Robust multi parameter immunomonitoring; Polyfunctional T cell analysis by FluoroSpot

From ELIspot to Fluorospot

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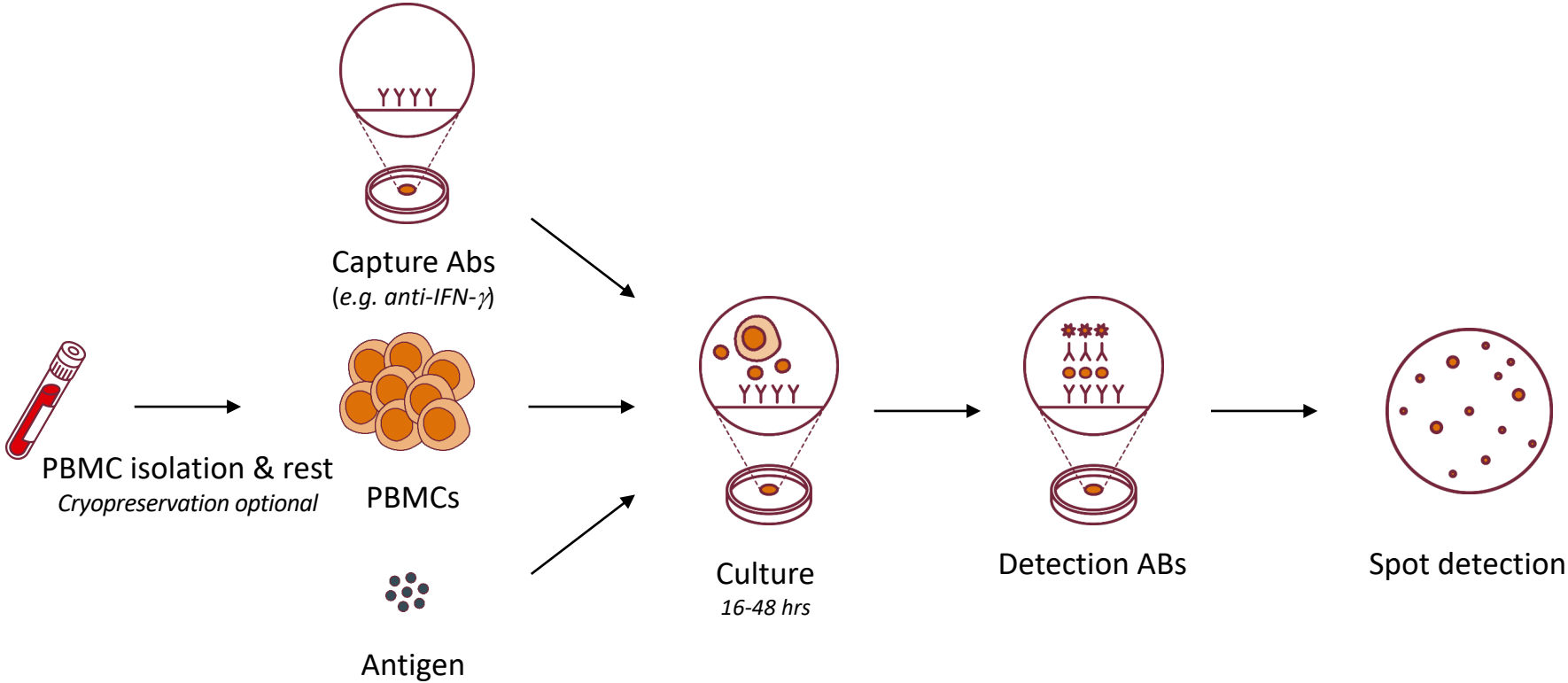
ELISpot / Fluorospot assay

- The ELIspot (enzyme-linked immunospot) assay is a test to determine the frequency of antigen-specific cells by determining effector molecule secretion at a single cell level
- The Fluorospot assay is a similar test
  - Fluorophores are used instead of using enzymatic detection, allowing for the readout of multiple parameters
- Common effector molecules used:
  - IFN-γ, Granzyme B, perforin, TNF-α – cytotoxic $CD8^+$ T cells
  - IL-2, IL-5, IL-10 and IL-17A – helper $CD4^+$ T cells
  - Antibody (and isotypes) – B cells
- Highly sensitive and high throughput

How do ELISpots/Fluorospot work?

