université PARIS-SACLAY
GRADUATE SCHOOL Health and Drug Sciences

*Master 1 D²HP*
*Development of Drugs and Health Products*

*Compulsory Teaching Unit 11: Pharmacology/Toxicology*

# Toxicological models : Immunotoxicology

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Faculté de Pharmacie Paris-Saclay
INSERM-996

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Année 2024-2025

# PLAN

## 1. DEFINITIONS

- The different cells
- The different organs
- The immune response

## 2. HYPERSENSITIVITIES

- Definition of hypersensitivity
- The different tests

## 3. IMMUNOSUPPRESSION

- Definition of immunosuppression
- The different tests

# PLAN

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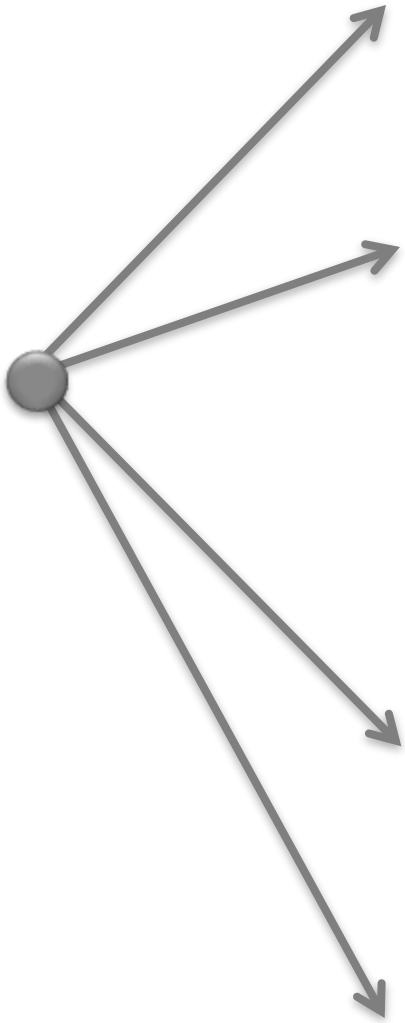
# IMMUNOTOXICOLOGY: DEFINITION

**Immunotoxicology** is a scientific discipline whose aim is to detect, quantify, and interpret direct or indirect alterations of the **immune system** that occur as a result of exposure to chemicals, pharmaceuticals, recombinant biologicals, or environmental and occupational pollutants.

# DÉFINITION DE L'IMMUNOTOXICOLOGIE

IMMUNOTOXICOLOGY



**Immunosuppression**

Infections, tumors

**Hypersensitivity** ('allergies')

Asthma, rhinitis,
Anaphylactic choc
Cytolysis (red blood cells, platelets, neutrophils),
hepatitis IA
immune complexes,
contact eczema

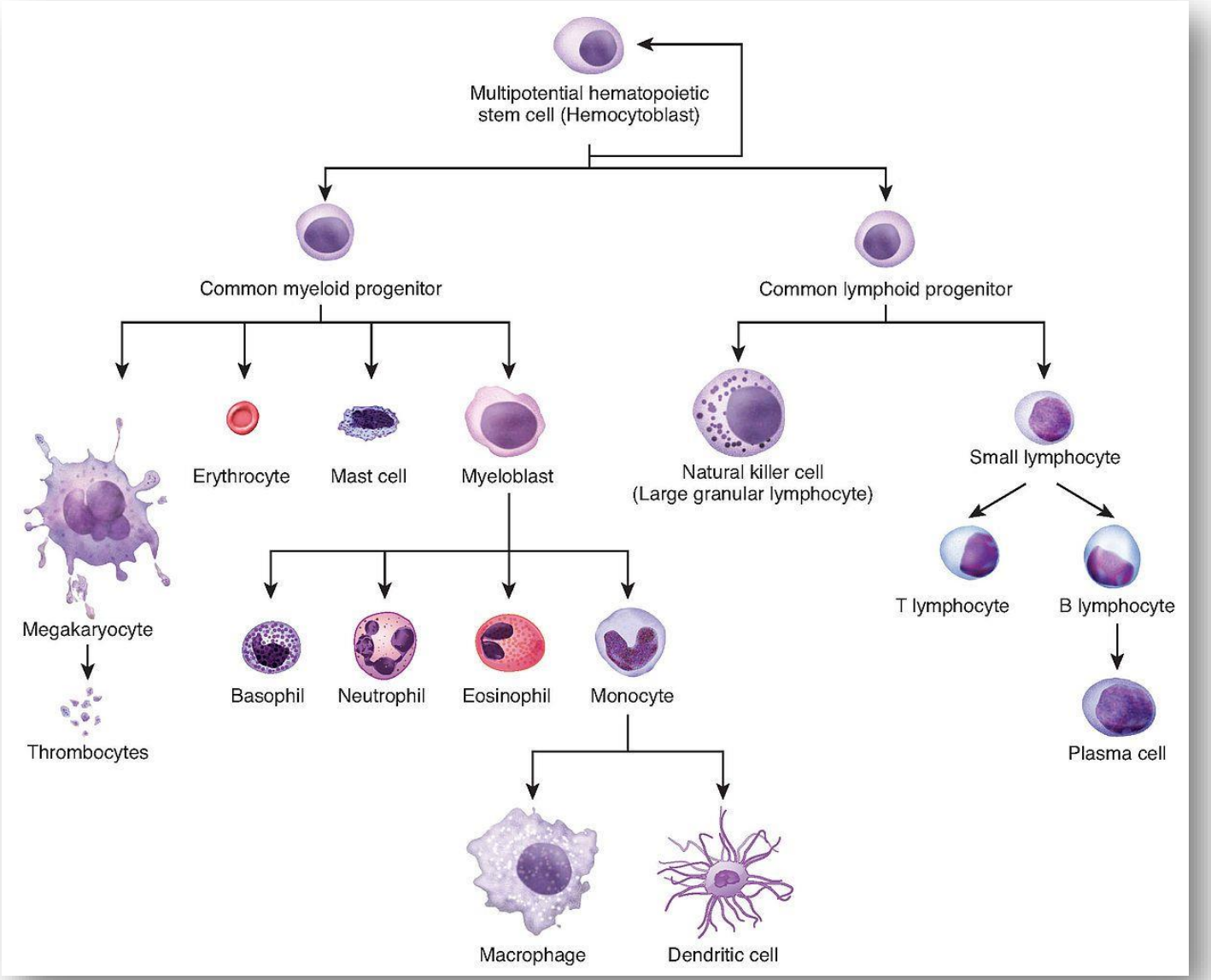
**Auto-immunity**

Autoimmune hepatitis, lupus

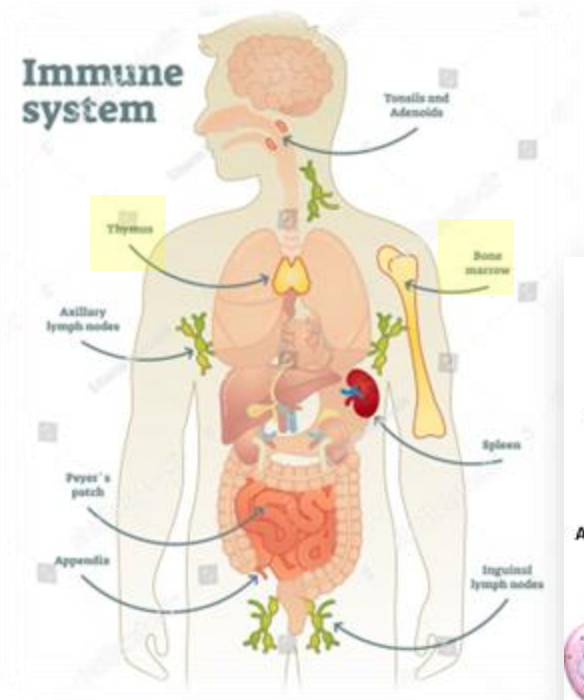
**Inappropriate stimulation of the immune system**

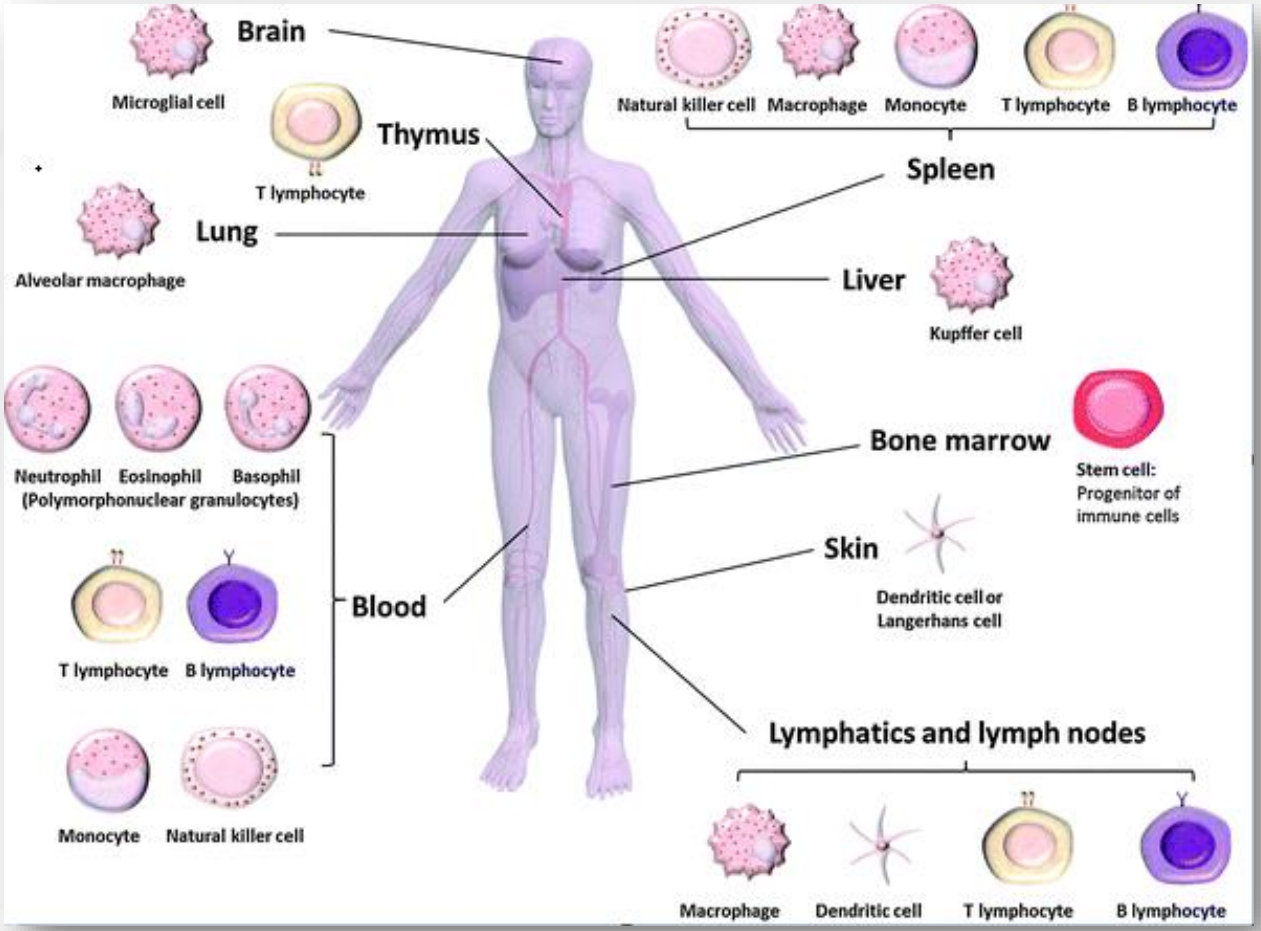
Cytokine shock
Influenza (flu)-like syndrome

# BLOOD CELLS

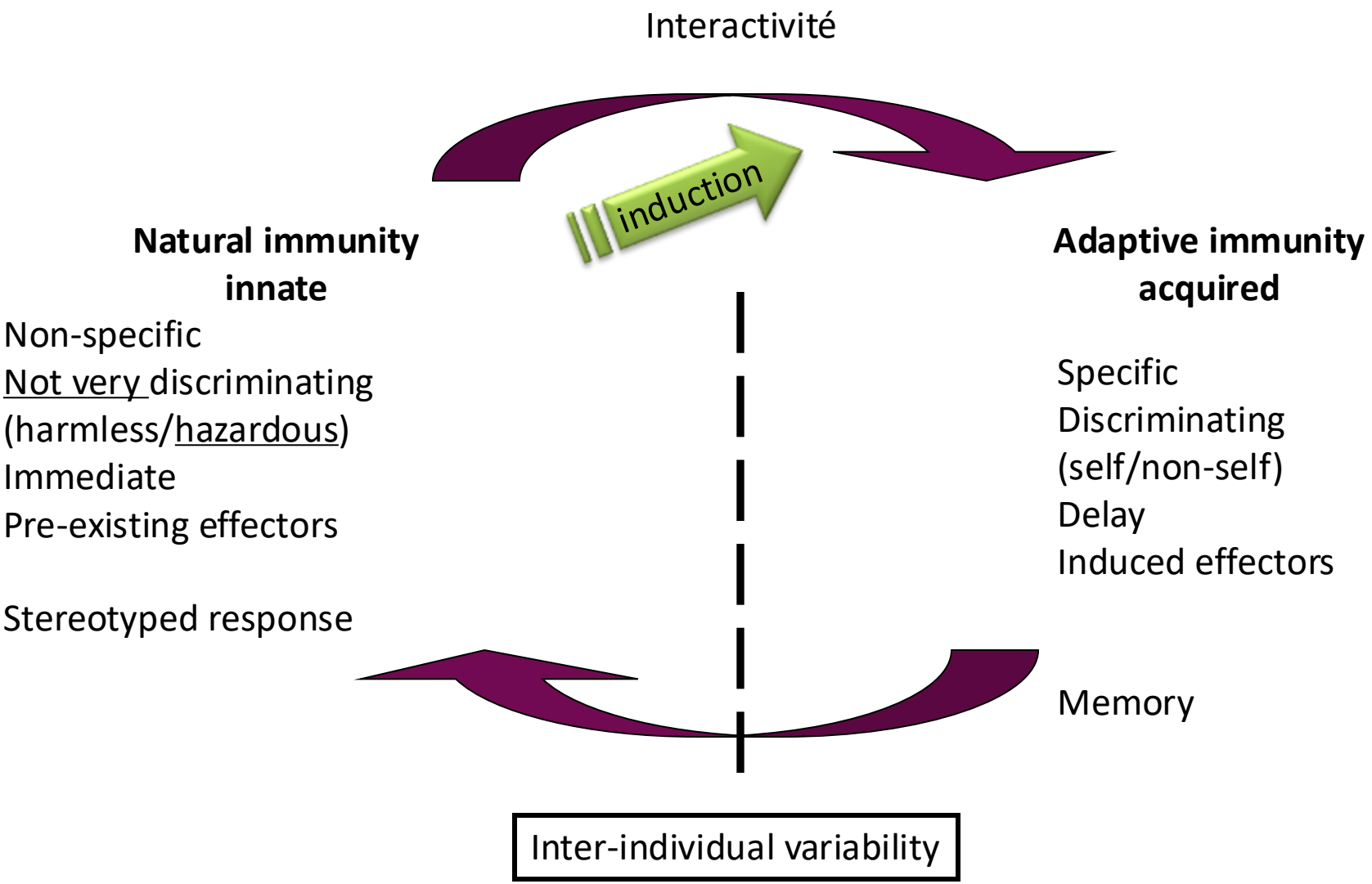

Multipotential hematopoietic stem cell (Hemocytoblast)
Common myeloid progenitor
Common lymphoid progenitor
Erythrocyte
Mast cell
Myeloblast
Natural killer cell (Large granular lymphocyte)
Small lymphocyte
Megakaryocyte
Thrombocytes
Basophil
Neutrophil
Eosinophil
Monocyte
T lymphocyte
B lymphocyte
Plasma cell
Macrophage
Dendritic cell

