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Electron Microscopy Imaging Reveals Unique Higher Order Structures of Adalimumab-TNFα and Infliximab-TNFα Complexes

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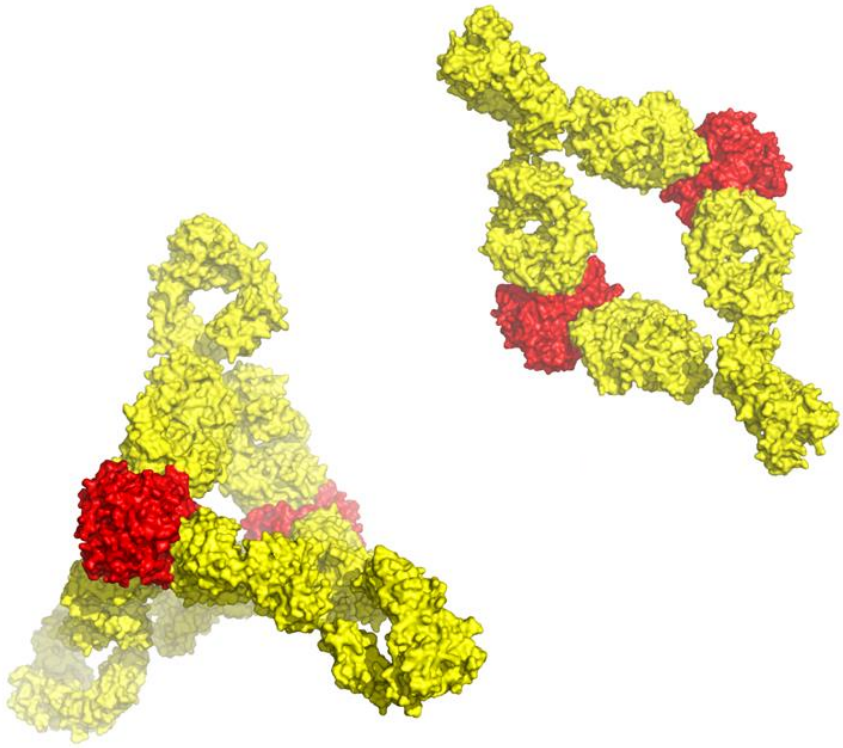
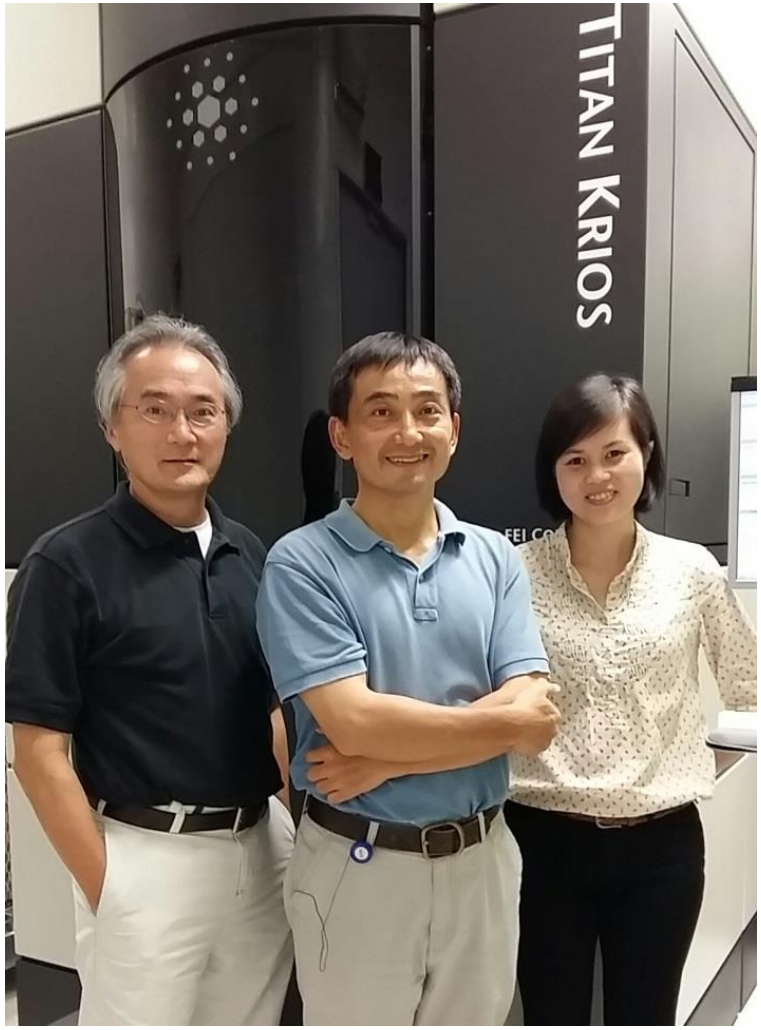


Acknowledgement



National University of Singapore
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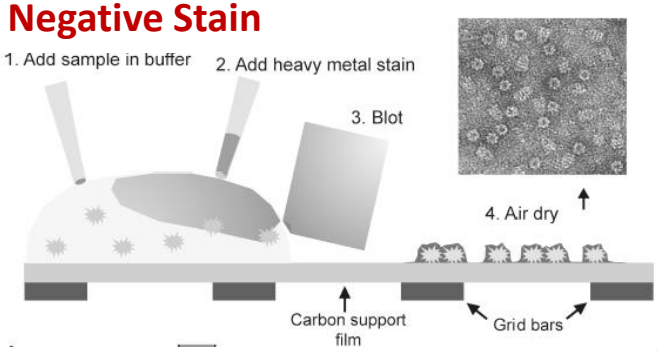
Content of today's talk

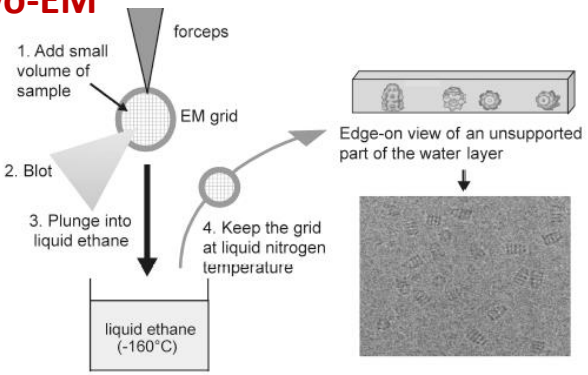
1. Introduction : Electron Microscopy, TNFα and its complexes with Adalimumab
2. Negative Stain EM and Cryo-EM of Adalimumab-TNFα complexes
3. Negative Stain EM of Infliximab-TNFα complexes
4. Summary

Electron Microscopy of Macromolecular Assemblies

Negative Stain

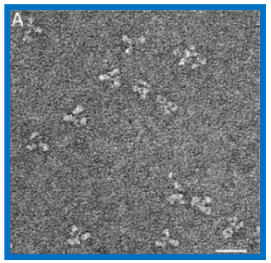


Cryo-EM



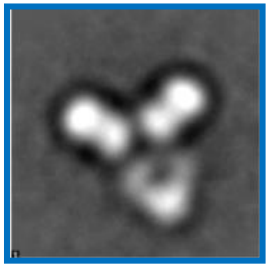
EM Imaging

Single Particle



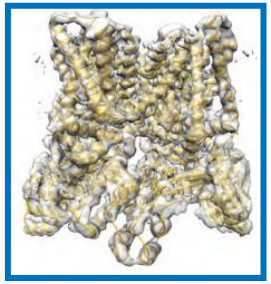
IgG

2D Class Average



IgG

3D Reconstruction

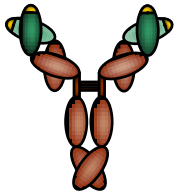


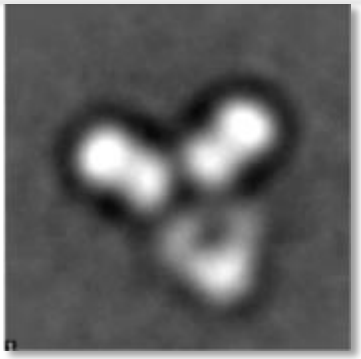
TrpV Ion Channel

E. V. Orlova and H. R. Saibil (2011) Structural Analysis of Macromolecular Assemblies by Electron Microscopy. Chem. Rev. 111: 7710–7748
M. Carroni and Helen Saibil (2016) Cryo electron microscopy to determine the structure of macromolecular complexes. Methods 95: 78–85

