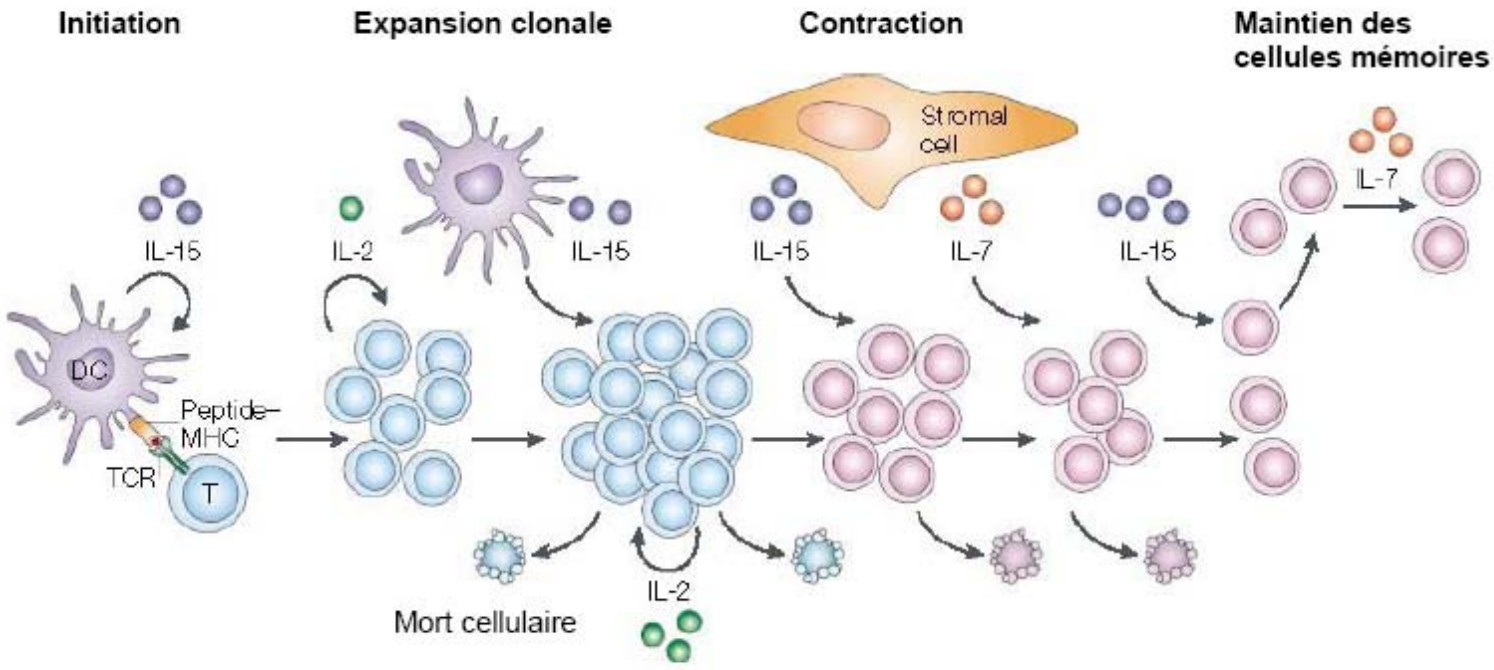
Important issues in immunotoxicity testing of chemicals

Pr Marc Pallardy, Toxicologie and INSERM UMR-S 749, Faculté de Pharmacie, Châtenay-Malabry, France

- Innate immune response: non antigen specific
  - Physical barriers
  - IgA, Complement
  - Neutrophils phagocytosis
  - Macrophages
  - NK cells cytotoxicity
- Adaptative immune response: antigen specific
  - Lymphocytes (T cells: TH1, TH2, TH17, Treg; B cells)

Adaptive immune response



D'après KS. Schluns et L. Lefrançois, Nature Review Immunology, 2003.

Occurs in response to pathogens and to chemicals

- Specific T-cell clones (ex: penicillin, nickel…)

IMMUNOTOXICITY

- Immunosuppression
  - Down-regulation of immune responses by chemicals
- Hypersensitivity
  - Innapropriate specific immune response to chemicals
- Stimulation of the immune system leading to pathology
  - Cytokine release
- Auto-immunity
  - Immune response to auto-antigens induced by chemicals

IMMUNOSUPPRESSION

Strategies for in vitro testing

Strategies for in vivo testing

based on the functional evaluation of
the different components of the immune system

In vitro testing for Immunosuppression

- Initial evaluation of myelotoxicity
  - Hematopoietic progenitors (CFU-GM assay)
- Determination of lymphotoxicity
  - Lymphocytes (trypan blue, MTT, LDH release)
- Determination of potential effects on NK cells
  - Cytotoxicity (^{51}Cr, flow cytometry) on selected target cells (YAC for rodent, K562 for human)
- Lymphocyte proliferation
  - Mitogens (ConA, PHA), Anti-CD3 + anti-CD28
  - ^{3}H-thymidine, flow cytometry **(PKH26, CFSE)**
- Cytokine production
  - Whole blood assay (IL-2, IFN-gamma)
- Determination of potential effects on antibody induction/production

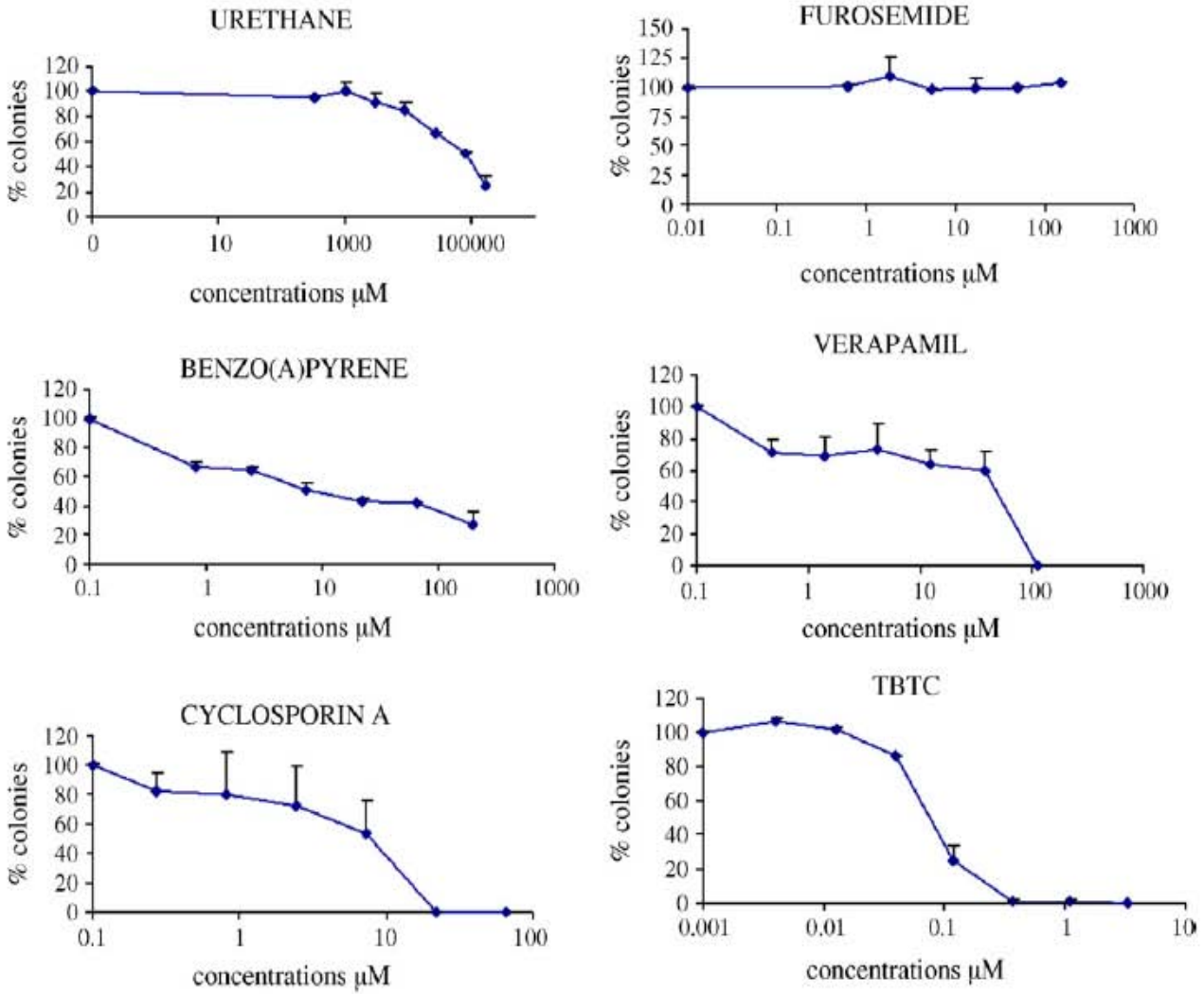


Fig. 1. CFU-GM test-graphs show the colonies number counted at different compound concentrations (µM). Colonies were counted after 14 days exposure.

M. Carfi et al, Toxicology 229, 11-22 (2007)

Table 2
Anti-CD3 antibody stimulation

Compounds	Anti-CD3 antibody stimulation: IC50 (μM)	
	Mouse lymphocytes	Human lymphocytes
Urethane	>10,000	>17,000
Furosemide	>1000	>100
Verapamil	30.27 (±3.5)	20.95 (±1.16)
Benzo(*a*)pyrene	>160	12.82 (±1.11)
Cyclosporin A	0.13 (±0.05)	1.00 (±0.13)
TBTC	>0.1	Not tested

Cells from mouse lymphocytes and from human peripheral blood have been stimulated with anti-CD3 antibody and treated with compounds. IC50 values (μM) have been calculated.

M. Carfi et al, Toxicology 229, 11-22 (2007)

IC50 obtained when combining all tests

IC50 comparison

Compounds	Mouse	Rat	Human
Urethane	No effect	No effect	No effect
Furosemide	No effect	No effect	No effect
Verapamil	13 < IC50 < 30	17 < IC50 < 30	20 < IC50 < 22
Benzo(*a*)pyrene	10 < IC50 < 18	9 < IC50 < 13	11 < IC50 < 12
Cyclosporin A	0.08 < IC50 < 0.3	0.16 < IC50 < 0.18	1 < IC50 < 8
TBTC	0.002 < IC50 < 0.003	0.007	0.07

The IC50 values (μM) obtained from each different test are reported as range. "No effect" means that the compound is not immunotoxic.

Verapamil, a calcium channel blocker, was found positive

True in the real life ?

Remember: no drugs have been withdrawn from the market due to immunosuppression; why ? impossible to detect !

M. Carfi et al, Toxicology 229, 11-22 (2007)

Chemical Hypersensitivity

Steps of chemical sensitization

- Pre-immunological phase
  - Chemical reactivity
  - Metabolism
  - Genetic polymorphism
- Sensitization phase
  - Dendritic cell activation
  - Hapten presentation to T-cells
  - T-lymphocyte activation and proliferation
- Effector phase
  - Th1 vs Th2 response

Strategies for detecting chemical

- Strategy for in vivo testing
  - Chemicals induce immunopathology (GMPT tests)
  - Chemicals induce an immune response (LLNA)
- In vitro
  - Try to mimick an immune response (ie lymphocyte proliferation) to chemical sensitizers using and in vitro approach ? Failed so far
  - Try to find unique properties of chemical sensitizers that distinghish them form other chemicals ?

