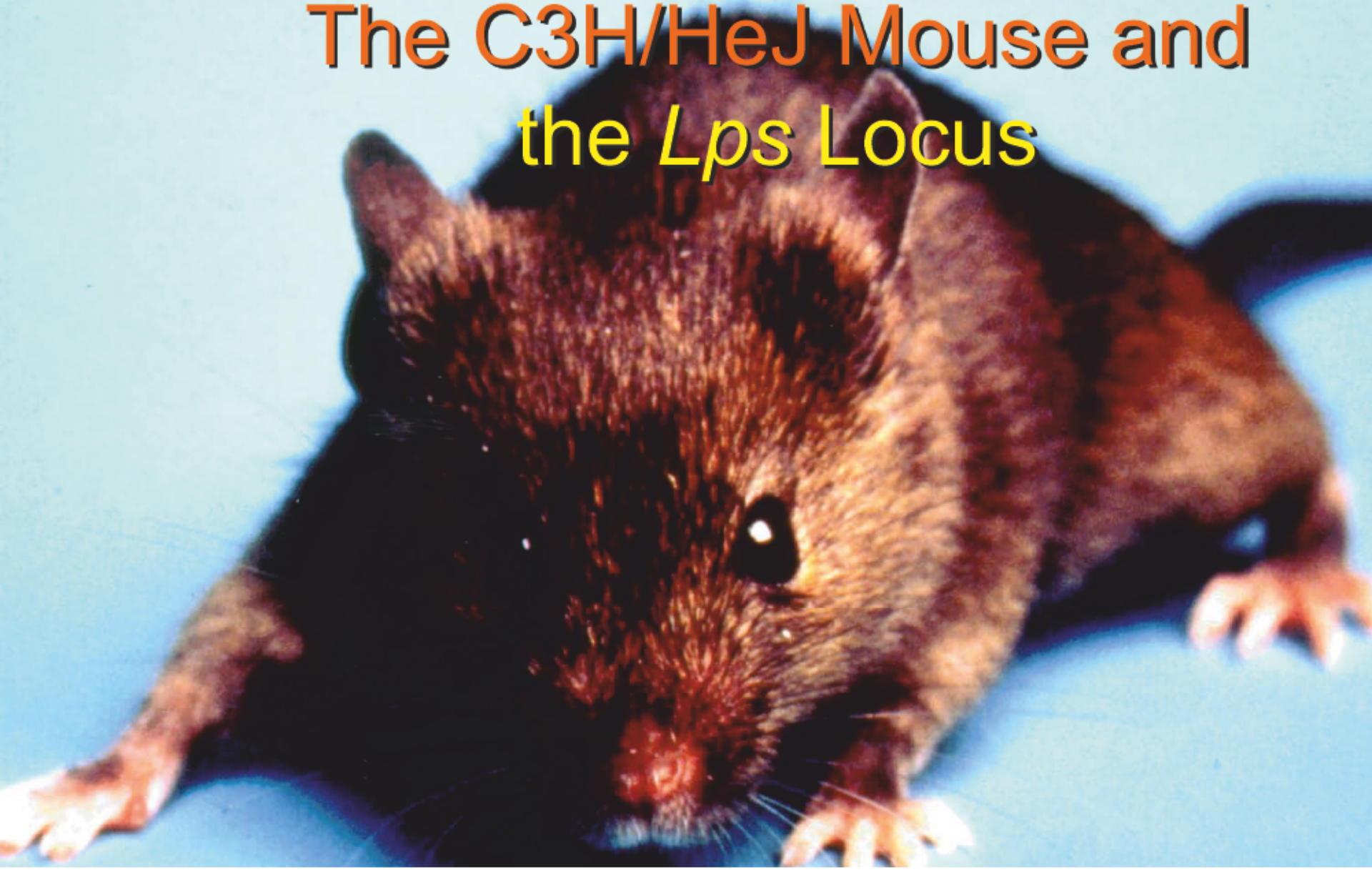
The C3H/HeJ Mouse and the *Lps* Locus



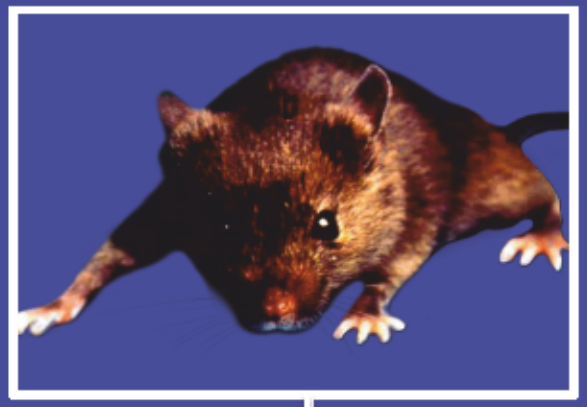
- Resistant to LPS (Heppner and Weiss, 1965)

The C3H/HeJ Mouse and the *Lps* Locus

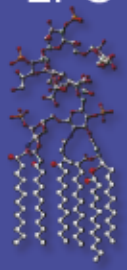


- Fail to make a cytokine response to LPS (for example, no TNF), suggesting a proximal defect.

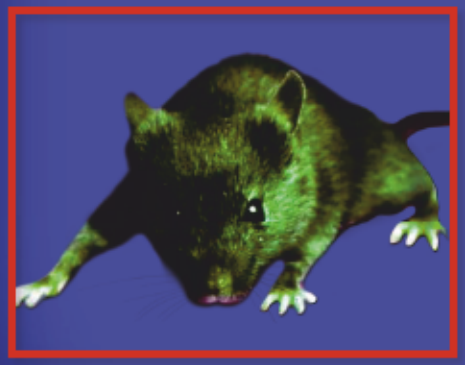
C3H/HeJ mice:
resistant to LPS
(and *only* LPS)



LPS

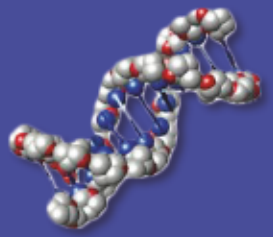


Gram negative bacteria

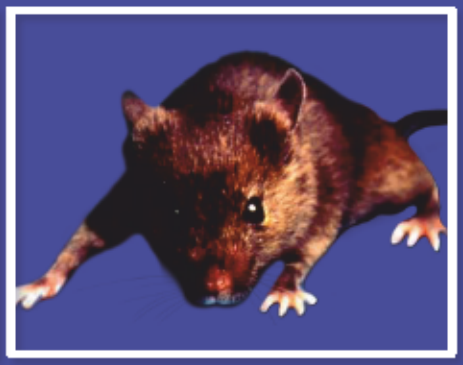


Unresponsive

Nucleic Acids



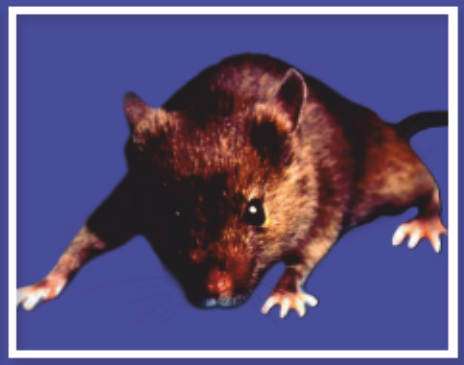
Viruses



Responsive

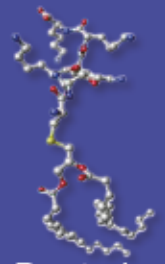
Flagellin

Bacteria

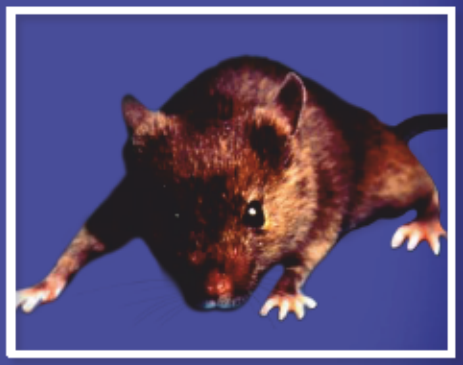


Responsive

Lipoproteins



Bacteria



Responsive

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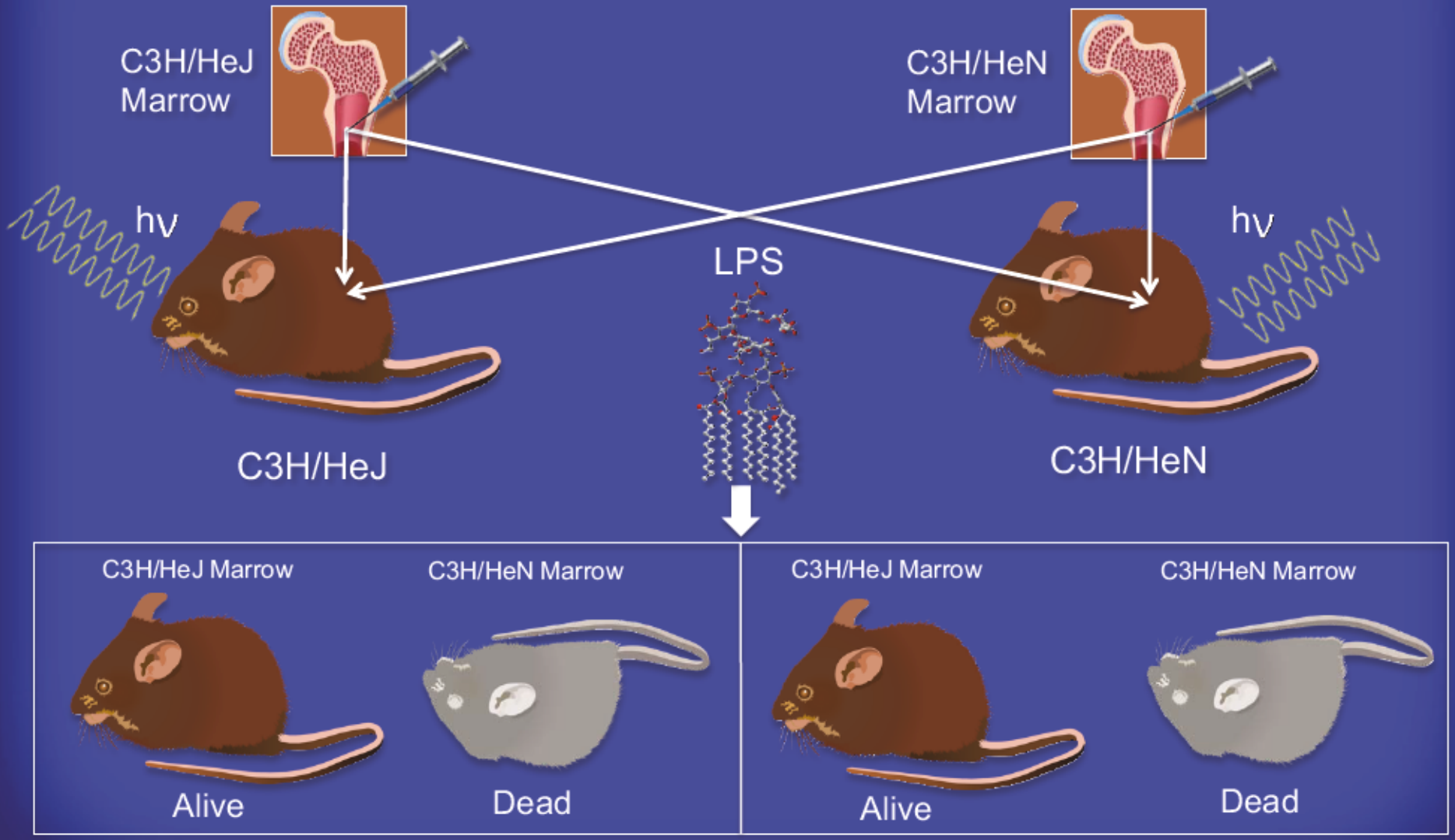
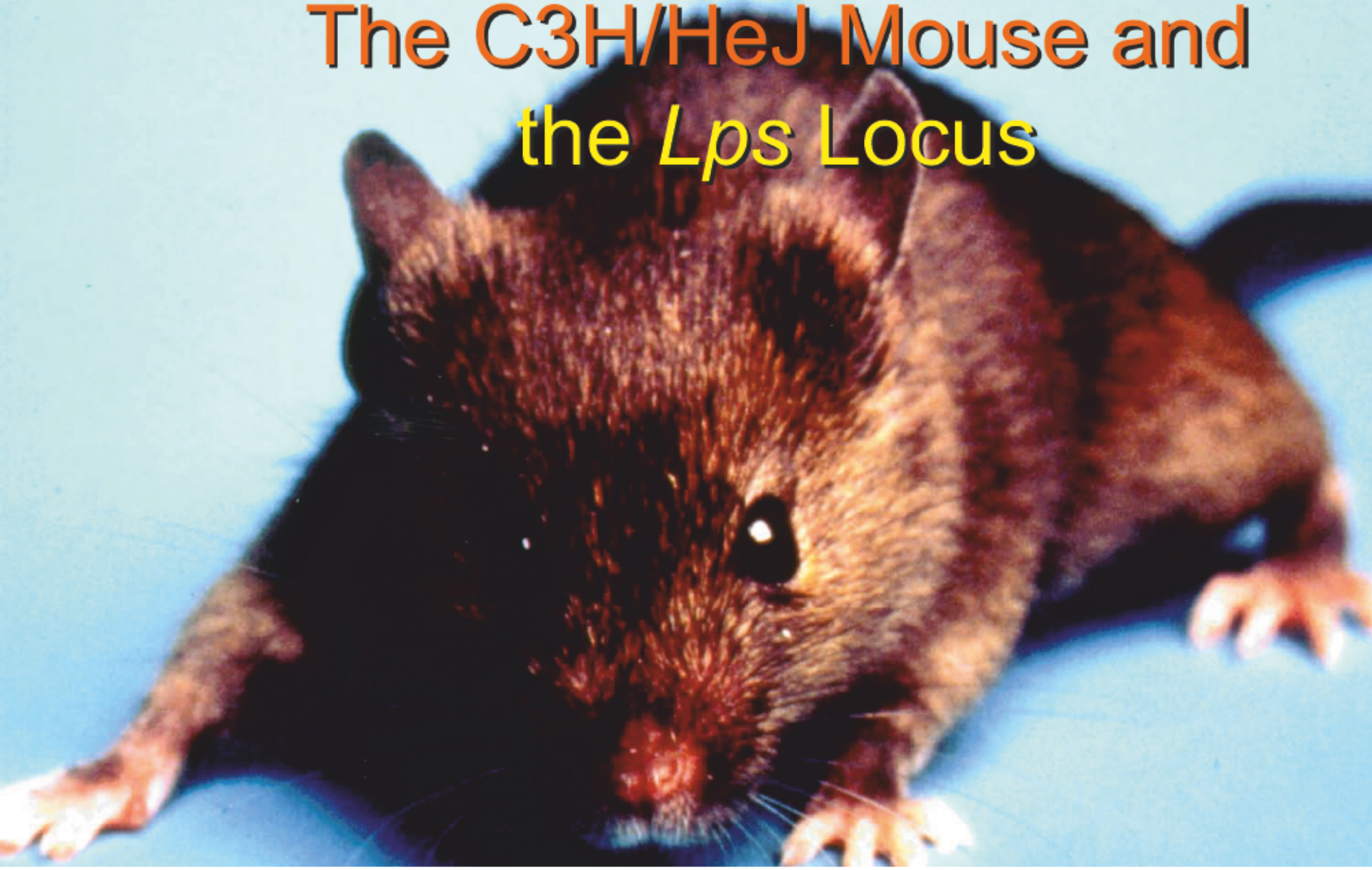
C3H/HeJ bone marrow transfer to C3H/HeN and vice versa: susceptibility to LPS-induced lethality is determined by the donor