# IMMUNE SYSTEM: ORGANS & CELLS

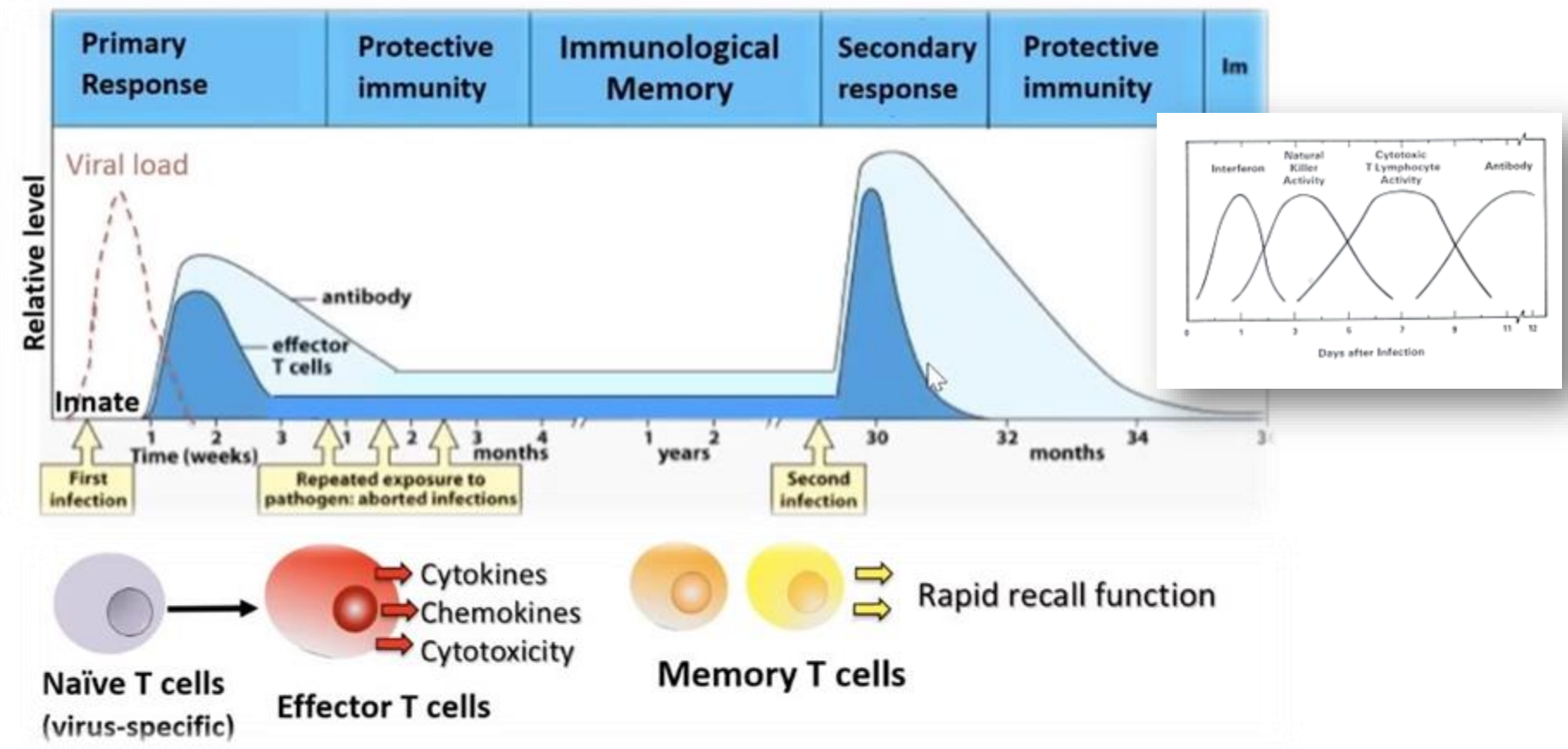


# IMMUNITY

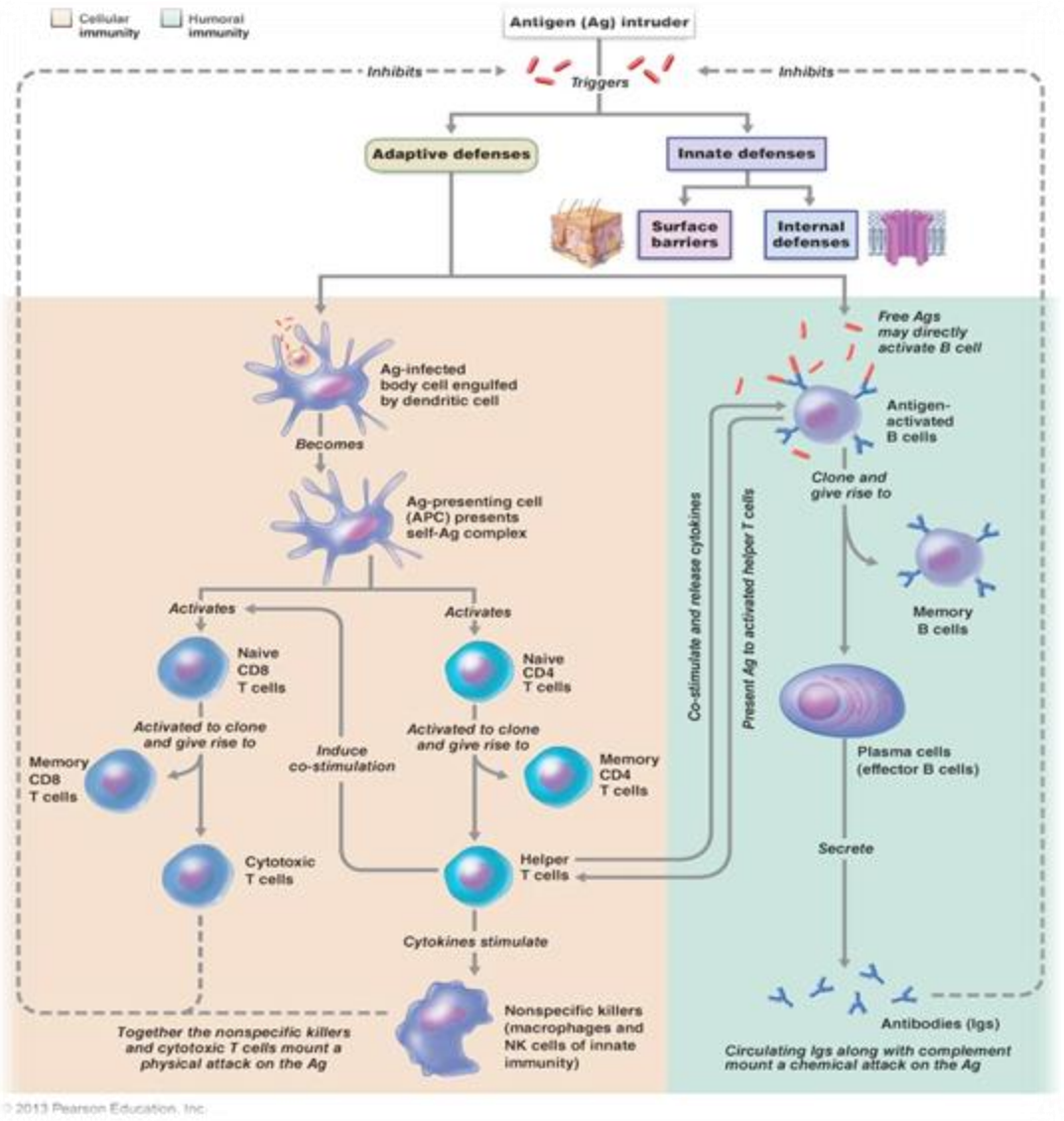

Interactivité
induction
Natural immunity
innate
Non-specific
Not very discriminating
(harmless/hazardous)
Immediate
Pre-existing effectors
Stereotyped response
Adaptive immunity
acquired
Specific
Discriminating
(self/non-self)
Delay
Induced effectors
Memory
Inter-individual variability

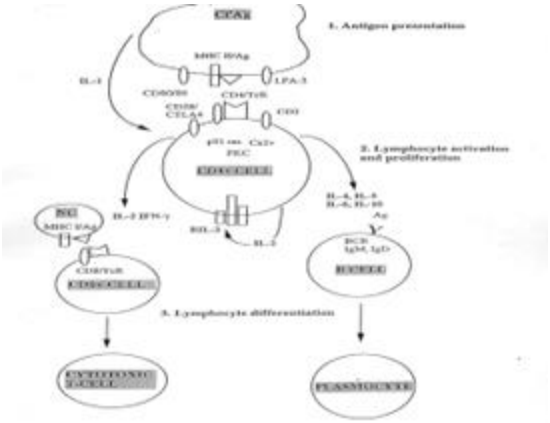
# IMMUNE MEMORY & PROTECTIVE IMMUNITY




The immune system, 2009, Garland Science

# SPECIFIC IMMUNE RESPONSE



CLASSIFICATION

IMMUNITY

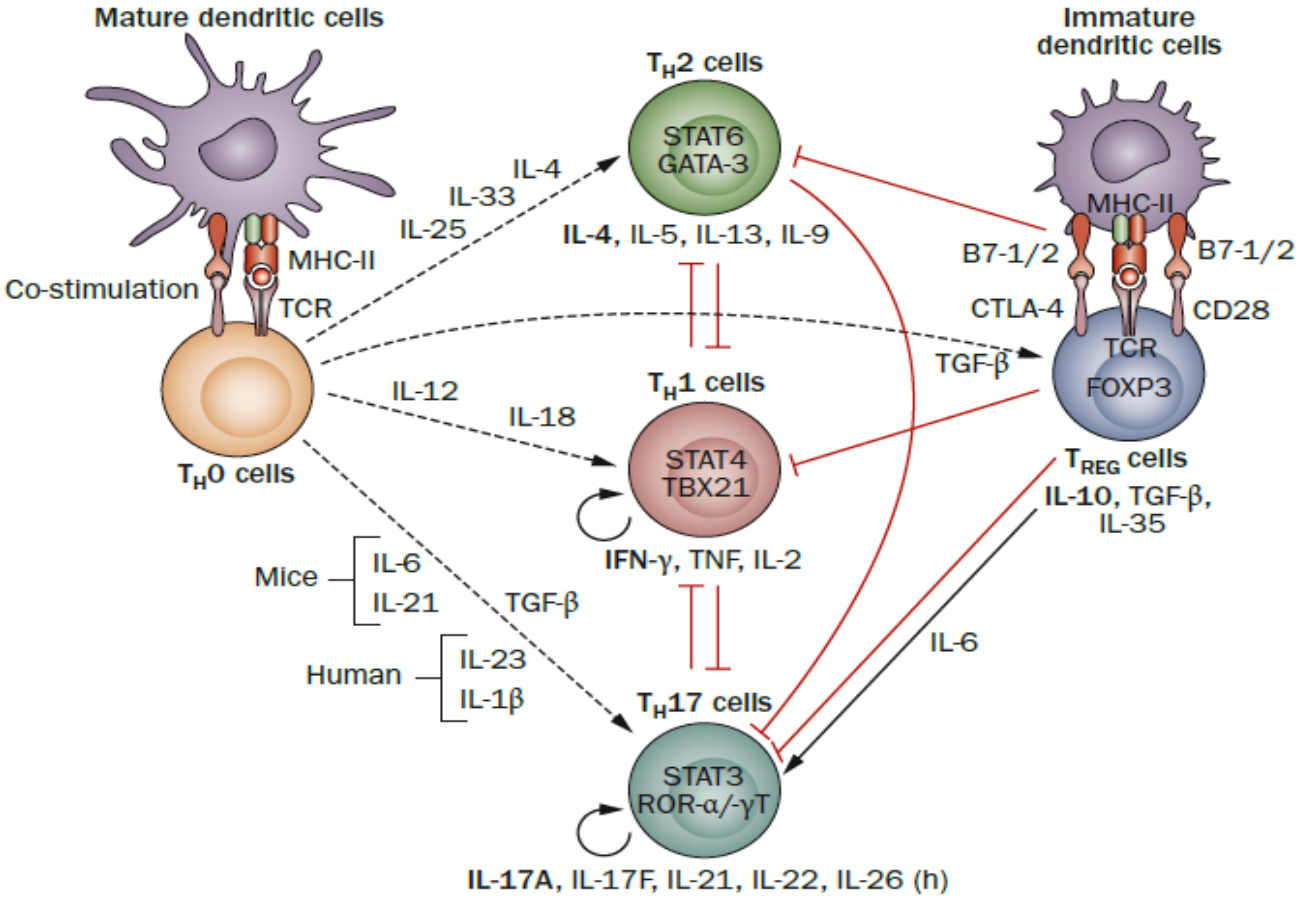
**Innate**
Immediate - First line of defense
Fixed and limited specificity
Same response with 2° exposure

