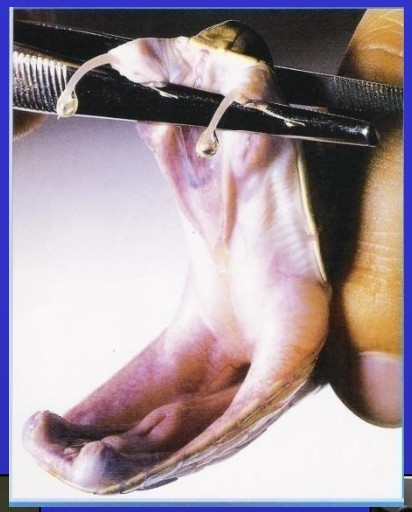
Produção de Vacinas no Brasil: Problemas, Perpectivas e Desafios Estratégicos

Monofosforil Lipídio A (MPLA) do Instituto Butantan

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2013



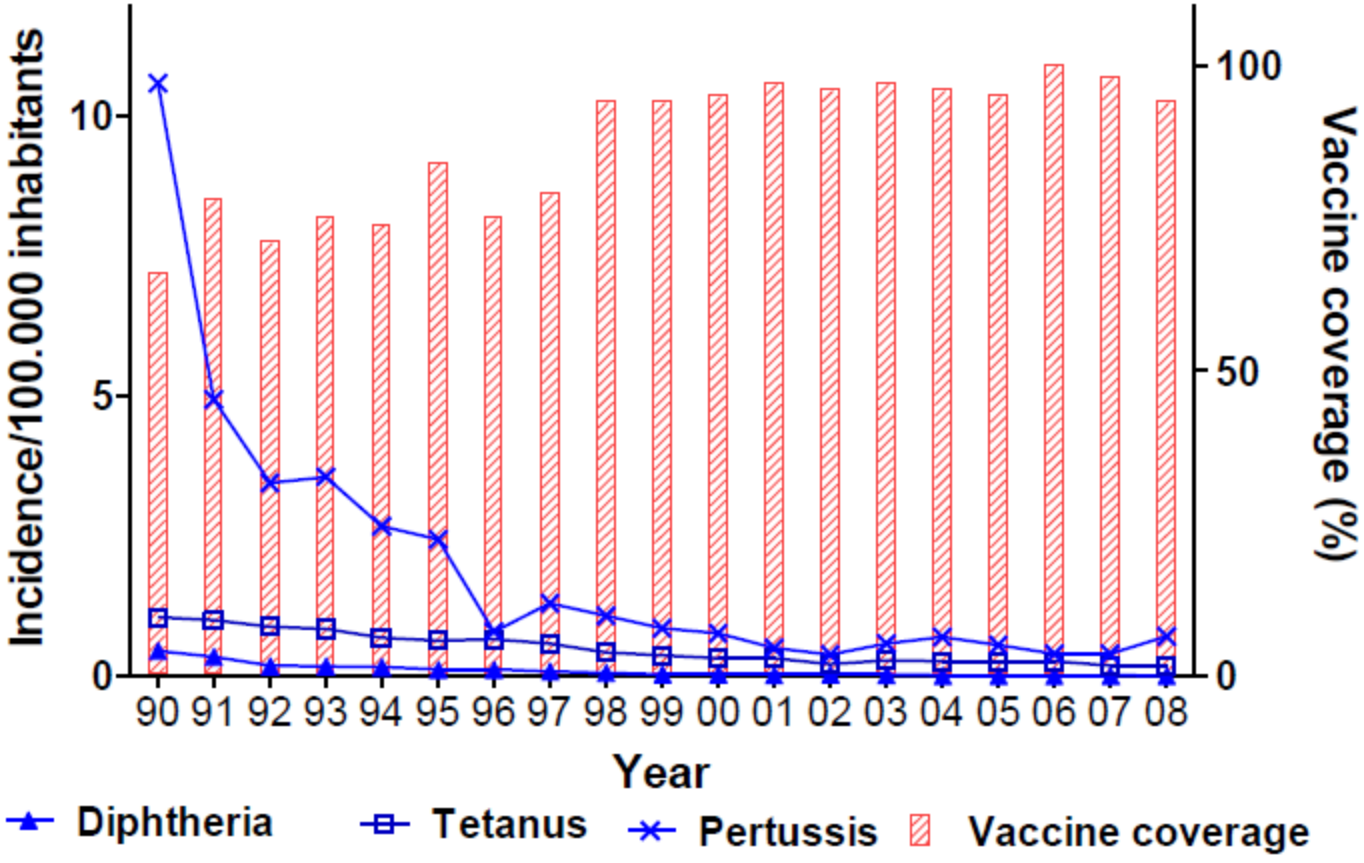




Instituto Butantan: An Important Public Manufacturer of Biologicals and Immunobiologicals in Brazil and Latin America

Instituto Butantan

Instituto Butantan:	Annual production of 400-700 thousands of ampule of sera
Vaccines produced:	Combined DPT – Diphteria, pertussis and tetanus. Hepatitis B (first recombinant vaccine in Brazil) *Rabies vaccine produced in VERO cells for human use, seasonal (and pandemic) influenza
	*The production was interrupted and a new production plant is being built and will start working on 2014.


Incidence/100.000 inhabitants
10
5
0
100
50
0
Vaccine coverage (%)
90 91 92 93 94 95 96 97 98 99 00 01 02 03 04 05 06 07 08
Year
Diphtheria
Tetanus
Pertussis
Vaccine coverage

Public mission:

Products of quality and affordable to the public system, to be provided as a benefit free of charge to all the Brazilians through the Public Health System (SUS) by the Ministry of Health.

Innovation and Development of new vaccines – Problem based solution

1) Problem identification (Ex.: seasonal and pandemic flu)
2) Components of a vaccine: antigen + adjuvant + delivery system
3) Search for an antigen (molecule that when innoculated, evoke an adaptative immune response such as antibody production;
4) Combination with adjuvant (molecule(s) that increases the immune response against the antigen);
5) Formulation with an appropriate delivery system.

adjuvare=help

Alexander Glenny 1926 –Search for compounds that would make antigens insoluble ended up with aluminum potassium sulfate

Adjuvant - substances when in combination with an antigen evokes a higher immune response against the antigen when compared the response induced against the antigen alone (antibody - humoral or cellular mediated immune response)

Attenuated live vaccines – do not need adjuvant
Vaccinia, BCG

Subunit vaccines – not efficient antigen w/o adjuvant
Tetanus, diphteria

Development of new adjuvants and new vaccine by Instituto Butantan

Adjuvant approved for human use: aluminium salts, monofosforil lipídiA MF59

MF59 – Emmulsion oil/water + squalene + Tween 80 + Span 85 (flu)

AS04 – Susp. aluminium hydroxide + MPLA (Hepatitis B e HPV)

AS02 – Emmulsion oil/water MPLA + QS21 (malaria, TB, cancer, HIV)

From empirical observations to rational develpoment of new adjuvants (based on scientific knowledge)

Table 3. Adjuvants in development for human vaccines

Adjuvants	Formulation	In pre-clinical or clinical trials
Montanides	Water-in-oil emulsions	Malaria (Phase I), HIV, Cancer (Phase I/II)
Saponins (QS-21)	Aqueous	Cancer (Phase II), Herpes (Phase I), HIV (Phase I)
SAF	Oil-in-water emulsion containing squalene, Tween™ 80, Pluronic™ L121	HIV (Phase I – Chiron)
AS03	Oil-in-water emulsion containing α-tocopherol, squalene, Tween™ 80	Pandemic Flu (GSK)
MTP-PtdEtn	Oil-in-water emulsion	HSV
Exotoxins	*P. aeruginosa*	*P. aeruginosa*, cystic fibrosis (AERUGEN – CrucellBerna)
	E. coli heat-labile enterotoxin LT	ETEC (Phase II – Iomai Corp.)
ISCOMs	Phospholipids, cholesterol, QS-21	Influenza, HSV, HIV, HBV, Malaria, Cancer
TLR ligands		
MPL®-SE	Oil-in-water emulsion	*Leishmania* (Phase I/II - IDRI)
Synthetic Lipid A	Oil-in-water emulsion	Various indications (Avanti/IDRI)
MPL®-AF	Aqueous	Allergy (ATL); Cancer (Biomira)
AS01	Liposomal	HIV (Phase I), Malaria (ASO1, Phase III, GSK) Cancer (Phase II/III, Biomira/MerckKGaA)
AS02	Oil-in-water emulsion containing MPL® and QS-21	HPV (Cervarix), HIV, Tuberculosis, Malaria (Phase III), Herpes (GSK)
AS04	Alum + aqueous MPL®	HPV, HAV (GSK)
AS15	AS01 + CpG	Cancer therapy (GSK)
RC529	Aqueous	HBV, pneumovax
TLR-9 (CpG)	n/a	Cancer (ProMune – Coley/Pfizer) HCV (ACTILON – Coley)
TLR-9 ISS series	n/a	HIV, HBV, HSV, Anthrax (VaxImmune Coley/GSK/Chiron) HBV (HEPLISAV, Phase III - Dynavax) Cancer (Phase II, Dynavax)
TLR-9 IMO series (YpG, CpR motif)	n/a	Cancer (IMOxine, Phase I, Hybridon Inc.) Cancer (IMO-2055, Phase II, Idera Pharm.) HIV (Remune, Phase I, Idera/IMNR)
TLR-9 agonist (MIDGE®)	n/a	Cancer (Phase I, Mologen AG)
TLR-7/8 (Imiquimod)	n/a	Melanoma (3M Pharmaceutical) HIV (preclinical), Leishmaniasis
TLR-7/8 (Resiquimod)	n/a	HSV, HCV (Phase II - 3M Pharmaceuticals)

Abbreviations: ETEC, Enterotoxigenic *Escherichia coli*; HBV, Hepatitis B virus; HCV, Hepatitis C virus; HPV, human papilloma virus; HSV, Herpes simplex virus.

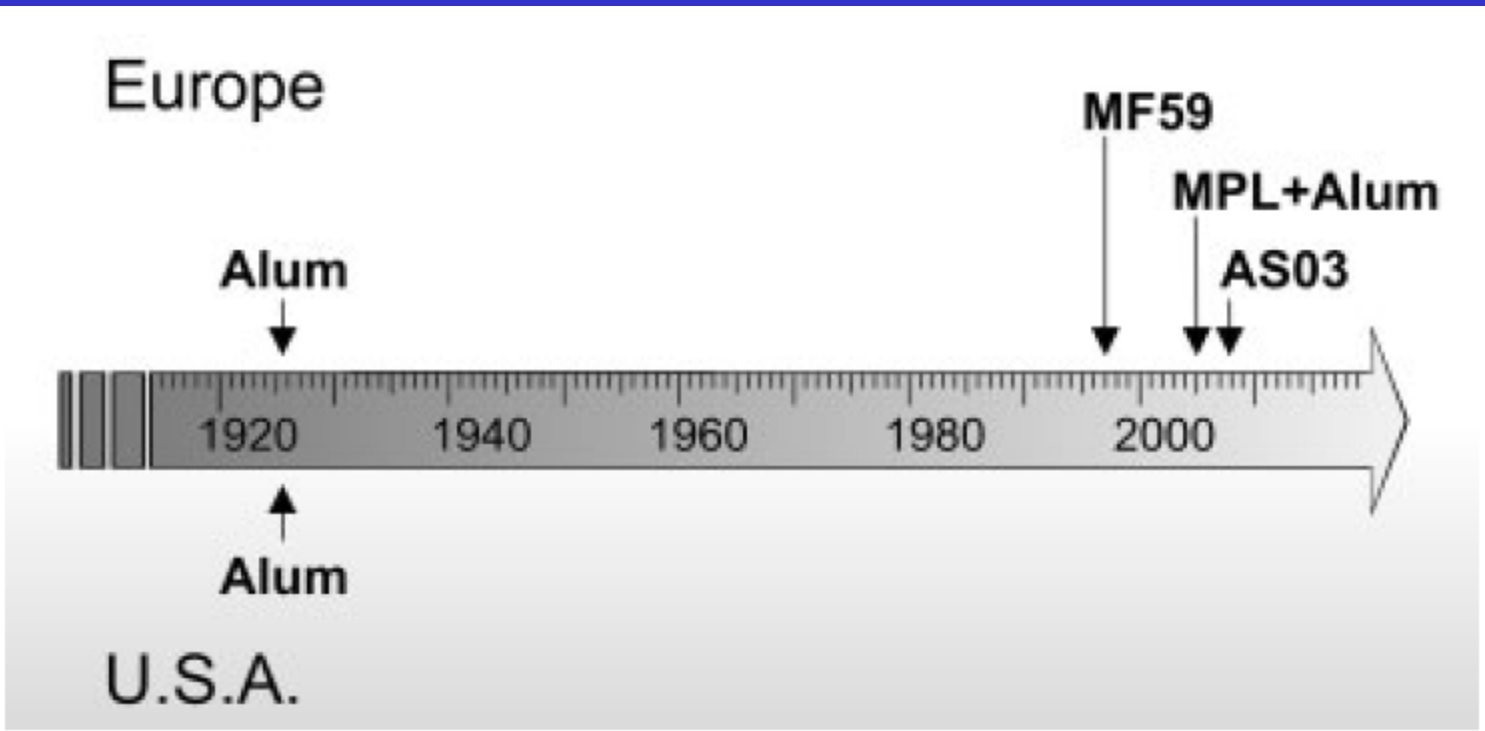


Figure 1. The slow process of adjuvant discovery. Alum was the first adjuvant to be licensed in the 1920s and is still the only adjuvant approved for human use in the USA. The squalene-based oil-in-water emulsion MF59 was first licensed in Europe for a flu vaccine (FLUAD) in 1997. The LPS analog monophosphoryl lipid A (MPL) formulated with alum (AS04) was first approved for an HBV vaccine (Fendrix) in Europe in 2005. The oil-in-water emulsion AS03 was approved for a pandemic flu vaccine (Prepandrix) in 2008.

-The Immune System is an essential part of an organism; without it the life would not be possible;

- The Immune System is unique and works coordinately;

- For didatic purposes, it can be divided in innate and adaptative immune system

- Innate immunity: Physical barriers (skin), chemical (secretions) and those medited by cells that defend the host from infections using a generic and non specific response (macrophages, fagocitose, complement);

- Adaptative immunity: Immune response that allows the recognition of specific antigens and pathogens, eliciting a strong and specific immune response, anytime when the related antigen or pathogen are encountered, possessing a memory response through genetic recombination and clonal expansion.

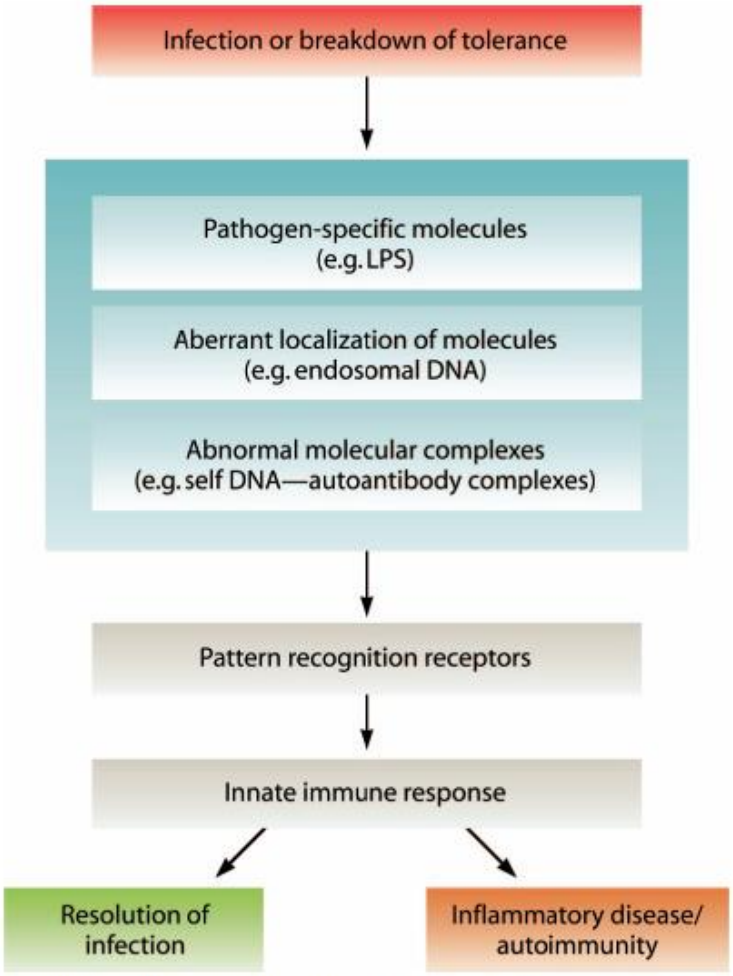


FIG. 1. Principles in innate immune recognition by PRRs. During microbial infection or breakdown of tolerance, pathogen-specific molecules, aberrant localization of foreign or self molecules, or abnormal molecular complexes are recognized by PRRs. This event triggers PRR-mediated signaling and induction of an innate immune response, which ultimately results in resolution of infection but also may cause inflammatory diseases or autoimmunity.

