Single-cell deep phenotyping of IgG-secreting cells for high-resolution immune monitoring

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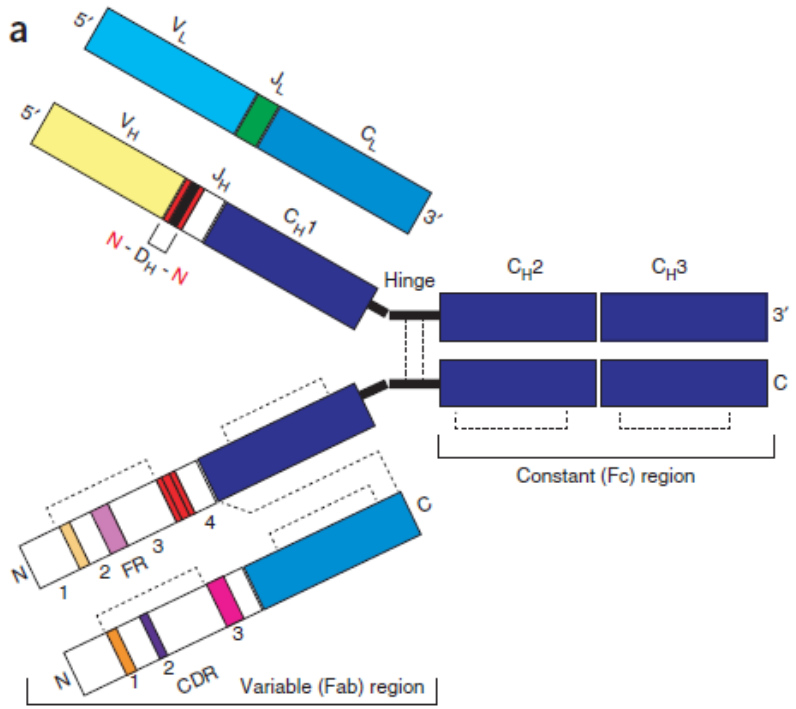
Introduction

nature biotechnology

The promise and challenge of high-throughput sequencing of the antibody repertoire

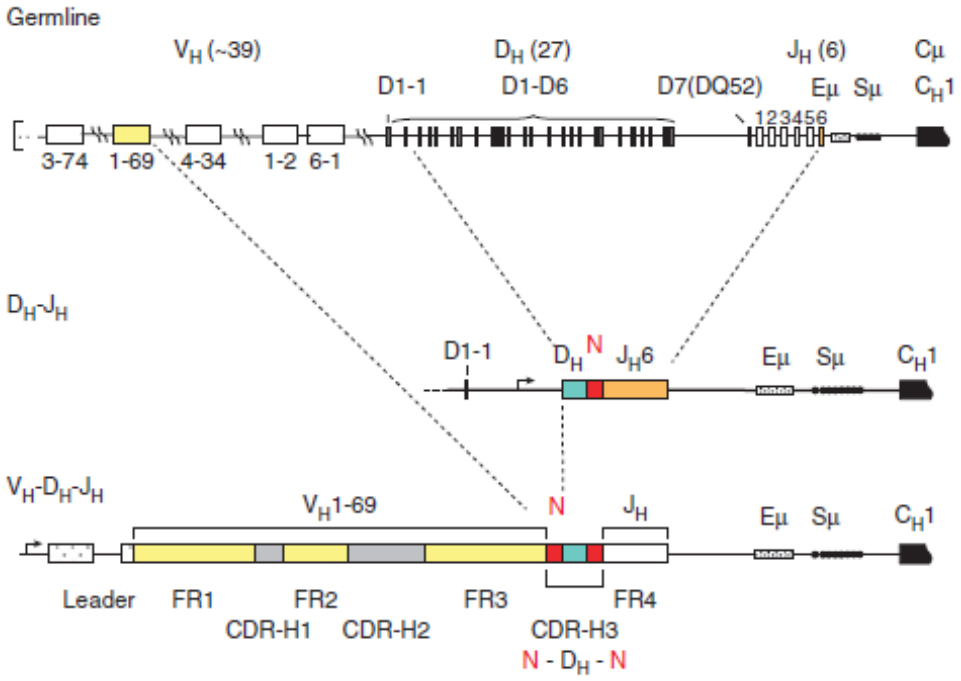
George Georgiou[1–4], Gregory C Ippolito[3,4], John Beausang[5,6], Christian E Busse[7], Hedda Wardemann[7] & Stephen R Quake[5,6,8,9]

Introduction: Generation of antibodies



- The adaptive immune system relies upon the generation of a diverse repertoire of B cell receptors (BCRs) = membrane-bound form of immunoglobulins (Ig) expressed on the surface of B cells
- Igs consist of a heavy chain (μ, α, γ, δ, ε) and a light chain (κ, λ)

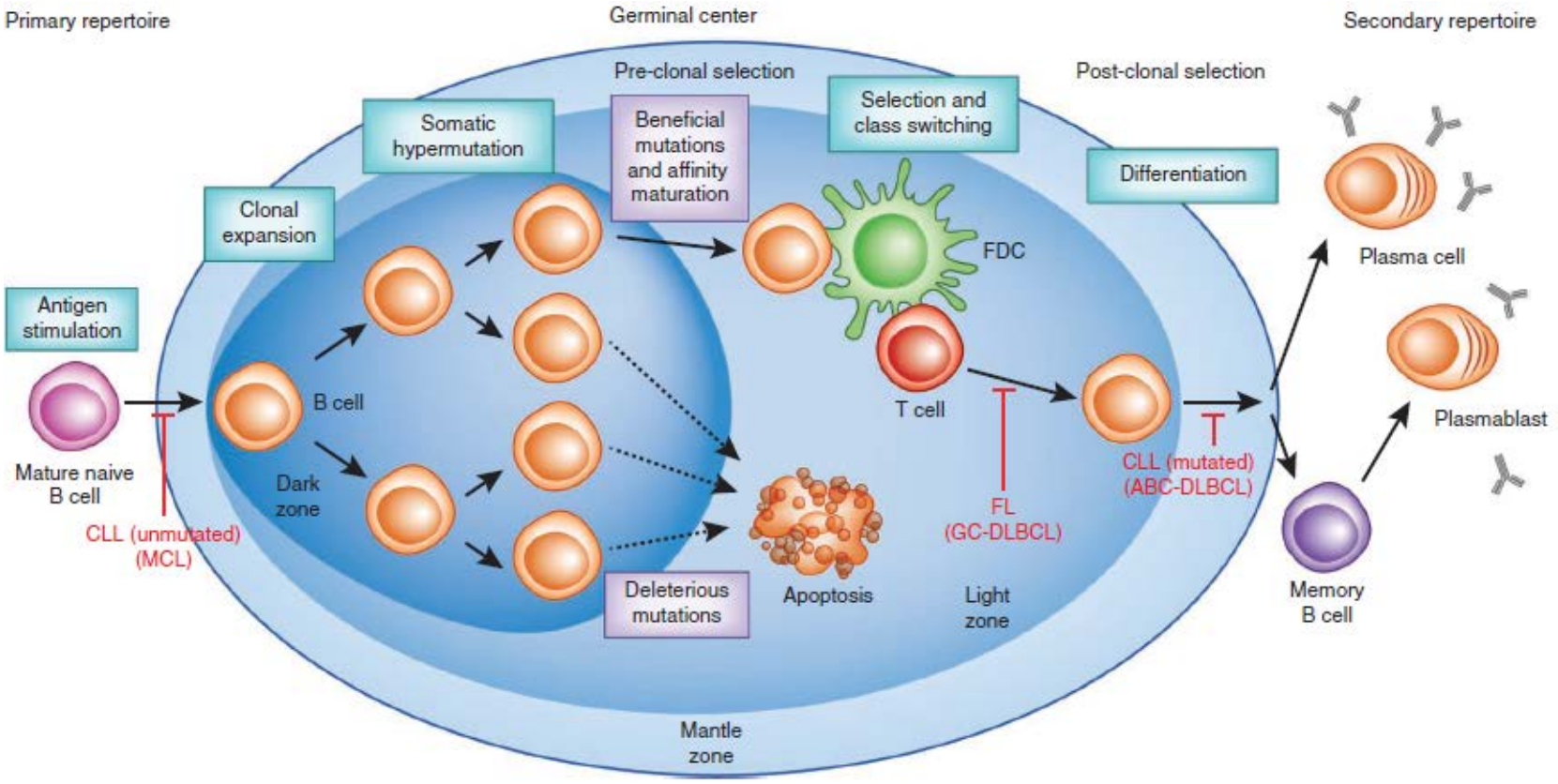
Introduction: Generation of antibodies



- Igs are assembled by somatic V(D)J recombination of a large number of Ig gene segments
  - heavy chain: ~40 V_H, 27 D_H, 6 J_H segments
  - light chain: 40 Vκ, 5 Jκ + 31 Vλ, 4 Jλ segments
  - deletion of nucleotides at the junctions between ligated segments by DNA exonucleases and insertion of palindromic/random nucleotides by DNA polymerases and transferases
  - sites of highest diversity (sequence and length): Complementarity-defining regions (CDRs)
- Successful rearrangement -> expression of BCR on B cell surface

Introduction: Maturation of antibodies

- B cell proliferation requires three signals:
  - BCR binds an antigen
  - Presentation to T helper cell via MHC Class II + activation by T cell using CD40L
  - Costimulatory cytokines



- clonal expansion in germinal centres of secondary lymphoid organs
  - somatic hypermutation of the variable domains by activation-induced cytidine deaminase (cytosine -> uracil, which is recognised as thymidine by DNA repair enzymes)
  - affinity maturation: B cells with highest-affinity BCR undergo preferential expansion and survival
  - class-switch from IgM to other Ig isotype (e.g. IgG)

Introduction: Maturation of antibodies

- B cells expressing high-affinity BCRs can terminally differentiate into
  - long-lived memory B cells: rapid recall response to rechallenge with antigen
  - plasma cells: downregulate BCR expression, reside in bone marrow / MALT, secrete protective antibody at very high rate (10’000-20’000 Ig/s) -> may persist indefinitely

➢ >10^{13} possible antibody molecules

  ➢ How does one analyse such a diverse repertoire?