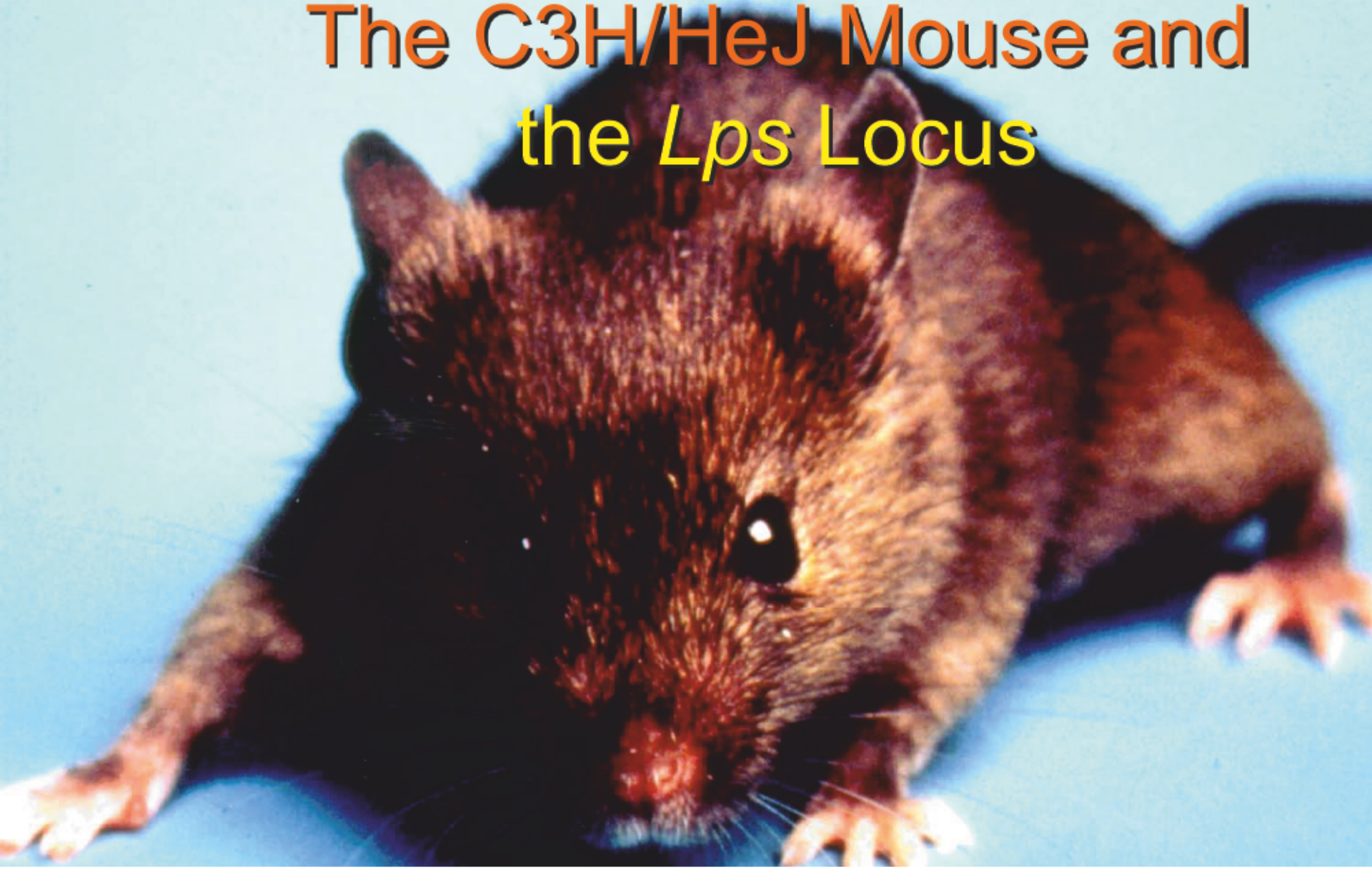


The C3H/HeJ Mouse and the *Lps* Locus



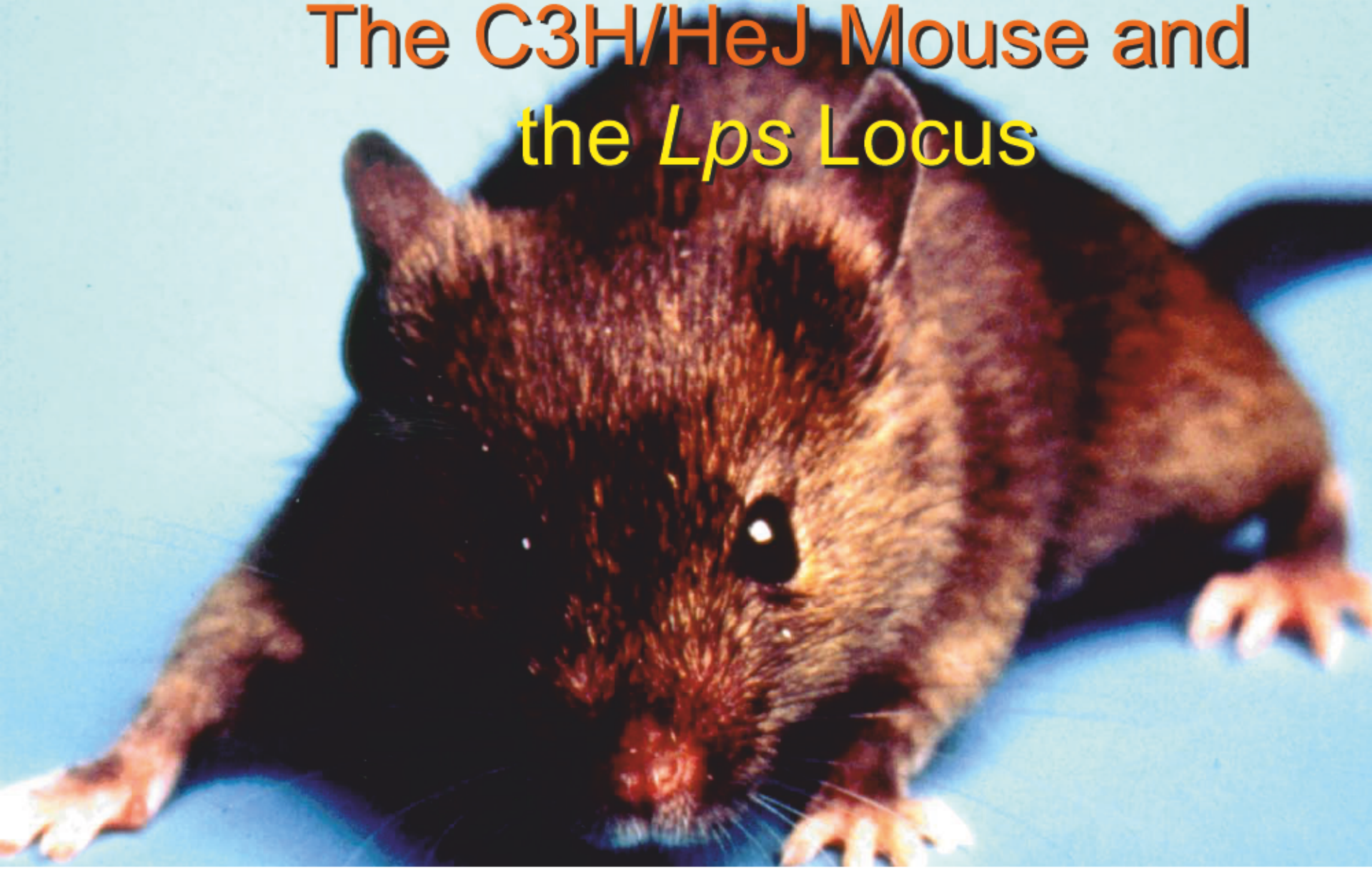
- Hypersusceptible to authentic G(-) infections (O'brien, et al., 1980; Svanborg-Eden, et al., 1983)

The C3H/HeJ Mouse and the *Lps* Locus



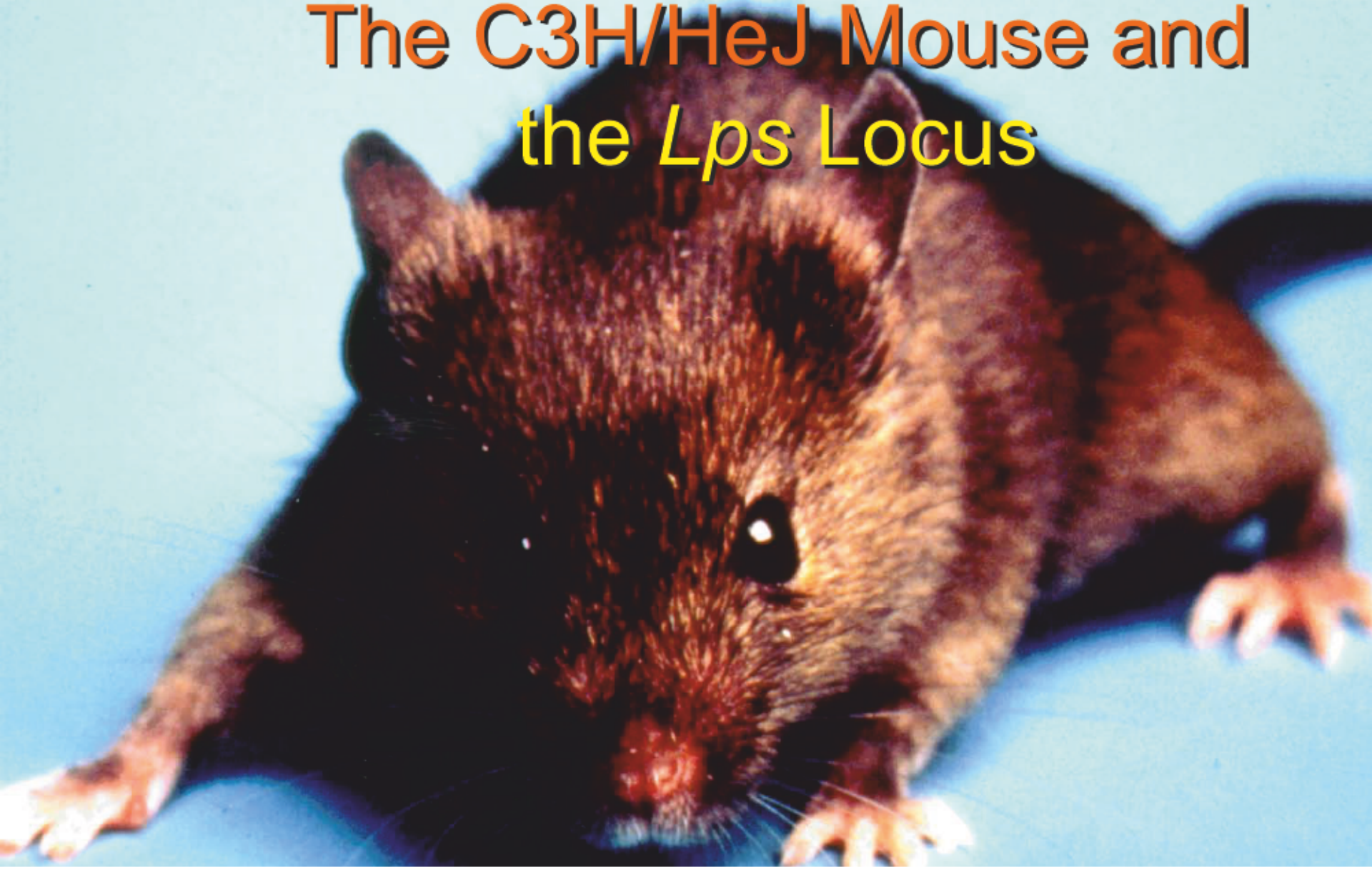
- LPS does not work as an adjuvant in C3H/HeJ mice (B.J. Skidmore et al, 1976)

The C3H/HeJ Mouse and the *Lps* Locus

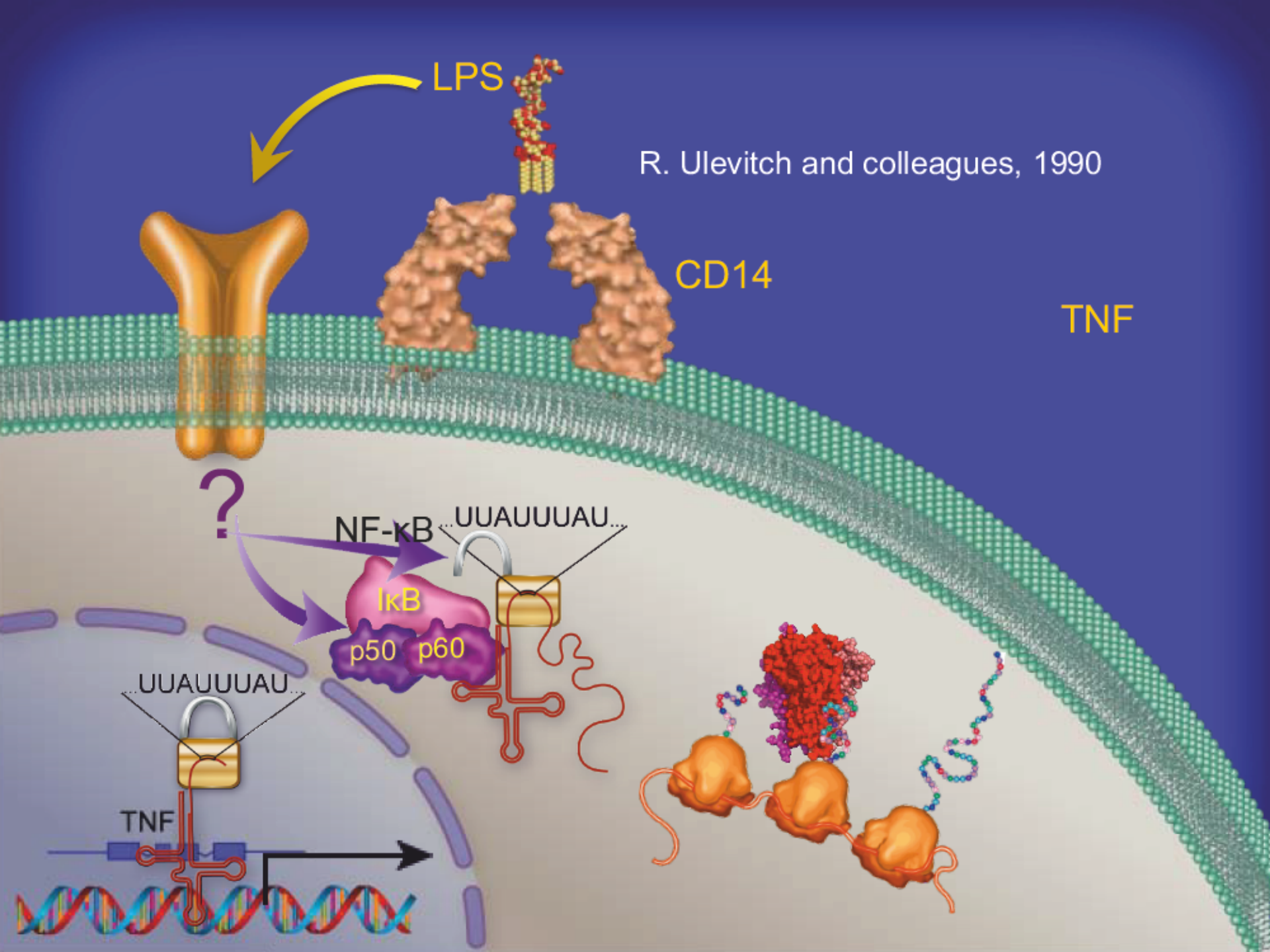


- Single locus (*Lps*); allelic to a mutation in the LPS-refractory C57BL/10ScCr strain (Coutinho and Meo,1978).

The C3H/HeJ Mouse and the *Lps* Locus

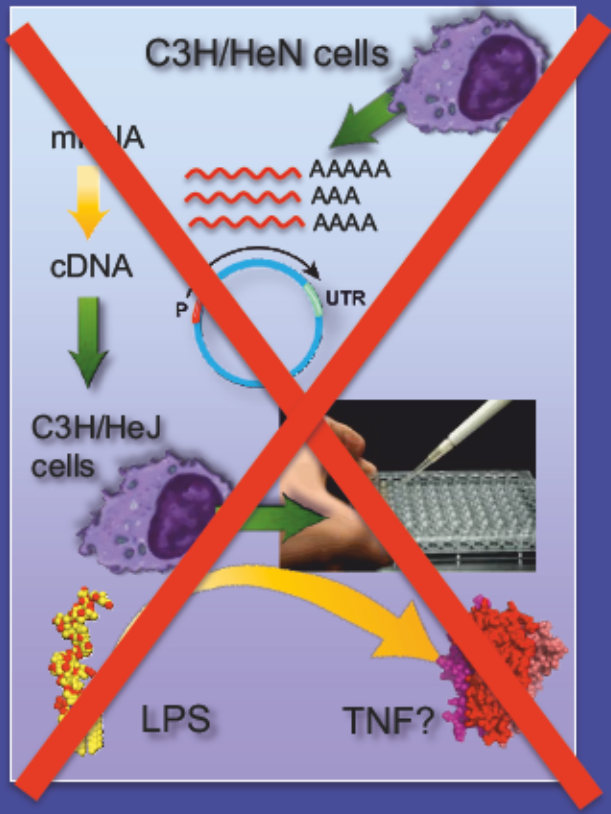


- *Lps* mapped to Chr. 4 between *Mup1* and *Polysyndactyly* loci by Watson et al. in 1978.


LPS
R. Ulevitch and colleagues, 1990
CD14
TNF
?
NF-κB
UUAUUUAU
IκB
p50
p60
UUAUUUAU
TNF

Conventional searches for a difference between C3H/HeJ and C3H/HeN

1. Cross immunization of C3H/HeJ and C3H/HeN mice.



2. Transfect cDNA from C3H/HeN to C3H/HeJ cells.

3. Comparisons at the protein level.

Betsy Layton

Alexander Poltorak

Christophe Van Huffel

Irina Smirnova

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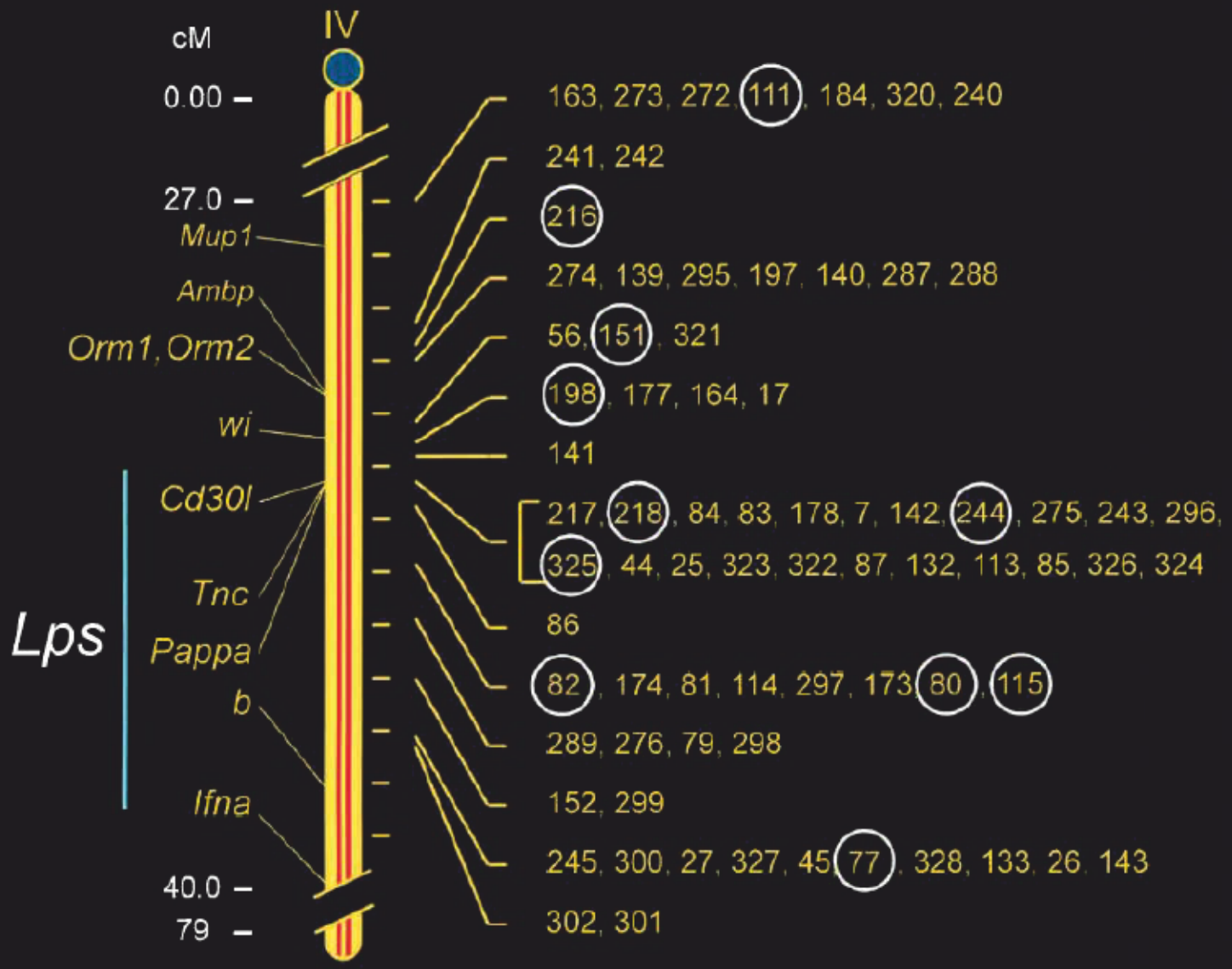
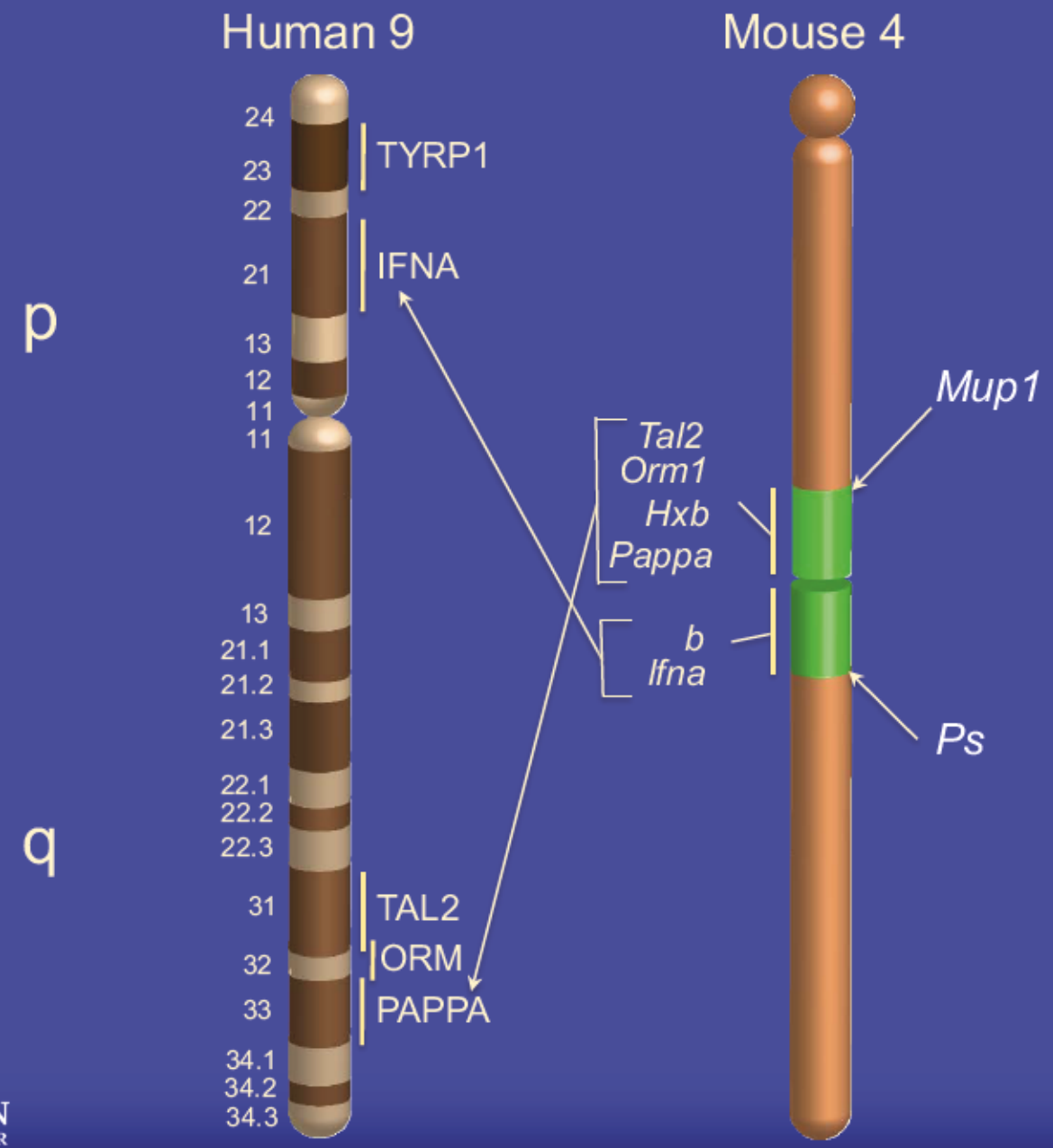
Positional cloning entails…

