Positivity assessment for intracellular cytokine staining (ICS)

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Measurement of antigen-specific T cells

- Tetramer staining, but limited to selected epitopes in the context of selected MHC types.
- IFN-γ ELISpot assay following *ex vivo* stimulation with antigen. Examines total PBMC and a single cytokine.
- Intracellular cytokine staining (ICS) by flow cytometry examines multiple cytokines and identifies T cell subset.
- CFSE proliferation expands cells over 6 days.

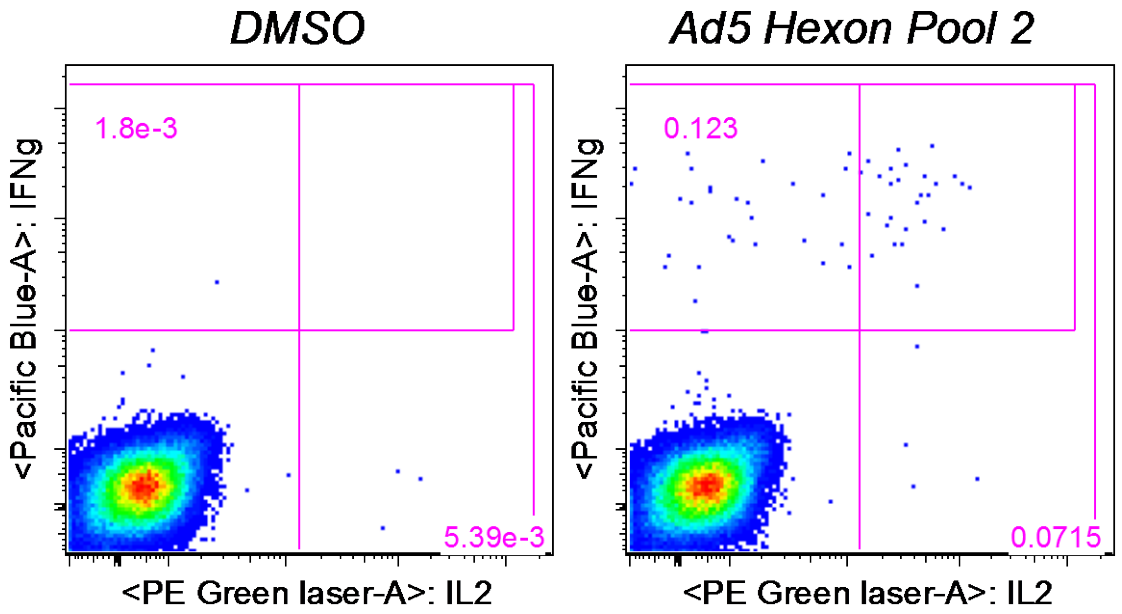
Intracellular cytokine staining (ICS)

- Peripheral blood mononuclear cells (PBMC) stimulated *ex vivo* with antigens of interest, (protein, 15mer peptide pools)
- Relevant negative control, e.g., the peptide diluent, DMSO, for peptide pools
- Results reported as percent of CD4+ or CD8+ T cells producing cytokine of interest
- Also useful to report percentage of trial participants with a positive response

ICS positivity

- Compare proportion of cells producing cytokine in antigen-stimulated samples vs. the unstimulated negative control (background)
- Often, positivity determined as 3-fold over the background, sometimes also with a minimum threshold (e.g., at least 0.05%)
- HVTN has used a Fisher's exact test comparing number of cells gated positive or negative (Horton et al, JIM, 2007, 323, p39)
- New MIMOSA Bayesian method

