Aggregation of therapeutic antibodies enhances dendritic cell uptake and T-cell responses

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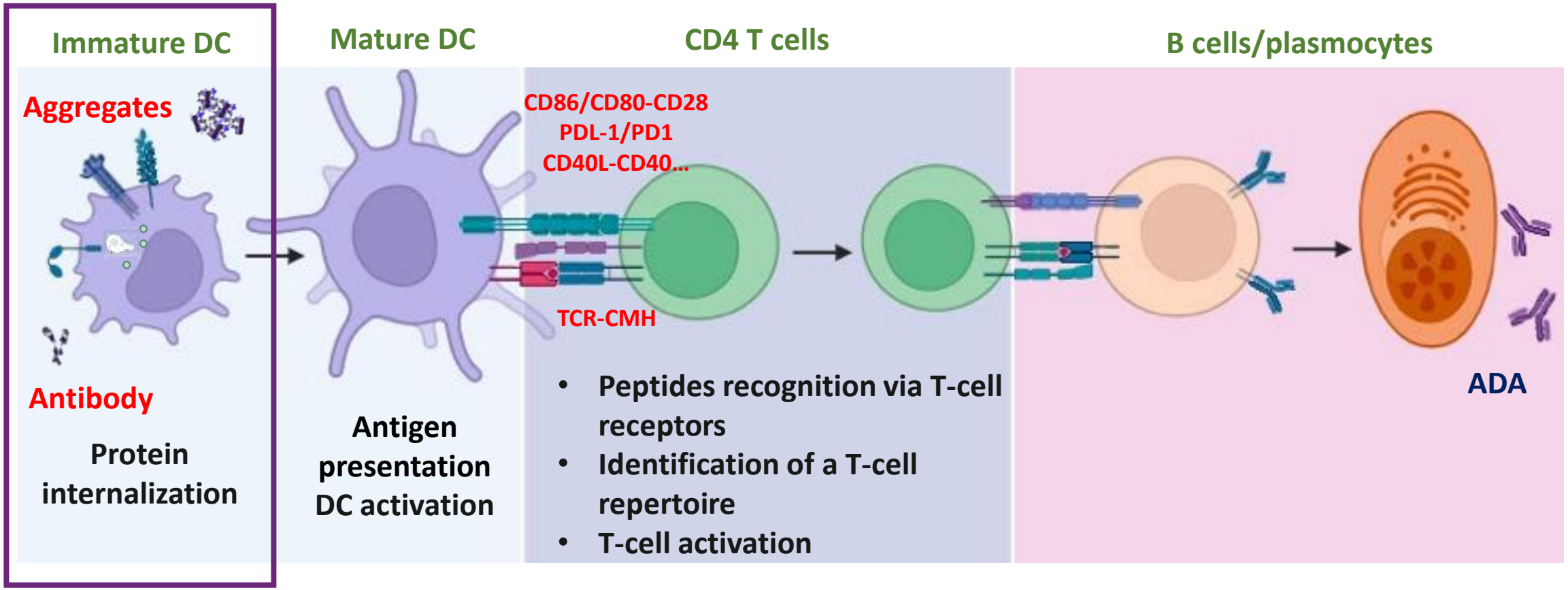
INSERM UMR 996

Inflammation, Microbiome, Immunosurveillance

Immunogenicity = recognition by the immune system of defined structures = needs mobilization of the adaptive immune response = T-cells



Internalization of proteins in DCs is a key step for the initiation of a T-cell response = dictates the quantity and the quality of presented peptides

Aggregates in biological products

Production process: *bioreactor, purification, formulation*
⇨ Aggregates elimination well controlled

Injected volume	≤ 100 mL (particles/container)		> 100 mL (particles/mL)	
Particle size	≥ 10 µm	≥ 25 µm	≥ 10 µm	≥ 25 µm
Light obscuration	6000	600	25	3
Microscopy	3000	300	12	2

European (Ph. Eur. 2.9.19)
US (USP <788>) Pharmacopea

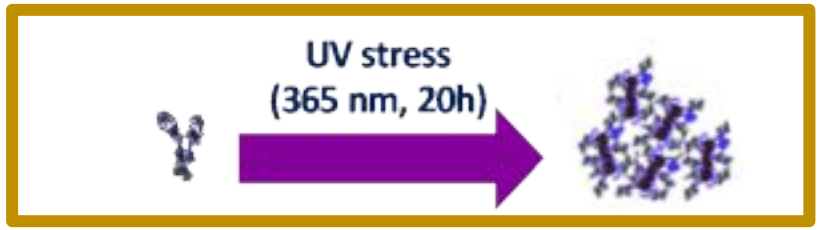
Handling & administration: T°C variations, shaking, light stress…
⇨ visual control & filtration

"Rapid aggregation after mixing Avastin® (bevacizumab) or Herceptin® (trastuzumab) with 5% dextrose and human plasma under *in vitro* conditions that simulate the interface of IV infusion" **Arvinte et al. (2013)**
"Nanometer, submicron, and micron protein particles have been evidenced in intravenous saline bags that could inadvertently be delivered to patients" **Pardeshi et al. (2020); Kannan et al. (2020)**

Antibodies to evaluate different routes of entry into DC

Antibody	Isotype	Characteristics	Target expression on DC	Possible route of entry	ADA incidence
Infliximab (anti-TNF)	IgG1, chimeric	Glycosylated, high mannose (5.5%); galactosyl (40%); low level of sialic acid (<1.3%)	TNF (+/-)	• FcγR • CLR (mannose receptors) • Target mediated endocytosis	17-58% (IgG1, IgG4, IgE) T-epitopes identified in healthy donors
Atezolizumab (anti-PDL-1)	IgG1, humanized	N297A mutation→ non glycosylated to abrogate Fc function Favorable for aggregation ?	PD-L1 (+)	• Membrane interaction • Target mediated endocytosis	13-36% Cancer patients

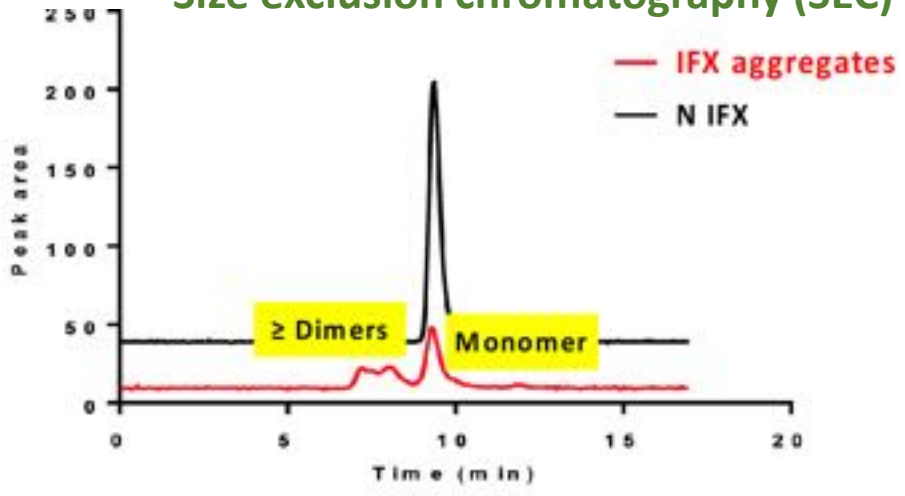
Nanosized aggregates characterization



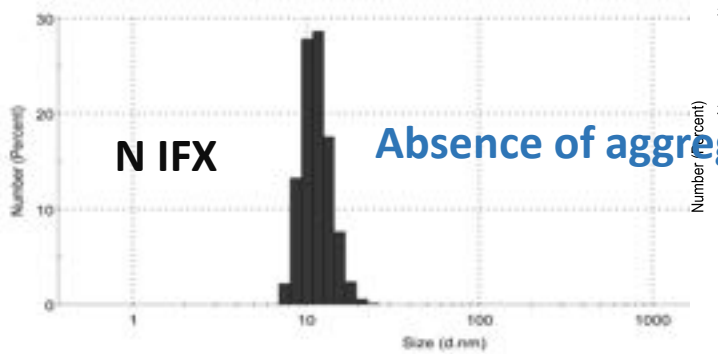
Native mAbs mAbs aggregates

Oxidation stress is a common chemical degradation process of mAbs

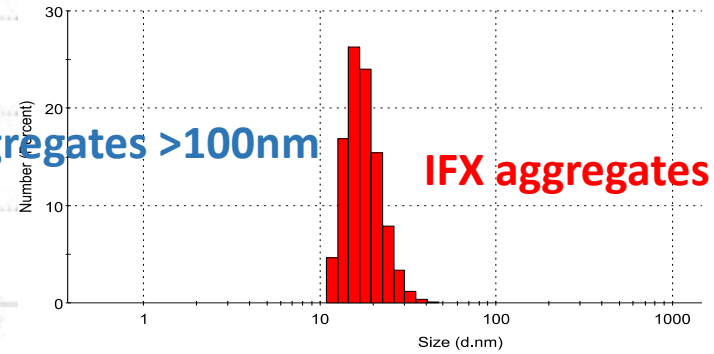
Size exclusion chromatography (SEC)*



Dynamic light scattering (DLS)

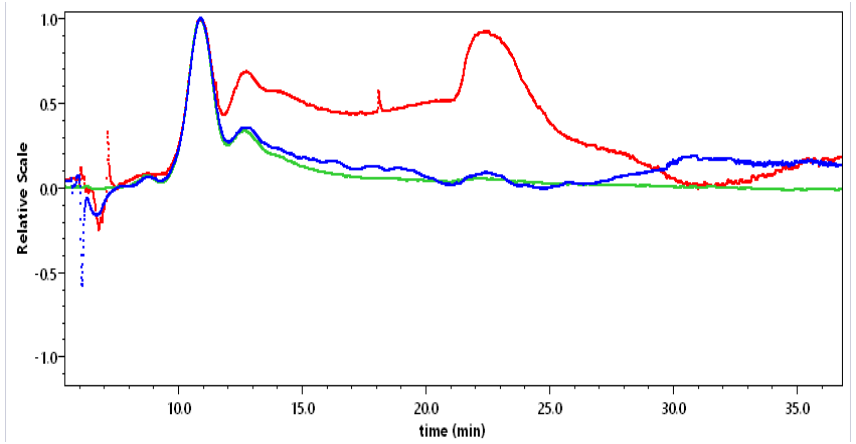


Absence of aggregates >100nm



IFX aggregates

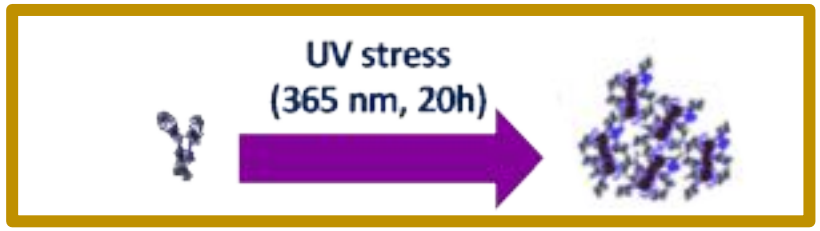
Field-flow fractionation - multi angle light scattering (MALS)



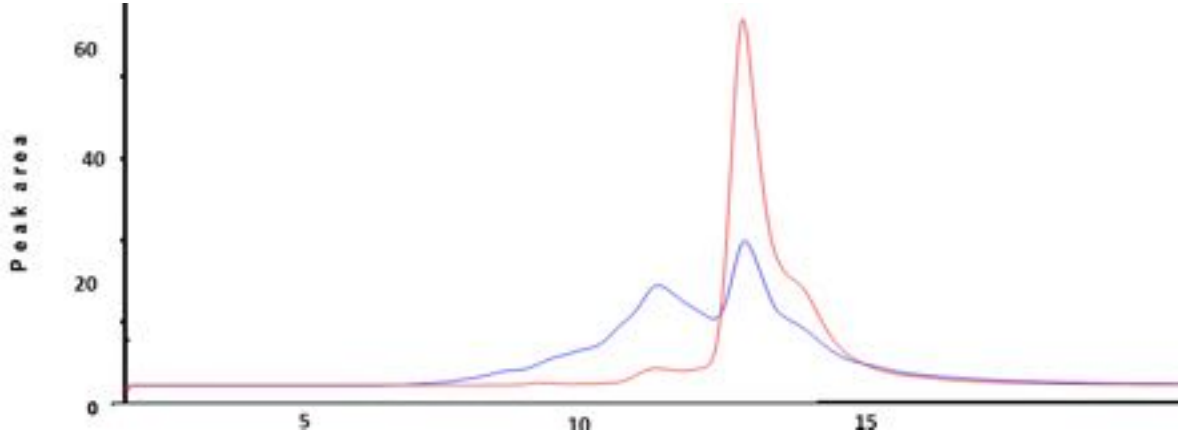
	Percentage
Monomer	52.25
Dimers	19.27
Trimers	26.41
Fragment	2.07