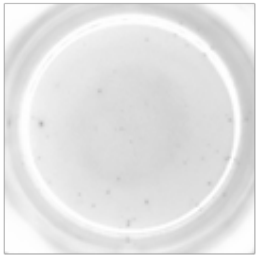


Example images

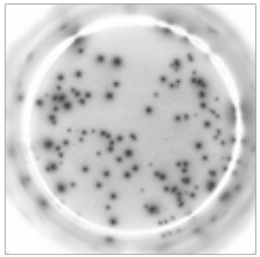
IFN-γ | IL-2
TNF-α | Triple

ELIspot

Neg control

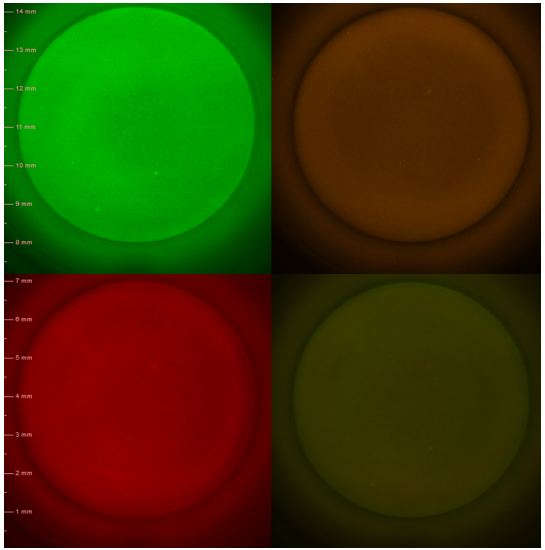


Pos control



Fluorospot

Neg control



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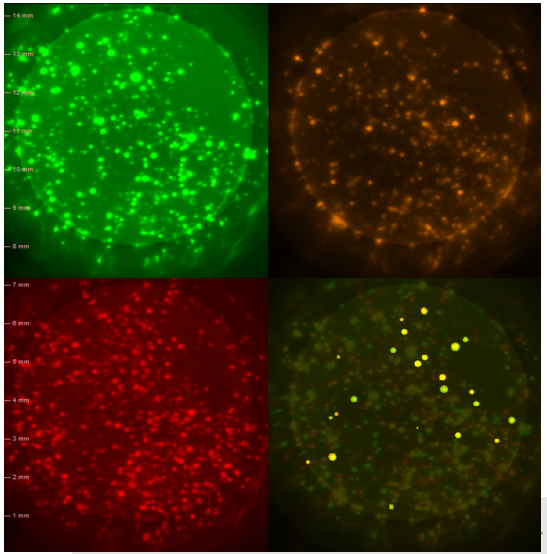


Focus of this talk: From ELIspot to Fluorospot

Assay optimization

Remains the same: Medium, thawing, resting, peptide stimulation

Needs optimization: incubation time, determining upper limit of quantification

Upper and lower limit of quantification

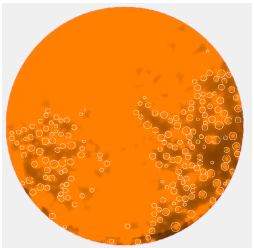
Incubation time, some cytokines need longer than others

Example data:

SARS-CoV-2 T cell responses using fluorospot

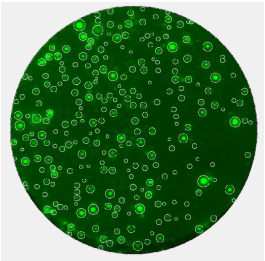
Range of detection

- **Lower limit**: each spot is 1 cell producing a cytokine, lower limit depends on donor specific background
- **Upper limit**: how many cells can still be analyzed, too close together and the software can't distinguish separate spot
  - Depends on size of the spots (donor dependent, assay length, coating concentration)
  - Fluorospot setting (some readers do this automatically)