Fisher's exact test ICS positivity

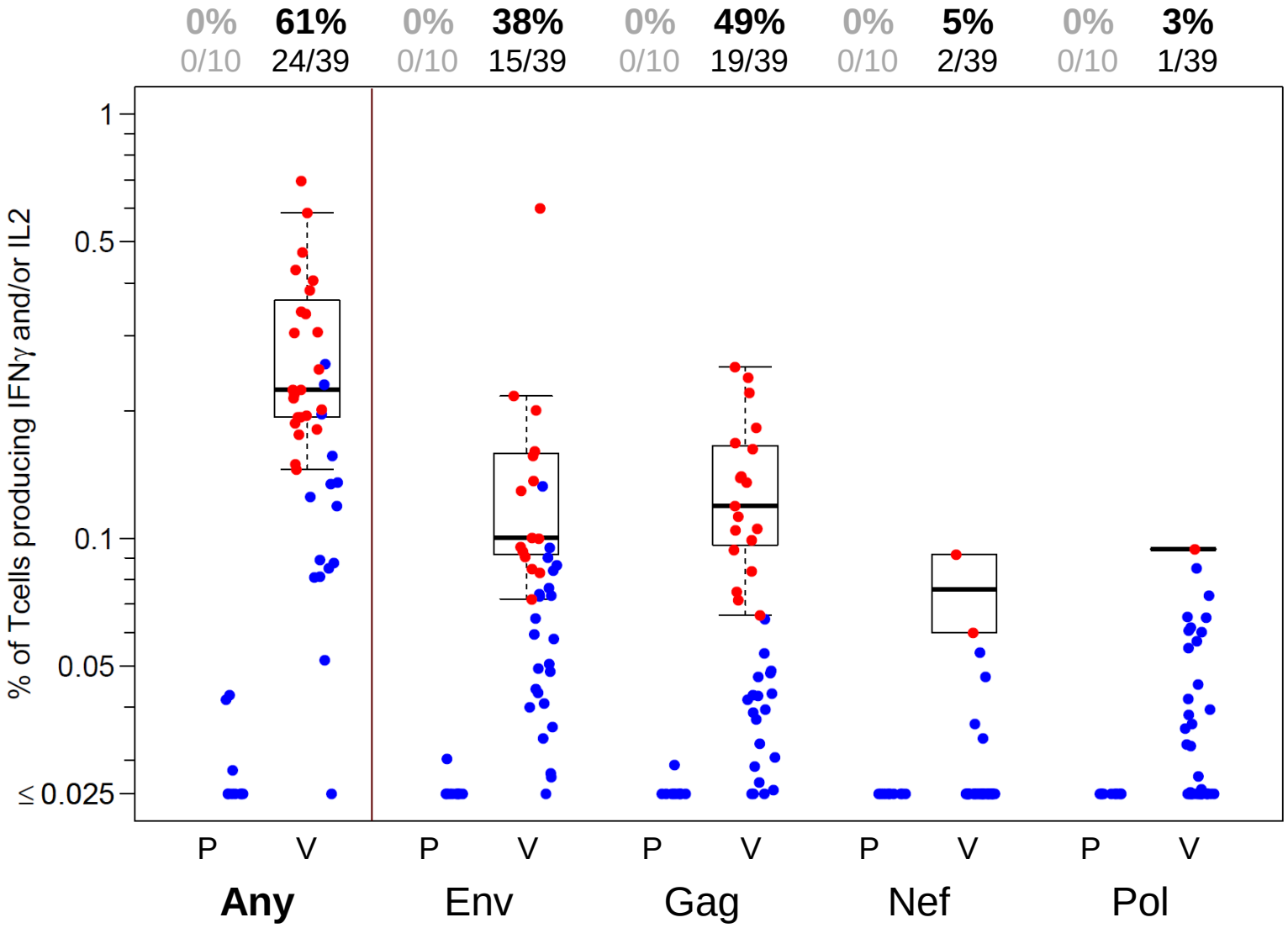


	DMSO	Hexon 2
Cytokine+	4	59
Cytokine-	55627	44712
	0.007%	0.13%

One-sided Fisher's exact test, discrete Bonferroni adjustment; $p \leq 0.00001$ for positivity

Applied for cells producing IFN-γ and/or IL-2.
Threshold chosen based on assay validation data.