Development of In silico/in vitro methods

Chemical reactivity

- QSAR
  - Patlewicz G, Aptula AO, Uriarte E, Roberts DW, Kern PS, Gerberick GF, Kimber I, Dearman RJ, Ryan CA, Basketter DA. An evaluation of selected global (Q)SARs/expert systems for the prediction of skin sensitisation potential. SAR QSAR Environ Res. 2007 Jul-Sep;18(5-6):515-41.
- Peptide reactivity assay
  - 2006, 82 molecules tested
- Cell-based models
  - Human dendritic cells (murine, human)
  - Cell lines (THP-1, U937, MUTZ-3)

Chemical reactivity: peptide reactivity assay

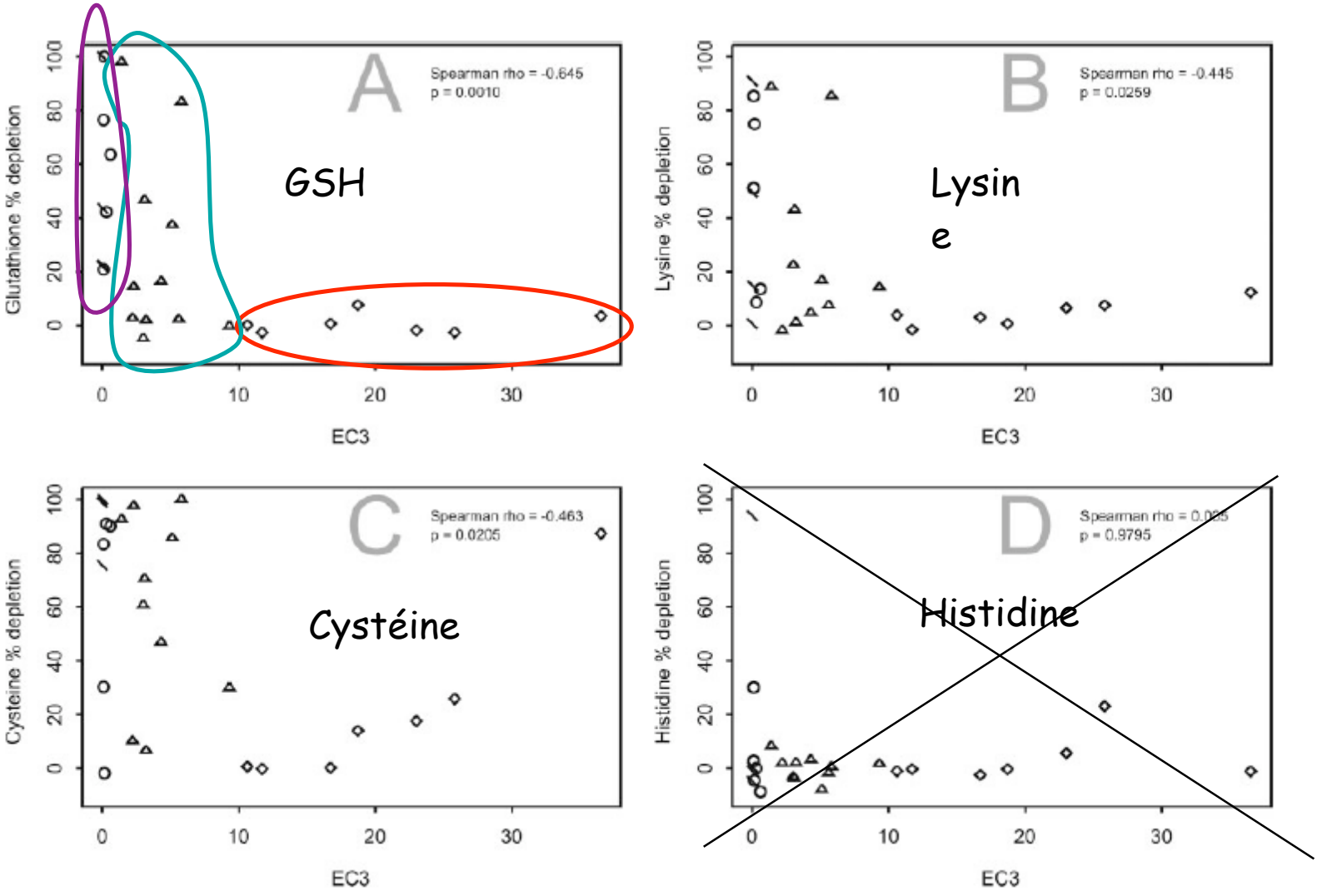


FIG. 4. Spearman correlation of LLNA EC3 data and peptide reactivity. LLNA potency category is denoted as Extreme (□), Strong (○), Moderate (△), or Weak (◇). Peptides include glutathione (A), lysine (B), cysteine (C), and histidine (D).

From Gerberick GF et al, Tox Sci 2004

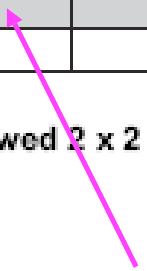
Statistics for non-sensitizers vs sensitizers (cys 1/10 and lys 1/50 prediction model)

		Predicted Classification (based on classification tree model)		
		Non-Sensitizer	Sensitizer	total
Chemical Classification[a]	Non-Sensitizer	26	3	29
	Sensitizer	6	46	52
	total	32	49	81

table statistics for the shadowed 2 x 2 table

sensitivity:	88%
specificity:	90%
positive predictivity:	94%
negative predictivity:	81%
accuracy:	89%

[a]Based primarily on LLNA data



6 molecules failed

Gerberick et al, 2006

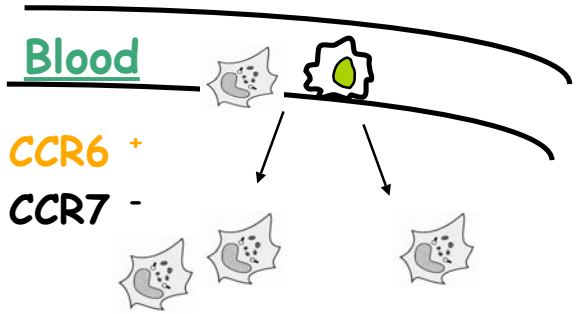
Cell-based assays

- Human dendritic cells (CD34-DC, Mo-DC)

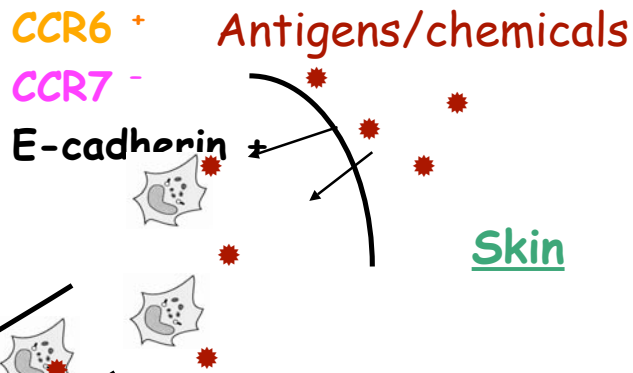
- Cell lines
  - THP-1
  - MUTZ-3
  - U937

Dendritic cell migration after antigen uptake

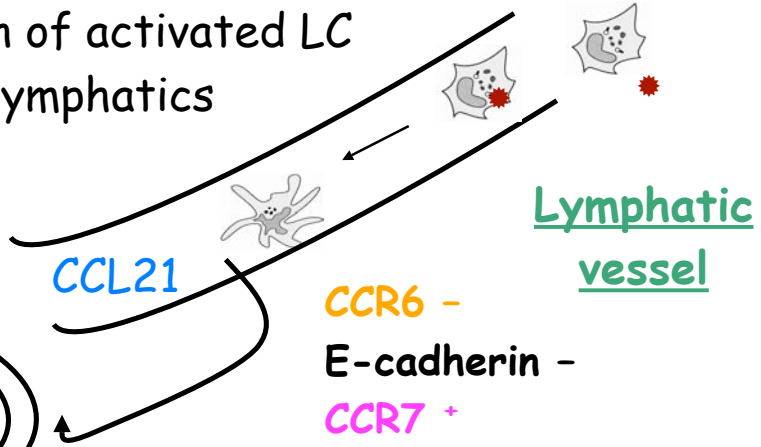
① Recruitment of LC precursors

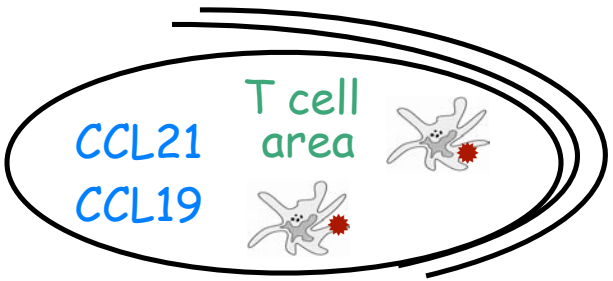


② CCL20 production by keratinocytes



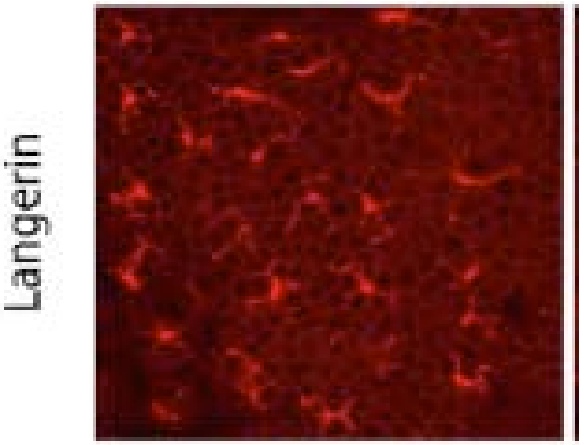
③ Migration of activated LC through lymphatics





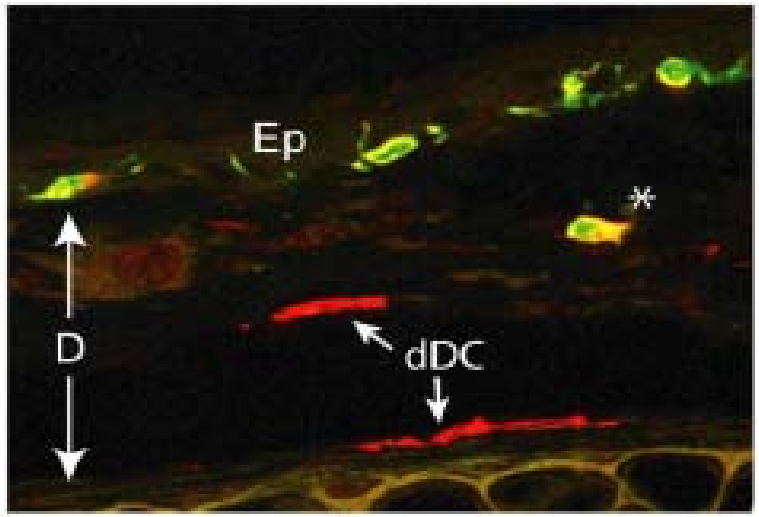
④ Homing in T cell areas of maturing LCs in reponse to CCL19 and CCL21