Granzyme B

- Example: Too bright/ too many spots
- Under representation of number of spots
- Action: settings or cell number



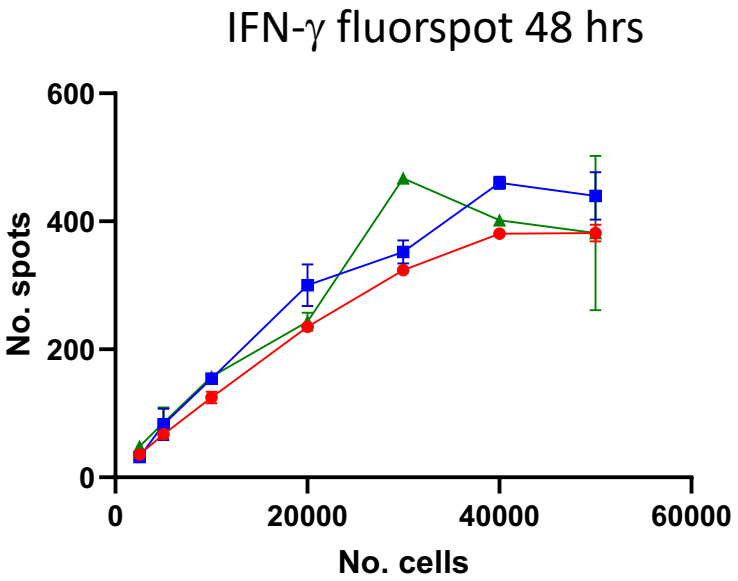
IFNy

- Example: looks alright
- Action: verify settings using negative controls

How to determine your upper limit of quantification?

Pan-CD3 stimulation to determine the range of spots that can be analysed

When the machine determines it is too high to count, we used the highest number of spots present in the plate to visualize the data



How many spots result in $R^2 > 0,95$?

How to determine your upper limit of quantification?

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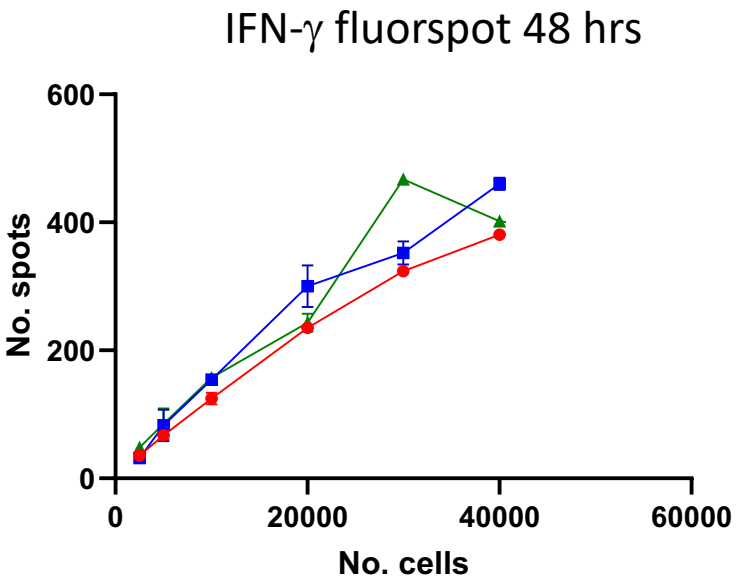