Innate immunity:

Based on the recognition of Pathogen-associated molecular patterns (**PAMPs**) – by Pattern recognition receptors (**PRR**)

PRRs: Pattern Recognition Receptors

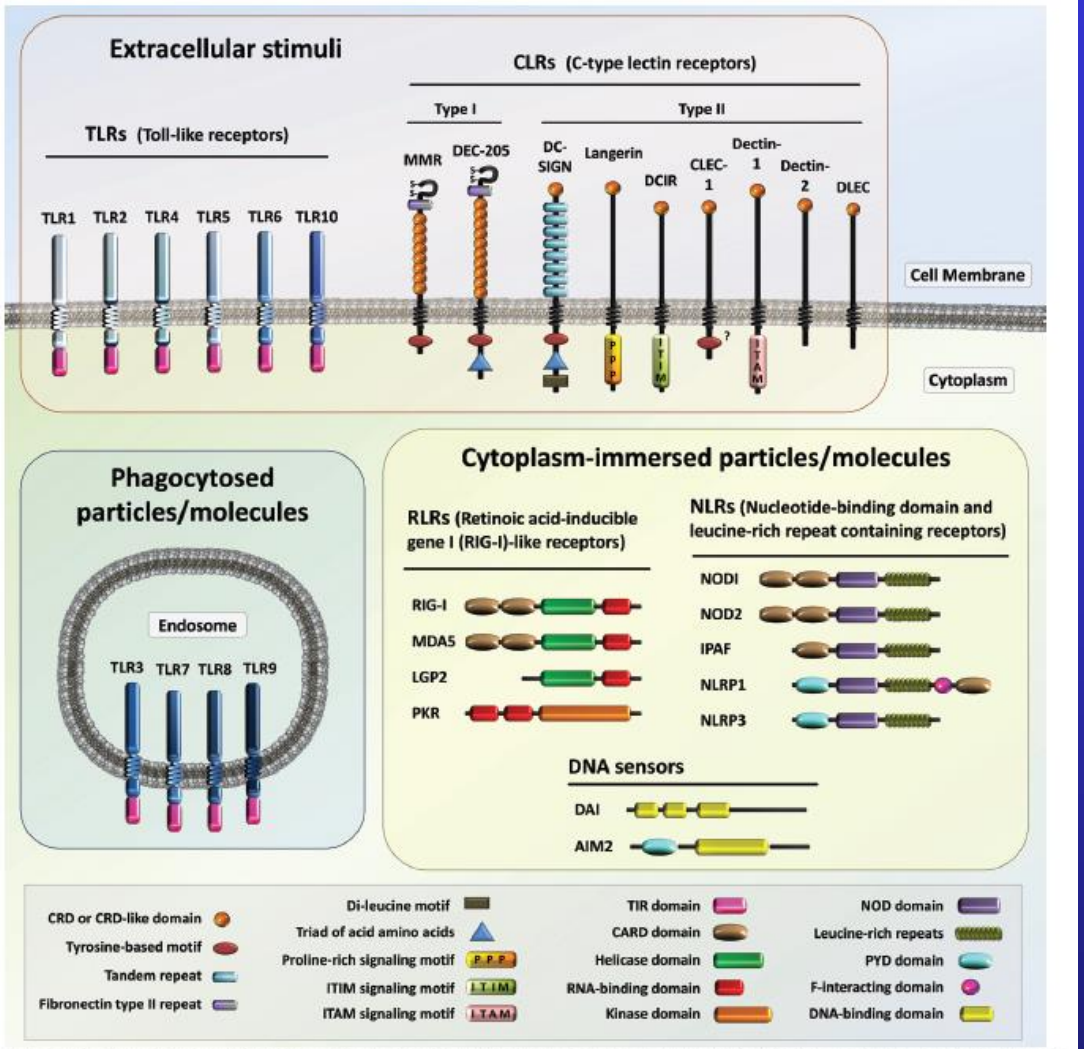


Figure 1. Schematic representation of the major classes of pattern recognition receptors (PRRs) of the innate immune system. Members of the Toll-like receptors (TLRs) can be found on the cell surface membrane (TLR1/2/4/5/6/10) as well as in the membrane of endosomes (TLR3/7/8/9); C-type lectins can be of type I or type II. Type I C-type lectins have an N-terminal domain displayed in the extracellular portion of the molecule, containing a cysteine-rich repeat (S-S), a fibronectin type II repeat and 8-10 carbohydrate recognition domains (CRDs), which bind ligands in a Ca^{2+}-dependent manner. Type II C-type lectins contain only one CRD at their carboxy-terminal extracellular domain. Nucleotide-binding oligomerization domain (NOD) leucine-rich repeat (LRR)-containing receptors (NLRs) are cytoplasmic PRR receptors and the central components of the inflammasomes; the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are also cytoplasmic PRR receptors. Retinoic acid-inducible protein I (RIG-I) is an interferon-inducible protein containing a caspase recruitment domain (CARD) and a DExD/H box helicase domain and has been described as a cytoplasmic dsRNA sensor and considered to be the prototype of this family of PRRs. Other miscellaneous PRRs are also shown (DNA sensors DAI and AIM2). The different kinds of motifs, domains, repeats, and features that compose modularly the PRRs are also shown. AIM2 = absent in melanoma 2; CLEC-1 = C-type lectin receptor 1; DAI = DNA-dependent activator of interferon-regulatory factors; DCIR = dendritic cell immunoreceptor; DC-SIGN = dendritic-cell specific ICAM-3 grabbing non-integrin; DEC-205 = dendritic cell receptor for endocytosis 205; DLEC = dendritic cell lectin; IPAF = ice protease-activating factor; ITAM = immunoreceptor tyrosine-based activation motif; ITIM = immunoreceptor tyrosine-based inhibitory motif; LGP2 = laboratory of genetics and physiology 2; MDA5 = melanoma differentiation-associated gene 5; MMR = macrophage mannose receptor; NLRP1/3 = nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 1/3; PKR = double-stranded RNA activated protein kinase; PYD = Pyrin domains; TIR = Toll/interleukin-1 receptor homology. Based on Refs. 10 and 32.

Table 1. Pattern-recognition receptors (PRRs).

PRRs	Ligands (DAMPs and PAMPs)		Cellular localization
	Exogenous	Endogenous	
Human TLRs (homo- or heterodimers)			
TLR1/TLR2	Triacyl lipopeptides (mycobacteria and *Neisseria*)	α-defensins	Cell surface
TLR2	Lipoproteins, peptidoglycan, lipoarabinomannan, porins, envelope glycoproteins, GPI-mucin, phospholipomannan, zymosan, α-glycan (various pathogens, such as Gram-positive and Gram-negative bacteria, mycobacteria, *Neisseria*, viruses, protozoa, *Candida*, fungi)	Surfactant protein A	Cell surface
TLR2/TLR4	Glucuronoxylomannan (*Cryptococcus neoformans*)	HSP60, HSP70, Gp96, HMGB1, surfactant protein D serum amyloid A, byglycan	Cell surface
TLR2/TLR6	Diacyl lipopeptides, lipoteichoic acid (*Mycoplasma*, Gram-positive bacteria)	Versican	Cell surface
TLR3	dsRNA	mRNA	Endosome
TLR4	LPS, envelope glycoproteins, glycoinositolphospholipids, mannan (Gram-negative bacteria, viruses, protozoa, *Candida*)	Fibronectin extra domain a, fibrinogen, tenascin-C, neutrophil elastase, lactoferrin, oxidized LDL, saturated fatty acids, heparan sulfate fragments, hyaluronic acid fragments	Cell surface
TLR5	Bacterial flagellin		Cell surface
TLR7/TLR8	ssRNA (RNA viruses)	ssRNA	Endosome
TLR9	CpG DNA, hemozoin (viruses, bacteria, protozoa)	Chromatin-IgG complexes	Endosome
TLR10	Unknown		Cell surface
RLRs			
RIG-I	Short dsRNA, 5'-triphosphate RNA (viruses)		Cytoplasm
MDA5	Long dsRNA (viruses)		Cytoplasm
NLRs			
NOD1	Peptidoglycans, diaminopimelic acid (Gram-negative bacteria)		Cytoplasm
NOD2	Peptidoglycans, muramyl dipeptide (Gram-negative and Gram-positive bacteria)		Cytoplasm
NLRP1	Muramyl dipeptide, *Bacillus anthracis* lethal toxin (Gram-negative and -positive bacteria, bacterial toxins)		Cytoplasm
NLRP3	RNA, DNA, muramyl dipeptide, bacterial toxins, silica, asbestos, UVB radiation, trinitrophenylchloride, trinitrochlorobenzene, dinitrofluorobenzene, (viruses, bacteria, environment)	ATP, hyaluronic acid, amyloid β peptide, urate crystals, elevated extracellular glucose, reactive oxygen species	Cytoplasm
IPAF	Cytosolic bacterial flagellins		Cytoplasm
C-type lectins			
MMR	Mannose, fucose	Mannose, fucose, sialyl Lewis X	Cell surface
Dectin-1	β-glucan		Cell surface
DC-SIGN	*N*-acetylglucosamine-mannose, mannan, HIV-1 (gp120), SIV	*N*-acetylglucosamine-mannose, ICAM-2, ICAM-3	Cell surface

Ligantes endógenos e exógenos.

Ativação, inibição, modulação

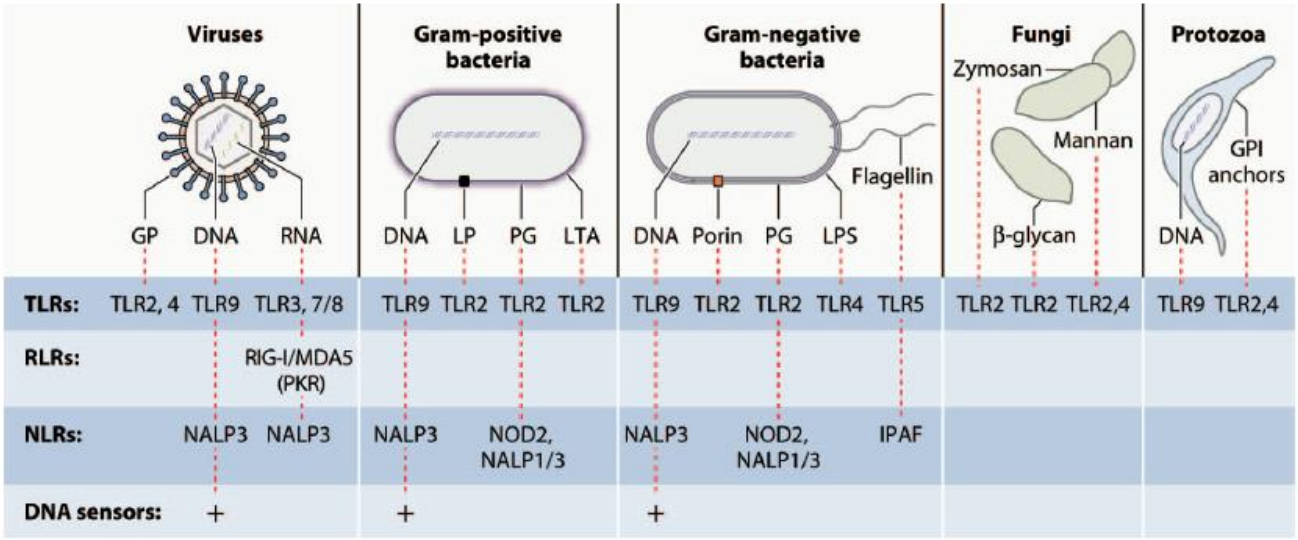
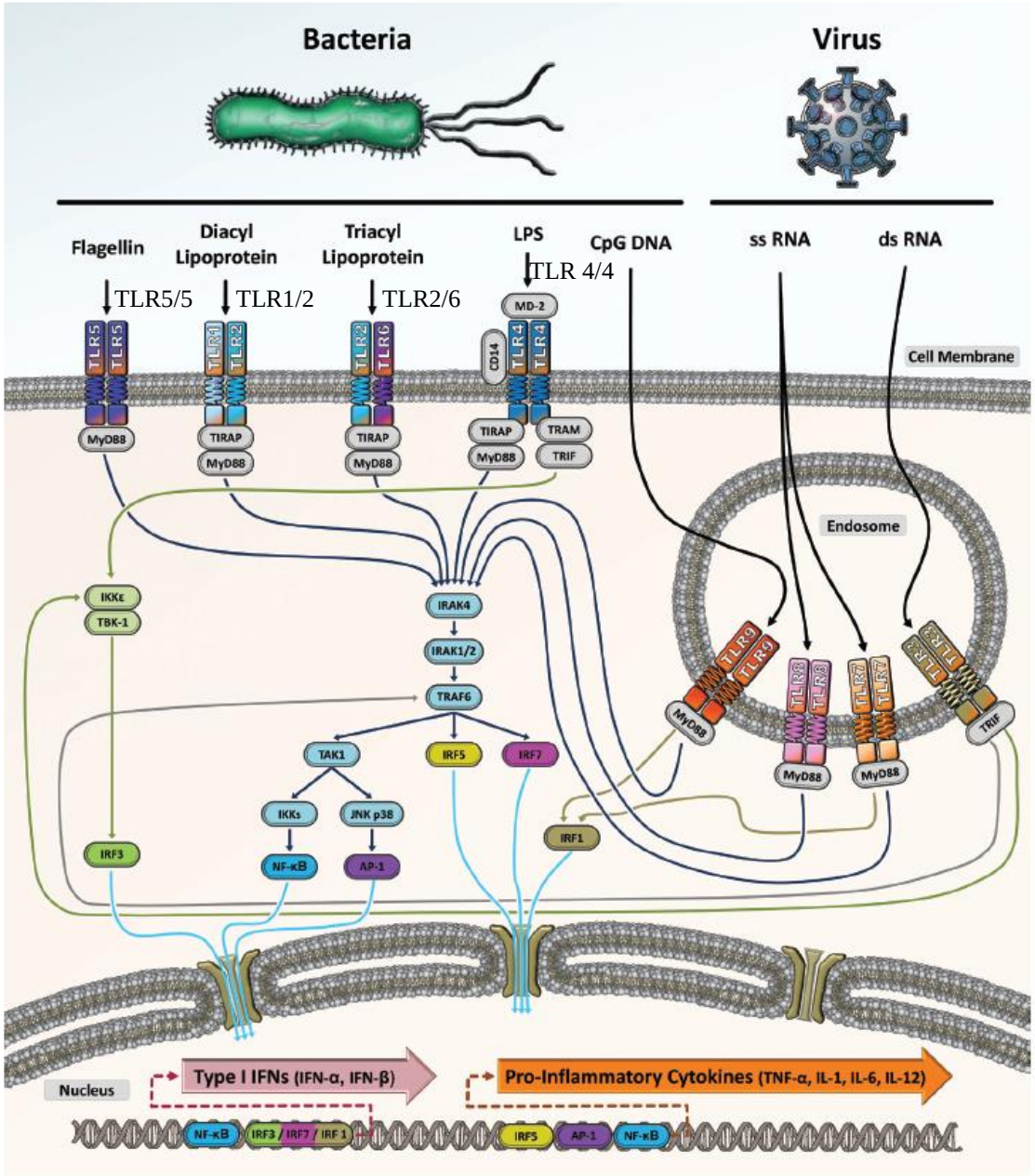


FIG. 3. Recognition of PAMPs from different classes of microbial pathogens. Viruses, bacteria, fungi, and protozoa display several different PAMPs, some of which are shared between different classes of pathogens. Major PAMPs are nucleic acids, including DNA, dsRNA, ssRNA, and 5′-triphosphate RNA, as well as surface glycoproteins (GP), lipoproteins (LP), and membrane components (peptidoglycans [PG], lipoteichoic acid [LTA], LPS, and GPI anchors). These PAMPs are recognized by different families of PRRs.

Recogniton of multiple PAMPs at the same time elicite a complex and coordinated response



Activation of multiple pathways during infection

Cross-talking among the pathways

Activation of mediators of pro-inflammetory and/or type I IFN genes

The response by Toll-like receptors is mediated by adaptor molecules that interacts with TIR (Toll/interleukin-1 receptor) homology domain

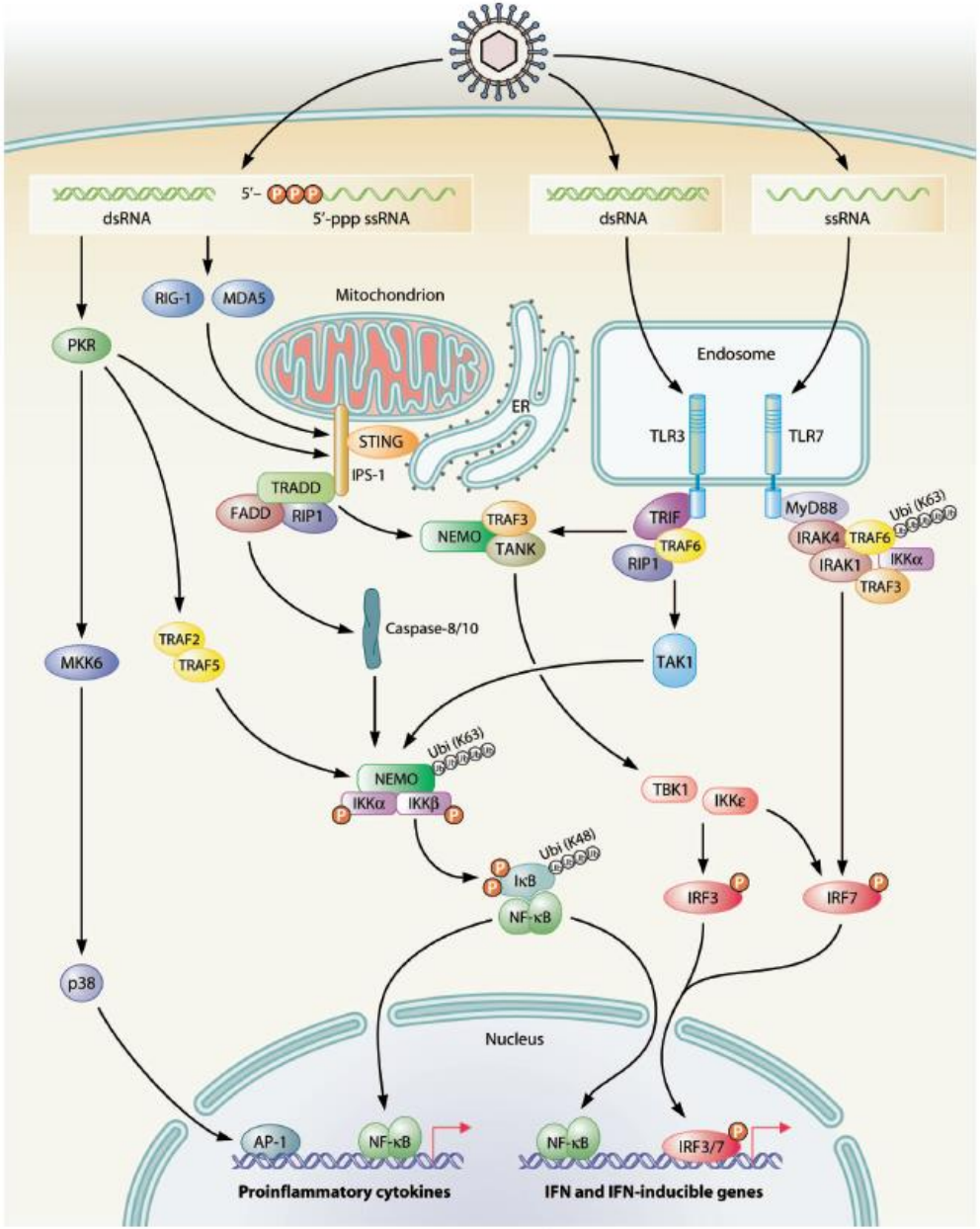


FIG. 6. Intracellular RNA recognition and signaling. Cytosolic dsRNA or 5′-triphosphate ssRNA is recognized primarily by the cytoplasmic RNA helicases RIG-I and MDA5, which mediate interaction with the adaptor IPS-1, localized to mitochondria, and trigger signaling to NF-κB and IRF3 via IKK and TBK/IKKε, respectively. dsRNA can also be recognized by TLR3 localized in the endosomal compartment or by cytosolic PKR, but whereas TLR3 triggers signaling to NF-κB and IRF3, PKR instead activates NF-κB and MAPKs. Finally, ssRNA is recognized by TLR7/8 in endosomes and induces signaling to IRF7 as well as to NF-κB and MAPKs (not shown in the figure).