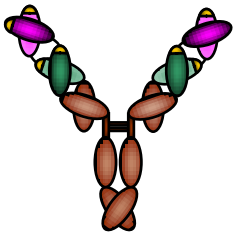
Electron Microscopy of Therapeutic Biologics

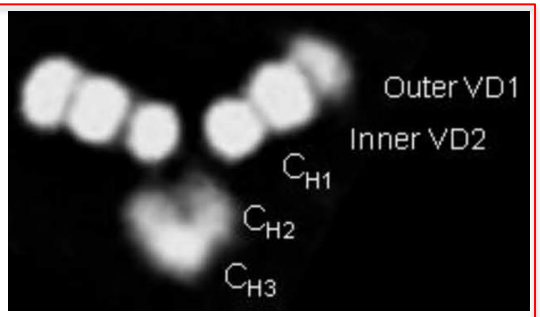
Negative Stain EM of mAb and Bispecific Antibody





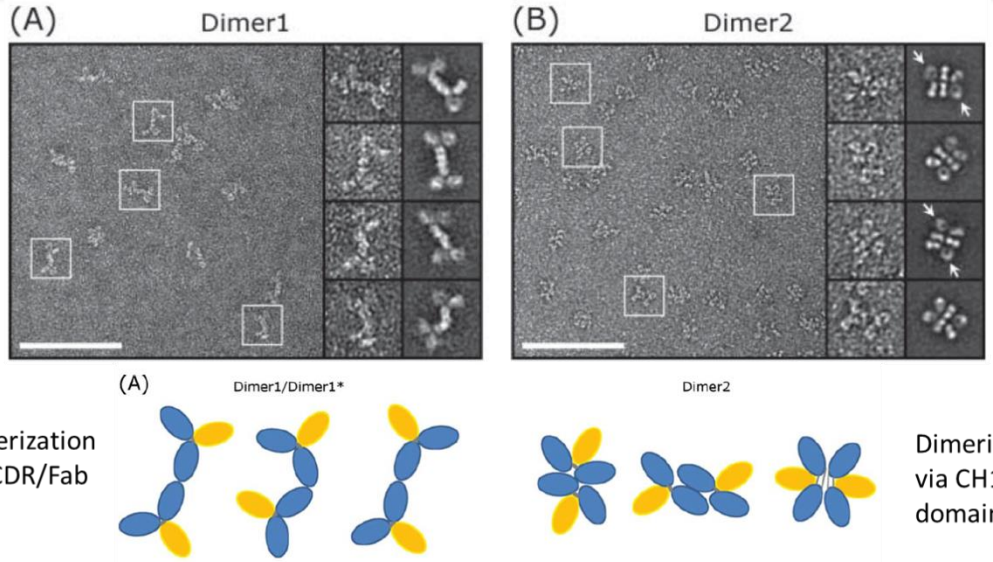
Adalimumab





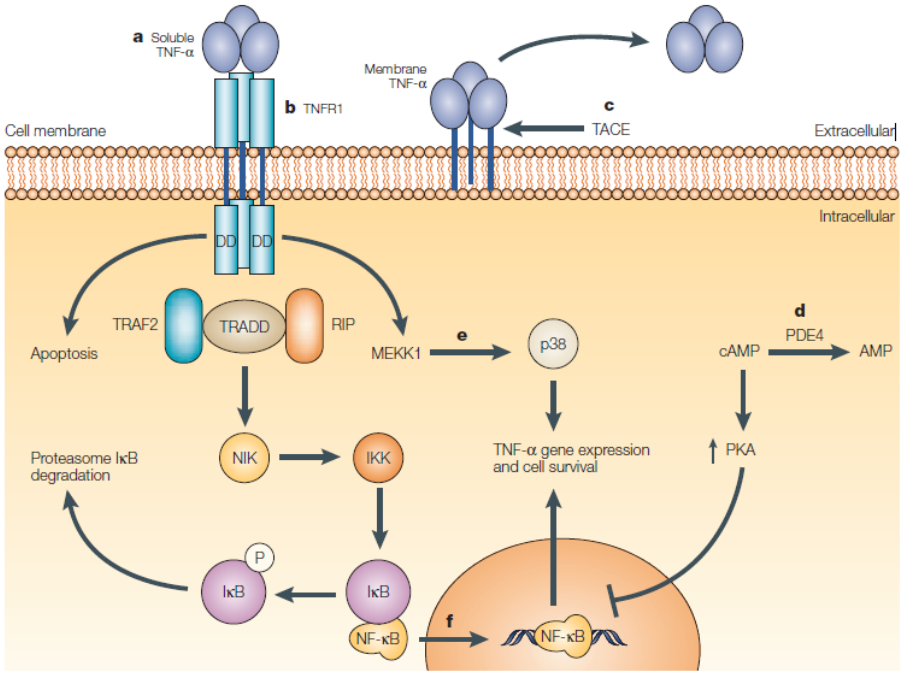
Dual-Variable Domain Ig (DVD-Ig)

Negative Stain EM of different dimer forms in mAbs



Tran B. et al. (2017) Protein Sci, 26:12, 2392-2398
Correia I. et al. (2013) mAbs, 5:3, 364-372
Plath F. et al. (2016) mAbs, 8:5, 928-940

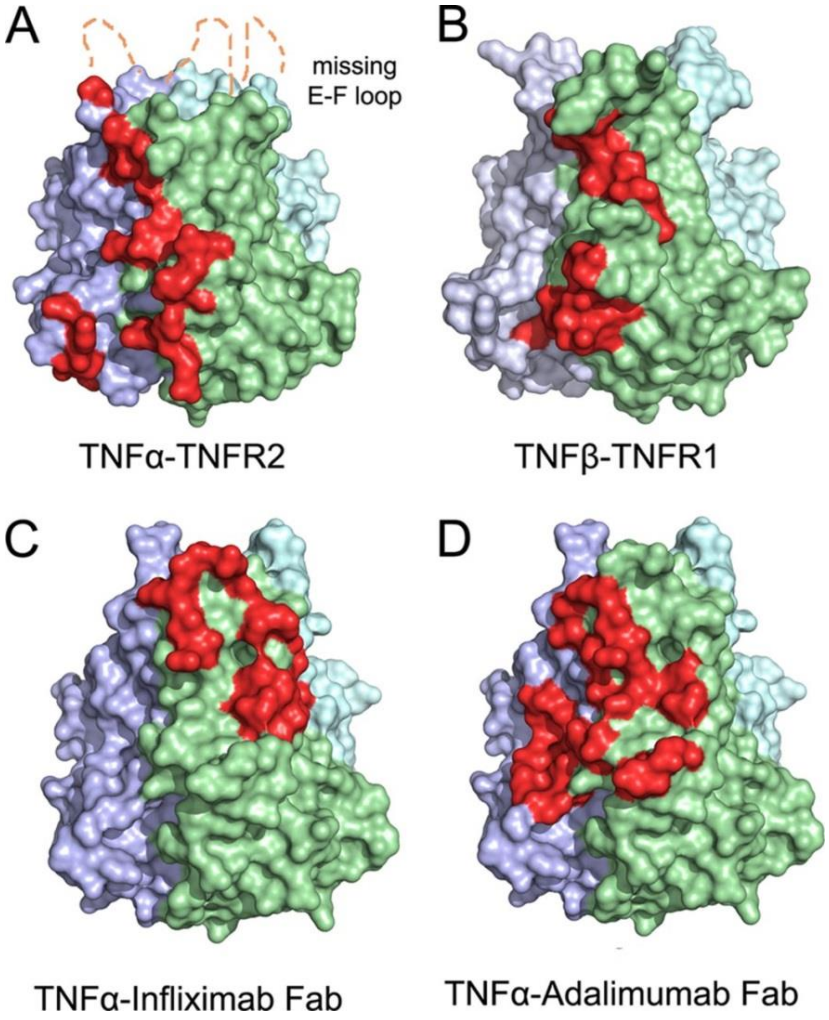
Introduction - TNFα



- TNFα: Tumor Necrosis Factor – alpha
- Central biological mediator that regulates inflammation responses
- Multi-functional cytokine mediates acute and chronic inflammation, anti-tumor responses and infection. Biological activity mediated through interaction with 2 distinct receptors, TNFR1 and TNFR2
- 17kDa mature protein, cell associated or secreted. Forms trimer, with 3 binding sites for TNF receptor (and targeting antibody)

