

“ROLE OF SMALL AGGREGATES ON IMMUNIZATION”

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INSERM UMR 996

13th Open Scientific Symposium on Immunogenicity of Biopharmaceuticals

European Immunogenicity Platform

Mechanisms of Immunogenicity

- **Patients can be immunized to proteins containing foreign epitopes**
 - Therapeutic antibodies (murine, chimerics (human/mouse), humanized antibodies, human antibodies)
- **Patients can be immunized following a break in immune tolerance**
 - The case of therapeutic proteins containing an identical sequence to the endogenous product
 - Need an additional signal or specific conditions of administration: danger signals, inflammation, infections ?
 - Erythropoietin
 - Factor VIII
 - IFN beta...

Influencing Factors

Patient features

- Immune status
- Pathology
- Genetic background

Treatment-related

- Length, Frequency
- Dose
- Route of administration
- Immunosuppressive medication

Immunogenicity

Product-related

- Degree of non-self, Presence of T or B-cell epitopes
- Post-translational modifications (glycosylation...)
- Formulation, production, purification, impurities
- Structural alterations
 - Oxidation, Deamidation and degradation, Conformational changes
- **Aggregation**

Multifactorial etiology of rhEPO-induced PRCA

Patient-related factors



patients

Product-related factors

rhEPO



**Formulation modifications =
potential emergence of immune
danger signals = Possible break in
Immune Tolerance**



T-cell
responds to



aggregated rhEPO

convergence of risk factors

rare case
of PRCA



rEPO case

- Replacement of HSA as a stabilizer by polysorbate 80 and glycine
- EPO marketed outside the USA (Eprex®)
- **Enhanced leaching** of organic compounds from uncoated rubber stoppers in the drug syringes
- Aggregates formation = adjuvant effect = danger signal

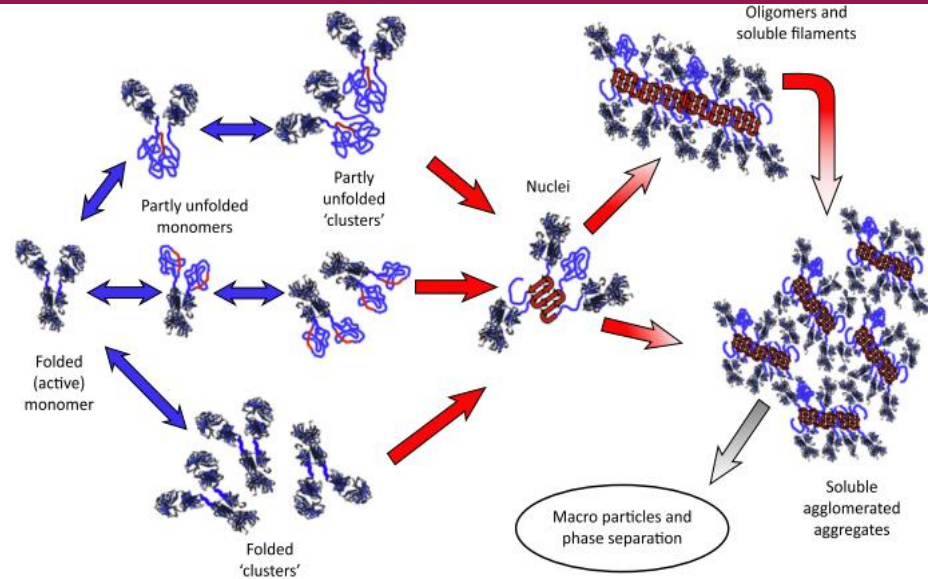
HX575 (EPO biosimilar) case

- **Sodium polytungstate** from needle of prefilled syringes bind rapidly and reversibly to rhEPO
- Strong denaturing effect on the secondary structure
- Induce rapid unfolding of the glycoprotein

Peginesatide (EPO receptor agonist)

- 49 cases of anaphylaxis, including 7 fatalities
- Commercial product = multiuse vial presentation
- Phase 3 clinical studies = single-use vial presentation
- Significantly higher concentration of **subvisible** particles in the multiuse vial

Aggregate formation



Roberts, TRENDS Biotech (2014)

Each type of stress can generate different aggregates: size, shape...

Insoluble aggregates

1 - 100 μm

Subvisible micron aggregates

> 100 μm

Visible aggregates

Soluble aggregates

< 100 nm

Oligomers

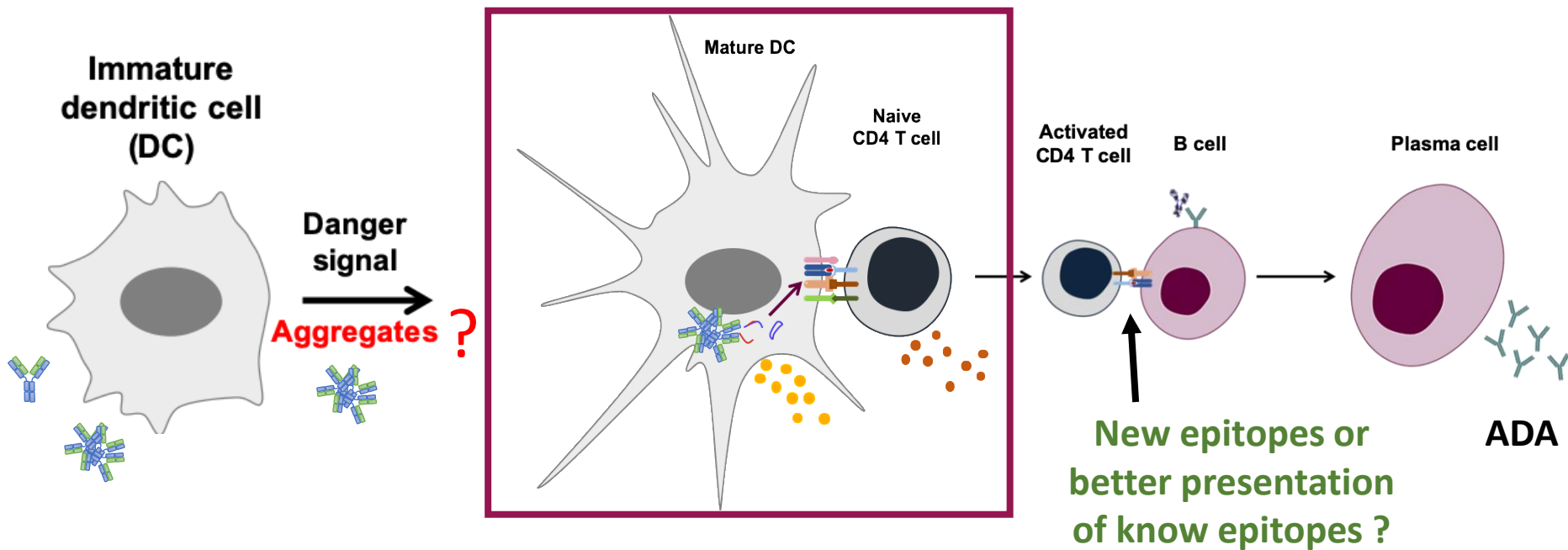
100 - 1000 nm

Submicron aggregates

- Handling & administration: $T^{\circ}\text{C}$ variations, shaking, light stress...
⇒ **ONLY** visual control & filtration

- Production process: *bioreactor, purification, formulation*
⇒ Aggregates well controlled

Role of aggregates in the initiation of a specific adaptive immune response

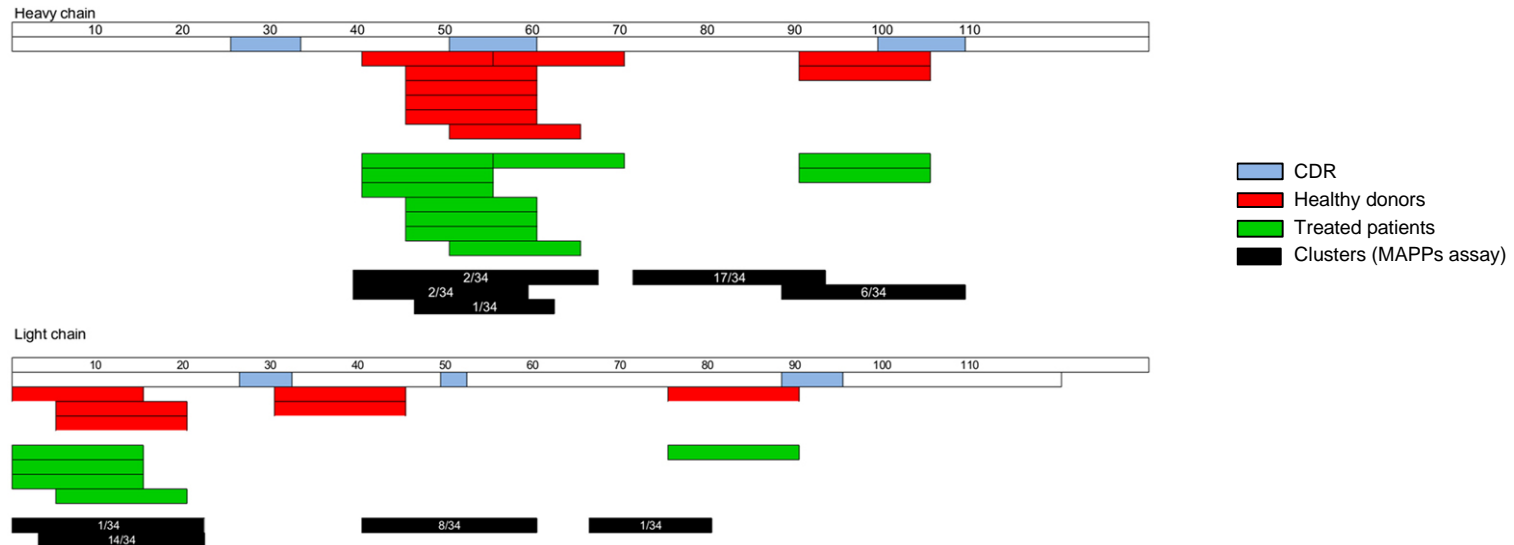


Hypothesis:

- Aggregates = immune danger signal ?
- Aggregates augment presentation of common epitopes to T-cells ?
- New epitopes: Identification of a T-cell repertoire recognizing neo-epitopes generated from aggregates ?