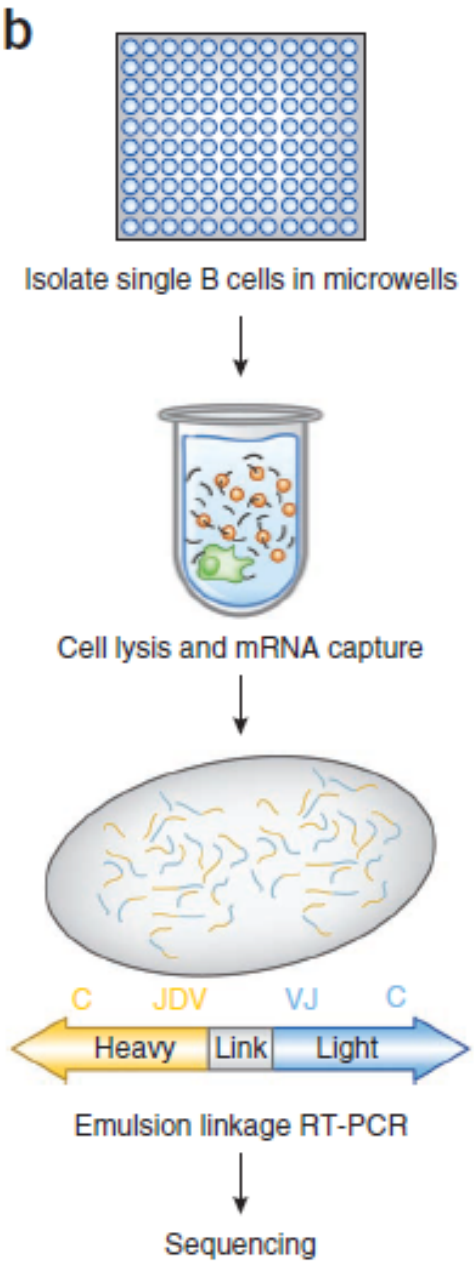
Ig-seq

- mRNA reverse transcription, then amplification of resulting cDNA using primers complementary to the rearranged V-region gene
- If done in bulk the information on which VH and VL chains were paired in the same cell is lost
  - many cells are lysed in bulk
  - VH and VL genes are amplified in separate reactions

➢ single-cell approach is required to accurately profile antibody response

Ig-seq: single cell approaches

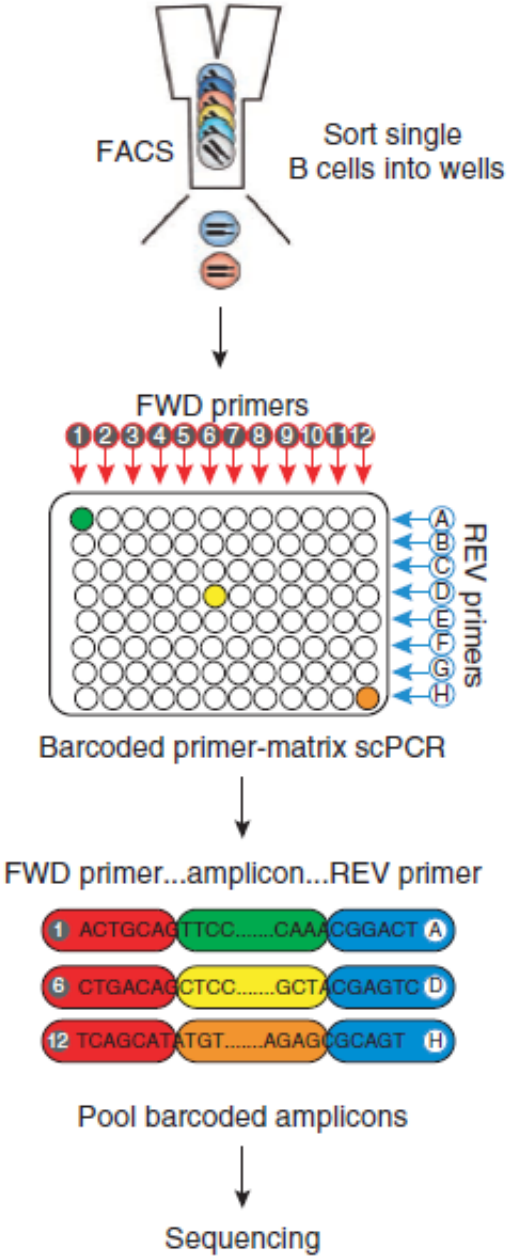
- single B cells are sequestered into subnanoliter volume wells
- cell lysis
- RNA capturing on magnetic poly-dT beads: bind polyadenylated mRNA
- Beads are collected and washed
- linked VH:VL segments are generated using overlap PCR



DeKosky *et al.*, *Nat Biotechnol*., 2013

Ig-seq: single cell approaches

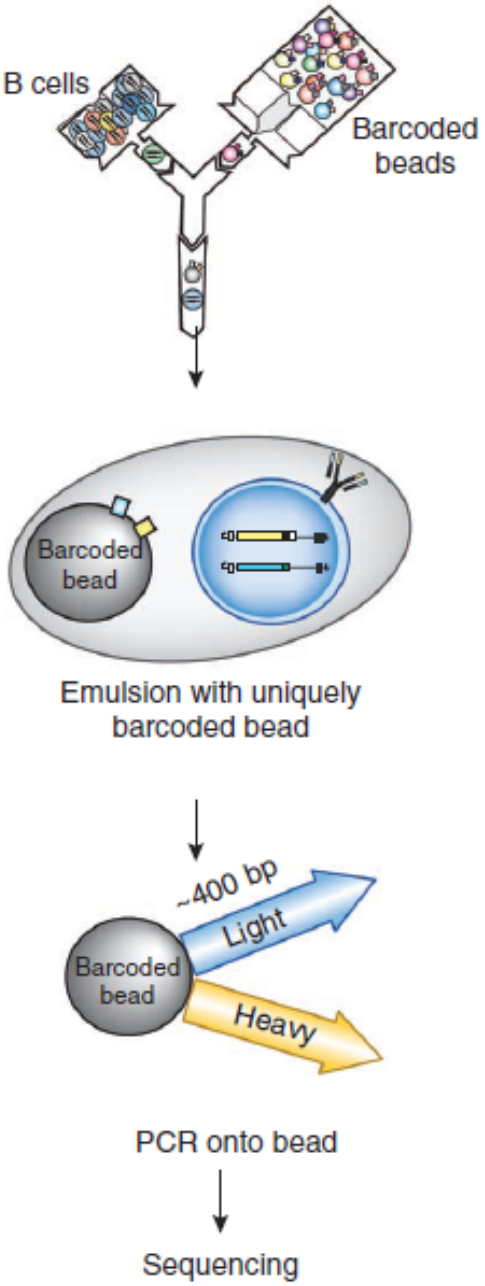
- Single B cells are sorted into wells and lysed
- Mixes of forward and reverse primers mixes are coupled to a set of DNA barcodes
  - All cells are exposed to the same primer mix
  - The amplicons from individual cells have unique FWD and REV barcode combinations
- Amplicons are pooled and sequenced



Busse *et al.*, *Eur. J. Immunol.*, 2013

Ig-seq: single cell approaches

- single cells are encapsulated in oil droplets, together with a unique DNA-barcoded bead
- The barcodes act as primers for VH and VL amplification
- Both amplification products remain linked, via the bead
- After sequencing, the barcodes allow pairing of VH and VL



Church *et al*., US patent US20130296535, 2013

Ig-seq: considerations

- Source of B cells
  - peripheral blood: **only 2%** of B cells present in human body
  - lymph nodes: 28%
  - spleen: 23%
  - red bone marrow: 17%

- Limitations of the presented methods: Information on Ig secretion rate is lost, no high-throughput analysis of antibody affinity

nature biotechnology

Single-cell deep phenotyping of IgG-secreting cells for high-resolution immune monitoring

Klaus Eyer[1], Raphaël C L Doineau[2,3,7], Carlos E Castrillon[4,5,7], Luis Briseño-Roa[3,6], Vera Menrath[3], Guillaume Mottet[4,5], Patrick England[6], Alexei Godina[2,6], Elodie Brient-Litzler[2,6], Clément Nizak[2], Allan Jensen[3], Andrew D Griffiths[2,8], Jérôme Bibette[1], Pierre Bruhns[4,5,8] & Jean Baudry[1,8]

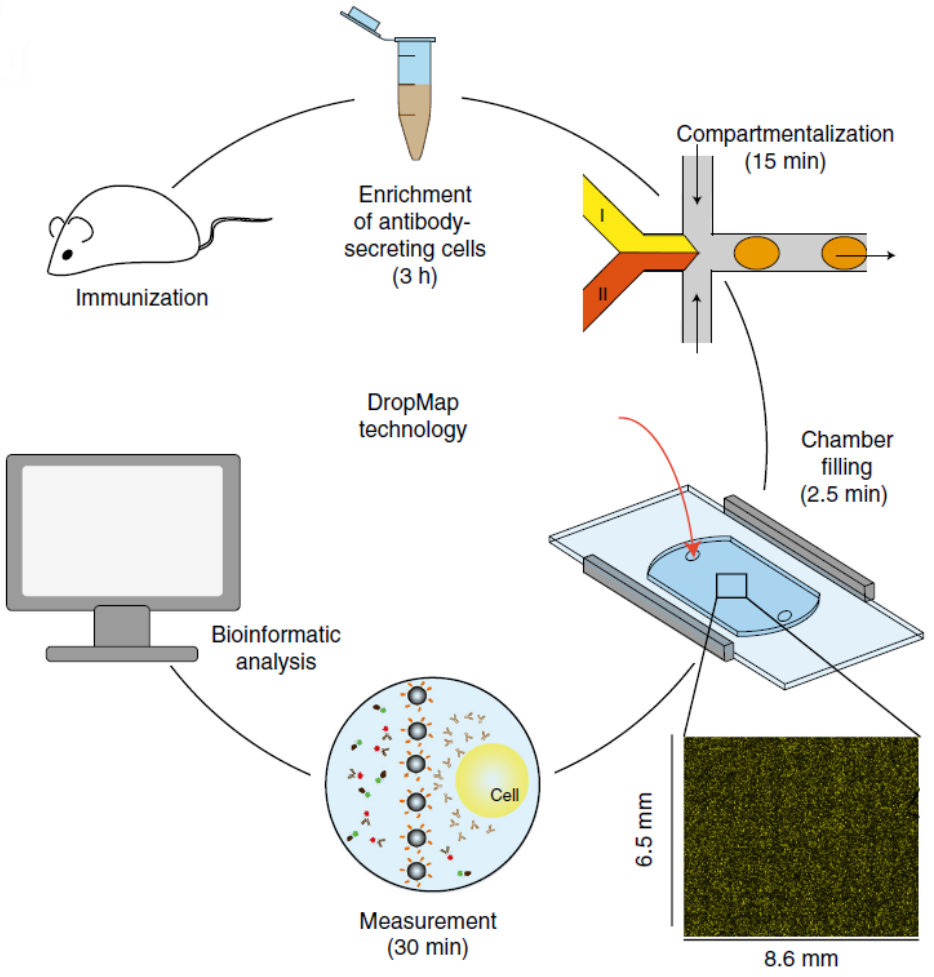
Published 11 September 2017

DropMap: Overview (1)