M. A. Palladino et al. (2003) Anti TNF-α Therapies : The Next Generation. Nat. Rev. Drug Discovery (2): 736

Crystal Structures Showed Adalimumab and Infliximab Bind Different Epitopes on TNFα

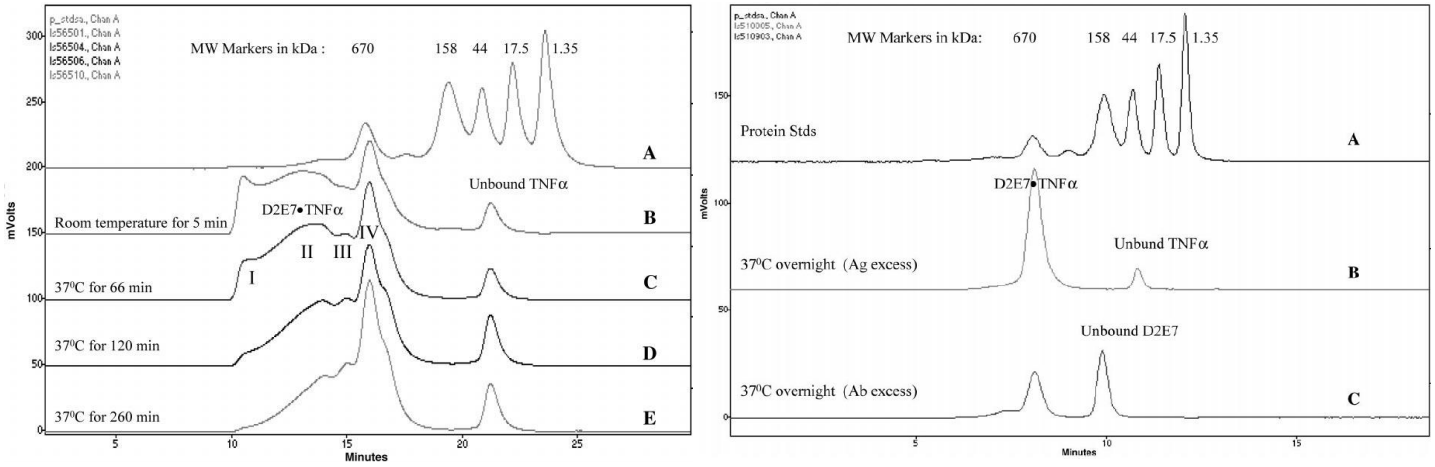


- Adalimumab Fab and Etanercept/TNFR2 bind to the interface of 2 adjacent TNFα protomers
- Infliximab Fab binds to the interface of 1 TNFα protomer
- Adalimumab Fab has larger binding surface to TNFα, and overlaps extensively with TNFα-TNFR2 interface.

	Binding surface to TNFα (Å^2)
Adalimumab	2,536
Infliximab	1,977
Etanercept	2,500

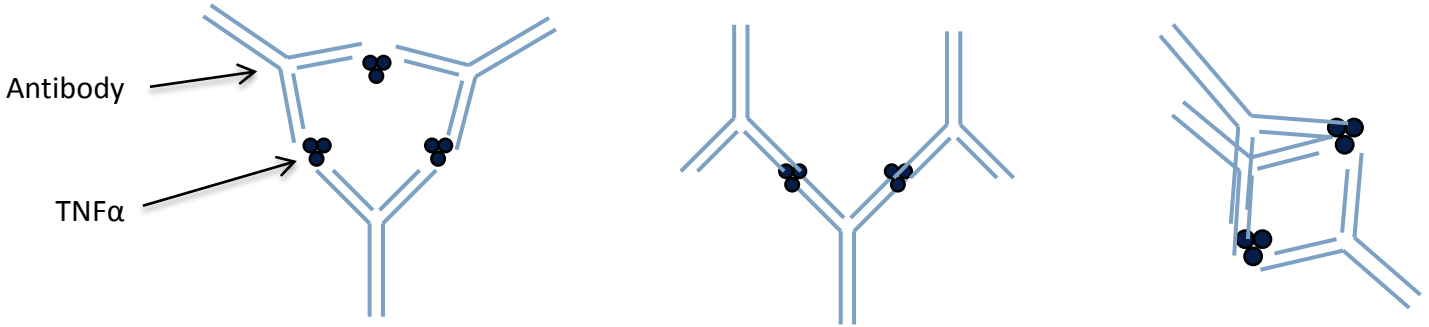
S. Hu et al. (2013) Comparison of the Inhibition Mechanisms of Adalimumab and Infliximab in Treating Tumor Necrosis Factor-Associated Diseases from a Molecular View. J. Biol. Chem. 288: 27059

Background - Stable Complex of Adalimumab (Intact IgG) with TNFα



- Adalimumab and TNFα form higher order aggregates upon initial complex formation.
- Adalimumab-TNFα forms stable complex of ~ 600 kDa (37°C overnight)

What is the structure of the stable complex between Adalimumab and TNFα ?



"Ring-like"
3:3 Complex
~600 kDa

or

"Open"
3:2 Complex
548 kDa

or

"Closed"
3:2 Complex
548 kDa

L. C. Santora (2001) Analytical Biochemistry 299, 119–129

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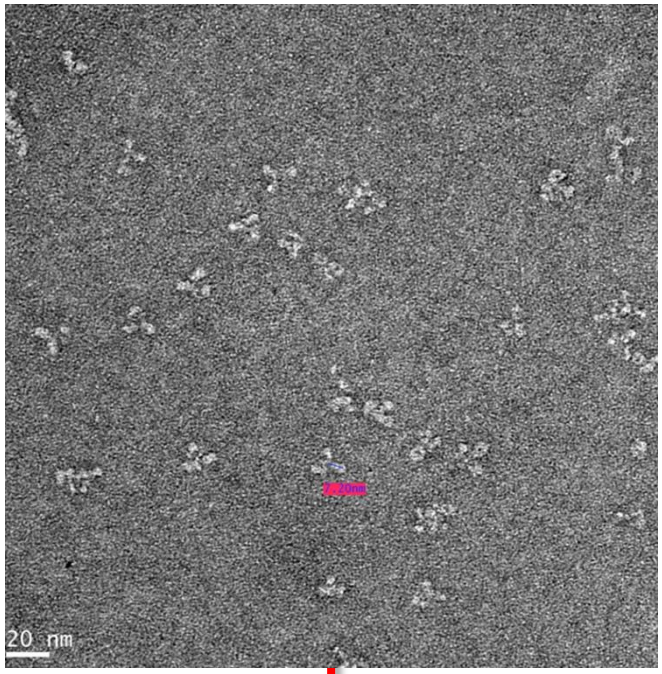
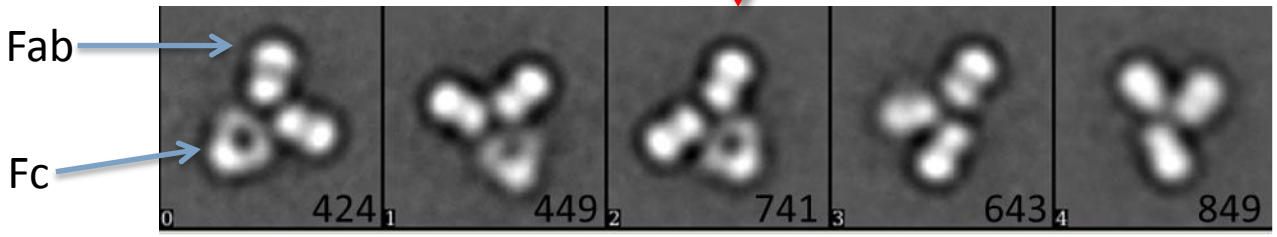
Adalimumab – TNFα Complex