Infliximab: a model to study aggregates immunogenicity

- Chimeric anti-TNF α monoclonal antibody (IgG1)
- Treatment of Crohn's disease & rheumatoid arthritis
- High potential for aggregation
- Highly immunogenic: 17 to 58 % of patients developing ADA (IgG1, IgG4, IgE)
- CD4 T-cell epitopes of infliximab identified in healthy donors



Infliximab aggregates induce moDC maturation (heat stress)



Surface activation
marker - 24h
(FACS)

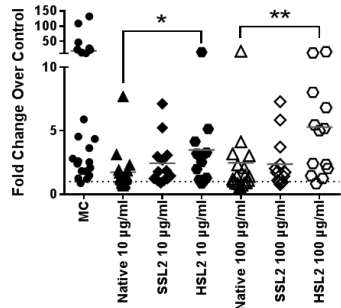
Cytokine
release - 48h
(multiplex assay)

mRNA
expression - 6h
(qPCR)

Cell Signaling
- 15mn
(Luminex)

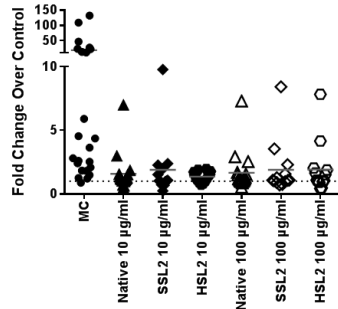
Infliximab

CD83 - % Positive Cells

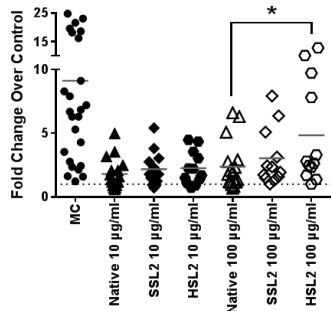


Natalizumab

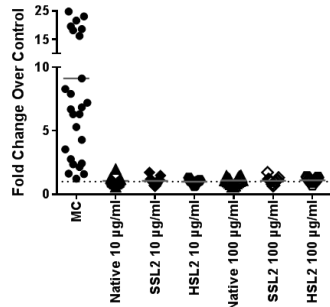
CD83 - % Positive Cells



CD86 - MFI

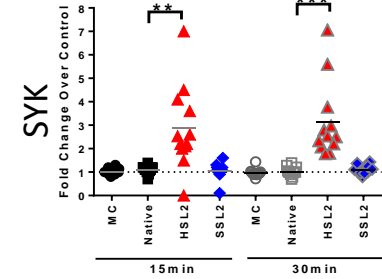


CD86 - MFI

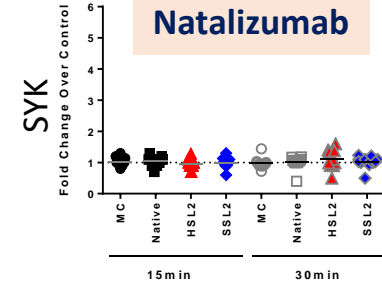


- Cytokine/chemokine signature induced by infliximab aggregates
 - *IL-1 β* , *IL-6*, *IL-8*, *IL-12p40*, *TNF- α* , *CCL2*, *CCL3* and *CCL4*
- No cytokine or chemokine up-regulation observed with natalizumab aggregates

Infliximab

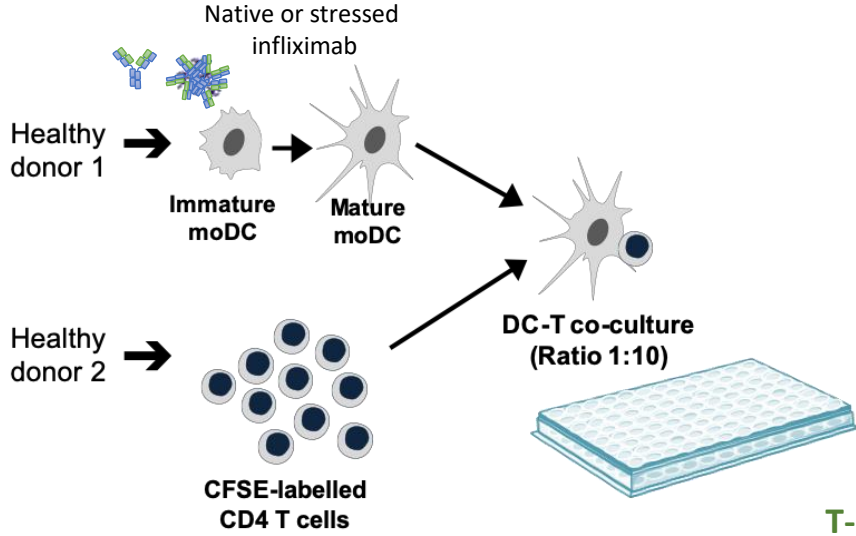


Natalizumab

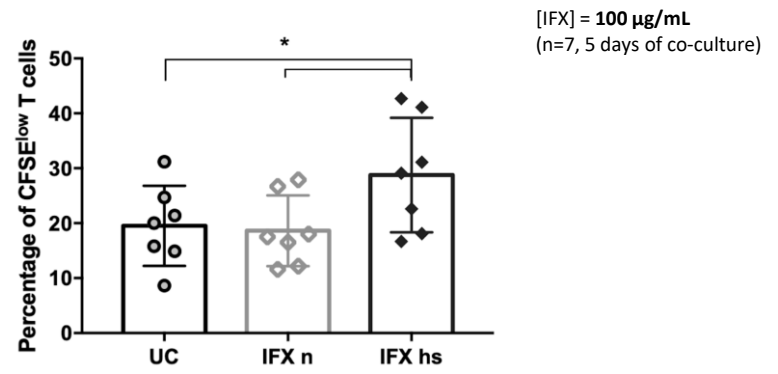


Infliximab aggregates induce Syk, ERK1/2 and Akt protein phosphorylation

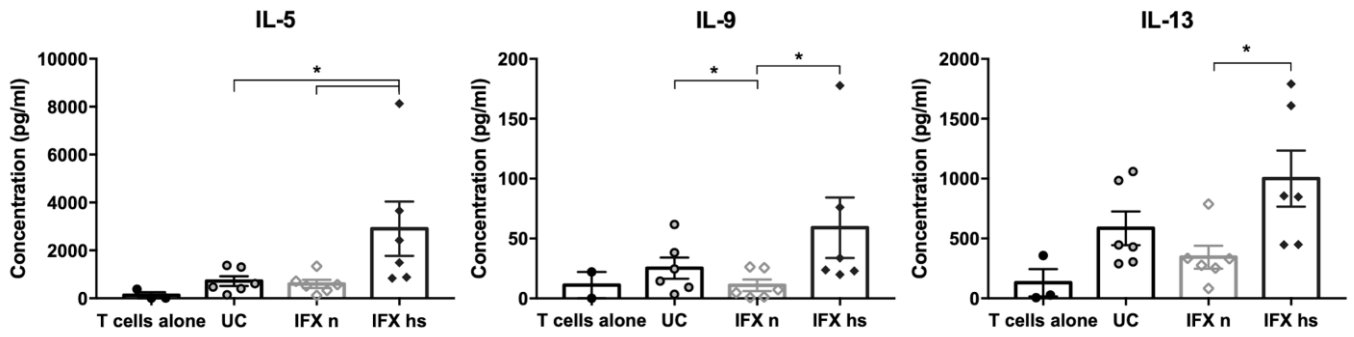
moDC maturation by infliximab aggregates triggers T-cell activation (allogenic co-culture model) / heat stress 55°C, 24hrs, 10 mg/mL solution



T-cell proliferation



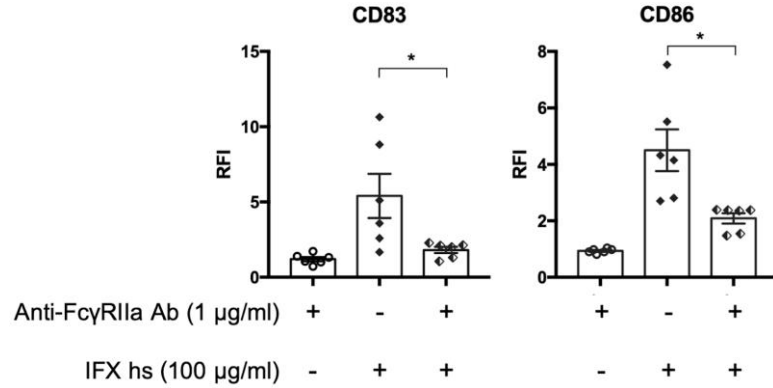
T-cell cytokine production



FcγRIIa implication in moDC response to infliximab aggregates

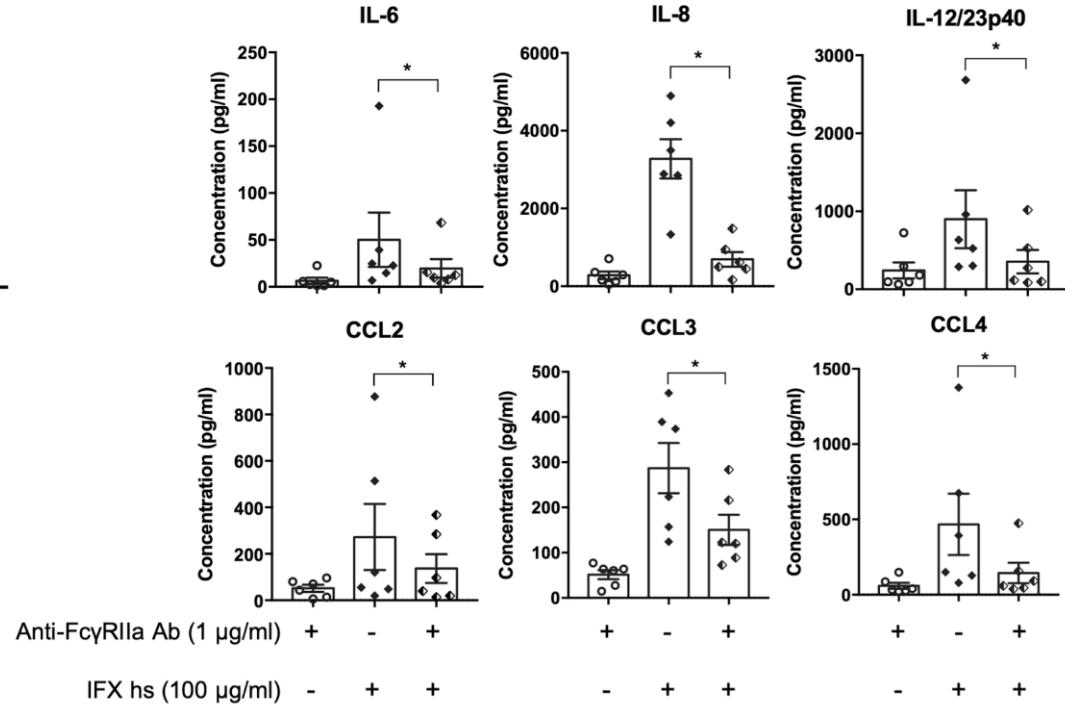
heat stress 55°C, 24hrs, 10 mg/mL solution

Maturation markers expression



[IFX] = 100 μg/ml
(n=6, 24 h of stimulation)

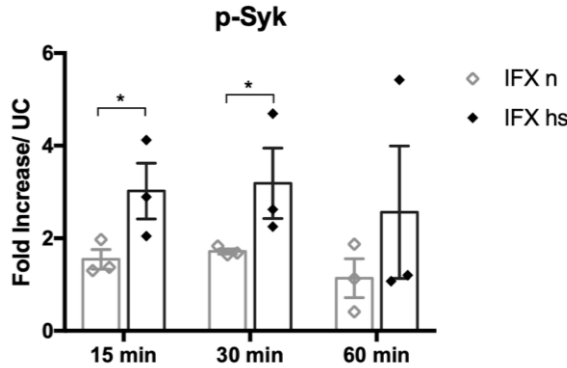
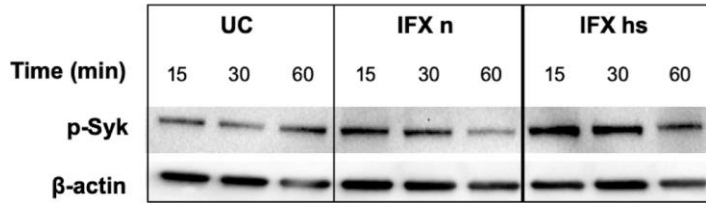
Cytokine & chemokine production