Murine epidermis

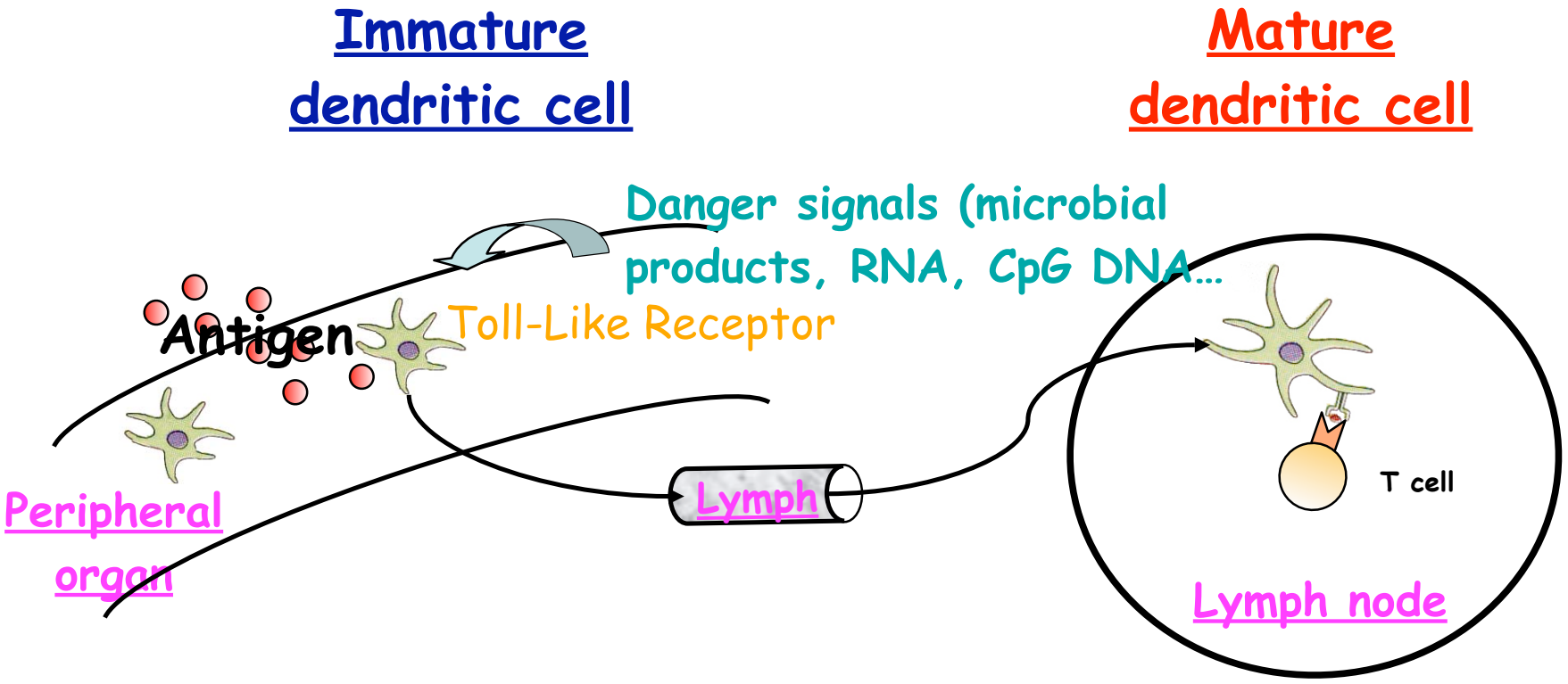


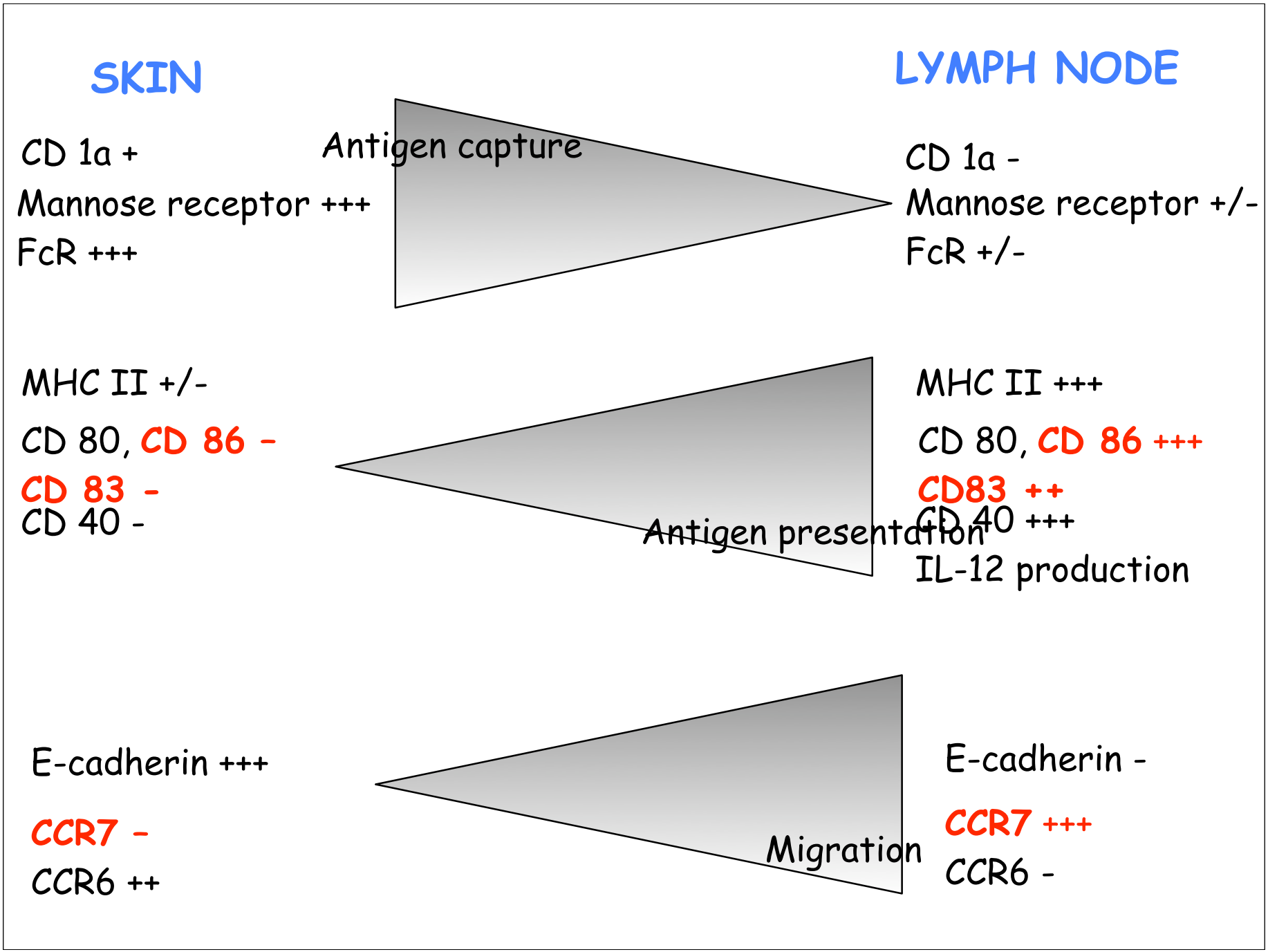
Murine skin
EGP-langerin

Green: LC
Red = CMH II
Yellow: LC/CMH II



Kissenpfenig et al, Immunity 2005


Immature dendritic cell
Mature dendritic cell
Danger signals (microbial products, RNA, CpG DNA…
Antigen
Toll-Like Receptor
Peripheral organ
Lymph
T cell
Lymph node
1- Antigen uptake and Activation
2- Migration
3- Antigen Presentation


SKIN
LYMPH NODE
CD 1a +
Mannose receptor +++
FcR +++
Antigen capture
CD 1a -
Mannose receptor +/-
FcR +/-
MHC II +/-
CD 80, CD 86 -
CD 83 -
CD 40 -
Antigen presentation
MHC II +++
CD 80, CD 86 +++
CD83 ++
CD 40 +++
IL-12 production
E-cadherin +++
CCR7 -
CCR6 ++
Migration
E-cadherin -
CCR7 +++
CCR6 -

Can chemical sensitizers play the role of danger signals and

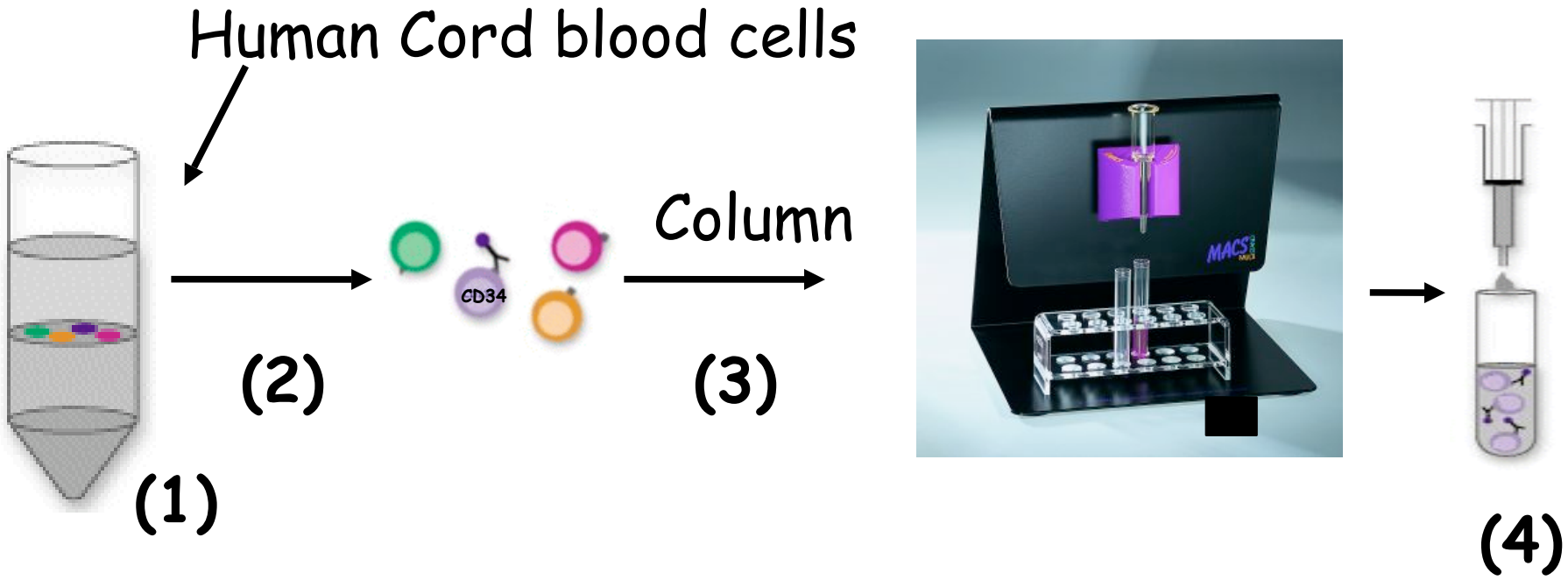
Hypothesis:

chemicals mimick « danger signals » (TLR agonists…) signalling in Dendritic Cells

or

Dendritic cells perceived chemical sensitizers as « danger »

CD34+ cells purification



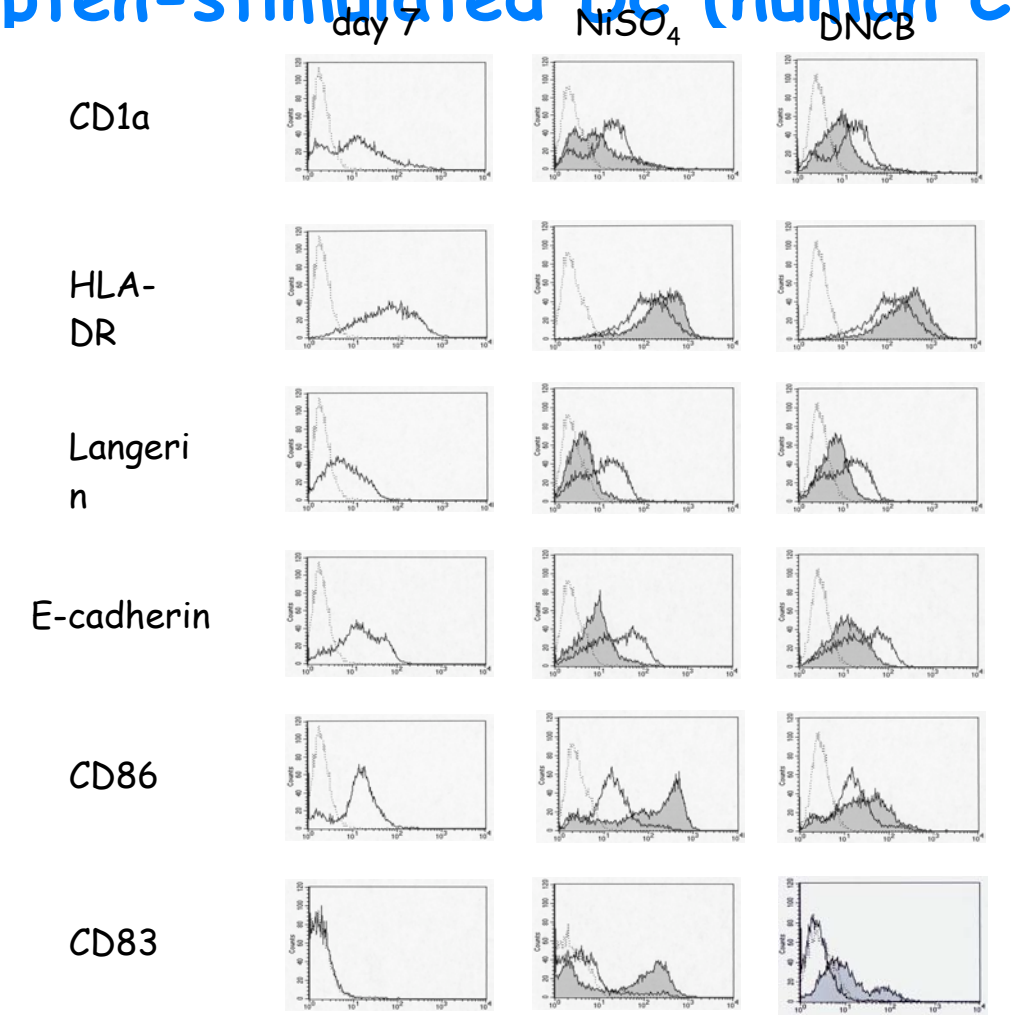
1: Collect human cord blood and isolate mononuclar cells using gradient density