Negative Stain Imaging of Adalimumab

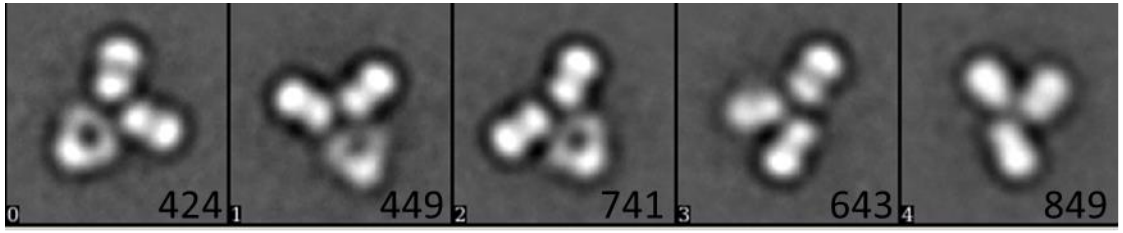


Class averaging with >3000 particles (67K magnification)

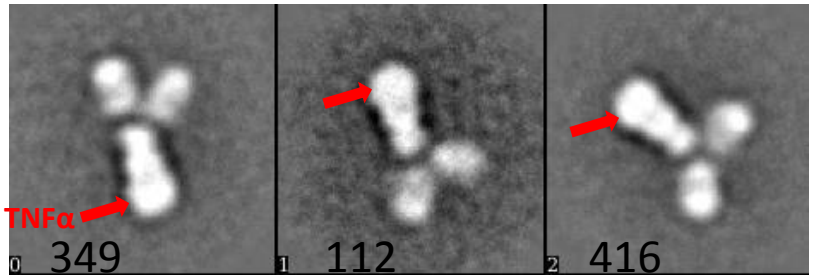


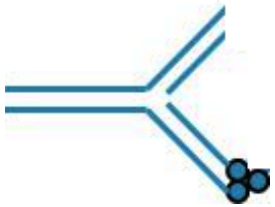
Tran B. et al. (2017) Protein Sci, 26:12, 2392-2398

Adalimumab-TNFα Complexes Observed Under EM (Negative Stain)



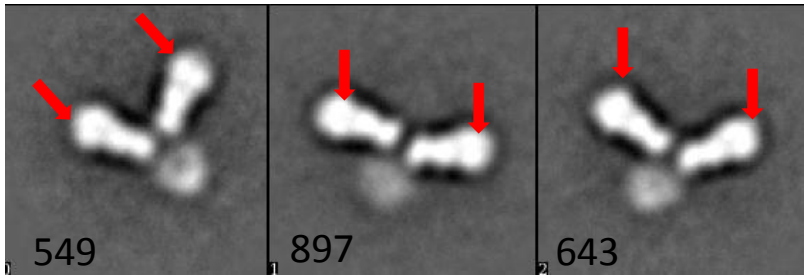
Adalimumab only

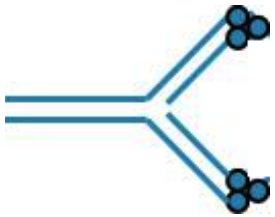




Adalimumab–TNFα
1:1

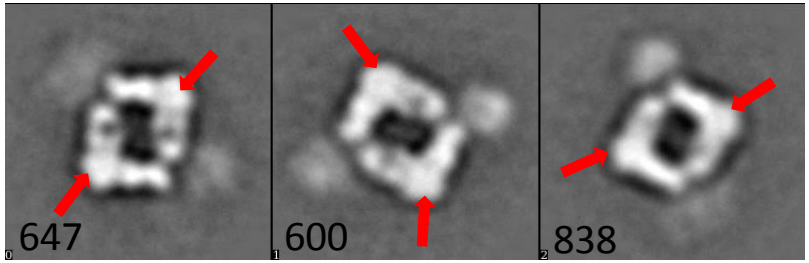
On-grid incubation

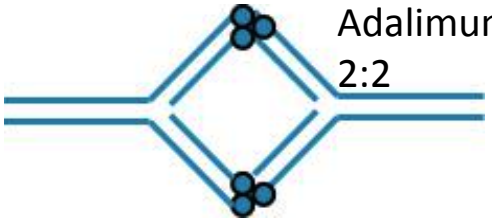




Adalimumab–TNFα
1:2

On-grid incubation





Adalimumab–TNFα
2:2

In-solution incubation (6X excess molar of TNFα)

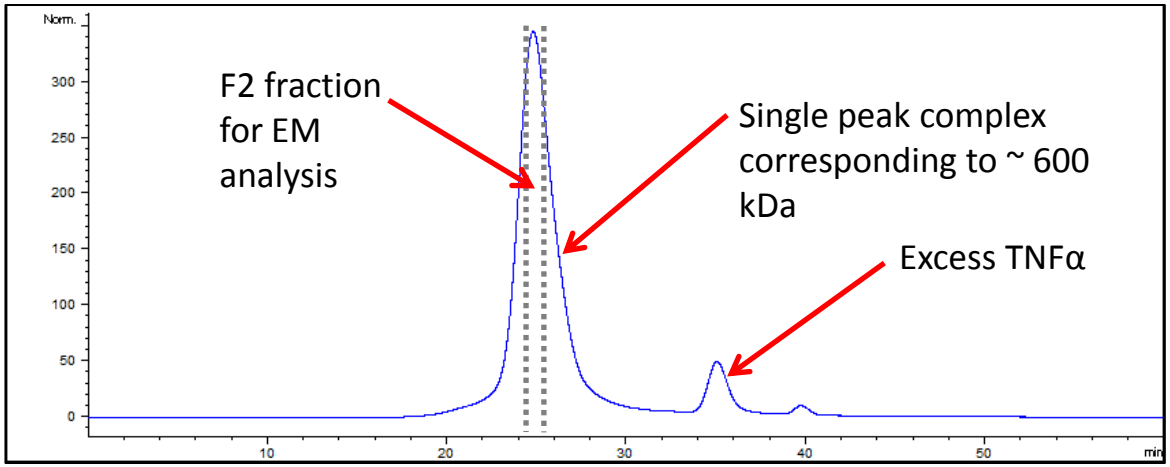
1:1, 1:2 and 2:2 Complexes Were Observed

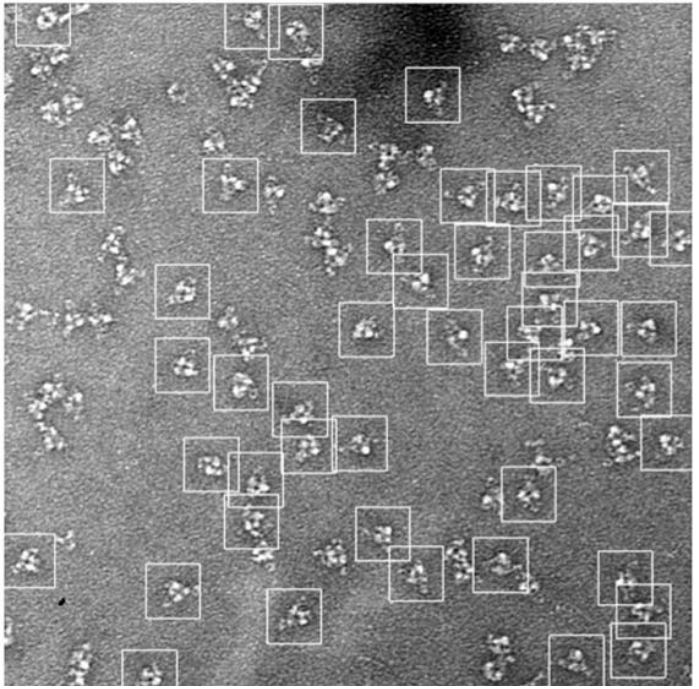
Tran B. et al. (2017) Protein Sci, 26:12, 2392-2398