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

Atezolizumab



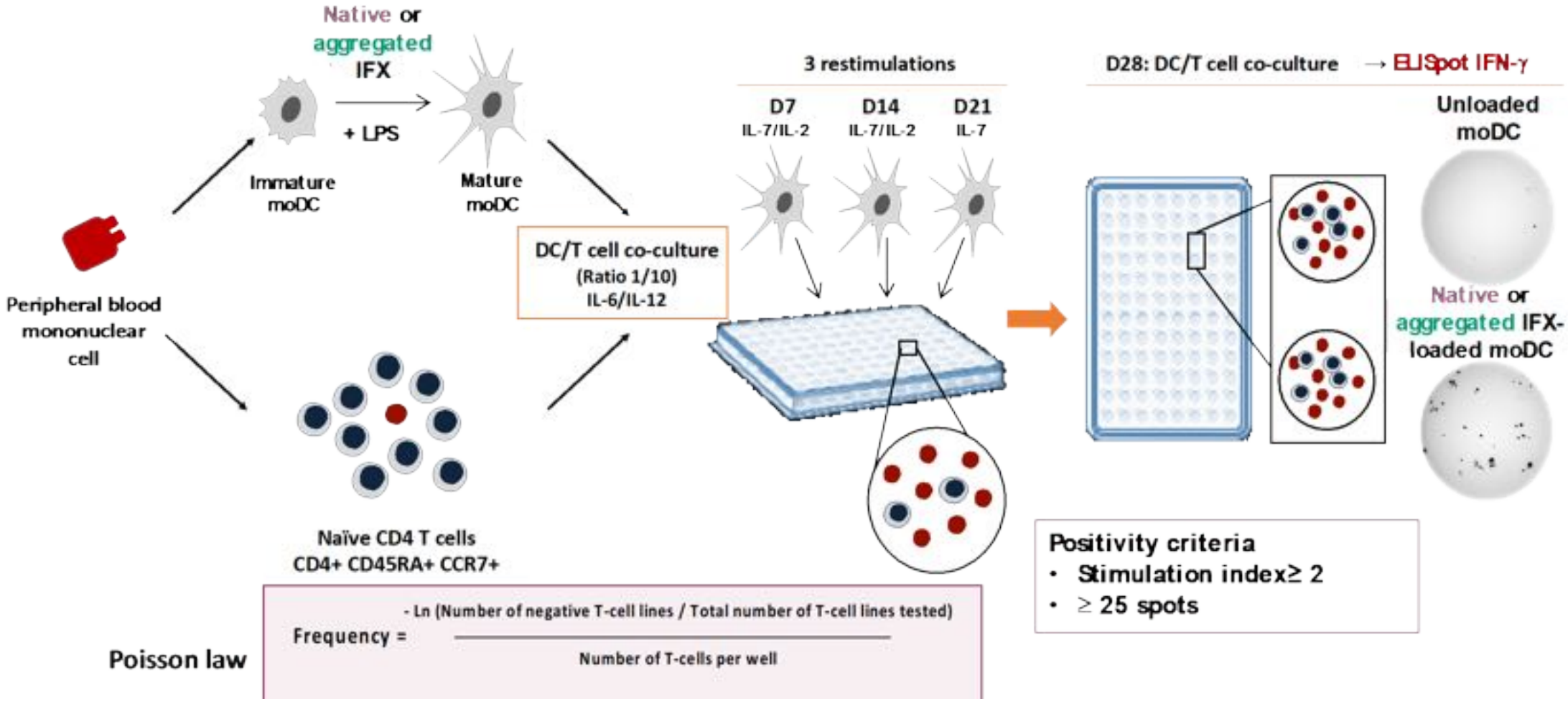
Native mAbs **mAbs aggregates**

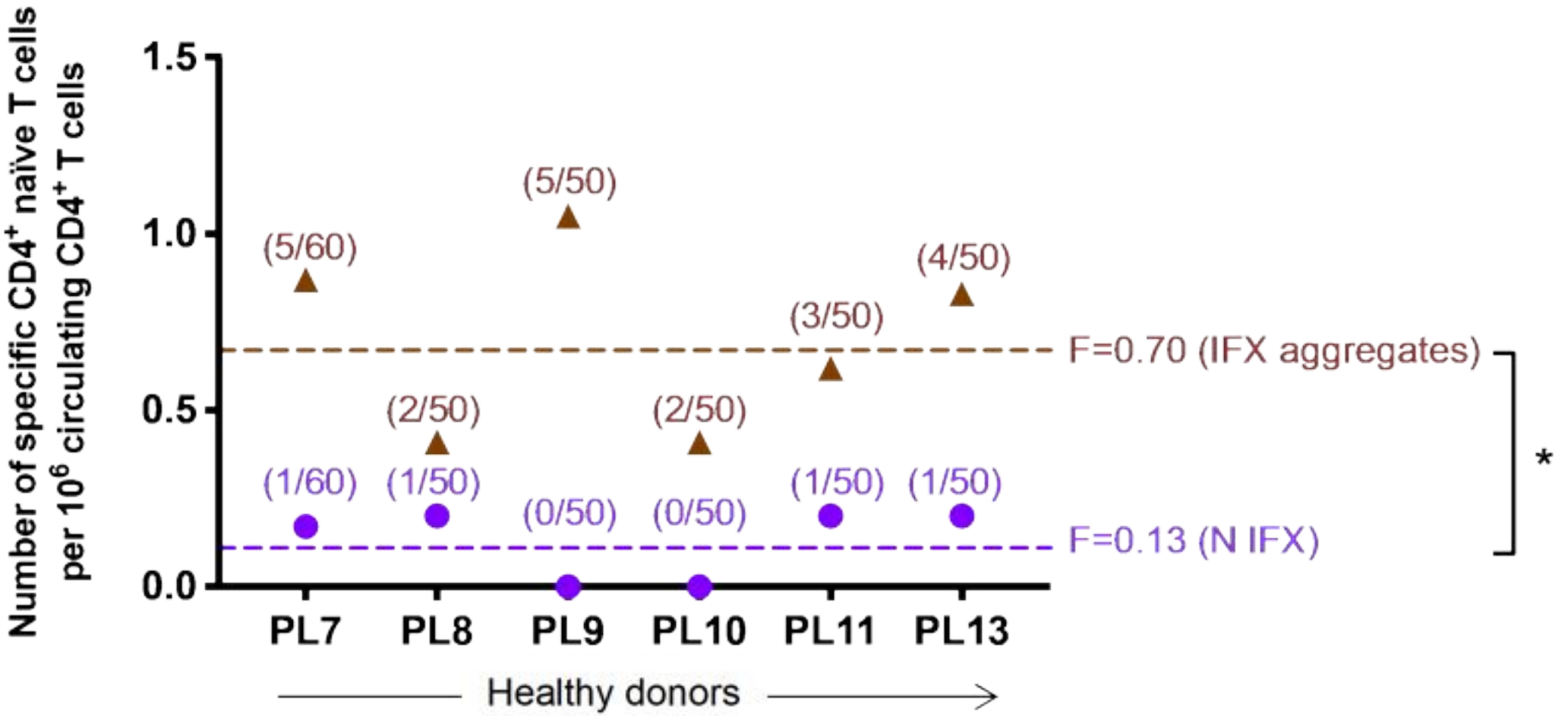


	Percentage
Monomer	50.05
Dimers	37.51
Trimers	12.44

T-cell responses to aggregates ?

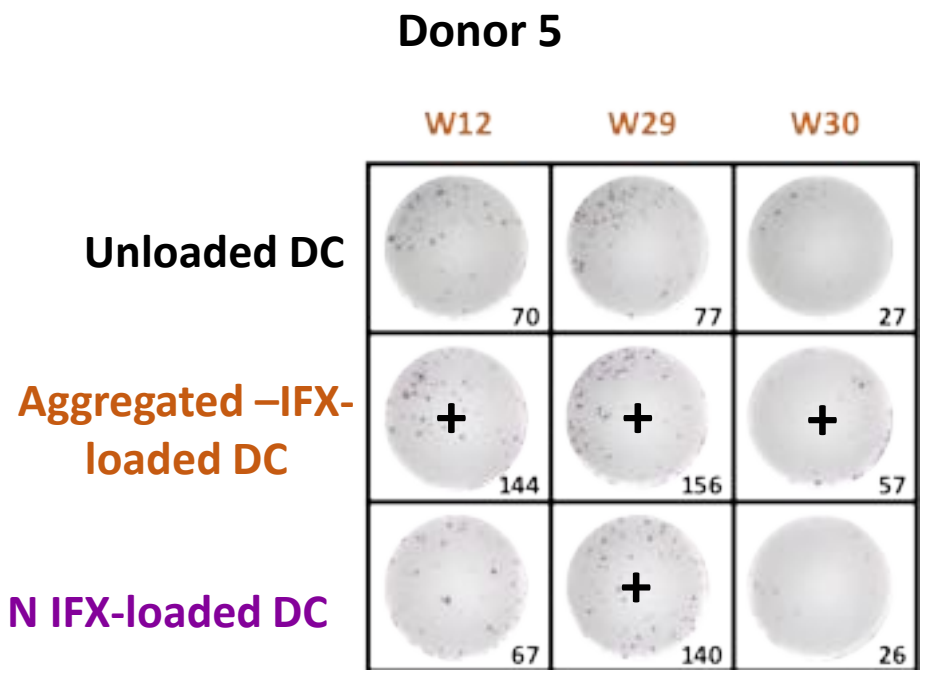
Autologous co-culture model to identify naïve T-cells recognizing Infliximab or aggregated Infliximab



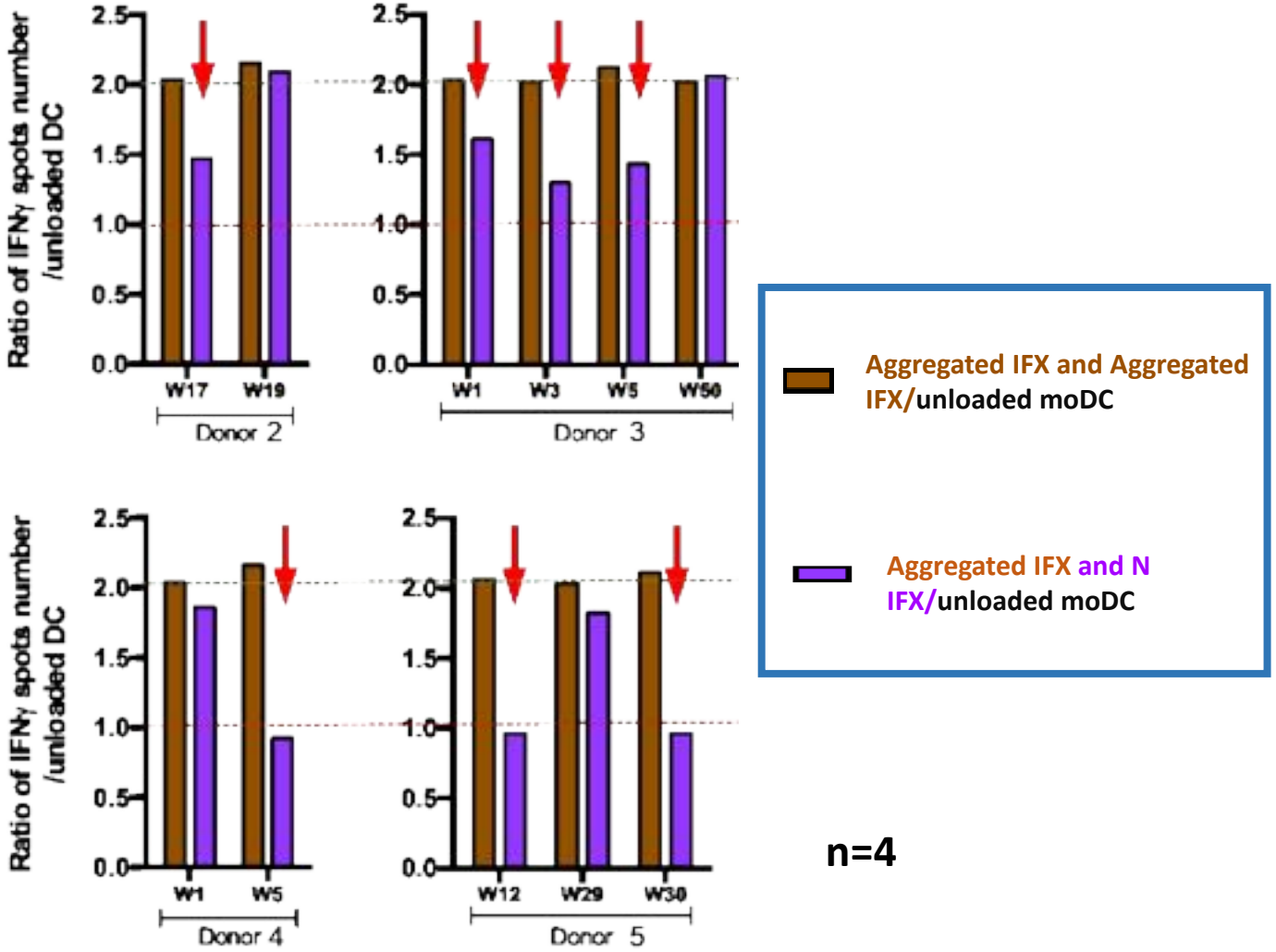


⇨ **Identification of a higher number of specific T cells in response to IFX aggregates for each donor**