2: Magnetic labelling using anti-CD34 antibody

3: Separate cells using a column with magnet

4: Collect CD34+ cells, purity should be >90%

Flow cytometry analysis of immature DC and hapten-stimulated DC (human CD34+/DC model)

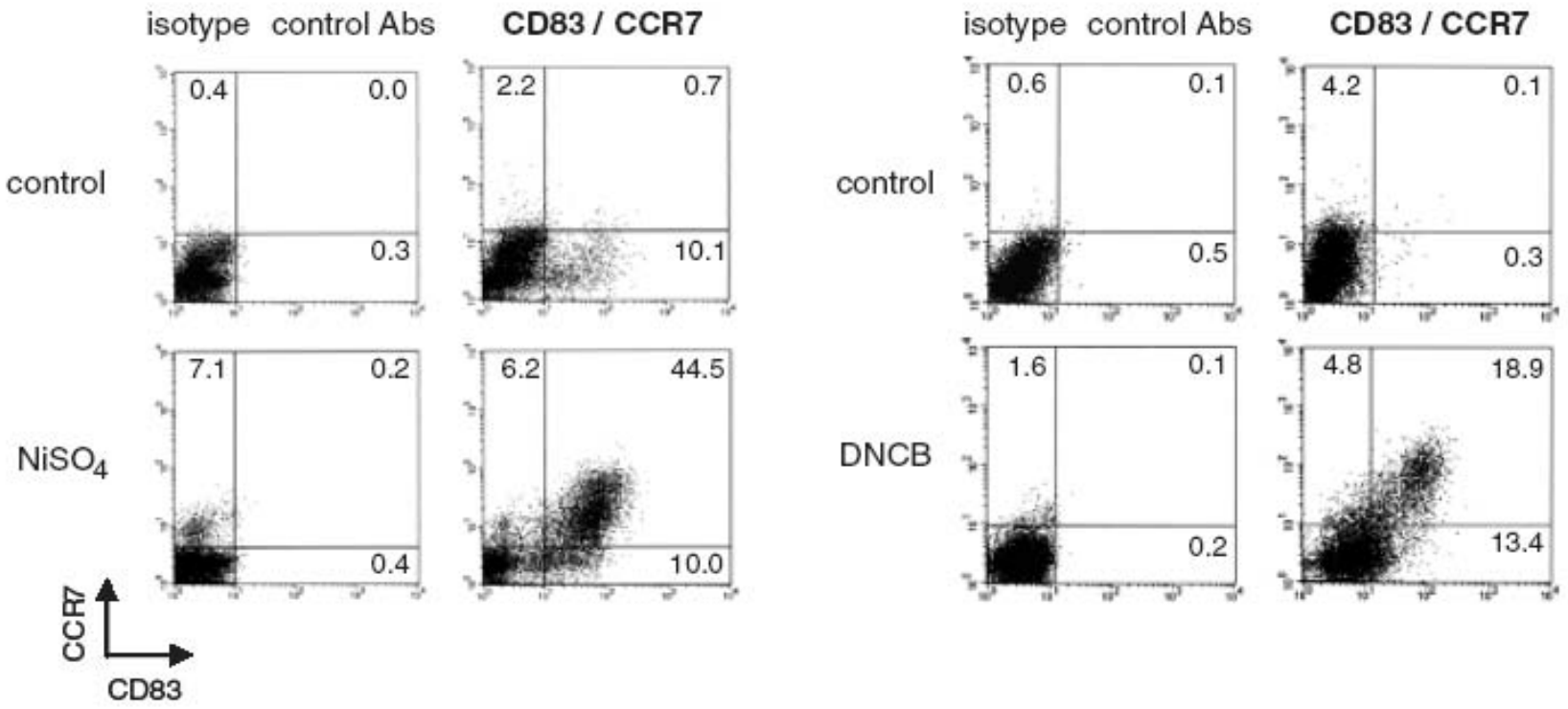


isotype control Ab
non stimulated-DC
hapten stimulated-DC

24 hrs treatment
NiSO4: 500 μM
DNCB: 25 μM

(Boisleve et al, Toxicology, 2005)

Haptens alter DC phenotype



Cytokine production by CD34-DC

ng/mL	Non treated	DMSO 0.05%	NiSO4 500 µM	DNCB 25 µM
TNF-α	ND	0.02	0.45	ND
IL-8	0. 44	0.45	98.7	13.1
IL-6	ND	ND	8.25	ND
IL-12p40	ND	ND	3.05	ND

- CD34-derived dendritic cells were treated with NiSO4 (500 µM) or with DNCB (25 µM).
- Cytokine productions were determined in supernatants using flow cytometry for TNF-α, IL-8, IL-6 and ELISA for IL-12.
- TNF-α production was measured after a 4 hours treatment whereas IL-8, IL-6 and IL-12p40 were measured after a 24 hours treatment
- ND: not detectable

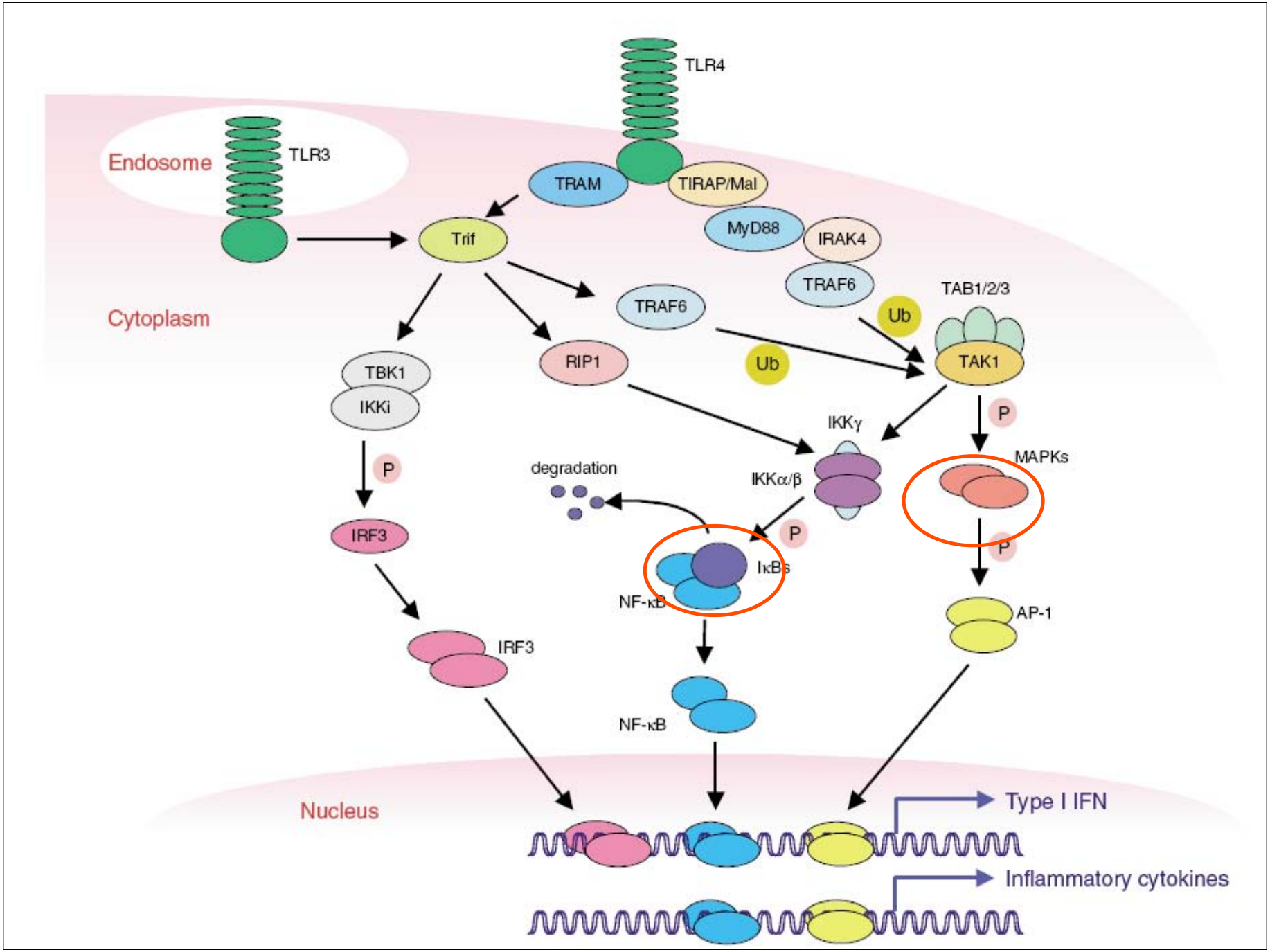
(Ade et al, Tox Sci, 2008)

Effects of chemical sensitizers on human CD34-DC phenotype

	Concentration	CD86	CD83	CCR7
$NiSO_4$	500 µM	4,2	10,1	2,8
DNCB	25 µM	2,3	6,6	2,4
pPD	75 µM	2,0	2,7	3,2
CIN	100 µM	1,5	0,7	0,8
HCHO	150 µM	4,4	10,8	2,7
BC	0,5 µg/ml	0,9	1,9	0,6
SDS	250 µM	0,9	0,7	0,9

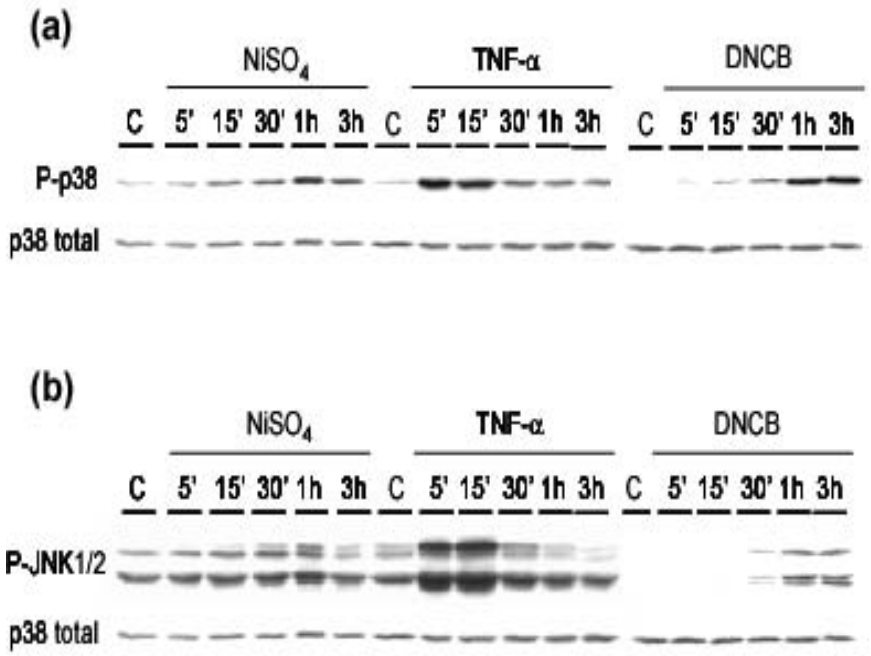
Results are expressed as fold induction
Mean of 3 independent experiments