Adalimumab – TNFα Stable Complex

Adalimumab-TNFα complex formation according to L. C. Santora, 2001 (Analytical Biochemistry 299: 119-129) :

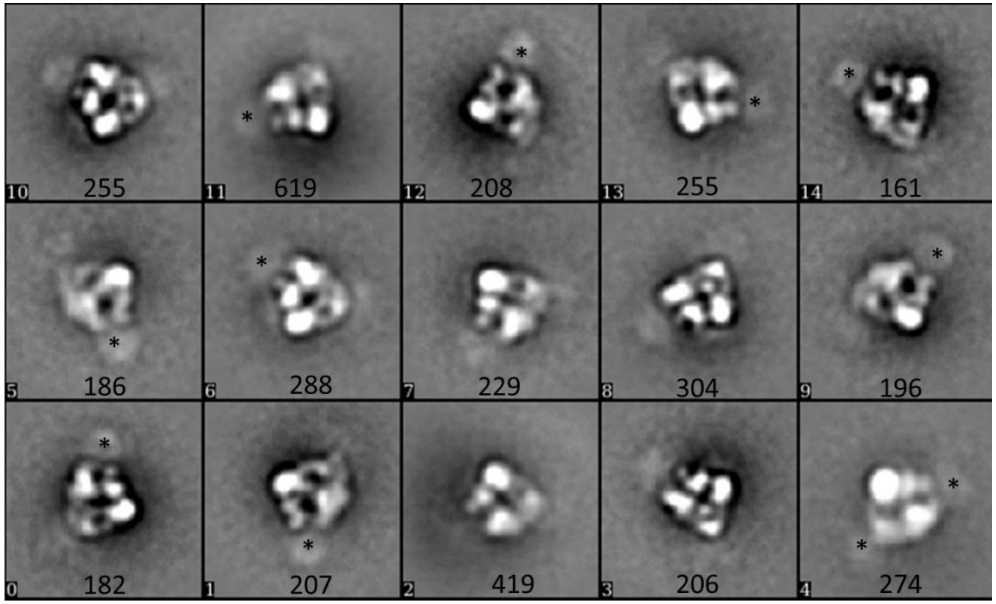
- Mixture of Adalimumab (2 mg/mL) and TNFα (0.8 mg/mL)
- Incubation at 37°C overnight , injected into SEC column (Superose 6 10/300 GL)
- Fraction of the main peak was collected for negative stain or Cryo-EM



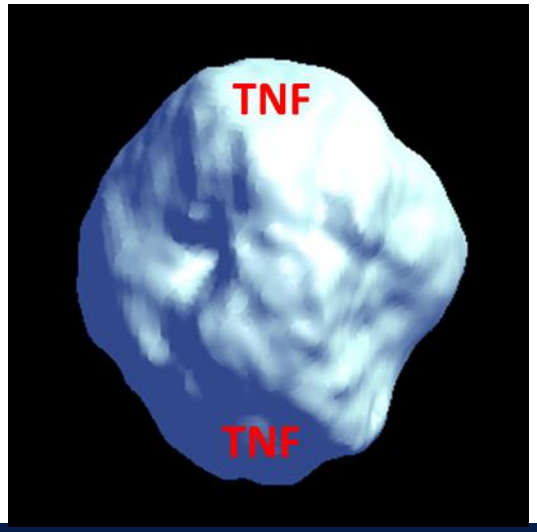


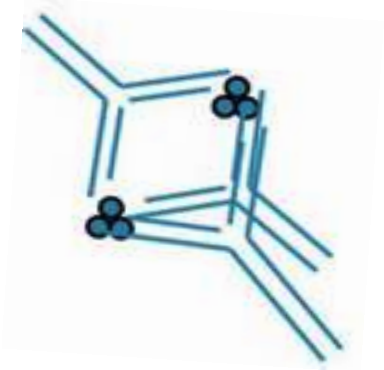
Negative Stain of SEC Fraction ~ 600kDa

Negative Stain EM of Stable Adalimumab-TNFα complex



* Fc





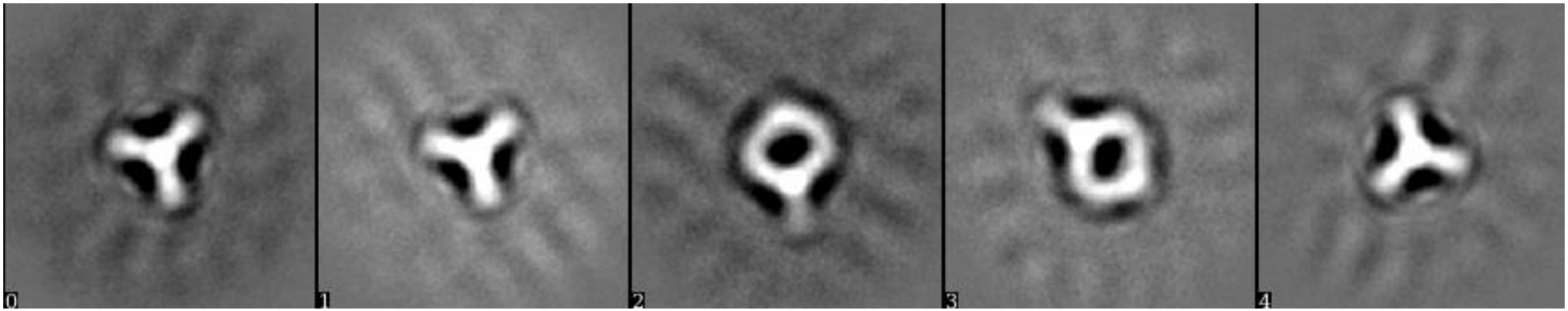
*Fc: not visible

- Single particle analysis Negative Stain EM Analysis
- Limited information obtained from negative stain imaging
- Class averaging from 100 frames at 67K magnification, 4004 particles
- Class averaging and low resolution sub-tomogram averaging suggest stable complex is 3:2 Adalimumab:TNFα complex, adopting a closed conformation
- Other 3:3 or 3:2 complex conformation were not observed under EM

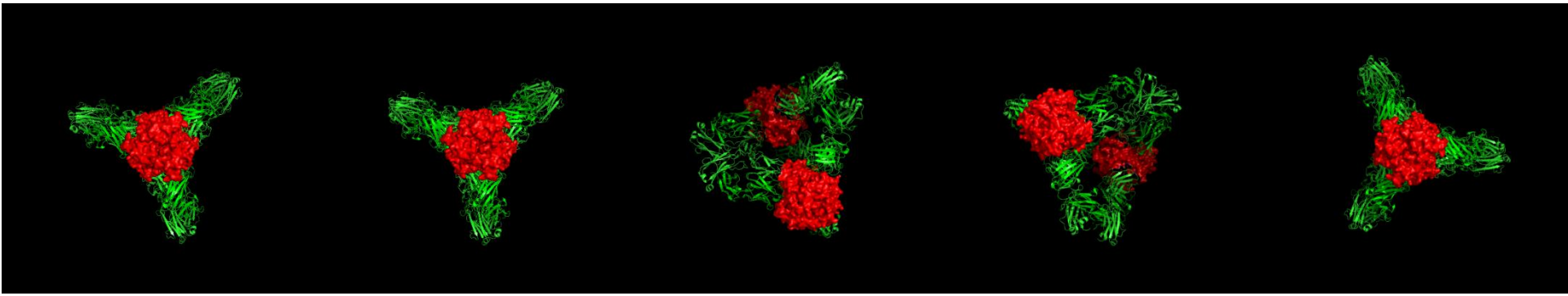
Cryo-EM of Adalimumab-TNFα stable complex