Complex interconected signals elicited by a pathogen (virus infection)

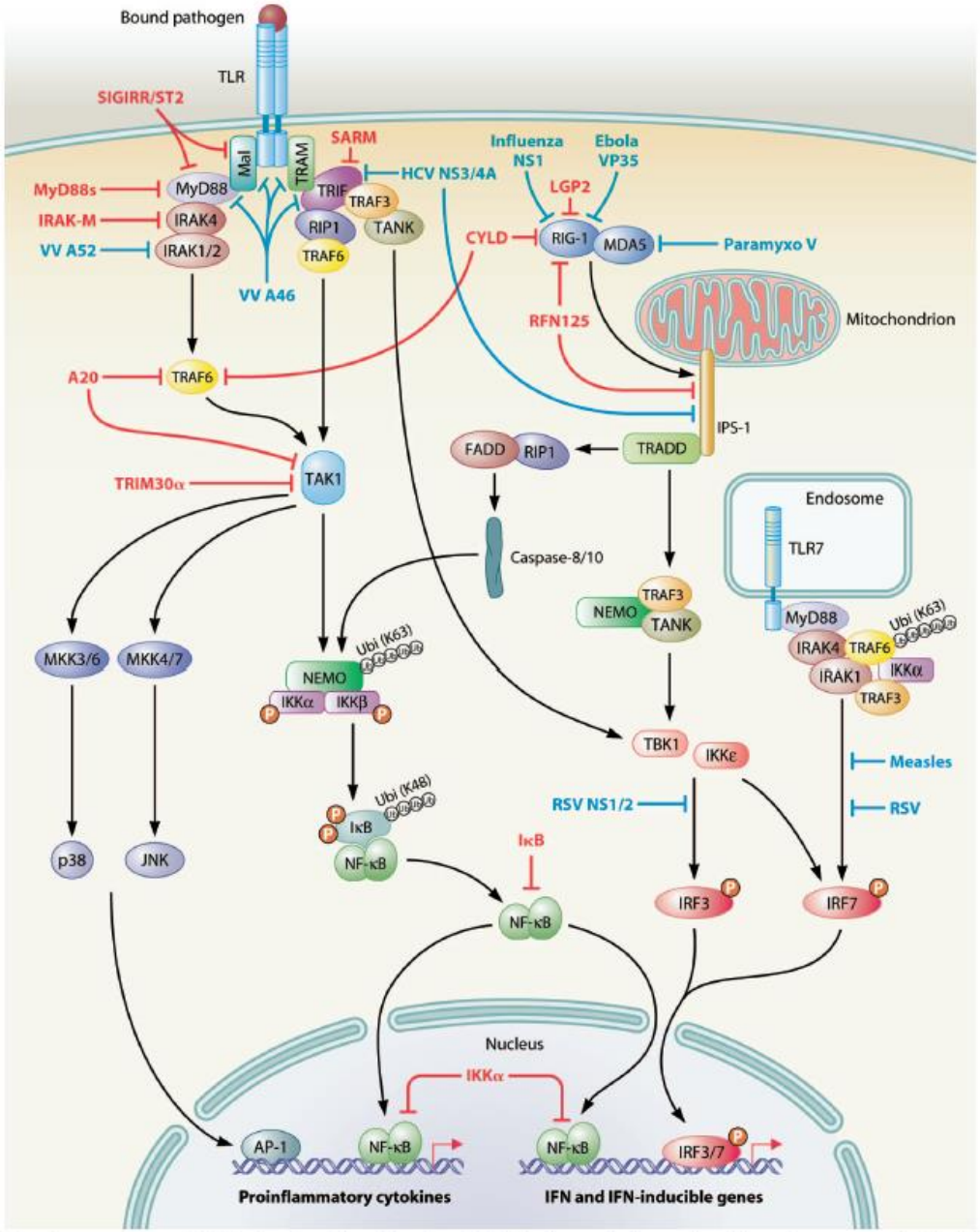


FIG. 7. Negative regulation of PRR signaling. Signaling through PRRs is negatively regulated by endogenous and microbial proteins. Endogenous negative control of PRR signaling (molecules depicted in red) serves the purpose of mediating negative feedback on the inflammatory response, whereas microbial interference with PRRs (viral molecules shown in green) constitutes a means to evade the host antimicrobial response. See text for further details.

The signal(s) has to be deactivated and controled!

The disbalance to one or to the other side may result in diseases.

Pathogens evolved mechanisms to evade the defense response of the host.

TABLE 2. Genetic disorders in innate immunity: associations with human diseases

Gene product[a]	Disease association	Reference(s)
TLRs		
TLR2	Susceptibility to leprosy and tuberculosis	33, 167
TLR2	Protection from Lyme disease; susceptibility to staphylococcal disease and tuberculosis	217, 278, 326
TLR2	Increased severity of genital herpes	37
TLR3	Susceptibility to HSV encephalitis	400
TLR4	Susceptibility to infection with gram-negative bacteria, malaria, and RSV	4, 218, 255, 360
TLR4	Susceptibility to meningococcal sepsis	337
TLR4	Protection from Legionnaires' disease	116
TLR5	Susceptibility to Legionnaires' disease	117
TLR7	Protection from hepatic fibrosis in chronic HCV infection	325
TLR9	Susceptibility to SLE	361
TLR9	Rapid progression of HIV infection	36
TLR9	Increased risk of low birth wt in malaria during pregnancy	256
Signaling proteins		
MyD88	Susceptibility to infection with pyogenic bacteria	376
Mal	Protection from invasive pneumococcal disease, malaria, and tuberculosis	185
IRAK1	Increased severity of sepsis	20
IRAK4	Susceptibility to infection with pyogenic bacteria	294
NEMO	EDA-ID; susceptibility to infection with pyogenic bacteria, mycobacteria, herpesviruses, fungi	78, 195, 282
IκBα	EDA-ID	62
IRF5	Increased risk of SLE	105, 183
NLRs		
NOD2	Susceptibility to Crohn's disease	144, 277
NOD2	Blau syndrome, early-onset sarcoidosis	166
NOD2	Susceptibility to psoriatic arthritis	299
NALP1	Susceptibility to vitiligo-associated multiple autoimmune diseases	160
NALP3	Periodic fever syndromes	86
Miscellaneous		
CD14	Susceptibility to RSV bronchiolitis	147
UNC93B	Susceptibility to HSV encephalitis	52

[a] In cases where the same PRR gene product is listed repeatedly, each entry represents a different mutation/SNP.

Mutations in the proteins involved in PRRs signalings may cause genetic disorders or predispose to diseases.

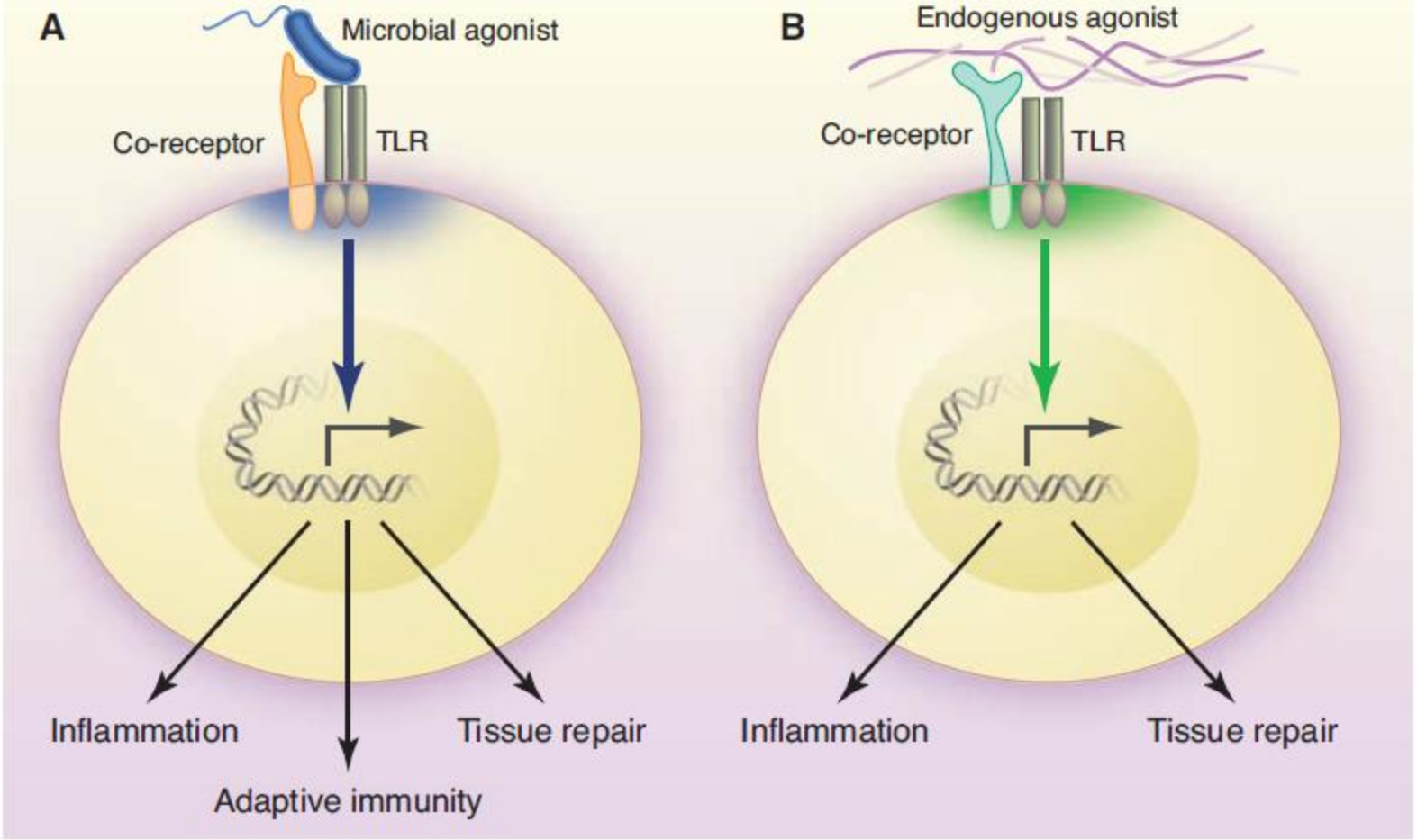


Fig. 4. Proposed consequences of TLR recognition of exogenous versus endogenous ligands. TLR engagement by exogenous (**A**) or endogenous (**B**) agonists leads to signaling from distinct subcellular compartments (indicated by blue and green, respectively) and/or engagement of co-receptors. Consequently, exogenous ligands induce transcription of genes leading to inflammation, tissue repair, and the initiation of adaptive immunity (A). In contrast, endogenous ligands induce TLR signaling for activation of inflammation and tissue repair but not the initiation of adaptive immunity (B).

The innate system also recognizes *self* e *non-self* ligands.

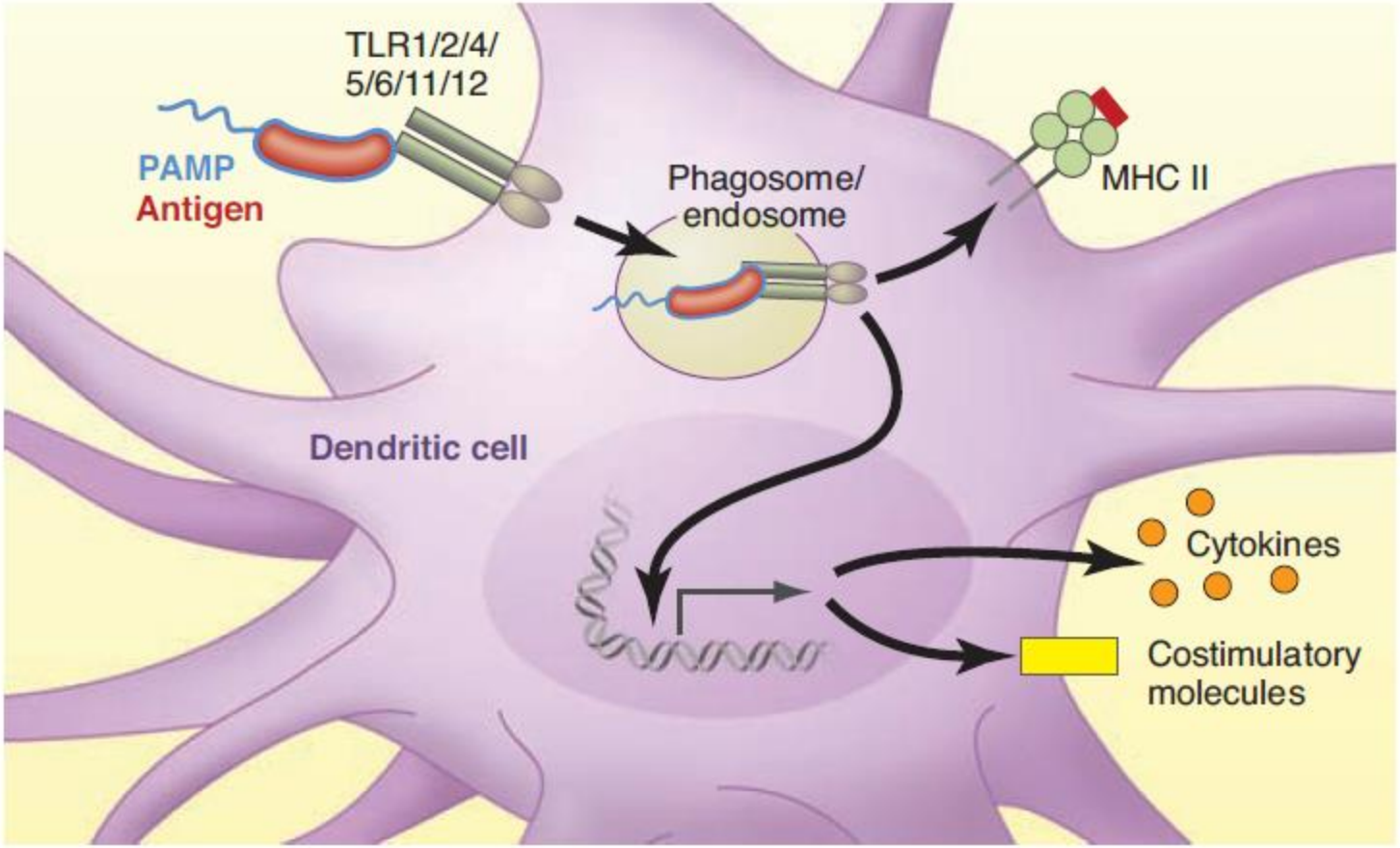


Fig. 1. Cell-extrinsic recognition of pathogens. Bacteria detected by DCs through TLRs are internalized into the phagosome where bacterial antigens are processed for presentation on MHC class II. Bacterial antigens (red) and PAMPs (blue) are present in the same phagosome, which indicates to the DC their common origin. TLR-mediated recognition of bacterial PAMPs promotes the selection of bacterial antigens for optimal presentation on MHC class II. TLR signaling also leads to the induction of costimulatory molecules and cytokines necessary for activation and differentiation of T lymphocytes.

Extra cellular recognition

↓

response

↓

Secretion of cytokines and co-stimulatory molecules
Antigen (epitope) presentation by MHC II/MHCI

Science 327: 291, 2010

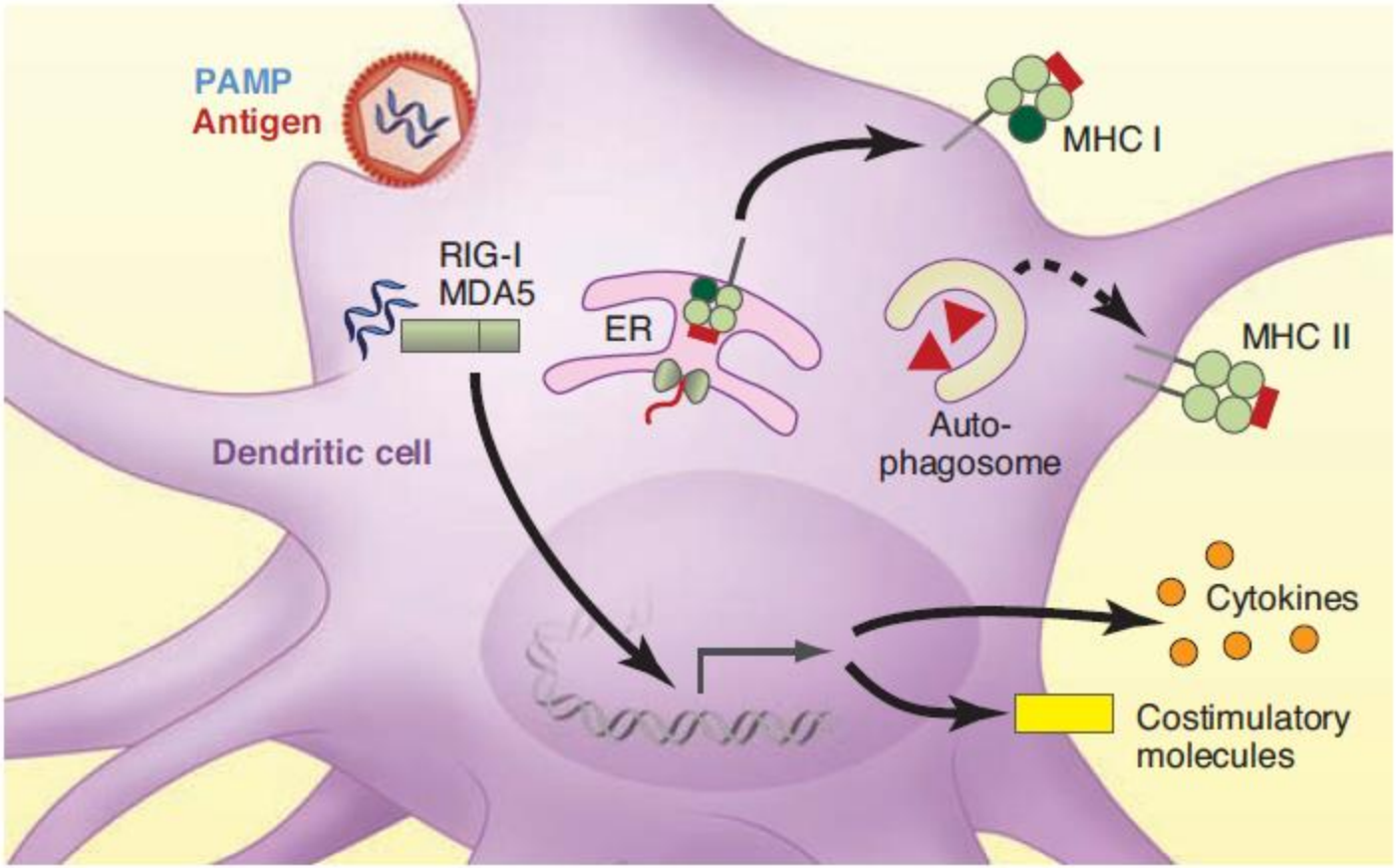
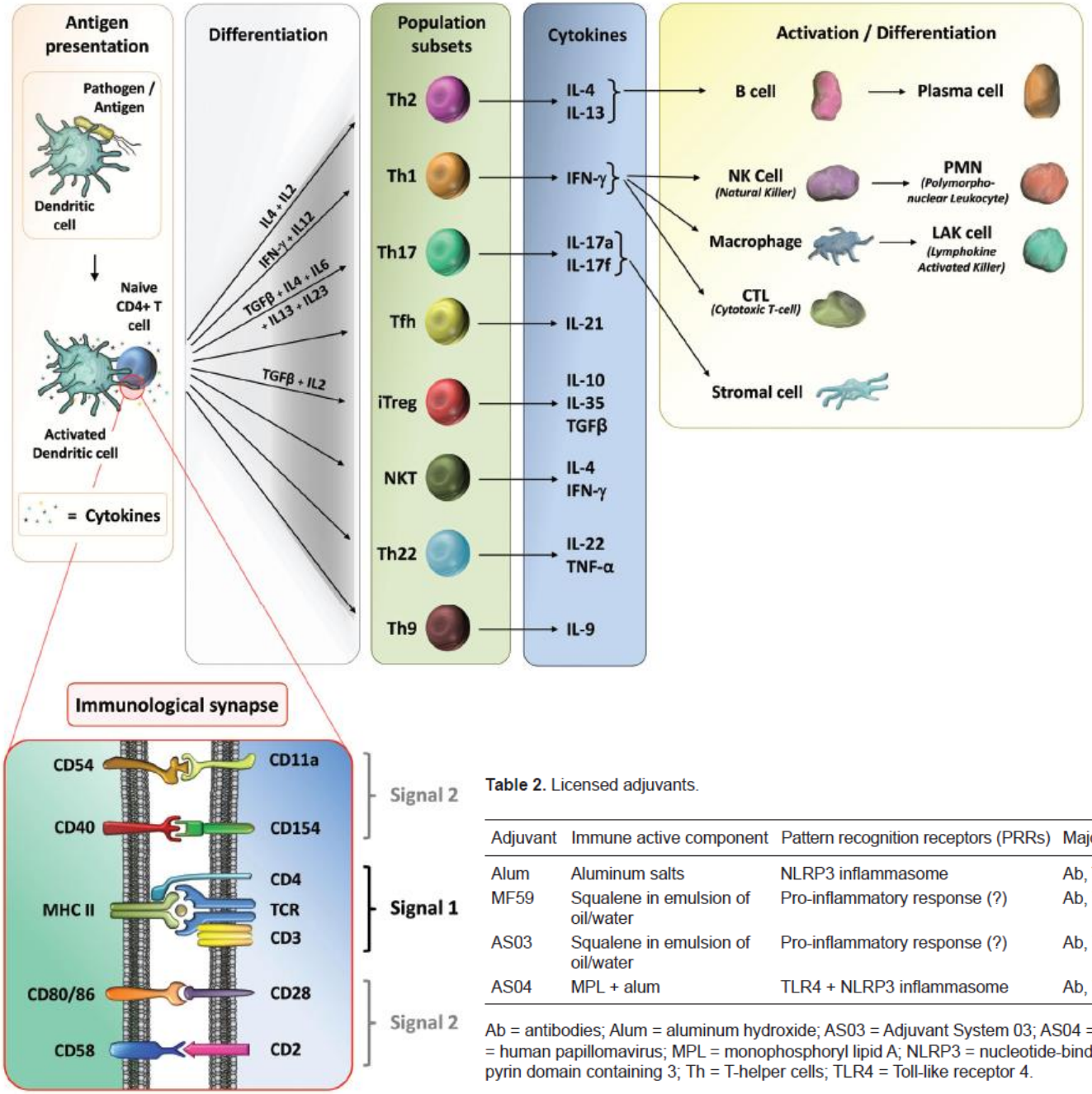