T-cells recognize UV-aggregated IFX



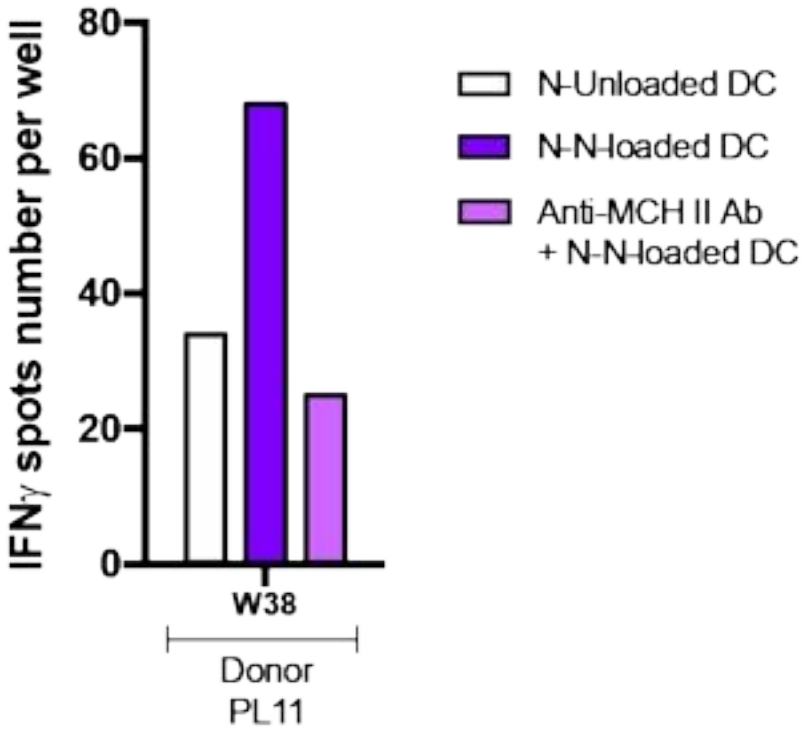
ELISpot 2: UV IFX-primed CD4 T cells (3/50)

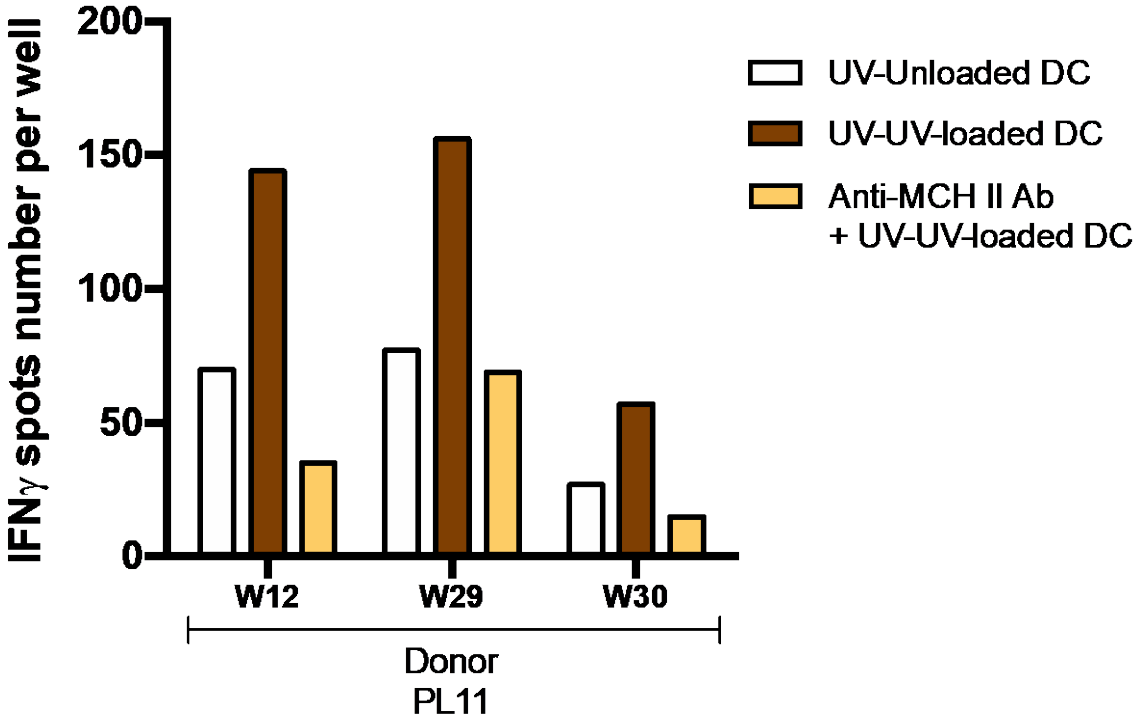


n=4

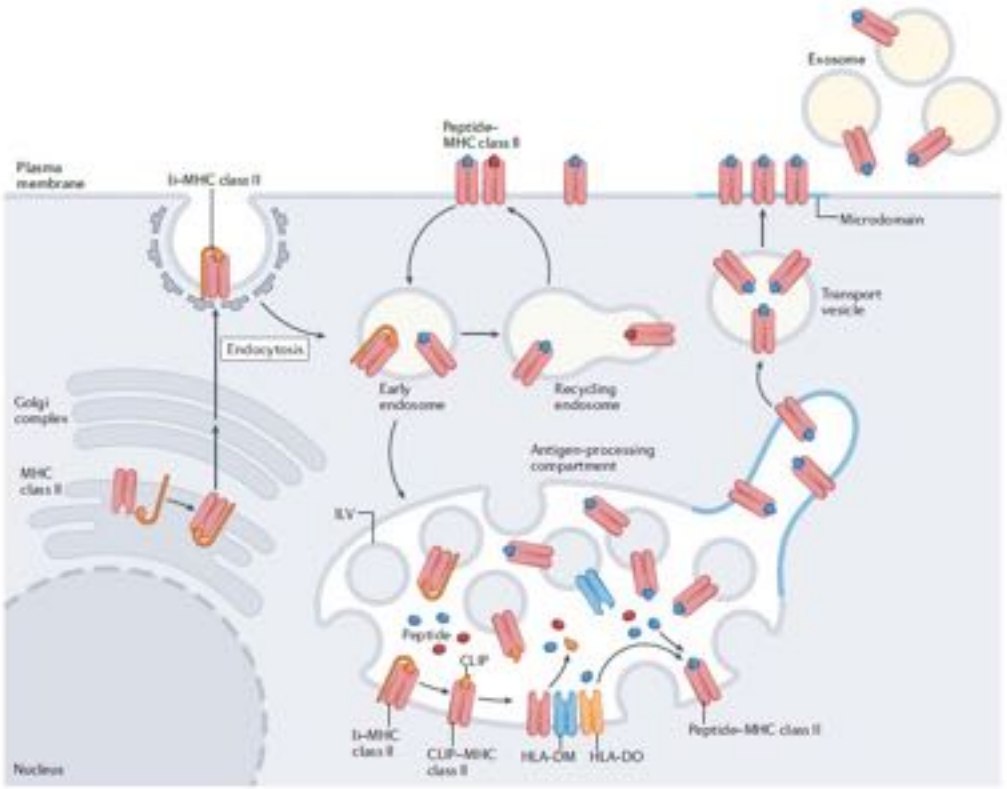
➪ Some aggregated IFX-specific T cells recognize ONLY aggregated-derived peptides

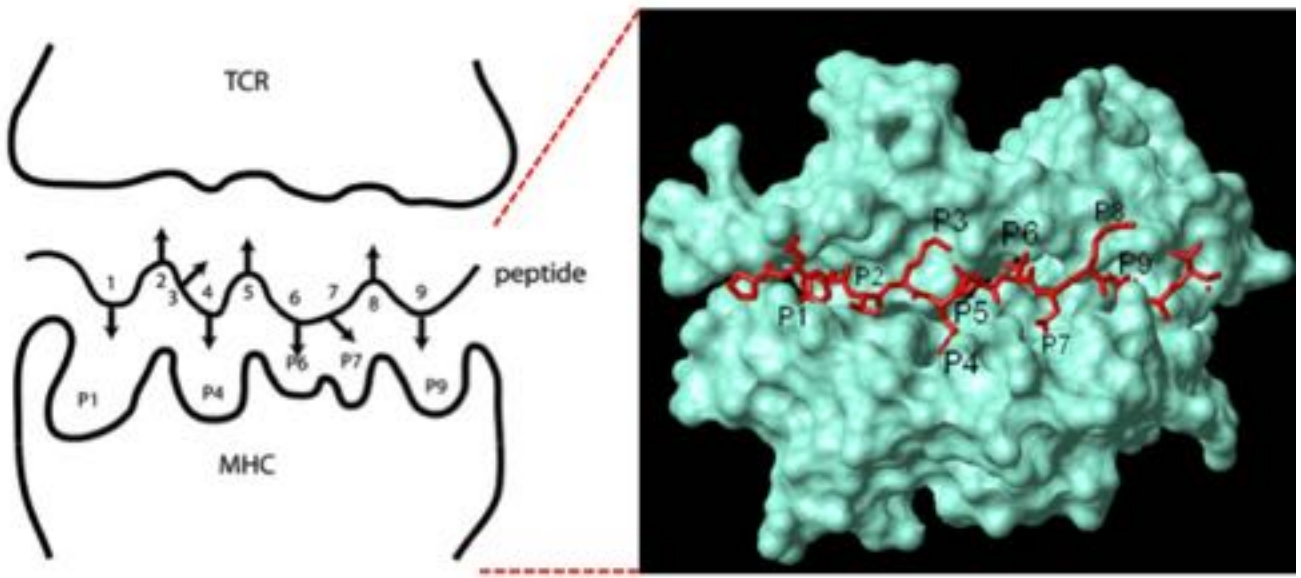
MHC class II molecules are required for the activation of naïve CD4 T cells recognizing native and aggregated infliximab.