- Quantitative, high-throughput system to analyse individual antibody-secreting cells

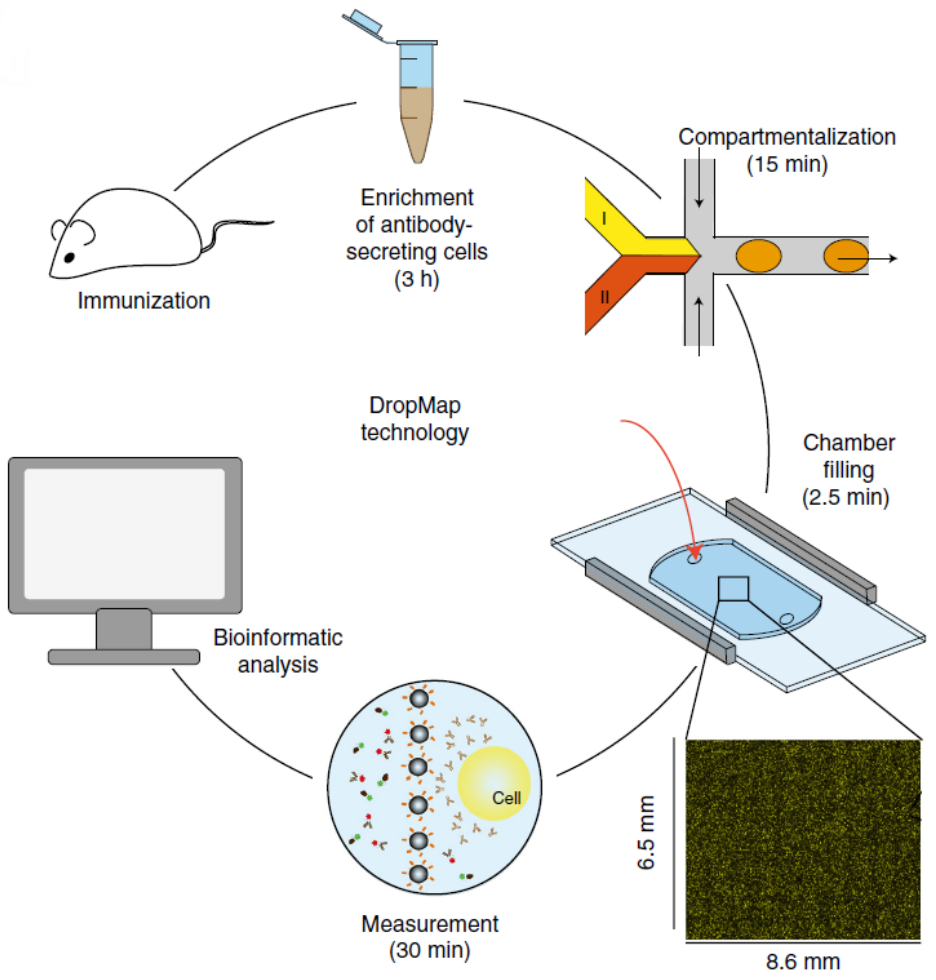
- Single-cell measurement of
  - IgG secretion rate (IgG molecules / s)
  - IgG reactivity + specificity to target antigen
  - IgG dissociation constant (Kd)

  - Here: characterisation of mouse humoral response to immunisation with tetanus toxoid (TT)



DropMap: Overview (2)

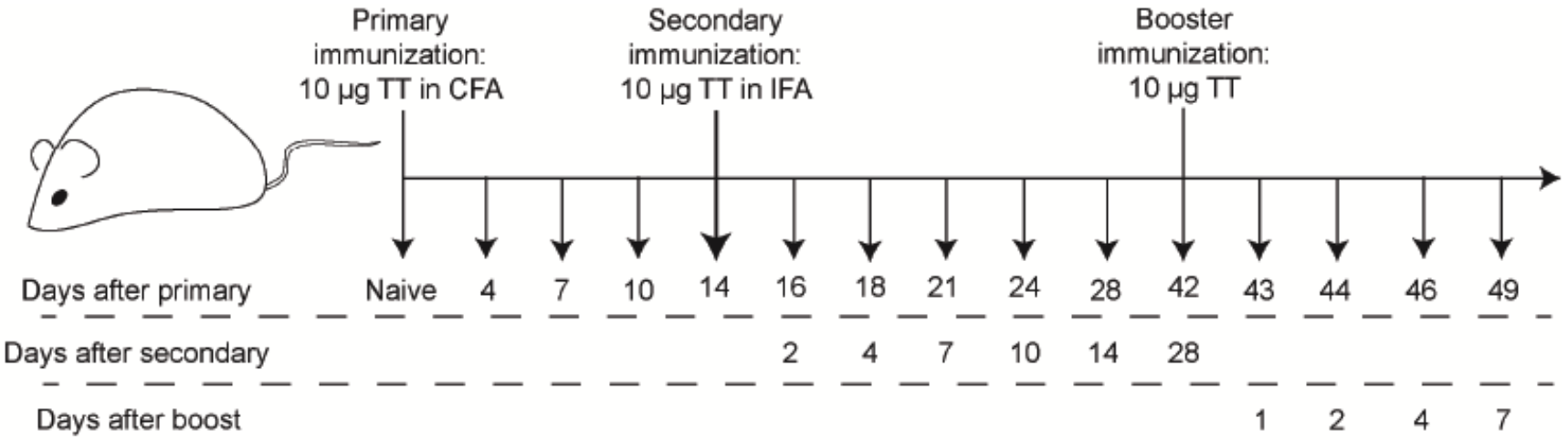
- Mice are immunised with antigen
- Cells are obtained from spleen and bone marrow and enriched using MACS
- Microfluidic system compartmentalises single IgG-secreting cells into oil emulsion droplets
- Droplet analysis in two dimensional arrays using a fluorescence relocation-based immunoassay
- Measurement of IgG secretion rate, affinity and specificity at a single-cell level



Immunization

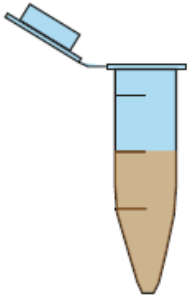
- Day 0: BALB-C Mice were immunized with inactivated tetanus toxoid (TT) in complete Freund's adjuvant
- Day 14: Secondary immunization, TT in incomplete Freund's adjuvant
- Day 42: Booster immunization, TT alone

- Control: same protocol, using BSA



Cell enrichment

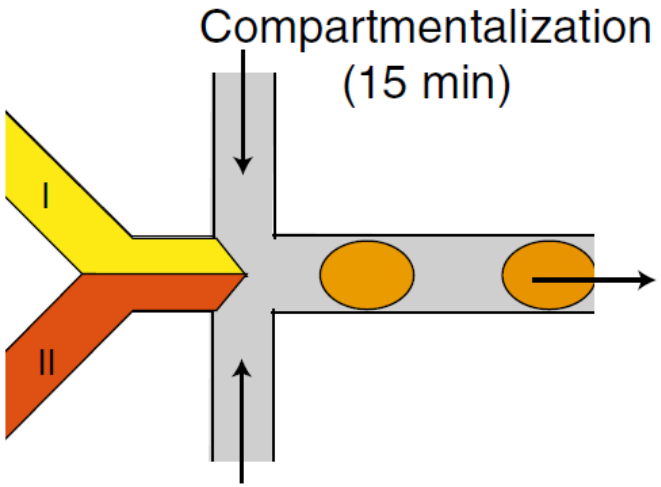
- Spleen and bone marrow cells were harvested from groups of 3 mice at 15 time-points, in 3-day intervals
- Non-target cells were depleted by MACS (duration: 3 h)
  - Depleted: T-Cells (CD3, CD4, CD8), mesenchymal cells (CD49b), erythrocytes (Ter119), granulocytes and macrophages (Gr-1)
  - remaining cells: «untouched» cells from B-cell lineage

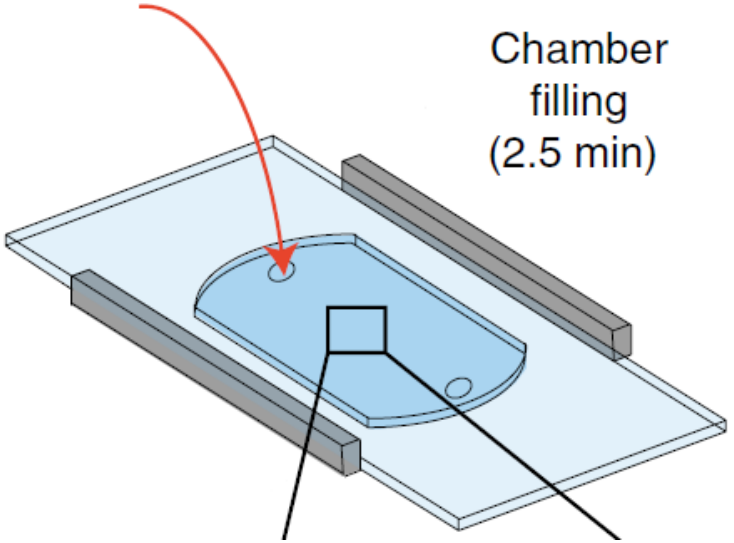


Enrichment of antibody-secreting cells (3 h)

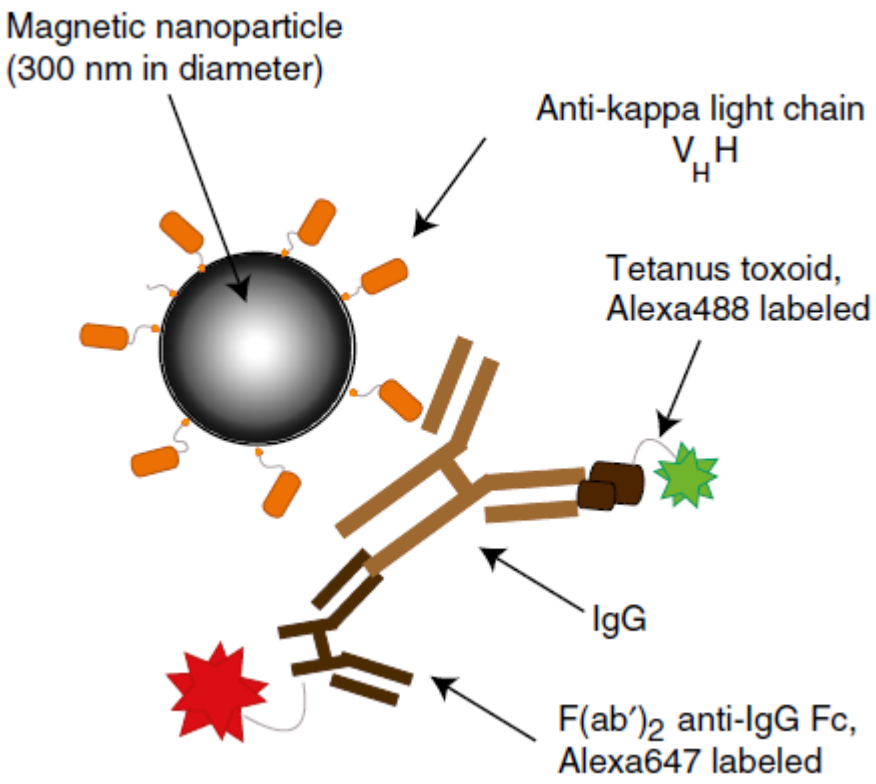
Compartmentalization

- cells are compartmentalized into 40 pL fluorinated oil droplets using a microfluidics system:
    I. cells or calibration antibodies
    II. fluorescently labelled detection reagents
- average of 0.2-0.4 cells per droplet (27% contain 1 cell, 6% contain >1 cell, 67% none)
- droplets are loaded into chambers, to create a 2D array containing 40’000 droplets