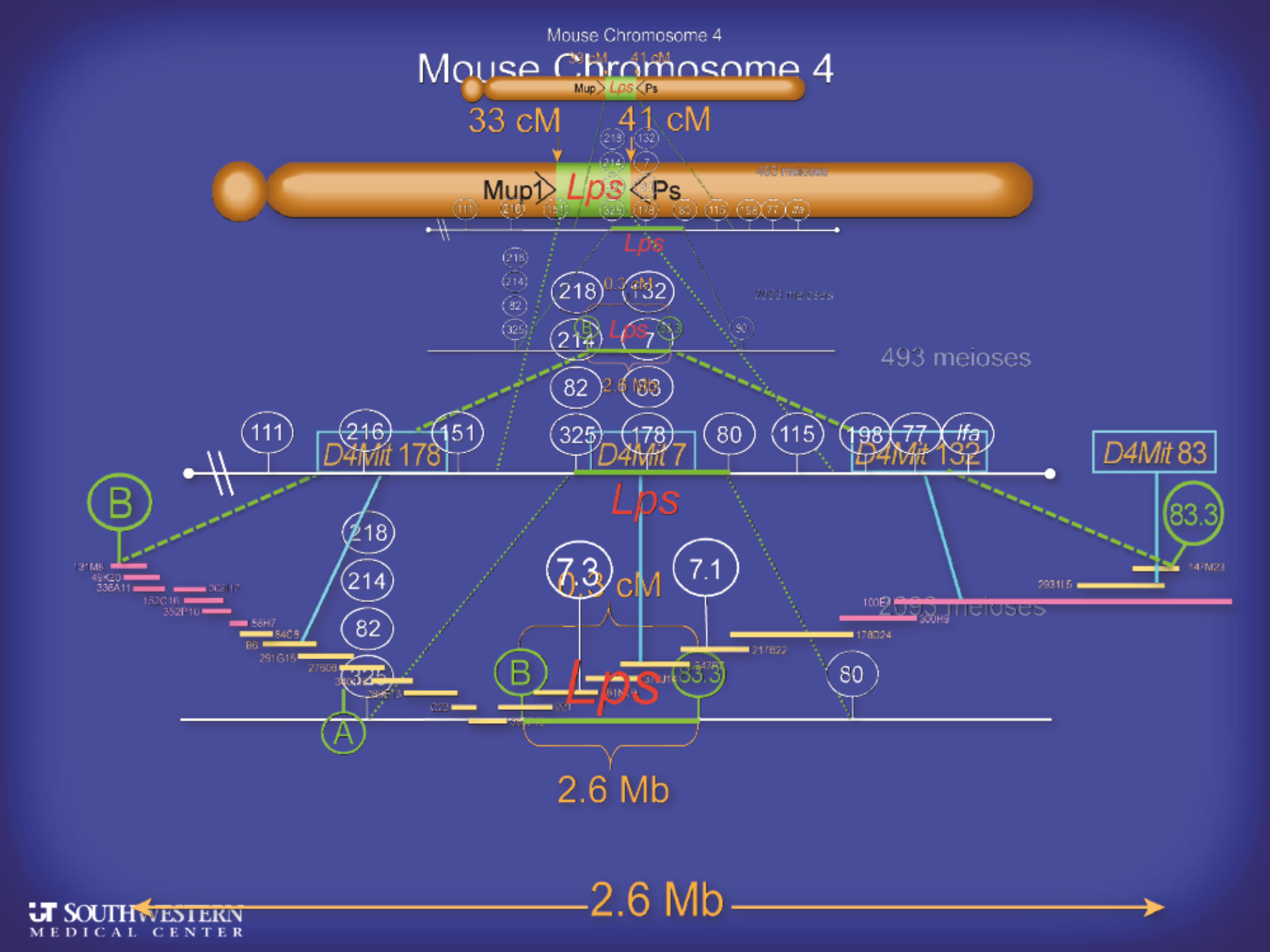
- *Genetic mapping* (in our case, on 2093 meioses)
- *Physical mapping* (in our case, entire interval cloned in 66 BACs and 2 YACs)
- *Exploration for genes* (in our case, 1 authentic genes and 7 pseudogenes)
- *Mutation identification* (find the one and only genetic change responsible for the phenotype.

MAPPING *Lps* WITH RESPECT TO D4MIT MARKERS

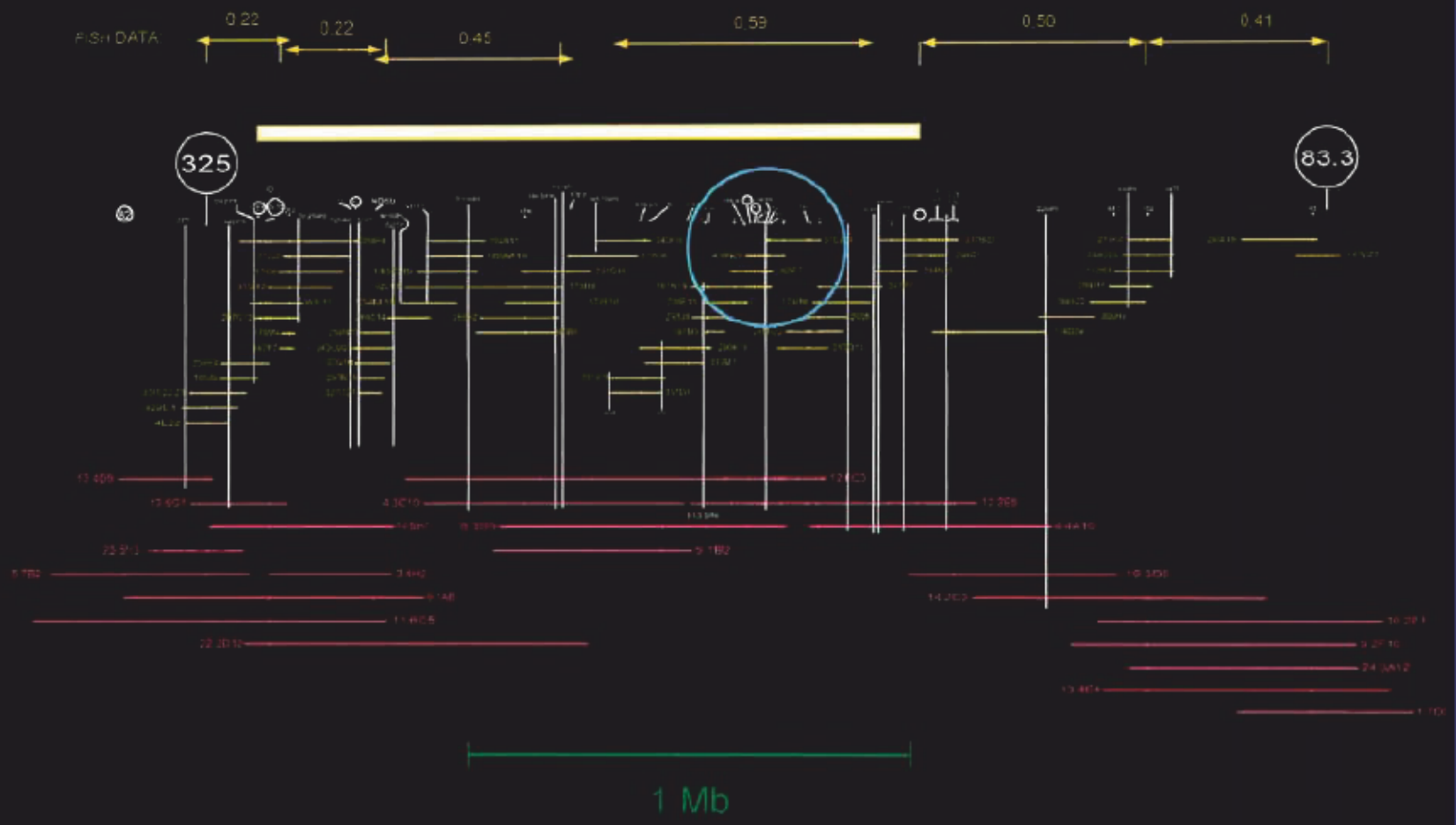


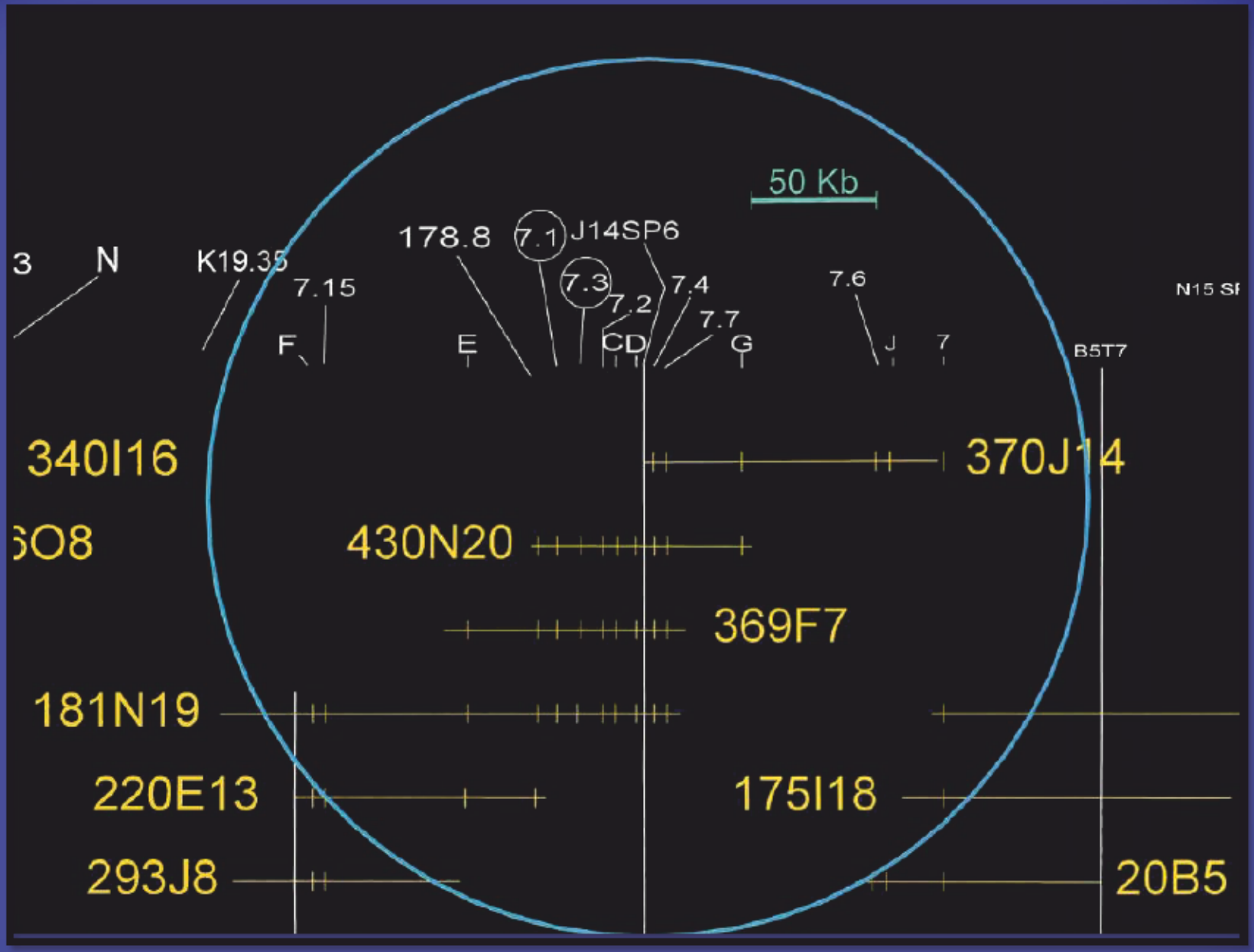
Comparison of Mouse and Human LPS Gene Locus




Mouse Chromosome 4
Mouse Chromosome 4
33 cM
41 cM
Mup
Lps
Ps
Mup1
493 meioses
2023 meioses
0.3 cM
2.6 Mb
D4Mit 178
D4Mit 7
D4Mit 132
D4Mit 83
111
216
151
325
178
80
115
198
77
Ifa
218
214
82
83
7
132
83.3
7.3
7.1
0.3 cM
A
B
2.6 Mb
SOUTHWESTERN
MEDICAL CENTER