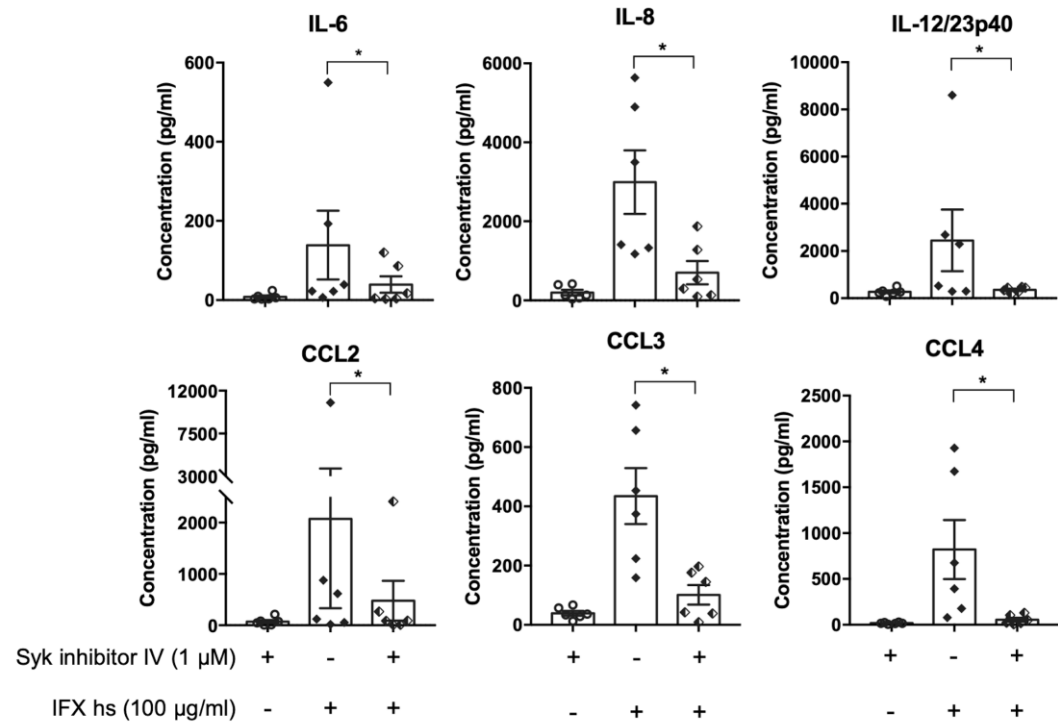
Syk implication in moDC response to infliximab aggregates

heat stress 55°C, 24hrs, 10 mg/mL solution

Syk phosphorylation



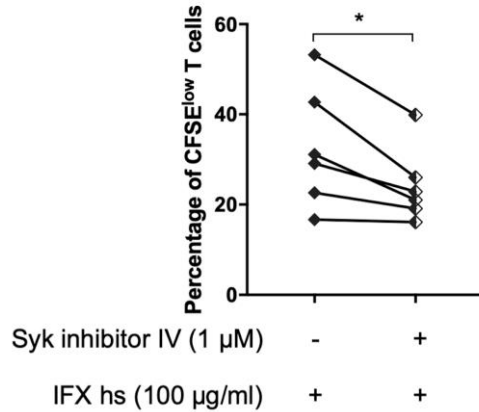
Syk-dependent cytokine & chemokine production



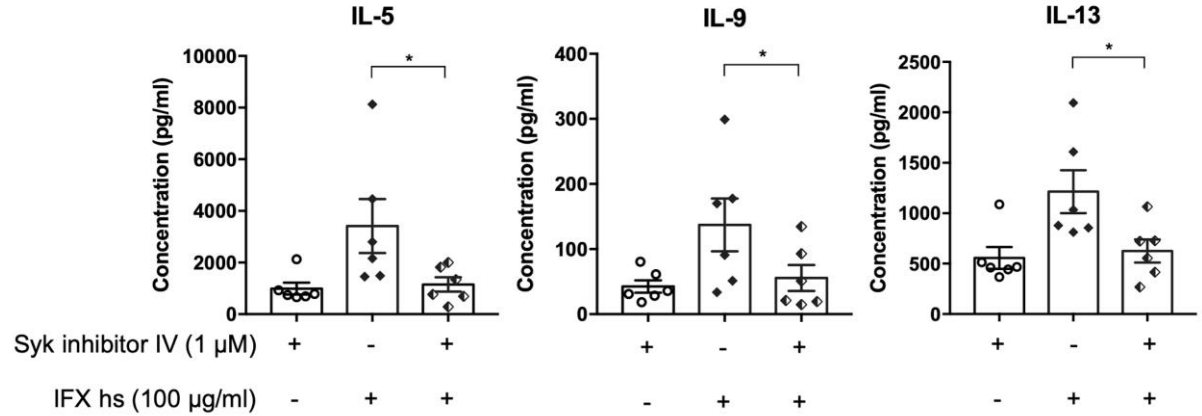
Syk implication in T-cell response to infliximab aggregates

heat stress 55°C, 24hrs, 10 mg/mL solution

T-cell proliferation



T-cell cytokine production

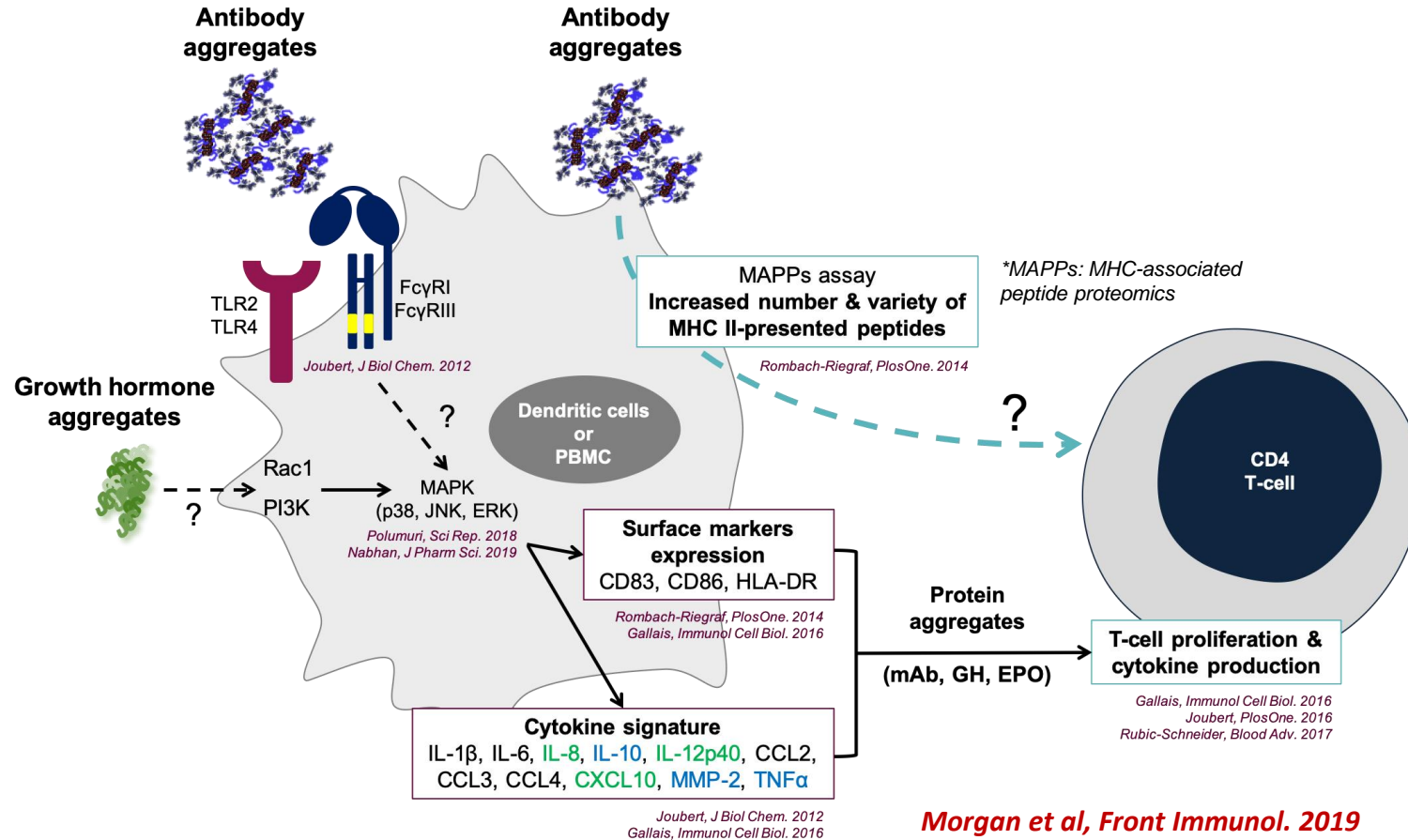


[IFX] = 100 μ g/ml
 (n=6, 5 days of co-culture)

⇒ Syk controls T-cell proliferation & cytokine production induced by aggregates-treated moDC

- High MW aggregates provoke DC phenotype modification with consequences on T-cell proliferation
- FcγRIIa and Syk are key components for aggregates recognition and cell signalisation
- Heat-stress aggregates can play the role of « immune danger signals » when present in the vicinity of DCs

In vitro studies: Aggregates act as danger signal

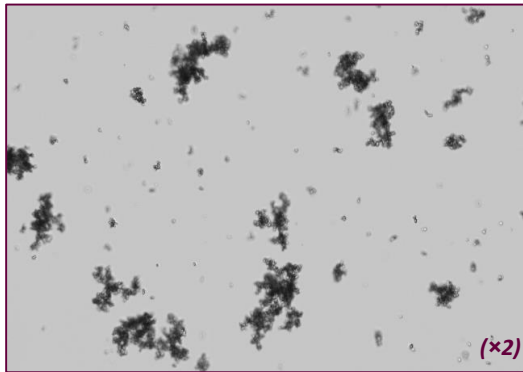


Morgan et al, Front Immunol. 2019

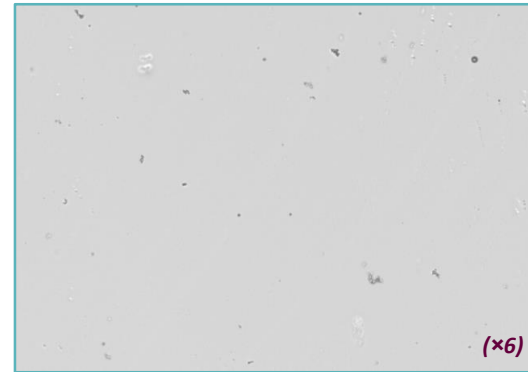
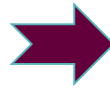
Refining antibody aggregate preparations

24-hour heat stress (55°C)