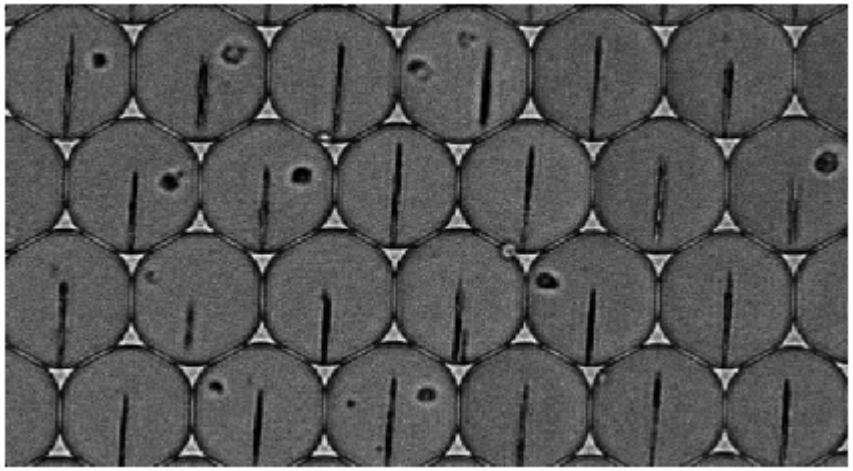


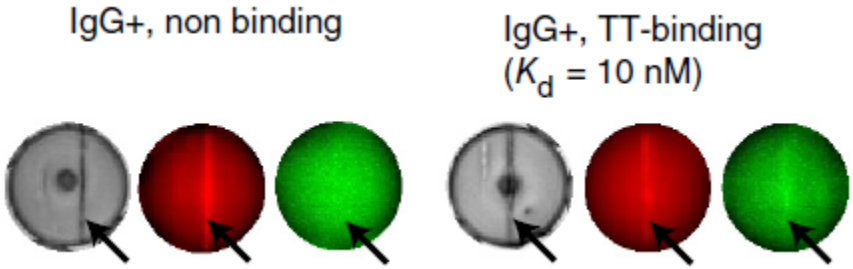
Detection assay: fluorescent labelling



- Anti-kappa light chain V_H-coated paramagnetic nanoparticles: bind antibodies in solution
- Fluorescent red anti-IgG Fc $F(ab')_2$ labels all antibodies
- Fluorescent green tetanus toxoid (TT) labels TT-specific antibodies

Detection assay: beadline

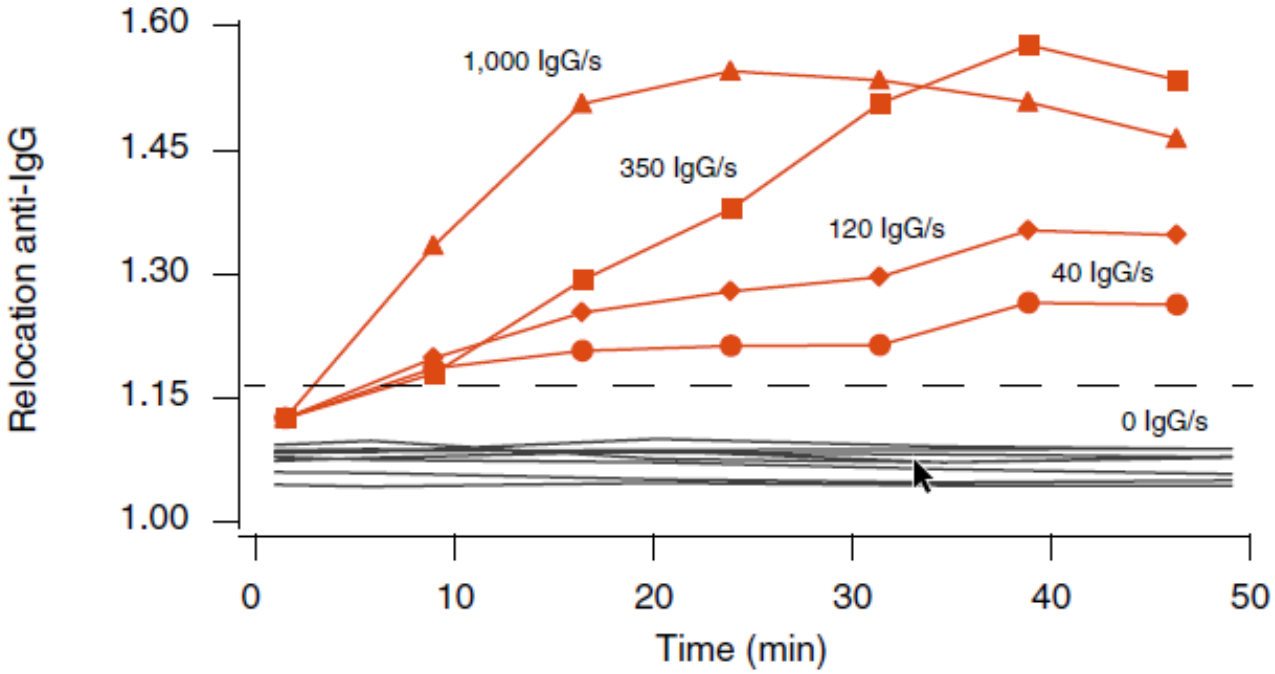




- A magnetic field is applied, to force the particles to form a beadline
- If particles have bound antibodies -> red fluorescence relocates to centre
- If bound antibodies are TT-specific -> both red and green fluorescence relocate

- ➢ IgG concentration was determined from the ratio of red fluorescence on the beadline to mean fluorescence in the droplet
- ➢ Green fluorescence ratio was compared to value obtained using calibrating anti-TT Ab

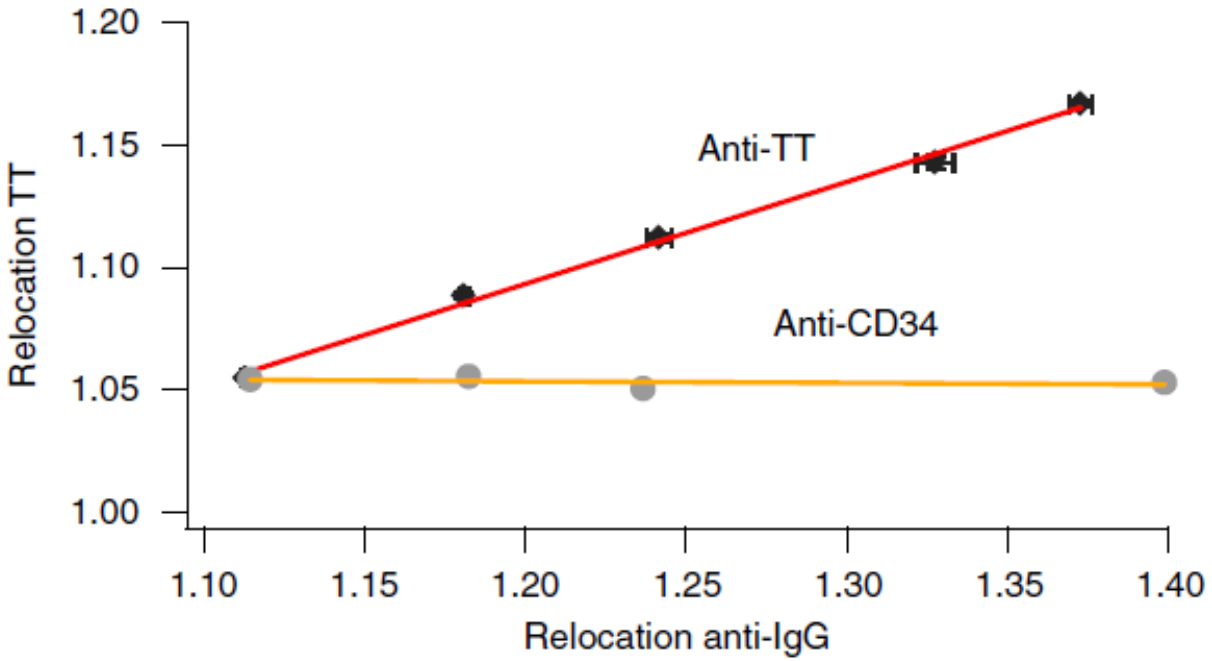
Detection assay: IgG secretion rate



- IgG secretion rate was calculated from the increase in the beadline/total well fluorescence ratio over time
- Red curves: individual splenocytes
- Grey curves: wells containing no cells