- Haptens are able to induce the expression of DC maturation markers involved in lymphocyte co-stimulation or DC migration.
- Same type of results have also been found by other goups (Gerberick, Aiba, Kimber, Verheyen, Aeby…)
- What are the signalling pathways triggered by haptens ? Are they similar


TLR4
Endosome
TLR3
TRAM
TIRAP/Mal
MyD88
IRAK4
TRAF6
Trif
TAB1/2/3
Cytoplasm
TRAF6
Ub
Ub
TAK1
RIP1
TBK1
IKKi
P
IKKγ
MAPKs
degradation
IKKα/β
P
P
IRF3
IκBs
NF-κB
AP-1
IRF3
NF-κB
Nucleus
Type I IFN
Inflammatory cytokines

Sensitizers activate MAPKs

Western-Blot

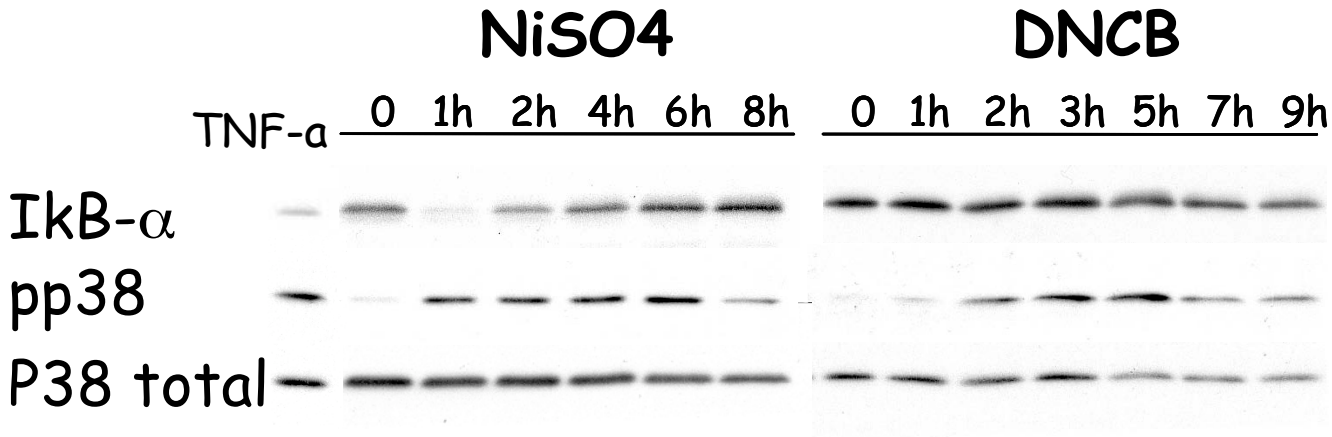


NiSO4: 500 μM
DNCB: 25 μM

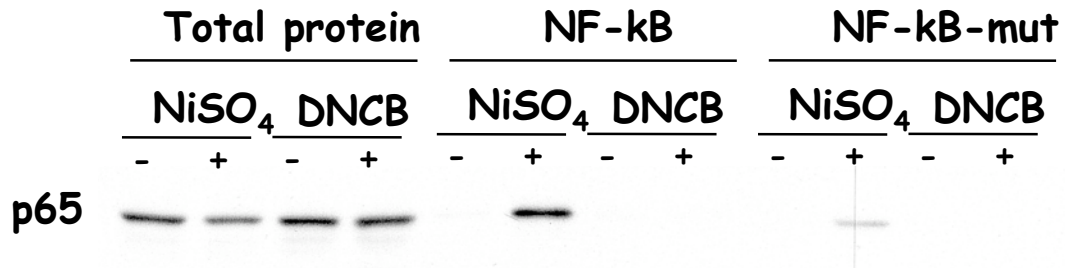
p38: P38 MAPK, P-p38: phospho-p38, JNK: Jun Kinase