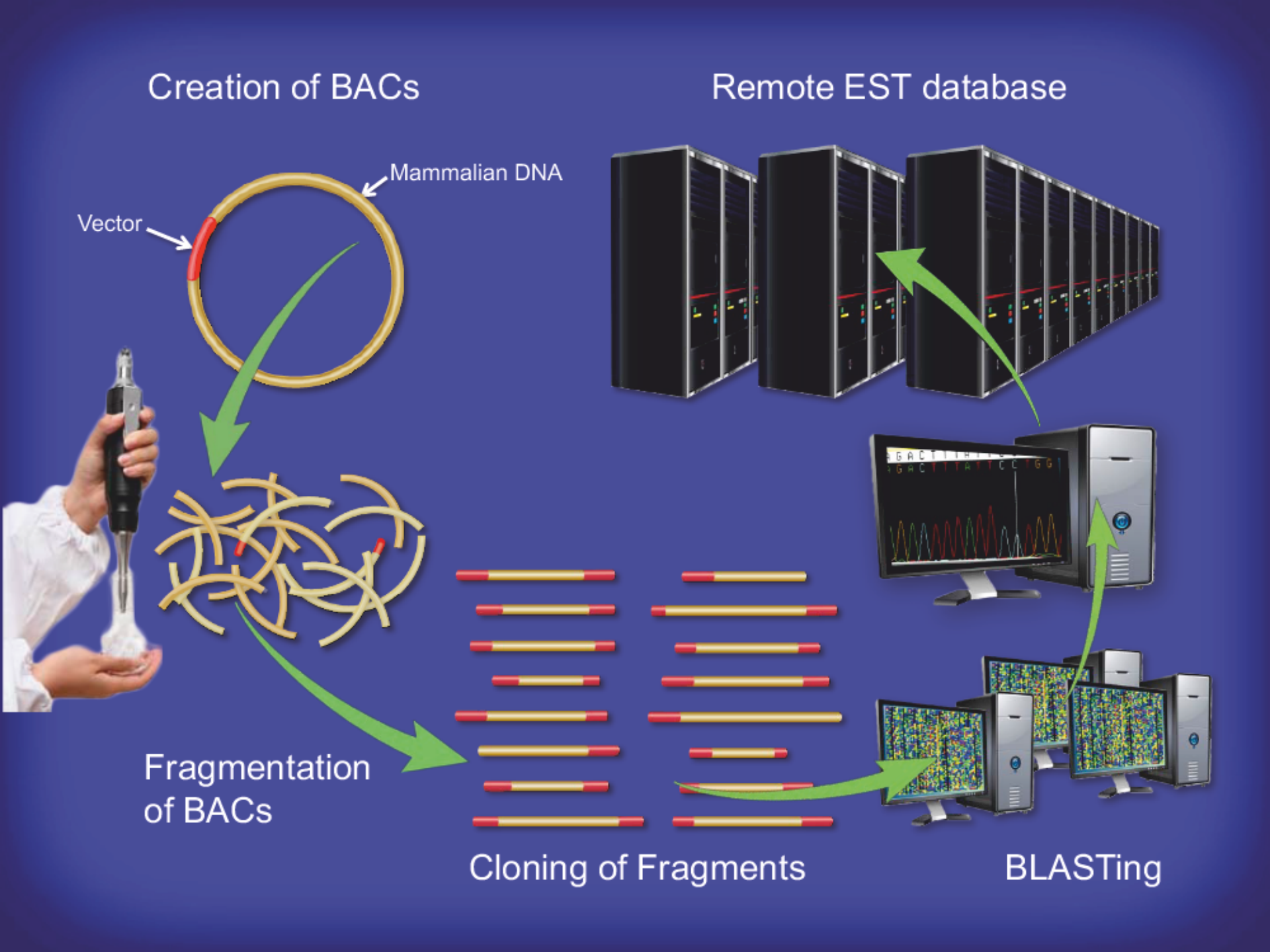
CONTIG: D4MIT325 THRU 83.3

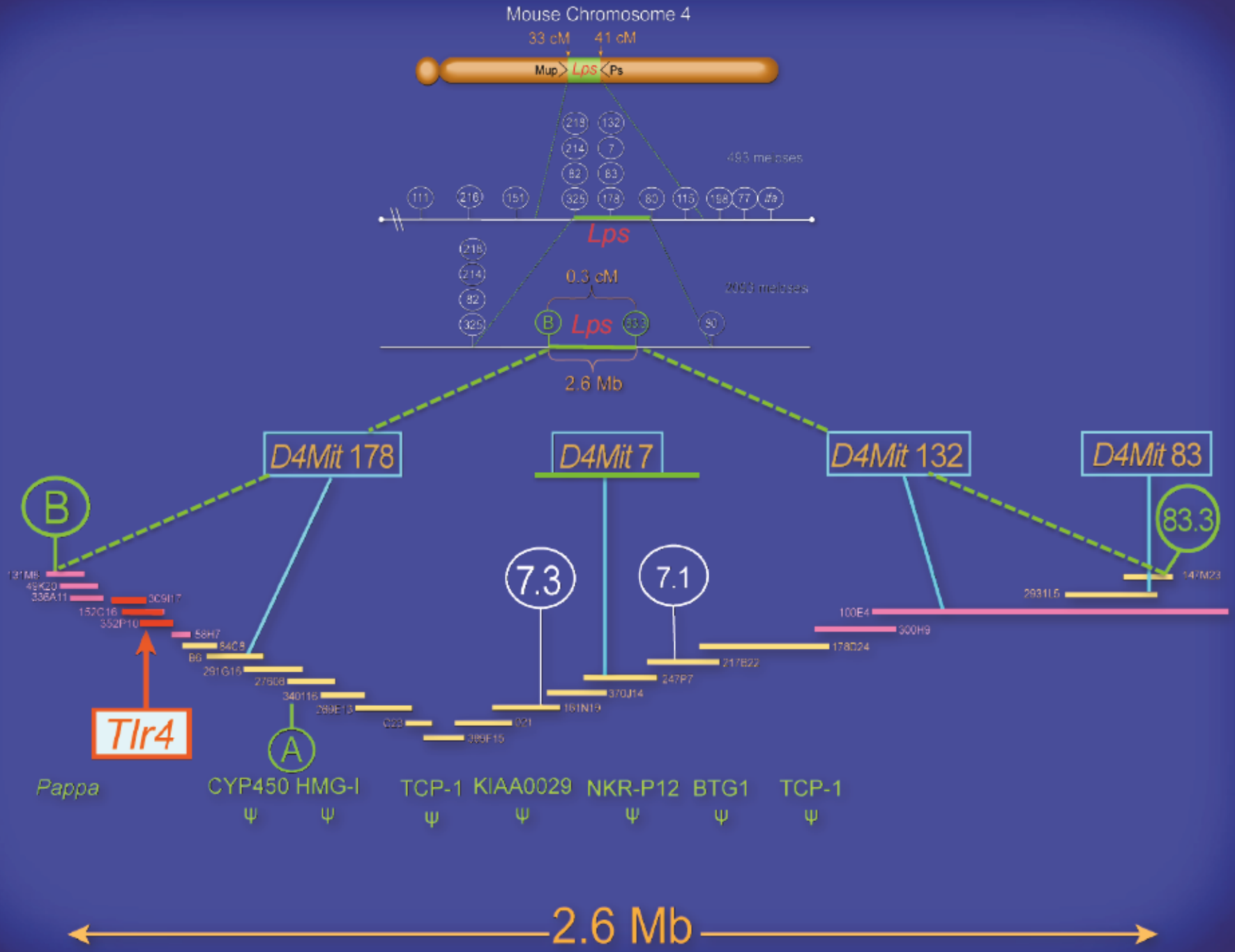


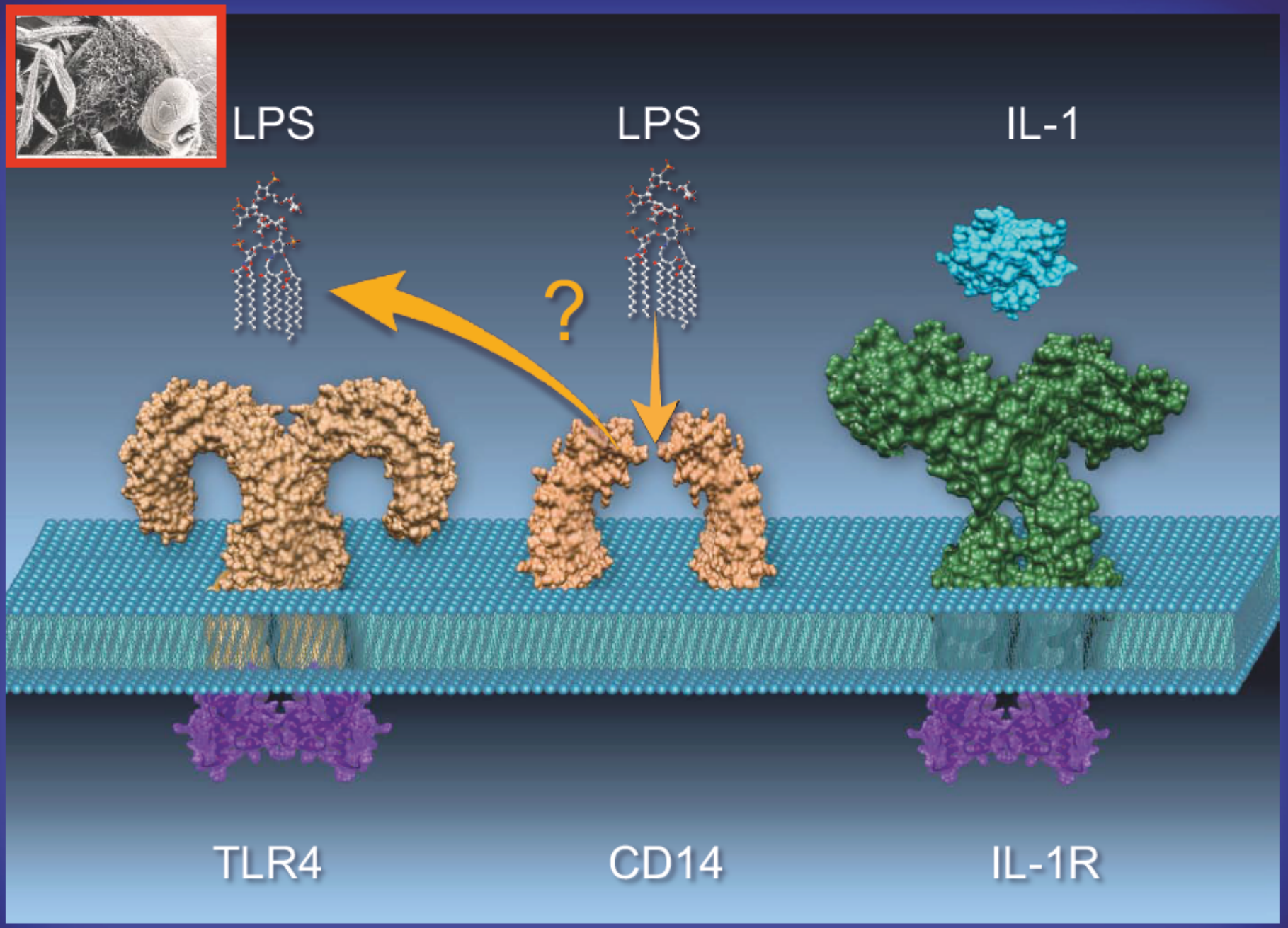

50 Kb
3
N
K19.35
7.15
178.8
7.1
J14SP6
7.3
7.2
7.4
7.7
7.6
F
E
CD
G
J
7
B5T7
N15 SI
340I16
370J14
GO8
430N20
369F7
181N19
220E13
175I18
293J8
20B5

Methods for finding genes within BACs

- Exon trapping
- Hybridization selection
- Computational prediction (GRAIL)
- Shotgun sequencing and EST database searching


Creation of BACs
Remote EST database
Mammalian DNA
Vector
Fragmentation of BACs
Cloning of Fragments
BLASTing


Mouse Chromosome 4
33 cM
41 cM
Mup
Lps
Ps
493 meioses
Lps
0.3 cM
2023 meioses
B
Lps
83.3
2.6 Mb
D4Mit 178
D4Mit 7
D4Mit 132
D4Mit 83
B
83.3
7.3
7.1
Tlr4
A
Pappa
CYP450
HMG-I
TCP-1
KIAA0029
NKR-P12
BTG1
TCP-1
Ψ
Ψ
Ψ
Ψ
Ψ
Ψ
Ψ
2.6 Mb


LPS
LPS
IL-1
?
TLR4
CD14
IL-1R

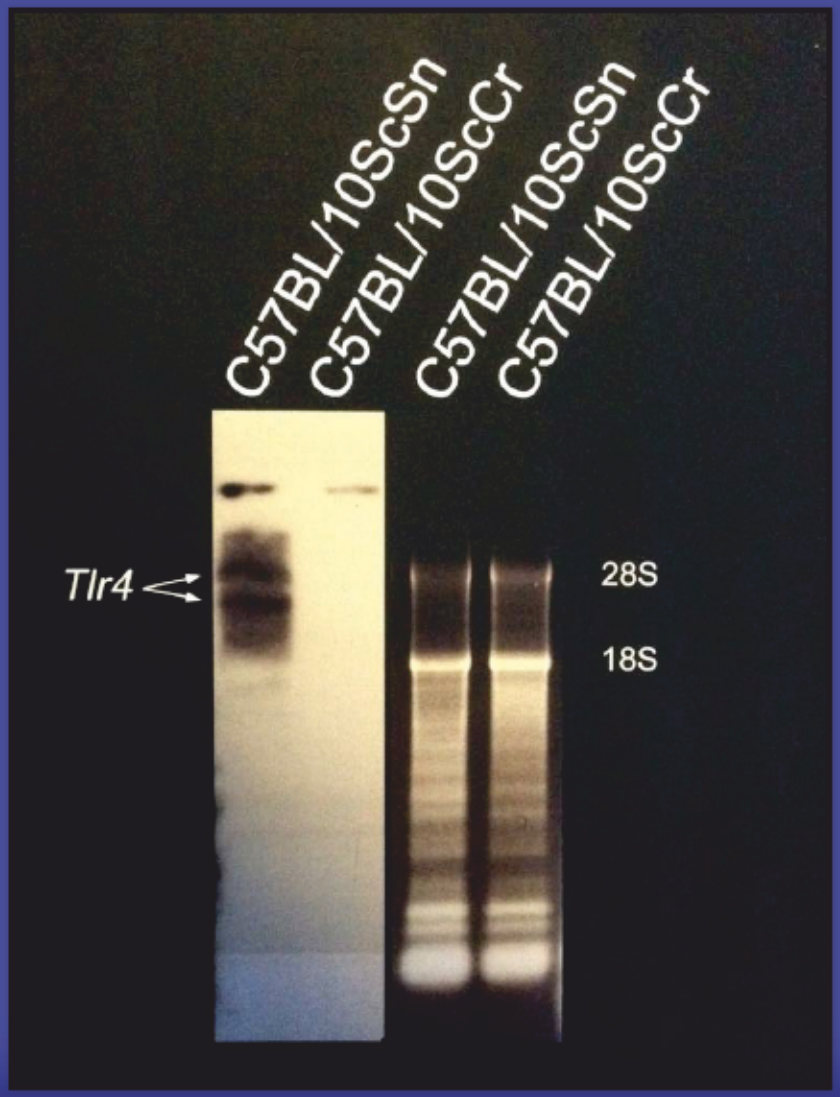
Screen shot of a mutation in TLR4 distinguishing C3H/HeJ from C3H/HeN mice

C3H/HeN

C3H/HeJ

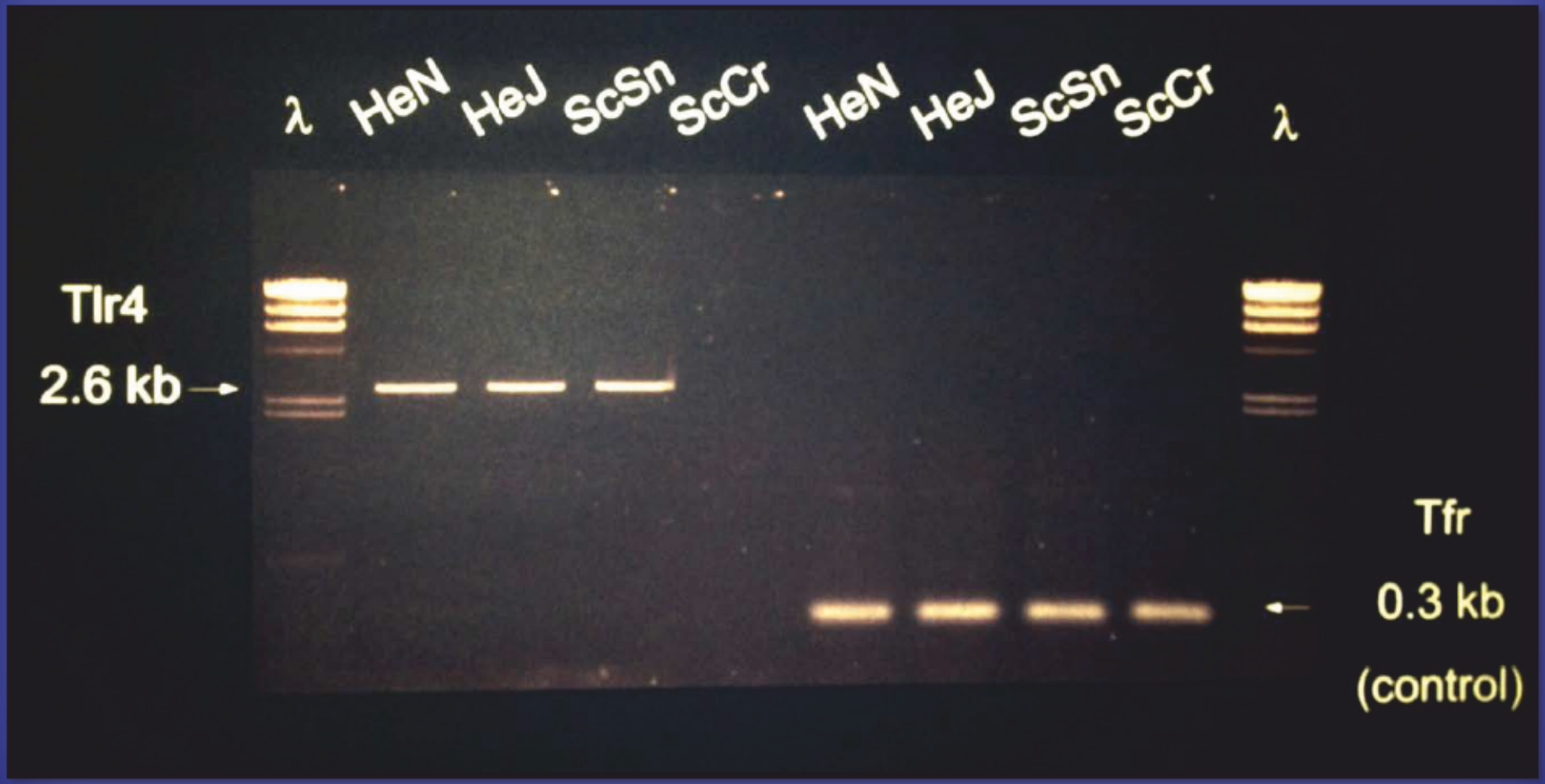
1998

On Northern blot analysis, C57BL/10ScCr mice appear not to express *Tlr4*



1998

RT-PCR also shows non-expression of Tlr4 in C57BL/10ScSr mice



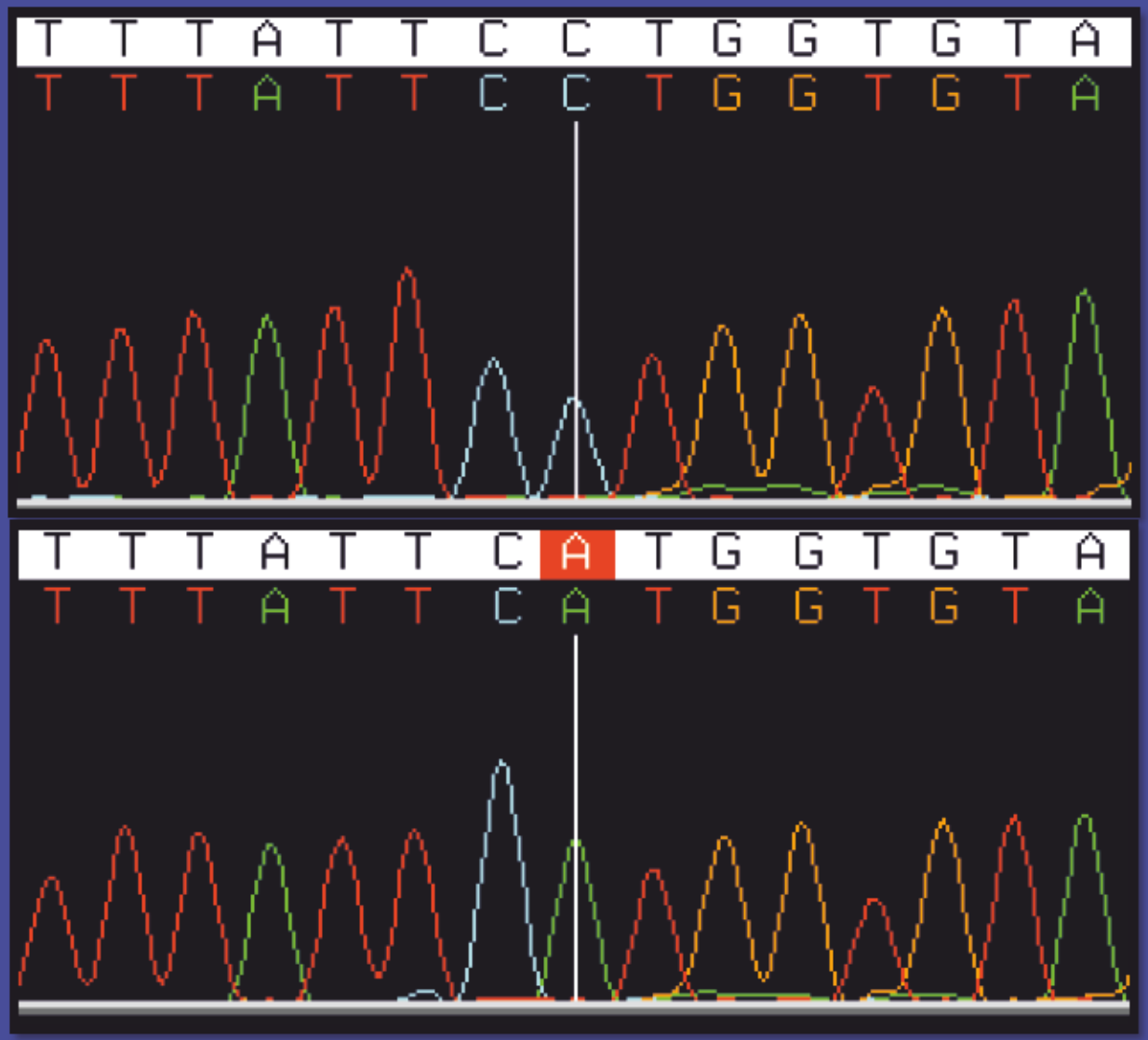
1998

712

… F I P G V …

C3H/HeN

Tlr4$^{lps\text{-}n}$



C3H/HeJ

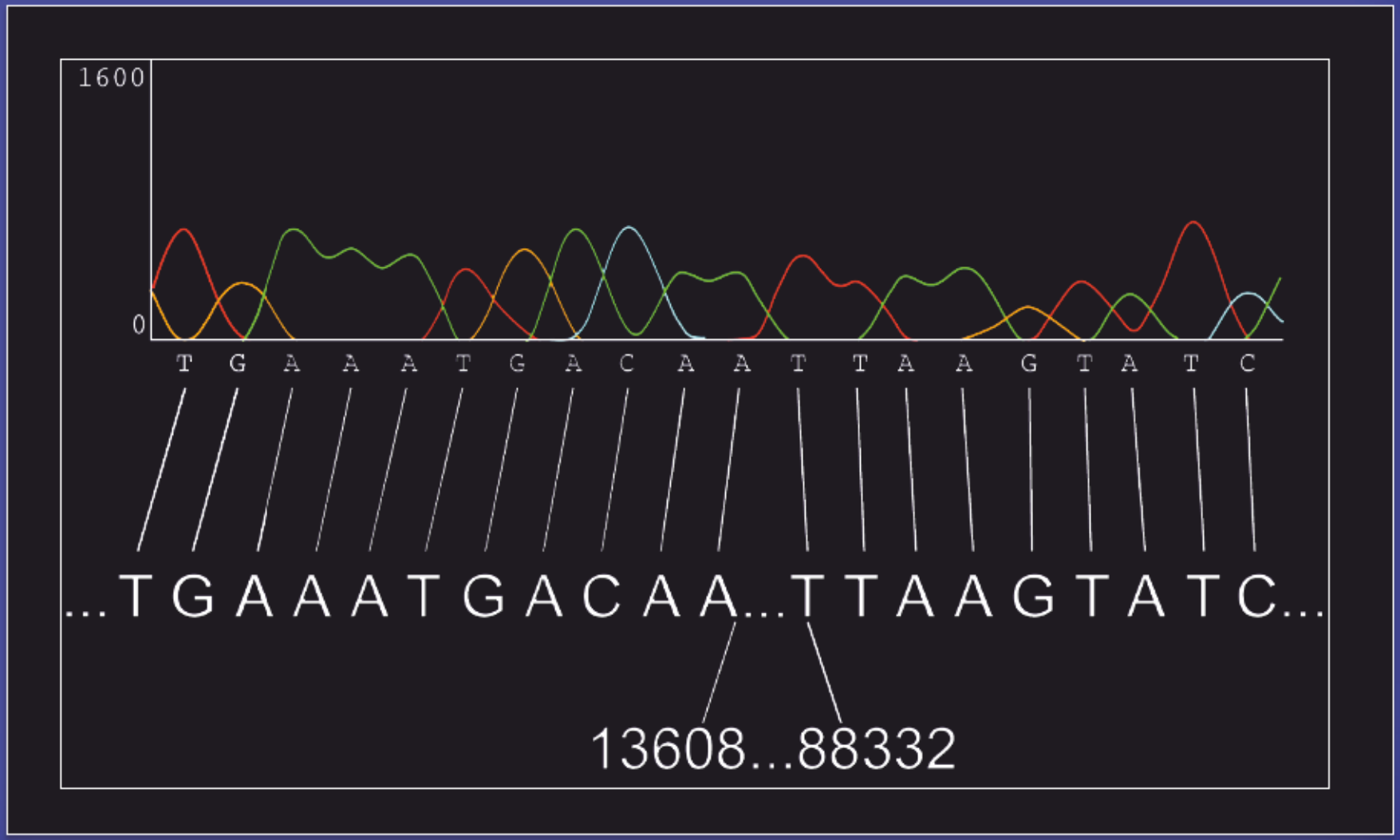
Tlr4$^{lps\text{-}d}$

… F I H G V …

1998

UT SOUTHWESTERN MEDICAL CENTER

Deletion of 74K in the C57BL/10ScCr mouse



26. F. Mutter, S. G. Clarkson, D. J. Galas, *Nucleic Acids Res.* **15**, 7191 (1987); A. Kressmann, S. G. Clarkson, V. Pirotta, M. L. Birnstiel, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1176 (1978).