**Adaptive**
Lag in response
Diverse with unique recognition
Faster 2° response and memory

**Humoral**
B cells & Antibody

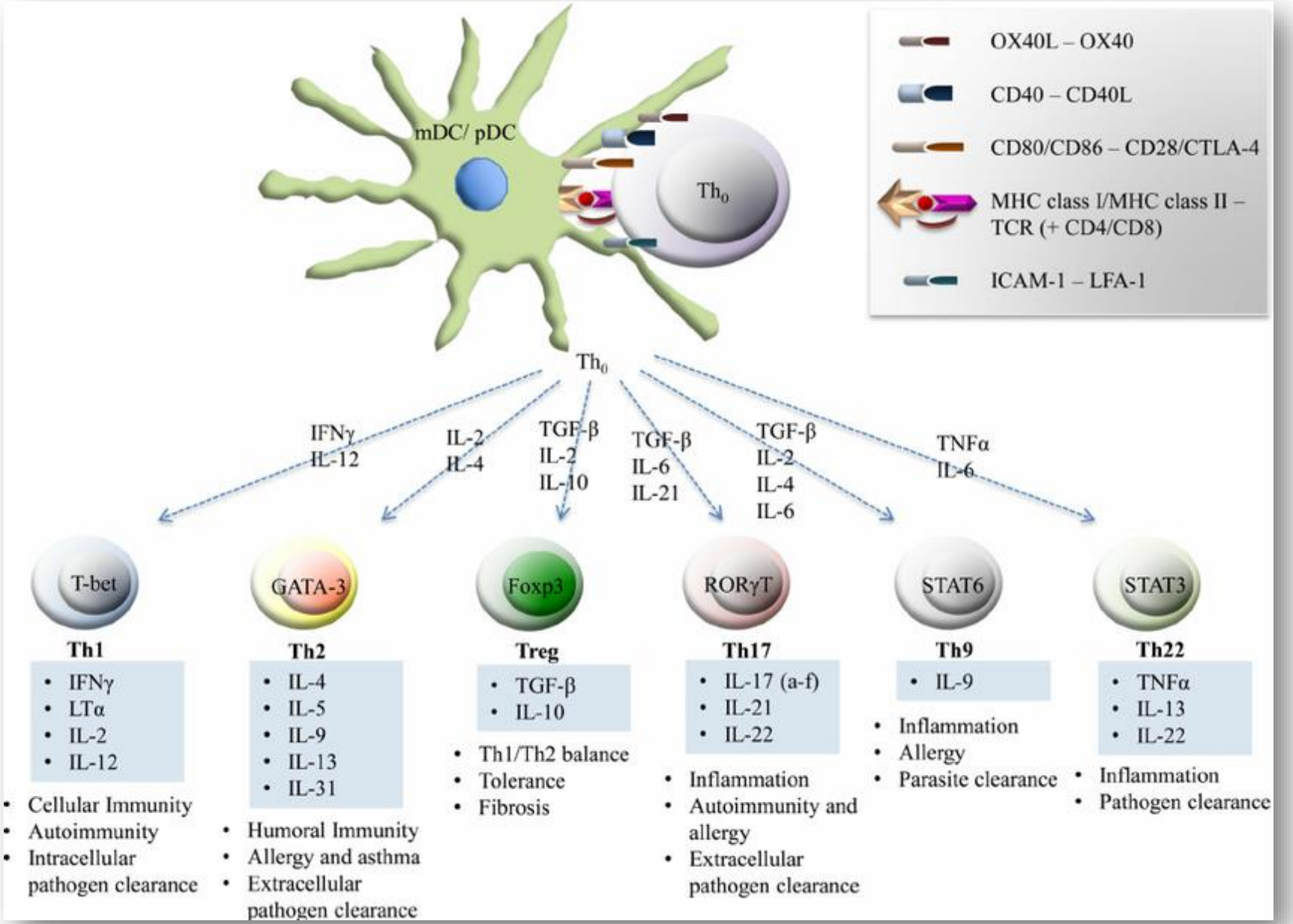
**Cell-Mediated**
T cells & Cytokines

# T CELL DIFFERENTIATION

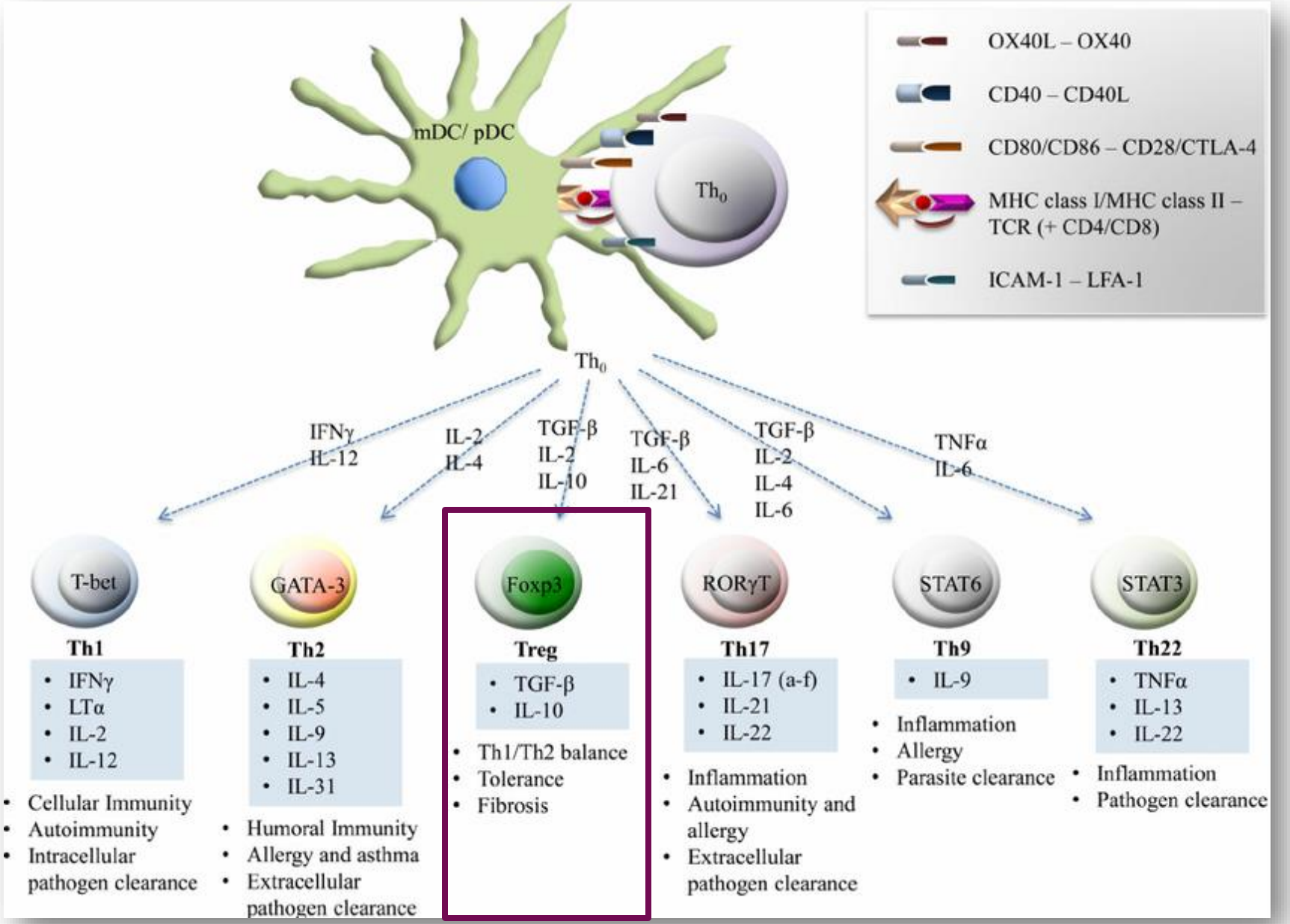



*Lahoute et al., 2011, Nature Rev. Cardiol.*

# T CELL DIFFERENTIATION



# T CELL DIFFERENTIATION



# IMMUNE TOLERANCE

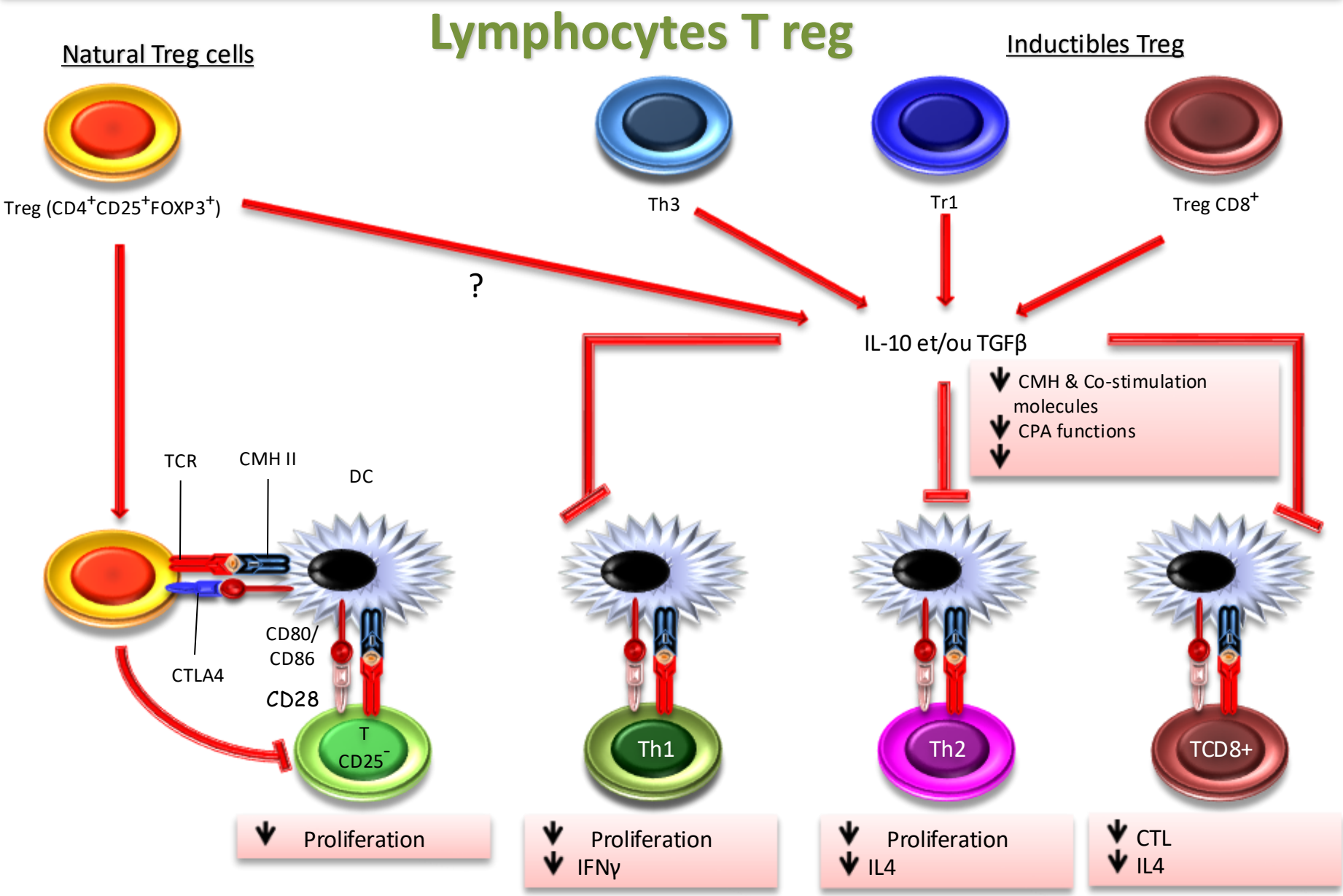
## CENTRAL TOLERANCE

- Thymic selection
- Eliminates lymphocytes that recognize self-antigens
- Avoids autoimmune responses directed against the "self"

## PERIPHERAL TOLERANCE

- Regulatory T cells
- Allows "tolerance" to external antigens that do not present a danger
- Allows "tolerance" to self antigens that have escaped thymic (central) selection

# T CELL DIFFERENTIATION



# PLAN

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   - **The different tests**

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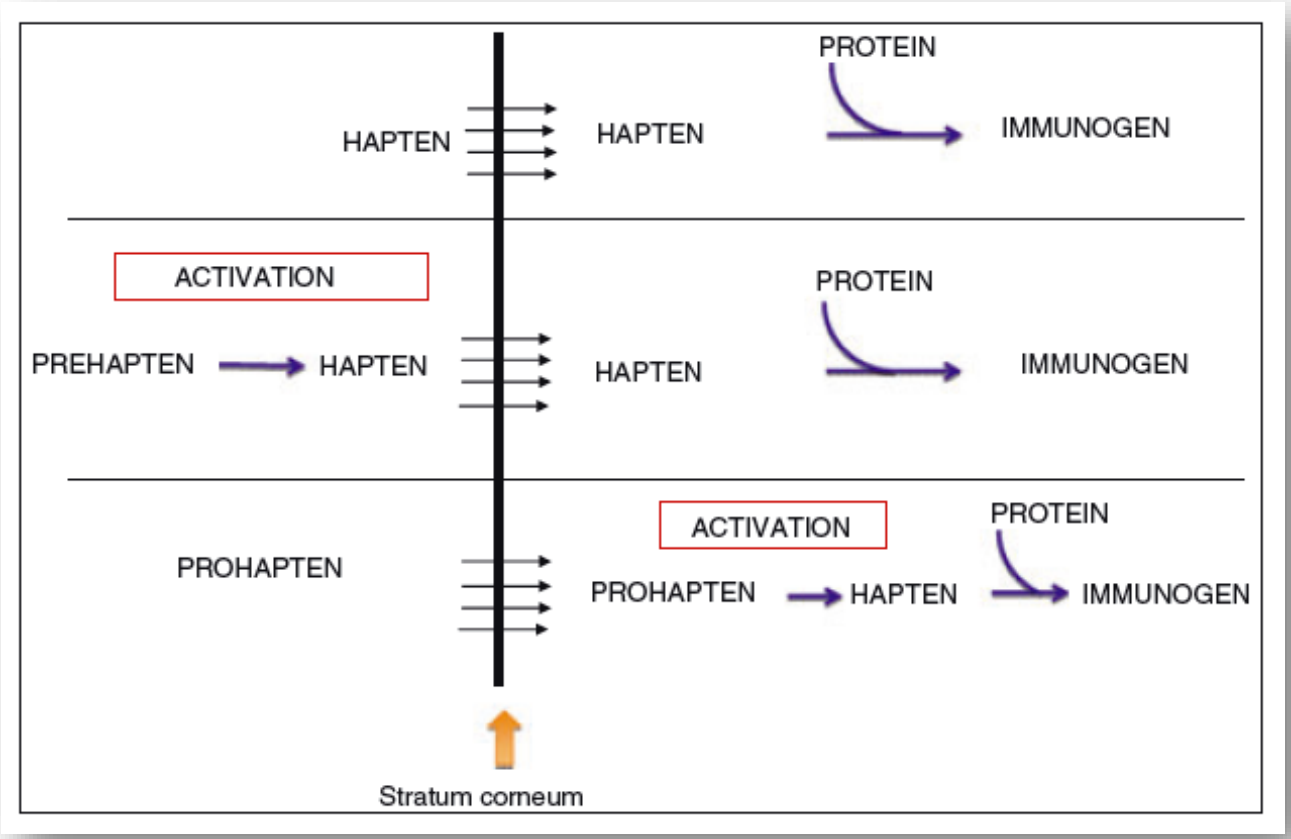
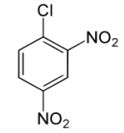
# DEFINITION

Allergic reactions are cellular or tissue lesions that are the adverse and sometimes fatal consequences of specific immune responses to xenobiotics (exogenous allergens or haptens).

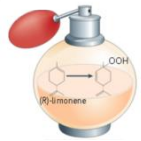
# HAPTENS



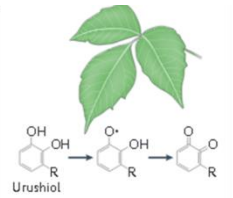




2,4 dinitrochlorobenzene

Haptens




Prehaptens




Prohaptens

Kaplan *et al.,* 2012; Karlberg *et al.*, 2013

# Hypersensitivity reactions

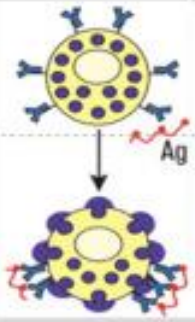
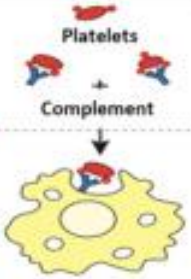
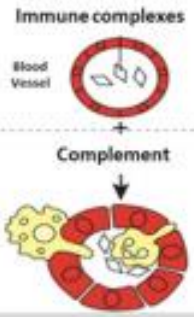
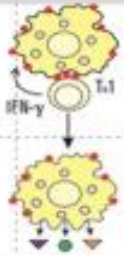
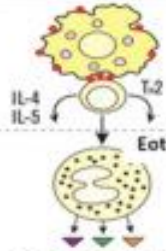
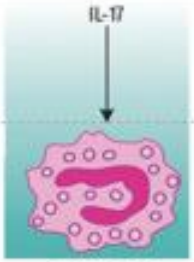
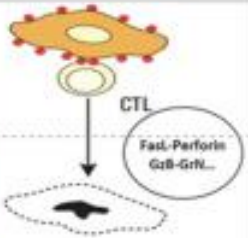
I = Allergic Anaphylaxis and Atopy

II = antiBody

III = immune Complex

IV = Delayed

## Hypersensitivity reactions

| Type | Type I | Type II | Type III | Type IV | | | |
|---|---|---|---|---|---|---|---|
| Main Effector | IgE | IgG | IgG | CD4 Th1 | CD4 Th2 | CD4 Th17 | Cytotoxic CD8 – Tc1 |
| Cell | Mastocyte | Complement, NK, macrophages | Complement, macrophages | Macrophages | Eosinophils | Neutrophils | Cytotoxicity |
| Mechanism |   |   |   |    Chemokins, cytokins, cytotoxic molecules |    Chemokins, cytokins, cytotoxic molecules |   Neutrophils |   Cell apoptosis, necroptosis... |
| | **Diseases** | | | | | | |
| Skin disease | Contact urticaria | Pemphigus, bullous pemphigoid | Vasculitis | Psoriasis, ACD | Atopic dermatitis | Psoriasis | ACD |
| Drug allergy | Anaphylaxis | Drug induced cytopenia | Drug induced vasculitis | MPE, DRESS | DRESS | AGEP | TEN |

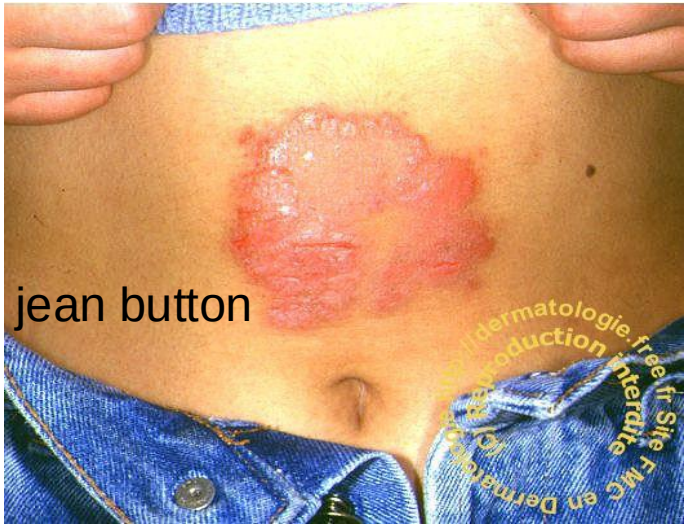
MPE: Maculo-Papular Exanthema
AGEP: Acute Generalised Exanthematous Pustulosis
TEN: Toxic Epidermal Necrolysis

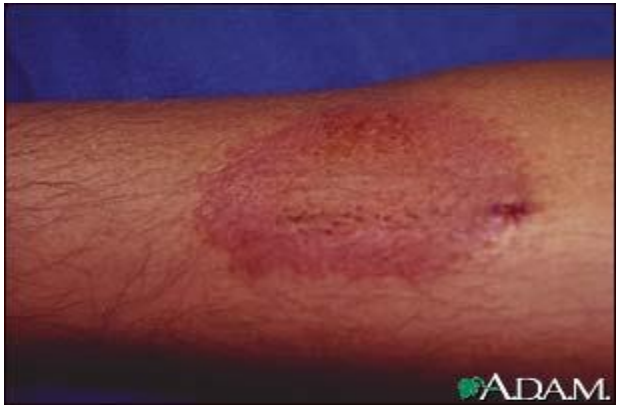
Villani *et al.*, 2918, Current Opinion in Toxicology

# PRODUCTS INVOLVED IN IMMUNOALLERGIC REACTIONS

| Classification | Molecules |
|---|---|
| ***Hypersensibibilty type I (HSI)*** | Beryllium (alloys), Isocyanates<br>(HSI) Trimellitic anhydride, β-lactams, sulfonamides |
| ***Hypersensibibilty type II (Type II)*** | Trimellitic anhydride, mercury<br>quinine, quinidine<br>nitrofurantoin<br>penicillin |
| ***Hypersensibibilty type III*** | Trimellitic anhydride, mercury<br>penicillins, sulfonamides, streptomycin |
| ***Hypersensibibilty type IV*** | DNCB, Beryllium, Chromium, Nickel<br>penicillins, sulfonamides |
| ***Others*** | Sulfamides, anticonvulsivants, *non*-steroidal *anti-inflammatory* (NSAIDS) |