Cryo-EM Revealed Assembly of Adalimumab-TNFα Stable Complex (3:2 Closed Conformation Complex)

>18K particles, 5 classes from 2D Class Averaging (Cryo-EM) (Fc is flexible and not visualized here)



Orientation of 3:2 Complex - Model adapted from crystal structure. (Fc is not visualized here)



Cryo-EM images clearly showed 3:2 closed conformation of Adalimumab-TNFα Stable Complex

Adalimumab Fab (Green); TNFα Trimer (Red)

Tran B. et al. (2017) Protein Sci, 26:12, 2392-2398

Cryo-EM Revealed Assembly of Adalimumab-TNFα Stable Complex (3:2 Closed Conformation Complex)

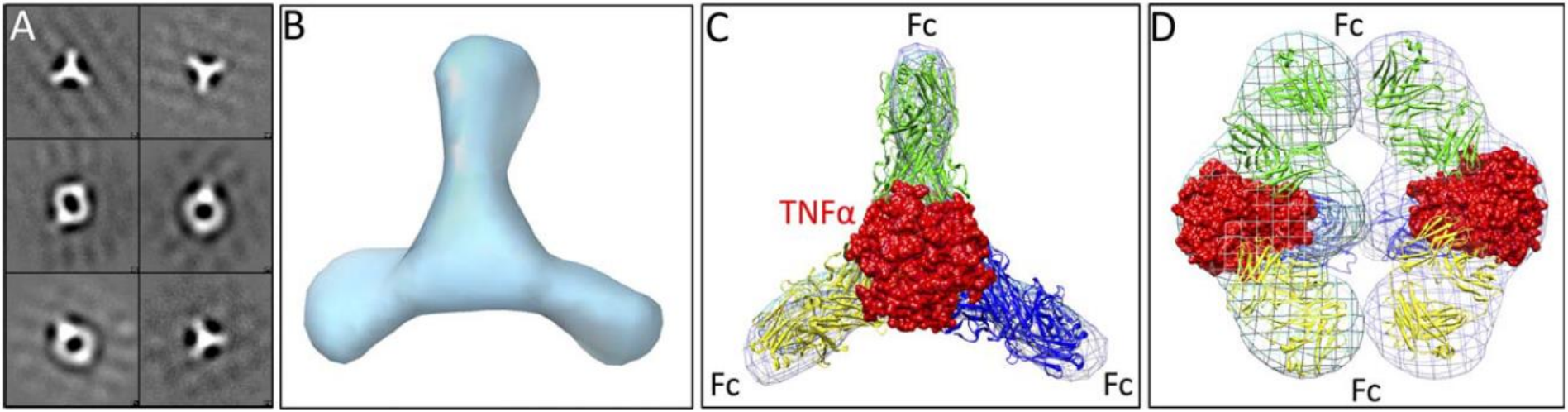


Figure 3. Analysis of Adalimumab-TFNα (3:2) complex by cryo-EM. (A) Representative classes of Adalimumab-TNFα (3:2) complex after 2D class averaging analysis. (B) 3D reconstruction of EM volume from 2D class averages showing the trigonal shape. (C) Superimposition of cryo-EM 3D volume (mesh figure) with X-ray model for Adalimumab-TNFα (3:2) complex, showing a structure with 3-fold symmetry. (D) Circular structure as viewed after 90° rotation along vertical axis of structure shown in (C). Fc is not visualized in the cryo-EM structure, with the approximate position of Fc depicted in the figure.

3:2 stable complex with closed conformation → All epitopes on Adalimumab and TNFα are fully occupied

Can we obtain higher resolution structure?

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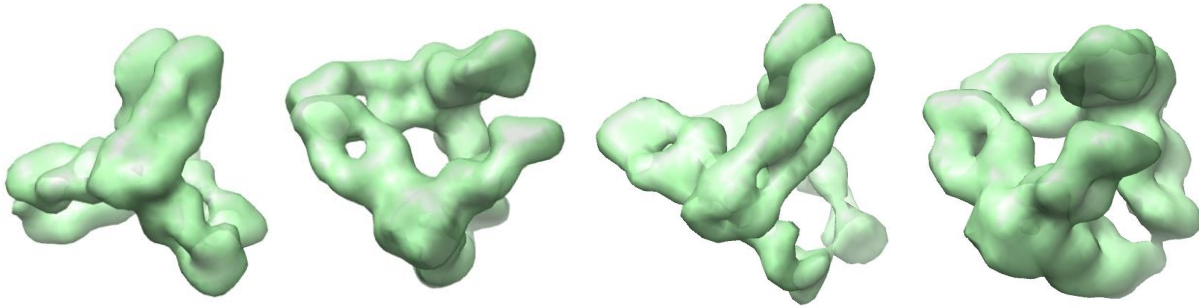
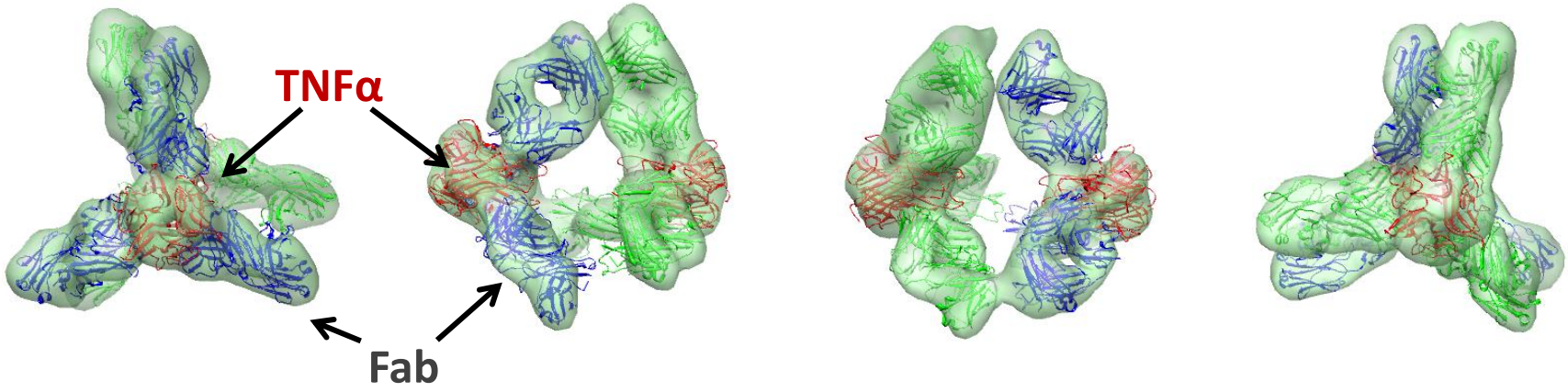
High Resolution Cryo-EM : Higher Order Complexes between Adalimumab and TNFα