Fig. 2. Cell-intrinsic recognition. DCs directly infected by viruses recognize PAMPs (blue) within the cytosol via RIG-I like receptors (RLRs). Cytosolic viral proteins (red) are processed and presented on MHC class I (via the conventional endoplasmic reticulum pathway) or MHC class II (via autophagy). RLR signaling leads to the induction of costimulatory molecules and cytokines necessary for activation and differentiation of T lymphocytes. How the origin of antigen is established in this instance is unclear. In the case of the MHC class I pathway, this may depend on the abundance of viral antigens; for MHC class II, it may depend on targeting of viral antigens (red triangles) by the autophagy machinery.

Intracelular recognition

↓

Idem

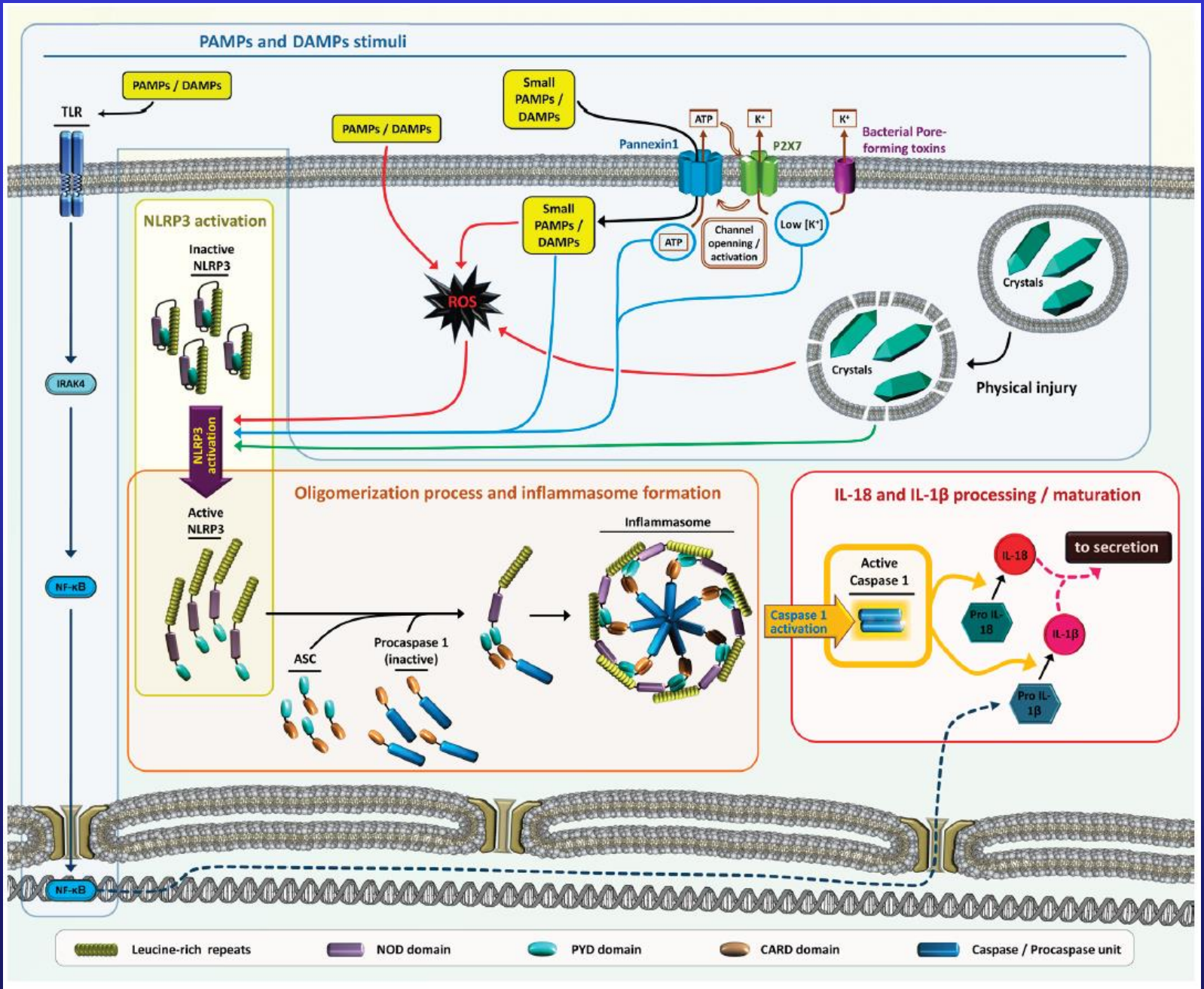


The immune response is the result of the activation and interaction of the innate and adaptative immune systems.

Table 2. Licensed adjuvants.

Adjuvant	Immune active component	Pattern recognition receptors (PRRs)	Major immune response	Vaccine
Alum	Aluminum salts	NLRP3 inflammasome	Ab, Th2 (mice) (+Th1 in humans)	Several
MF59	Squalene in emulsion of oil/water	Pro-inflammatory response (?)	Ab, Th1/Th2	Seasonal and pandemic influenza
AS03	Squalene in emulsion of oil/water	Pro-inflammatory response (?)	Ab, Th1/Th2	Pandemic influenza
AS04	MPL + alum	TLR4 + NLRP3 inflammasome	Ab, Th1	HBV, HPV

Ab = antibodies; Alum = aluminum hydroxide; AS03 = Adjuvant System 03; AS04 = Adjuvant System 04; HBV = hepatitis B virus; HPV = human papillomavirus; MPL = monophosphoryl lipid A; NLRP3 = nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing 3; Th = T-helper cells; TLR4 = Toll-like receptor 4.



Ativação da via do inflamassoma e conexão com a via de TLR

Conclusion: PAMPs (Pathogen-associated molecular patterns) and DAMPs (Danger-associated molecular patterns) that activate innate immune response are potential adjuvants! Metabolic activation is involved in immune modulation!

Problems to be solved: Besides adjuvant effects, some present undesirable and negative effects.

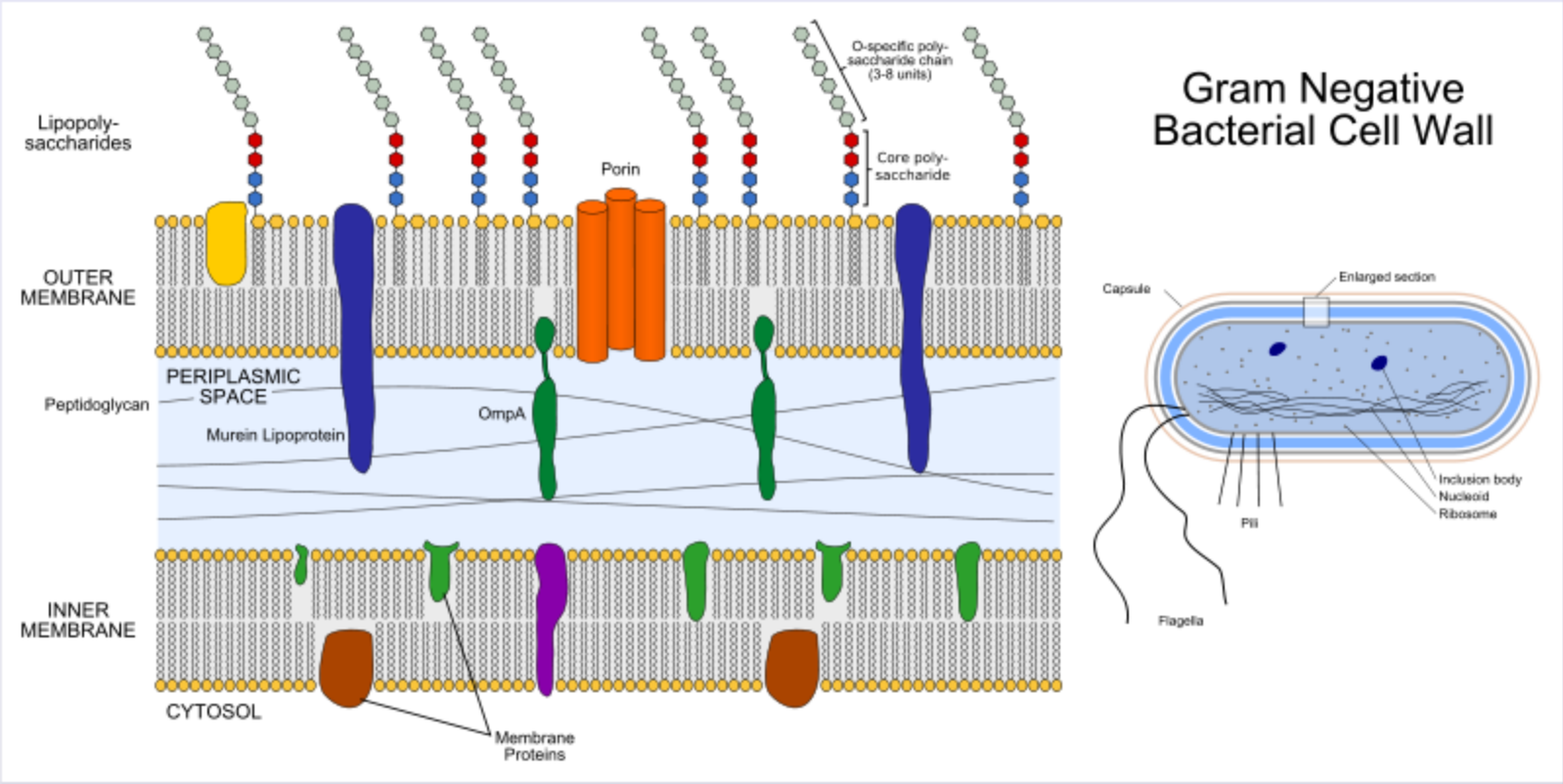
Instituto Butantan

Instituto Butantan:	Major producer of sera and vaccines in Latin America Annual production of 150-200 million doses of vaccines Annual production of 400-700 thousands of ampule of sera
Vaccines produced: em produção	Combined DPT – Diphteria, pertussis and tetanus. Hepatitis B (first recombinant vaccine in Brazil) *Rabies vaccine produced in VERO cells for human use
	*The production was interrupted and a new production plant is being built and will start working on 2014.

Example of an Adjuvant developed at Instituto Butantan

Pertussis vaccine:

- Bordetella pertussis inativated whole cell by formaldehyde
- Reatogenic, in part by the presenc of LPS



Development # 1

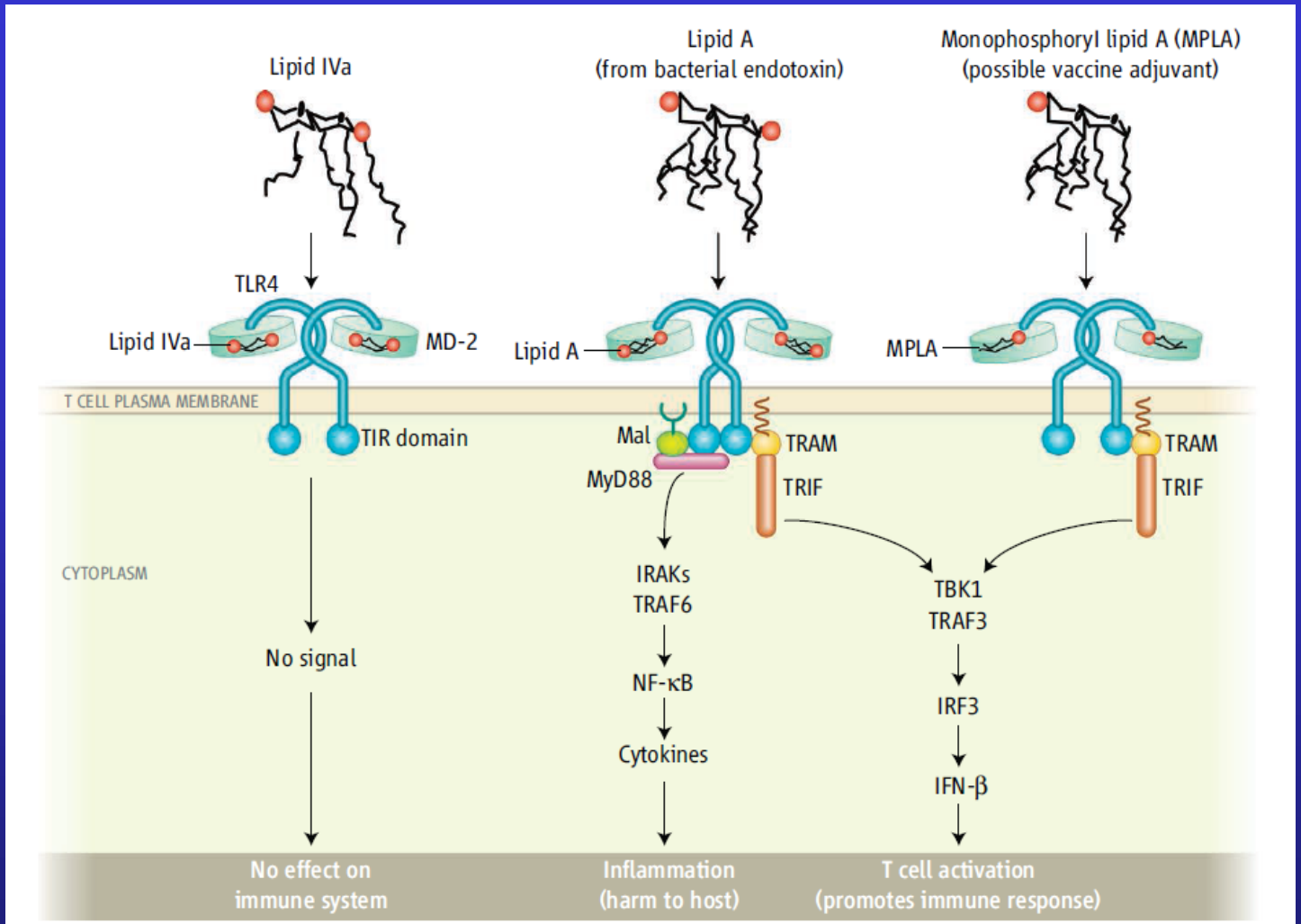
A new whole cell pertussis vaccine, less reactogenic by the removal of LPS

Vaccine DTPw → Vaccine DTP_{low}

Development # 2

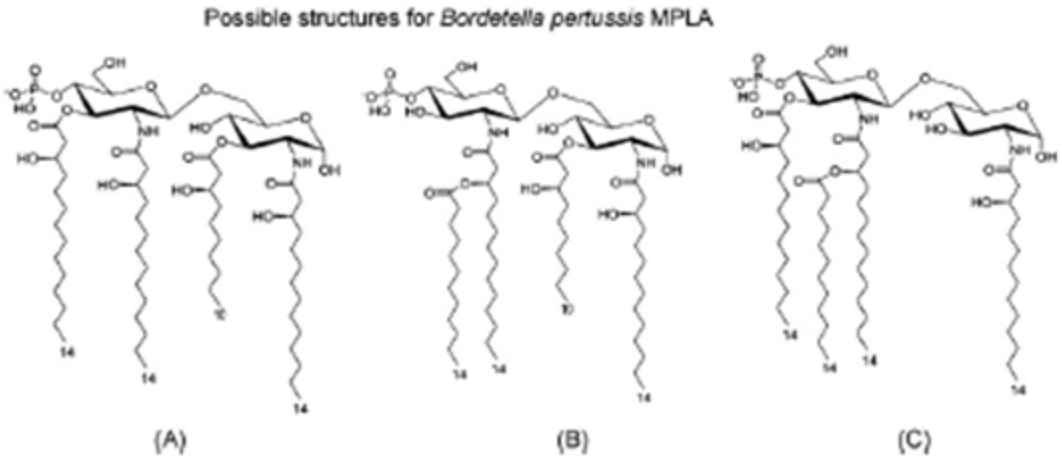
A new MPLA adjuvant produced from Bordetella pertussis LPS

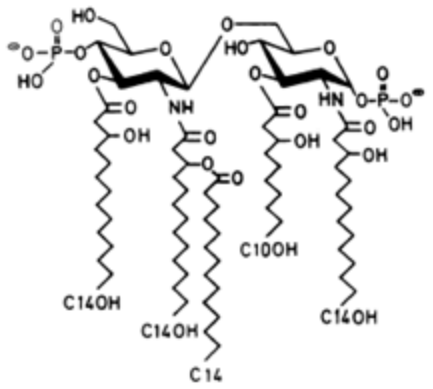
LPS → MPLA



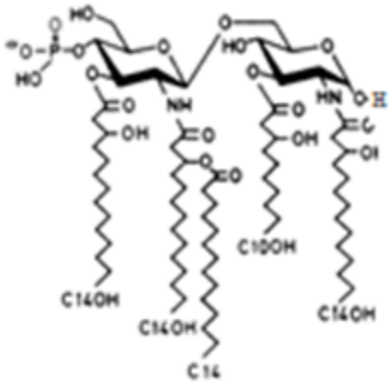
Rules of engagement. The TLR4–MD-2 complex is expressed as dimers on the surface of immune cells (T and B cells, phagocytes). Lipid A and two of its analogs, lipid IVa and monophosphoryl lipid A, bind to MD-2. There is no direct evidence that these lipids bind TLR4. The specific engagement of each lipid may cause structural changes in MD-2 and TLR4, resulting in new protein binding sites in the cytoplasmic domain of TLR4. The resulting recruitment of specific adapter proteins activates distinct signaling pathways and associated cellular responses.