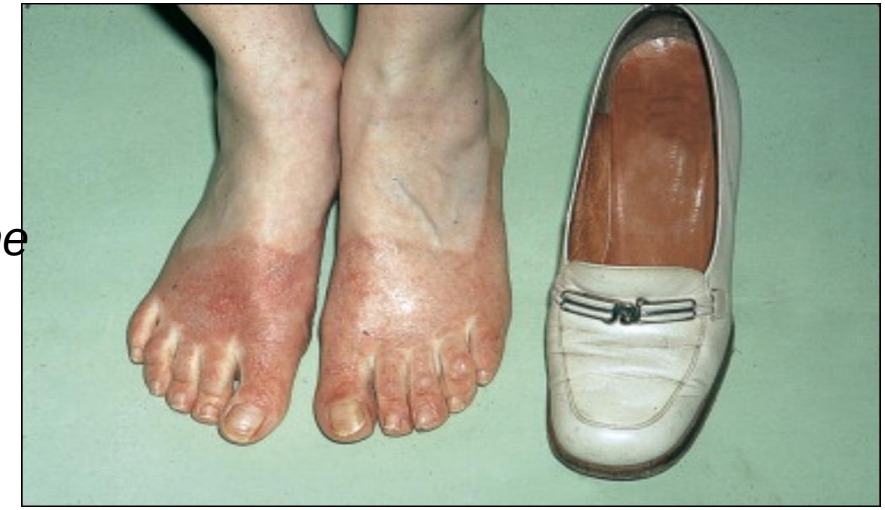
# EXEMPLES



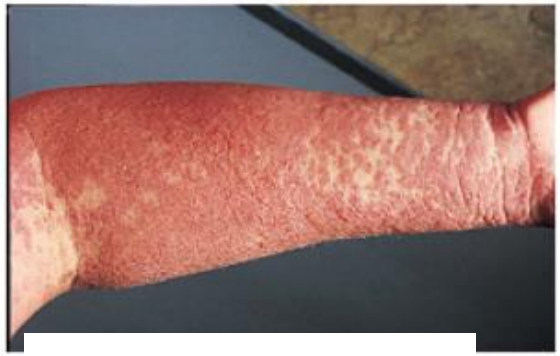

Plaster

Conservator
*Methylisothiazolinone*

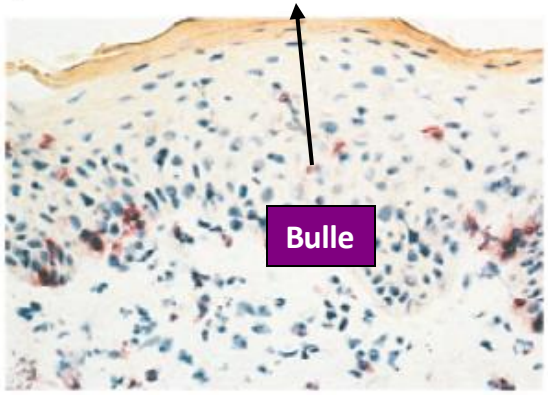


# EXEMPLES

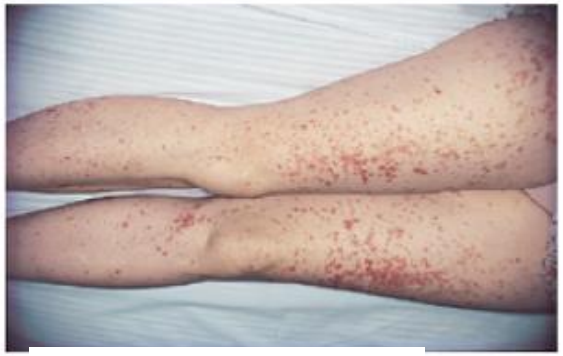
Bullous erythema



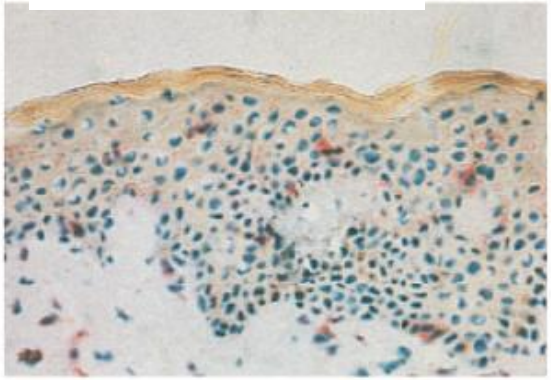
Cytotoxic CD8⁺ LT




Macopapular exanthema

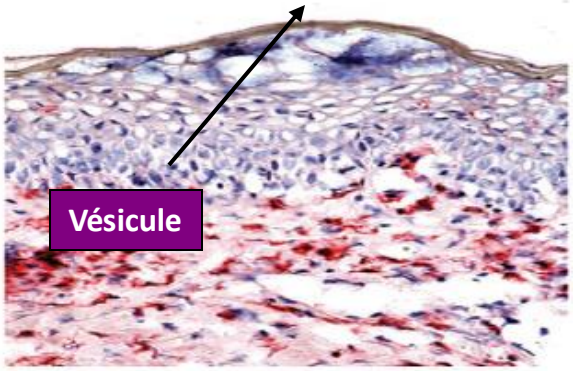


LT CD4⁺, IL-5



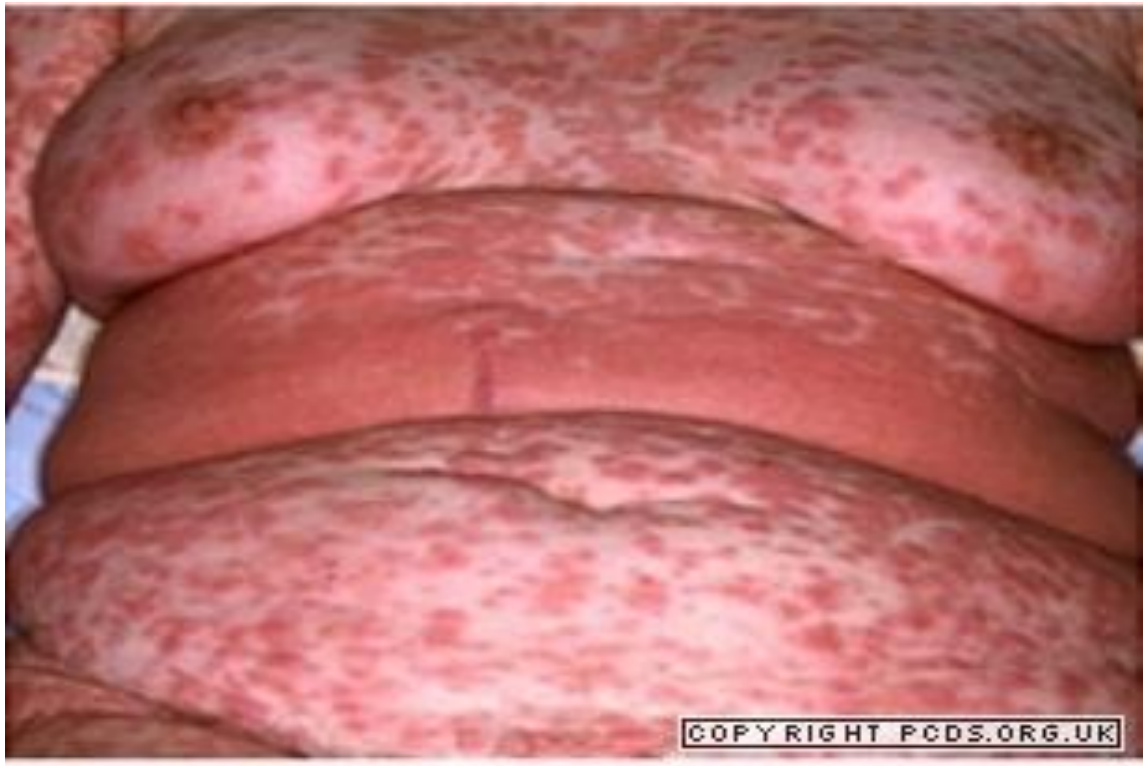
Pustular exanthema

LT CD4⁺, IL-8




Various drugs can elicit distinct forms of T-cell–mediated drug reactions. For example, amoxicillin causes bullous skin disease (*A*), maculopapular exanthema (*B*), and acute generalized exanthematous pustulosis (*C*). Analysis of skin infiltrates and functional analysis of drug-specific T-cell clones from these different forms of drug allergy revealed distinct contribution of $CD4^+$ and $CD8^+$ T cells to these disorders, as well as distinct functions of CD4 cells. In maculopapular exanthema, CD4 cells dominate. More $CD8^+$ T cells are found in patients with (mild) bullous skin disease, and these cells can kill keratinocytes (*D*). CD4 cells secrete high levels of interleukin-5 and substantial amounts of interferon-γ and can kill activated MHC class II–expressing keratinocytes (*E*). $CD4^+$ and $CD8^+$ T cells are found in patients with acute generalized exanthematous pustulosis (*F*); both these cells contribute to vesicle formation through their cytotoxic activity. CD4 cells secrete granulocyte-monocyte colony-stimulating factor and interleukin-8, which leads to the recruitment of neutrophils (54, 72, 74, 75, 78, 85). (Immunostaining by the avidin–biotin complex/alkaline phosphatase method; original magnification, ×250.)

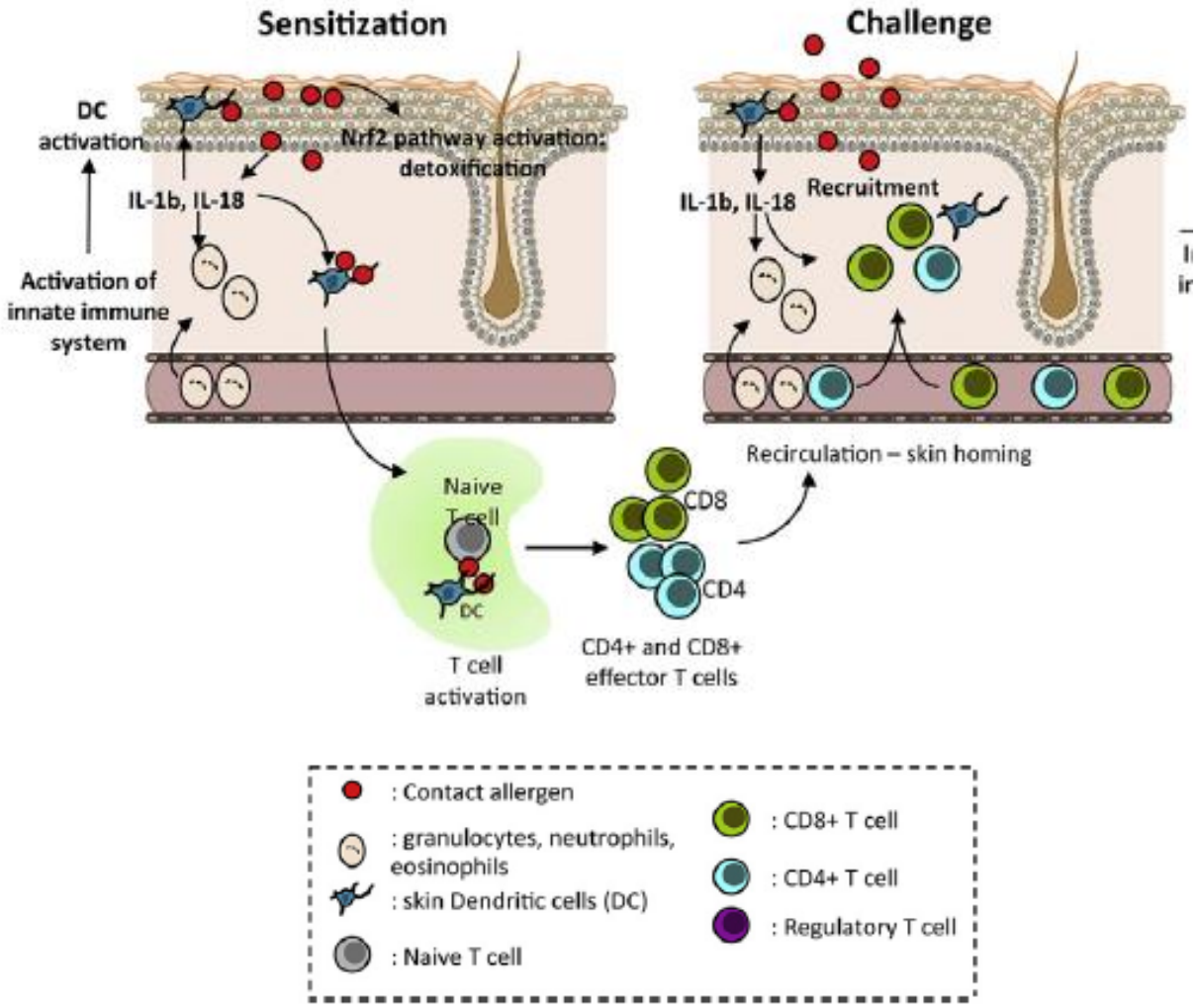
# EXEMPLES




## DRESS
## CD8+ cells/MHC class I association

DRESS : **D**rug **R**eaction with **E**osinophilia and **S**ystemic **S**ymptoms
Ex: allopurinol

# TYPE VI : ALLERGIC CONTACT DERMATITIS PATHOPHYSIOLOGY



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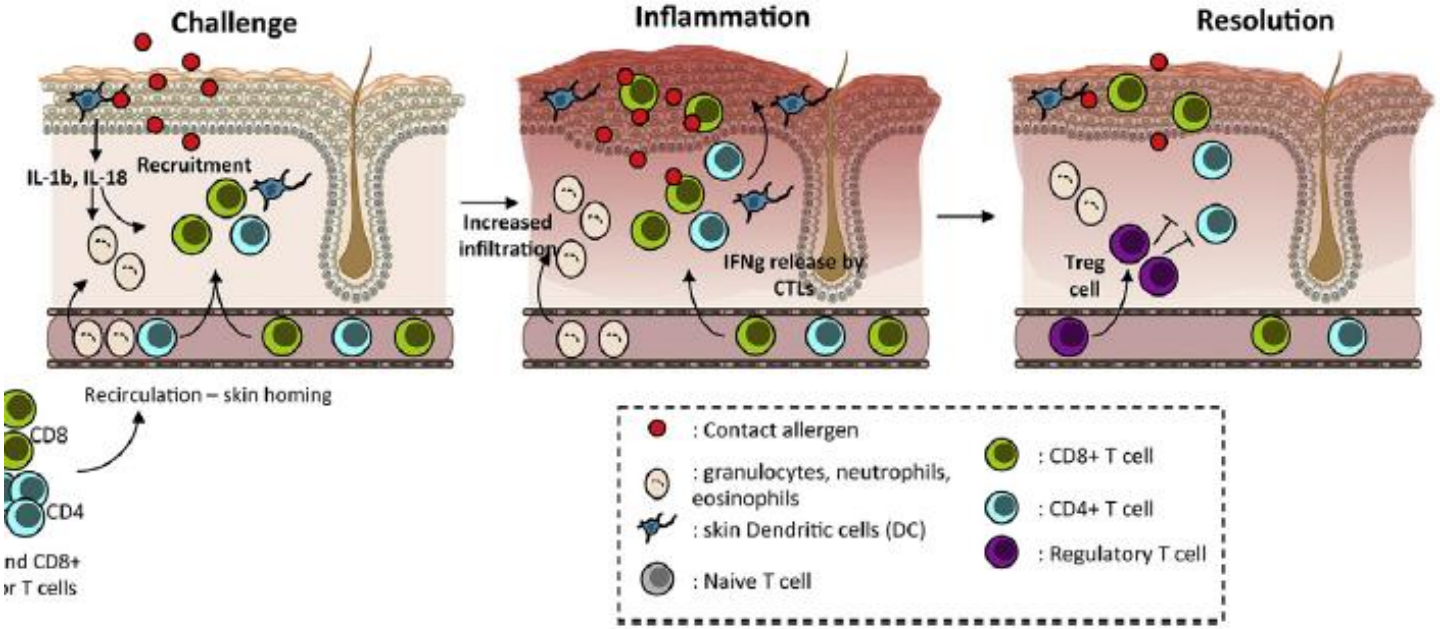
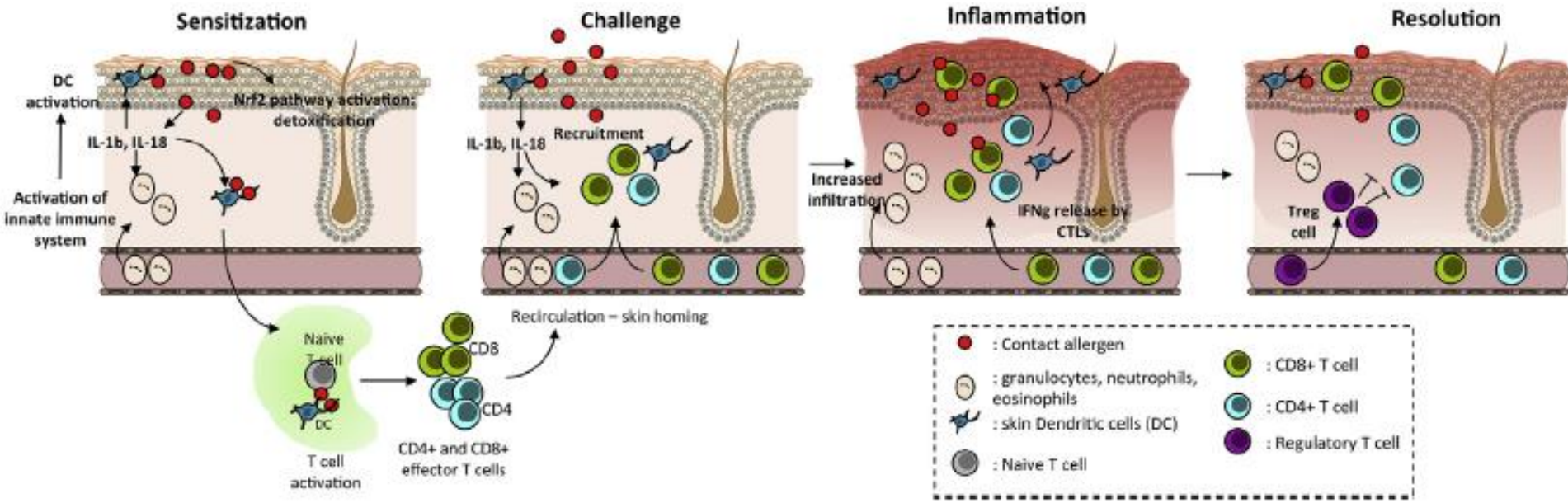
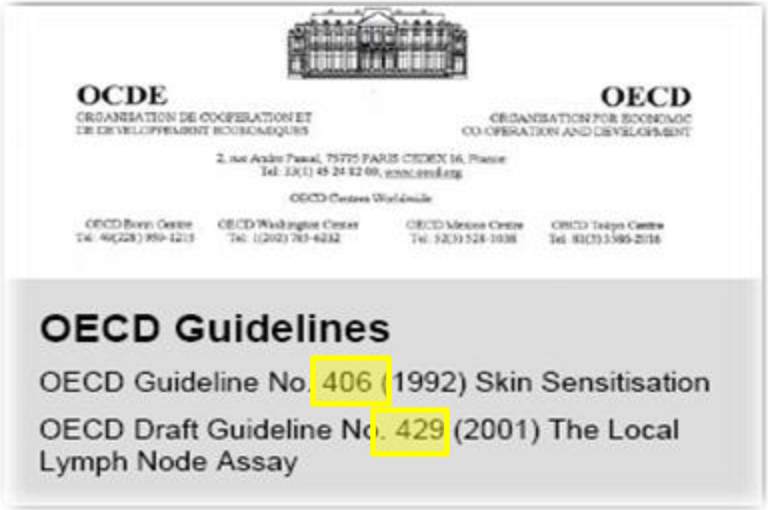