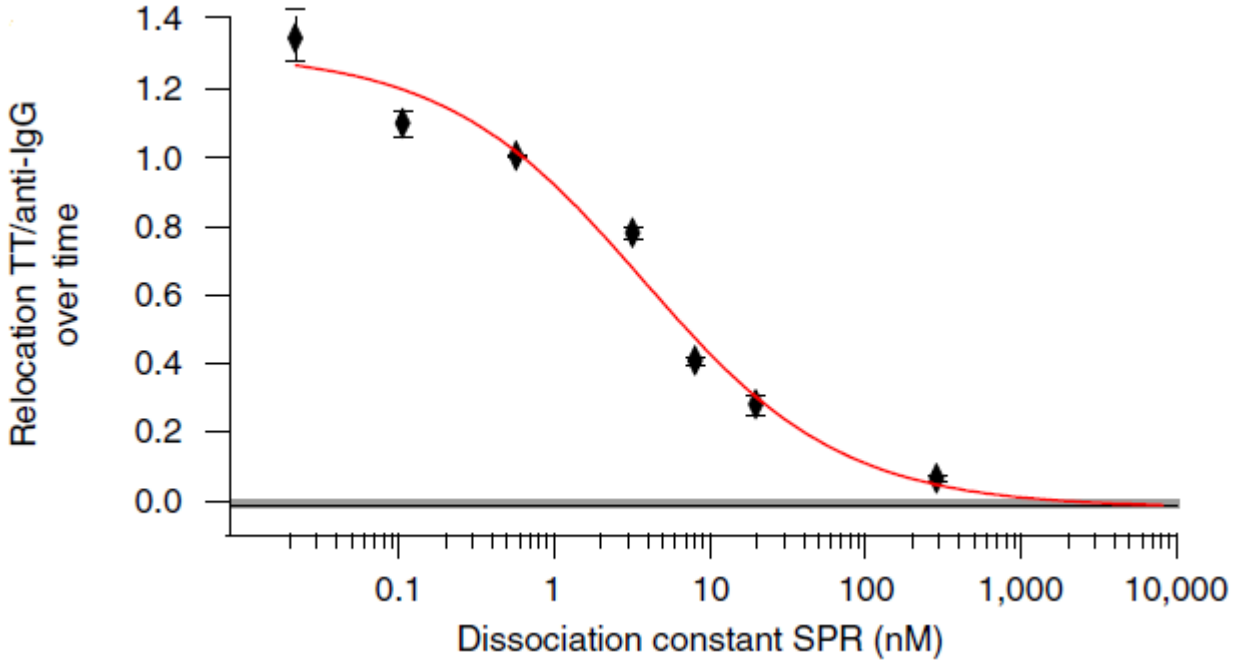
➢ Secretion rates of ~4 – 10’000 IgG/s : in accordance with literature

Detection assay: anti-TT calibration



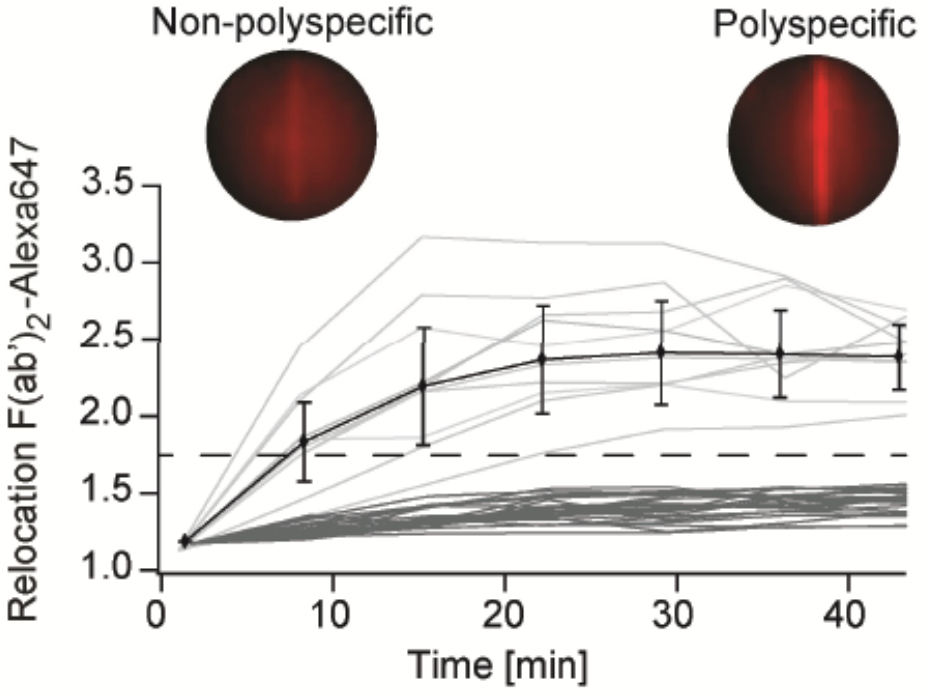
- Using multiple anti-TT Ab with known Kd values (determined using SPR), relocation of red fluorescent anti-IgG(Fc) was plotted against relocation of green fluorescent antigen (only one anti-TT Ab shown)

Detection assay: anti-TT calibration



- Multiple anti-TT Ab with known Kd values (determined using SPR) were used for calibration
- Ratio of relocation of green fluorescent antigen to relocation of red fluorescent anti-IgG(Fc) was plotted against Kd
  - Fitted sigmoidal calibration curve

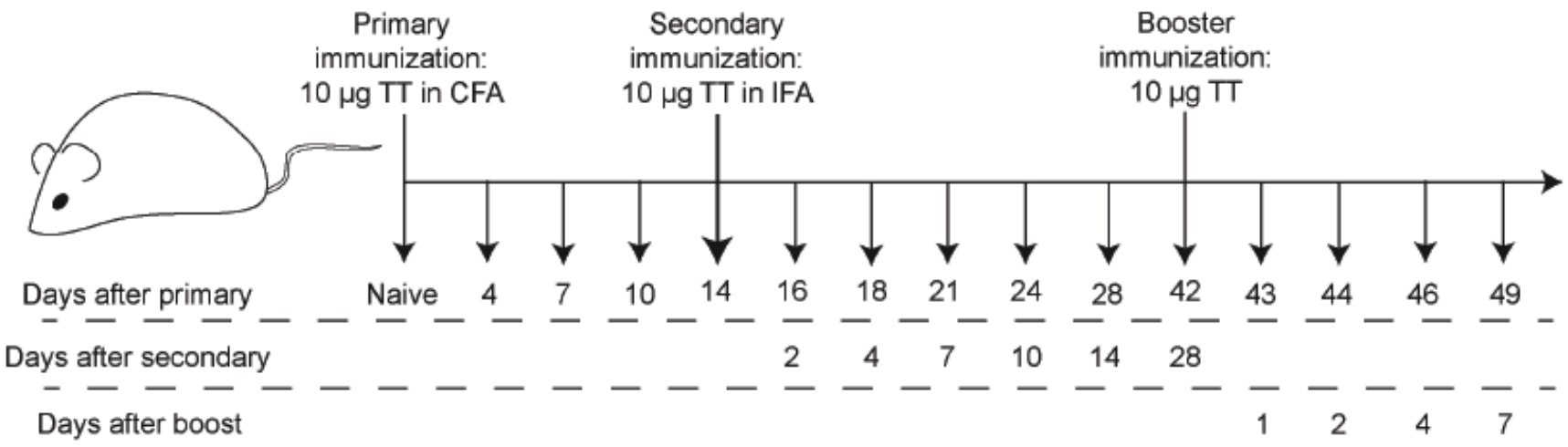
Are anti-TT antibodies specific?



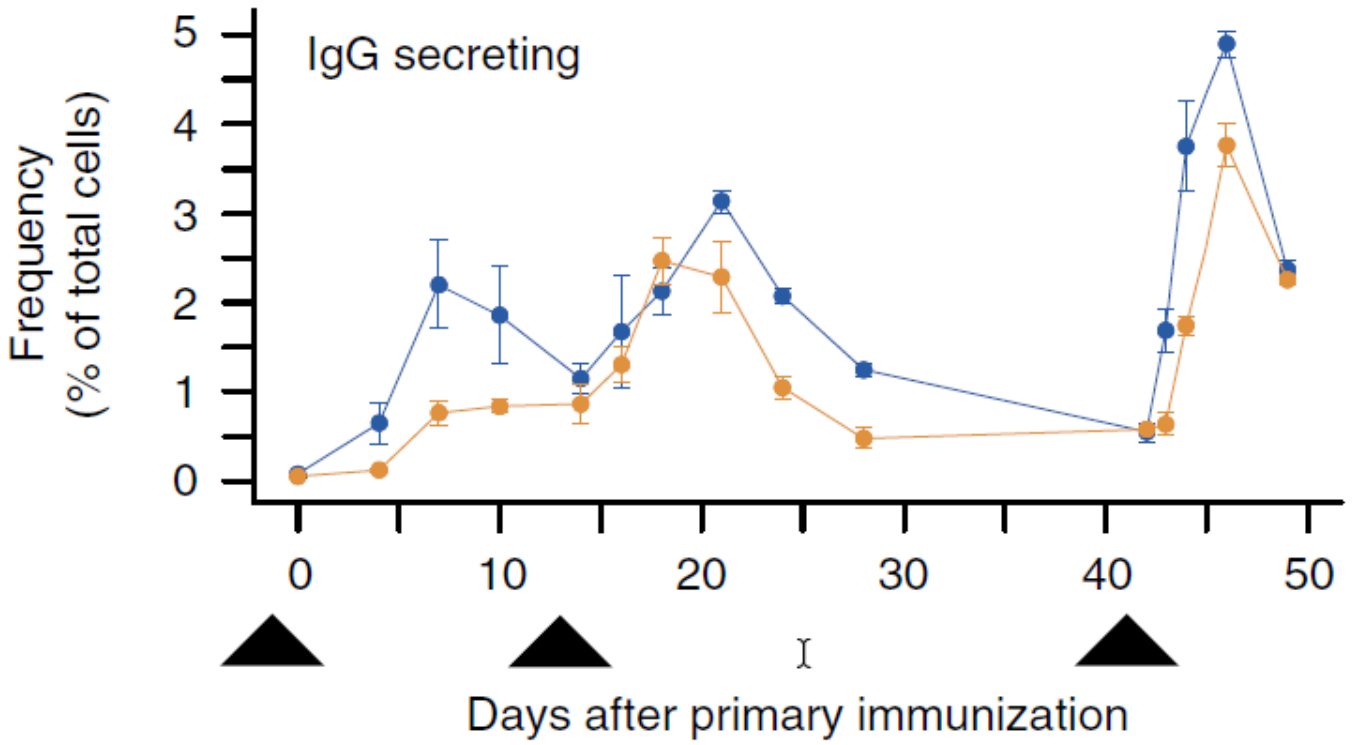
- For some cells (**light grey curves**) the red beadline fluorescence ratio was higher that the maximum obtained using purified IgG during calibration
- Polyreactivity, insolubility or cluster formation

- Control experiment: additional orange-labelled antigens (BSA, human IgG, ssDNA) were added to the droplet
  - 96% of cells with relocation > threshold (**dashed line**) showed relocation of orange fluorescence to beadline, indicating polyreactivity
  - Only 6% of cells below threshold showed the same