the aminoacyl-AMS compounds. Supported by NIH grant GM30220.

9 September 1998; accepted 2 November 1998

Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in *Tlr4* Gene

Alexander Poltorak, Xiaolong He,* Irina Smirnova, Mu-Ya Liu,† Christophe Van Huffel,‡ Xin Du, Dale Birdwell, Erica Alejos, Maria Silva, Chris Galanos, Marina Freudenberg, Paola Ricciardi-Castagnoli, Betsy Layton, Bruce Beutler§

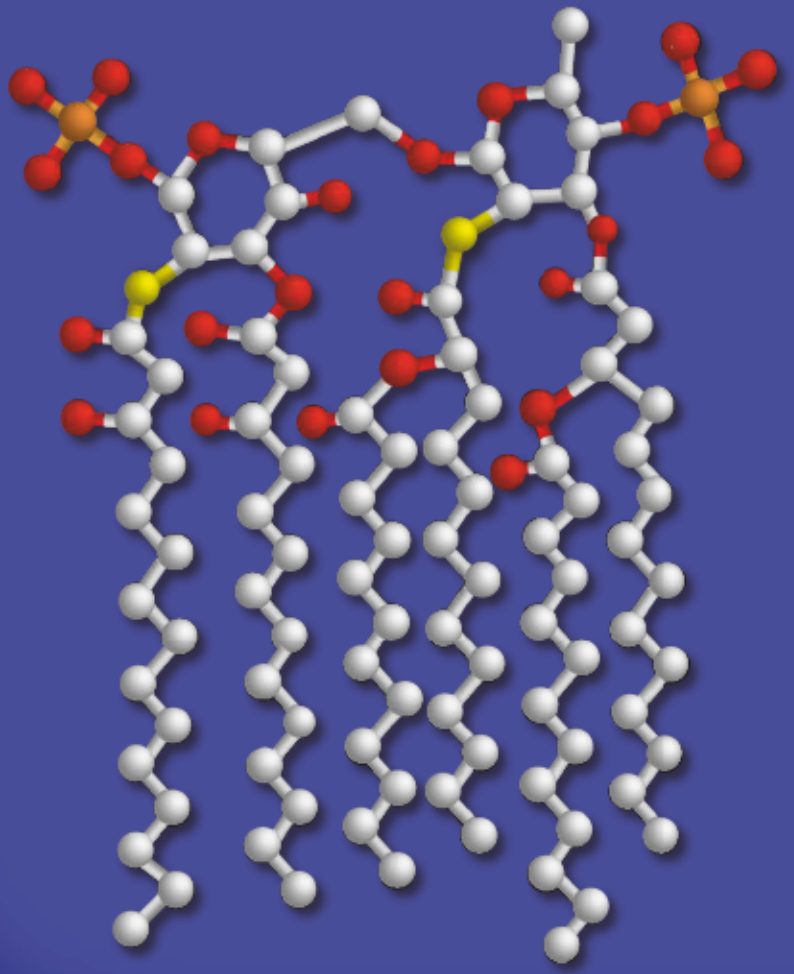
Mutations of the gene *Lps* selectively impede lipopolysaccharide (LPS) signal transduction in C3H/HeJ and C57BL/10ScCr mice, rendering them resistant to endotoxin yet highly susceptible to Gram-negative infection. The codominant Lps^d allele of C3H/HeJ mice was shown to correspond to a missense mutation in the third exon of the Toll-like receptor-4 gene (*Tlr4*), predicted to replace proline with histidine at position 712 of the polypeptide chain. C57BL/10ScCr mice are homozygous for a null mutation of *Tlr4*. Thus, the mammalian Tlr4 protein has been adapted primarily to subserve the recognition of LPS and presumably transduces the LPS signal across the plasma membrane. Destructive mutations of *Tlr4* predispose to the development of Gram-negative sepsis, leaving most aspects of immune function intact.

Conservative estimates hold that in the United States alone, 20,000 people die each year as a result of septic shock brought on by Gram-negative infection (*1*). The lethal effect of a Gram-negative infection is linked, in part, to the biological effects of bacterial lipopolysaccharide (endotoxin), which is produced by all Gram-negative organisms. A powerful activator of host mononuclear cells, LPS prompts the synthesis and release of tumor necrosis factor (TNF) and other toxic cytokines that ultimately lead to shock in

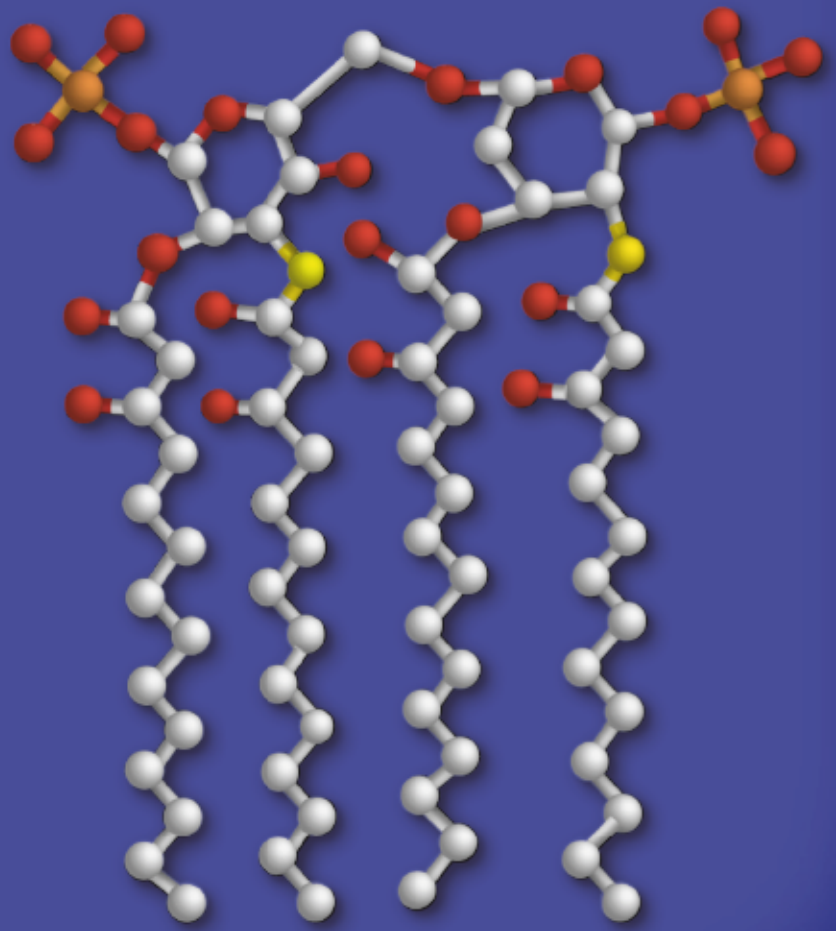
But the question remained:

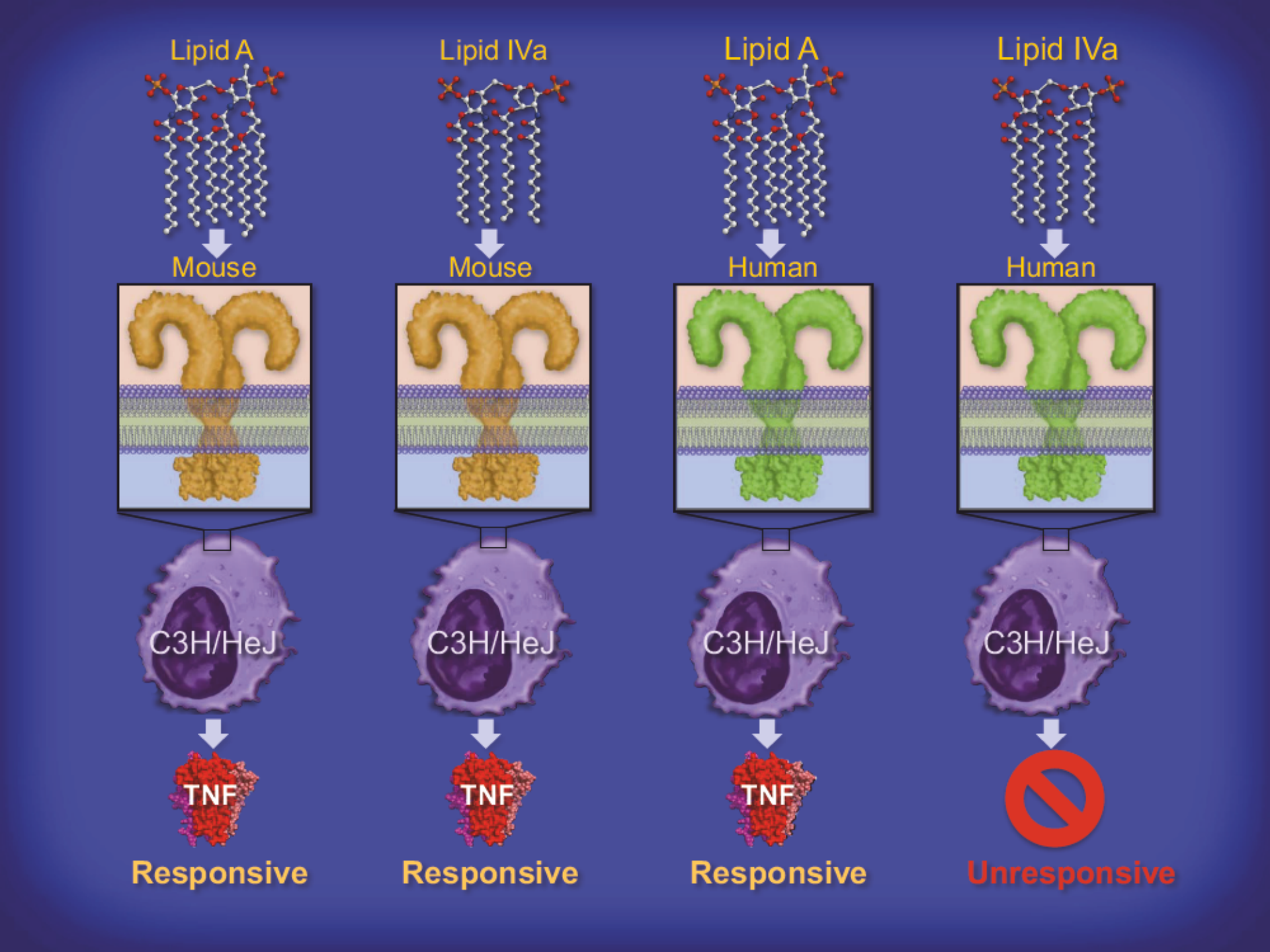
Was there direct contact between TLR and LPS?

Lipid A:
Agonist for both
mouse and human



Lipid IVa:
Agonist for mouse;
antagonist for human




Lipid A
Lipid IVa
Lipid A
Lipid IVa
Mouse
Mouse
Human
Human
C3H/HeJ
C3H/HeJ
C3H/HeJ
C3H/HeJ
TNF
TNF
TNF
Responsive
Responsive
Responsive
Unresponsive

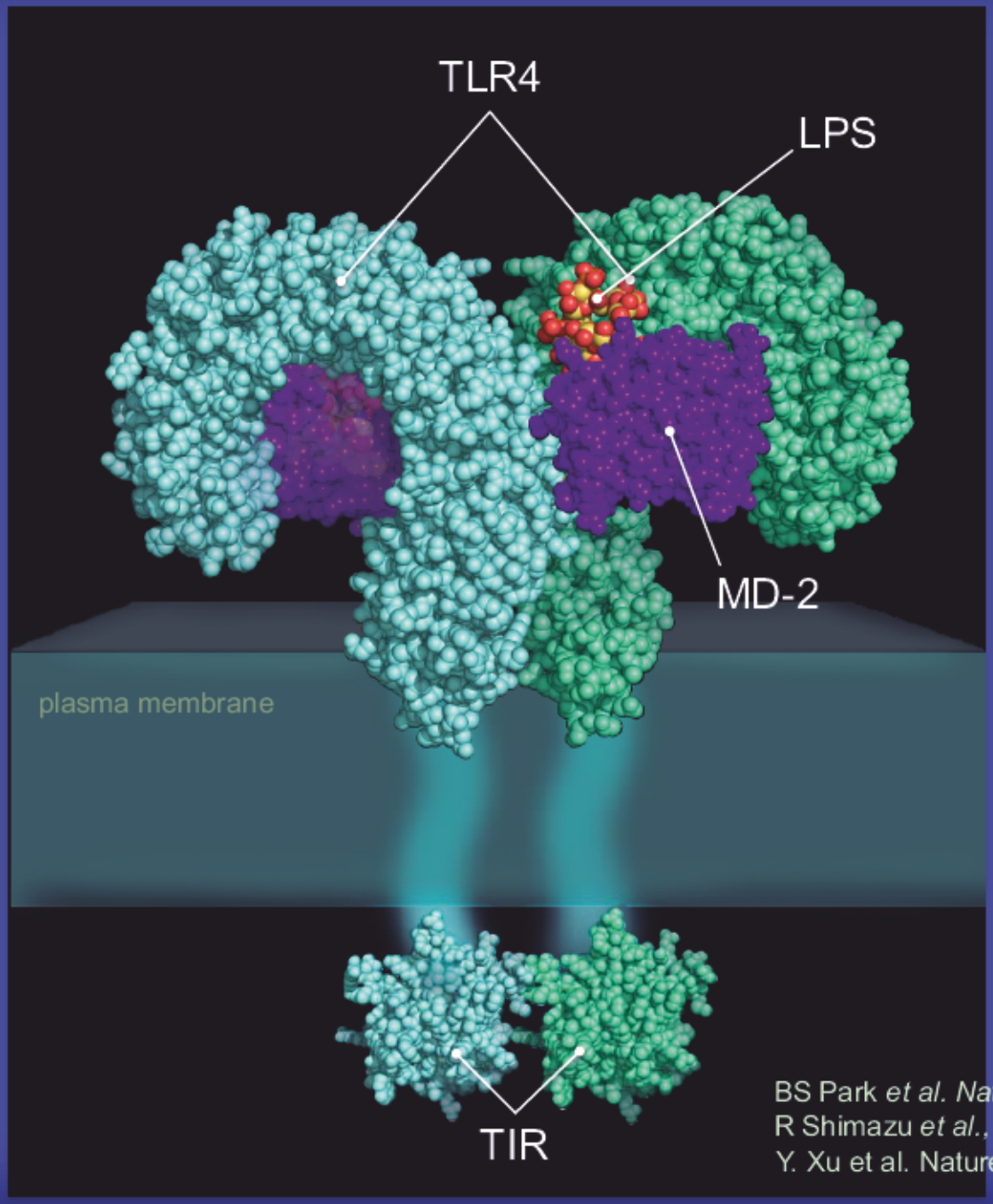
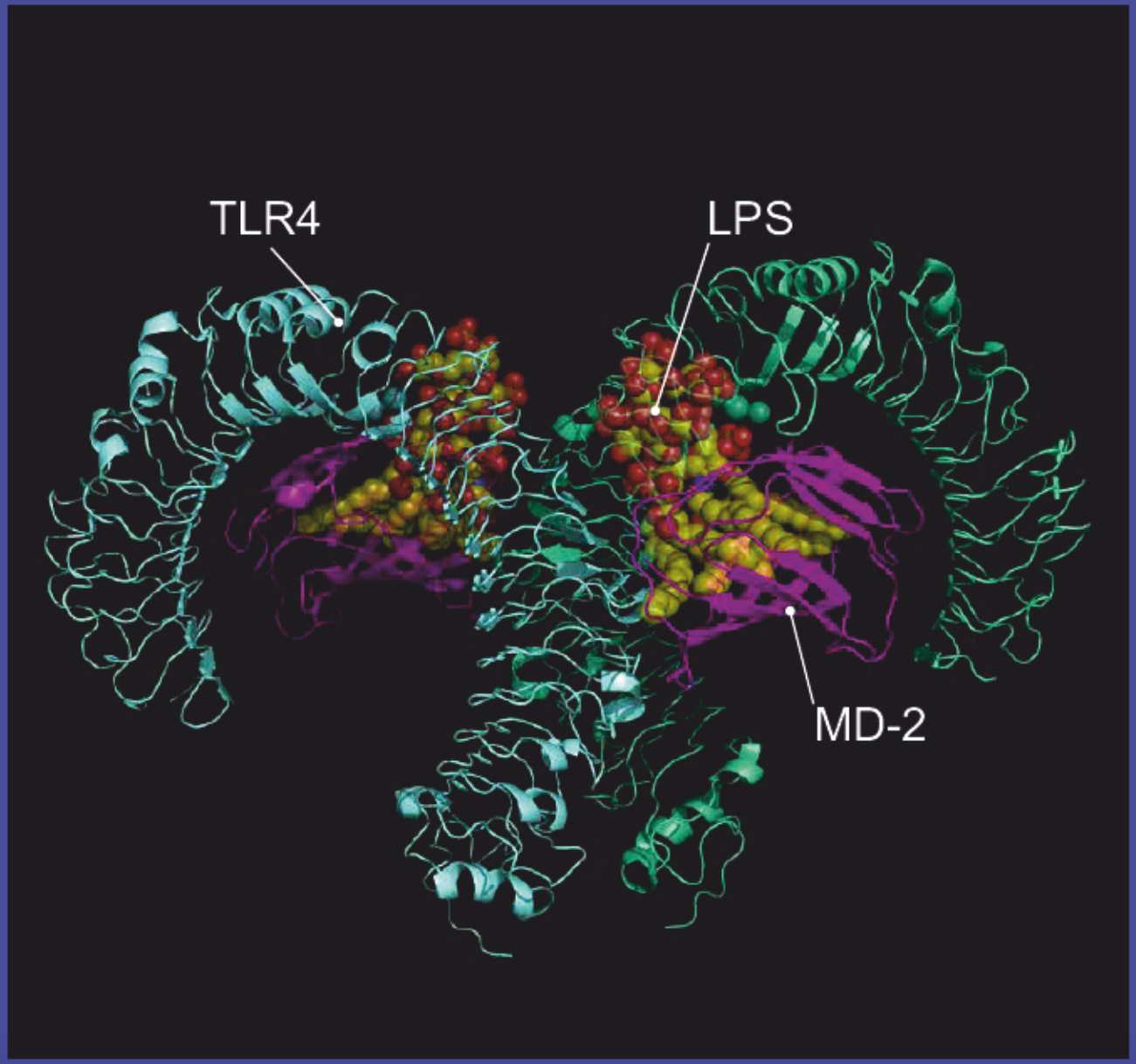
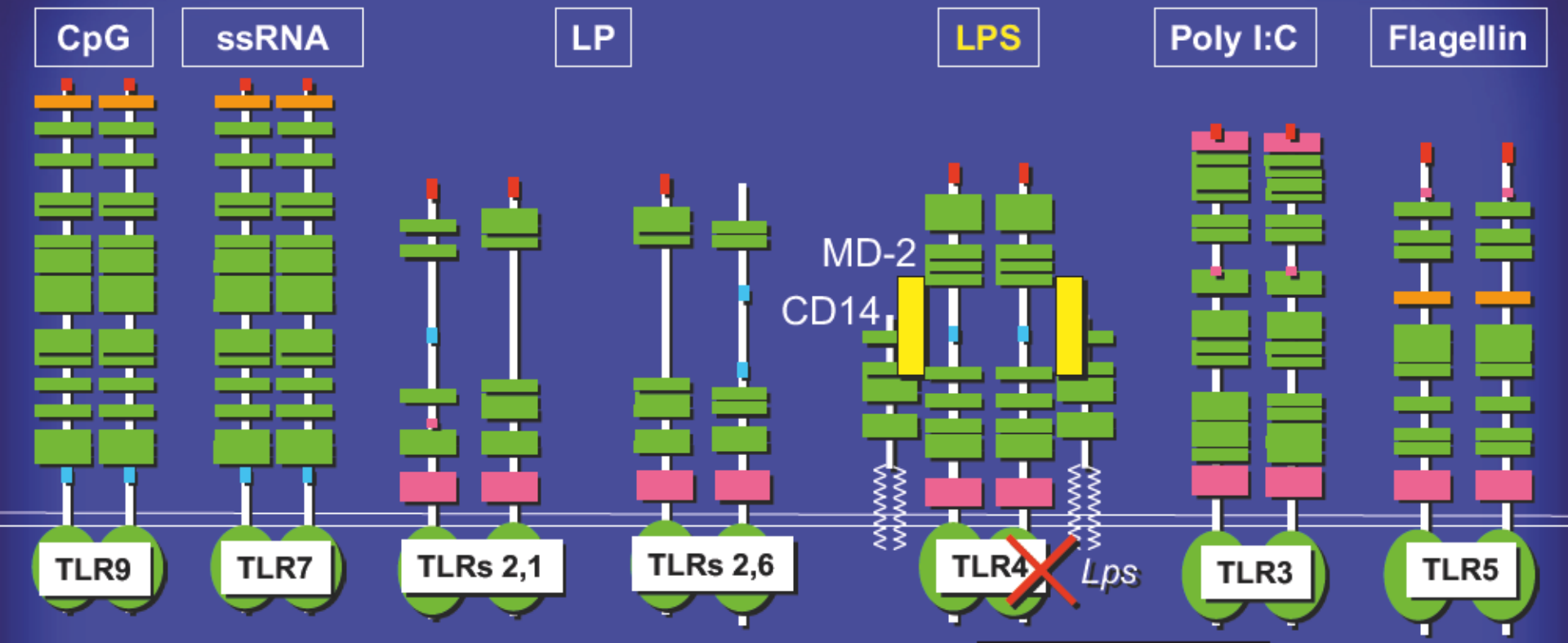