Example of ICS results for CD4+ T-cell responses in placebo vs. vaccine recipients in an HVTN study



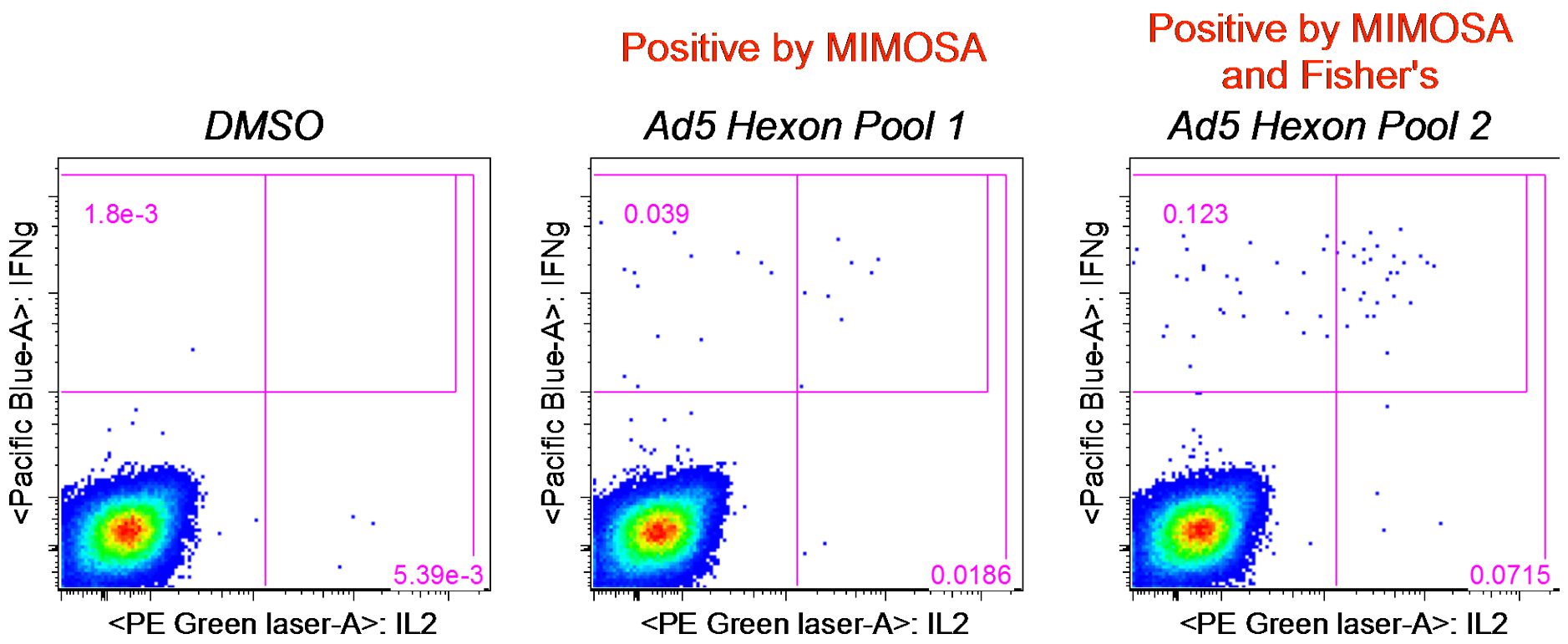
Positive responses shown in red

Mixture Models for Single-Cell Assays (MIMOSA)