# TYPE VI : ALLERGIC CONTACT DERMATITIS PATHOPHYSIOLOGY



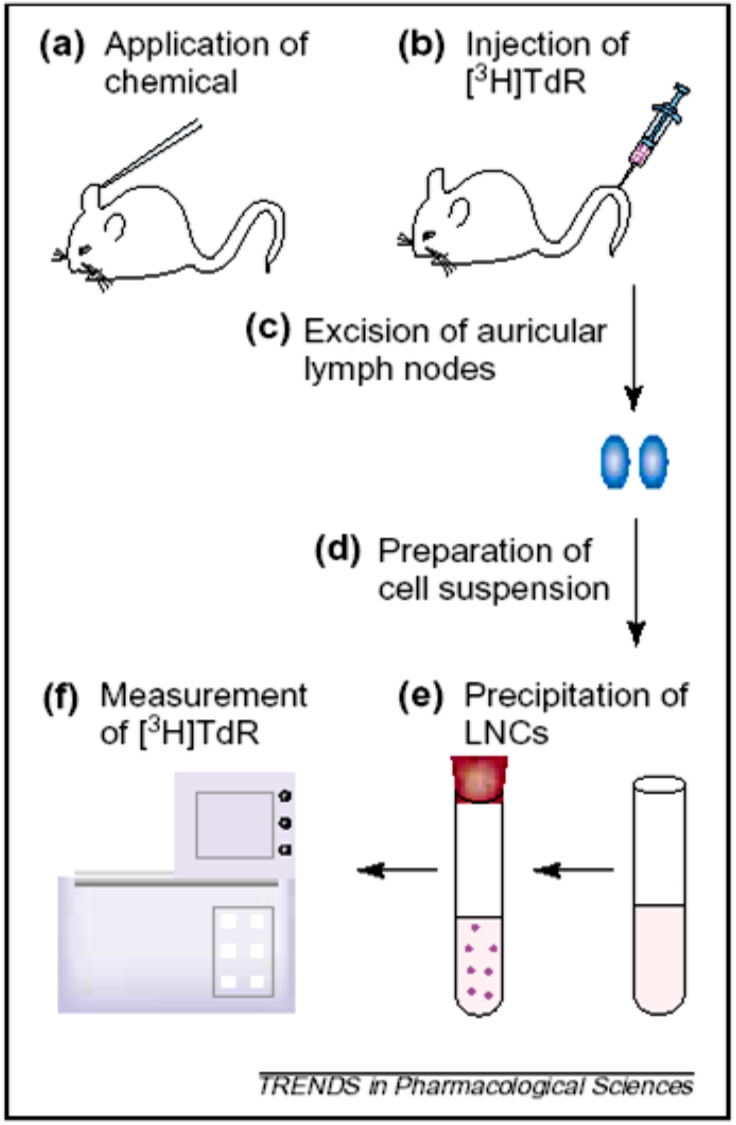

Villani *et al.*, 2918, Current Opinion in Toxicology

# EVALUATION METHODS: ANIMAL TESTING

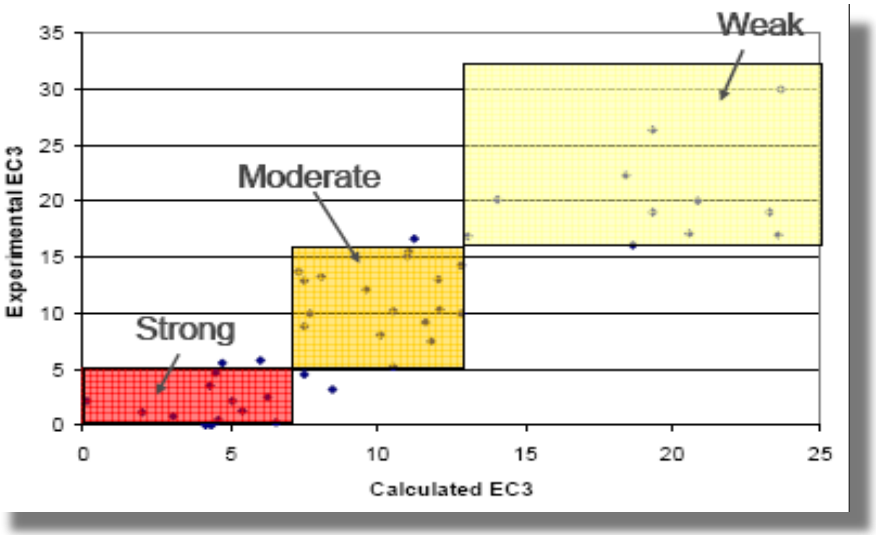



| Test | Positivity criterion |
|---|---|
| Guinea Pig Maximisation Test (GPMT) (OECD TG 406) | Positive response if ≥30% of animals tested |
| Buehler Test (OECD TG 406) | Positive response if ≥15% of animals tested |
| Mouse local lymph node assay (LLNA) (OECD TG 429) | Stimulation Index (IS)≥3 |
| LLNA: DA (OECD TG 442A) | IS≥1.8 |
| LLNA: BrdU-ELISA (OECD TG 442B) | IS≥1.6 |

# EVALUATION METHODS: LLNA




- It measures the sensitization phase
  (Guinea pig test: measures the effector phase)
- The criterion is the lymphocyte proliferation phase
- It allows a quantitative evaluation
  lymphocyte proliferation in cpm
- Positivity and evaluation criteria:
  Stimulation index ≥ 3
  Dose-response



# THE 3 ALTERNATIVE TESTS VALIDATED

**OECD/OCDE** **LD 442C**

Adoptée:
4 février 2015

**LIGNE DIRECTRICE DE L'OCDE POUR LES ESSAIS DE PRODUITS CHIMIQUES**

**Sensibilisation cutanée *in chemico* : essai de liaison directe sur la réactivité peptidique (DPRA)**

---

**OECD/OCDE** **TG 442D**

Adopted:
February 2015

**OECD GUIDELINE FOR THE TESTING OF CHEMICALS**

***In Vitro* Skin Sensitisation: ARE-Nrf2 Luciferase Test Method**

---

**OECD/OCDE** **442E**

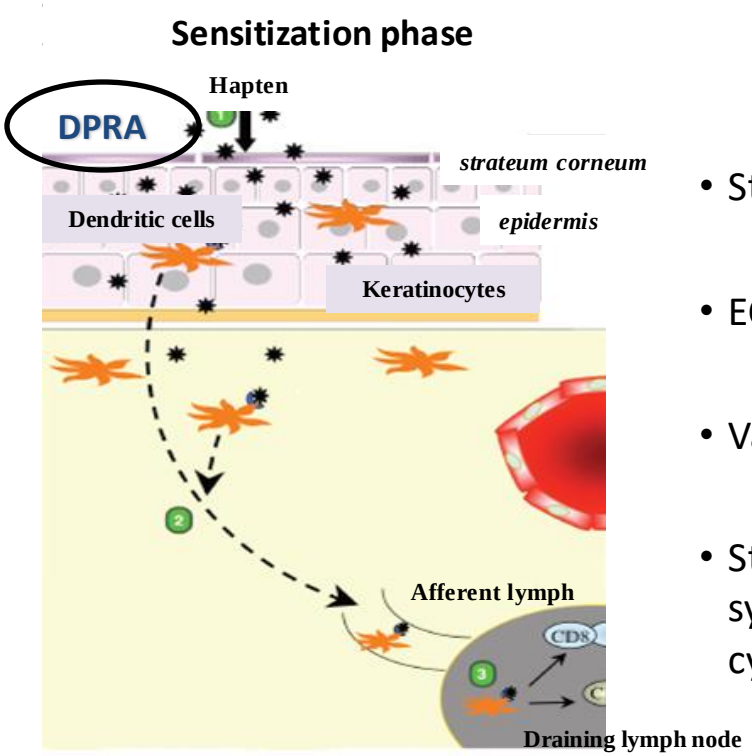
Adopted:
29 July 2016

**OECD GUIDELINE FOR THE TESTING OF CHEMICALS**

***In Vitro* Skin Sensitisation: human Cell Line Activation Test (h-CLAT)**

# DPRA

## Direct Peptide Reactivity Assay (DPRA, Procter & Gamble)



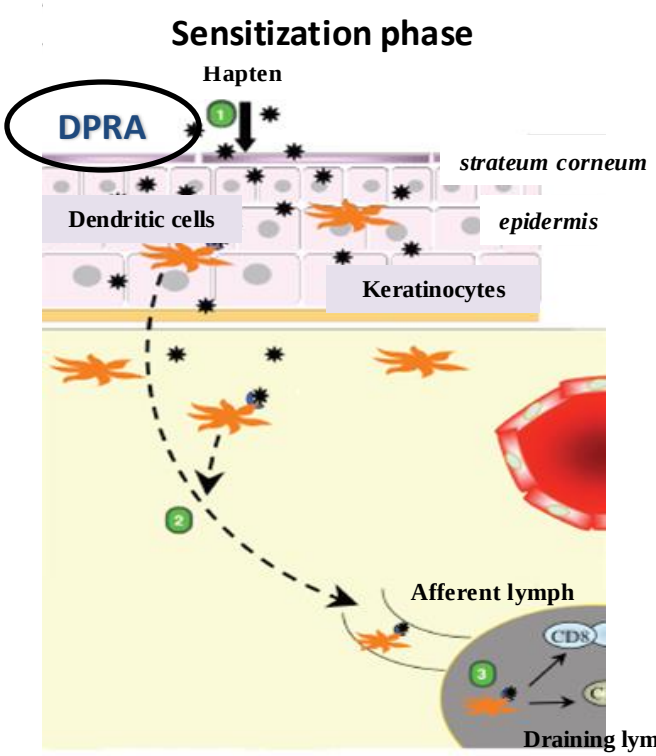

according to Saint-Mezard, et al., *Eur J Dermatol.* **2004**

### Test *in chemico*

- Study of the molecular event that initiates the AOP
- ECVAM recommendation in December 2013
- Validated by the OECD in February 2015 (TG N° 442C)
- Study of the reactivity of the tested chemicals towards synthetic peptide models containing either lysine or cysteine

# DPRA

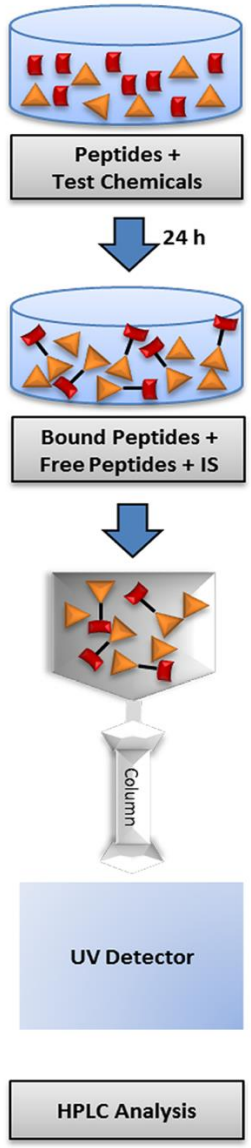
## Direct Peptide Reactivity Assay (DPRA, Procter & Gamble)




according to Saint-Mezard, et al., *Eur J Dermatol.* **2004**

### Test *in chemico*

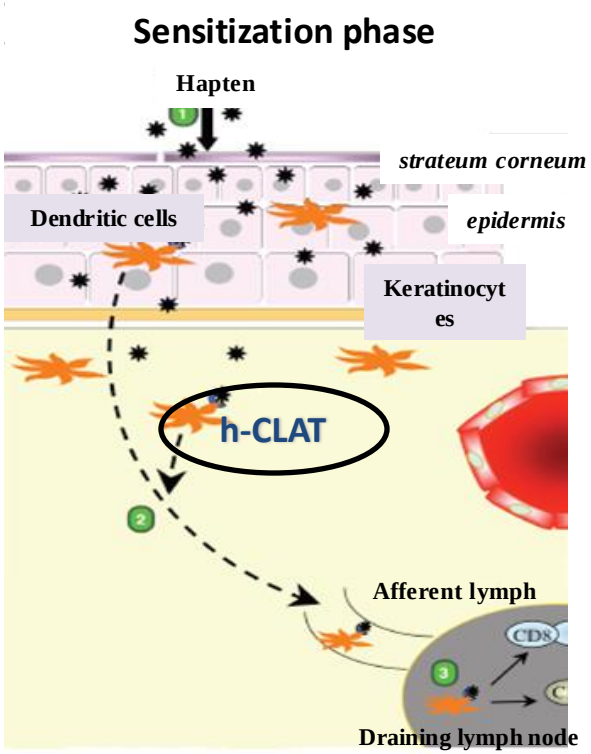
- Study of the molecular event that initiates the AOP
- ECVAM recommendation in December 2013,
- Validated by the OECD in February 2015 (TG N° 442C)
- Study of the reactivity of the tested chemicals towards synthetic peptide models containing either lysine or cysteine




according to Wong et al, *Frontiers in Pharmacology*, **2015**

# h-CLAT

## Human Cell Line Activation Test (KAO, Shiseido)




D'après Saint-Mezard, et al., *Eur J Dermatol.* **2004**

**In vitro test** : THP-1 cell line (pro-monocytic myeloid cell line)

- 3rd key event in PDO: dendritic cell activation
- ECVAM recommendations in March 2015
- Validated by the OECD in July 2016 (TG n° 442E)

**OBJECTIVE**

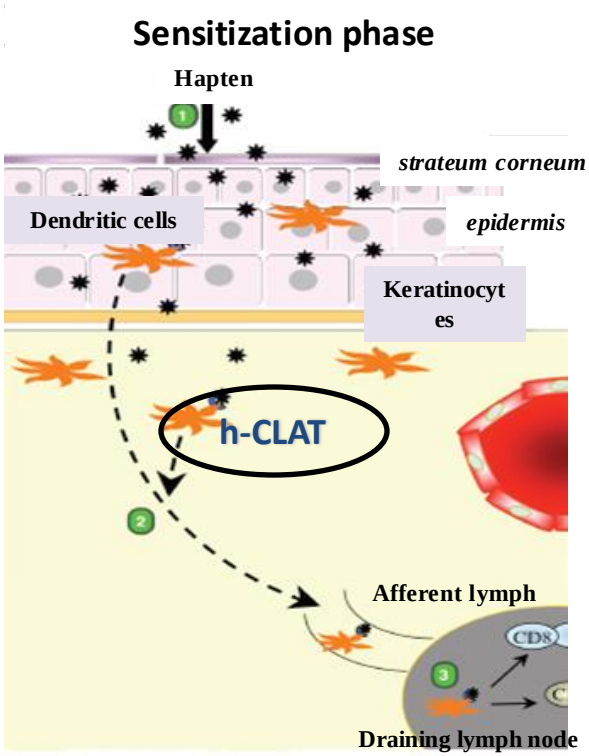
Measurement of cell surface marker expression:

CD86 = Facteur de co-stimulation lymphocytaire

CD54 = Molécule d'adhésion

# h-CLAT

## Human Cell Line Activation Test (KAO, Shiseido)




according to Saint-Mezard, et al., *Eur J Dermatol*. **2004**

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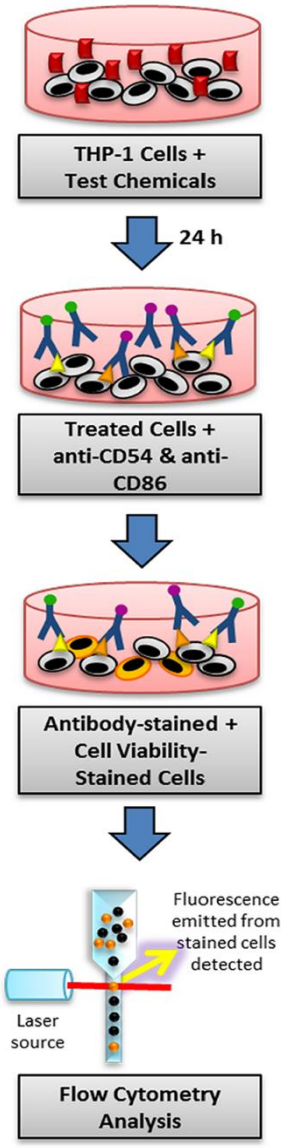
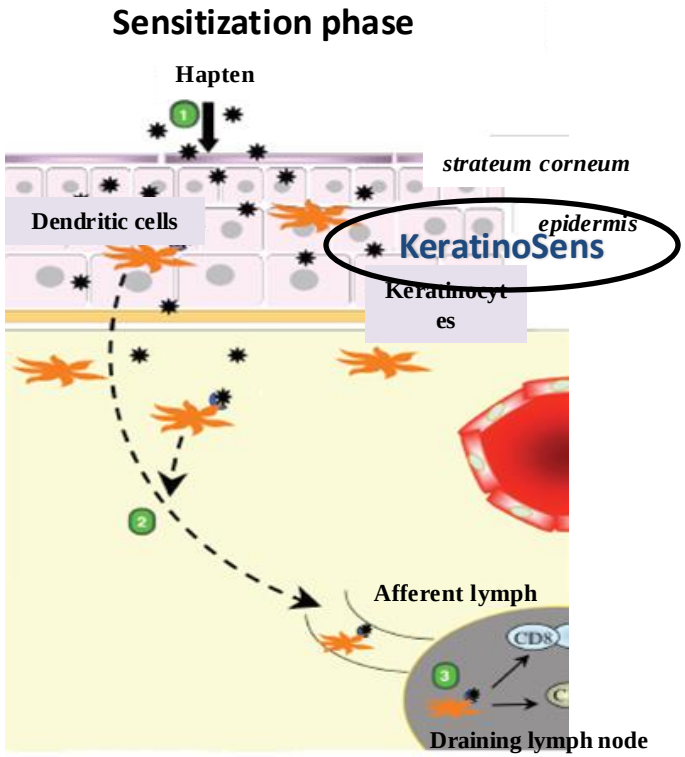



according to Wong et al, *Frontiers in Pharmacology*, **2015**

# KeratinoSens

## (Givaudan)



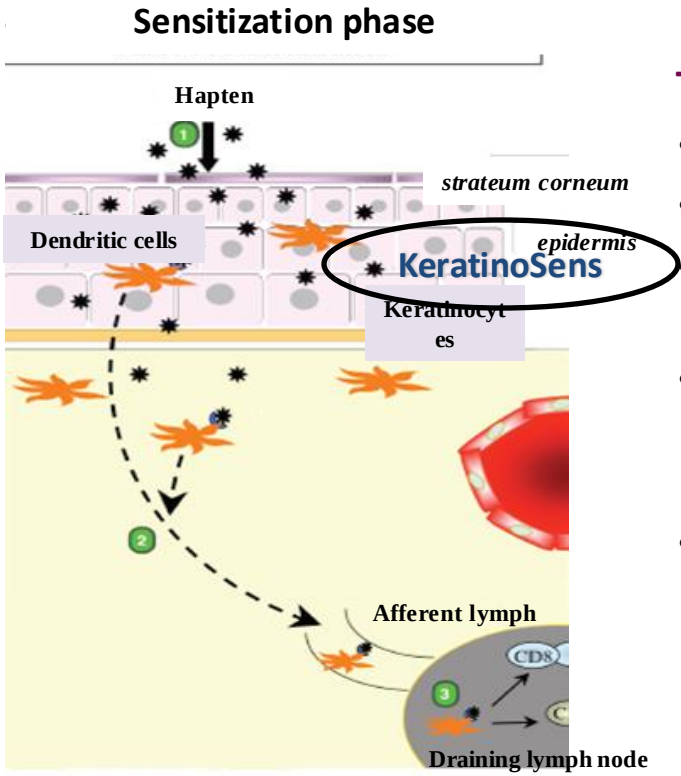

according to Saint-Mezard, et al., *Eur J Dermatol.* **2004**

### Test *in vitro*

- Study of the second AOP event
- Recommended by ECVAM in February 2014
- Validated by the OECD in February 2015 (TG N° 442D)
- Study of keratinocyte activation via the Keap1-Nrf2 pathway known to regulate the response to oxidative stress or electrophilic compounds,
- Use of the HaCaT cell line, immortalised human cells transfected with a selectable plasmid to quantify luciferase gene induction as a measure of Keap1-Nrf2 pathway activation