Hypothesis concerning the augmented recruitment of T-cells





ROUTING

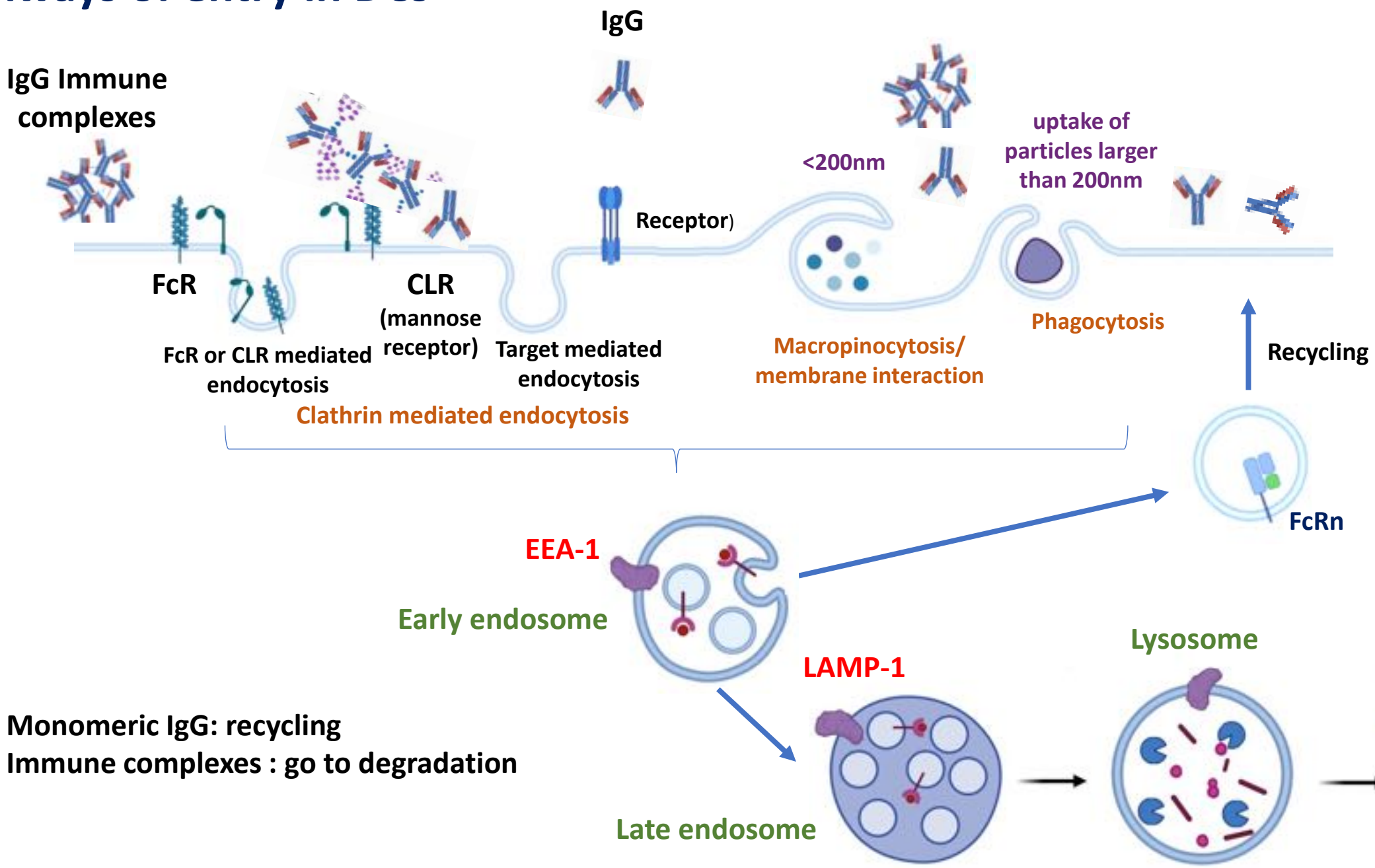
Antigen presentation
TCR recruitement

To be elucidated at two levels

Pishesha N et al (2022)

The quantity of IFX entering DCs plays a role in T-cell response ?

Pathways of entry in DCs

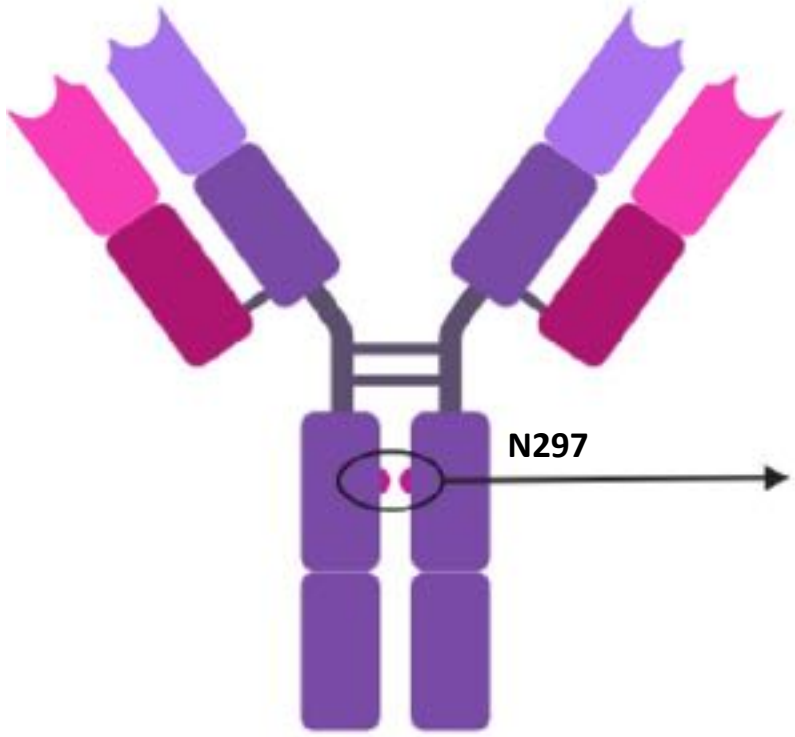


Monomeric IgG: recycling
Immune complexes : go to degradation

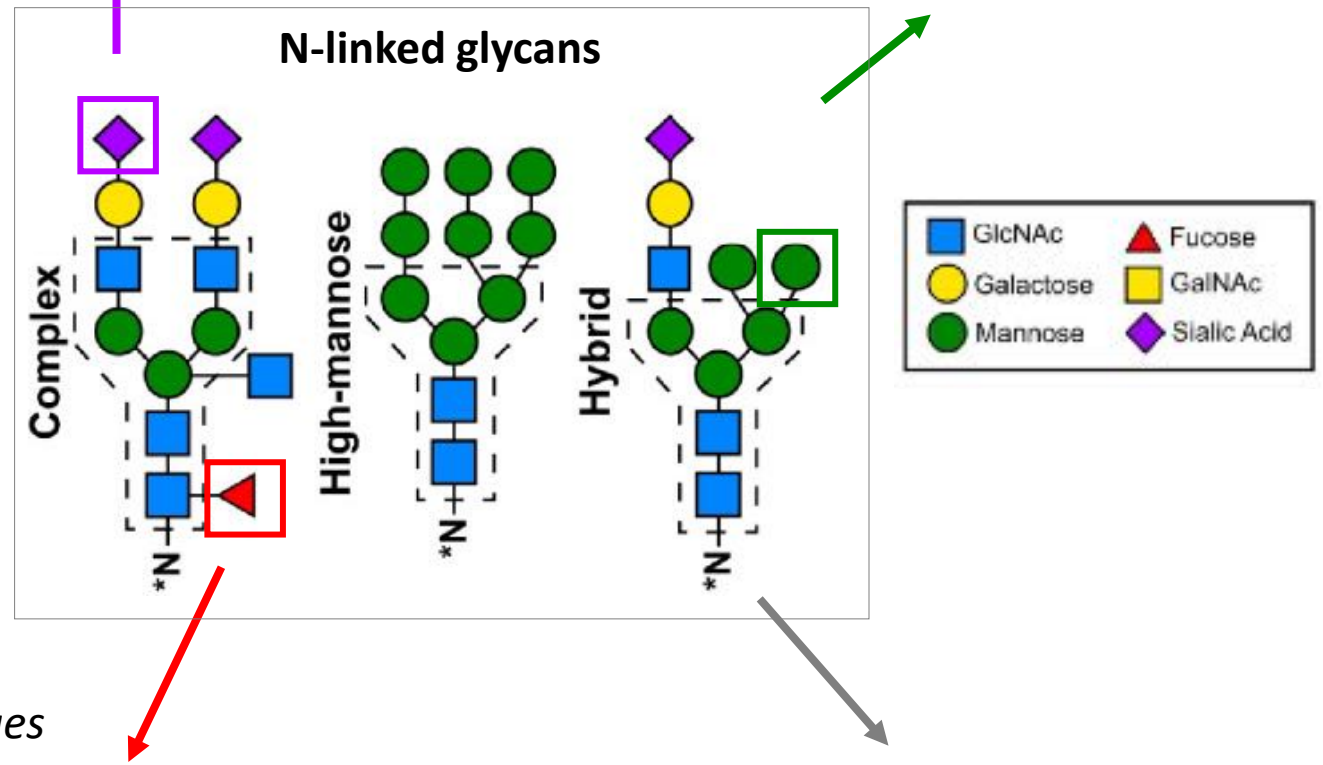
N-glycosylation of the Fc fragment

- Mannosylation :
- Increased serum clearance
- Mannose residues mainly recognized by mannose receptors (MR)/CD205, CD206 and 209 (DC-SIGN)

Sialylation :
Sialic acid mainly recognized by Siglecs and DC-SIGN on DC surface



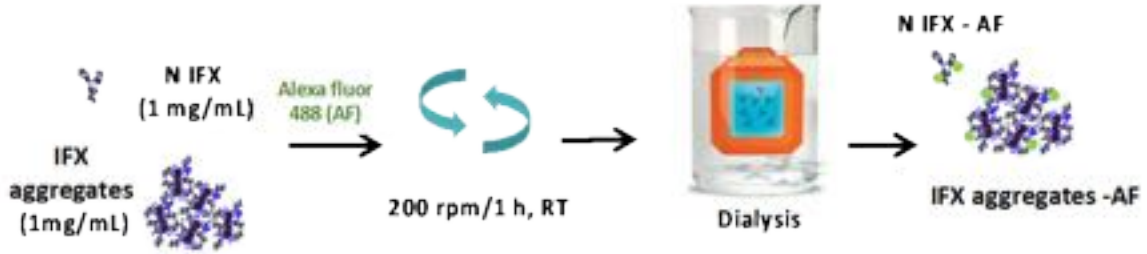
mannose, N-acetylglucosamine and fucose residues



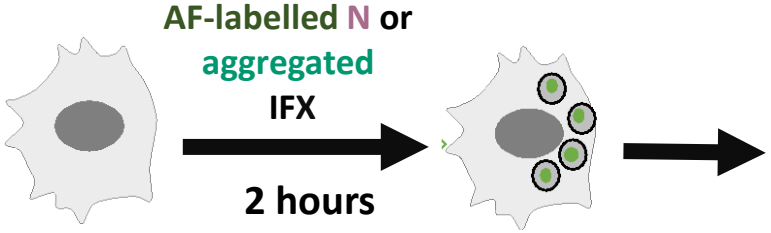
Fucose is recognized by mannose receptor

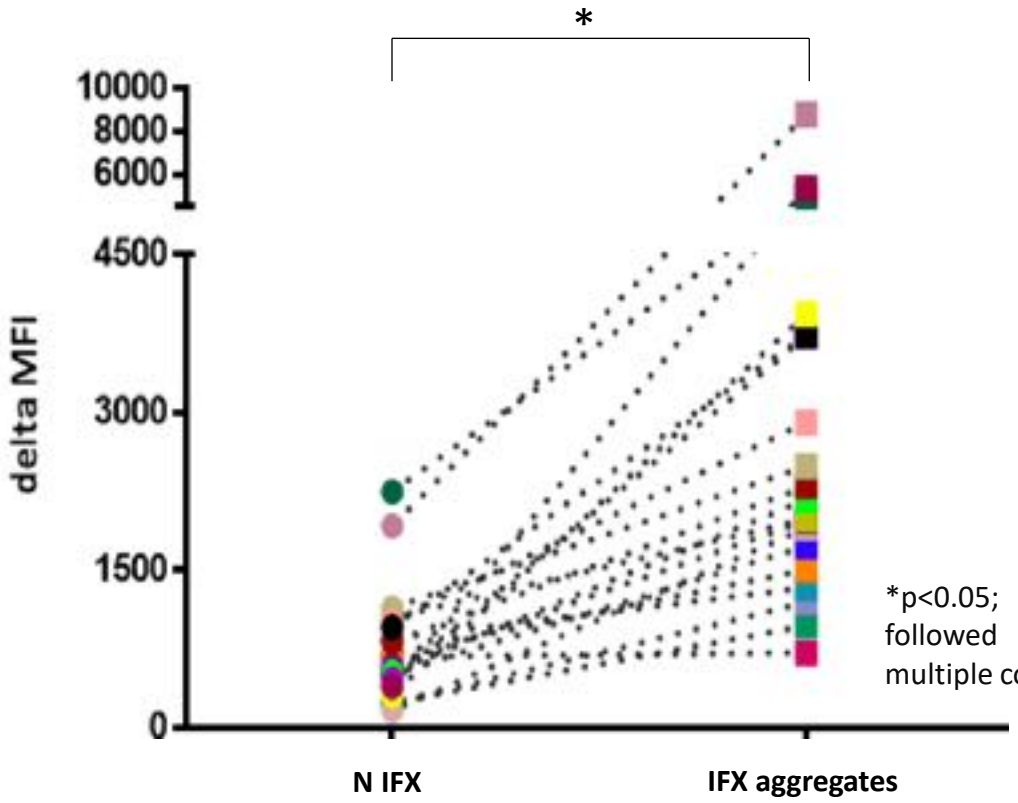
- N-glycosylation is necessary for the recognition by FcγR
- MR recognition