Image created using PyMol

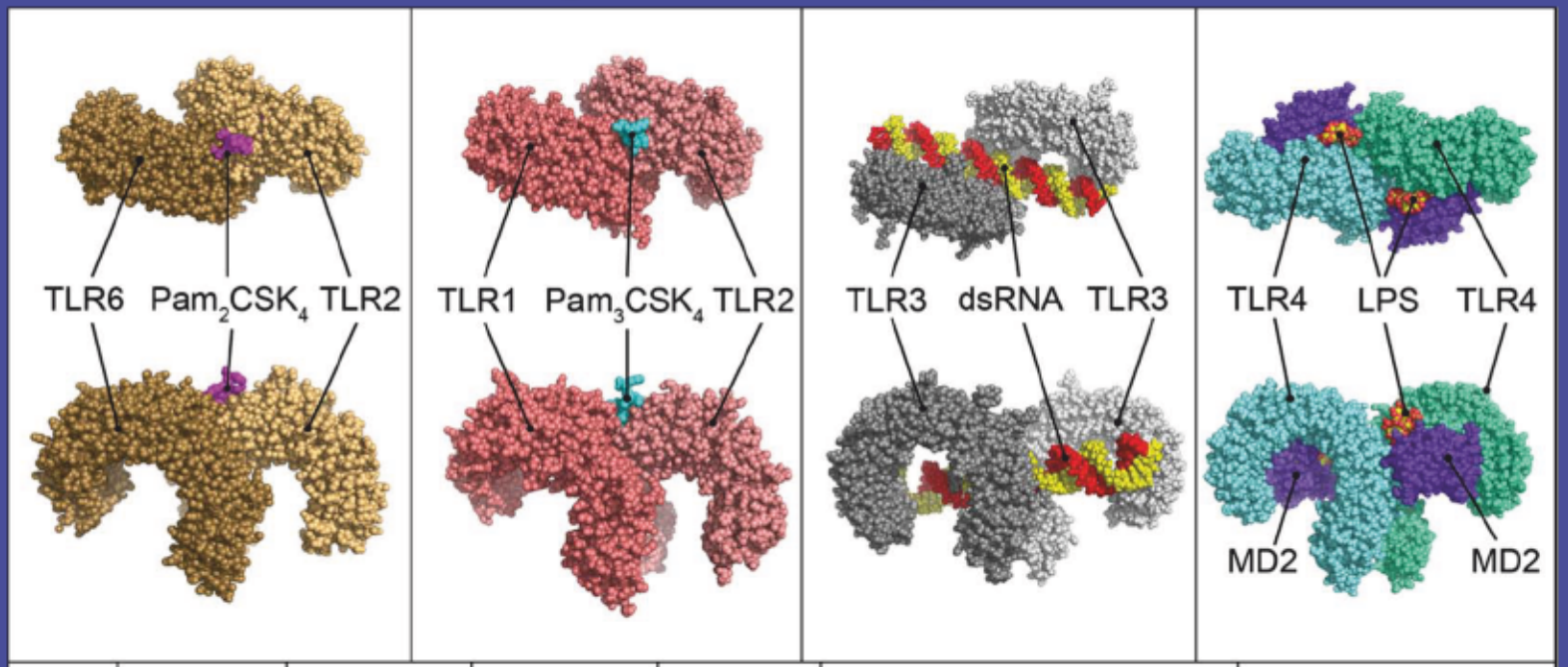
BS Park *et al. Nature* **458**, 7242 (2009)
R Shimazu *et al., J Exp Med* **189**, 11 (1999
Y. Xu et al. Nature 408, 6808 (2000)


TLR4
LPS
MD-2



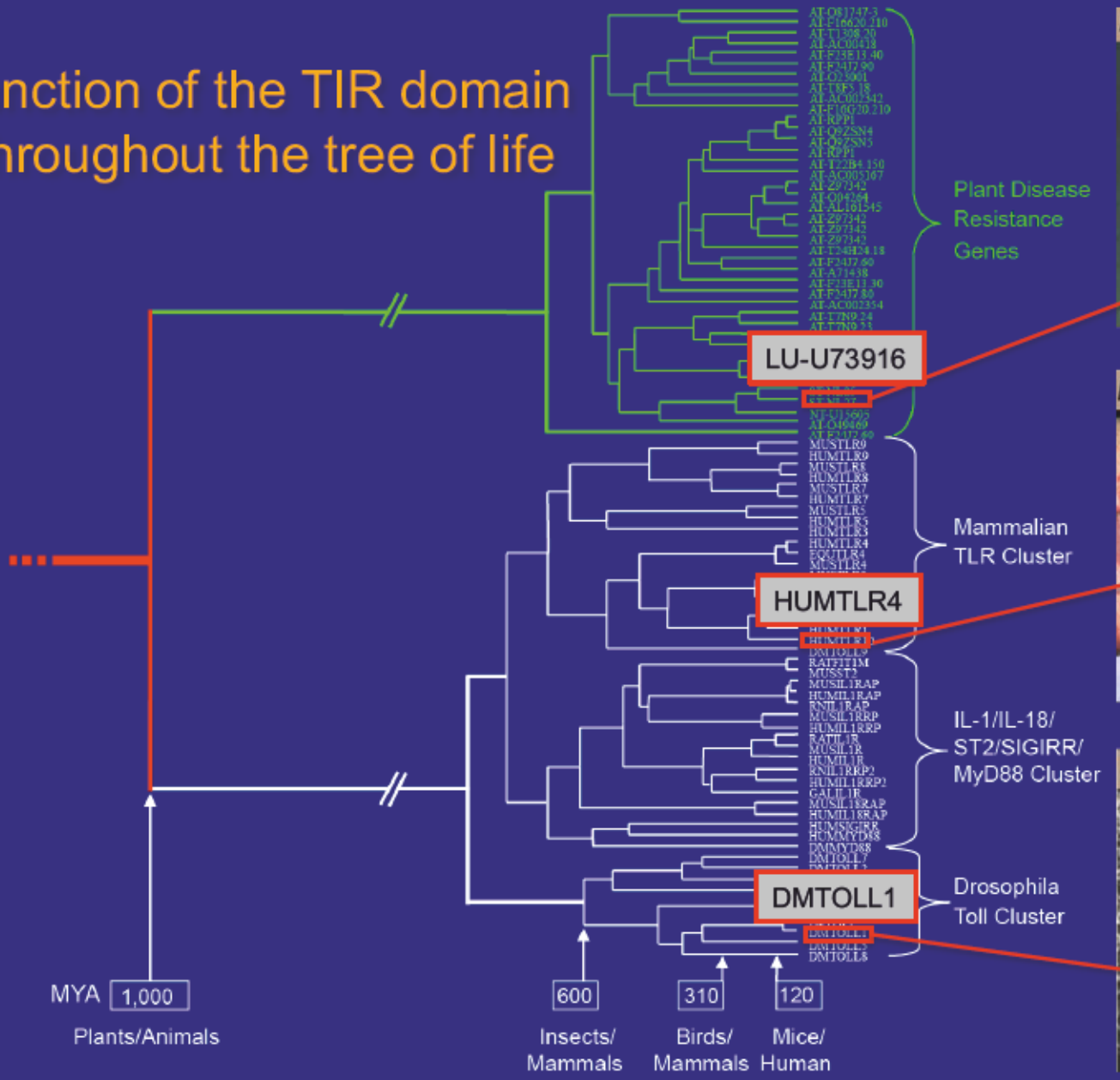
The role of TLR4 as a sensor of LPS suggested that other microbial ligands were sensed by other TLRs, and the specificity of most TLRs was revealed by knocking the genes out one at a time, largely in the Akira lab.

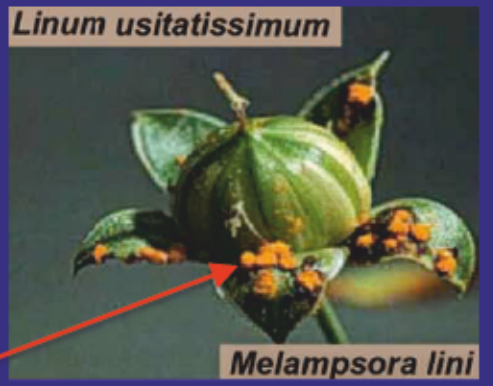
Now, the mode of binding of several ligands to TLRs is understood

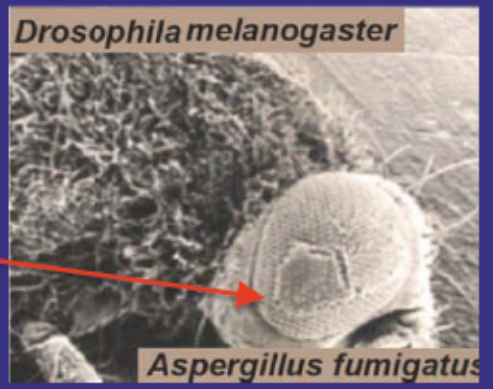


Receptor	TLR 2/1 or 2/6	TLR 3	TLR 4	TLR 5	TLR 7	TLR 9
Ligands	Lipopeptides	Poly I:C, dsRNA	LPS	Flagellin	ssRNA, resiquimod, imiquimod, loxoribine	Unmethylated DNA, CpG-DNA
Source	Gram-positive bacteria, fungi	Viruses	Gram-negative bacteria	Bacterial flagellum	Viruses	Bacteria
Examples	Pam_3CSK_4	dsRNA	LPS	Flagellin	ssRNA, Resiquimod, Imiquimod, Loxoribine	CpG-DNA