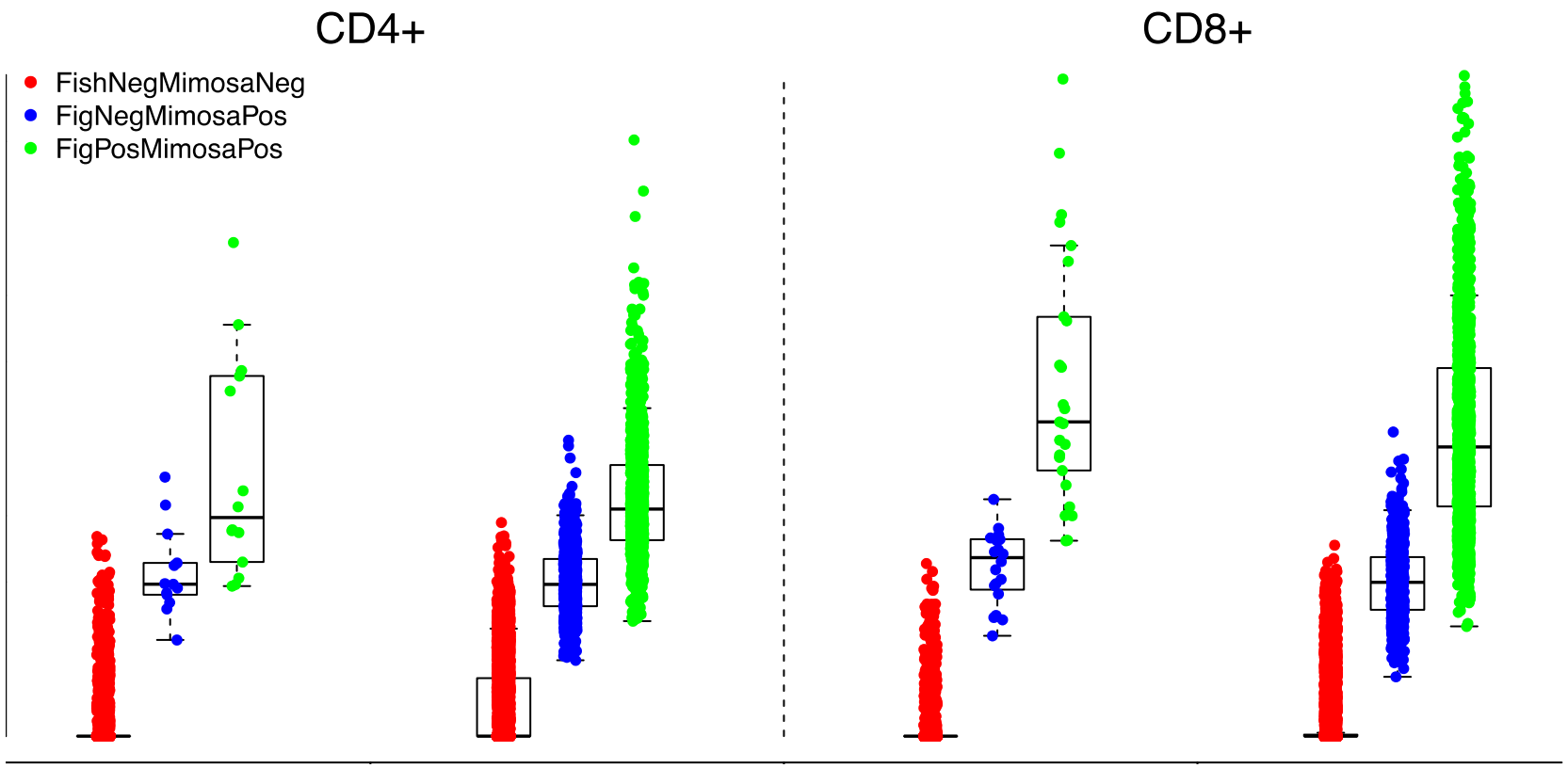
- Bayesian hierarchical mixture model framework where one component models the responders and the other models the non-responders
- Parameters defining these distributions, as well as probabilities of response/non-response, are estimated from observed data
- Thus, there is sharing of information across responders and non-responders
- False-discovery rate multiplicity adjustment (0.01, 1%)

Finak et al, Biostatistics, 2013, 15, p87

MIMOSA identifies low-level responses as positive



MIMOSA detects additional low-level responses as positive



Cross-protocol comparison

- ICS has been used across multiple clinical trials
- The ICS assay has been modified over time from 8 colors to 12 colors, but IFN-γ and IL-2 have been cross validated for each new assay
- Phase I to II trials, varying numbers of participants
- Primary immunogenicity time point generally 2 to 4 weeks post last vaccination
- False-positivity assessed in placebo recipients

MIMOSA positivity testing across HVTN protocols

- Implementation methods
  - MIMOSA performed separately for each trial
  - Antigen pooling for like antigens by summing cell counts; then standard “pooling” to ANY antigen
  - FDR calculation by pooling all subjects and fixing antigen
- Evaluation of different thresholds:
  - Instead of using FDR as threshold, use Pr(response). This is a fixed threshold for better comparability across studies
  - Test stringent thresholds to achieve low false positive rate