Data collection with Volta Phase Plate (VPP), with > 600K particles

2D class average

3D reconstruction model

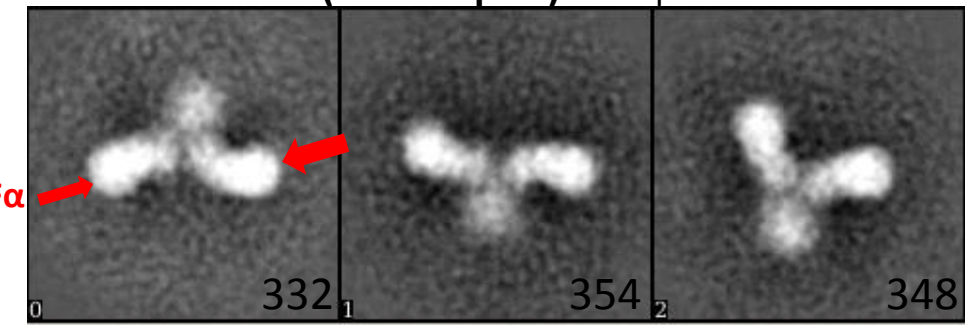




Infliximab (Remicade) – TNFα Complex

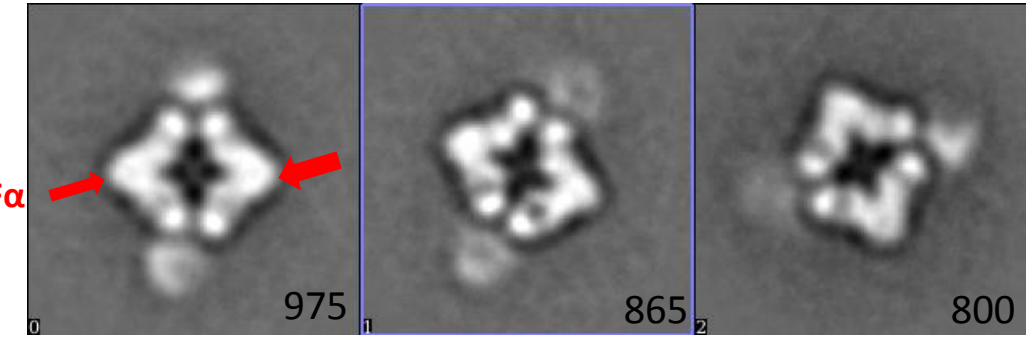
Infliximab-TNFα Complexes Observed Under Negative Stain EM

Infliximab-TNFα (1:2 complex) 1034 particles



On-grid incubation

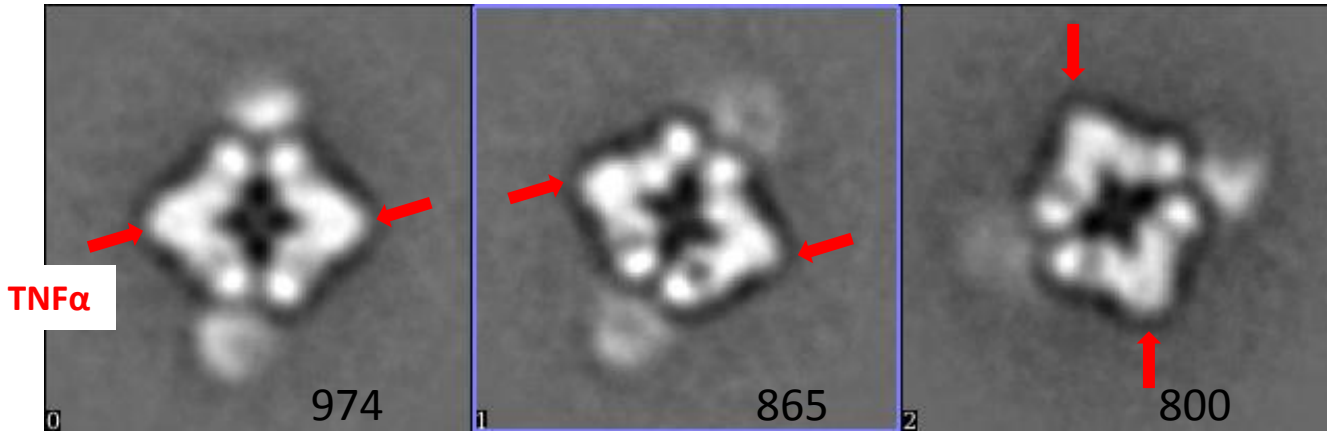
Infliximab-TNFα (2:2 complex) 2640 particles

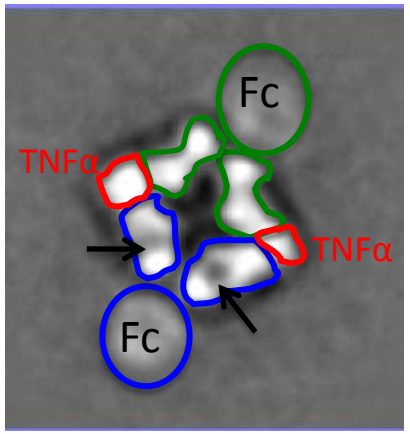


In-solution incubation (6X excess molar of TNFα)

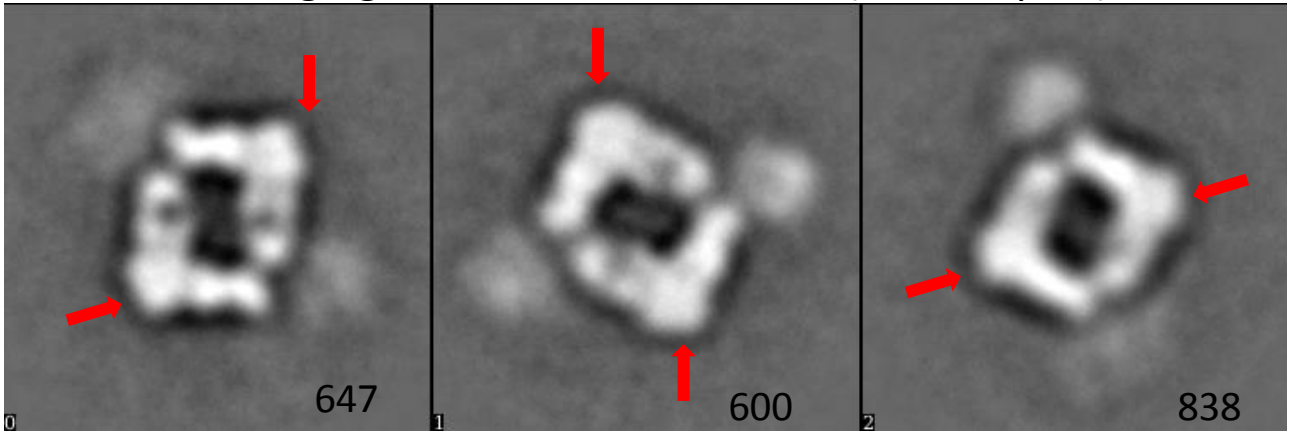
Infliximab-TNFα Complexes Observed Under Negative Stain EM

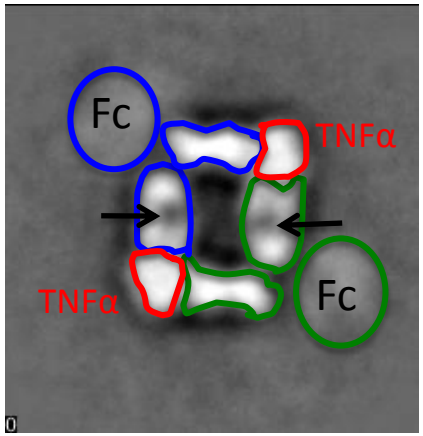
2D class averaging for **Infliximab-TNFα** (2:2 complex)





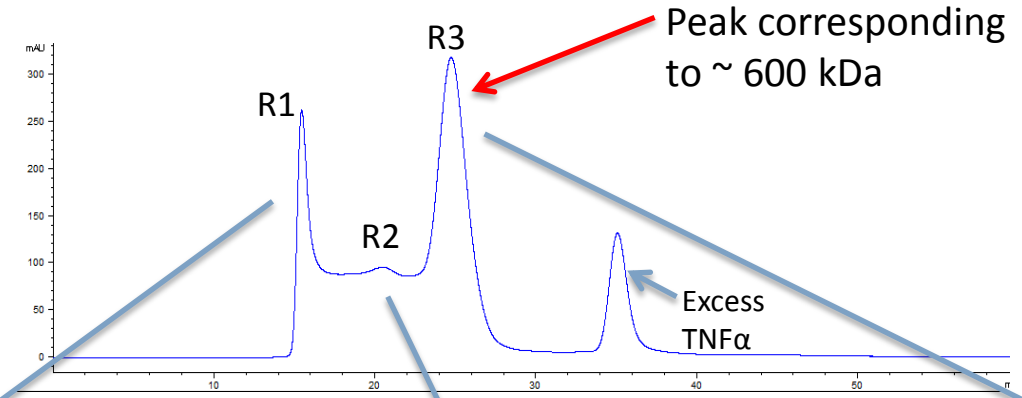
2D class averaging for **Adalimumab-TNFα** (2:2 complex)