Flow imaging microscopy images

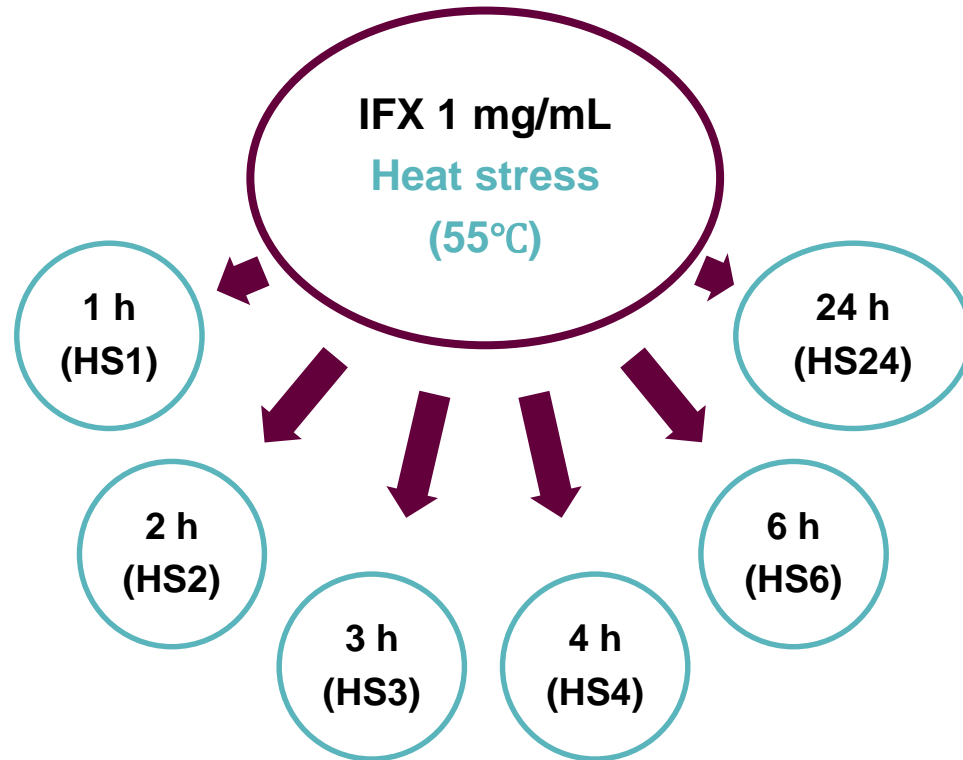


Solution at **10 mg/mL**



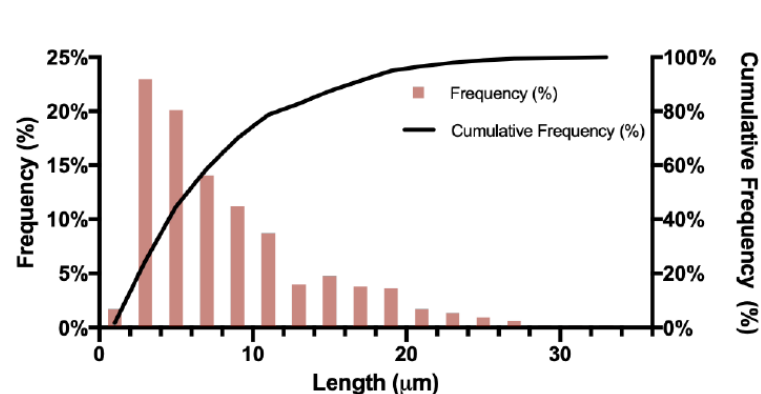
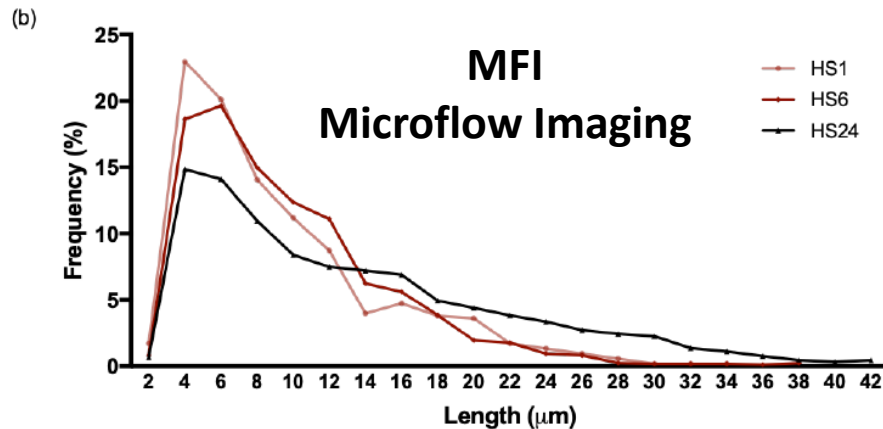
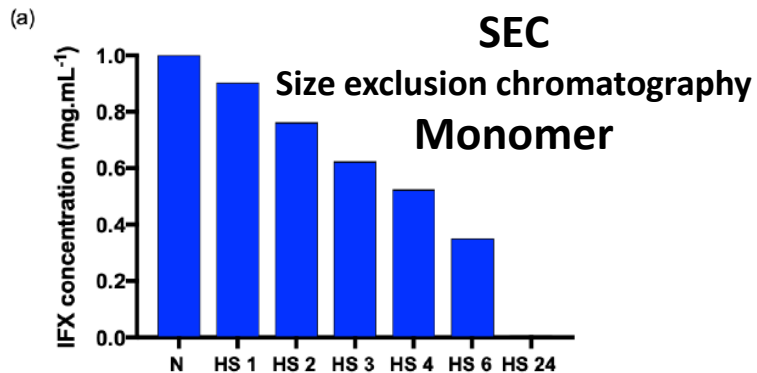
Solution at **1 mg/mL**

Refining antibody aggregate preparations



Refining antibody aggregate preparations

Heat-stress: 55°C, 1h



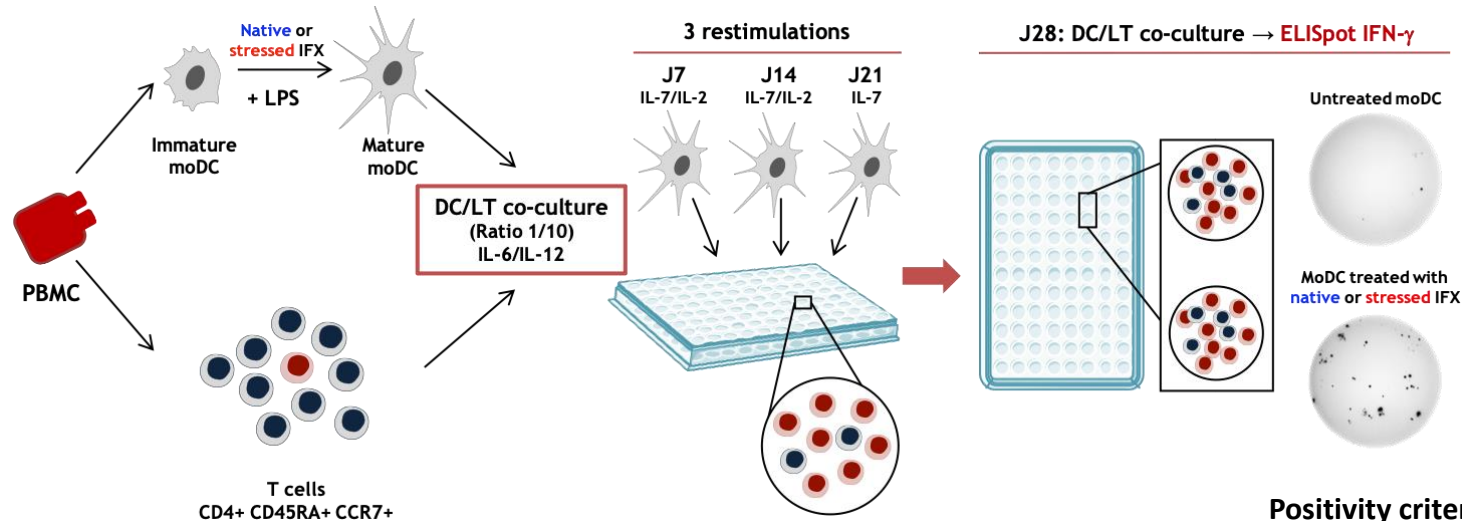
10% aggregates, with 75% between 1 and 10 μm

10% aggregation

- No mo-DC phenotype modifications = No effect
- Percentage of aggregation is playing a major role

Infliximab aggregates: identification of T-cells (*autologous co-culture model*)

Heat-stress: 55°C, 1h



Positivity criteria

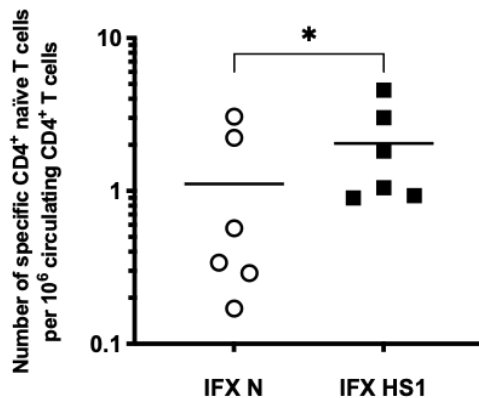
- Index of stimulation ≥ 2
- Spot number ≥ 25

- Native infliximab (IFX n) = 100 $\mu\text{g/ml}$
- Stressed infliximab = 100 $\mu\text{g/ml}$

$$\text{Frequency} = \frac{-\ln(\text{Number of negative T-cell lines} / \text{Total number of T-cell lines tested})}{\text{Number of T-cells per well}}$$

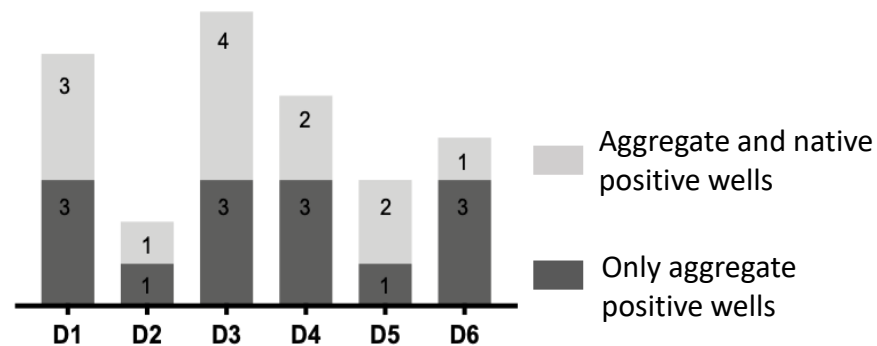
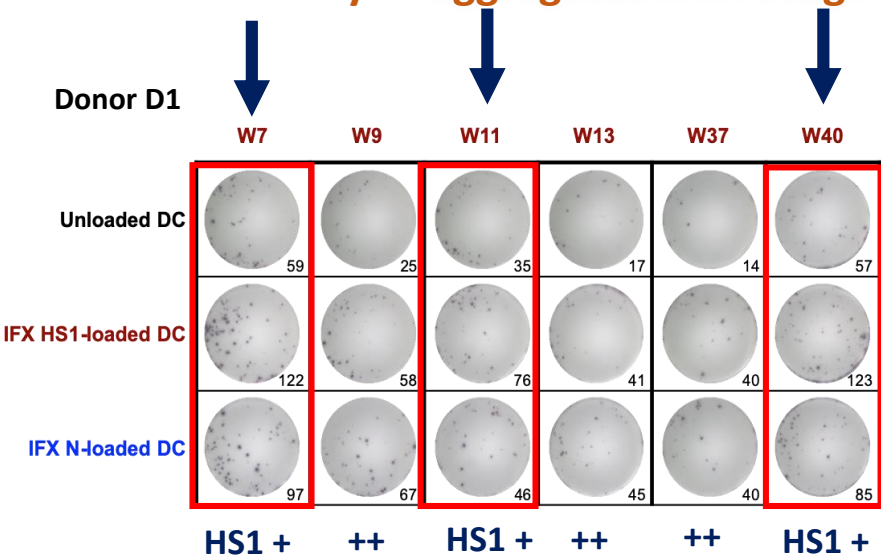
*Poisson distribution