MPLA present adjuvant response, LPS is a endotoxin (pirogen), one of the mediators of septic syndrome

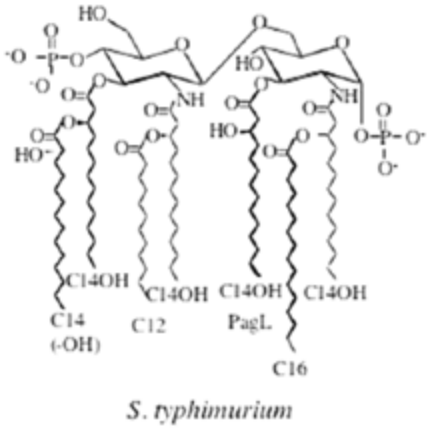




Lipídio A Bordetella pertussis

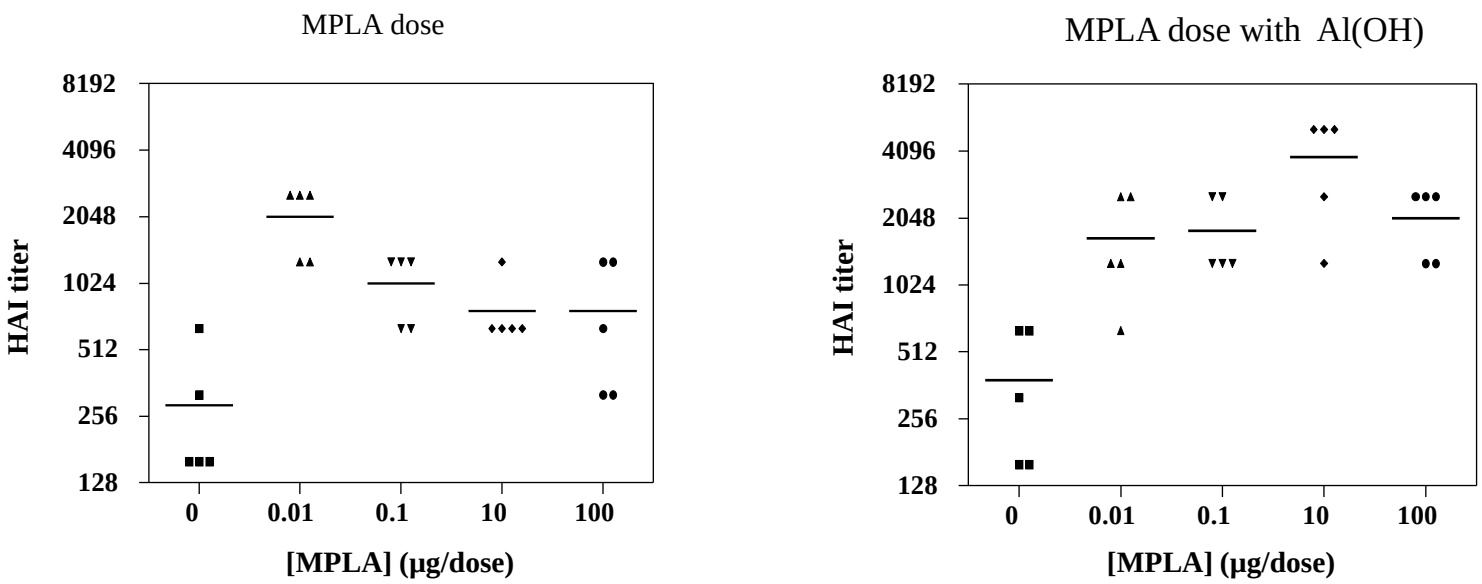


MPLA Bordetella pertussis



Lipídio A de S. typhimurium

Adjuvant activity of MPLA on H3N2 split virion seasonal influenza strain vaccine using ¼ of a dose



HAI titers of 1:40 is considered protective

Figure 4. Investigation of MPLA dose in the presence or absence of $Al(OH)_3$. Sera from individual mice immunized with 3.75 µg HA/dose of H3N2 split virion influenza vaccine were analyzed for HAI titers when increasing concentrations of MPLA were combined with no other adjuvant (A), or with $Al(OH)_3$ (B). — indicates HAI GMT.

H5N1

Split virion

Table 4. HAI titers induced by H5N1 split virion vaccine with different adjuvant formulations

Vaccine dose (HA content)	No adjuvant	$Al(OH)_3$	MPLA (aqueous suspension)	$Al(OH)_3$+MPLA (aqueous suspension)
3.75 µg	1:20	1:289	1:40	1:200
7.5 µg	1:127	1:260	1:164	1:320
15 µg	1:136	_	_	_

HAI titers were measured using sera collected 21 days after the inoculation of the vaccine. GMT of 5 animals is shown. HAI titers of 1:40 is considered protective

Whole Virus

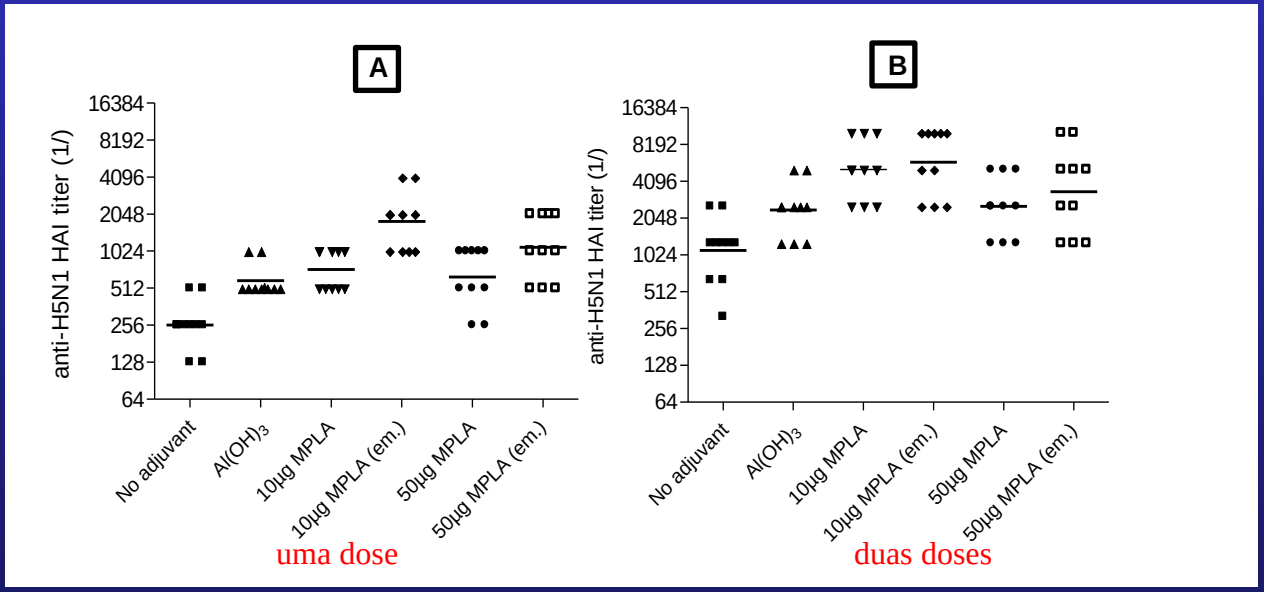


Figure 5. HAI titers in immunized mice. Mice were immunized with one (A) or two (B) 3.75 µg doses of H5N1 whole virus vaccine using the indicated adjuvant formulations. Sera were collected 21 days after each immunization and tested for HAI. Individual values and GMT are shown.

- Higher production of influenza vaccine (seasonal and pandemic) (3,75 ug of antigen instead of 15 ug, 4X more vaccines)

- Lower cost (Less antigen, cheap adjuvant – MPLA from B. pertussis)



Avaliação da imunogenicidade e proteção conferida pela vacina KSAC em cães

Apresentação do estudo realizado em canil de experimentação

Steve Reid (IDRI)
Antônio Campos Neto (The Forsyth Institute)
Equipe Butantan
Universidade Federal do Piaí, Maranhão e Ceará

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INSTITUTO BUTANTAN

Antigeno KSAC, mais efetiva contra leishmaniose visceral (*Goto Y, et al, 2011).* A vacina candidata contra a leishmaniose visceral (Leishmania donovani e Leishmania infantum) utiliza como antígeno vacinal, uma poliproteína composta pela proteína 11 da membrana do kinetoplastídio de Leishmania donovani (**K**MP-11), correspondente aos nucleotídios 1-276 (Genbank: XM_001468995.1) , proteína esterol 24 -c-metiltransferase (**S**MT) de Leishmania infantum, correspondente aos nucleotídios 4-1059 (Genbank: XM_001469795.1), proteína antígeno S homólogo estágio específica A2 (**A**2) de Leishmania donovani correspondente aos nucleotídios 4-779 (Genbank: S69693.1) e cisteína protease tipo I de Leishmania infantum (**C**PB) correspondente aos nucleotídios 738-1687 (Genbank: AJ420286.1).

ANTÍGENO KSAC

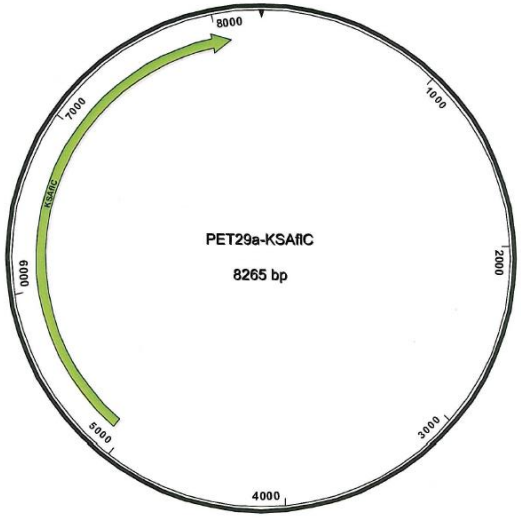


Figura 1- Proteína multi-epitopo KSAC clonado no vetor pET29a.
Expressão em bactéria E. coli HMS174 (DE3).
Banco semente produzido pela Charles River Laboratory (USA).
Antígeno produzido em BPF pela Merial (Fr).

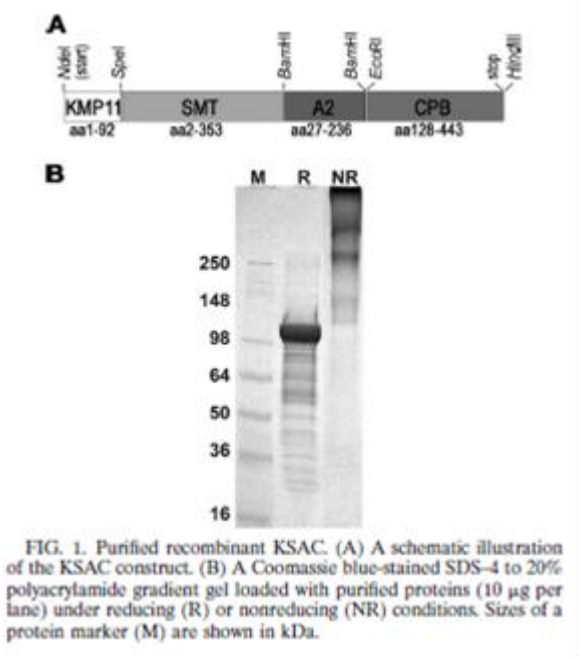
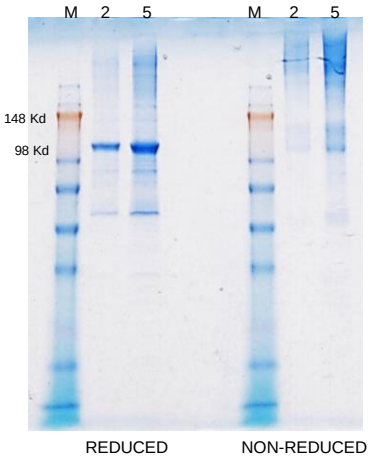


FIG. 1. Purified recombinant KSAC. (A) A schematic illustration of the KSAC construct. (B) A Coomassie blue-stained SDS–4 to 20% polyacrylamide gradient gel loaded with purified proteins (10 μg per lane) under reducing (R) or nonreducing (NR) conditions. Sizes of a protein marker (M) are shown in kDa.

RECOMBINANT PROTEIN CERTIFICATE OF ANALYSIS

Recombinant Name:	**KSAC**
Protein Description:	Recombinant *Leishmania* fusion antigen
Lot Number:	440-92 (KSAC-81)
Date of production:	06-JUL-2011
Protein Concentration (mg/ml):	0.398 mg/ml (BCA)
Vials (#):	11 vials (1 ml/vial)
Total Amount (mg):	5.6 mg
Buffer:	20 mM Tris pH 8.0
Endotoxin:	**3480 EU/mg (Endosafe)**
SDS-PAGE Analysis (Final Coomassie gel)	
Expression vector and host:	pPDM, HMS-174
Purification method:	**DE52 - QHP - HA – S100**
Predicted size & pI:	105052 Da, pI 5.9
Location of primers/sequences	N/A

Note: All bands detected by Western Blot



4-20% Tris-Glycine
M: SeeBlue Plus2 MW Marker
2 and 5 ug KSAC loaded

Animais

Canis familiaris



Critérios de inclusão:

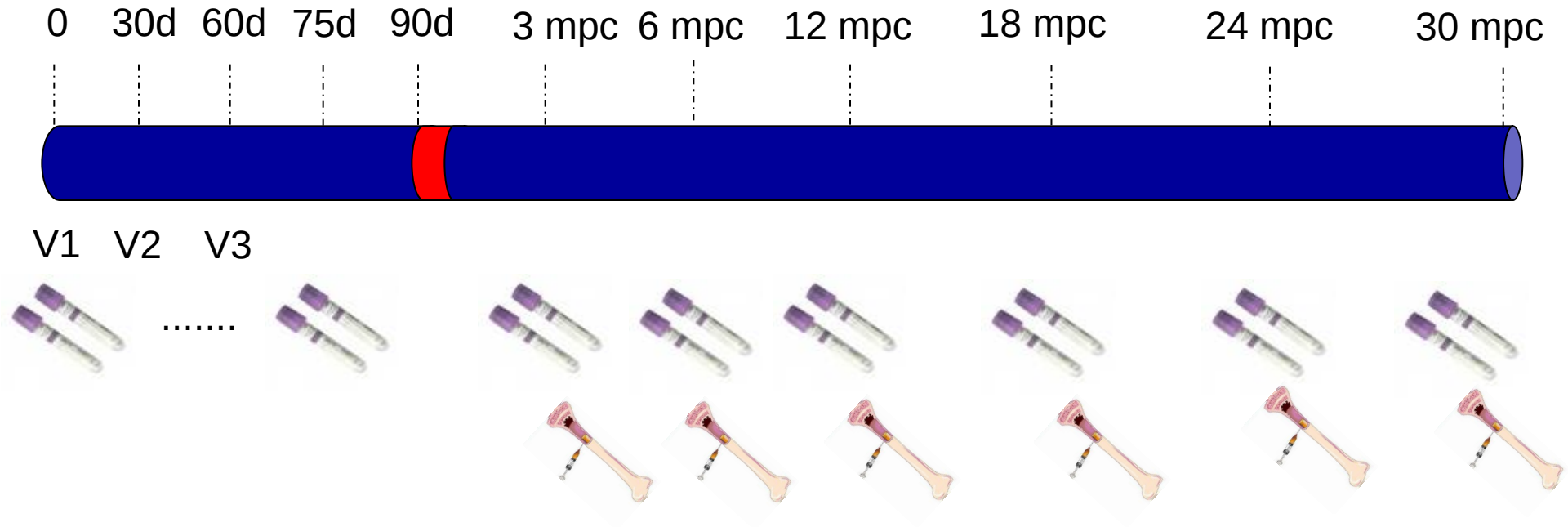
- Sem histórico de infecção por *Leishmania*
- Sorologia negativa
- PCR negativo (medula óssea)
- Hemograma, função renal e hepática com valores normais

- Machos e fêmeas
- Idade mínima de 4 meses
- Ração e água *ad libitum*
- Previamente submetidos a tratamento anti-helmíntico e vacinação (parvovirose, cinomose, adenovírus, hepatite, adenovírus tipo 2, parainfluenza, coronavirose, leptospirose, tosse canina e *Bordetella bronchiseptica*) (Fujiwara, 2003)

Delineamento experimental

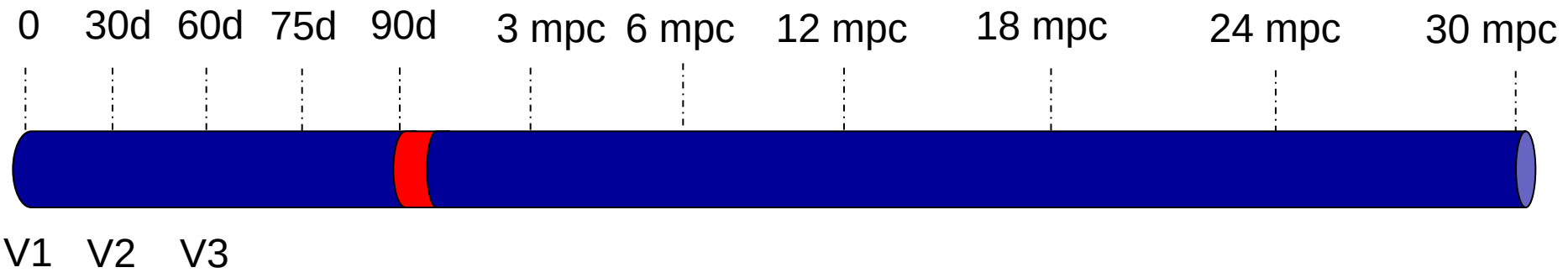
Grupos	Concentração de adjuvante (µg)	Concentração de KSAC (µg)	Número de animais
Salina	-	-	6
KSAC + Salina	-	10	6
KSAC + MPLA	10	10	8
KSAC + MPLA	50	10	8
KSAC + GLA	10	10	6
KSAC + GLA	50	10	6