Analysis of anti-TT responses over time after immunization



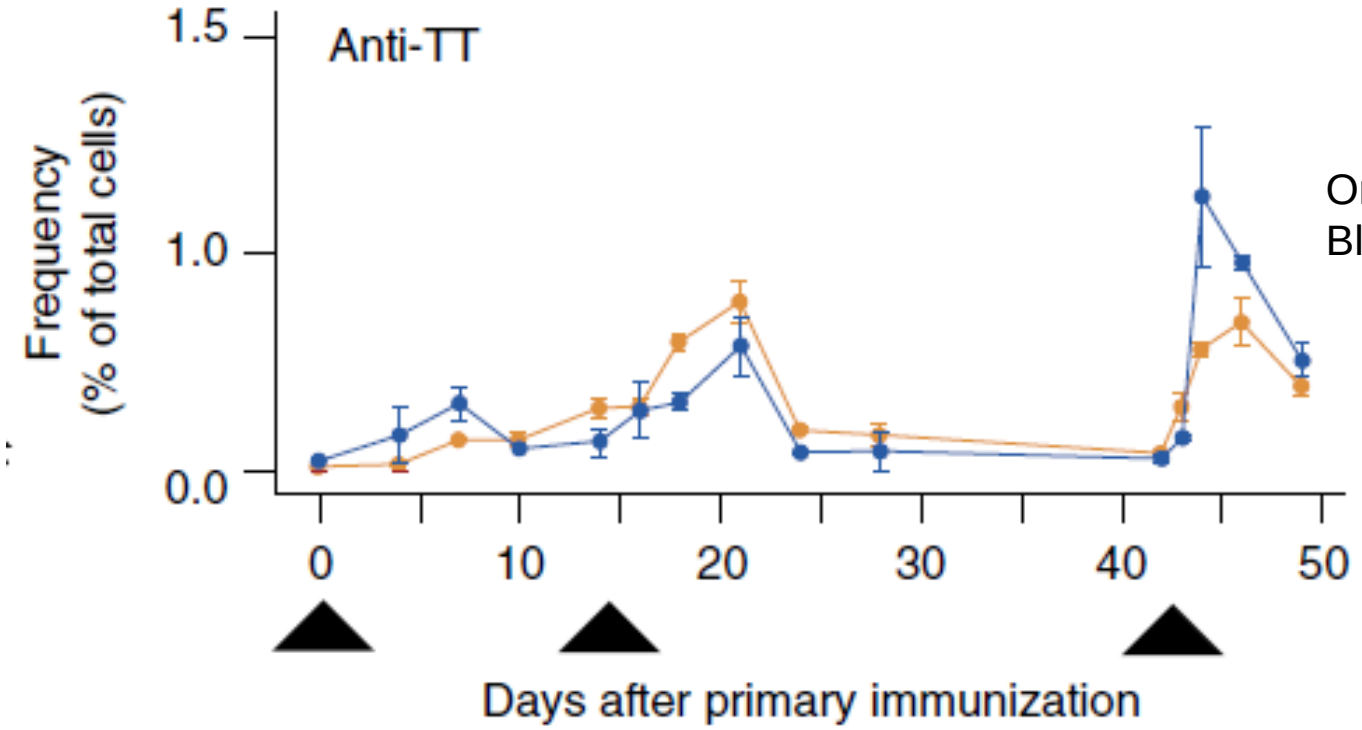
Frequency of IgG-secreting cells (IgG-SC)



Orange: spleen
Blue: bone marrow

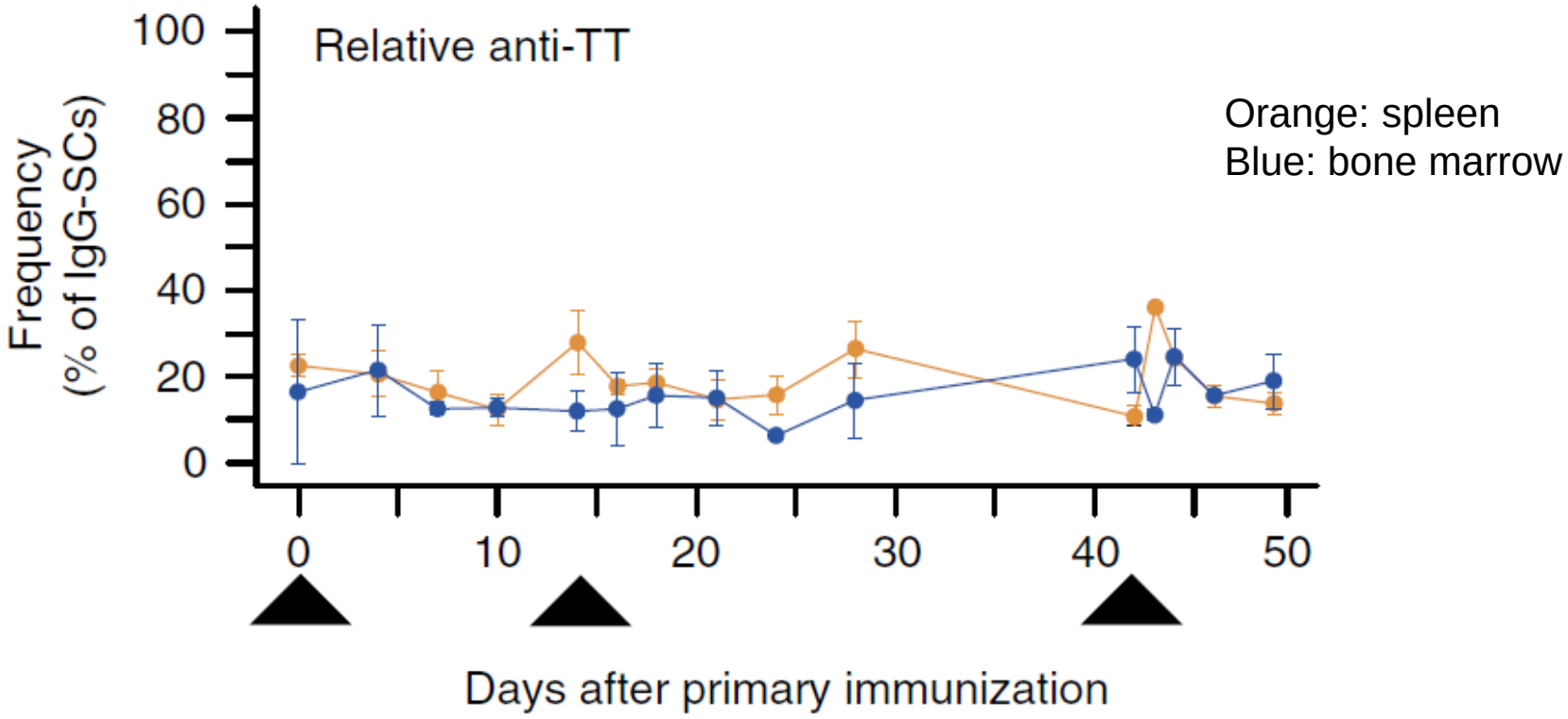
- Frequency of IgG-SC in spleen and bone marrow samples increased 80-fold in the spleen and 45-fold in the bone marrow
- Peak 4-7 d after each immunization

Frequency of anti-TT IgG-SC (% of **total cells**)



- Frequency of anti-TT IgG-SC evolved similarly to percentage of total cells that were IgG-SC
- Similar response for each of the 3 mice in individual groups
- Frequency of cells declined rapidly after each boost, but serum anti-TT titers remained high

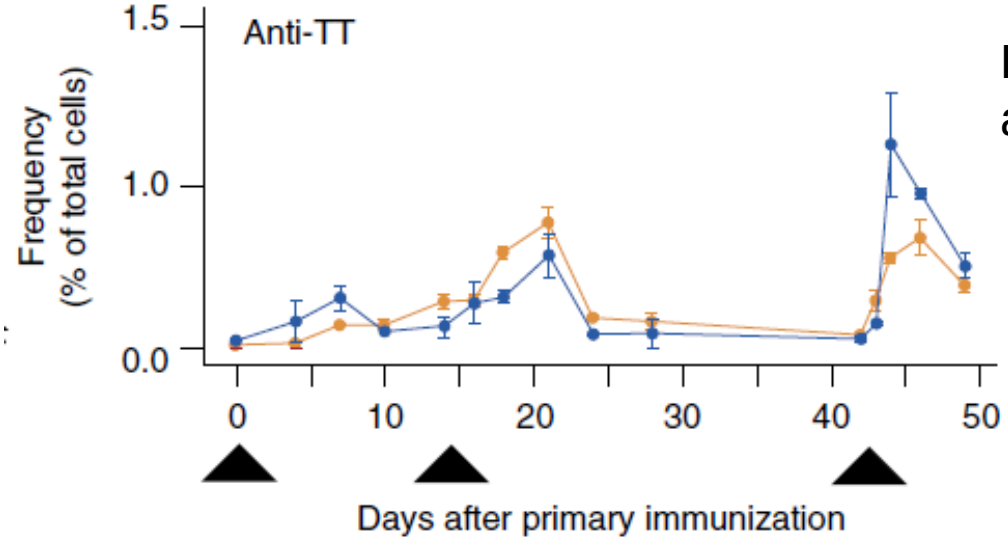
Frequency of anti-TT IgG-SC (% of **IgG-SC**)



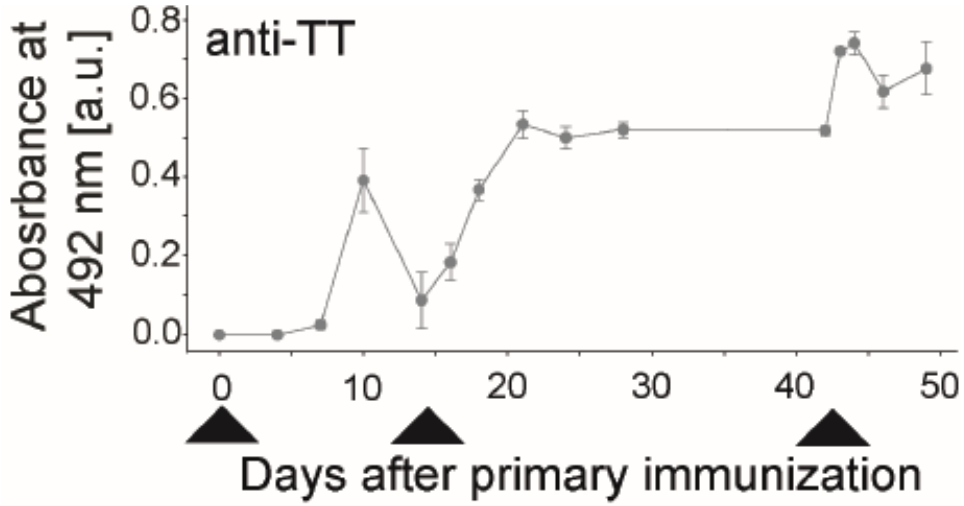
- Fraction of IgG-SC that produced anti-TT was relatively stable

➢ Coactivation of IgG-SC without TT-binding by adjuvants / cytokines?