N AND AGGREGATED IFX LABELLING BY ALEXA FLUOR



N AND AGGREGATED IFX UPTAKE BY MoDC

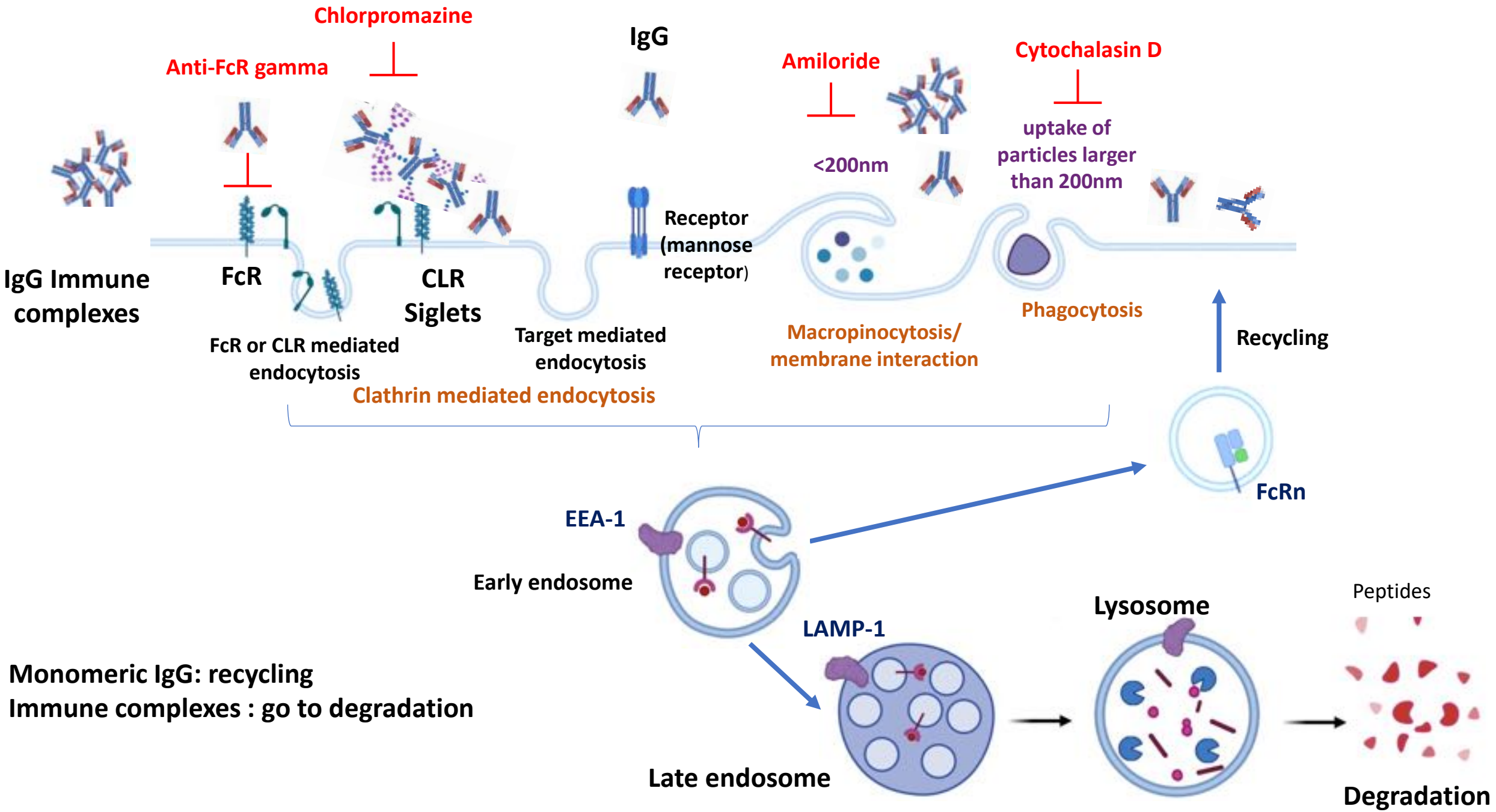




*p<0.05; Friedman test followed by Dunn's multiple comparison

Uptake calculation: difference between mean fluorescence intensity of surface-localized (4°C) and internalized (37°C) N or aggregated IFX.

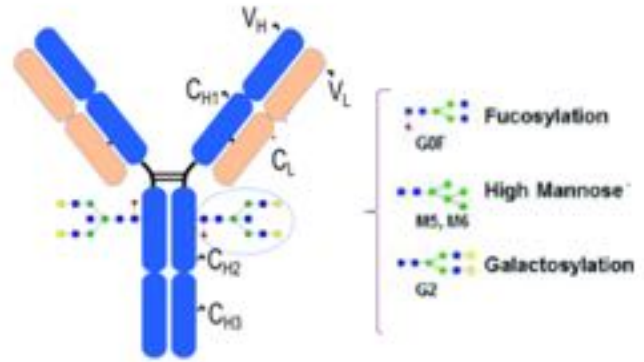
IFX aggregates are more internalized in comparison to native IFX

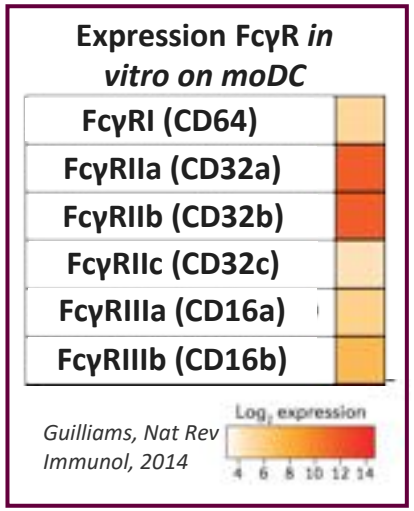


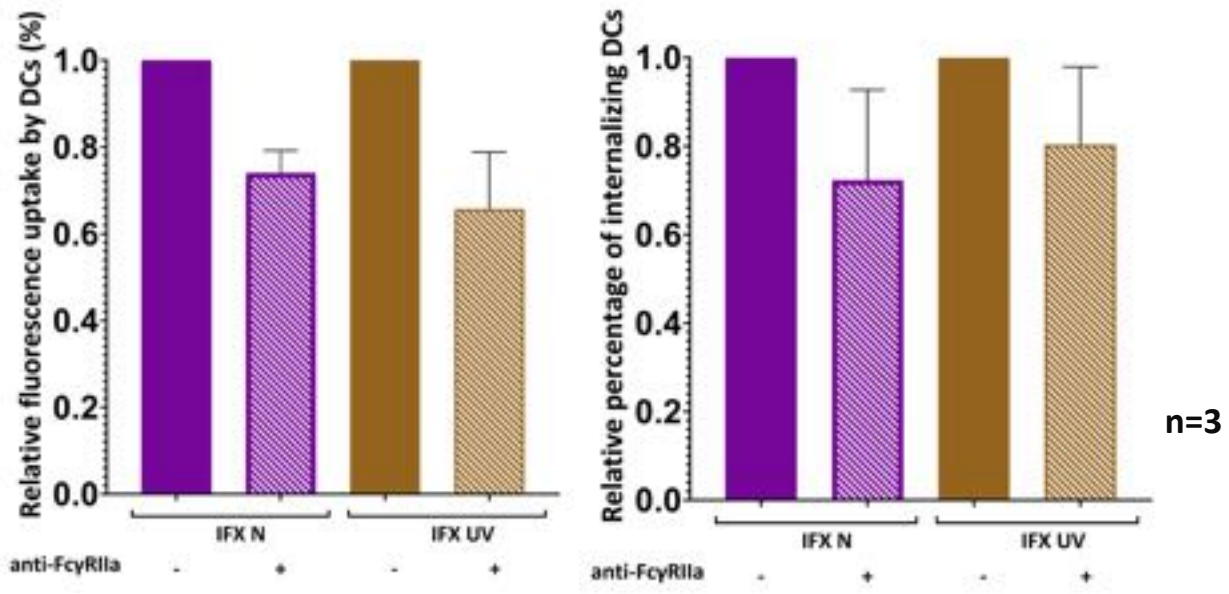
Monomeric IgG: recycling
Immune complexes : go to degradation

Receptors implicated in IFX N or IFX-UV uptake by DC

IFX is glycosylated on its Fc part
→ interaction with FcR and CLR



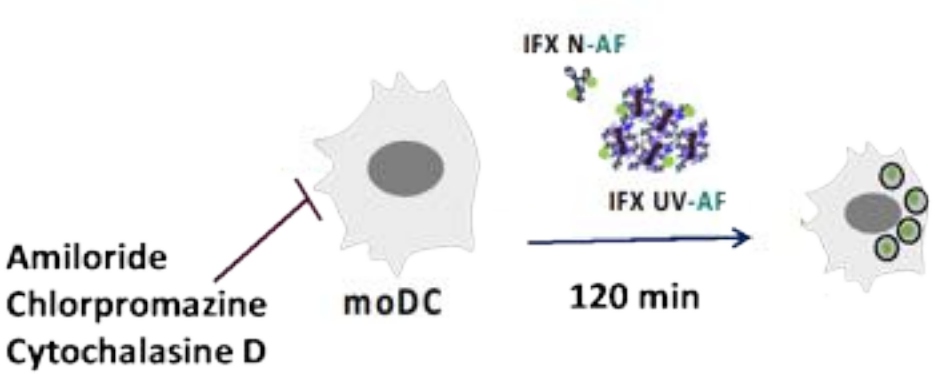




n=3

FcR gamma RIIa is not playing a major role in native and aggregates IFX internalization