Delineamento experimental



Desafio com 1 x 10^7 promastigotas (via endovenosa)
Cepa MHOM/BR/1972/BH46

Delineamento experimental



Imunogenicidade vacinal

Imunogenicidade e eficácia vacinal

Determinação da produção de anticorpos

Produção de IgG induzida pela vacinação (imunogenicidade)

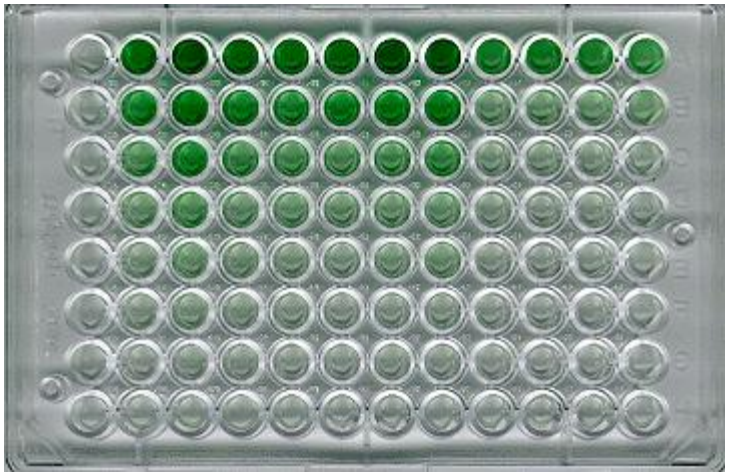
Produção de IgG após infecção-desafio (proteção)

KSAC

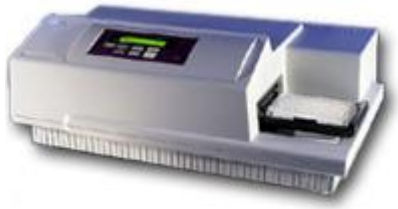
Kit EIE-LVC (Biomanguinhos)

rK39 (IDRI)

ELISA indireta



(Fujiwara et al., 2005; Fujiwara et al., 2007)



Avaliações hematológicas e bioquímicas

Hematológicas



Auto Hematology Analyzer BC-2800Vet

Número de leucócitos/mm^3
Níveis de hemoglobina
Contagem de plaquetas



Contagem diferencial de leucócitos

Bioquímicas

Dosagem
- Alanina aminotransferase (ALT)
- Aspartato aminotransferase (AST)
- Uréia
- Creatinina
- Albumina
- Proteínas totais
- Bilirrubina total
- Cálcio
- Fosfatase alcalina
- Globulina
- Fósforo
- Gama GT
- Glicose
- Sódio
- Amilase

Avaliação parasitológica

Avaliação da infecção e carga parasitária

PCR quantitativa (qPCR)





Extração de DNA



PCR em tempo real

DNA polimerase
- Amplificação de fragmento de 90 bp
- Curva padrão com plasmideo de *Leishmania*
- *Foward 5′ TGT CGC TTG CAG ACC AGA TG 3′*
- Reverse *5′ GCA TCG CAG GTG TGA GCA C 3′*

β-actin
- Amplificação de fragmento de 307 bp
- Forward, *5′ CTTCTACAACGAGCTGCGCG 3′*
- Reverse, *5′ TCATGAGGTAGTCGGTCAGG*.

(Mary et al., 2004; Selvapandyian et al., 2009; Alves et al., 2009)

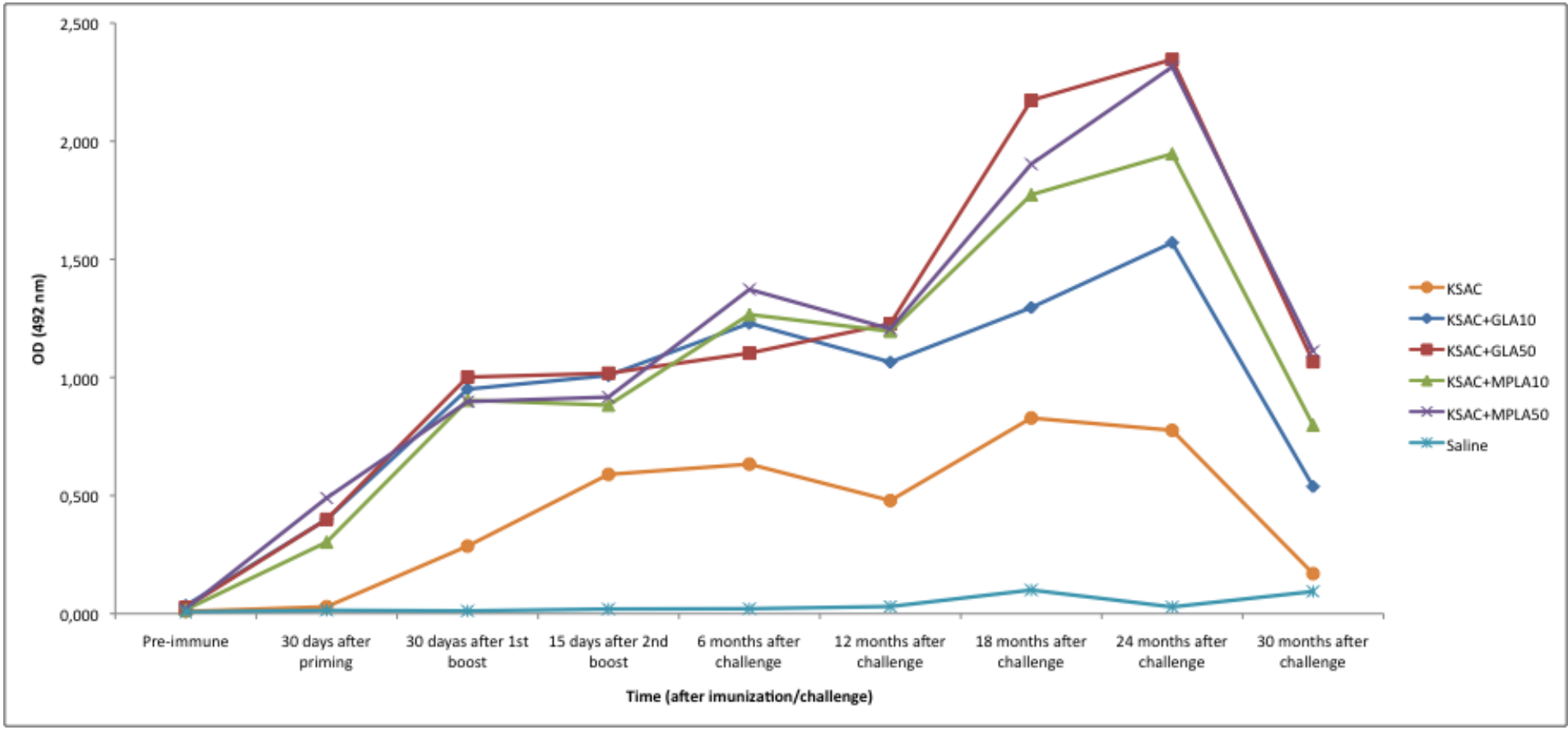
Acompanhamento clínico

Escore clínico

Categorias:	Número de pontos		
	0	1	2
Pele	Normal	Anormal (Áreas de alopécia, hiperqueratose, descamação)	Lesões severas
Condição corporal	Normal ou Obeso	Magro	Caquexia
Peso	Sem alteração ou ganho de peso	5,0% - 7,5% de perda	Maior que 7,5%
Unhas	Normal	Anormal (Discretamente aumentada)	Onicogrifose
Linfonodos	Normais ou não palpaveis	Até 2 aumentados	3 ou mais aumentados
Lesões Nasais/Orais	Ausente	Uma pequena lesão	2 ou mais lesões
Conjutivite	Ausente	Discreta	Grave

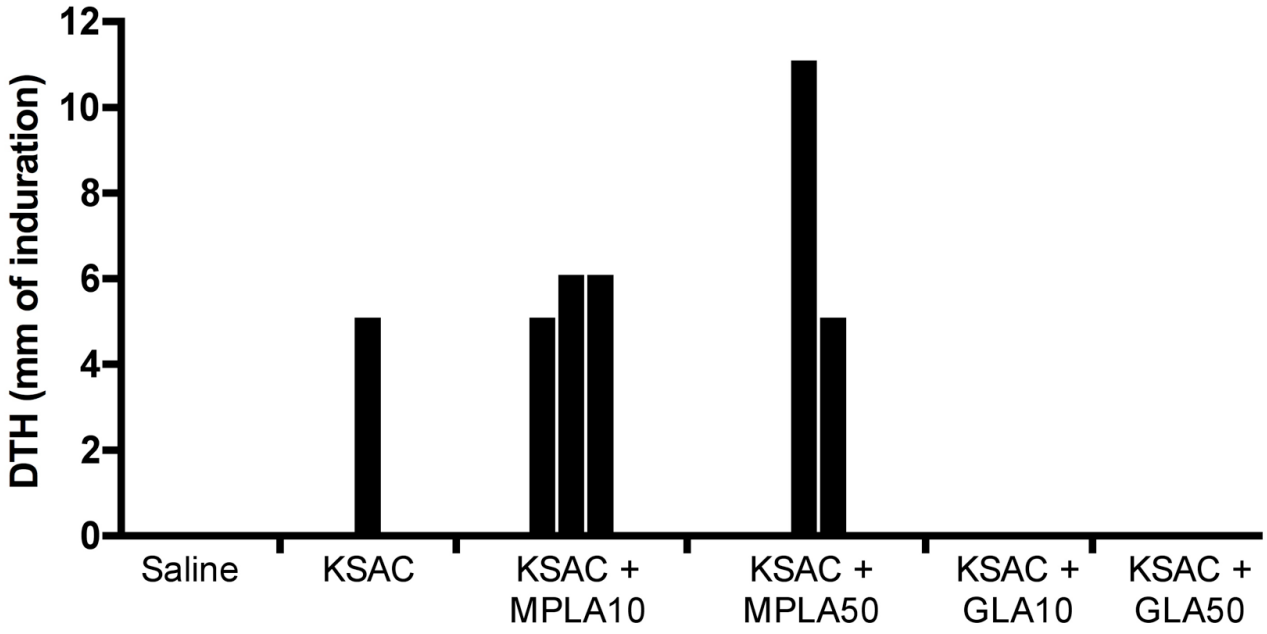
Necropsia e Laudo clínico*

Produção de anticorpos Anti-KSAC



Antibody response of beagle dogs immunized with KSAC. Animals were immunized three times (one month apart) with 10µg of KSAC mixed with either the adjuvant GLA (10 and 50 µg), or the adjuvant MPLA (10 and 50µg) or saline. Dogs were bled before the immunizations (Pre-immune), 30 days after the priming and first boost, 15 days after the second boost, and 6, 12, 18, 24 and 30 months after challenge. Antibody response was measured by conventional serological ELISA with sera diluted at 1/100. Bars represent the SD for the OD obtained per group.

Hipersensibilidade tardia frente ao KSAC (30 dias) pós-vacinação



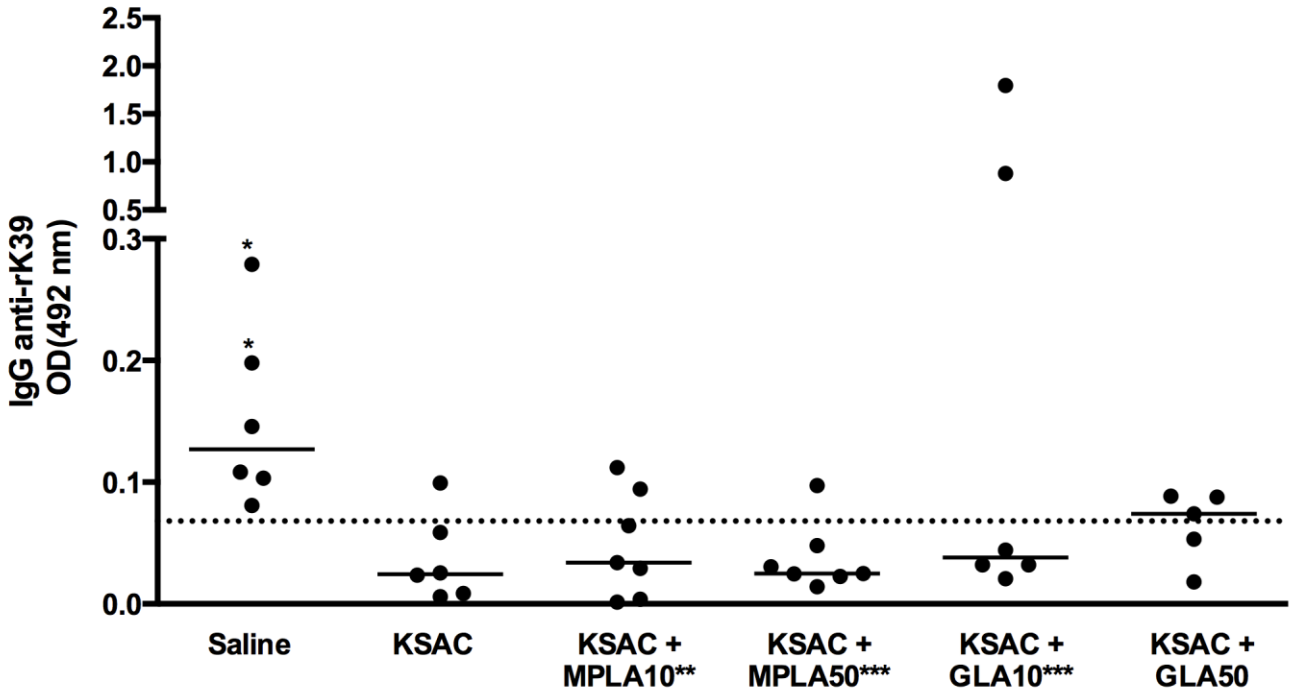
DTH reaction in KSAC immunized and control dogs. Groups of dogs were immunized three times (one month interval) with either saline, or with 10µg of KSAC mixed with saline, or with two concentrations of MPLA (10 and 50µg) or with two concentrations of GLA (10 and 50 µg). Thirty days after the last immunization the animals were skin tested with 5 µg of KSAC using the Mantoux technique (100µl of antigenic solution inoculated intradermally). Induration reactions were read 48 hours later. Bars represent individual animals.

Hipersensibilidade tardia frente ao KSAC



DTH reaction in KSAC immunized dog (KSAC + MPLA50)

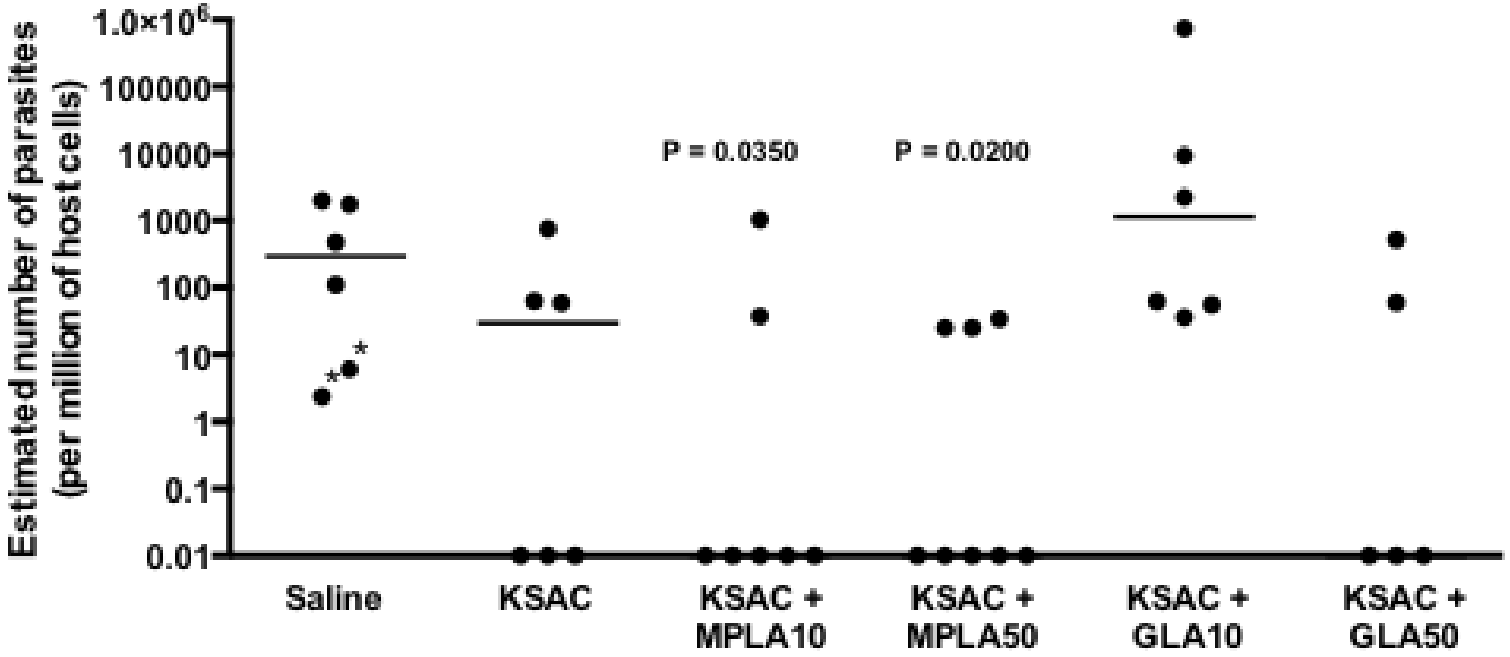
Produção de anticorpos Anti-rK39



Antibody production (IgG anti-rK39) in immunized and control dogs 30 months after challenge with *L. infantum*. Solid lines represent median and dotted line represent the calculated cut-off to distinguish positive animals (cut-off = 0.065, considering 3 standard deviation).
*Result using serum obtained during euthanasia/necropsy
**Loss of animals with no association with leishmaniasis (fight)
***Loss of animal with association with leishmaniasis

Detecção de *Leishmania* por qPCR

Carga parasitária



Parasite burden in immunized and control dogs 24 months after challenge with *L. infantum*. Solid line represent median of parasites per million of host cells.
*Results from samples obtained during euthanasia/necropsy