Serum anti-TT titer



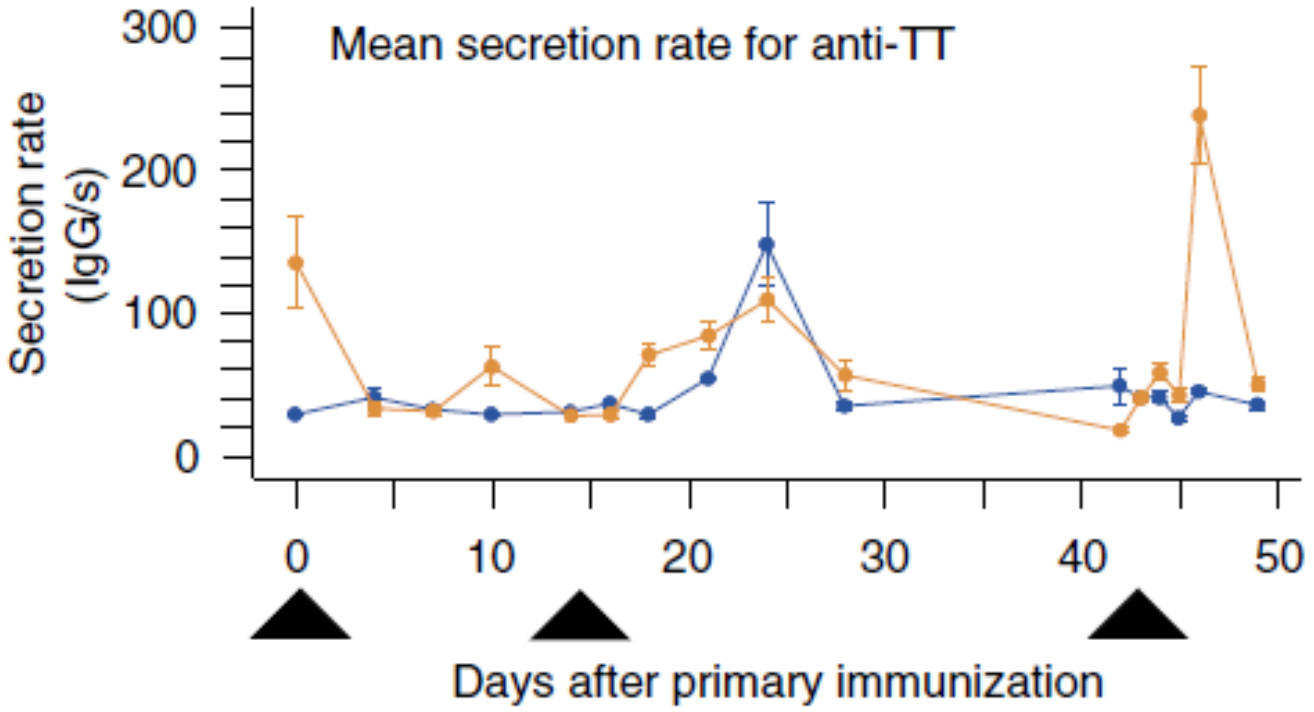
Frequency of anti-TT IgG-SC



anti-TT antibody titer

Supplementary Fig. 10. Titer measurements in immunized mice. Serum titers of anti-TT IgGs (measured by ELISA, absorbance at 492 nm at a 1/600 dilution).

Secretion rate of anti-TT IgG-SC



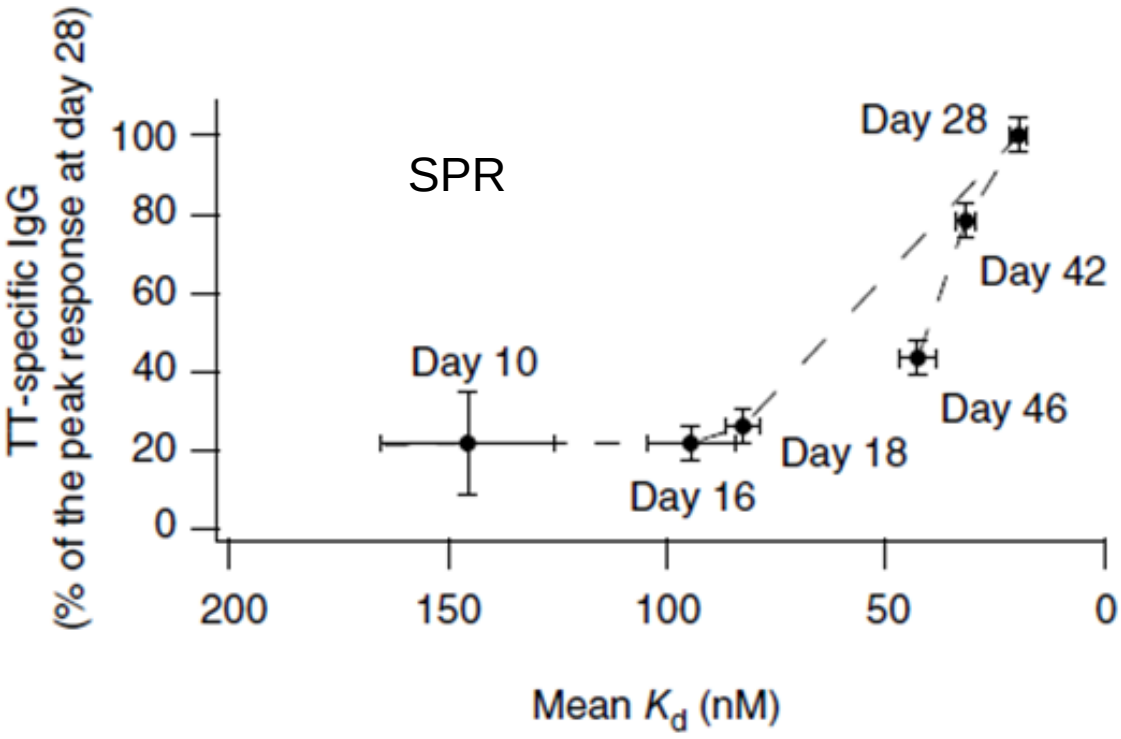
Orange: spleen
Blue: bone marrow

- Changes in mean IgG secretion rates in spleen and bone marrow are modest after immunization
- Probably can’t compensate for the contraction of anti-TT IgG-SC

Why did serum anti-TT remain high?

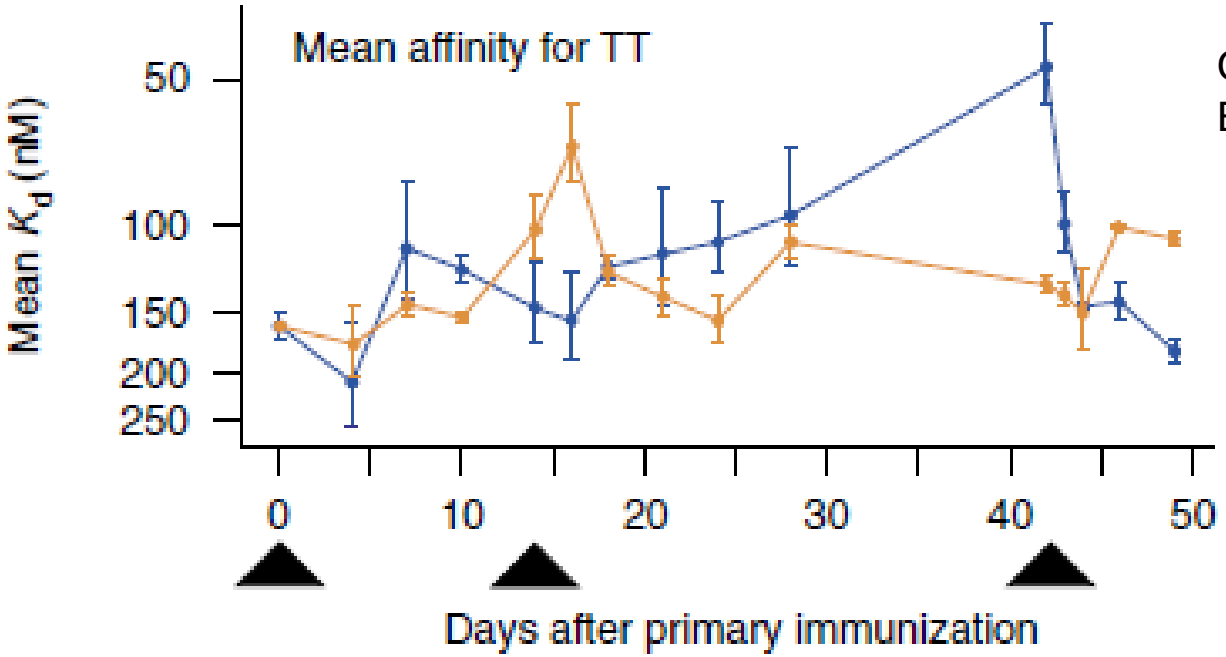
- Frequency of anti-TT IgG-SC in spleen and bone marrow declined rapidly after the peak following secondary immunization and boost
- Serum half-life of IgG in mice is 4-8 d
- Maintenance of serum IgG titers can’t be explained by increased secretion
  - Serum titer could be maintained by a reservoir of IgG-SC niching outside the spleen and bone marrow in other organs

Evolution of Kd: **SPR of serum IgG**



- **SPR**: mean affinity of serum TT-specific IgG increased 7-fold along the immunization scheme, peaking 14 d after the secondary immunization (day 28) and remaining high
- **DropMap**: 2-4-fold increase in affinity of TT-binding IgG from single-cell analysis
- **Primary immun**.: day 0; **secondary immun**.: day 14; **boost: day** 42