# KeratinoSens assay

## (Givaudan)

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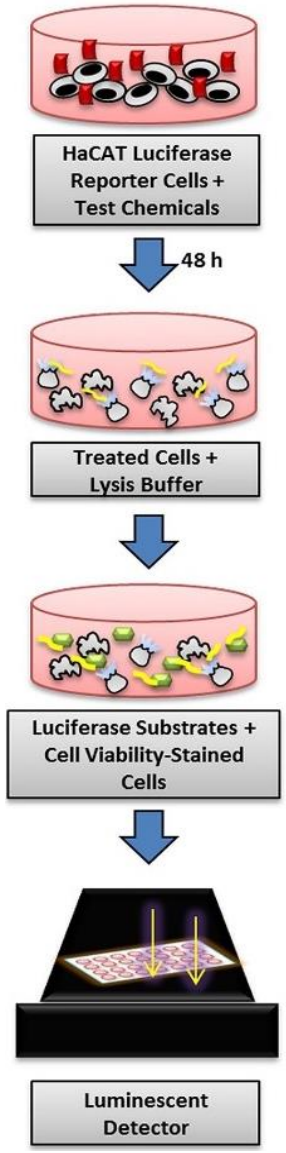
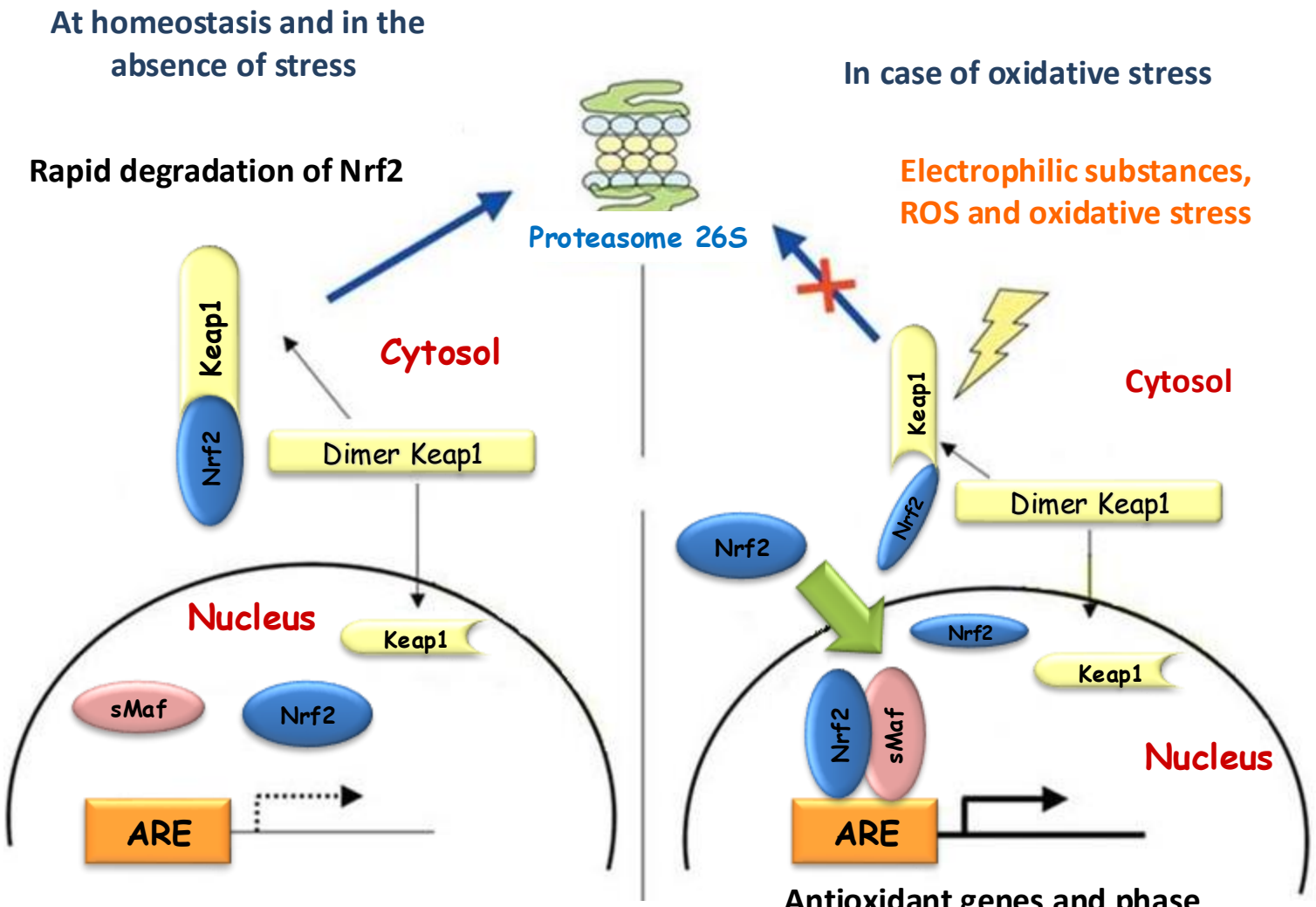



according to Wong et al, *Frontiers in Pharmacology*, **2015**

# Nrf2/Keap1 pathway




*Nrf2 : Nuclear factor-erythroid 2 (NF-E2)-related factor 2
*Keap-1 : Kelch-like ECH-associated protein 1
*Maf : Musculoaponeurotic fibrosarcoma
*ARE : Antioxydant Responsive Element

From Watai Y et al., Genes to Cells 2007

# KeratinoSens assay

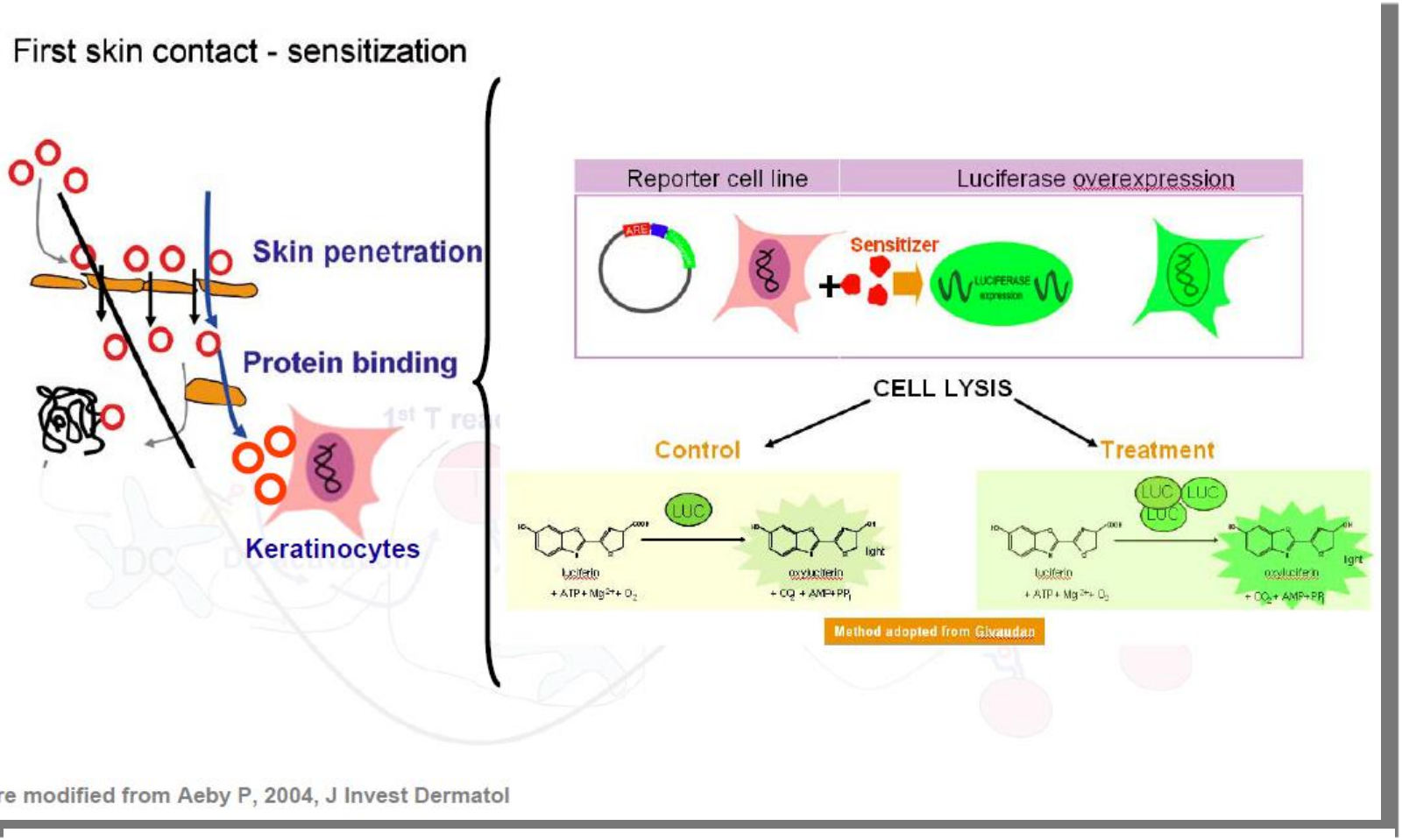



Figure modified from Aeby P, 2004, J Invest Dermatol

15. Test chemicals are considered positive in the KeratinoSens™ if they induce a statistically significant induction of the luciferase gene above a given threshold (e.g., > 1.5 fold or 50% increase in the case of KeratinoSens™), below a defined concentration which does not significantly affect cell viability (e.g., below 1000 µM and at a concentration at which the cellular viability is above 70% in the case of KeratinoSens™ (9) (12)). For this purpose, the

# PLAN

1. DEFINITIONS
   - The different cells
   - The different organs
   - The immune response

2. HYPERSENSITIVITIES
   - Definition of hypersensitivity
   - The different tests

**3. IMMUNOSUPPRESSION**
   - **Definition of immunosuppression**
   - **The different tests**

## Clinical consequences

- Infectious diseases
- Certain types of cancer

These pathologies are the consequence of a lack of regulation by the immune system.

# IMMUNOSUPPRESSION

## COMMON TARGETS FOR LMWM ARE MOSTLY INTRACELLULAR ≠ BIOLOGICS

- **T-cell Receptor signalisation**
  - Cyclosporine A, Tacrolimus: Calcineurin inhibitors
- **IL-2 signalisation** : transplantation
  - Sirolimus: mTOR (IL-2 response)
- **Alteration of lymphocyte trafficking**: multiple sclerosis
  - Fingolimod (Gilenya®): sphingosine-1-phosphate receptor
- **Inhibition of lymphocyte proliferation**: auto-immune diseases, cancer
  - Azathioprine: purine metabolism
  - 6-mercaptopurine: purine metabolism
  - Mycophenolate mofetil: purine metabolism
  - Methotrexate: dihydrofolate reductase

## IMMUNOSUPPRESSION AND BIOLOGICS : TARGETS ARE EXTRACELLULAR

- **Soluble products: auto-immune diseases**
  - Anti-TNF-alpha: adalimumab, infliximab, golimumab, certolizumab…
- **T-cell Receptor signalisation**: transplantation, cancer
  - Anti-CD3: Muromonab
- **Cytokine signalisation** : transplantation, autoimmune diseases
  - Anti-CD25: daclizumab, basiliximab
  - TNF-alpha: adilimumab, infliximab, certolizumab, golimumab
  - Anti-IL6 R: Tocilizumab
- **Alteration of lymphocyte trafficking**: multiple sclerosis
  - Anti- integrin a4 subunit : Natalizumab
- **Lymphocyte proliferation/apoptosis**: auto-immune diseases, cancer
  - Anti-CD20: Rituximab

# IMMUNOSUPPRESSION

## TUMORS WITH HIGH INCIDENCE IN KIDNEY TRANSPLANTED PATIENTS

HHV: Human Herpes Virus
HSV: Herpes Simplex Virus
EBV: Epstein Barr Virus
HPV: Human Papilloma Virus

- **Skin and lips cancer** (HSV1 = HHV1)
  - 38% of total cancer in this population
  - **4 to 7 higher incidence**/normal population
  - Sun exposure augments the incidence

- **Non-Hodgkin Lymphoma** (EBV = HHV4)
  - 14 to 18% of total cancer in this population
  - **40 higher incidence**/normal population

- **Kaposi Sarcoma** (HSV8 = HHV8)
  - 4 to 6% of total cancer in this population
  - **400 à 500 higher incidence**/normal population

- **Cervical cancer** (HPV16)
  - 15% of total cancer in this population
  - **14 higher incidence**/normal population

# IMMUNOSUPPRESSION

## one of the Key Characteristic (KE) of carcinogens

**Table 1.** Key characteristics of carcinogens.

| Characteristic | Examples of relevant evidence |
|---|---|
| 1. Is electrophilic or can be metabolically activated | Parent compound or metabolite with an electrophilic structure (e.g., epoxide, quinone), formation of DNA and protein adducts |
| 2. Is genotoxic | DNA damage (DNA strand breaks, DNA–protein cross-links, unscheduled DNA synthesis), intercalation, gene mutations, cytogenetic changes (e.g., chromosome aberrations, micronuclei) |
| 3. Alters DNA repair or causes genomic instability | Alterations of DNA replication or repair (e.g., topoisomerase II, base-excision or double-strand break repair) |
| 4. Induces epigenetic alterations | DNA methylation, histone modification, microRNA expression |
| 5. Induces oxidative stress | Oxygen radicals, oxidative stress, oxidative damage to macromolecules (e.g., DNA, lipids) |
| 6. Induces chronic inflammation | Elevated white blood cells, myeloperoxidase activity, altered cytokine and/or chemokine production |
| 7. Is immunosuppressive | Decreased immunosurveillance, immune system dysfunction |
| 8. Modulates receptor-mediated effects | Receptor in/activation (e.g., ER, PPAR, AhR) or modulation of endogenous ligands (including hormones) |
| 9. Causes immortalization | Inhibition of senescence, cell transformation |
| 10. Alters cell proliferation, cell death or nutrient supply | Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis |

Abbreviations: AhR, aryl hydrocarbon receptor; ER, estrogen receptor; PPAR, peroxisome proliferator–activated receptor. Any of the 10 characteristics in this table could interact with any other (e.g., oxidative stress, DNA damage, and chronic inflammation), which when combined provides stronger evidence for a cancer mechanism than would oxidative stress alone.

Perfluorooctanoic acid (PFOA) is immunosupressive and now classified as carcinogen to human (group 1, IARC)

Smith MT, Guyton KZ, Gibbons CF, Fritz JM, Portier CJ, Rusyn I, DeMarini DM, Caldwell JC, Kavlock RJ, Lambert P, Hecht SS, Bucher JR, Stewart BW, Baan R, Cogliano VJ, Straif K. 2016. Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. Environ Health Perspect 124:713–721; http://dx.doi.org/10.1289/ehp.1509912

Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are extremely persistent chemicals that are widely distributed in the environment as a result of high chemical stability under normal environmental conditions and extensive use over the last 50 years in commercial and industrial applications including fluoropolymer manufacturing, food packaging, lubricants, water-resistant coating, and fire-fighting foams.