Detecção de *Leishmania* por qPCR

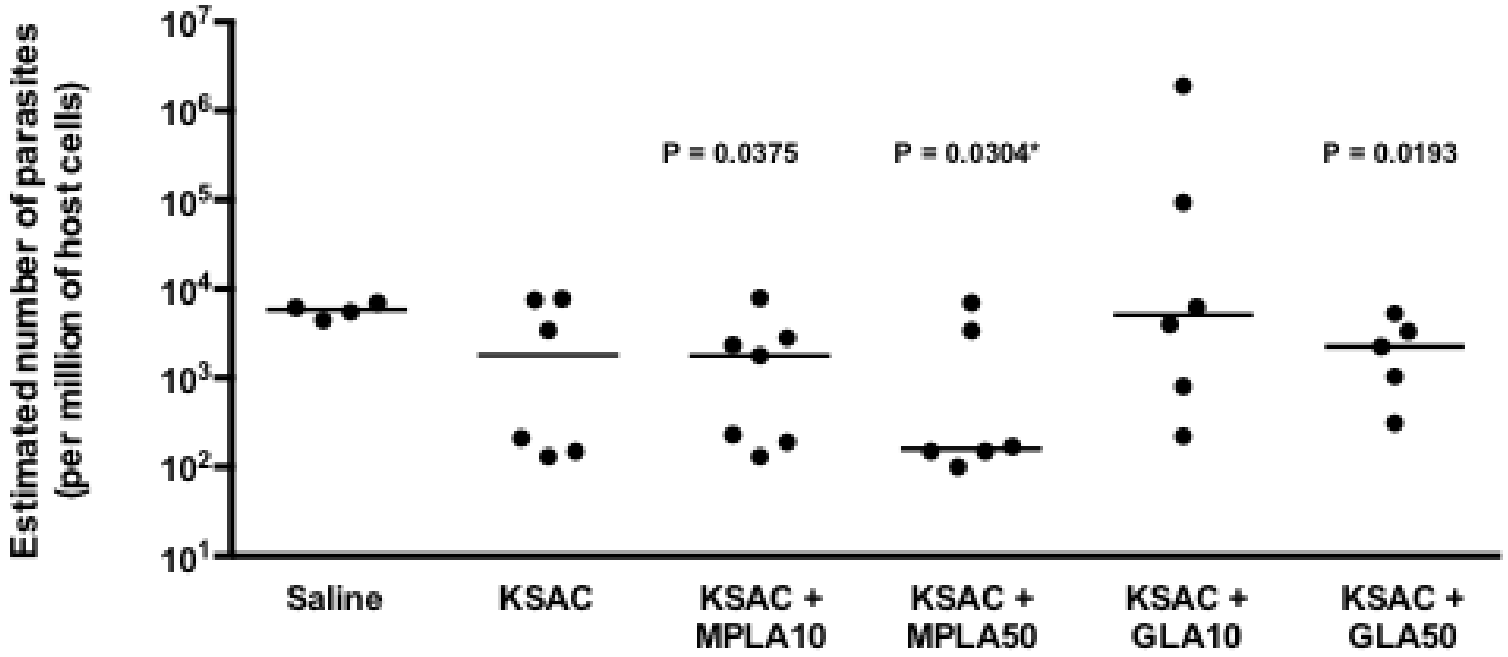
Número de animais positivos após 24 meses de infecção

Grupos	Número de animais do grupo	Mortes associadas à infecção	Número de animais PCR positivos (%)
Salina	6	2	4/4 (100%)
KSAC + Salina	6	-	3/6 (50%)
KSAC + MPLA10	8*	-	2/7 (28.5%)
KSAC + MPLA50	8	-	3/8 (37.5%)
KSAC + GLA10	6	-	6/6 (100%)
KSAC + GLA50	6*	-	2/5 (40%)

***Perda de animais não relacionadas ao processo de infecção

Detecção de *Leishmania* por qPCR

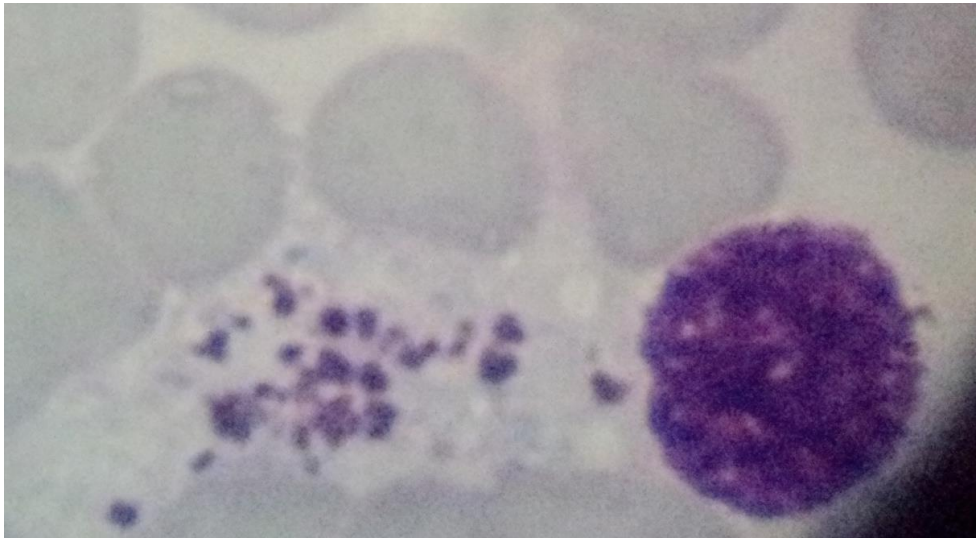
Carga parasitária



Parasite burden in immunized and control dogs 30 months after challenge with *L. infantum*. Solid line represent median of parasites per million of host cells.

Detecção de *Leishmania* por qPCR

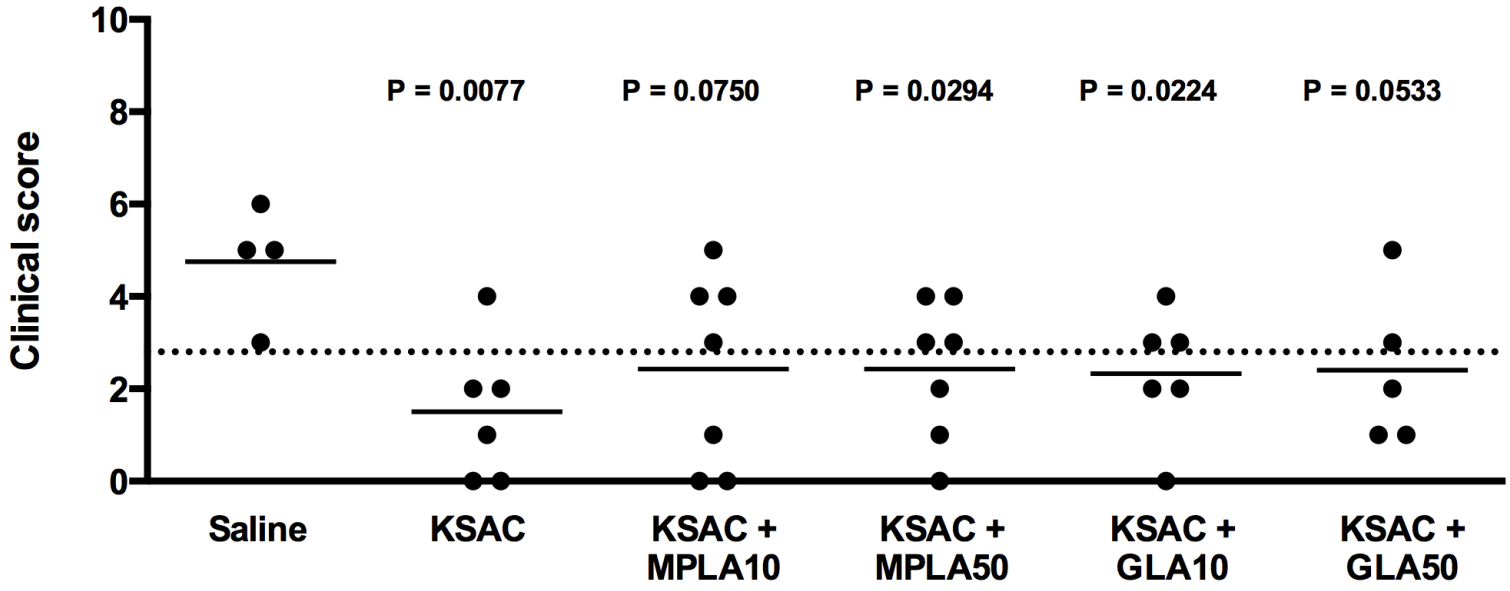
Carga parasitária



Bone marrow smear with macrophage parasitized with *L. infantum* (Animal 873 – Saline) during follow up of 12 months after challenge

Acompanhamento clínico

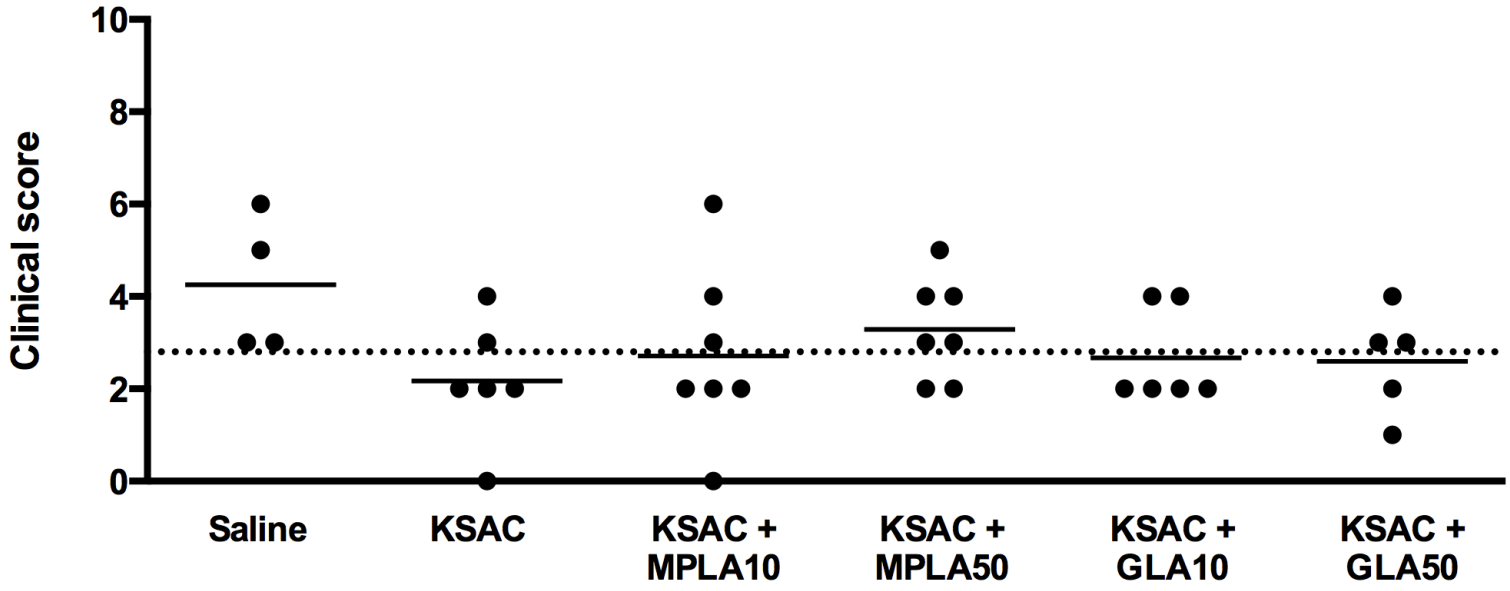
Escore clínico



Clinical score observed in immunized and control dogs 24 months after challenge with *L. infantum*. Solid line represent mean average of clinical score. Values represent differences to the Saline group (P values were provided).

Acompanhamento clínico

Escore clínico



Clinical score observed in immunized and control dogs 30months after challenge with *L. infantum*. Solid line represent mean average of clinical score.

Acompanhamento clínico

Laudos clínicos (necropsia)

Animal 996 (Saline). Opacidade de córnea e aumento do abdômen, alterações estas associadas a resposta após desafio. Após os primeiros sintomas, o animal apresentou perda de peso progressiva. Foi observado o óbito do animal 996 (Saline) no dia 09/12/2011, com uveite, aumento do abdômen, perda de peso progressiva, sem nenhuma relação com processo vacinal. Positividade para qPCR.

Animal 900 (Saline). Tumor mamário de crescimento explosivo, com óbito 21 dias depois do diagnóstico e tratamento intensivo com quimioterápicos. Durante necropsia, metástase nos linfonodos subiliacais foram encontradas, envolvendo e obstruindo os ureteres do animal. Sinais associados à leishmaniose. Positividade para qPCR.

Animal 874 (KSAC + MPLA10). Briga foi decorrente do estado de cio. Feridas estavam concentradas na região inguinal e de membros posteriores.

Animal 822 (KSAC + GLA50). Briga foi decorrente do estado de cio. Feridas estavam concentradas na região inguinal e de membros posteriores.

Acompanhamento clínico







Avaliações hematológicas e bioquímicas

Hematológicas



Auto Hematology Analyzer BC-2800Vet

Número de leucócitos/mm^3
Níveis de hemoglobina
Contagem de plaquetas



Contagem diferencial de leucócitos

Bioquímicas

Dosagem
- Alanina aminotransferase (ALT)
- Aspartato aminotransferase (AST)
- Uréia
- Creatinina
- Albumina
- Proteínas totais
- Bilirrubina total
- Cálcio
- Fosfatase alcalina
- Globulina
- Fósforo
- Gama GT
- Glicose
- Sódio
- Amilase

Resumo dos resultados e considerações

• A produção de anticorpos anti-KSAC foi patente até 24 meses após imunização, com considerável queda após 30 meses de acompanhamento

• Considerando produção de anticorpos e resposta de hipersensibidade tardia (Teste de Montenegro), as vacinas KSAC+$MPLA_{10}$, KSAC+$MPLA_{50}$ demonstraram melhor potencial de imunogenicidade

• As vacinas KSAC+$MPLA_{10}$, KSAC+$MPLA_{50}$ e KSAC+GLA_{50} apresentaram redução significativa de carga parasitária 30 meses após a infecção desafio

• A longa patência da infecção foi decorrente da cepa utilizada da infecção (MHOM/BR/1972/BH46) e deve ser reduzida pela utilização da cepa CL14 (Wild type strain isolada em campo), atualmente em teste no laboratório (patência de 12 meses).

Tradition : A revolution incorporated in the routine life of the people

Revolution: Something that will be incorporated as tradition

Each vaccine development can be a revolution in public health.

If it works and if it is acessible to all,
the developed vaccine becomes a tradition!

Thanks!

Perspectivas

- Xenodiagnóstico
- Histopatologia
- Disponibilidade de teste de vacinas ou adjuvantes em modelos (cães, coelhos, primatas – *Callithrix* sp, etc)

- Apoio laboratorial aos centros de teste em campo
- Banco de amostras e controle de qualidade

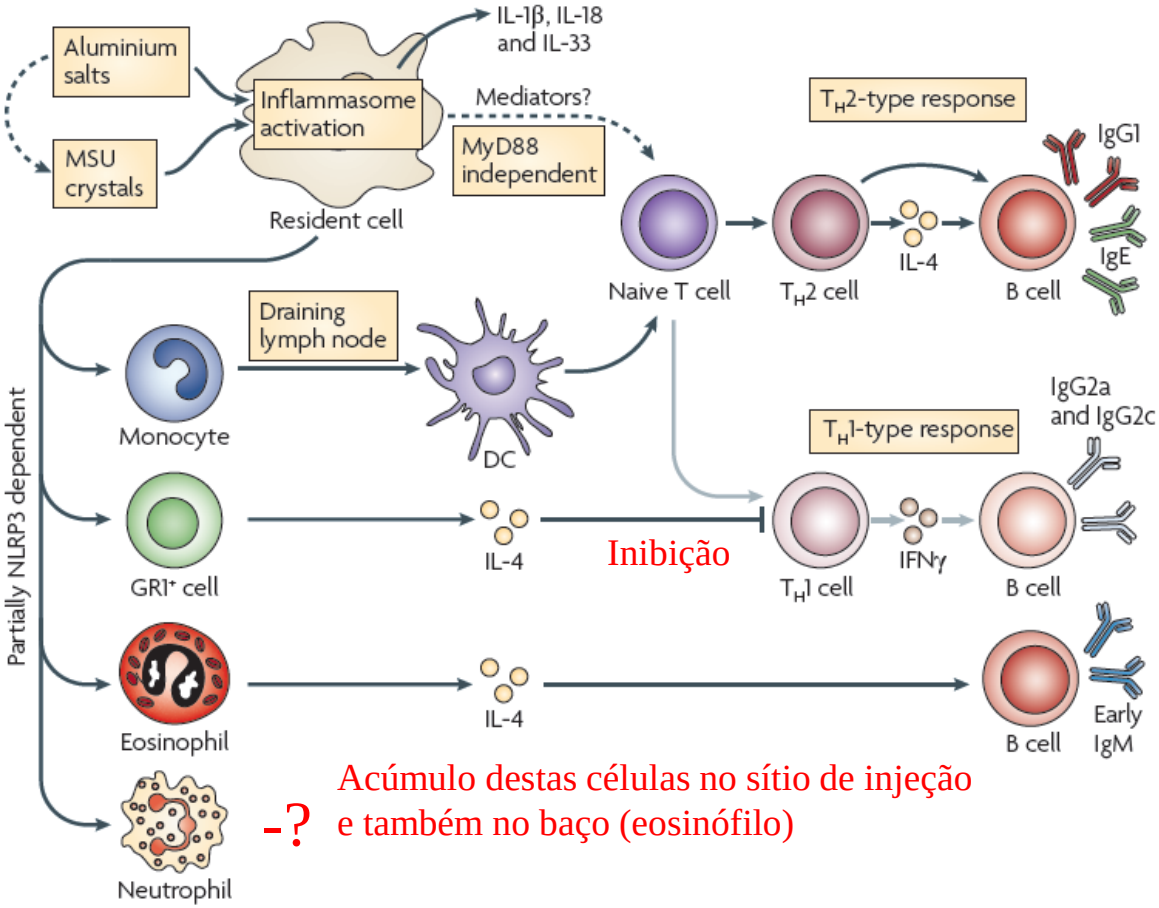


Figure 2 | **Stimulation of adaptive immune responses by aluminium salts.** Aluminium salts and/or monosodium urate (MSU) crystals that are induced by aluminium salts activate resident phagocytes partly through the NLR family, pyrin domain containing 3 (NLRP3; also known as NALP3) inflammasome. If and how NLRP3 inflammasome activation contributes to induction of adaptive T helper 2 (T_H2)-type responses, such as interleukin-4 (IL-4) production by T_H2 cells and IgG1 and IgE production by B cells, is still controversial and under investigation. Aluminium salts also induce the accumulation of monocytes, GR1$^+$ cells, eosinophils and neutrophils to the site of injection, and eosinophils also accumulate in the spleen. Monocytes mature into CD11c$^+$ dendritic cells (DCs) as they traffic to the draining lymph nodes, where they activate naive CD4$^+$ T cells. GR1$^+$ cells secrete IL-4, which suppresses T_H1-cell responses, and eosinophils enhance early IgM responses by B cells. Although neutrophils accumulate at the injection site, they have no known role in adaptive immunity. IFNγ, interferon-γ; MyD88, myeloid differentiation primary-response gene 88.