Evolution of Kd: **DropMap**



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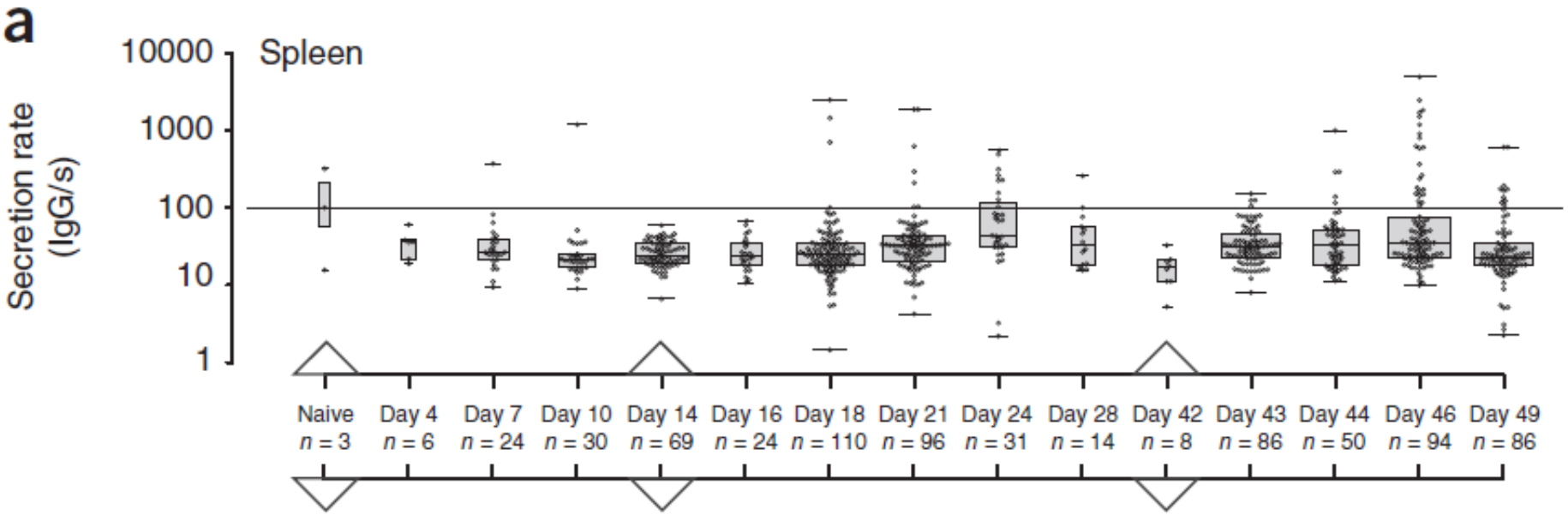
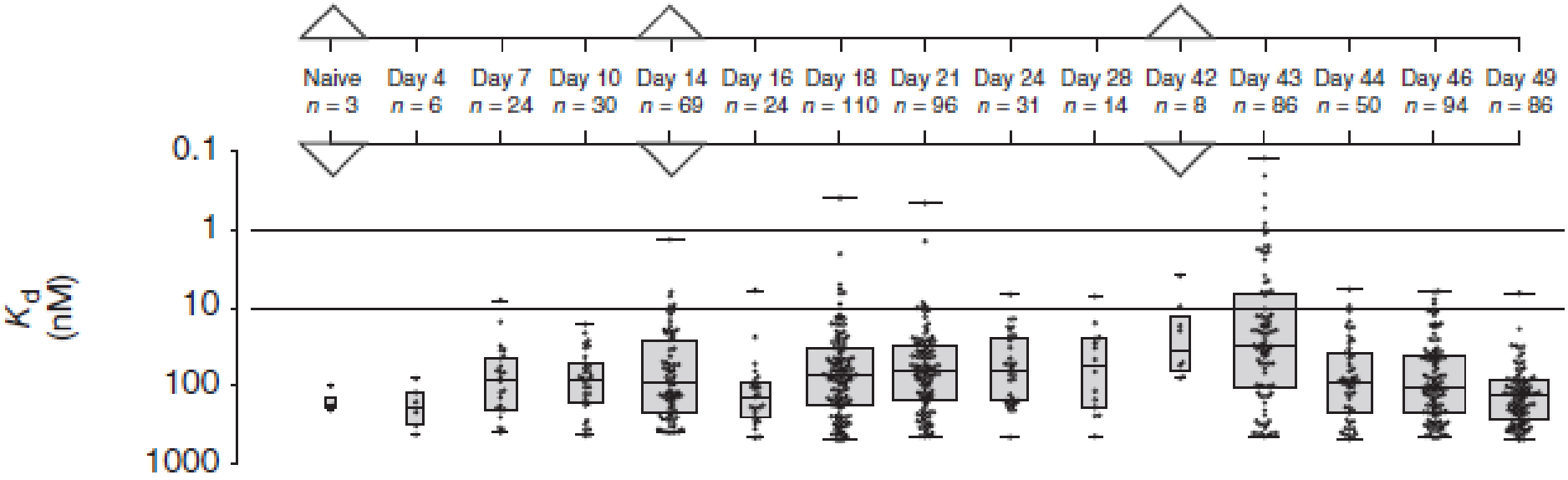
Anti-TT cells: Influence of immunizations/boost on **IgG secretion rate**



- Dots: single cells
- *n* = number of detected IgG-SC
- Box plots: middle line = median, upper/lower box = quartiles, horizontal lines = maximum/minimum

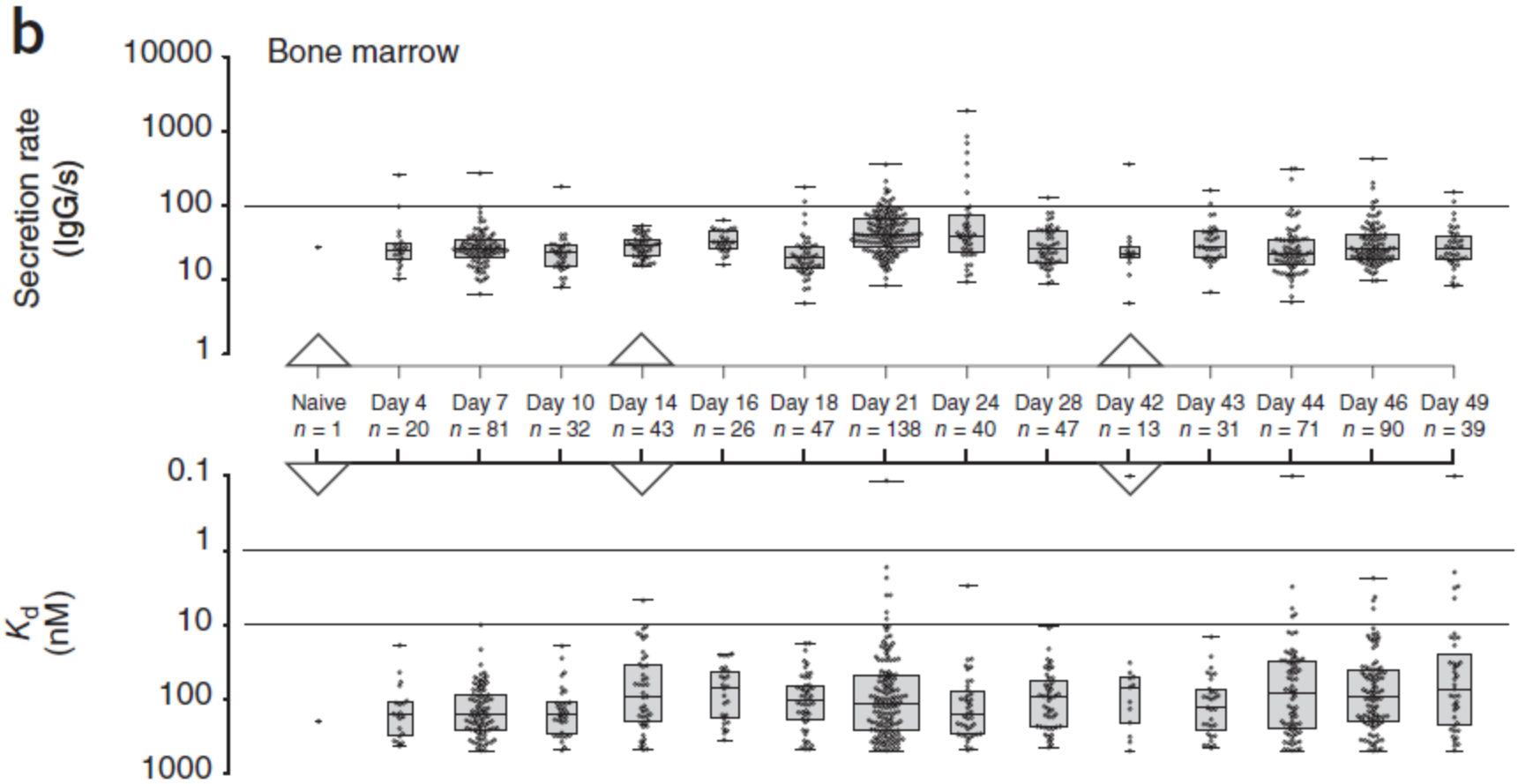
- Antigen challenge (triangles) increased the **mean secretion rates** and caused large increases in the **range** of secretion rates

Anti-TT cells: Influence of immunizations/boost on **Kd**



- Antigen challenge caused a large increase in the **range of affinities** at single-cell level
- Largest fraction of cells secreting high-affinity anti-TT Ab : **spleen 1 d after the boost** (**day 43**)
  - 26% of anti-TT cells with Kd < 10 nM, 7% with Kd < 1 nM
- **Day 44**: Kd range and high-affinity cells had already decreased
  - 4% of anti-TT cells with Kd < 10 nM, 0% with Kd < 1 nM
- Calculated sample Kd values span the typical range of affinities found for humoral responses (~4 logs)

Anti-TT cells in bone marrow: secretion and Kd



Spleen vs. bone marrow: IgG secretion and Kd

- After antigen challenge, the spleen and bone marrow showed a similar increase in single-cell IgG secretion rates
- High IgG secretors (>100 IgG/s) appeared only after the second immunization (day 14) in both organs
- Ultra-high IgG secretors (>1000 IgG/s) appreared mostly in the spleen (maximum: 0.1% of all cells)

- After 2nd immunization, the Kd range increased more strongly in the spleen, the increase in the bone marrow also occurred later (day 21 vs day 18)

Influence of immunizations/boost on IgG secretion rate and Kd

Conclusions:

- IgG secretion rate and affinity increase after antigen challenge
- However, no correlation was observed between IgG secretion rate and Kd at the single-cell level

- In most hybridoma protocols (references), splenocytes are harvested 2-4 d after the boost
  - This approach maximizes the number of high-secreting cells recovered
  - However, this may be too late to recover IgG-SC secreting high-affinity IgG (here: Kd maximum 1 d after the boost)

Yokoyama *et al., Curr. Protoc. Immunol*., 2013
Greenfield, *Antibodies: A laboratory Manual*, 2014

Summary

- DropMap allowed quantitative, time-resolved and high-throughput single-cell analysis of the humoral immune response
- ➢ Allows optimization of immunization and vaccination protocols + exclusion of polyreactive antibodies from screening experiments

- After immunization, average IgG secretion rates and affinities increased only modestly
- The range of individual secretion rates and affinities increased dramatically

- Both antigen-specific and non specific IgG-SCs increased in frequency and increased their secretion rates after antigen challenge
  - Including after the boost with antigen without adjuvant
  - May be caused by extensive activation and/or stimulation of bystander B cells
- No correlation between IgG secretion rate and affinity

Summary (2)

- Highest-affinity anti-TT cells were found in the spleen 1 d after the boost

- The peak in the number of cells producing high-affinity, TT-specific IgG was 3 d later in bone marrow than in spleen

➢ B-cell expansion and somatic hypermutation in the spleen may generate high-affinity IgG-SC, which then niche in the bone marrow

Unresolved questions

- Can cross-reactive / unspecific IgG be removed by repetitive boosting with pure antigen?
- Do less inflammatory adjuvants (alum, TLR agonists) cause a more “focused” immune response?
- How accurately do DropMap measurements reflect *in vivo* behaviour of B cells?