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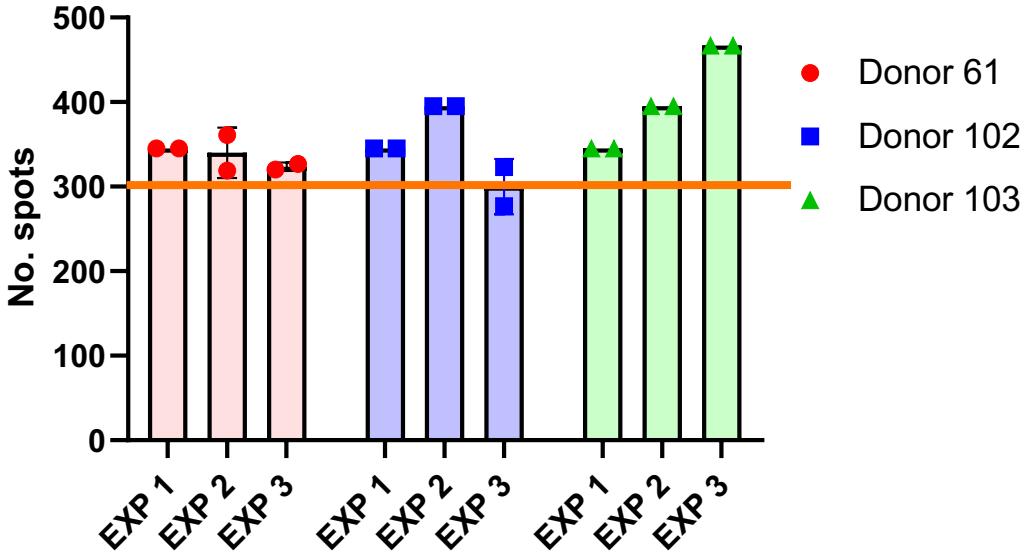


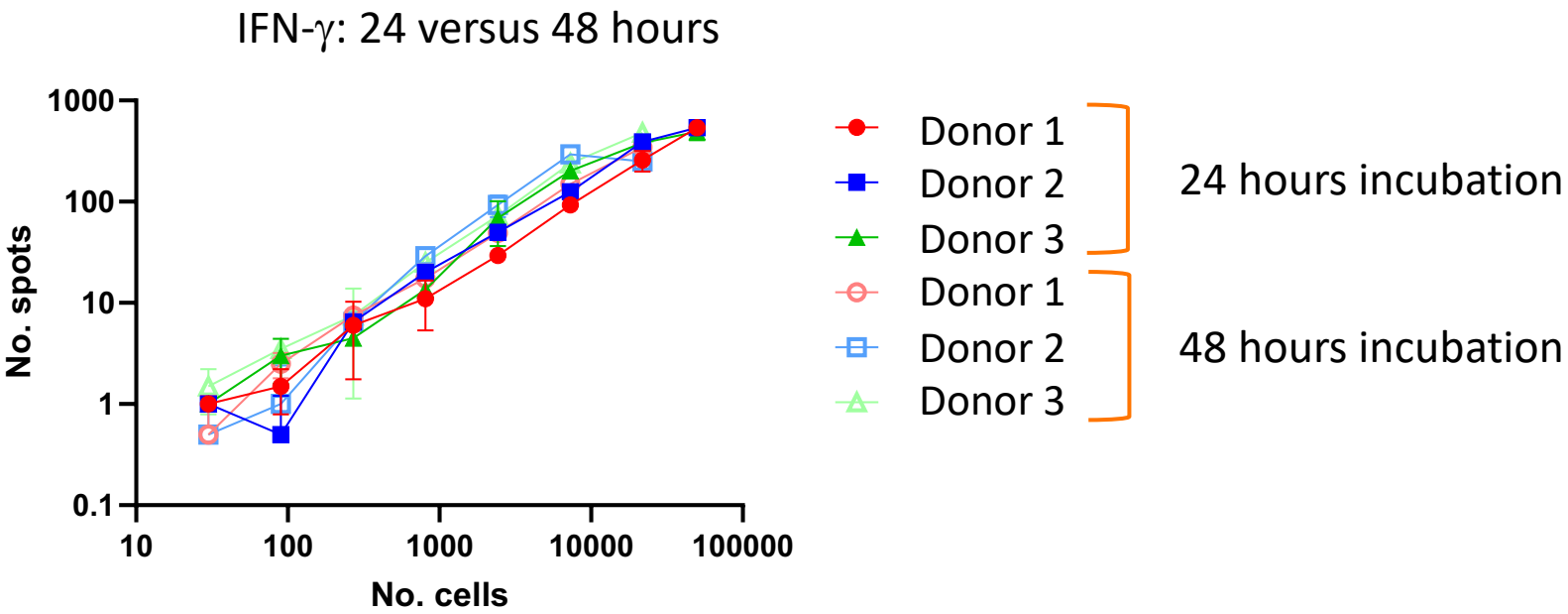
- IFN-γ fluorspot 48 hrs
- Similar results for IL-2
- Higher cell numbers for TNF-α
- Smaller spot size?