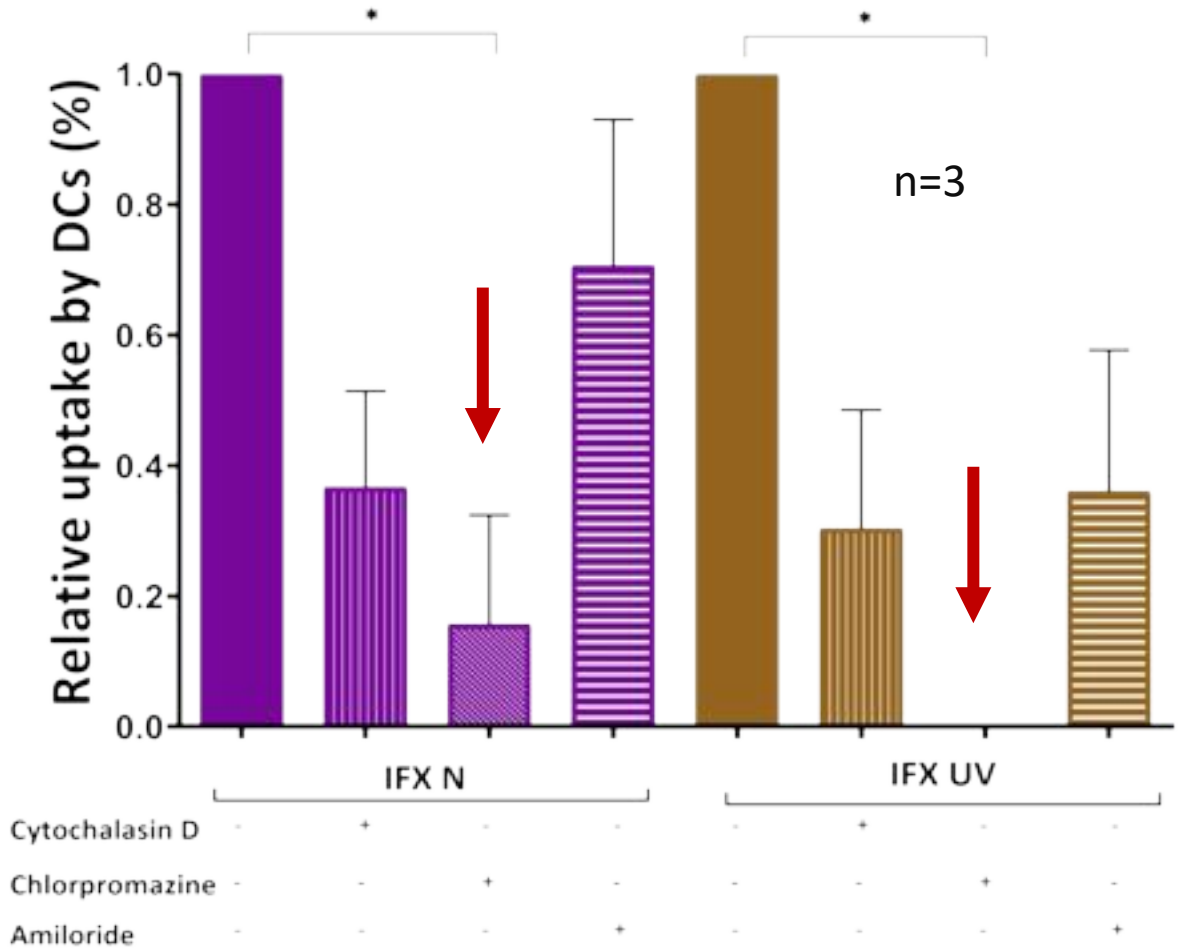
Internalization pathways implicated in IFX N or UV uptake by DC



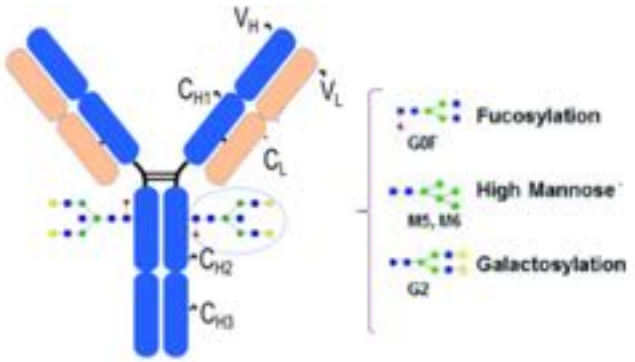
Chlorpromazine: inhibits **endocytosis**
Amiloride: inhibits **macropinocytosis**
Cytochalasine D: inhibits **phagocytosis**

Relative fluorescence

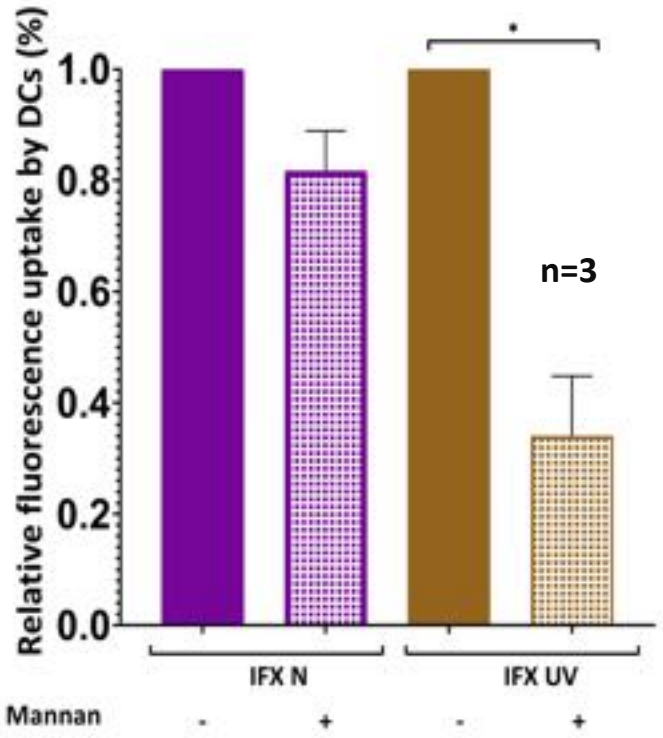
$$\frac{(\text{delta MFI without inh} - \text{delta MFI with inh}) \times 100}{\text{delta MFI sans inh}}$$

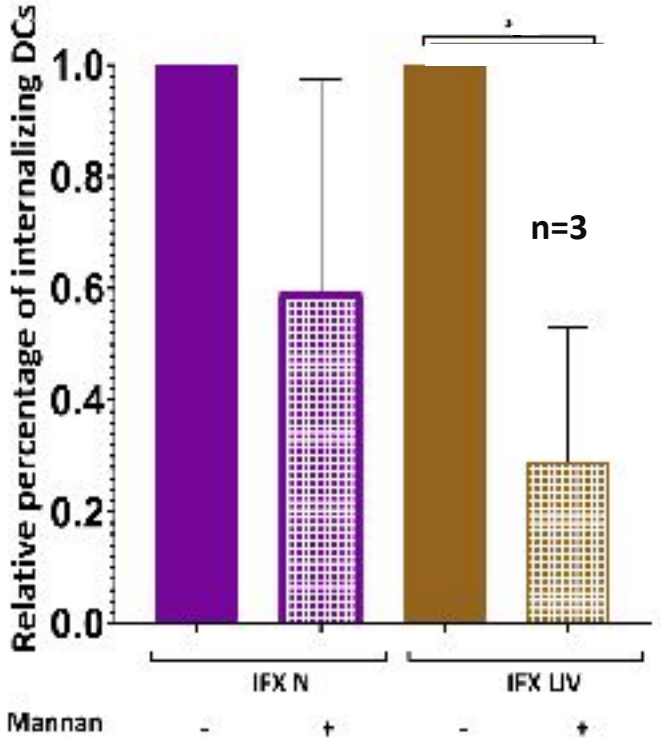


Receptors implicated in IFX N or UV-IFX uptake by DC



IFX: 5,5% high mannose in its Fc portion→ interaction with mannose recognizing CLR

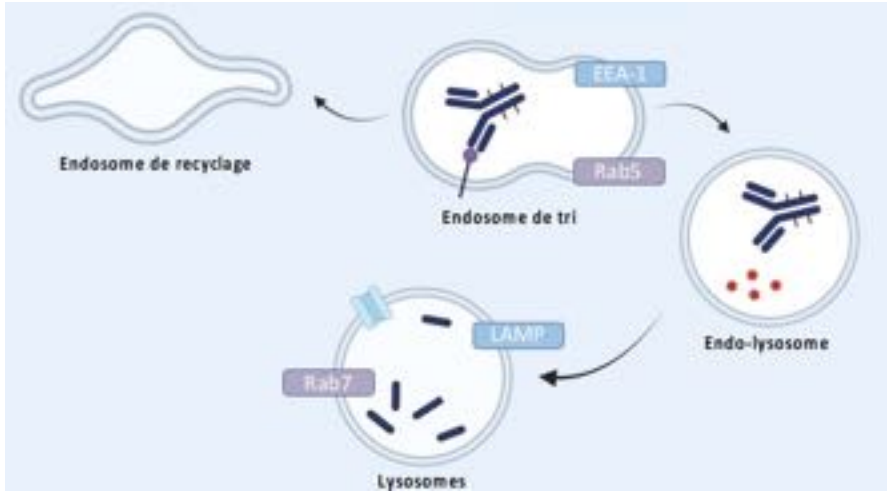


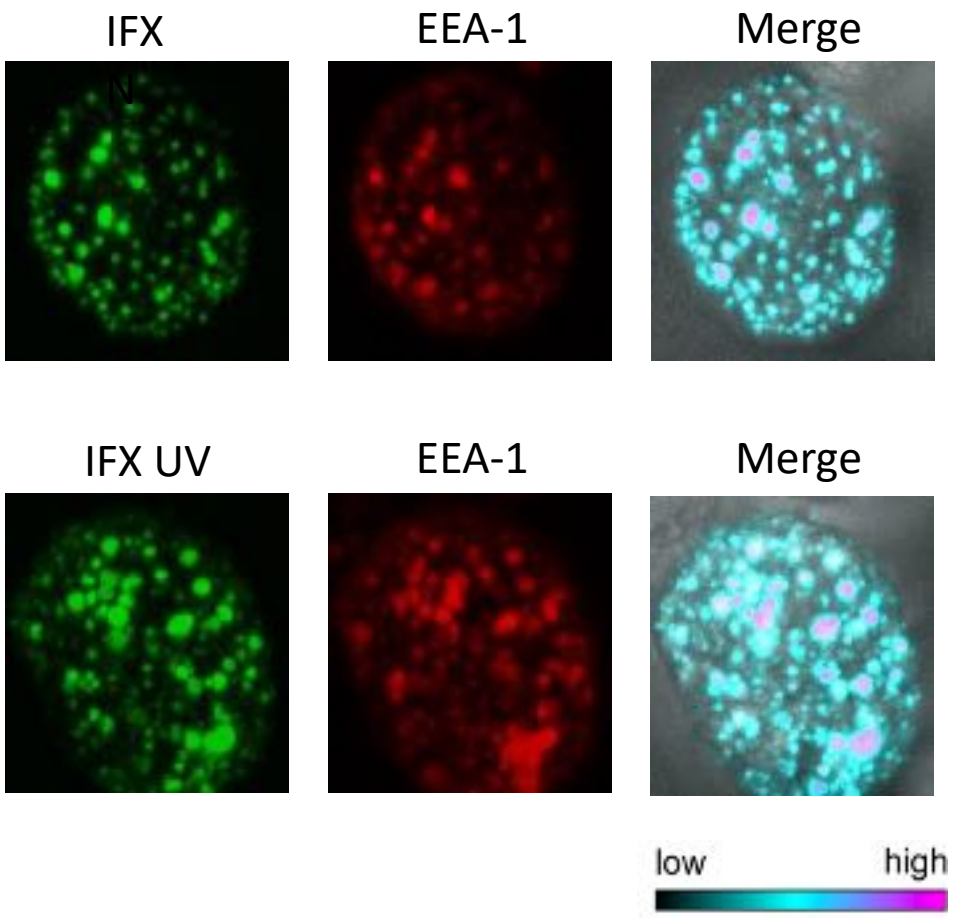


Internalization of aggregated IFX is inhibited by mannan (67% vs 22% for IFX N)
Mannose-dependent endocytosis is one of the major pathways for IFX aggregates internalization
Probably not CD209 (DC-SIGN; results with DG-75 transfected cell line are negative)
CD206 ? CD205 ?

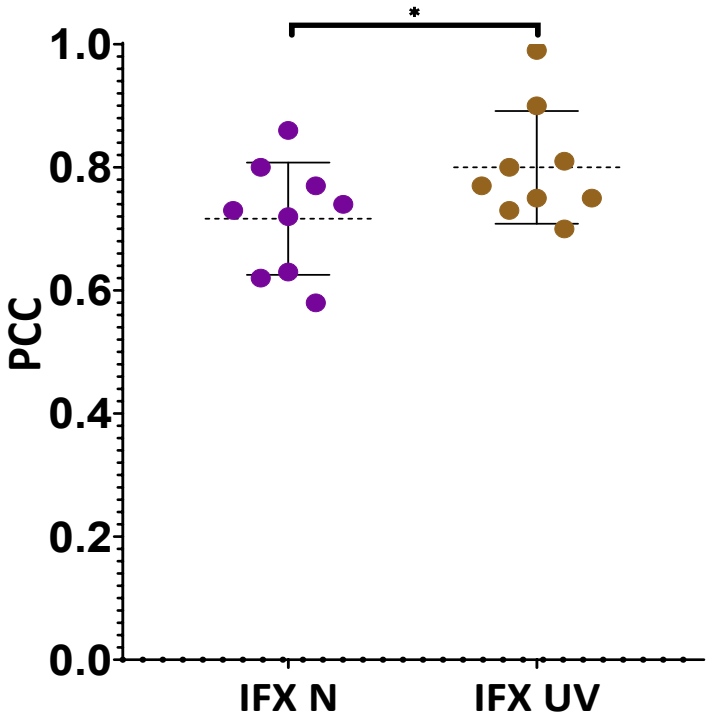
Routing of IFX N or UV in endosomal compartments of DC

Augmentation of IFX-UV aggregates intake leads to an increase in cellular compartments ?