T-cell frequencies



⇒ Higher frequency of naive CD4 T cells recognizing peptides deriving from stressed IFX compared to native IFX (n=6)

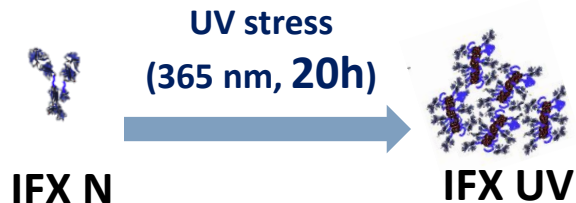
Cross-reactivity of aggregated IFX-recognizing T cells with native IFX



⇒ Some aggregated IFX-specific T cells can recognize ONLY aggregated-derived peptides

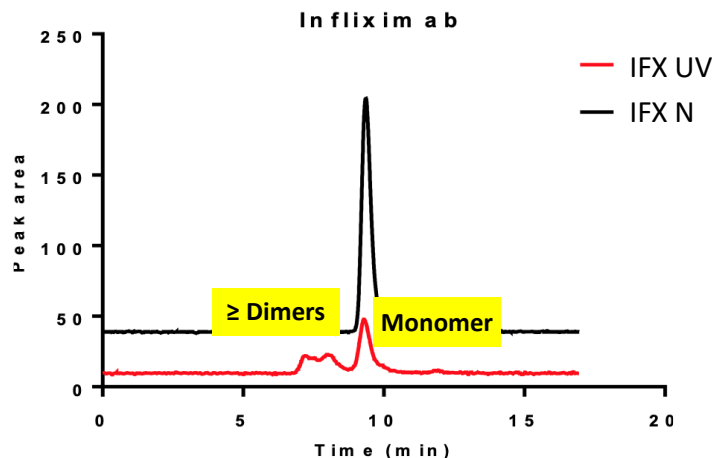
- **A percentage-dependent effect of HMW aggregates on DC phenotype was found**
- **In the absence of « immune danger signals » effects, a T-cell response is still found**
- **Some T-cells are responding only to peptides generated from infliximab aggregates by DCs**

UV-light stress-induced aggregates



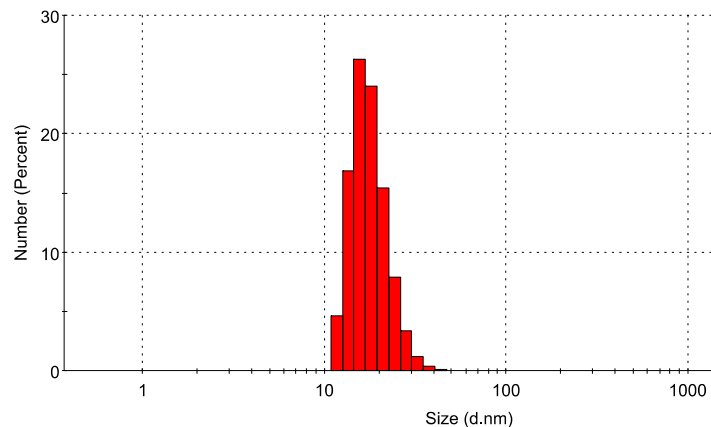
⇒ 20 hours of UV stress induce the formation of dimers & soluble oligomers

Size exclusion chromatography



- Soluble dimers and oligomers <100 nm
- Mixture monomer/agregates

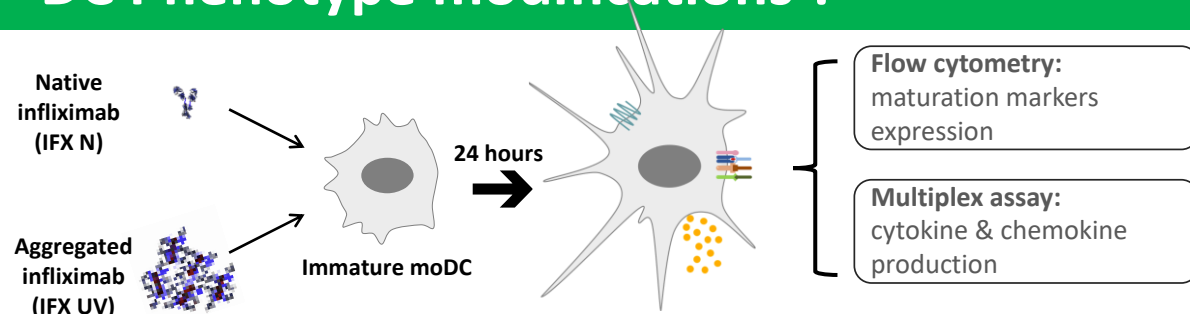
Dynamic light scattering



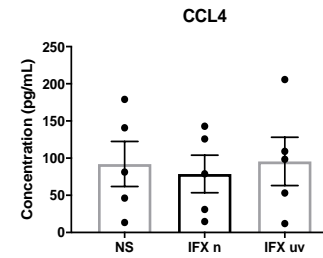
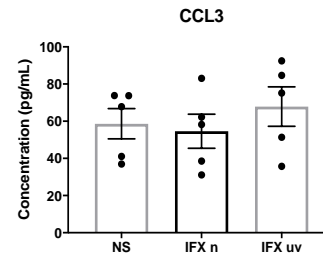
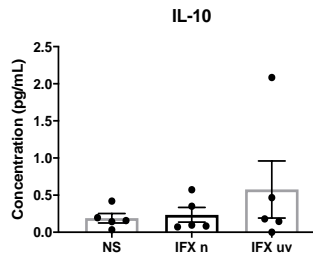
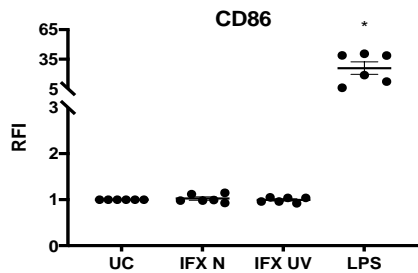
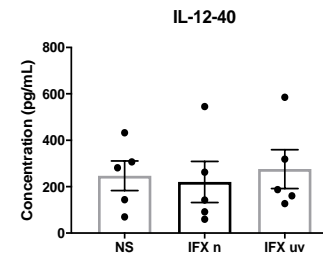
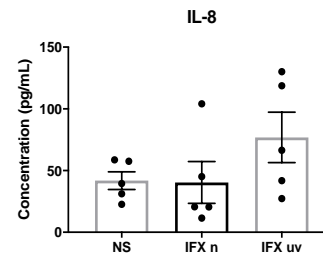
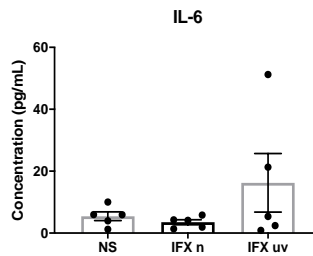
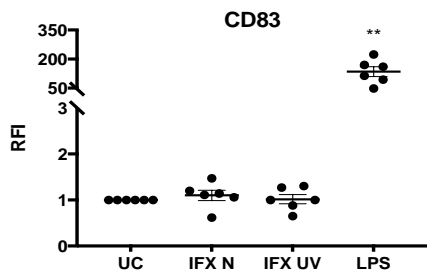
- Absence of aggregates >100nm

DC Phenotype modifications ?

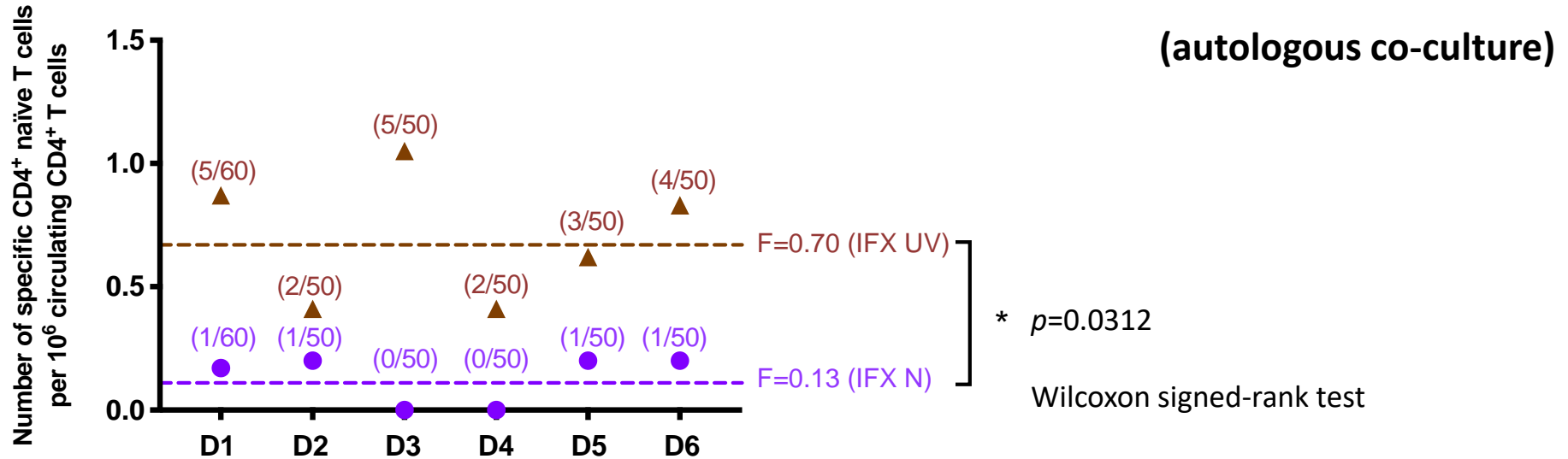
- MoDC stimulation & readouts



⇒ No increase of CD83 and CD86 expression nor increase of inflammatory cytokine and chemokine secretion



Identification of naïve CD4 T-cells recognizing native or UV-aggregated IFX



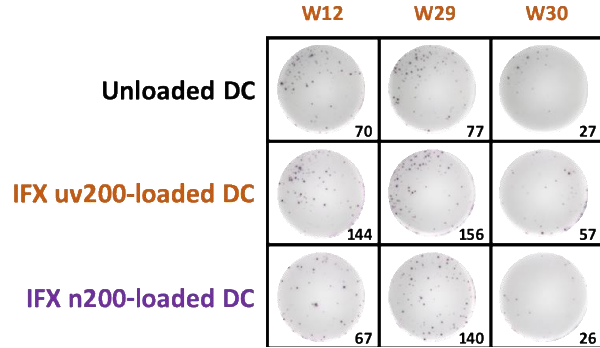
⇒ Identification of a higher number of specific T cells in response to IFX UV for each donor