Combining cytokines

- Cytokines can have different dynamics
  - IFN-γ : within 24 hours (based on ELIspot)
  - IL-2 and TNF-α: 24 to 48 hours recommended
  - IL-17 and IL-5: 48 hours recommended

- To be able to combine the cytokines we need to compare 24 hours incubation to 48 hours incubation

Comparing 24 to 48 hours of incubation IFN-γ

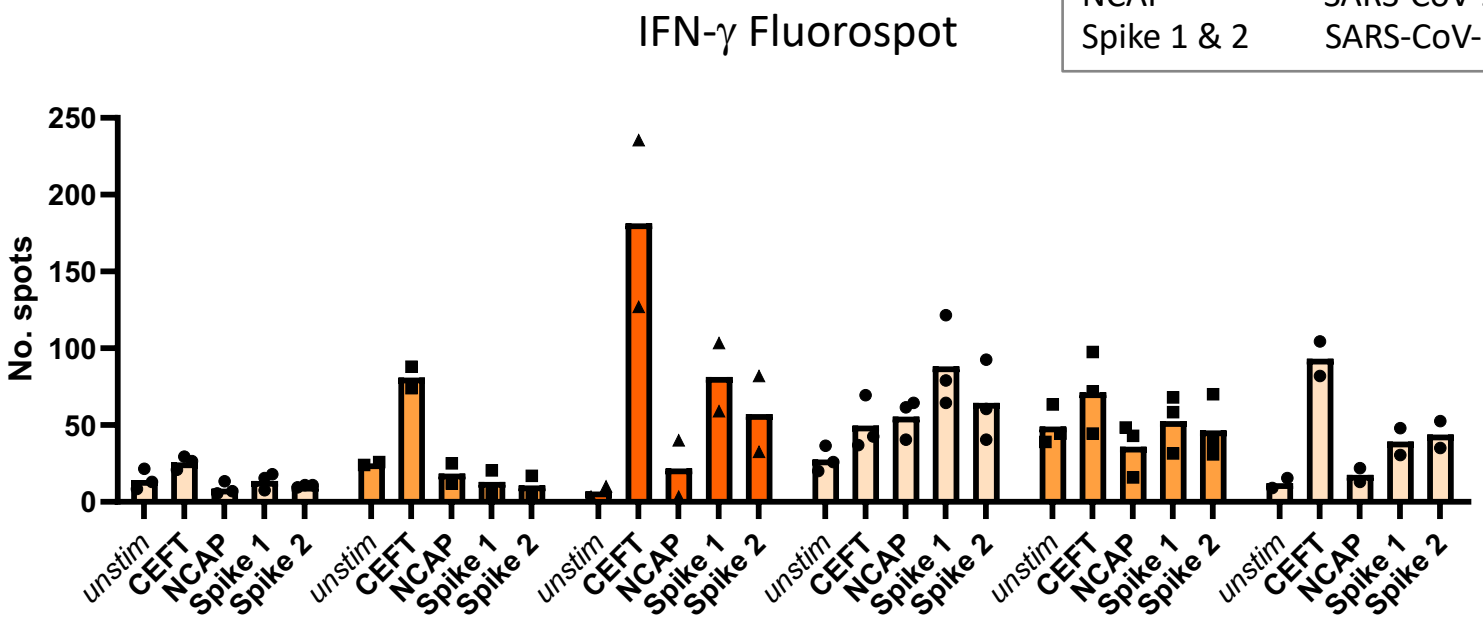


- **So now we are ready to look into some real data…**

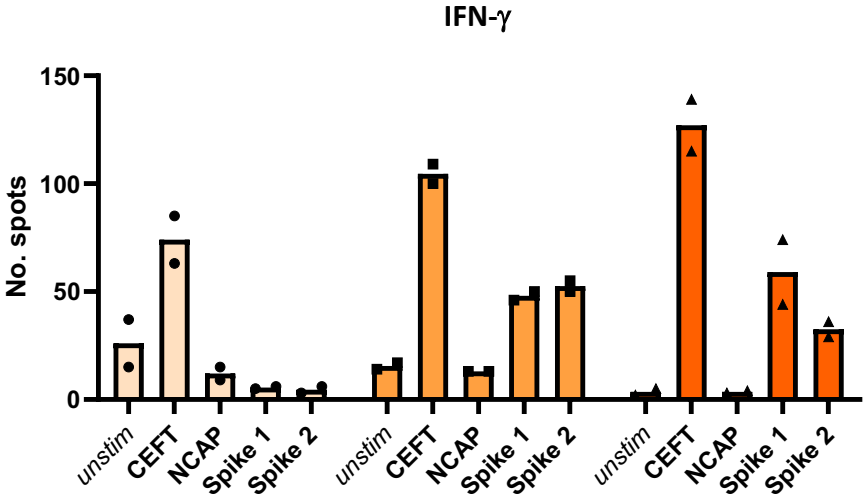
SARS-CoV-2 data

Conditions:

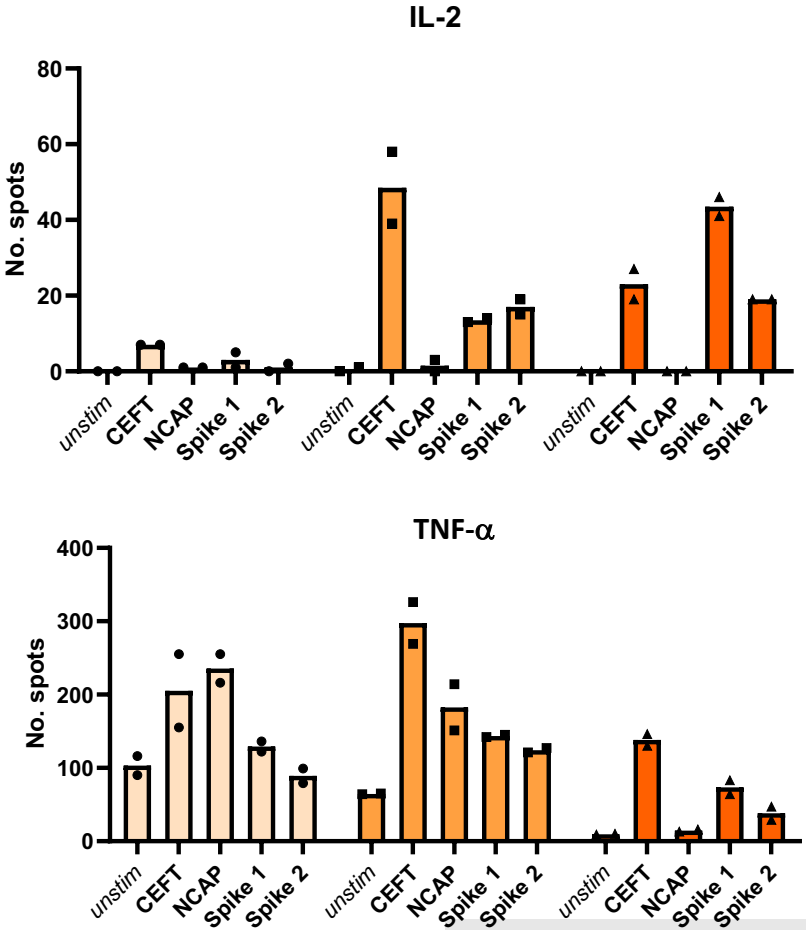
Unstim	Cells only / neg contr
CEFT	CMV/EBV/FLU/TT peptide pool
NCAP	SARS-CoV-2 peptide pool
Spike 1 & 2	SARS-CoV-2 peptide pools