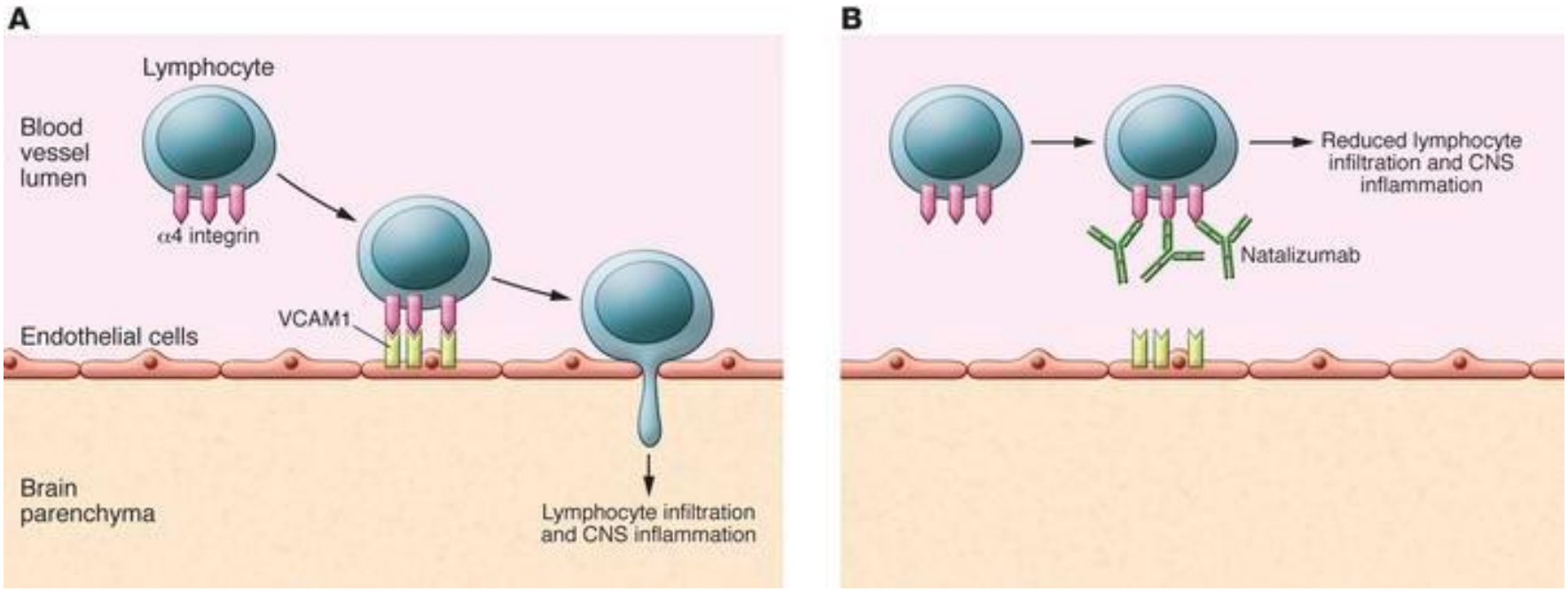
Non-stick products, waterproof and heat-resistant, PFAS have been widely used since the 1950s in a variety of industrial fields and everyday consumer products: textiles, food packaging, fire-fighting foams, non-stick coatings, cosmetics, phytosanitary products…

## THE NATALIZUMAB (TYSABRI®) CASE: POST-MARKETING IMMUNOSUPPRESSION

- **Humanized antibodies directed to α4 integrin sub-unit**
  - α4β7 (VCAM, fibronectine) inhibition
- **Indications**: Multiple sclerosis
- **NDA (New Drug Application) in November 2004**
  - 3 months post-marketing: 2 cases of PML (progressive multifocal encephalopathy) with one death in patients receiving natalizumab + Avonex® (Interferon-beta) since 2 years
  - 3rd case in a clinical trial in Crohn disease
  - Cause = reactivation of the JC (John Cunningham) virus
  - Temporary withdrawn from the market and reintroduced in 2006
- **2017**: cumulative risk of PML with a positive JCV serostatus following 72 natalizumab infusions is roughly **27 per 1000 with prior immunosuppressant exposure**, and **17 per 1000 without**

# IMMUNOSUPPRESSION

## THE NATALIZUMAB (TYSABRI®)




A) α4 integrin binds to vascular cell adhesion molecule 1 (VCAM1) on inflamed brain endothelium. This interaction gives lymphocytes access to the CNS. The presence of immune cells in the brain is a prominent feature of MS.

B) Natalizumab, a humanized antibody against α4 integrin, blocks binding of lymphocytes to VCAM on inflamed brain endothelium, thereby preventing lymphocyte entry into the CNS.

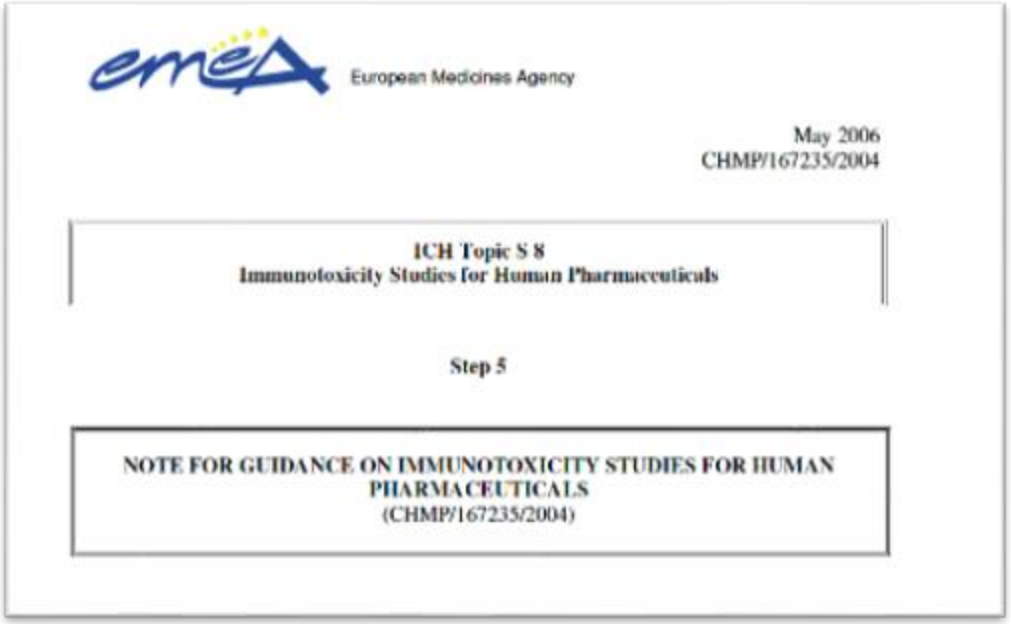
*2012 Steinman. Journal of Cell Biology. 199:413–416. doi: 10.1083/jcb.201207175*

## IMMUNOSUPPRESSION AND TESTING

- ICHS8 **"Immunotoxicity studies for Human pharmaceuticals"** focuses on providing recommendations on nonclinical testing for unintended immunotoxicity induced by human pharmaceuticals
- Published 2005 to 2006
- Immunotoxicity is defined as "unintended immunosuppression or enhancement"
  - Hypersensitivity and auto-immunity are excluded
- Biologics are not included (discussed in ICHS6)
- ICHS8 is using a "weight of evidence approach"
  - If cause of concern conduct additional tests
  - Best before large scale clinical trials

ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

# PROTOCOLS - PRACTICAL CONSIDERATIONS



European Medicines Agency

May 2006
CHMP/167235/2004

ICH Topic S 8
Immunotoxicity Studies for Human Pharmaceuticals

Step 5

NOTE FOR GUIDANCE ON IMMUNOTOXICITY STUDIES FOR HUMAN PHARMACEUTICALS
(CHMP/167235/2004)

**Objectives of the guideline**

The objectives of this guideline are to provide

(1) recommendations on nonclinical testing approaches to identify compounds which have the potential to be immunotoxic, and

(2) guidance on a weight-of-evidence decision making approach for immunotoxicity testing.

Immunotoxicity is, for the purpose of this guideline, defined as unintended immunosuppression or enhancement. Drug-induced hypersensitivity and autoimmunity are excluded

- CHOICE OF ANIMAL SPECIES
- LENGTH OF THE EXPERIMENT
- PHARMACOKINETIC PARAMETERS
- DOSES (3 doses, maximum dose less than or equal to the DMT)
- ROUTE OF ADMINISTRATION
- EX VIVO TESTS
- IN VITRO APPROACH POSSIBLE (MOUSE, RAT, MONKEY, HUMAN)

Rats and/or mice

28 days study

ICH: *International Council for Harmonisation*

# IMMUNOSUPPRESSION: TESTS

- **STATIC SETTINGS/PARAMETERS**
  - Blood count, blood formulation
  - Thymus and spleen weight
  - Cellularity thymus and spleen
  - Histology of lymphocyte organs
  - Lymphocyte subpopulations

- **DYNAMIC SETTINGS**
  - Cell proliferation (T cells, B cells)
  - Cytotoxic T cells
  - Antibody production
  - Natural Killer activity
  - Phagocytosis activity

# EVALUATION OF IMMUNOSUPPRESSION: METHODS

## Screen – Tier I

| | |
|---|---|
| **Immunopathology** | Hematology<br>Weights: body, spleen, thymus<br>Histology – spleen, thymus, LN |
| **Innate nonspecific immunity** | Natural Killer (NK) cell activity |
| **Cell-mediated immunity** | Mixed lymphocyte reaction (MLR)<br>Lymphocyte blastogenesis to mitogens |
| **Humoral-mediated immunity** | Enumeration of IgM antibody PFC<br>Lymphocyte blastogenesis to LPS |

# EVALUATION OF IMMUNOSUPPRESSION: METHODS

## Comprehensive – Tier II

| | |
|---|---|
| **Immunopathology** | Quantitation of splenic B & T cells |
| **Innate nonspecific immunity** | Macrophage function; quantitation of resident peritoneal cells and phagocytic activity |
| **Humoral-mediated immunity** | Enumeration of IgG antibody PFC |
| **Cell-mediated immunity** | Cytotoxic T lymphocyte (CTL) assay |
| | Delayed-type hypersensitivity (DTH) |
| **Host resistance challenge models** | Viral, bacterial, parasite, and syngeneic tumor cell models |

# EVALUATION OF IMMUNOSUPPRESSION: METHODS

- **IMMUNOPATHOLOGY**
  - Numération, blood count, bone marrow examination
  - Thymus and spleen weight.
  - Thymus and spleen cellularity
  - Histology of thymus and spleen
  - Lymphocyte subpopulations

- **IMMUNE RESPONSE**
  - T cell proliferation (ConA, PHA, anti-CD3, allogeneic cells)
  - Differentiation (cytotoxic lymphocytes)
  - Production of antibodies
  - B cell proliferation (LPS)
  - Natural Killer activity
  - Phagocytosis macrophages

- **PATHOLOGICAL ANIMAL MODELS**
  - Infectious (listeria monocytogenes, influenza...)
  - Tumour

# TESTS: IMMUNOSUPPRESSION

# PROLIFERATION TEST

Splenocytes (2.10$^6$ cellules/ml)

↓

Add mitogens or allogeneic cells
(PHA, CONA, anti-CD3, LPS)

↓

Incubation 48 H, 37° C, 5%$CO_2$

↓

Add Thymidine 3H-T (1mCi/puits)
Incubation 6 à 8 heures

↓

Measurement of 3H-T incorporation into DNA
Proliferation curve

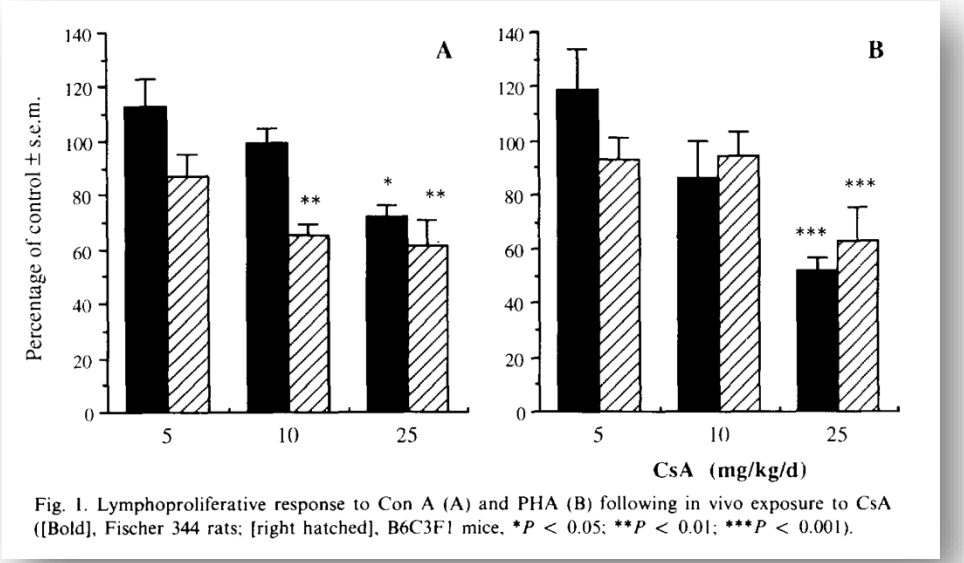



Fig. 1. Lymphoproliferative response to Con A (A) and PHA (B) following in vivo exposure to CsA ([Bold], Fischer 344 rats; [right hatched], B6C3F1 mice, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

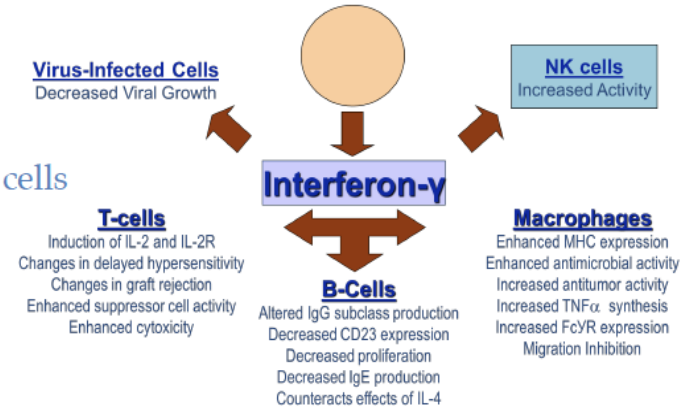
*Concanavalin A (ConA) is a glycoprotein of the lectin family. It binds specifically by affinity to D-mannose and D-glucose*

*Phytohemagglutinin is a lectin present in plants.*

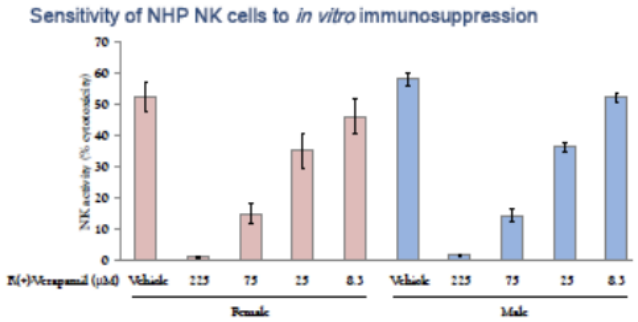
# FUNCTIONAL TESTS - NK

WHY NK cells?

- Evaluate innate immunity
  - NK cells kill tumor & virus-infected cells




- Serve as a "bridge" between innate and acquired immunity
  - NK cells are involved in a number of immunological processes



# FUNCTIONAL TESTS - NK

Splenocytes
$^{51}$Cr-labelled YAC cells (30 min)

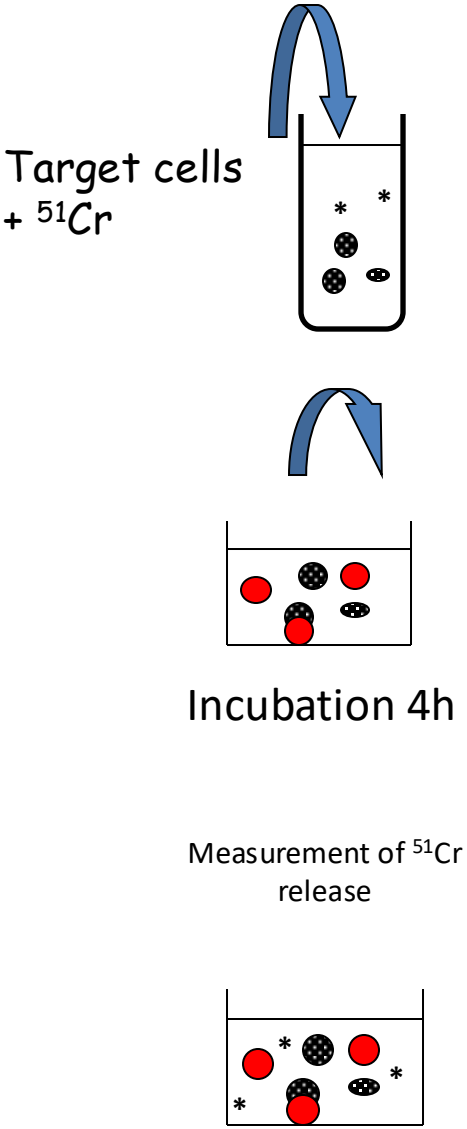
↓

Incubation of splenocytes with YAC cells for **4 hours**, 37° C, 5% Co2
3 ratios (100:1, 66:1, 33:1)