Chemical sensitizers activate NFKB

Western-Blot

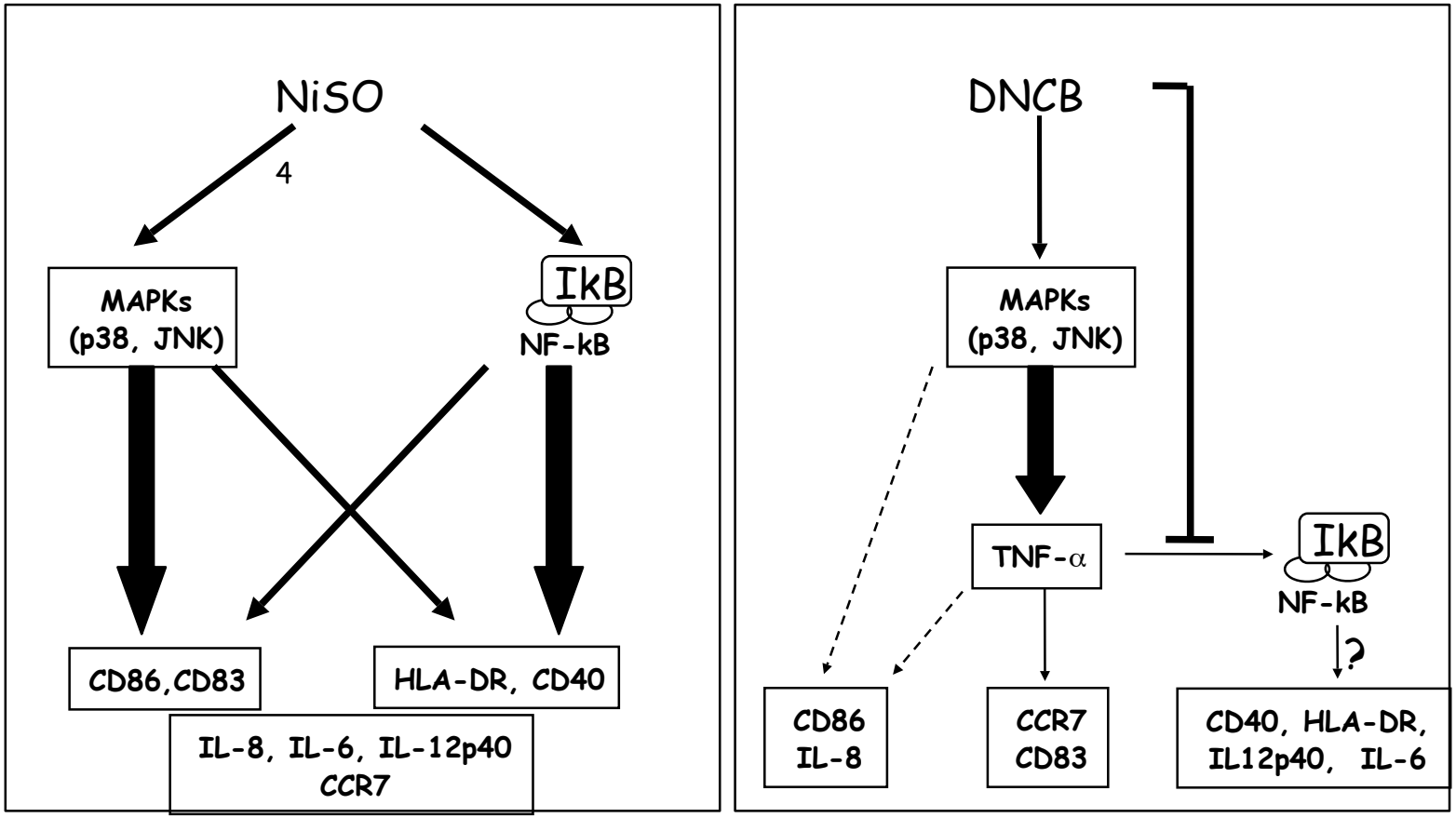


DNA-binding activity



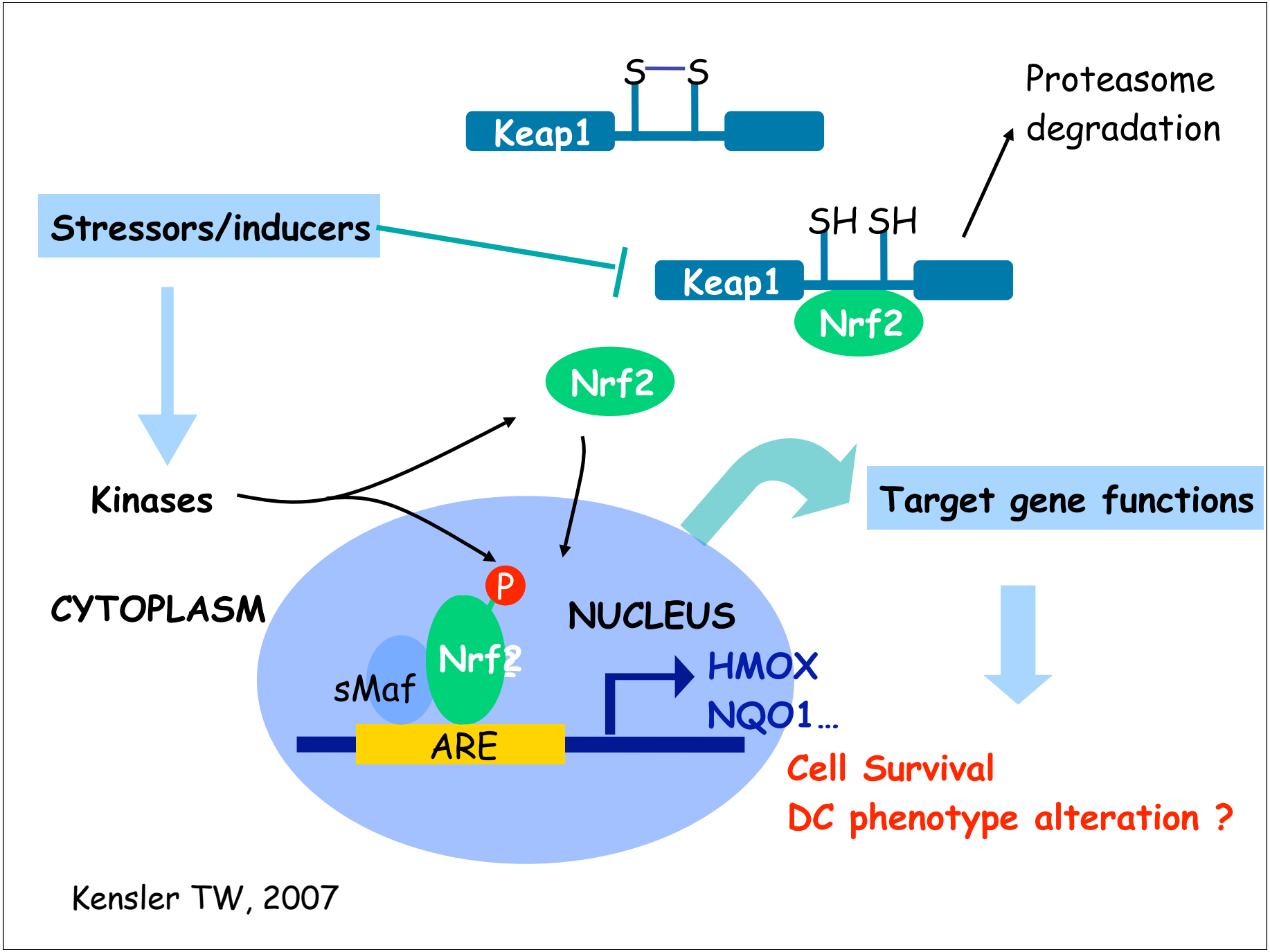
NiSO4: 500 μM
DNCB: 25 μM

Signaling pathways in CD34-DC activated by $NiSO_4$ or DNCB

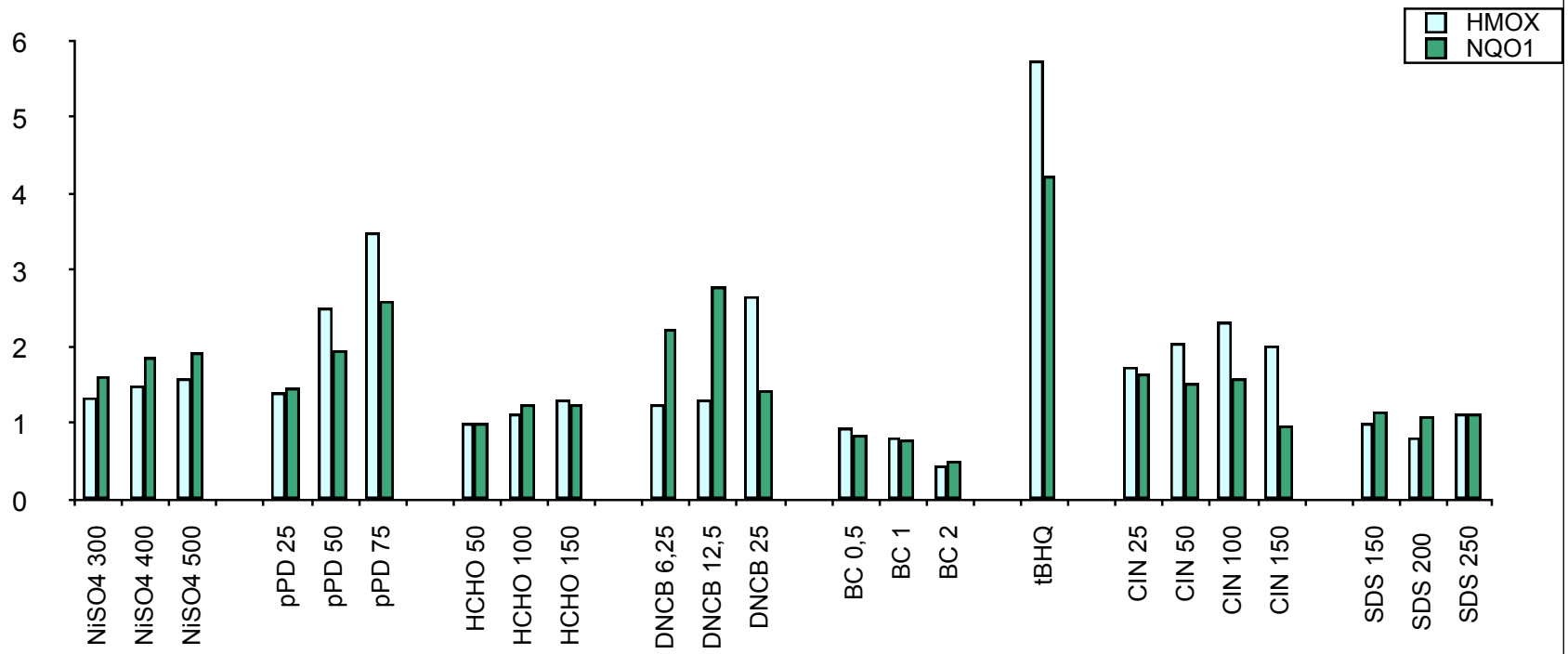


How DC handle the chemical stress ?

- Chemical sensitizers modify the DC phenotype
- Current hypothesis: chemical reactivity is necessary for a chemical to be a sensitizer
- However, chemical stress is known to induce cell signaling leading to cell death
- Necessity for the DC to handle the chemical stress to survive

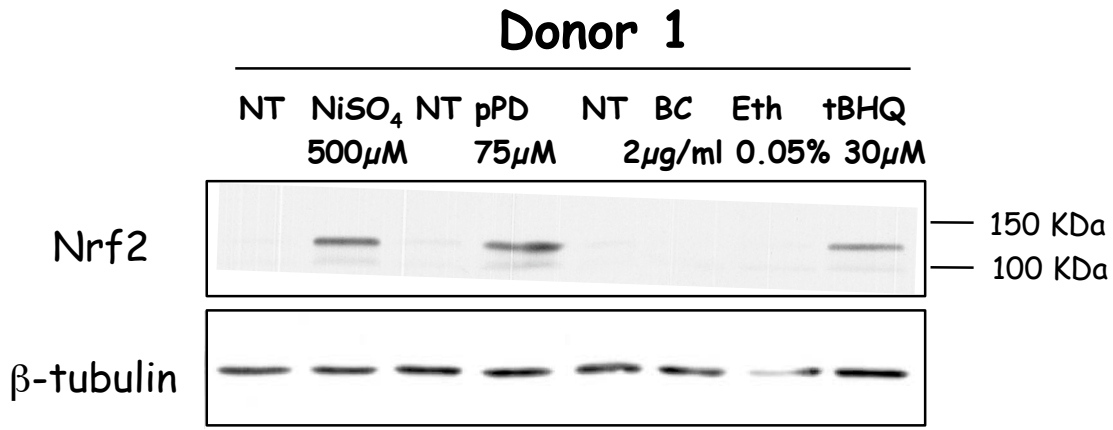

S—S
Keap1
Proteasome
degradation
Stressors/inducers
SH SH
Keap1
Nrf2
Nrf2
Kinases
Target gene functions
CYTOPLASM
P
NUCLEUS
Nrf2
sMaf
HMOX
NQO1…
ARE
Cell Survival
DC phenotype alteration ?
Kensler TW, 2007

mRNAs expression of NQO1 and HMOX1 after chemical treatment (CD34-DC model)

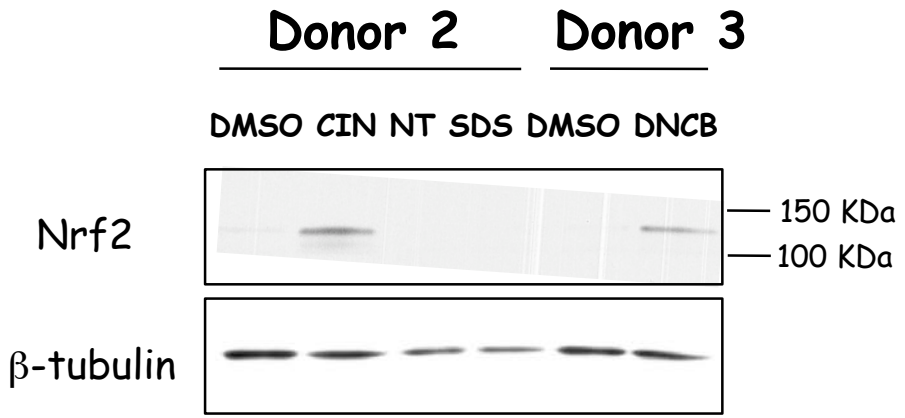


Results of a representative experiment

Nrf2 protein level in response to chemicals (CD34-DC model)



Cells were treated with chemicals for 5h
$3x10^6$ cells lysed in Laemmli Buffer
Incubation at 100°C during 5 minutes
Centrifugation 20 min at 15,000 g
Migration of 20 µL of template



Models for in vitro evaluation

Cell lines: THP-1

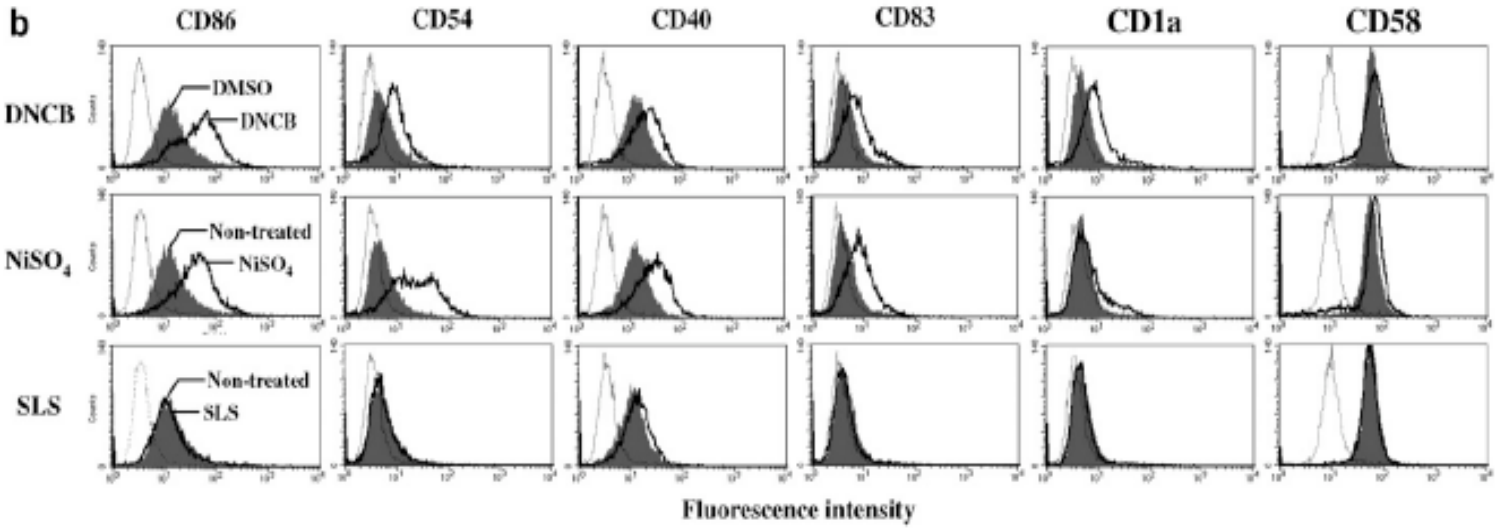


Fig. 1. Phenotypic alterations induced by DMSO (vehicle), DNCB (2.5 μg/mL), $NiSO_4$ (85 μg/mL), and SLS (45 μg/mL). The data shown represent middle concentrations used in the study. These concentrations were selected as representative data that provide the maximum effect. (a) FSC/SSC data: living cells (gray dots) and dead cells (black dots), (b) Histograms for each surface marker evaluated in THP-1 cells: Isotype control (dotted line), vehicle-control (either DMSO or untreated; shaded peak), and chemical-treated (dark solid line). Since untreated control and DMSO-treated control gave the same results, untreated and DMSO-treated results were interchanged. These are representative data from three independent experiments. MFI = mean fluorescence intensity.

Miyazawa M et al, Tox in vitro (2007)