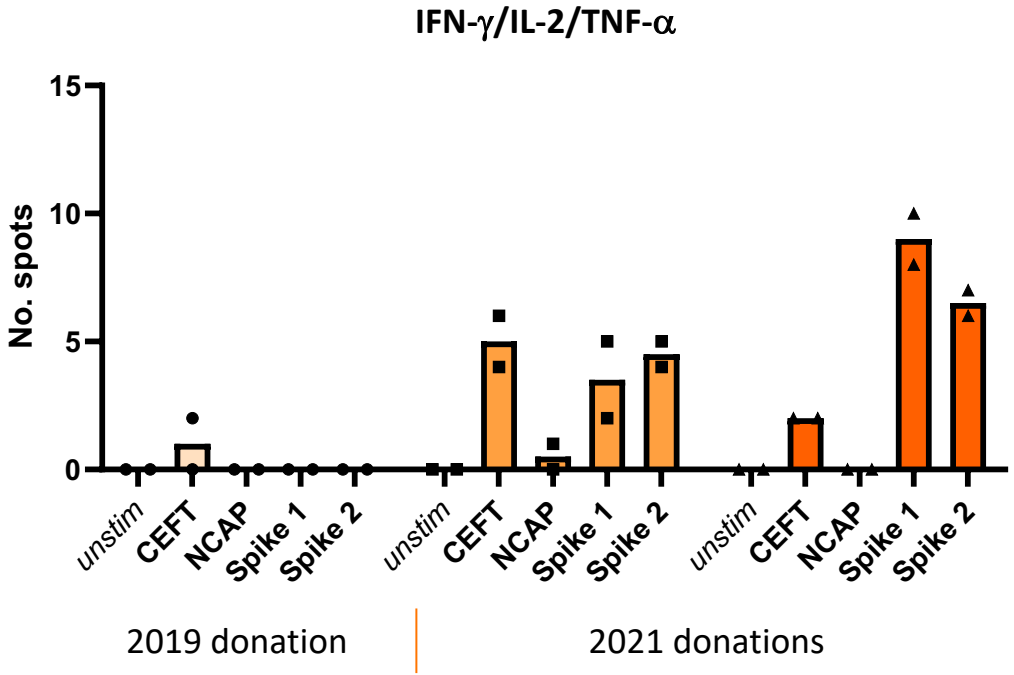
SARS-CoV-2 data



2019 donation | 2021 donations

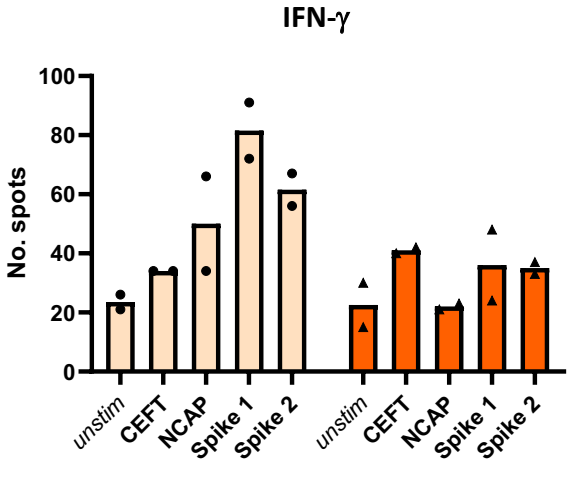


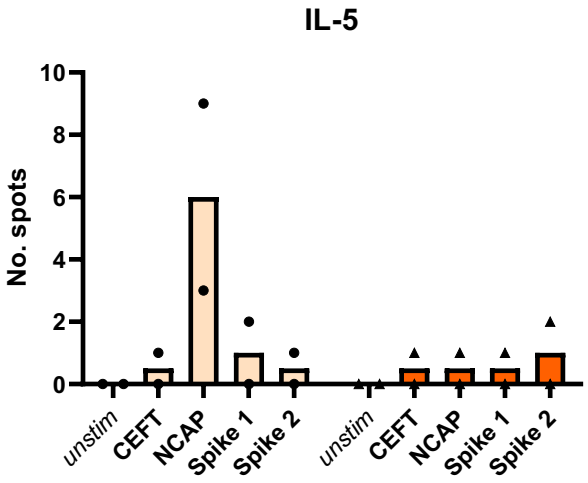
SARS-CoV-2 data

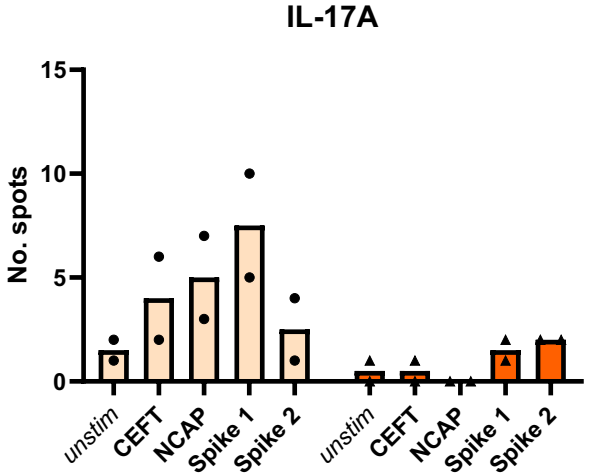


- Polyfunctional cells present
  - Dual and triple responses similar

SARS-CoV-2 data - T cell skewing cytokines







- Nice single cytokine responses
- No double/triple cytokines responses

Conclusion / Discussion

- Standard IFN-γ ELIspot can be easily extended with multiple cytokines with Fluorospot