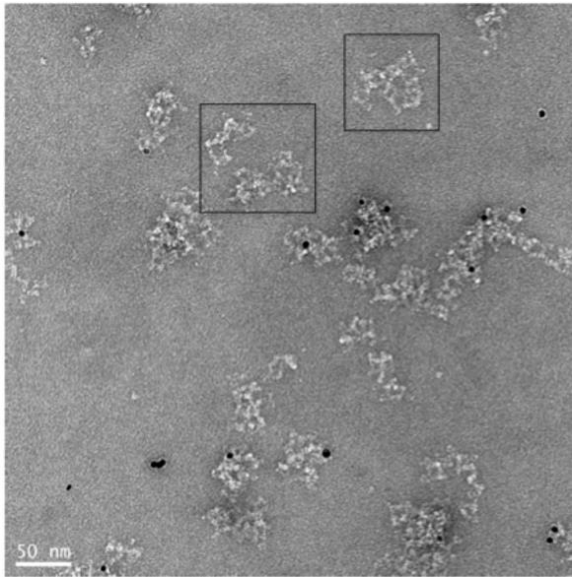


Different Fab orientation when comparing 2:2 complexes between Infliximab-TNFα and Adalimumab-TNFα

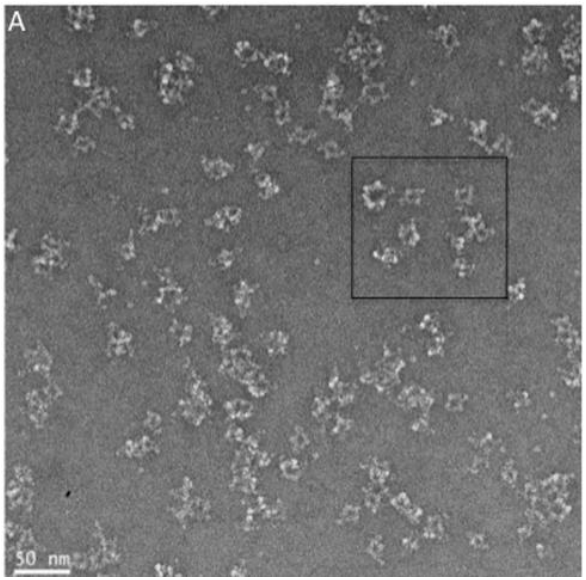
Infliximab - TNFα Stable Complex – Higher Order Aggregates Observed



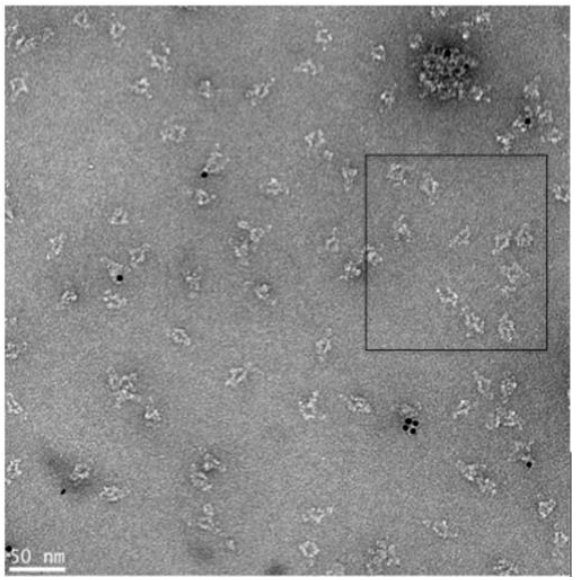
Infliximab-TNFα (R1 fraction)



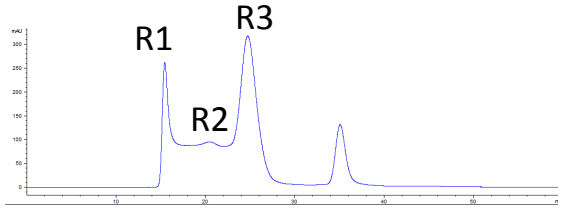
Infliximab-TNFα (R2 fraction)



Infliximab-TNFα (R3 fraction)



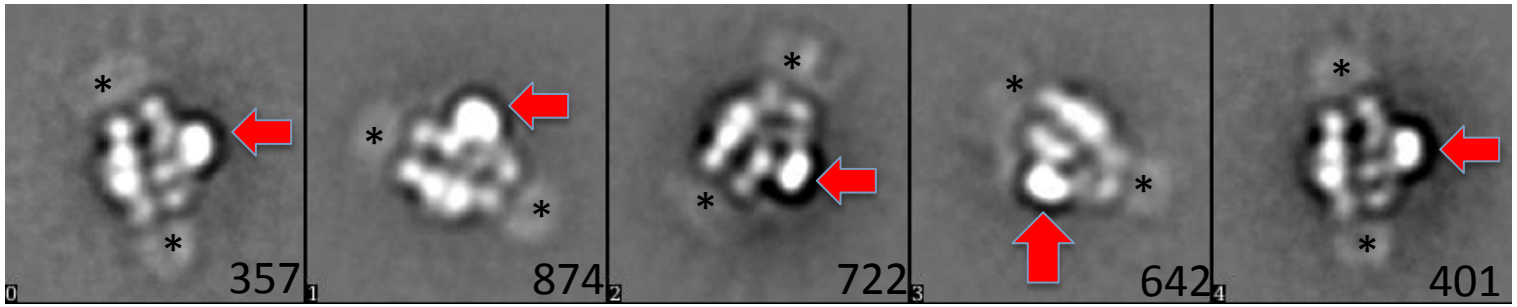
Infliximab - TNFα Stable Complex (Negative Stain)



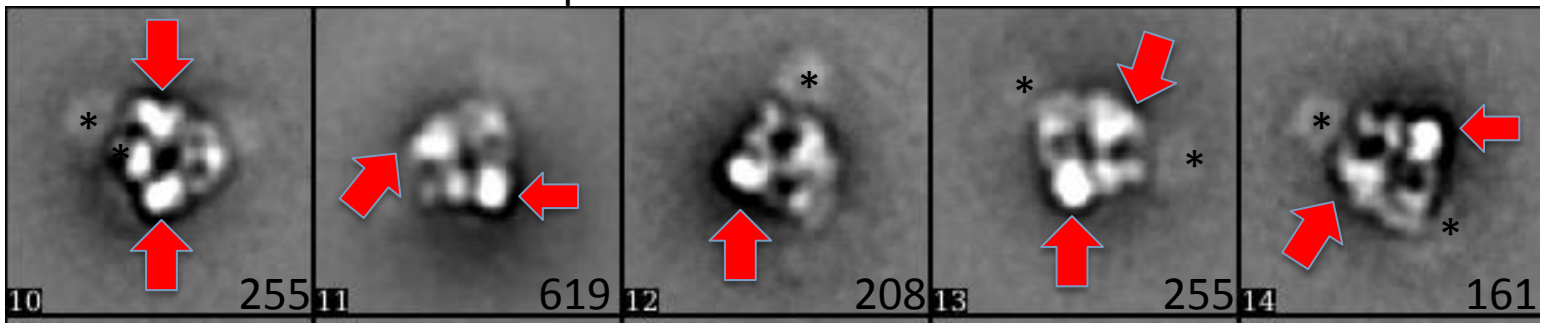
Infliximab-TNFα (R3 fraction)

* Fc

TNFα or IgG



Adalimumab-TNFα 3:2 complex



Limited structural information due to low resolution and preferred orientation

SUMMARY

- Negative Stain EM class averaging able to produce low resolution images for mAb, bispecifics and complexes with antigens

- Structures of Adalimumab-TNFα complexes.
  - Observed 1:1. 1:2, 2:2 and 3:2 complexes between Adalimumab and TNFα
  - Different 2:2 structures when comparing Adalimumab-TNFα and Infliximab-TNFα
  - Closed conformation of the 3:2 stable complex

- Cryo-EM imaging provided high resolution structures, to support 3:2 closed conformation complex structure of Adalimumab-TNFα