Cell lines: THP-1

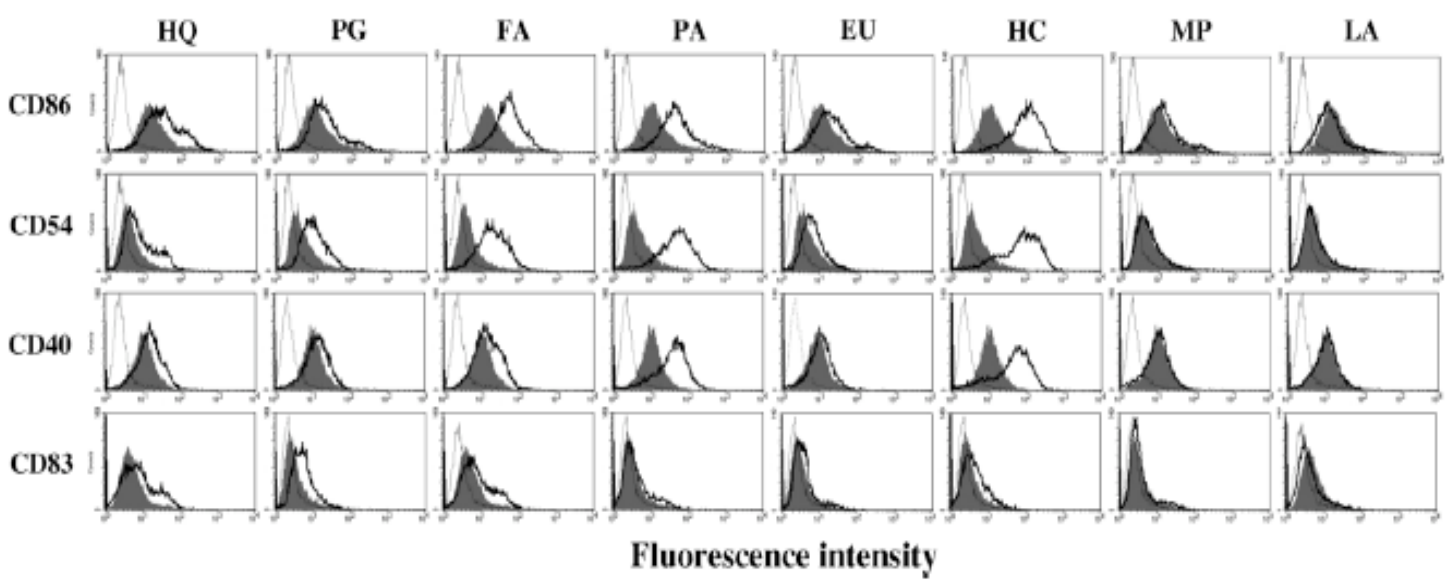


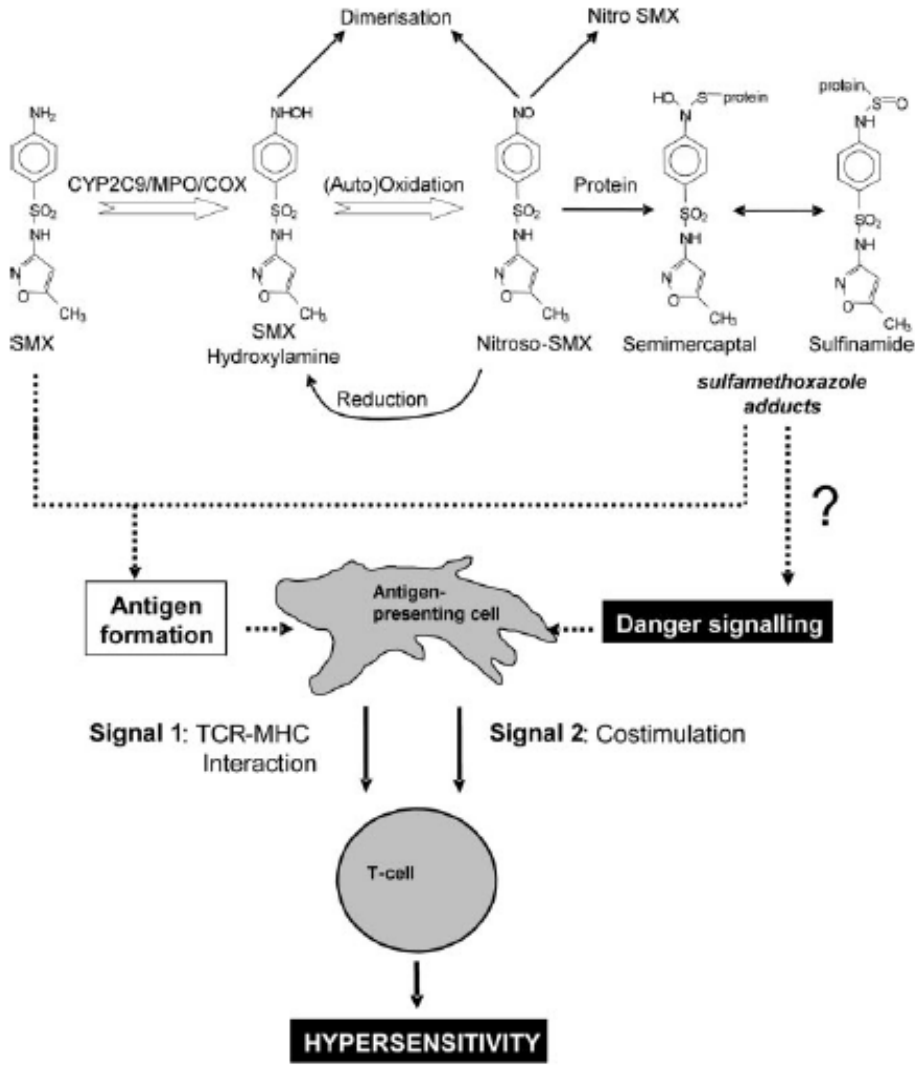
Fig. 4. Effect of six allergens and two non-allergens on surface phenotypic changes. The histograms are representative data at the highest concentration from three independent experiments. Isotype control (dotted line), vehicle-control (shaded peak), and chemical-treated (dark solid line).

Miyazawa M et al, Tox in vitro (2007)

- THP-1 based assay = hCLAT (human cell line activation test)
  - Evaluated by 5 labs since 2004: P&G, Shiseido, Kao, Henkel, L'Oréal (2nd ring study ongoing)
- U937/CD86
  - Originally developed by L'Oréal and Cosmital SA (now P&G) with LVMH. Recently transferred in Shiseido and

And drugs (1) ?

Most of the time
not chemically reactive

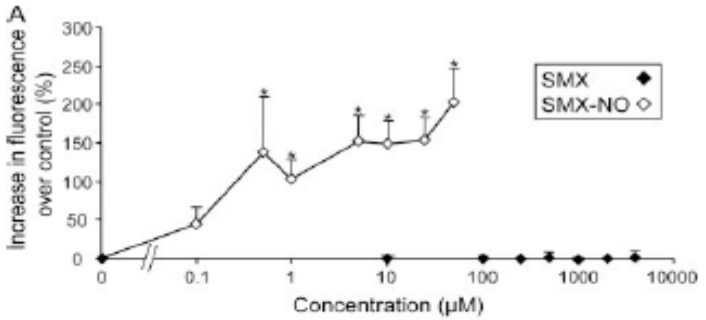


Sanderson JP et al, 2007

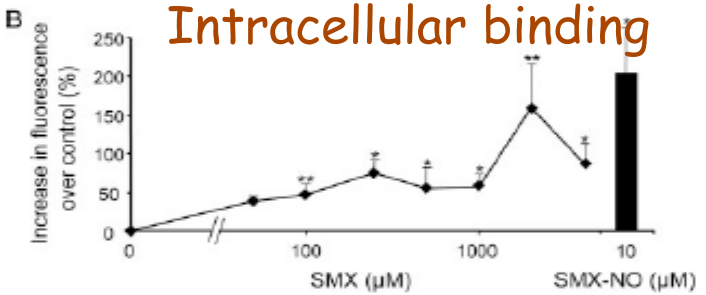
And drugs (2) ?

Detection of SMX adducts in Mo-DC following incubation with both SMX and SMX-NO

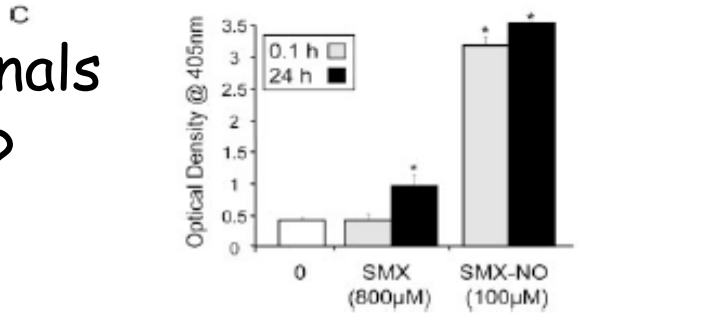
Cell surface covalent binding



Intracellular binding



Are these adducts providing signals in DC allowing their maturation ?



Protein-SMX adducts

Sanderson JP et al, 2007

Prediction of chemical sensitization

- In vivo preclinical test are in the process of prevalidation for the detection of contact sensitizers
- Prediction of the potential of chemical to be a sensitizer may be possible based on the following basis:
  - Protein reactivity is necessary for a majority of molecules
  - This property may be obtain only after metabolisation (the case for drugs ?)
  - A chemical sensitizer = danger danger signal for DC
- And of course: more work is needed.

Conclusions and perspectives

- The amount of results obtained in the field of immunotoxicology is highly dependent on the regulatory activity
  - Ex: EMEA guideline in immunotoxicology and TDR test
- The 7th amendment to the cosmetics directive and the REACH program are at the initiative of a great **industrial** and academic efforts in the search for alternative/in vitro tests
- Always important to keep in mind that to generate new tests it is necessary to

Success ?

- **Hypersensitivity**
  - The peptide reactivity assay and cell assays are very promising; prevalidation studies are ongoing
  - Combination of results from these assays and others (QSAR…) may provide a robust decision tree
- **Immunosuppression**
  - Efforts are ongoing to validate well-described existing tests
  - Work is needed to develop new tests and to refine the existing ones
- **Immunostimulation**
  - TG1412 accident due to CRS (cytokine release syndrome: there is a need for tests predicting this type of events

SUBSETS OF DCs

Myeloid lineage

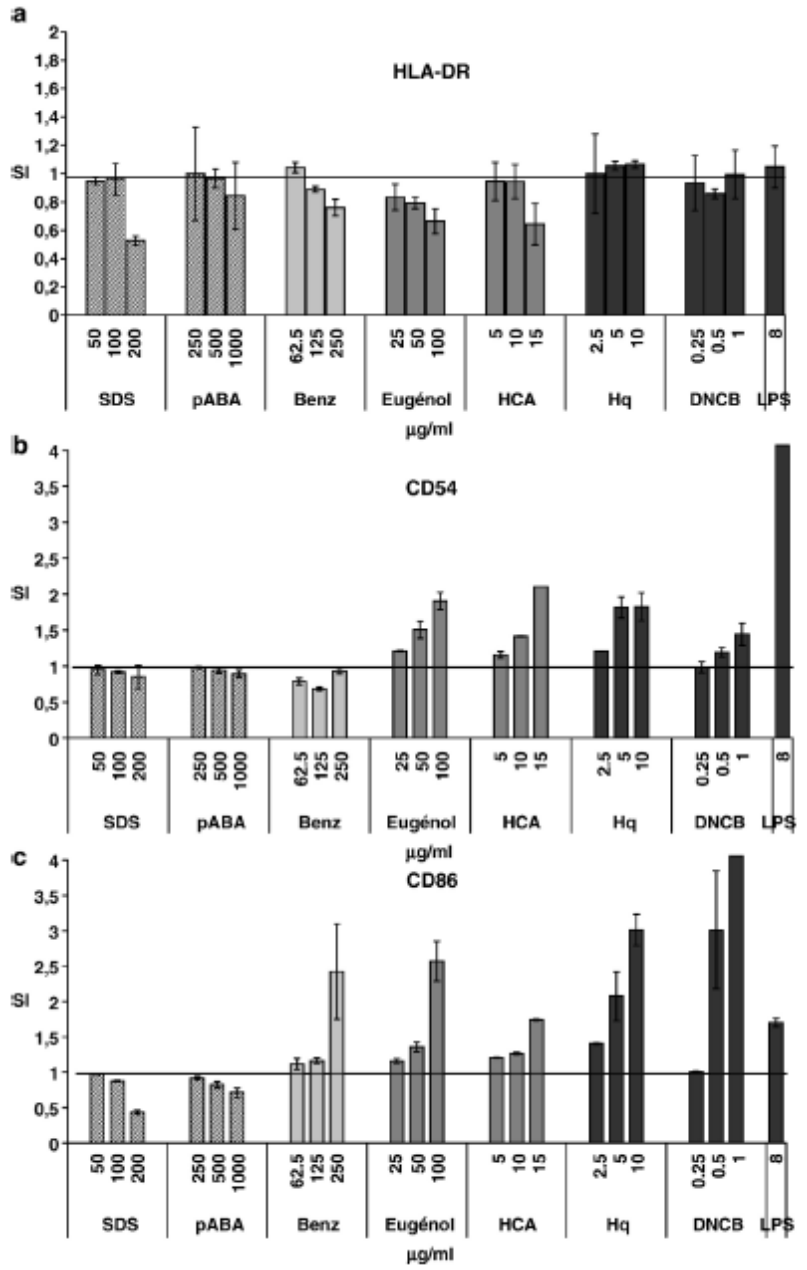
- Langerhans cells
- Dermal DC

Lymphoid lineage

- Plasmacytoid DC
- Thymic DC

MUTZ-3 cell line

The MUTZ-3 cell line can also differentiate in LC or in DC



Azam P et al, Tox Appl Pharm (2005)