- Different cytokines result in different upper limits of quantification
- Polyfunctional T cells can be analysed by looking for double or triple positive spots
- T cell skewing cytokines IL-5 and IL-17A could also be found, but rarely co-express IFN-γ
- Reproducibility should be further investigated with validation study

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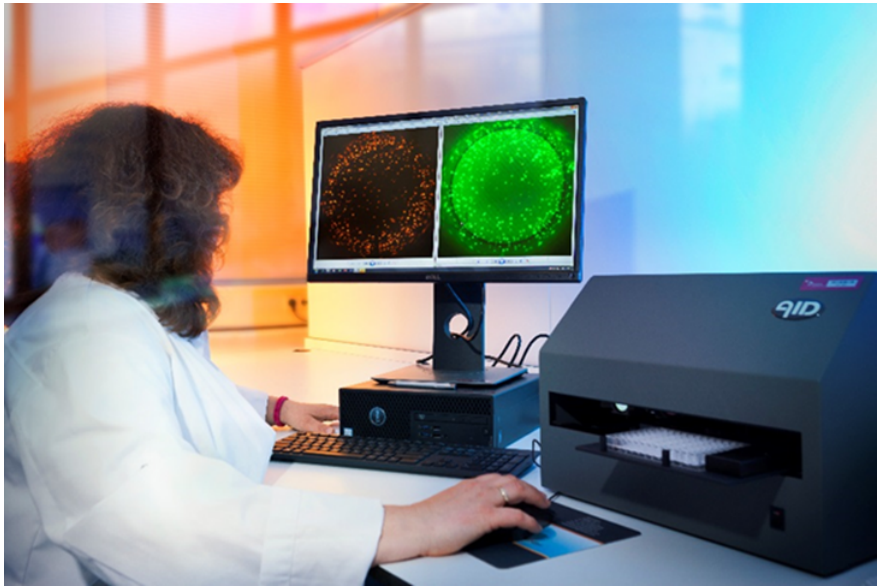
  - Anja ten Brinke
  - Annick de Vries

Special thanks to

- Blood bank donors



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