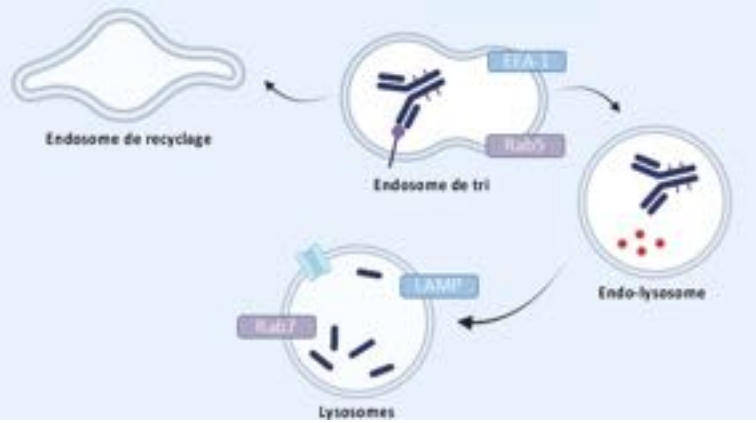


Higher co-localization rate between IFX UV and EEA-1 in comparison to IFX N

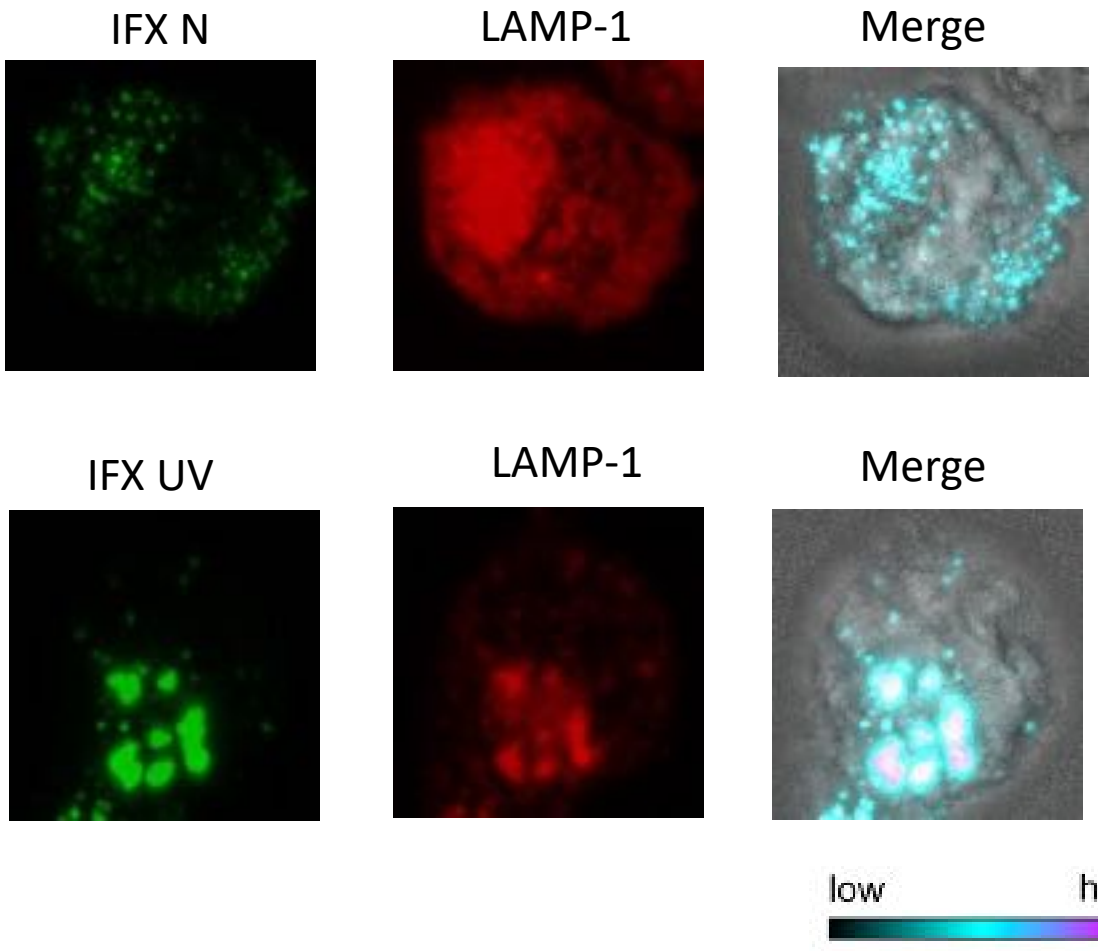


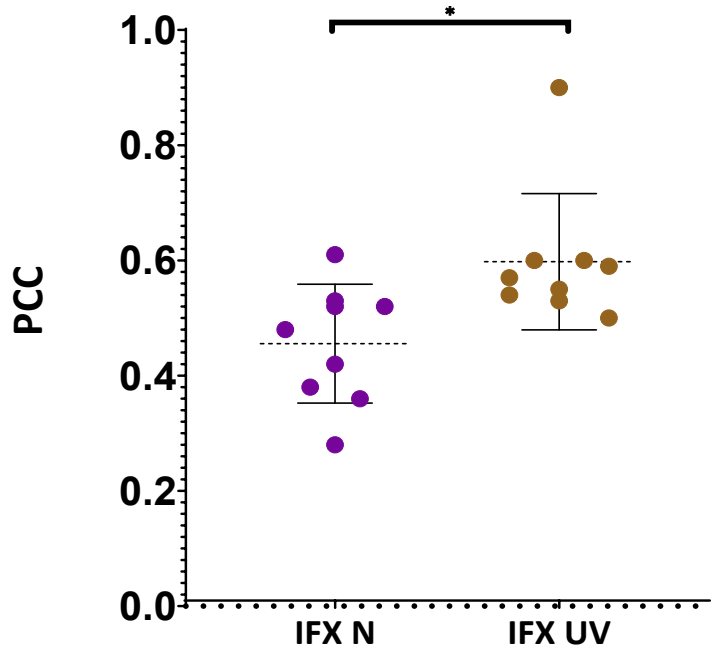
PCC: Pearson correlation coefficient = co-localization

15 min incubation with IFX N or IFX UV aggregates



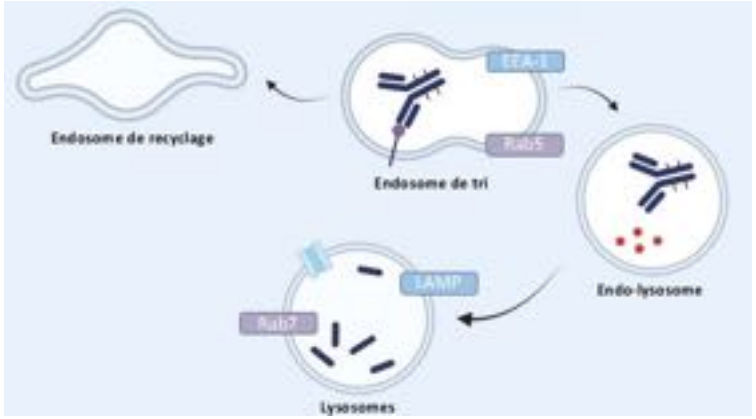
Higher colocalization rate between IFX UV and LAMP-1 in comparison to IFX N





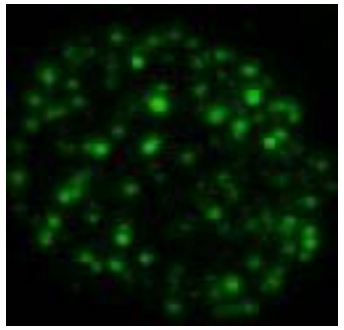
PCC: Pearson correlation coefficient = co-localization