Function of the TIR domain throughout the tree of life

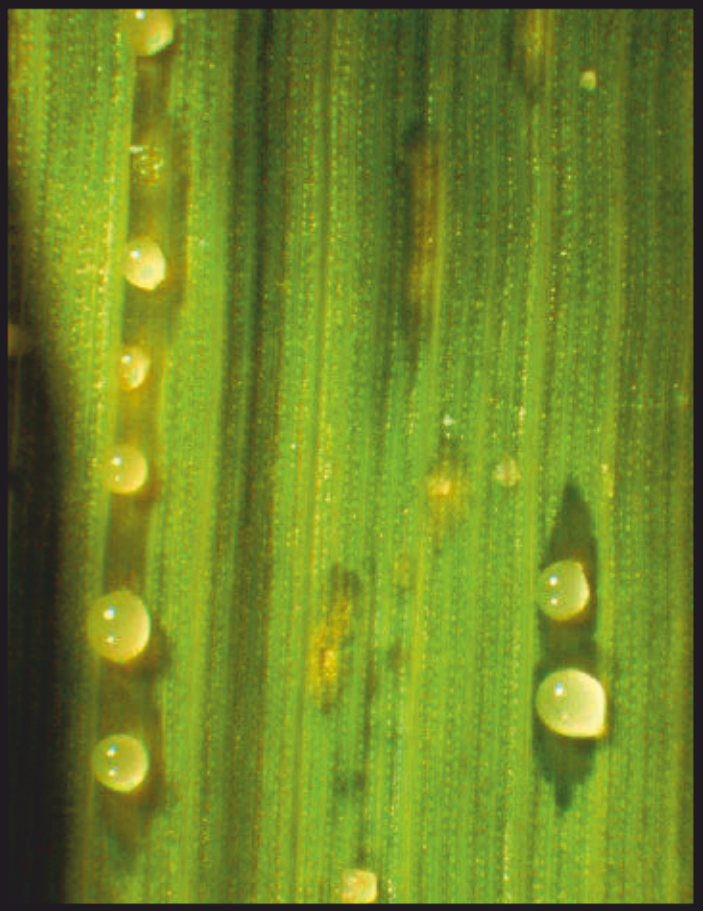






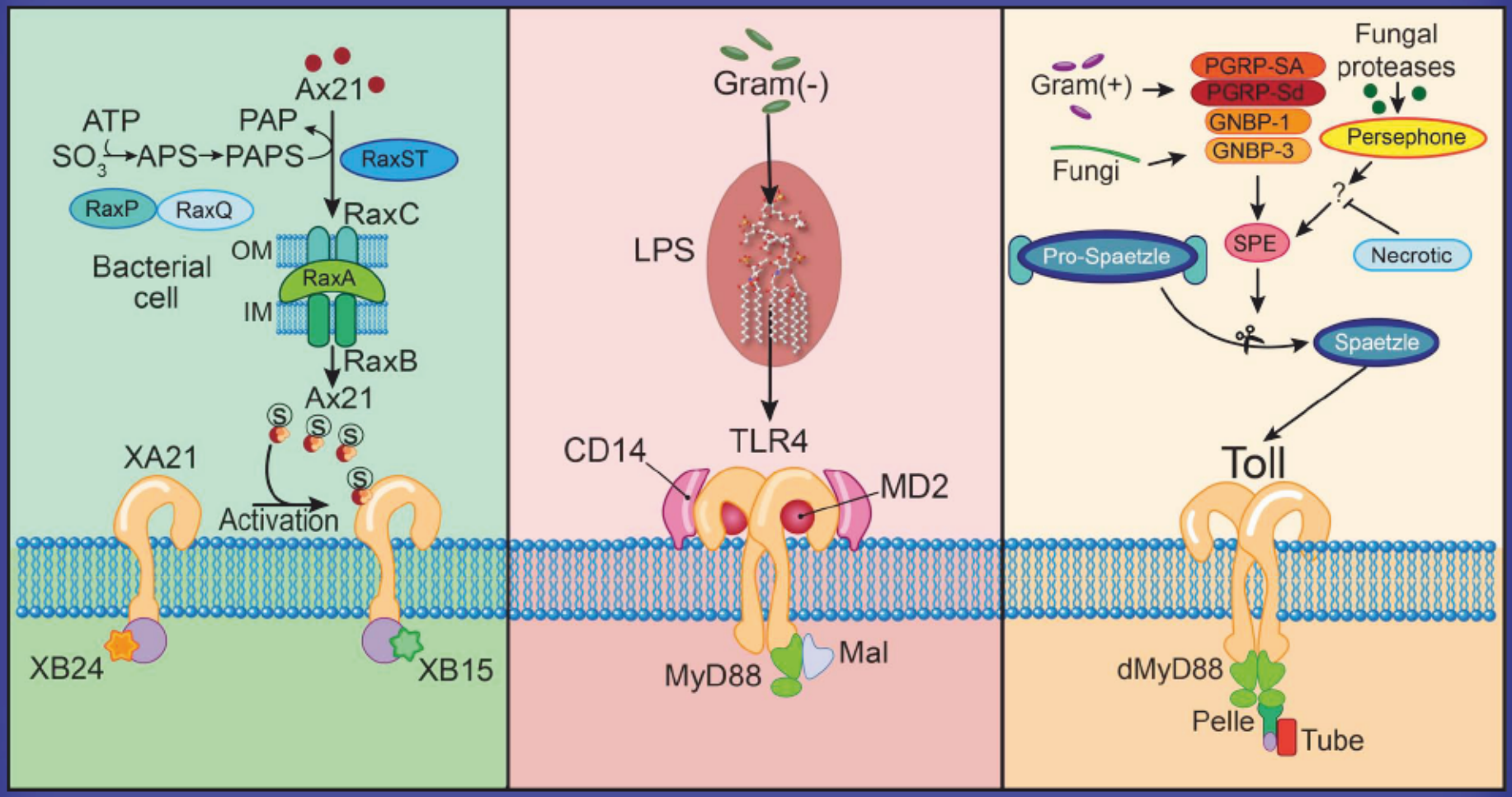
Bacterial blight of rice

Pamela Ronald
Discoverer of XA21

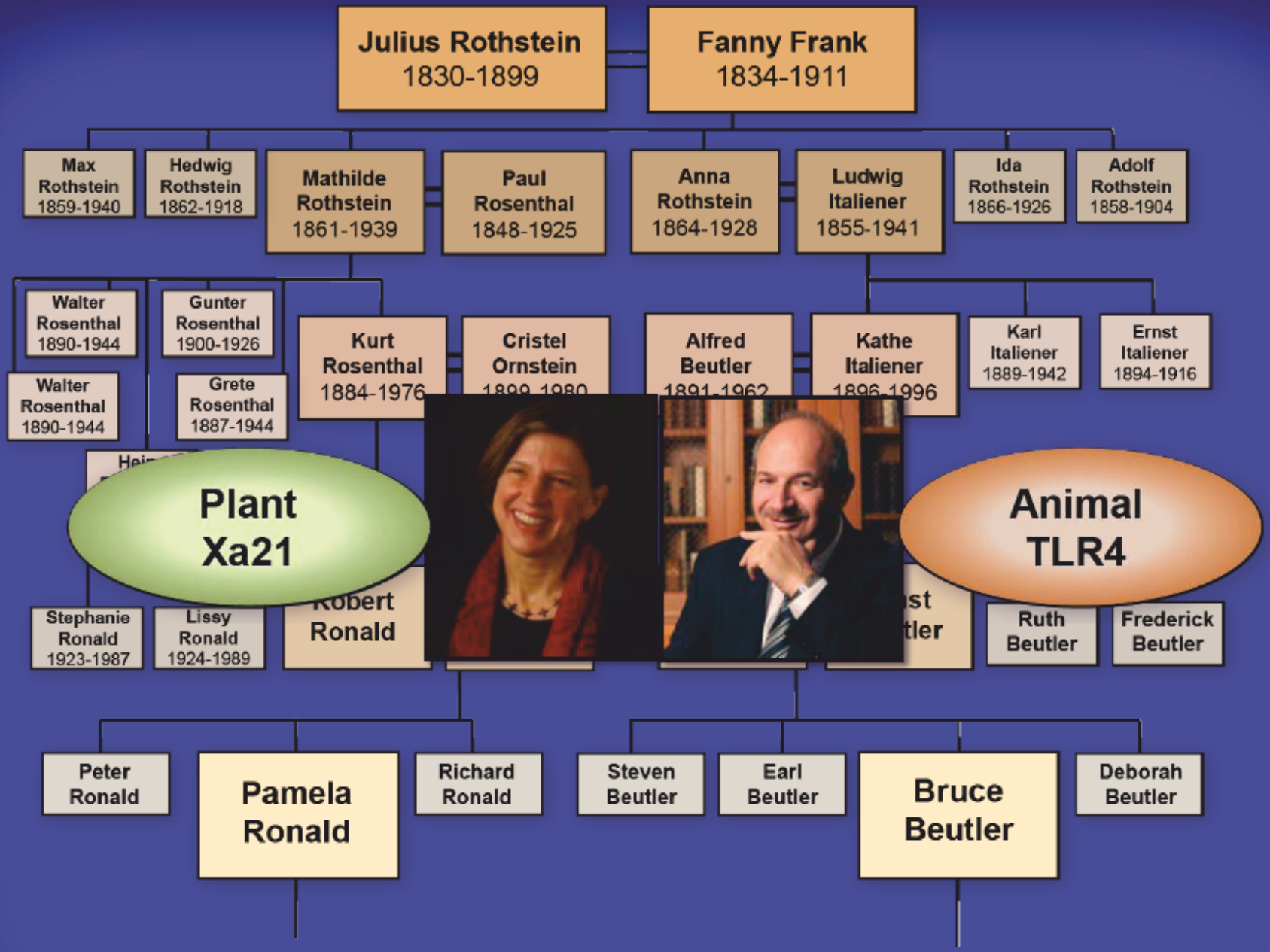


Xanthomonas oryzae (Xoo)

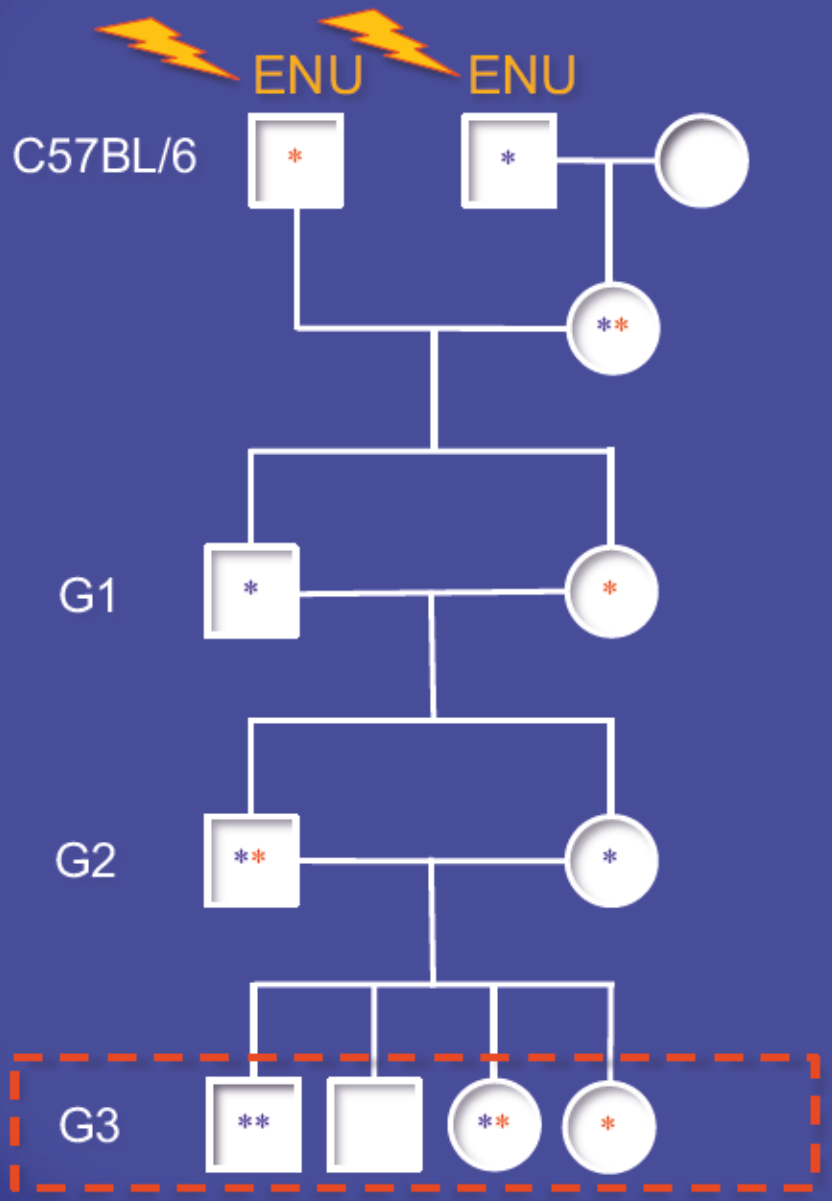
Images courtesy Dr. Pamela Ronald

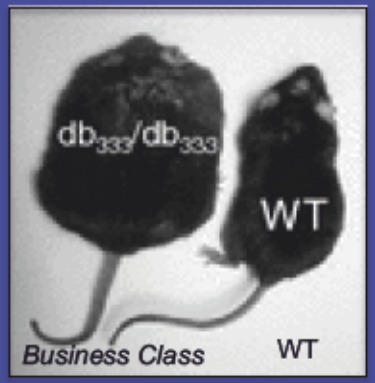


Monocots (Rice) Vertebrates (Mouse) Insects (Drosophila)


Julius Rothstein
1830-1899
Fanny Frank
1834-1911
Max Rothstein
1859-1940
Hedwig Rothstein
1862-1918
Mathilde Rothstein
1861-1939
Paul Rosenthal
1848-1925
Anna Rothstein
1864-1928
Ludwig Italiener
1855-1941
Ida Rothstein
1866-1926
Adolf Rothstein
1858-1904
Walter Rosenthal
1890-1944
Gunter Rosenthal
1900-1926
Walter Rosenthal
1890-1944
Grete Rosenthal
1887-1944
Kurt Rosenthal
1884-1976
Cristel Ornstein
Alfred Beutler
Kathe Italiener
1896-1996
Karl Italiener
1889-1942
Ernst Italiener
1894-1916
Plant
Xa21
Animal
TLR4
Stephanie Ronald
1923-1987
Lissy Ronald
1924-1989
Robert Ronald
Ruth Beutler
Frederick Beutler
Peter Ronald
Pamela Ronald
Richard Ronald
Steven Beutler
Earl Beutler
Bruce Beutler
Deborah Beutler

Making new phenotypes in mice



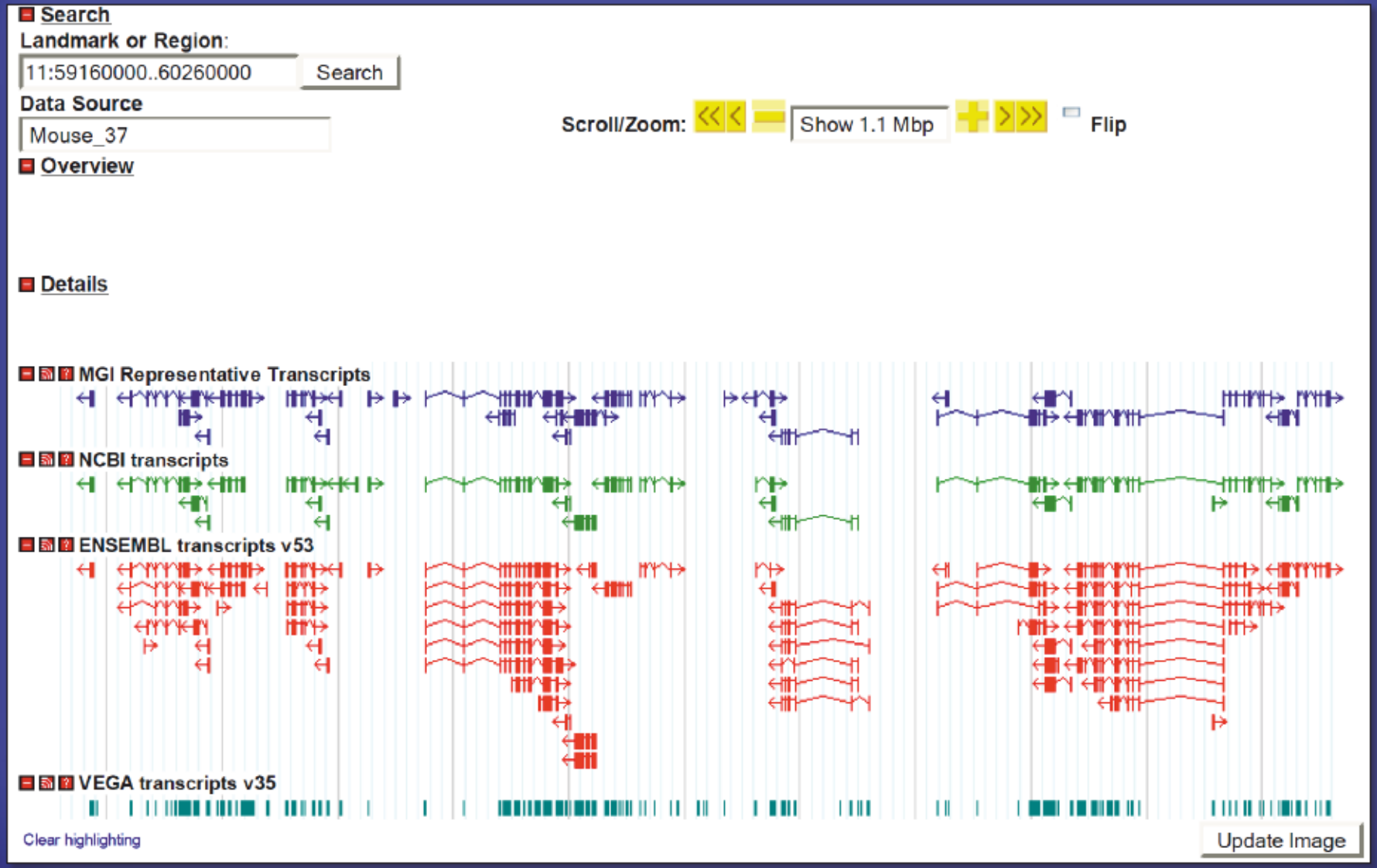


As of 9/20/11, >151,000 G1 + G3 mice produced. ~60 coding/splicing changes per sperm.

The process of finding mutations was greatly accelerated by the sequencing and annotation of the mouse genome…

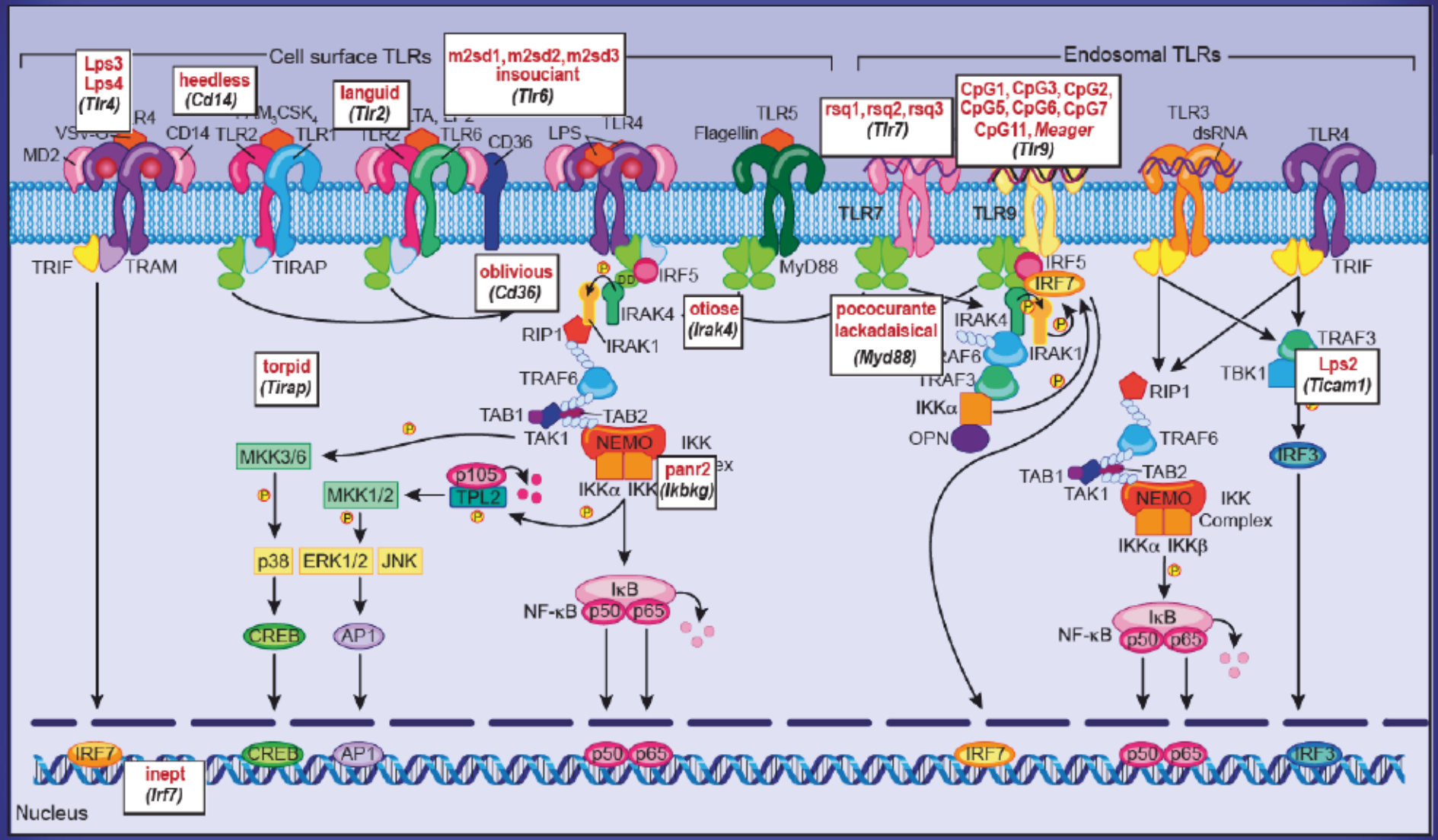
Never again was it necessary to make a BAC contig or search for genes.

MGI genome browser (typical view)

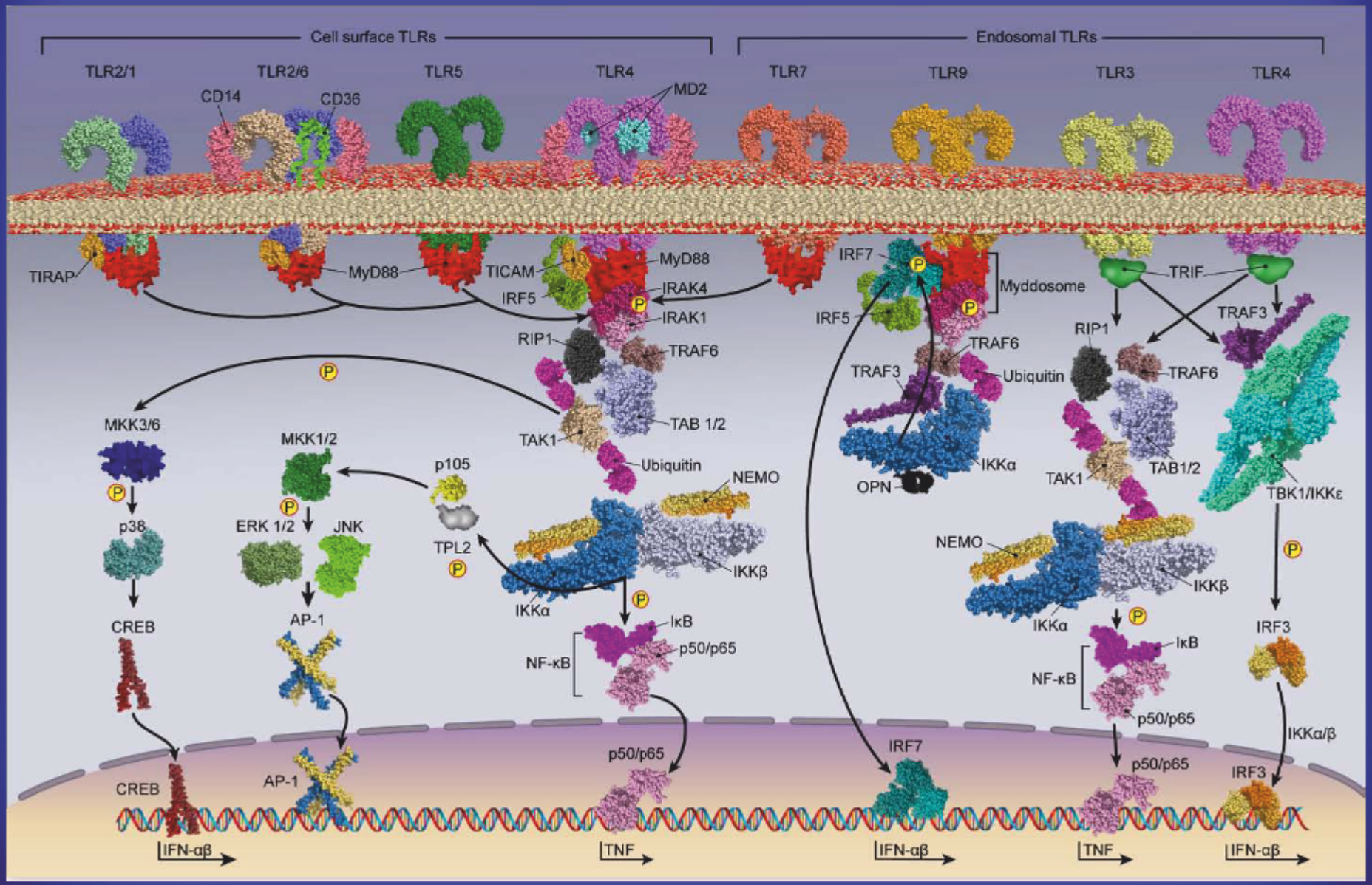


Massively parallel short read sequencing has made targeted exon sequencing unnecessary in most cases.