↓

Measurement of $^{51}$Cr release in the supernatant
(results expressed in cpm, % specific lysis, lytic unit)

$$\% \text{ cytotoxicity} = [(cpm_e - cpm_{sr})/cpm_{mr}-cpm_{sr})]x100$$




YAC cells : NK cells

# FUNCTIONAL TESTS – TDAR (T-DEDENDENT ANTIGEN RESPONSE)

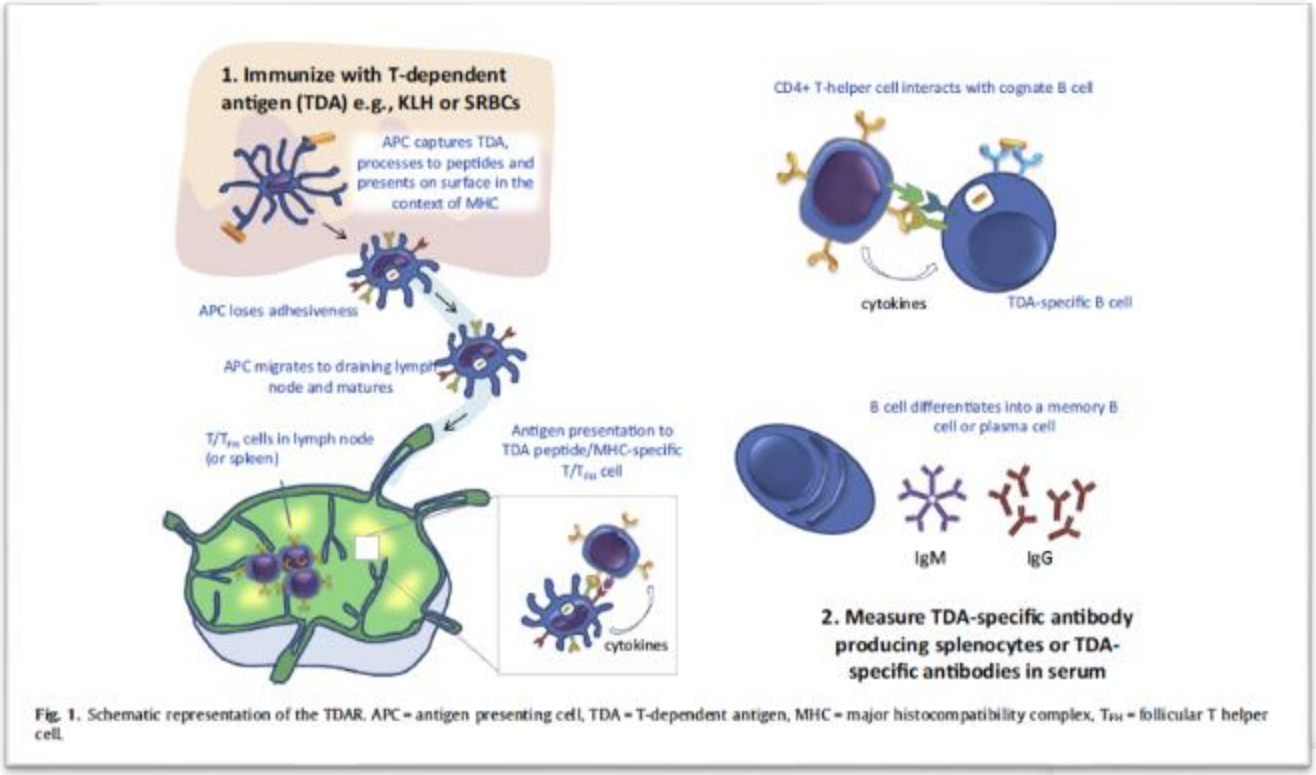
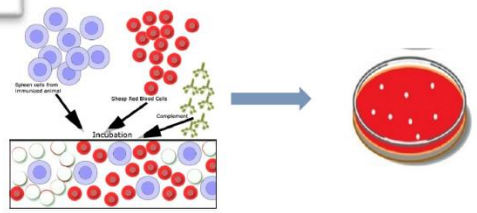



Fig. 1. Schematic representation of the TDAR. APC = antigen presenting cell, TDA = T-dependent antigen, MHC = major histocompatibility complex, $T_{FH}$ = follicular T helper cell.

*Lebrec et al., Reg. Tox. Pharmcol, 2014*

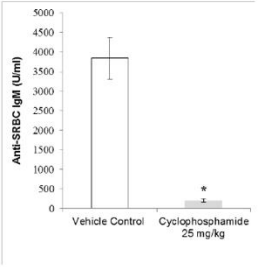
Keyhole limpet hemocyanin (KLH) is a large, multisubunit, oxygen-carrying, metalloprotein that is found in the hemolymph of the giant keyhole limpet *Megathura crenulata*

- PFC Assay

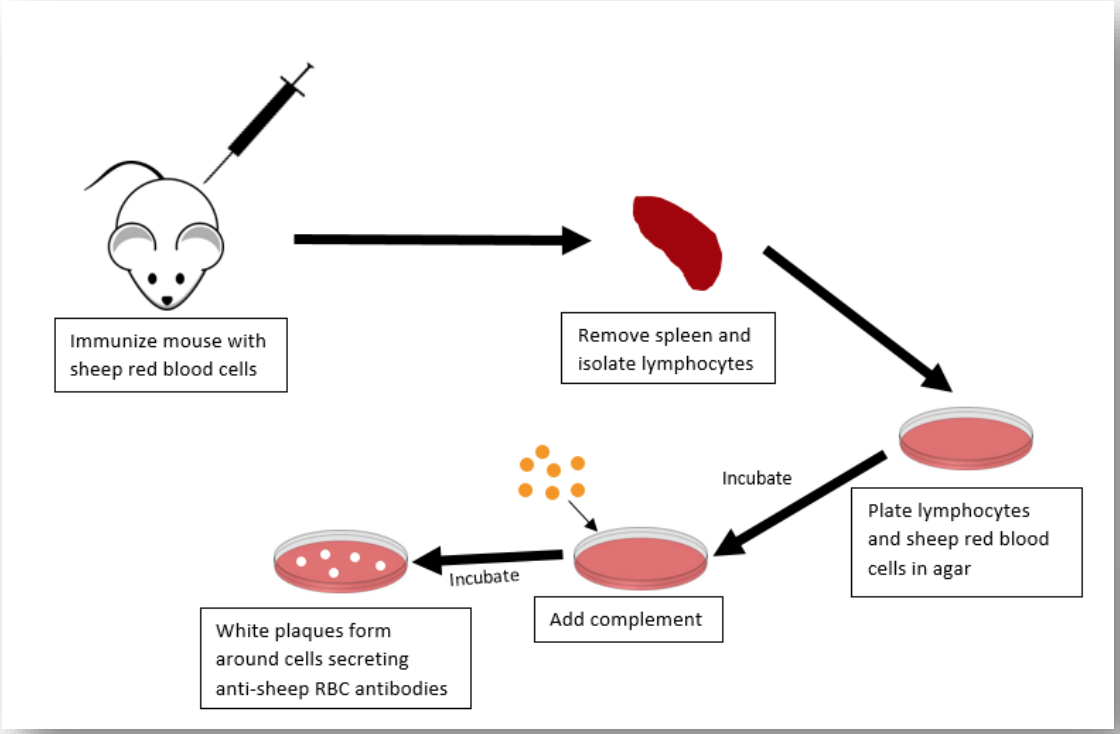



- ELISA

| Treatment Group | Anti-SRBC IgM (U/ml) | SE |
|---|---|---|
| Vehicle Control | 3849 | 530 |
| Cyclophosphamide 25 mg/kg | 210 | 46 |



# PLAQUE FORMING CELL ASSAY



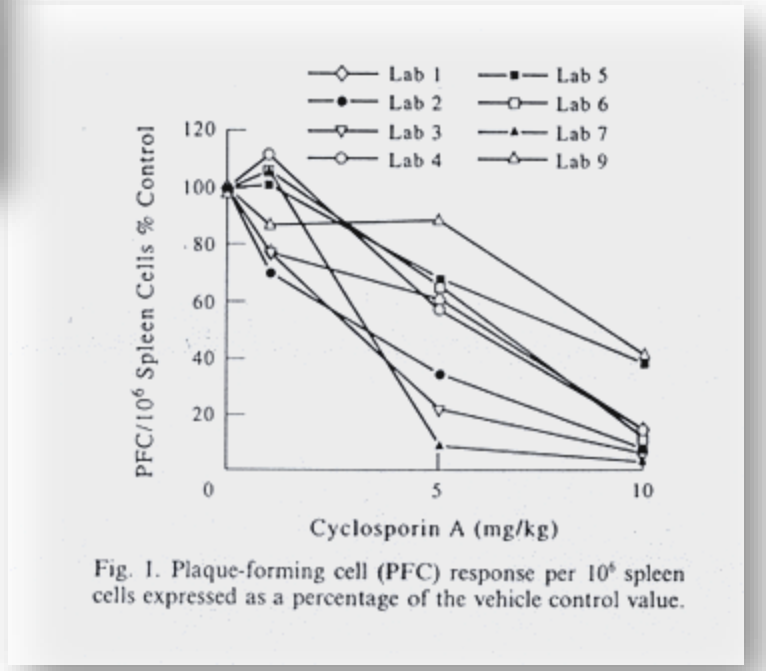




Fig. 1. Plaque-forming cell (PFC) response per $10^6$ spleen cells expressed as a percentage of the vehicle control value.

# FUNCTIONAL TESTS – CTL

1. Splénocytes, lymphocytes

2. Culture of splenocytes in the presence of inactivated cells P815 (mouse mast cells) or WFUG1 (rat lymphoma) or Jurkat (human leukemia) **FOR 5 DAYS**

3. Recover activated lymphocytes.
   Incubate these lymphocytes with $^{51}Cr$ labelled target cells
   3 ratios of lymphocytes to target cells (50:1, 25:1, 12.5:1)

4. Incubate for 4 hours. Measure the radioactivity in the supernatants

5. Results are expressed as % cytotoxicity
   % Cytotoxicity = $(cpm_e - cpm_{min})/(cpm_{max} - cpm_{min})$

# Exemples

# IMMUNOSUPPRESSION (I)

Table 3
Body weights, organ weights and cellularity

| Group | Survival (%) | Body weight | | Organ weights | | | | Cellularity ($\times 10^6$ total nucleated cells/organ) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Average (g) | Change (g) | Spleen | | Thymus | | Spleen | Thymus | Bone marrow |
| | | | | Average[a] | Relative[b] | Average | Relative | | | |
| Naïve | 100 | 164 | 29 | 0.384 | 0.235 | 0.252 | 0.154 | 240.8 | 297.4 | 38.2 |
| Vehicle | 100 | 161 | 27 | 0.412 | 0.255 | 0.239 | 0.148 | 250.0 | 336.8 | 36.0 |
| AZA | 93 | 141[+] | 10[+] | 0.335 | 0.239 | 0.091[+] | 0.065[+] | 190.8[+] | 47.9[+] | 32.1 |
| CY | 100 | 143[+] | 11[+] | 0.273 | 0.190 | 0.048[+] | 0.033[+] | 52.7[+] | 8.8[+] | 28.1[+] |
| CsA | 100 | 164 | 32 | 0.416 | 0.254 | 0.221 | 0.134 | 246.5 | 343.2 | 39.0 |

[a] Average organ weight ($n = 14$, except AZA $n = 13$).
[b] Average organ weight relative to body weight ($n = 14$, except AZA $n = 13$).
[+] Statistically significant change from naïve control ($P < 0.05$).

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Azathioprine: inhibits nucleotide biosynthesis
Cyclophosphamide: alkylating agent
Cyclosporine: cyclic immunomodulating polypeptide

# IMMUNOSUPPRESSION (II)

Table 5
Blood, spleen and thymus phenotype results

| Tissue | Test article | Relative | | | | Absolute ($\times 10^7$ cells) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Average CD3 (%) | CD4 (%) | CD8 (%) | CD45R (%) | Average CD3 | CD4 | CD8 | CD45R |
| Blood | Naïve | 60.1 | 40.1 | 22.4 | 13.0 | 3.4 | 2.3 | 1.3 | 0.8 |
| | Vehicle | 59.8 | 40.5 | 22.5 | 15.6 | 3.4 | 2.3 | 1.3 | 0.9 |
| | AZA[a] | 59.7 | 39.7 | 21.2 | 11.5 | 2.3[+] | 1.5[+] | 0.8[+] | 0.5[+] |
| | CY[b] | 26.0[+] | 14.9[+] | 13.9[+] | 0.8[+] | 0.2[+] | 0.1[+] | 0.1[+] | 0.0[+] |
| | CsA[c] | 39.0[+] | 26.8[+] | 12.6[+] | 11.5 | 1.2[+] | 0.8[+] | 0.4[+] | 0.4[+] |
| Spleen | Naïve | 44.0 | 25.2 | 21.0 | 19.5 | 4.5 | 2.6 | 2.1 | 2.0 |
| | Vehicle | 43.6 | 25.0 | 20.7 | 20.3 | 4.6 | 2.6 | 2.2 | 2.1 |
| | AZA | 43.3 | 24.5 | 20.3 | 20.6 | 3.2[+] | 1.8[+] | 1.5[+] | 1.6 |
| | CY | 36.3[+] | 17.8[+] | 20.7 | 3.6[+] | 0.3[+] | 0.1[+] | 0.2[+] | 0.0[+] |
| | CsA | 29.3[+] | 15.9[+] | 13.8[+] | 23.9 | 2.5[+] | 1.4[+] | 1.2[+] | 2.0 |
| Thymus | Naïve | 13.7 | 11.6 | 11.2 | NA[d] | 2.6 | 2.2 | 2.0 | NA |
| | Vehicle | 13.6 | 11.7 | 11.0 | NA | 3.0 | 2.5 | 2.4 | NA |
| | AZA | 35.5[+] | 26.6[+] | 19.5[+] | NA | 0.5[+] | 0.4[+] | 0.3[+] | NA |
| | CY | 24.5[+] | 21.0[+] | 22.3[+] | NA | 0.0[+] | 0.0[+] | 0.0[+] | NA |
| | CsA | 1.4[+] | 1.2[+] | 1.3[+] | NA | 0.3[+] | 0.3[+] | 0.3[+] | NA |

[a] Azathioprine.
[b] Cyclophospharnide.
[c] Cyclosporin A.
[d] Not applicable.
[+] Statistically significant difference from naïve control ($P < 0.05$).

# EXEMPLES

Table 6
Natural killer activity in B6C3F1 mice and Fischer 344 rats following in vivo exposure to CSA

| CsA (mg/kg/day) | Fischer 344 rats | | B6C3F1 mice | |
|---|---|---|---|---|
| | CsA blood concentration ($M \times 10^{-8}$) | $LU_{10}$[a] ($n = 6$) | CsA blood concentration ($M \times 10^{-8}$) | $LU_{10}$[a] ($n = 7$) |
| 0 | 0 | 26 ± 3 | 0 | 28 ± 3 |
| 5 | 2 ± 0.2 | 21 ± 1 (83%)[b] | 0.6 ± 0.1 | 19 ± 2* (68%) |
| 10 | 16 ± 2 | 24 ± 4 (93%) | 1 ± 0.1 | 17 ± 4* (61%) |
| 25 | 50 ± 11 | 26 ± 3 (102%) | 12 ± 1.6 | 19 ± 1* (68%) |

[a]Values are expressed in lytic units 10% ($LU_{10}$) ± S.E.M.
[b]Percentage of control ± S.E.M.
*$P < 0.05$.

Table 7
Natural killer activity in B6C3F1 mice and Fischer 344 rats following in vitro exposure to CSA

| | Cyclosporin A (M) | | | | $IC_{50}$ (M) |
|---|---|---|---|---|---|
| | Vehicle | $10^{-7}$ M | $10^{-6}$ M | $10^{-5}$ M | |
| Fischer 344 rats | 37 ± 8[a,c] | 25 ± 4 (68%)[b] | 19 ± 2* (51%) | 15 ± 5*** (40%) | $1.7 \times 10^{-6}$M |
| B6C3F1 mice | 24 ± 8[a,c] | 12 ± 4* (50%) | 8 ± 1** (33%) | 7 ± 1*** (29%) | $1.0 \times 10^{-7}$ M |

[a]Values are expressed in lytic units 10% ($LU_{10}$) ± S.E.M.
[b]Percentage of control ± S.E.M.
[c]Mean ± S.E.M. of three independent experiments.
*$P < 0.05$;
**$P < 0.01$;
***$P < 0.001$.

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# EXEMPLES

Table 4
Cytotoxic T-lymphocyte activity in B6C3F1 mice and Fischer 344 rats following in vivo exposure to CSA

| CsA (mg/kg/day) | Fischer 344 rats | | B6C3F1 mice | |
|---|---|---|---|---|
| | CsA blood concentration ($M \times 10^{-8}$) | CTL activity[a] ($n = 6$) | CsA blood concentration ($M \times 10^{-8}$) | CTL activity[a] ($n = 7$) |
| 5 | 6 ± 0.7 | 97 ± 11 | 0.6 ± 0.1 | 109 ± 6 |
| 10 | 23 ± 3 | 112 ± 5 | 1 ± 0.1 | 96 ± 9 |
| 25 | 83 ± 7 | 66 ± 8** | 12 ± 1.6 | 107 ± 4 |

[a]Values are expressed as a percentage of control ± S.E.M.
**$P < 0.01$.

Table 5
Cytotoxic T-lymphocyte activity in B6C3F1 mice and Fischer 344 rats following in vitro exposure to CSA

| | Cyclosporin A (M) | | | $IC_{50}$ (M) |
|---|---|---|---|---|
| | $10^{-7}$ M | $10^{-6}$ M | $10^{-5}$ M | |
| Fischer 344 rats | 96 ± 18[a,b] | 55 ± 8** | 12 ± 3*** | $1.27 \times 10^{-6}$ |
| B6C3F1 mice | 87 ± 3[a,b] | 57 ± 2** | 4 ± 2*** | $0.96 \times 10^{-6}$ |

[a]Values are expressed as a percentage of control ± S.E.M.
[c]Mean ± S.E.M. of three independent experiments.
**$P < 0.01$;
***$P < 0.001$.

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obrigado
Dank U
Merci
mahalo
Köszi
спасибо
Grazie
Thank you
mauruuru
Takk
Gracias
Dziękuję
Děkuju
danke
Kiitos