60 min incubation with IFX N or IFX UV aggregates

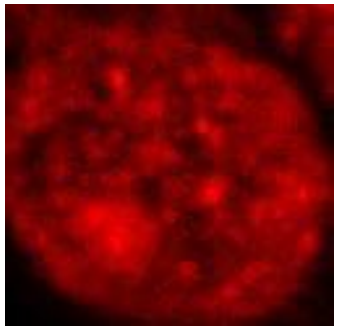


Higher co-localization rate between IFX UV and HLA-DR in comparison to IFX N

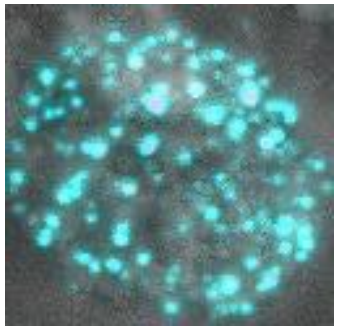
IFX N



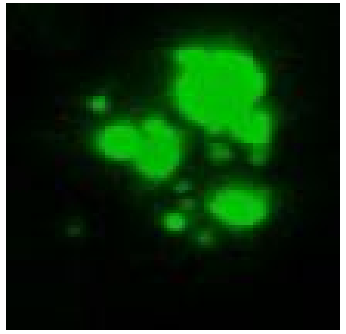
HLA-DR



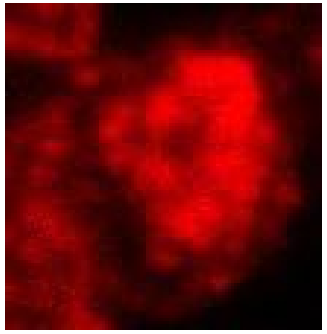
Merge



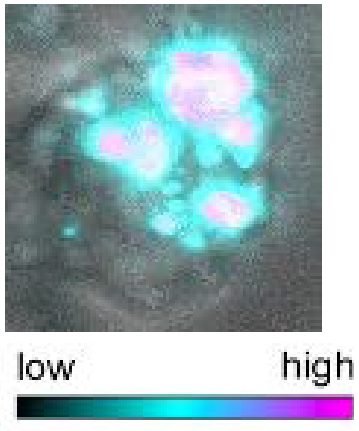
IFX UV

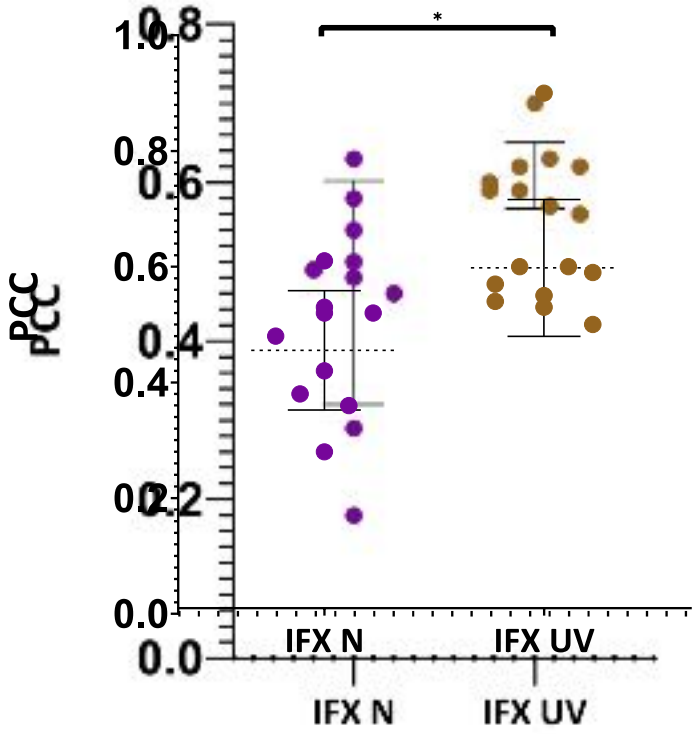


HLA-DR



Merge



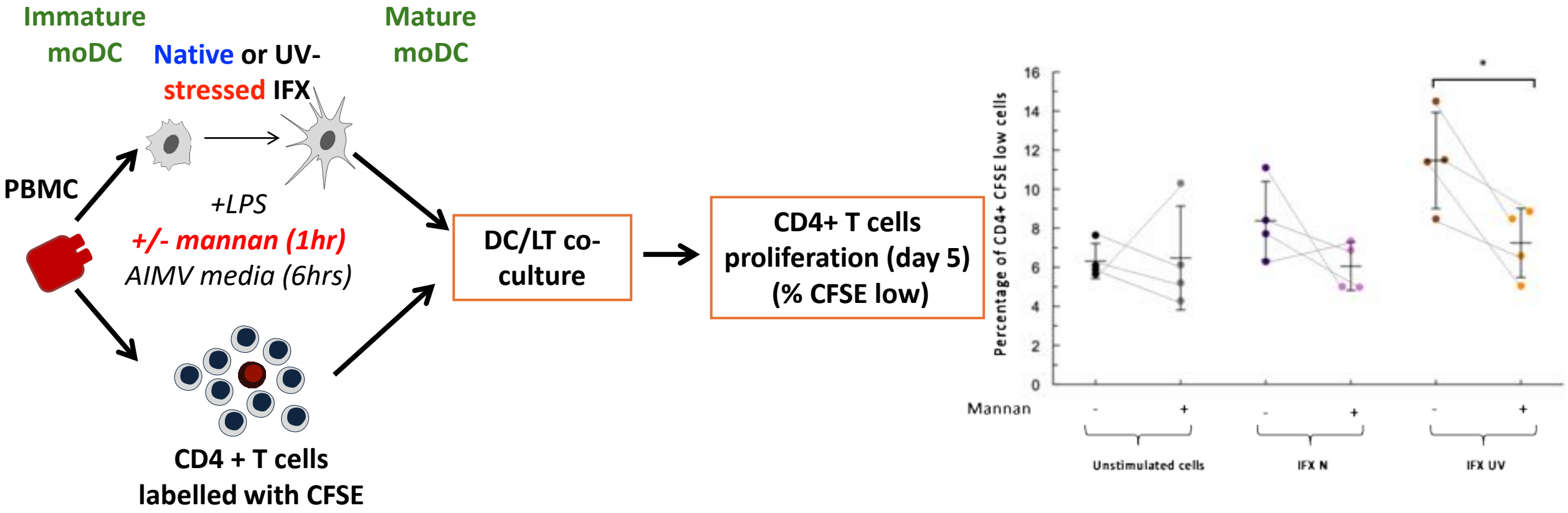


PCC: Pearson correlation coefficient = co-localization

120 min incubation with IFX N or IFX UV aggregates

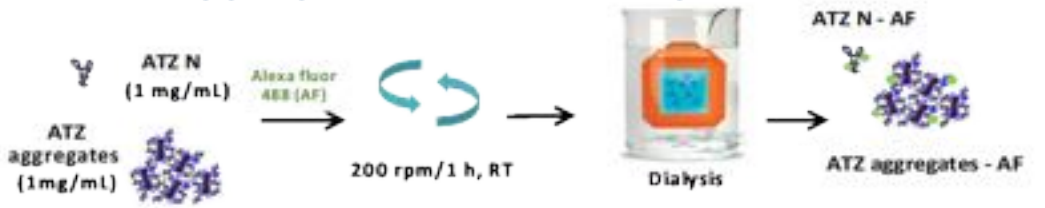
Effect of CLR mediated endocytosis on T cell activation

Autologous DC-T cell co-culture model: measure of T cell proliferation via CFSE staining

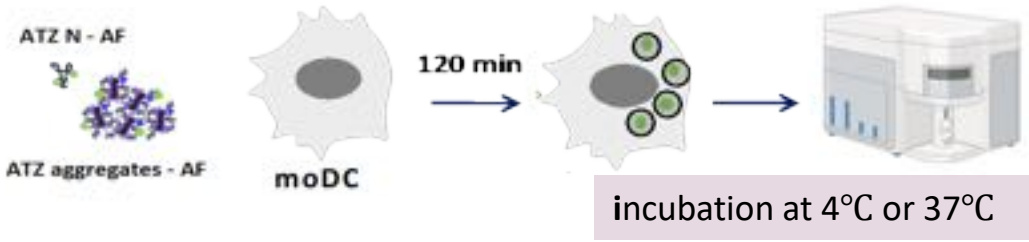


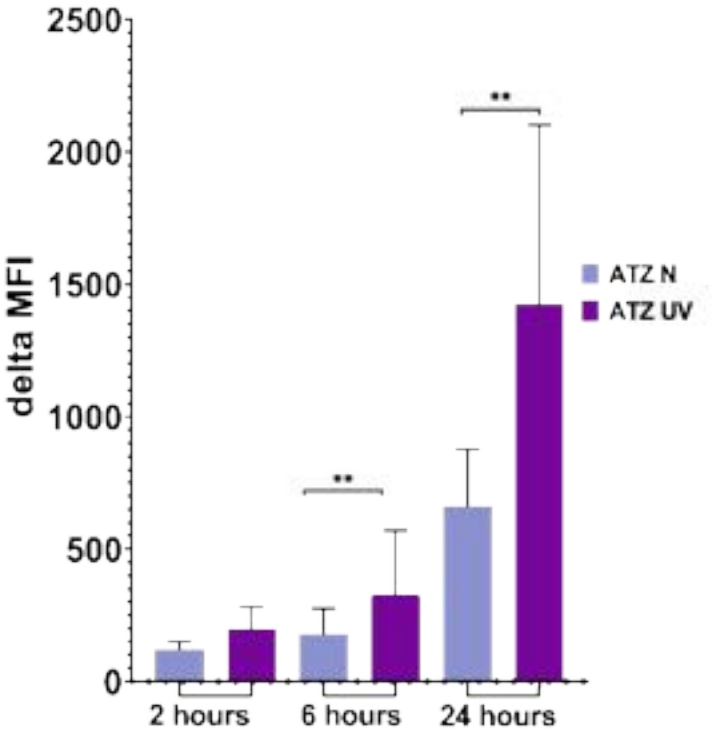
Quantification of ATZ N or ATZ-aggregates uptake by DCs : direct labeling

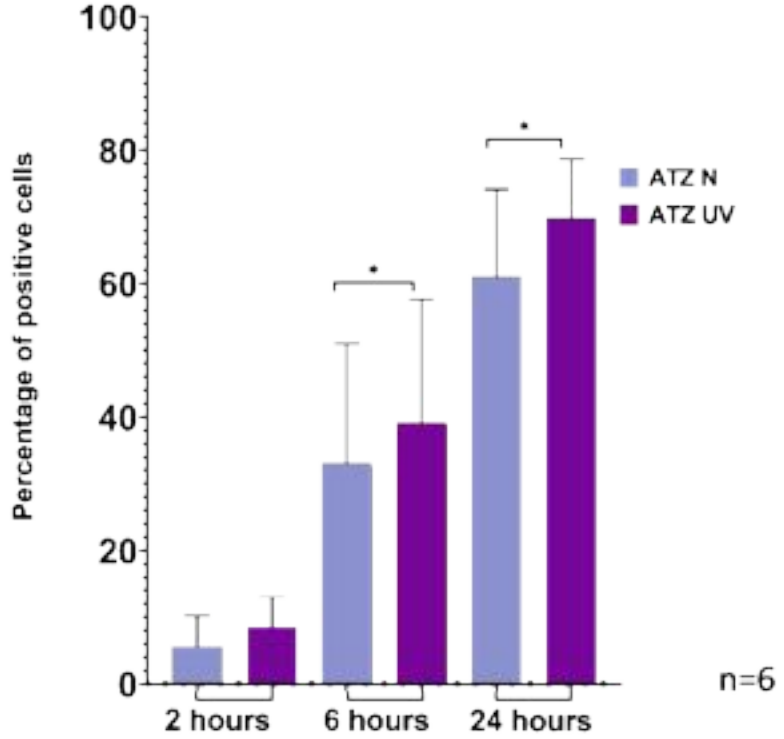
N and aggregated ATZ labeling with alexa fluor



N and aggregated ATZ uptake by MoD







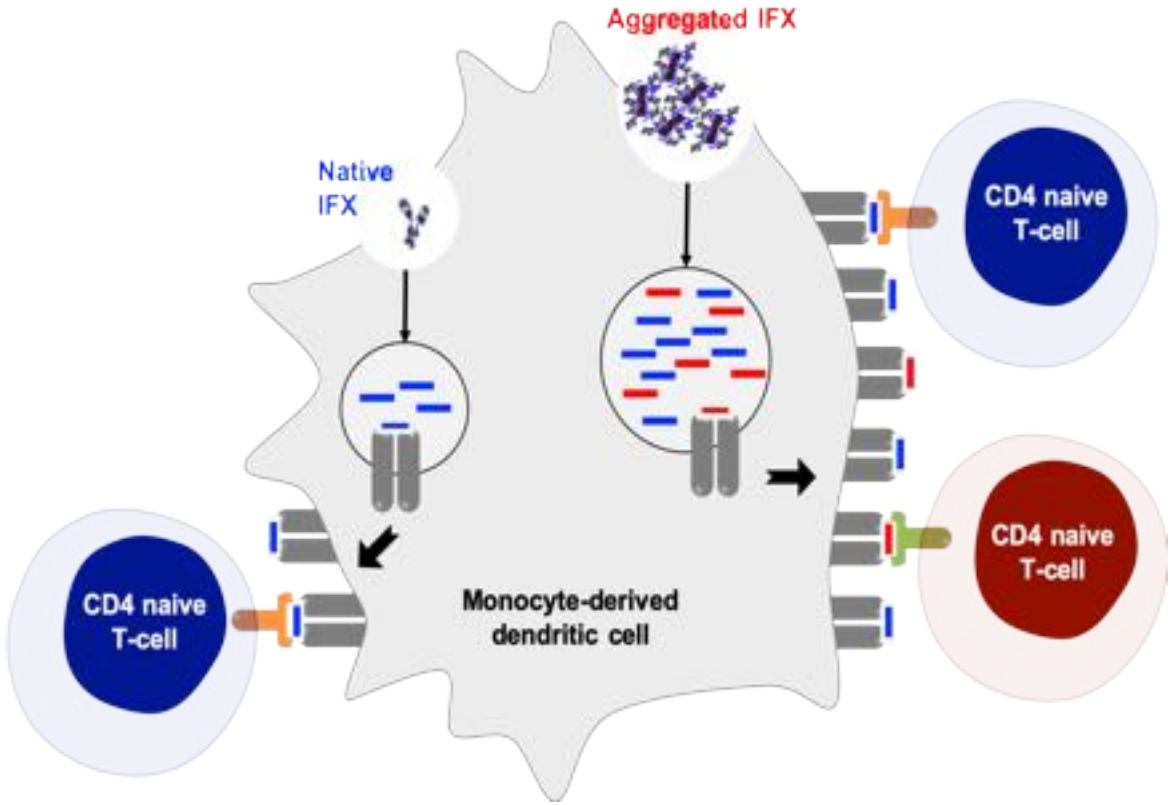
- ATZ aggregates are more internalized compared to native ATZ
- Aggregates internalization is independent of glycosylation
- Target oriented ?

Uptake calculation: difference between mean fluorescence intensity of surface-localized (4°C) and internalized (37°C) N or aggregated ATZ

In conclusion: nanosized aggregated

Dendritic cell

- No modification moDC phenotype
- Higher internalization of mAb aggregates by moDC
- Internalization of aggregates via a mannose-dependent endocytosis for IFX
- Target-oriented for ATZ ?
- Impact on peptide presentation and T cell response, mechanisms ?



T cell response

- Increased recruitment of T-cells with aggregates
- Detection of naïve T-cell recognizing mAb aggregates but not the native protein

- **Aggregation increase the number of presented peptides derived from the antibody ?**
- **Peptides with low avidity participate to T-cell recruitment ?**
- **Increase of the number of T-cells recruited ?**

and/or

- **Neo-peptides are generated by the aggregation process ?**
- **New T-cells recruited ?**

Next steps

- **Identification of the pathways involved in aggregates internalization**
  - Relationship with the structure and/or mode of entry (MR, target-oriented, FcR, mixed)
    - Use of other antibodies
  - Quantification of mAbs internalization *(see Estefania Tumbaco-Valarezo poster, collaboration with Servier)*
- **In search of the mechanisms leading to increase of naïve T-cells recruitment**
  - Augmentation of immunodominant epitopes ?
  - Specific aggregate epitopes ?
- **Strategy**
  - MHC associated peptide proteomics (MAPPS) using aggregates
  - Identify TCR clonotypes (increase ? specificity ?)

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