⇒ Mean frequencies (6 donors):

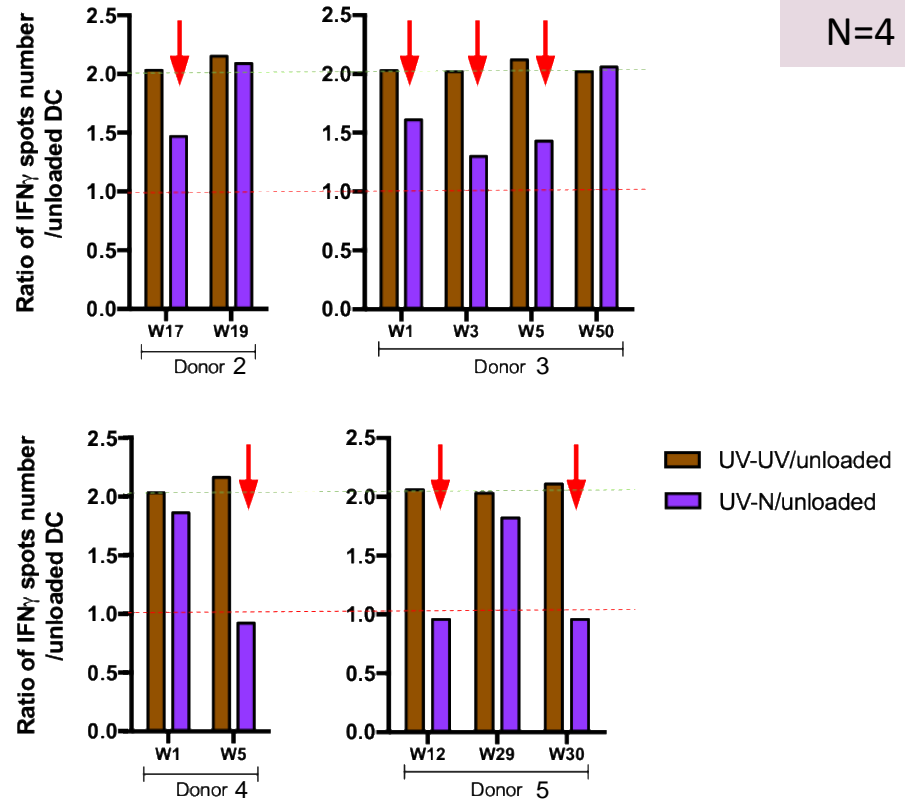
- 200 $\mu\text{g/ml}$: $F(\text{IFX N}) = 0.13$ vs $F(\text{IFX UV}) = 0.70$

Donor 5

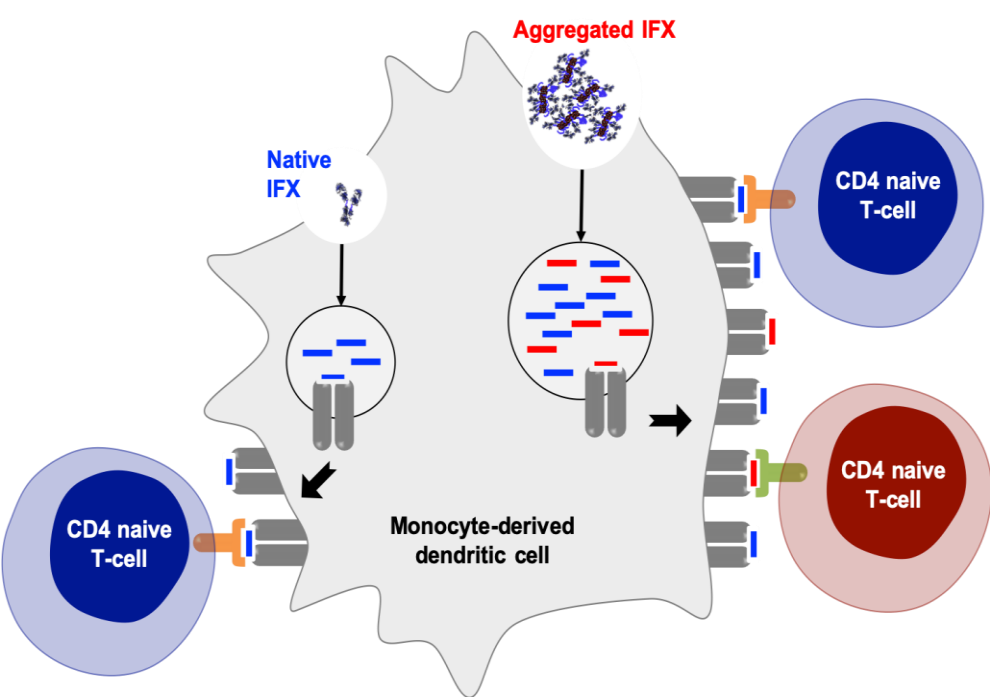
ELISpot 2: UV IFX-primed naive CD4 T cells (3/50)



IFX=200 µg/ml



- ⇒ Some aggregated IFX-specific T cells can recognize ONLY aggregated-derived peptides
- ⇒ Suggesting the formation of neo-epitopes through aggregation and/or a better presentation of native IFX-generated peptides

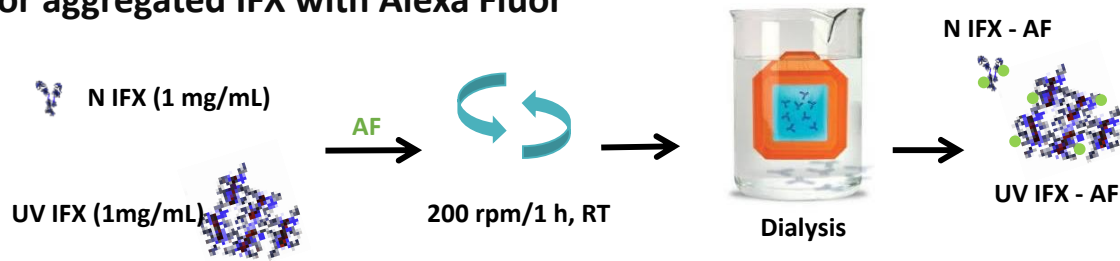


Increased number of presented peptides derived from the native antibody?

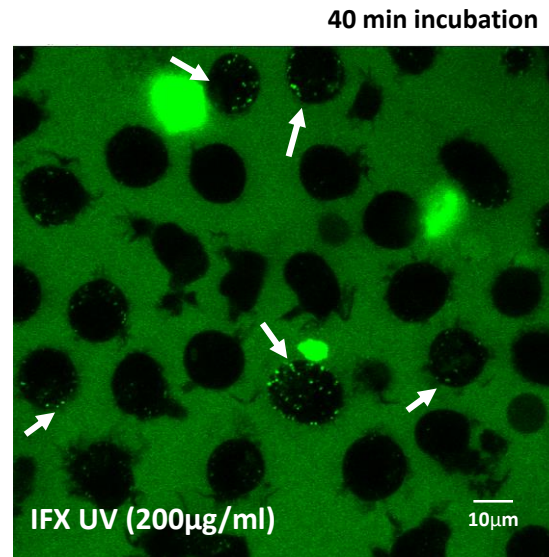
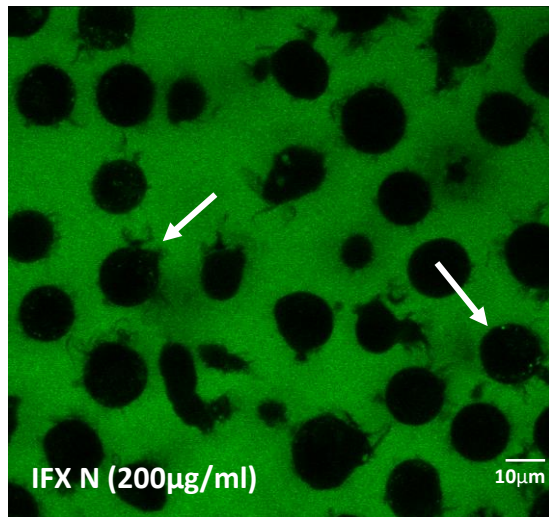
Neo peptides generated by the aggregation process?

Interaction between UV aggregates and DCs

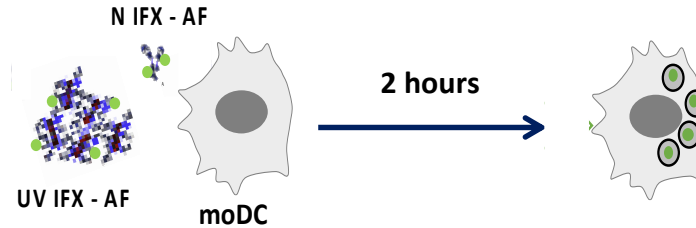
1- Labeling of native or aggregated IFX with Alexa Fluor



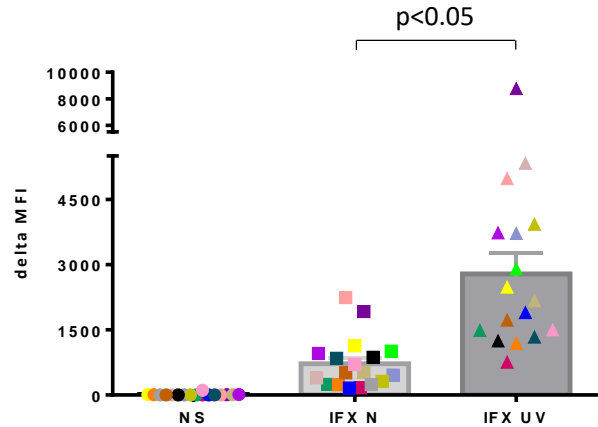
2- Antigen uptake assay



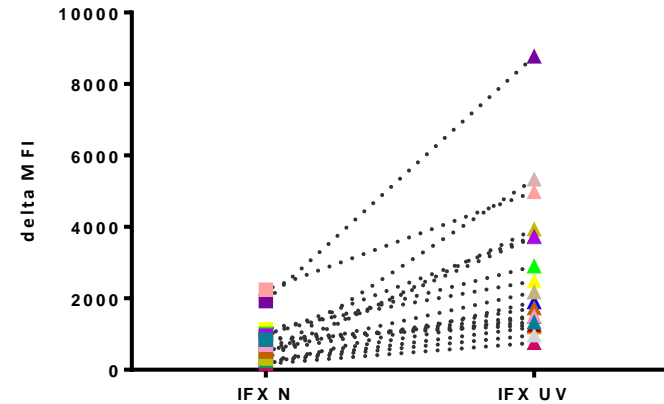
Internalization of native and UV-treated Infliximab