MUTZ-3 cell line

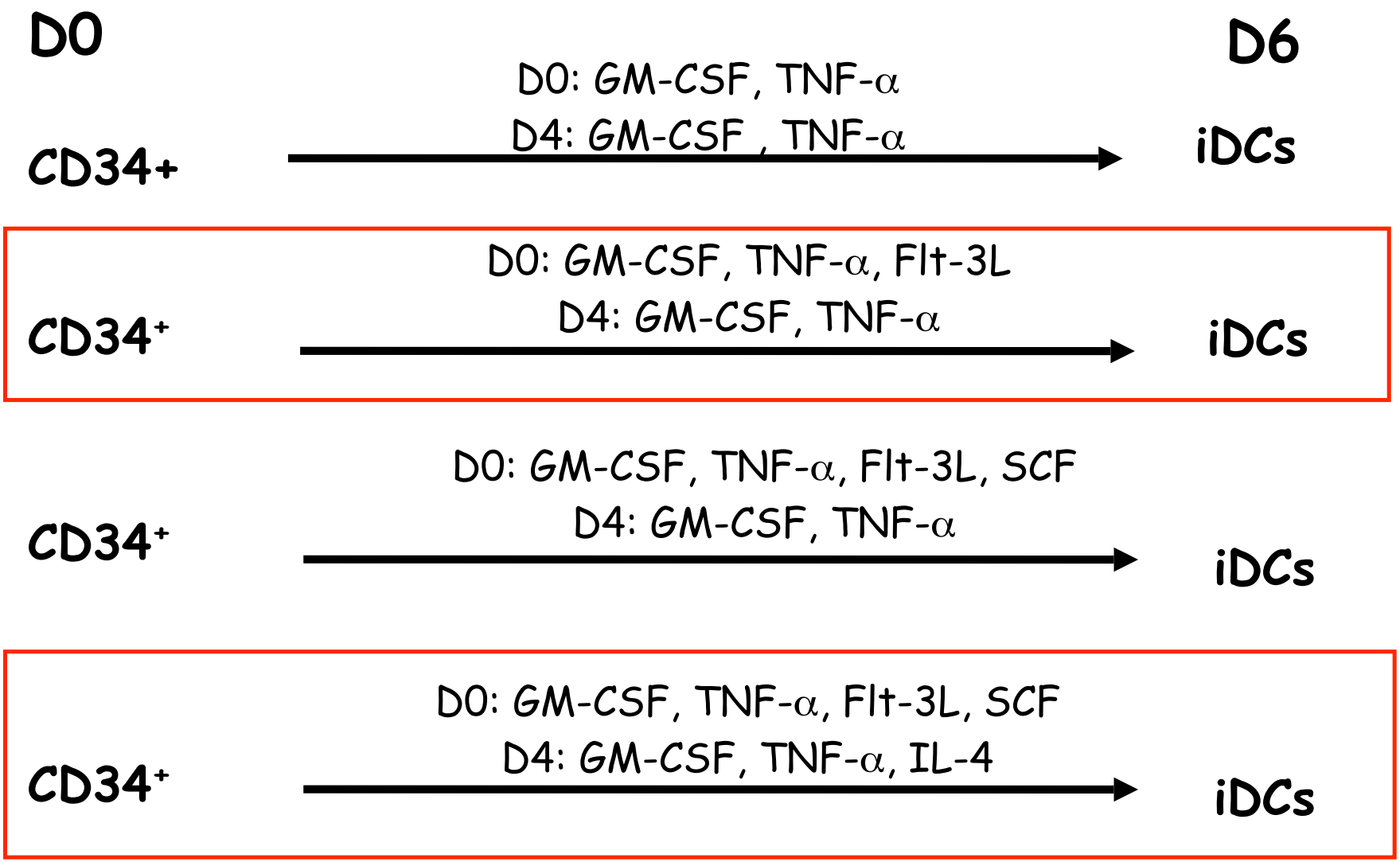
Table 3
Comparison of MUTZ-3 performances with LLNA

Chemical	EC[a] calculated (µg/ml)	LLNA values[b] (%, w/v)	LLNA classification[c]	LLNA references
DNCB	0.3	0.08	Extreme	Kimber et al. (2003)
Hydroquinone	0.6	0.15	Strong	Lea et al. (1999)
HCA	1.3	8	Moderate	Kimber et al. (2003)
Eugenol	16.5	13	Weak	Kimber et al. (2003)
Benzocaine	45.9	NS[d]	NS[d]	Gerberick et al. (2000)

[a] Effective concentrations (µg/ml) calculated from CD86 overexpression in MUTZ-3 (Fig. 2c).
[b] Standard radioactive local lymph node assay values found in literature (EC3 values).
[c] Classification based on the ECETOC members proposal presented in Kimber et al. (2003). To note, this classification differs slightly from the one used in ECETOC (1999).
[d] Non-sensitizer.

Azam P et al, Tox Appl Pharm (2005)

DC generation from CD34+ cells



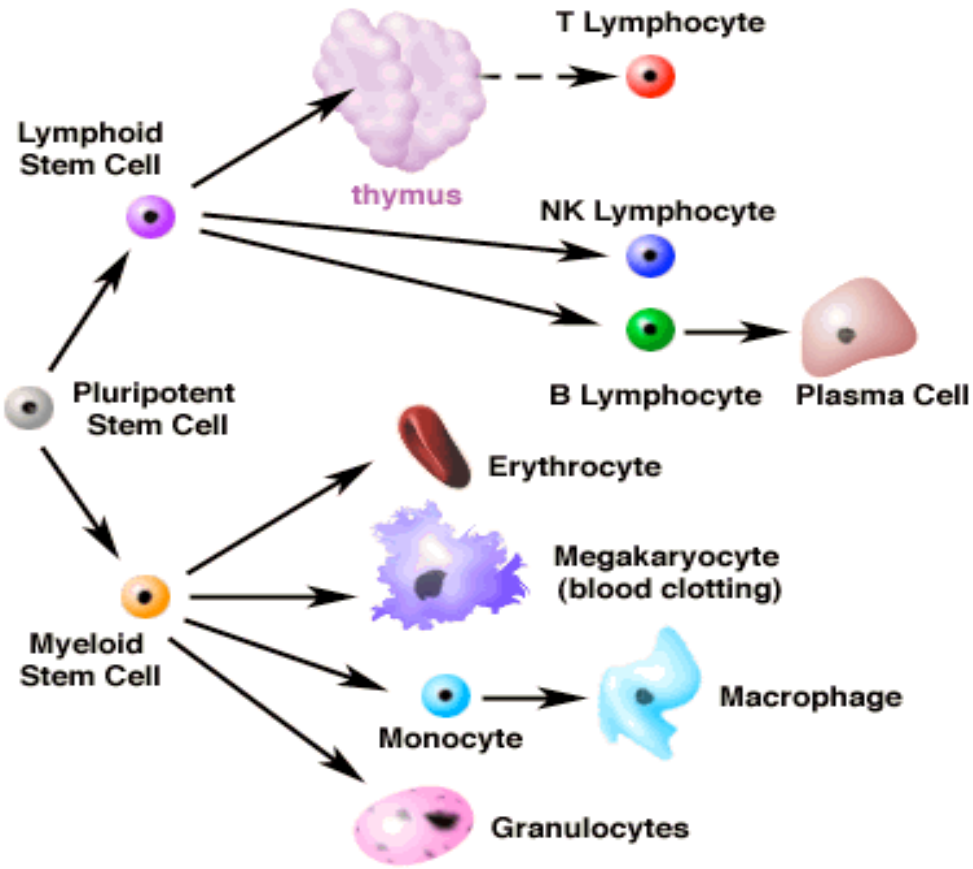

T Lymphocyte
Lymphoid
Stem Cell
thymus
NK Lymphocyte
B Lymphocyte
Plasma Cell
Pluripotent
Stem Cell
Erythrocyte
Megakaryocyte
(blood clotting)
Myeloid
Stem Cell
Monocyte
Macrophage
Granulocytes

Table 1
Viability data

Compounds	Cytotoxicity tests: IC50 (μM)		
	Mouse	Rat	Human
Urethane	>10,000	>10,000	>10,000
Furosemide	>1000	>1000	>1000
Verapamil	>15	>15	>100
Benzo(*a*)pyrene	>200	>200	>50
Cyclosporin A	15.61 (±1.9)	>6	>5
TBTC	0.046 (±0.06)	0.02 (±0.001)	>0.1

This table summarizes cytotoxic test results performed by all laboratories participating on this study.

M. Carfi et al, Toxicology 229, 11-22 (2007)

Regulatory tests for the detection of chemical sensitizers

- Guinea pig tests: mimick the elicitation phase of contact dermatitis
  - Buëhler test
  - Magnusson & Kligman test (+ adjuvant, GPMT)
  - OCDE 406, FDA, US-EPA, EMEA (local tolerance guideline)
- Local Lymph Node Assay: measure the sensitization phase of contact allergy
  - OCDE 429, EMEA (local tolerance guideline), FDA, US-EPA
  - A revolution : a test not based on the evaluation of

ECVAM exploratory study

- RIVM (H. van Loveren, R. Vandenbriel)
- University of Milan (E. Corsini)
- Bayer (HW. Vohr)
- University of Utrecht (R. Pieters)
- University of Paris 11 (M. Pallardy, A. Biola)

- ECVAM (L. Gribaldo, M. Carfi)

Sources of cells

- Mouse and rats
  - Splenocytes
  - CD34+ cells (hematopoietic progenitor)
- Human
  - Peripheral Blood Monocyte Cells after density gradient purification
  - CD34+ cells (hematopoietic progenitor)

- DC were first identified in the epidermis: Langerhans cells (1868)
- Their presence in other tissues was identified in 1973 (Steinman and Cohn)
- Early 1990s: in vitro generation of human DCs from CD34+ progenitor cells
- Mid 1990s: human DC can also be

DC migration after hapten stimulation

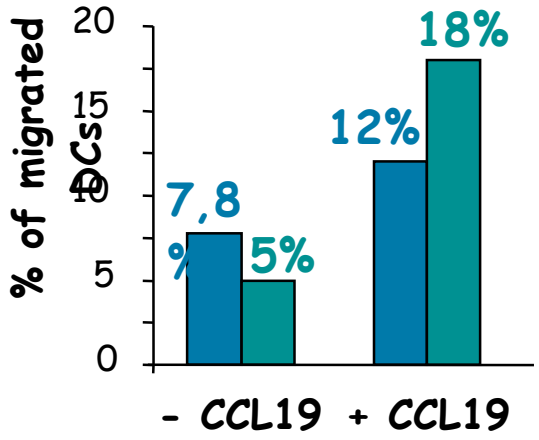
Transwell® system

200 000 cells

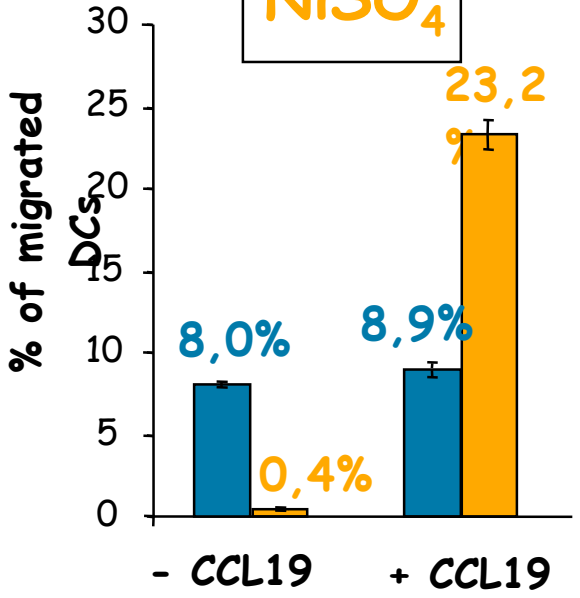
culture medium
+/- CCL19

DNCB

control cells 24h
DNCB 24h
$NiSO_4$ 24h



$NiSO_4$



→ Up-regulation of CCR7 allows the migration of DC after CCL19 addition (Boisleve et al, J Invest Dermatol, 2004)