Modulation of adaptative immunity by aluminium salts, by stimulating the production of antibodies (Th2 response) and inhibiting Th1 response

The FASEB Journal • Research Communication

Antigen depot is not required for alum adjuvanticity

Sharon Hutchison,[1] Robert A. Benson,[1] Vivienne B. Gibson, Abigail H. Pollock, Paul Garside, and James M. Brewer[2]

Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK

ABSTRACT Alum adjuvants have been in continuous clinical use for more than 80 yr. While the prevailing theory has been that depot formation and the associated slow release of antigen and/or inflammation are responsible for alum enhancement of antigen presentation and subsequent T- and B-cell responses, this has never been formally proven. To examine antigen persistence, we used the chimeric fluorescent protein EαGFP, which allows assessment of antigen presentation *in situ*, using the Y-Ae antibody. We demonstrate that alum and/or CpG adjuvants induced similar uptake of antigen, and in all cases, GFP signal did not persist beyond 24 h in draining lymph node antigen-presenting cells. Antigen presentation was first detectable on B cells within 6–12 h of antigen administration, followed by conventional dendritic cells (DCs) at 12–24 h, then finally plasmacytoid DCs at 48 h or later. Again, alum and/or CpG adjuvants did not have an effect on the magnitude or sequence of this response; furthermore, they induced similar antigen-specific T-cell activation *in vivo*. Notably, removal of the injection site and associated alum depot, as early as 2 h after administration, had no appreciable effect on antigen-specific T- and B-cell responses. This study clearly rules out a role for depot formation in alum adjuvant activity.—Hutchison, S., Benson, R. A., Gibson, V. B., Pollock, A. H., Garside, P., Brewer, J. M. Antigen depot is not required for alum adjuvanticity. *FASEB J.* 26, 1272–1279 (2012). www.fasebj.org

Nature. 2008 Jun 19;453(7198):1122-6. doi: 10.1038/nature06939. Epub 2008 May 21.

Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants.

Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA.

Department of Immunobiology, Yale University School of Medicine, New Haven, Connecticut 06520, USA.

Abstract

Aluminium adjuvants, typically referred to as 'alum', are the most commonly used adjuvants in human and animal vaccines worldwide, yet the mechanism underlying the stimulation of the immune system by alum remains unknown. Toll-like receptors are critical in sensing infections and are therefore common targets of various adjuvants used in immunological studies. Although alum is known to induce the production of proinflammatory cytokines in vitro, it has been repeatedly demonstrated that alum does not require intact Toll-like receptor signalling to activate the immune system. Here we show that aluminium adjuvants activate an intracellular innate immune response system called the Nalp3 (also known as cryopyrin, CIAS1 or NLRP3) inflammasome. Production of the pro-inflammatory cytokines interleukin-1beta and interleukin-18 by macrophages in response to alum in vitro required intact inflammasome signalling. Furthermore, in vivo, mice deficient in Nalp3, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) or caspase-1 failed to mount a significant antibody response to an antigen administered with aluminium adjuvants, whereas the response to complete Freund's adjuvant remained intact. We identify the Nalp3 inflammasome as a crucial element in the adjuvant effect of aluminium adjuvants; in addition, we show that the innate inflammasome pathway can direct a humoral adaptive immune response. This is likely to affect how we design effective, but safe, adjuvants in the future.

J Immunol. 2009 October 1; 183(7): 4403–4414. doi:10.4049/jimmunol.0900164.

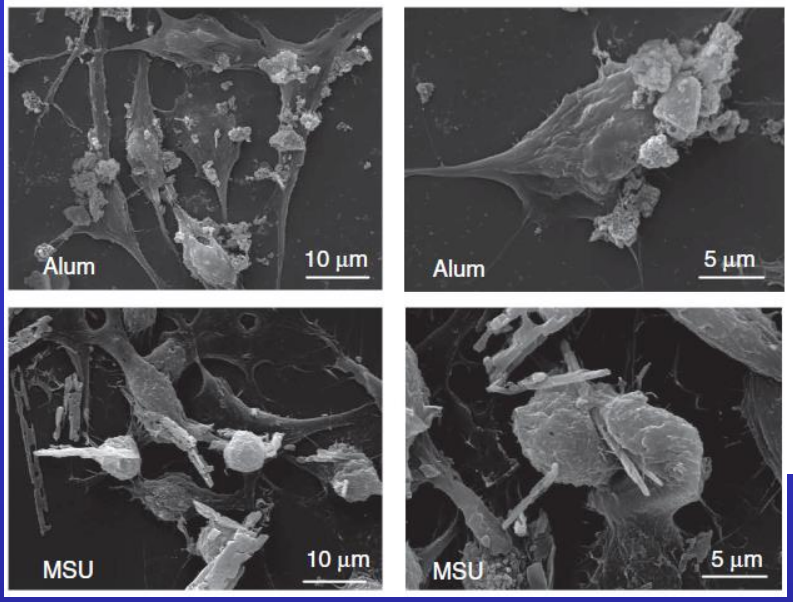
Alum induces innate immune responses through macrophage and mast cell sensors, but these are not required for alum to act as an adjuvant for specific immunity.[1,2]

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Abstract

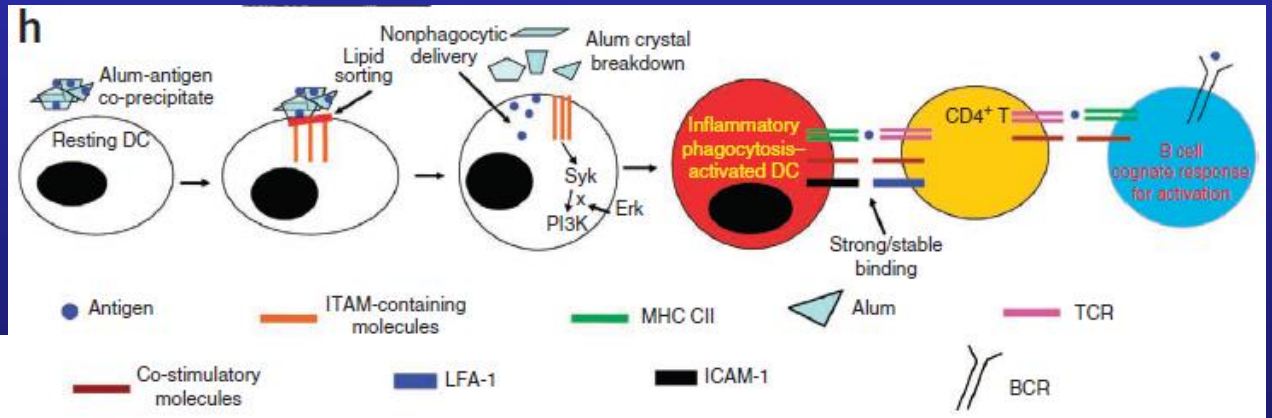
To understand more about how the body recognizes alum we characterized the early innate and adaptive responses in mice injected with the adjuvant. Within hours of exposure, alum induces a type 2 innate response characterized by an influx of eosinophils, monocytes, neutrophils, DCs, NK cells and NKT cells. In addition, at least thirteen cytokines and chemokines are produced within 4 hours of injection including IL-1β and IL-5. Optimal production of some of these, including IL-1β, depends upon both macrophages and mast cells, while production of others, such as IL-5, depends on mast cells only, suggesting that both of these cell types can detect alum. Alum induces eosinophil accumulation partly through the production of mast cell derived IL-5 and histamine. Alum greatly enhances priming of endogenous CD4 and CD8 T cells independently of mast cells, macrophages and of eosinophils. In addition, antibody levels and Th2 bias was similar in the absence of these cells. We found that the inflammation induced by alum was unchanged in caspase-1 deficient mice, which cannot produce IL-1β. Furthermore, endogenous CD4 and CD8 T cell responses, antibody responses and the Th2 bias were also not impacted by the absence of caspase-1 or NLRP3. These data suggest that activation of the inflammasome and the type 2 innate response orchestrated by macrophages and mast cells in vivo are not required for alum's adjuvant effects on endogenous T and B cell responses.



Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity

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As an approved vaccine adjuvant for use in humans, alum has vast health implications, but, as it is a crystal, questions remain regarding its mechanism. Furthermore, little is known about the target cells, receptors, and signaling pathways engaged by alum. Here we report that, independent of inflammasome and membrane proteins, alum binds dendritic cell (DC) plasma membrane lipids with substantial force. Subsequent lipid sorting activates an abortive phagocytic response that leads to antigen uptake. Such activated DCs, without further association with alum, show high affinity and stable binding with CD4+ T cells via the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function–associated antigen-1 (LFA-1). We propose that alum triggers DC responses by altering membrane lipid structures. This study therefore suggests an unexpected mechanism for how this crystalline structure interacts with the immune system and how the DC plasma membrane may behave as a general sensor for solid structures.



used. (**h**) A proposed mechanism of how alum invokes the humoral immune response in vaccination. In response to the alum-antigen mixture, DC membrane lipids serve as a surrogate receptor to the crystal surface. The ensuing lipid sorting triggers the aggregation of ITAM containing molecules, Syk recruitment and PI3K activation. However, alum does not enter DCs; it instead delivers the antigen into the DCs via endocytic uptake. DCs process the antigen in their MHC class II compartment and at the same time become activated as a consequence of the inflammatory phagocytosis. The activated DCs strongly engage CD4+ cells via ICAM-1 and LFA-1 binding, leading to the subsequent cognate B cell activation. Here cross-presentation of MHC class I antigens is absent, resulting in no CTL induction.

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Host DNA released in response to aluminum adjuvant enhances MHC class II-mediated antigen presentation and prolongs CD4 T-cell interactions with dendritic cells.

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Abstract

Many vaccines include aluminum salts (alum) as adjuvants despite little knowledge of alum's functions. Host DNA rapidly coats injected alum. Here, we further investigated the mechanism of alum and DNA's adjuvant function. Our data show that DNase coinjection reduces CD4 T-cell priming by i.m. injected antigen + alum. This effect is partially replicated in mice lacking stimulator of IFN genes, a mediator of cellular responses to cytoplasmic DNA. Others have shown that DNase treatment impairs dendritic cell (DC) migration from the peritoneal cavity to the draining lymph node in mice immunized i.p. with alum. However, our data show that DNase does not affect accumulation of, or expression of costimulatory proteins on, antigen-loaded DCs in lymph nodes draining injected muscles, the site by which most human vaccines are administered. DNase does inhibit prolonged T-cell-DC conjugate formation and antigen presentation between antigen-positive DCs and antigen-specific CD4 T cells following i.m. injection. Thus, from the muscle, an immunization site that does not require host DNA to promote migration of inflammatory DCs, alum acts as an adjuvant by introducing host DNA into the cytoplasm of antigen-bearing DCs, where it engages receptors that promote MHC class II presentation and better DC-T-cell interactions.

Alum adjuvant effect is complex and may involve several different pathways and mechanisms !!

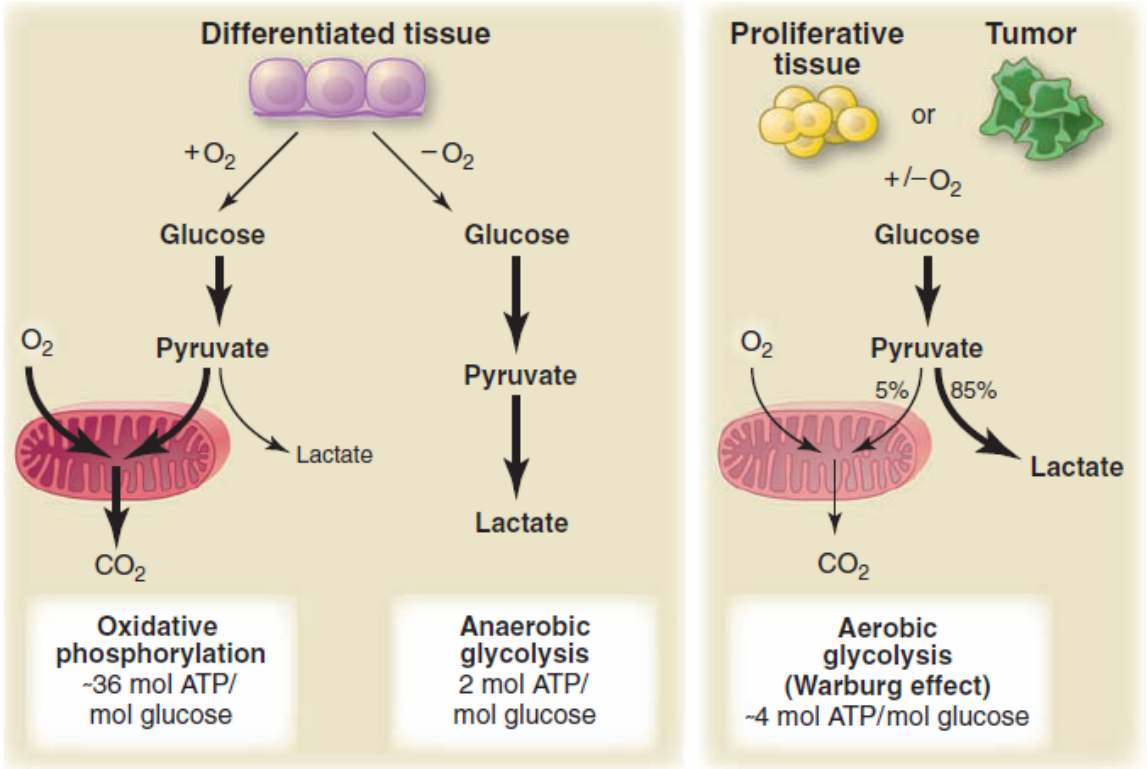
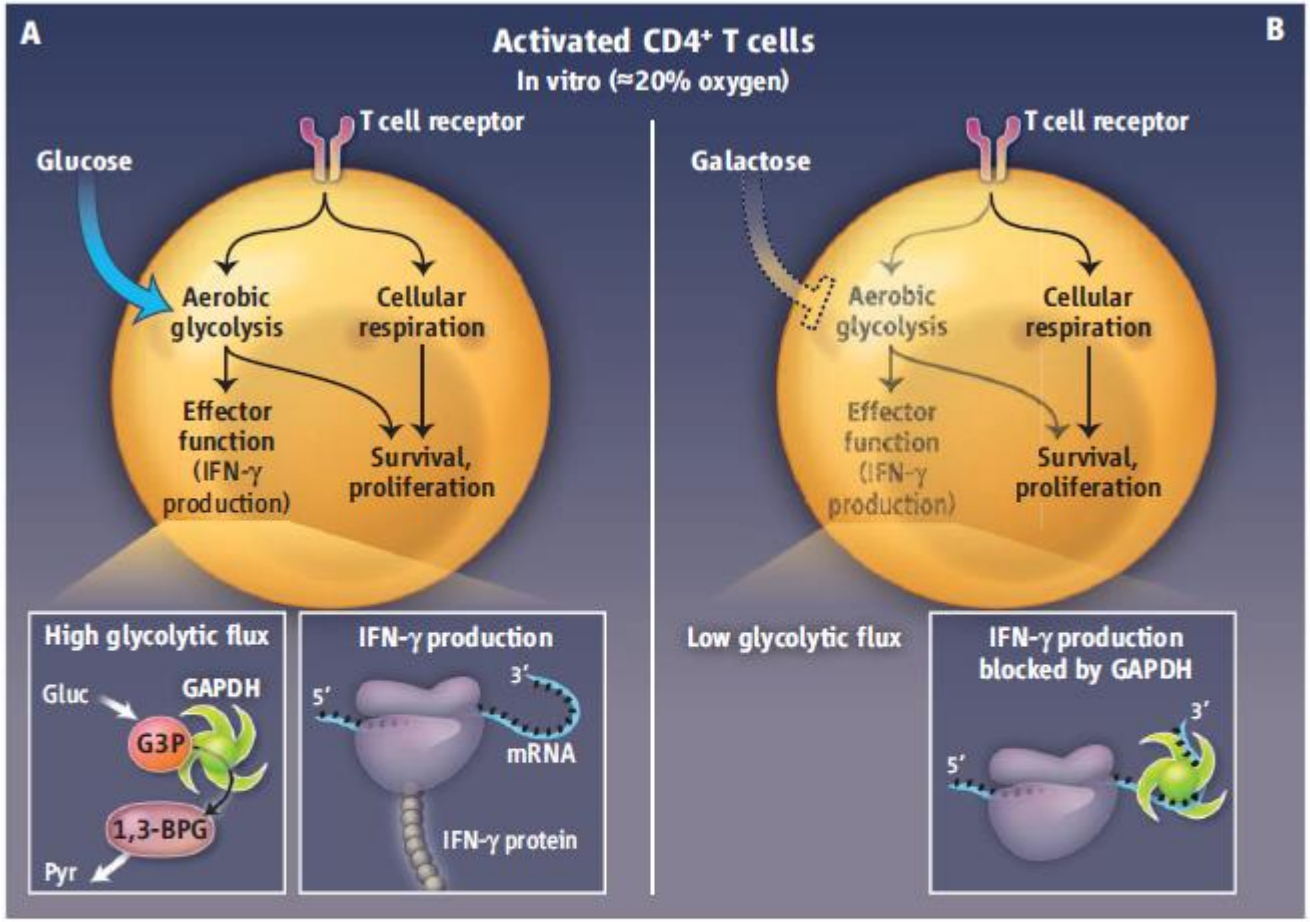


Fig. 2. Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect). In the presence of oxygen, nonproliferating (differentiated) tissues first metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO_2 during the process of oxidative phosphorylation. Because oxygen is required as the final electron acceptor to completely oxidize the glucose, oxygen is essential for this process. When oxygen is limiting, cells can redirect the pyruvate generated by glycolysis away from mitochondrial oxidative phosphorylation by generating lactate (anaerobic glycolysis). This generation of lactate during anaerobic glycolysis allows glycolysis to continue (by cycling NADH back to NAD^+), but results in minimal ATP production when compared with oxidative phosphorylation. Warburg observed that cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis). This property is shared by normal proliferative tissues. Mitochondria remain functional and some oxidative phosphorylation continues in both cancer cells and normal proliferating cells. Nevertheless, aerobic glycolysis is less efficient than oxidative phosphorylation for generating ATP. In proliferating cells, ~10% of the glucose is diverted into biosynthetic pathways upstream of pyruvate production.

Metabolismo e resposta imune



T cell metabolism. In activated CD4+ T cells, T cell receptor signaling drives aerobic glycolysis and cellular respiration. When cultured in 20% O_2, T cell survival and proliferation can be supported by either metabolic process, whereas the production of effector cytokines such as IFN-γ requires aerobic glycolysis. **(A)** When cultured in glucose, glycolytic flux is high, GAPDH acts on the glycolytic substrate G3P, mRNA, and IFN-γ mRNA is translated. **(B)** When cultured in galactose, glycolytic flux is inhibited, and the lack of glycolytic intermediates causes GAPDH to bind to IFN-γ mRNA, repressing its translation. Gluc, glucose; Pyr, pyruvate; 1,3-BPG, 1,3-biphosphoglycerate.

Influenza and MPLA

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Pneumoccocus and whole cell pertussis

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Auxílio financeiro

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Table 1 Key steps of the development of the recombinant pertussis vaccine

1984	The project starts
1985	Cloning and sequencing of the pertussis toxin gene
1987	Identification of the key amino acids to be changed by mutagenesis to make a nontoxic derivative of pertussis toxin
1988	Sequence of the gene coding for filamentous hemagglutinin
1989	Construction of a *Bordetella pertussis* strain producing a nontoxic mutant of pertussis toxin
	Phase I clinical trials
1990	Phase II trials with acellular pertussis vaccine, alone (aP) or combined with diphtheria and tetanus (DTaP)
1992	Beginning of the phase III efficacy trial with a DTaP vaccine
1993	Approval and marketing of the aP vaccine in Italy
1995	End of the phase III efficacy trial, approval and marketing of the DTaP vaccine in Italy
1996–97	Filing of the product license application worldwide