Internalisation \rightarrow Δ of mean fluorescent intensity (MFI) = MFI at 37°C - MFI at 4°C



n=18



Friedman test followed by Dunn's multiple comparison

Conclusions

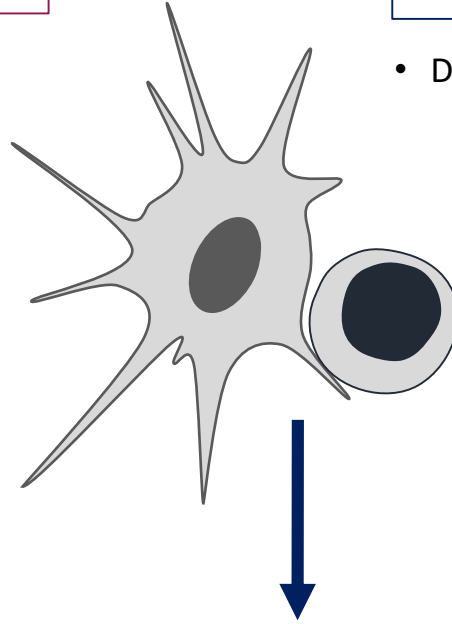
Therapeutic antibody aggregates

Adjuvant/Danger signal

T-cell triggering

- Dendritic cell maturation
- Cytokine and chemokine production
- Implication of FcγRIIa and Syk
- Highly dependent on size and aggregate proportion

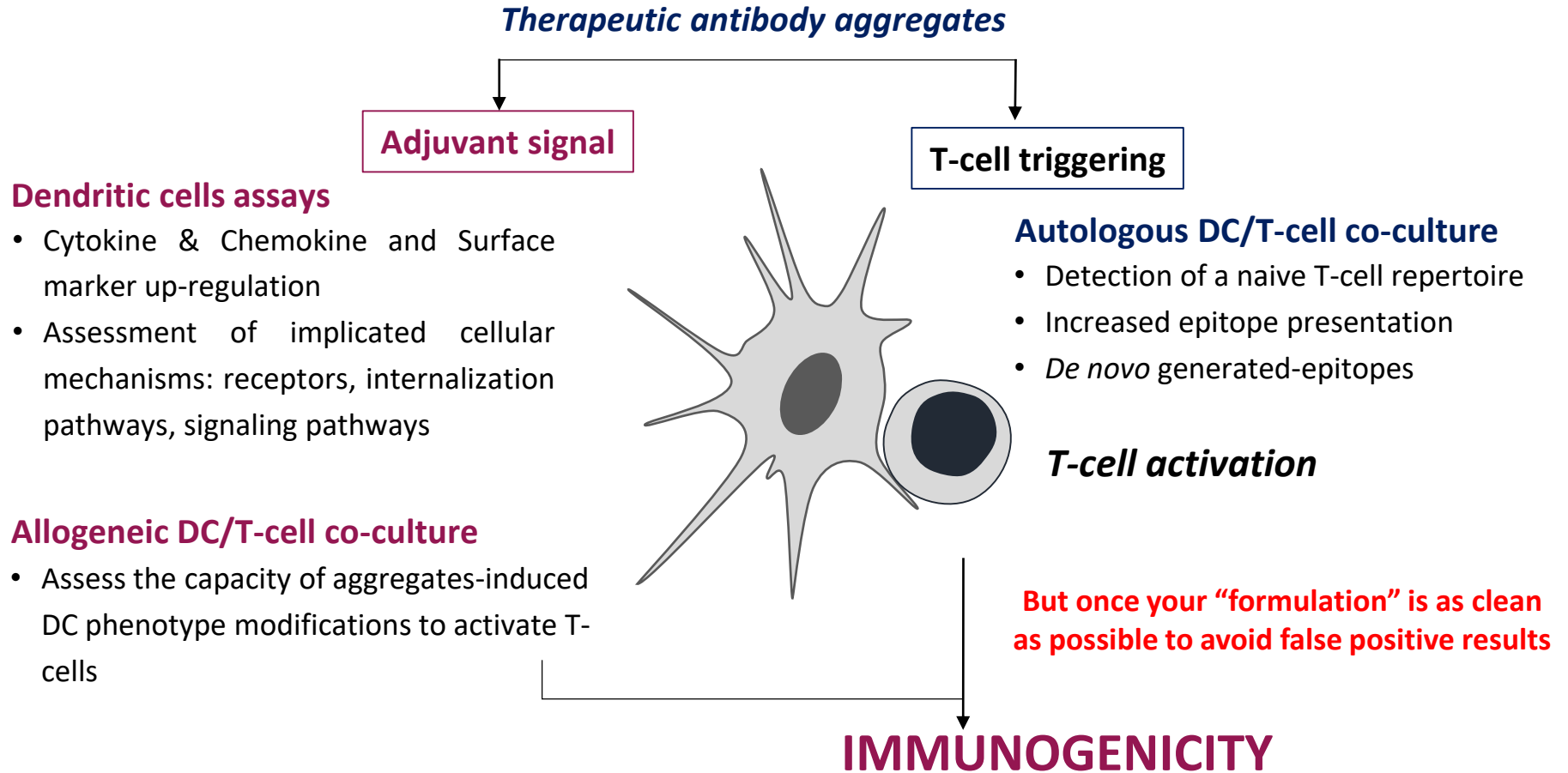
- Detection of T-cells recognizing « aggregates »
 - Increased epitope presentation ?
 - *De novo* generated-epitopes ?



Specific T-cell activation

IMMUNOGENICITY

Conclusion - Models



Acknowledgments



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Baptiste Robin

François-Xavier Legrand

Nicolas Huang



CEA

Bernard Maillère



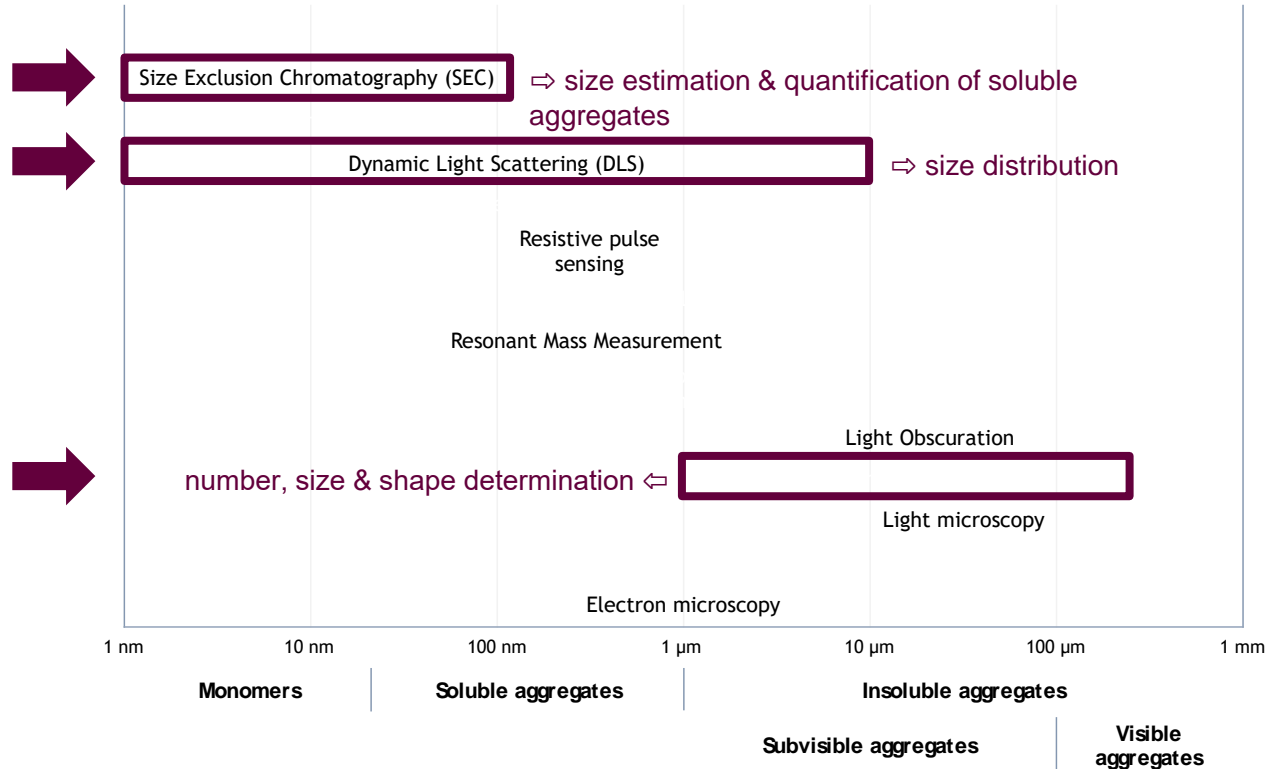
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PARIS-SACLAY**