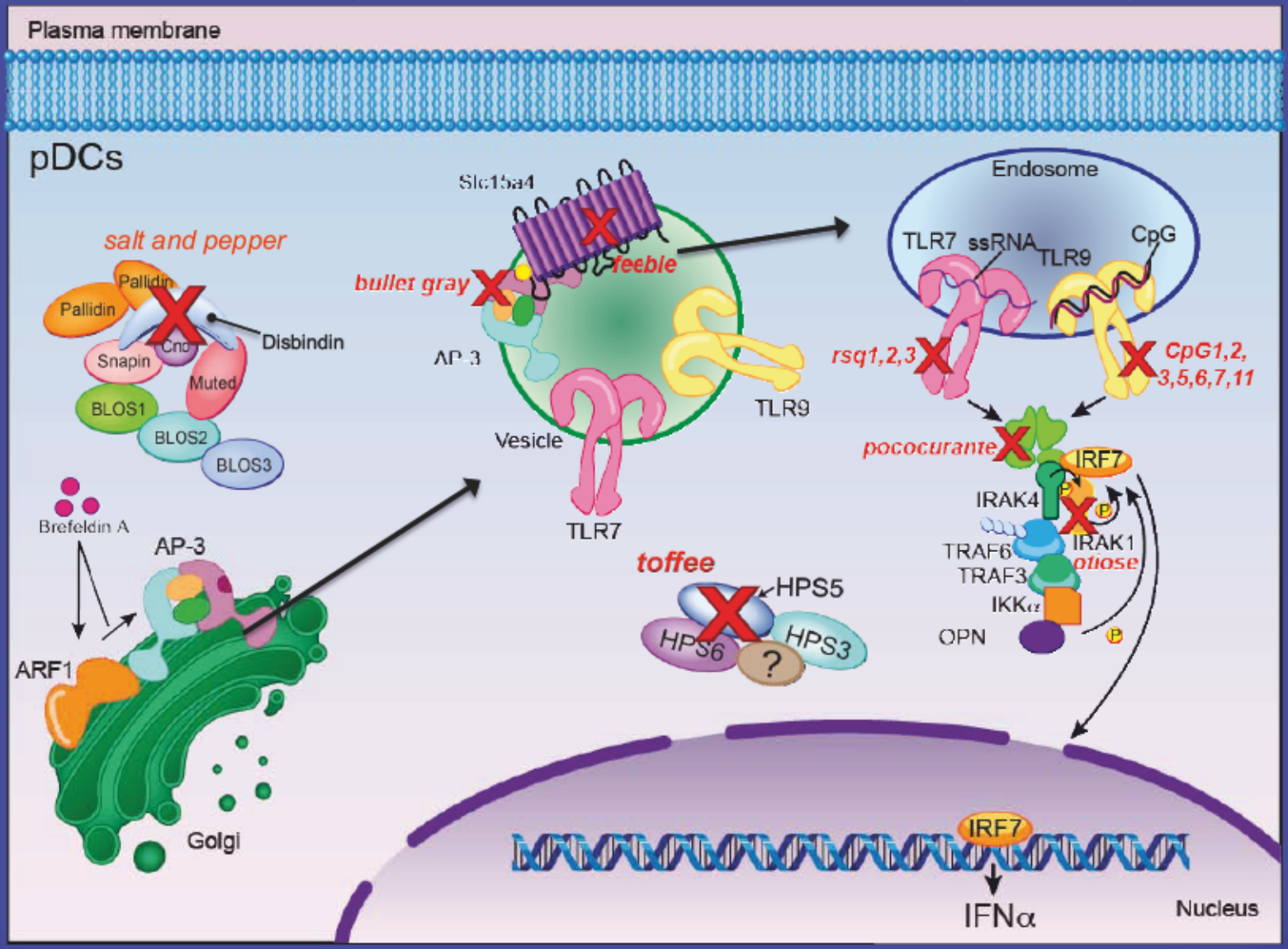
- The cost of sequencing a mouse genome to >90% coverage is now about $3,000. About 4 mouse genomes can be sequenced per week in our lab.
- The price per base pair continues to drop by about 80% each year.
- With minimal mapping, it is possible to find mutations extremely quickly.

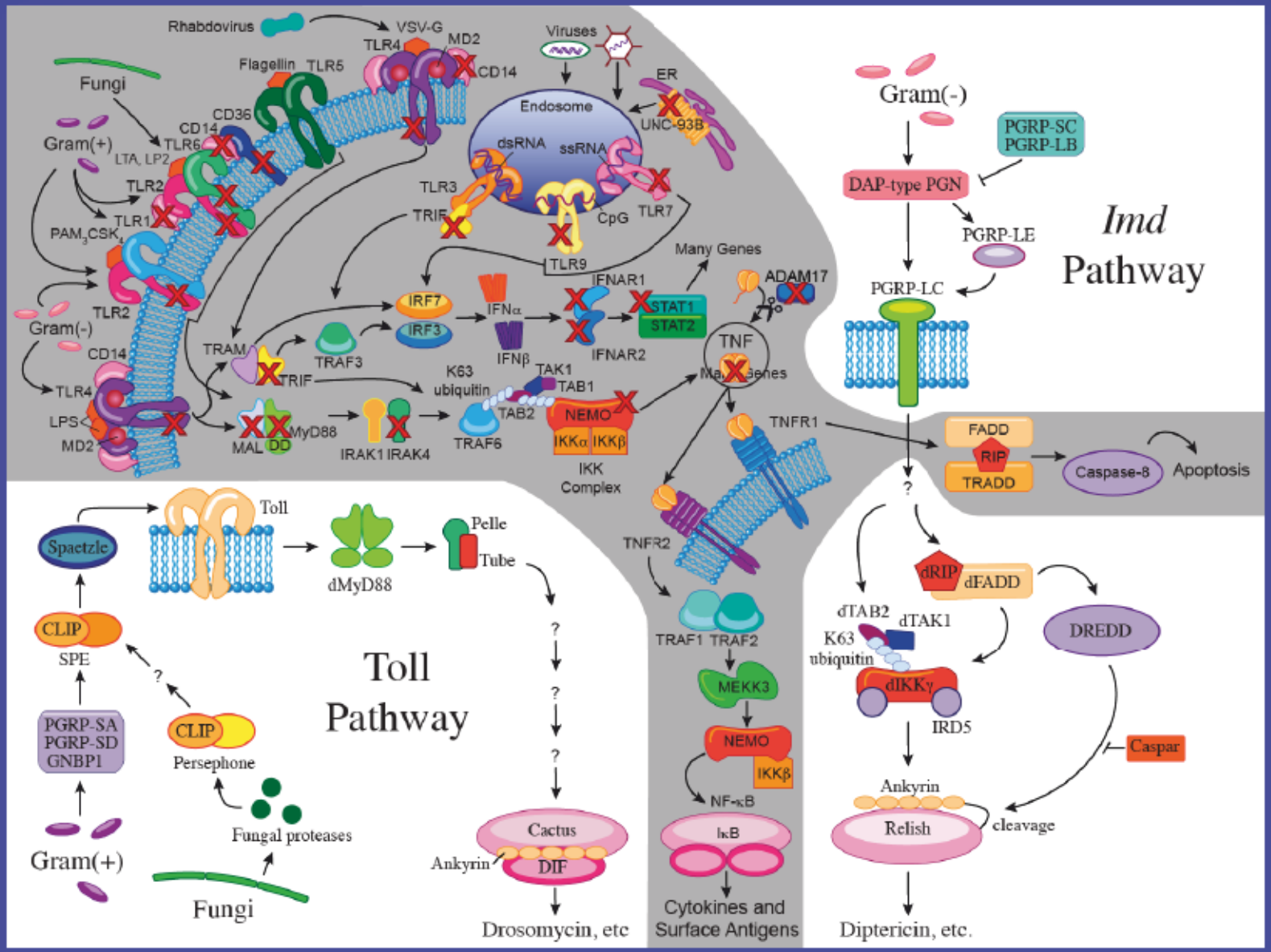


From genetics to structures to mechanism…

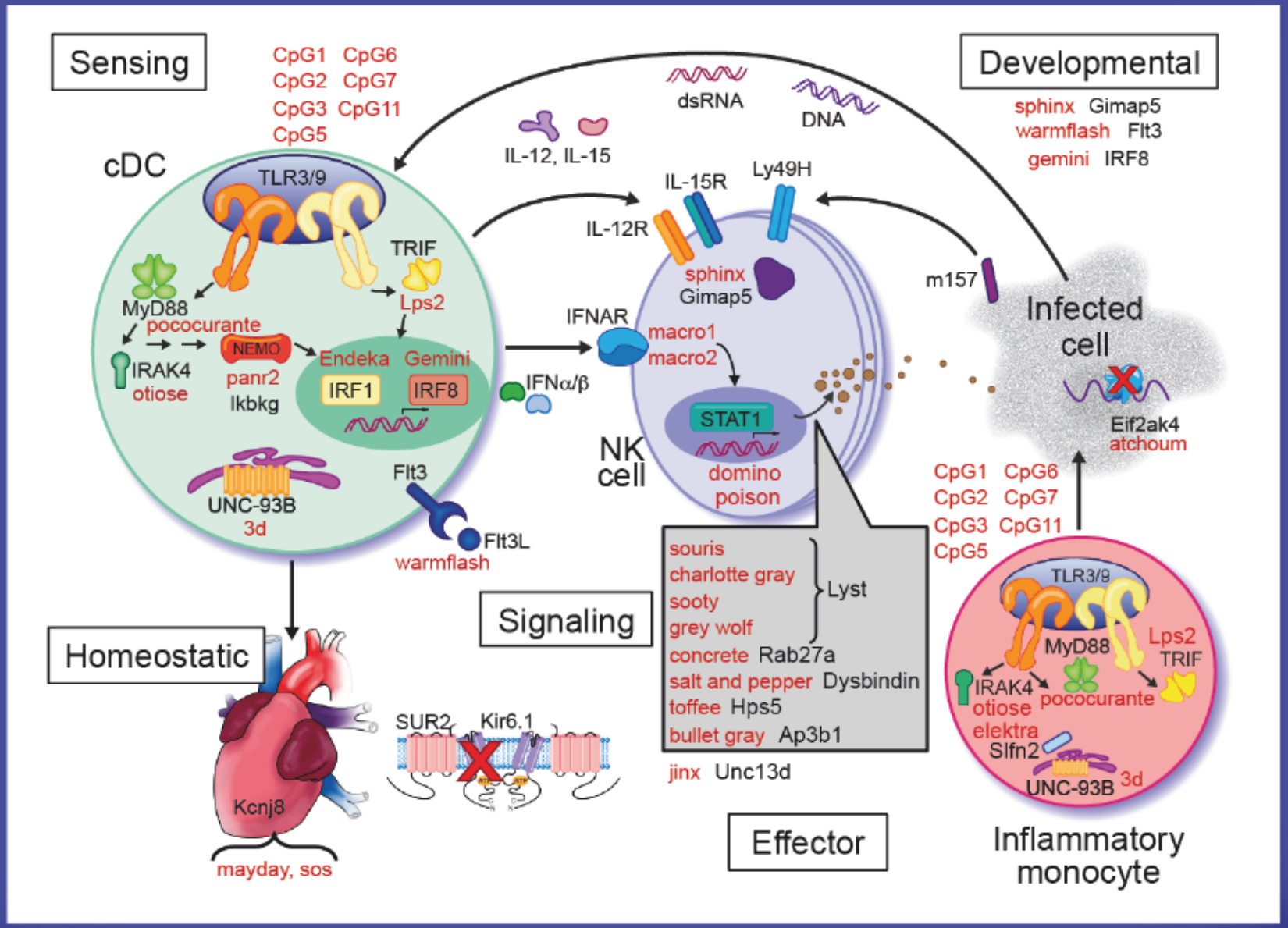


In plasmacytoid dendritic cells, specialized machinery is needed for TLR signaling

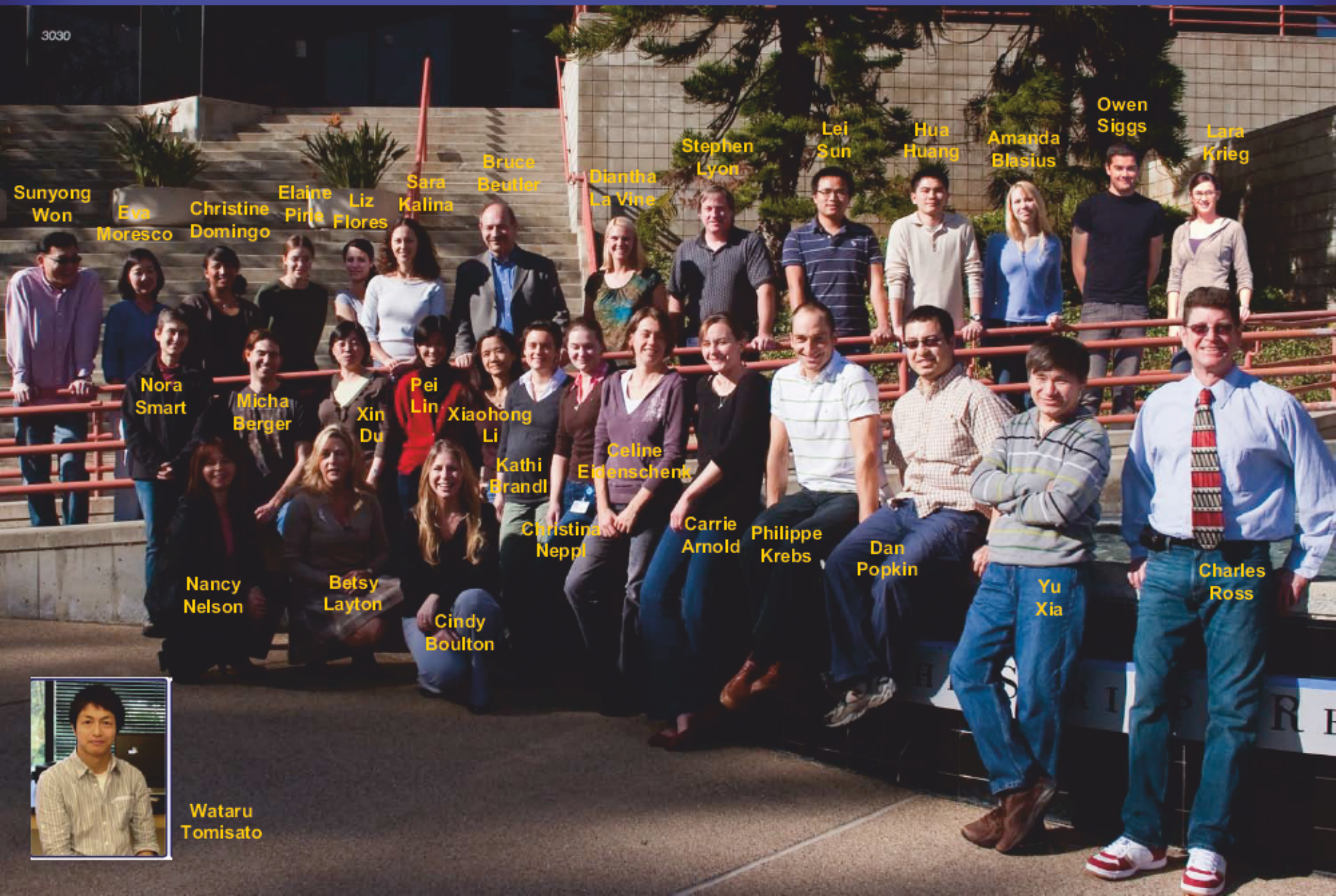



Rhabdovirus
VSV-G
TLR4
MD2
CD14
Flagellin
TLR5
Fungi
CD36
CD14
TLR6
Gram(+)
LTA, LP2
TLR2
TLR1
PAM3CSK4
TLR2
Gram(-)
CD14
TLR4
LPS
MD2
Viruses
ER
Endosome
UNC-93B
dsRNA
ssRNA
TLR3
TRIF
CpG
TLR7
TLR9
TRAM
TRIF
TRAF3
IRF7
IRF3
IFNα
IFNβ
IFNAR1
IFNAR2
STAT1
STAT2
Many Genes
ADAM17
TNF
Many Genes
K63
ubiquitin
TAK1
TAB1
TAB2
NEMO
IKKα
IKKβ
IKK
Complex
MAL
MyD88
DD
IRAK1
IRAK4
TRAF6
TNFR1
TNFR2
TRAF1
TRAF2
MEKK3
NEMO
IKKβ
NF-κB
IκB
Cytokines and
Surface Antigens
FADD
RIP
TRADD
Caspase-8
Apoptosis
?
Gram(-)
PGRP-SC
PGRP-LB
DAP-type PGN
PGRP-LE
PGRP-LC
Imd
Pathway
dRIP
dFADD
dTAB2
dTAK1
K63
ubiquitin
dIKKγ
IRD5
DREDD
Caspar
Ankyrin
Relish
cleavage
Diptericin, etc.
Toll
Spaetzle
CLIP
SPE
PGRP-SA
PGRP-SD
GNBP1
CLIP
Persephone
Fungal proteases
Gram(+)
Fungi
Toll
Pathway
dMyD88
Pelle
Tube
?
?
?
Cactus
Ankyrin
DIF
Drosomycin, etc

Mutations we have found to cause MCMV susceptibility



Beutler Lab, December 15, 2009



Others not shown in the last slide

- Jiangfan Jiang
- Kasper Hoebe
- Ben Croker
- Koichi Tabeta
- Karine Crozat
- Sophie Rutschmann
- Philippe Georgel

For the cloning of the *Lps* locus

- Alexander Poltorak
- Irina Smirnova
- Christophe Van Huffel
- Betsy Layton
- Xiaolong He
- Mu-Ya Liu
- Xin Du
- Dale Birdwell
- Erica Alejos

- Chris Galanos and Marina Freudenberg (Max Planck Institute fur Immunbiologie, Freiburg)

- Paola Ricciardi-Castagnoli (University of Milan)