**FACULTÉ DE
PHARMACIE**

Back-Up slides

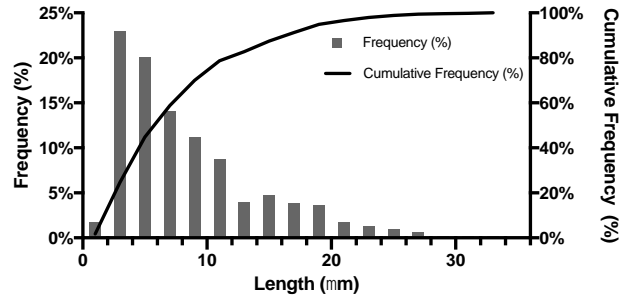
Protein aggregation

Aggregates characterization



Refining antibody aggregates preparations

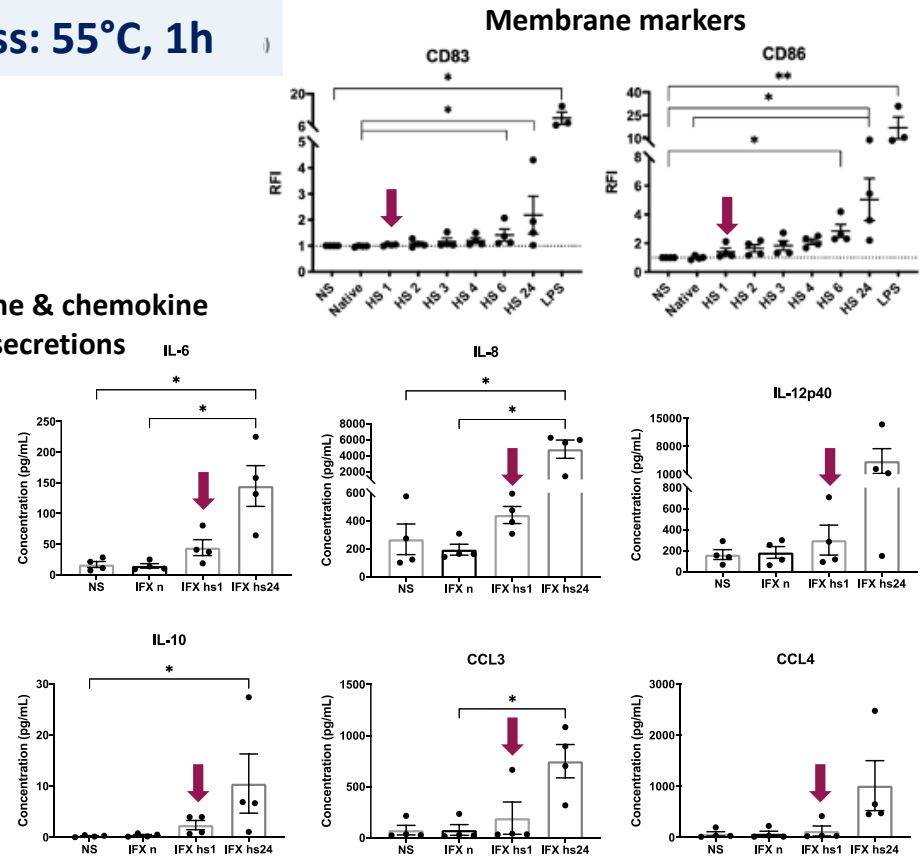
Heat-stress: 55°C, 1h

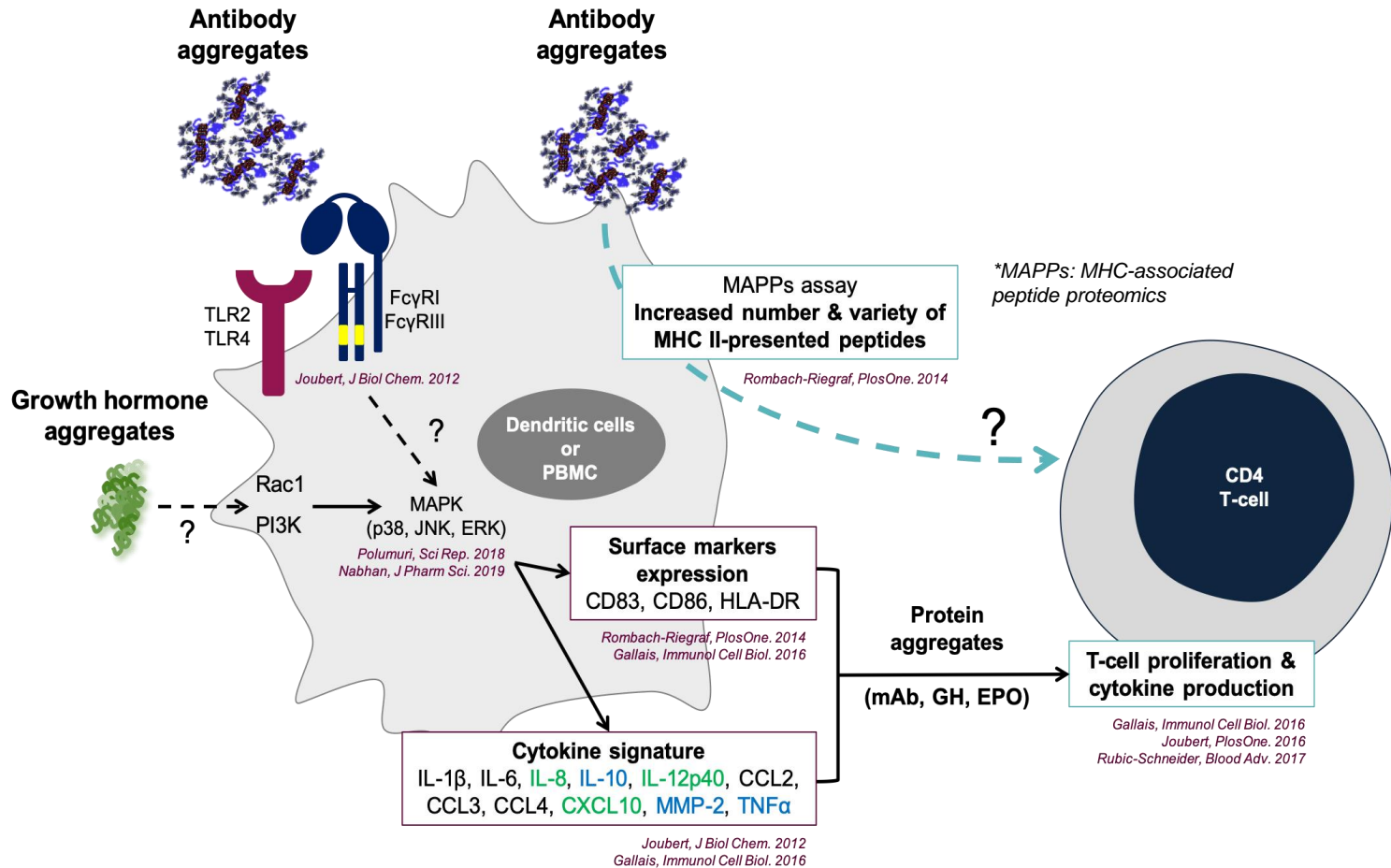


10% aggregates, with 75% between 1 and 10 μ m

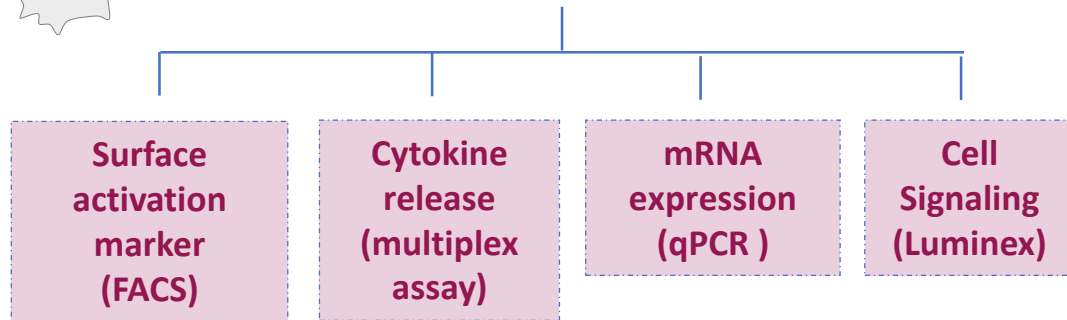
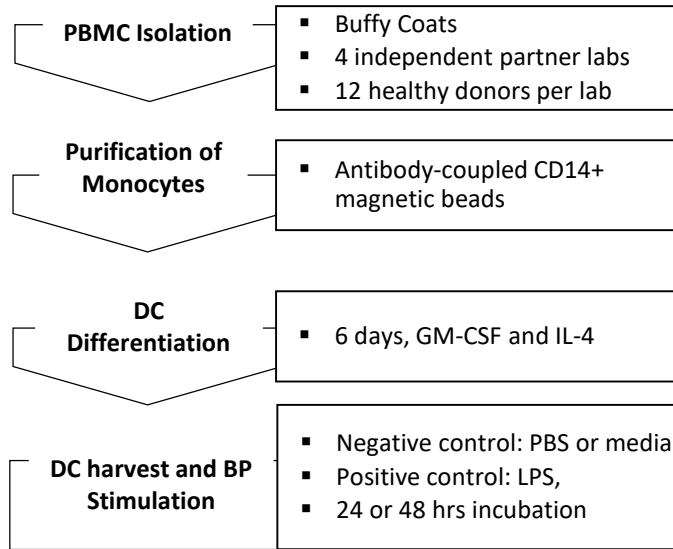
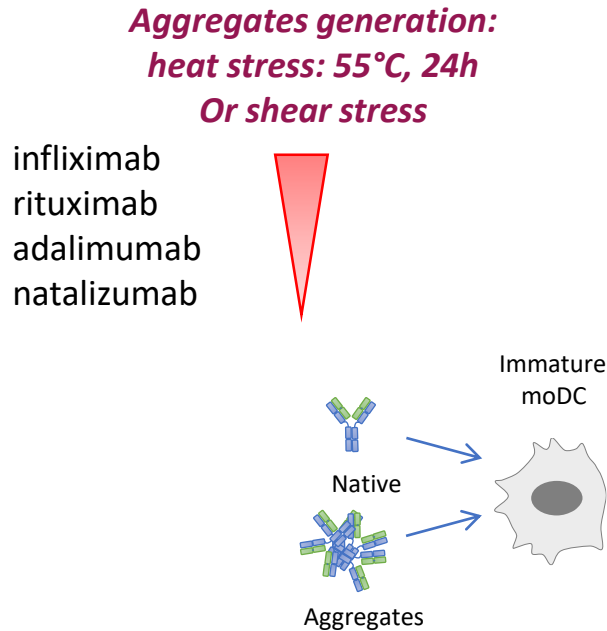
Don't induce moDC phenotypic maturation

Cytokine & chemokine secretions



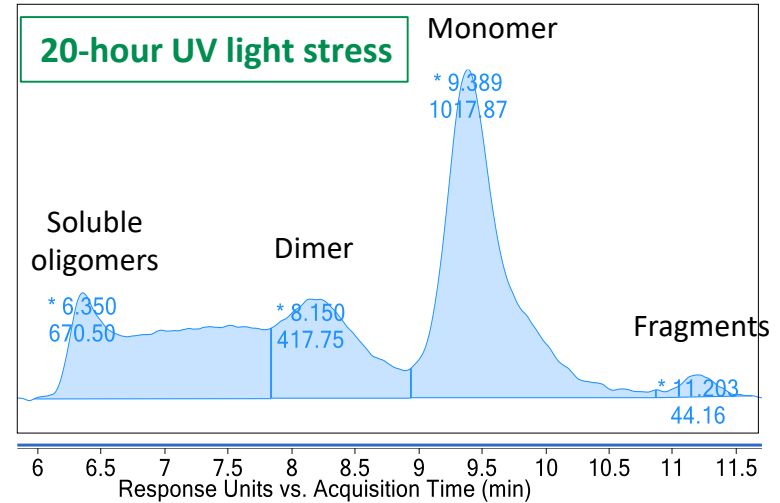
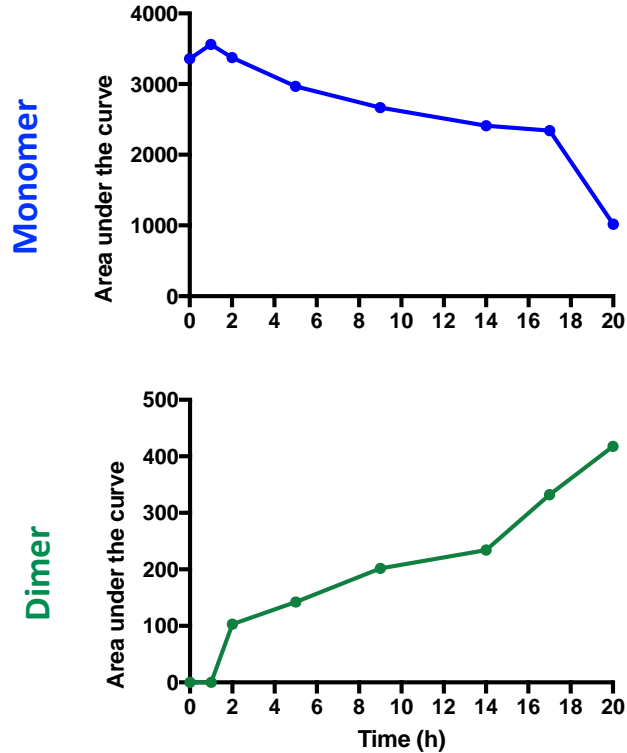
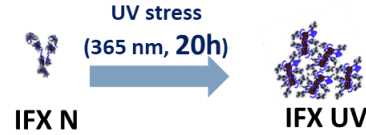


Human in vitro dendritic cell (DC) assay



UV-light stress-induced aggregates

Size exclusion chromatography



- ⇒ Time-dependent decrease of monomer concentration & increase of dimer formation
- ⇒ 20 hours of UV stress induce the formation of dimers & soluble oligomers