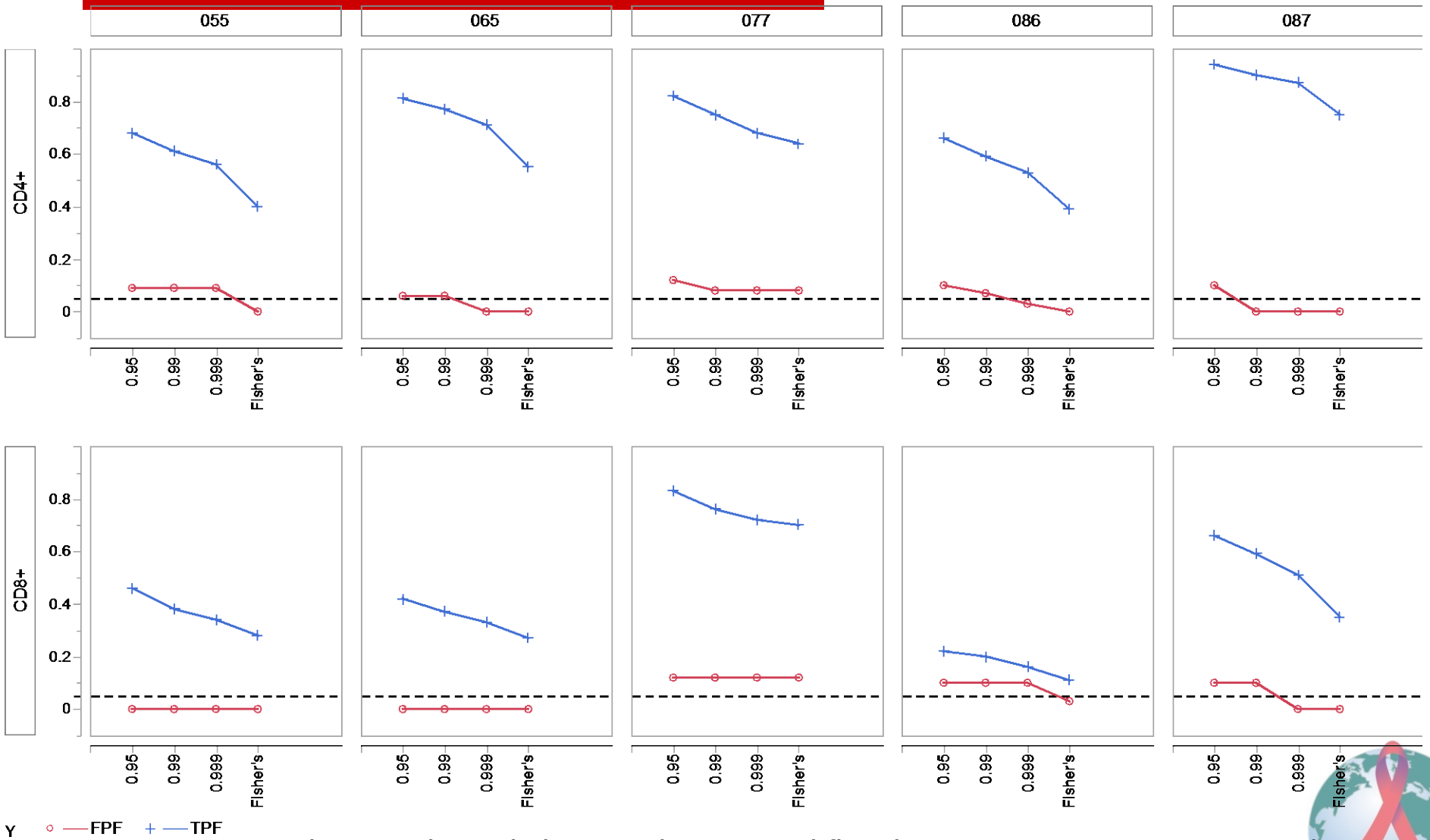
Protocol	Control	Treatment
055	22	104
065	18	89
077	24	132
086	30	132
087	10	80
096	16	80
097	19	78
204	172	165
502	173	306
503	141	154
505	49	189

- Number of subjects included in analysis
- Chose a single visit with the most data

Terminology

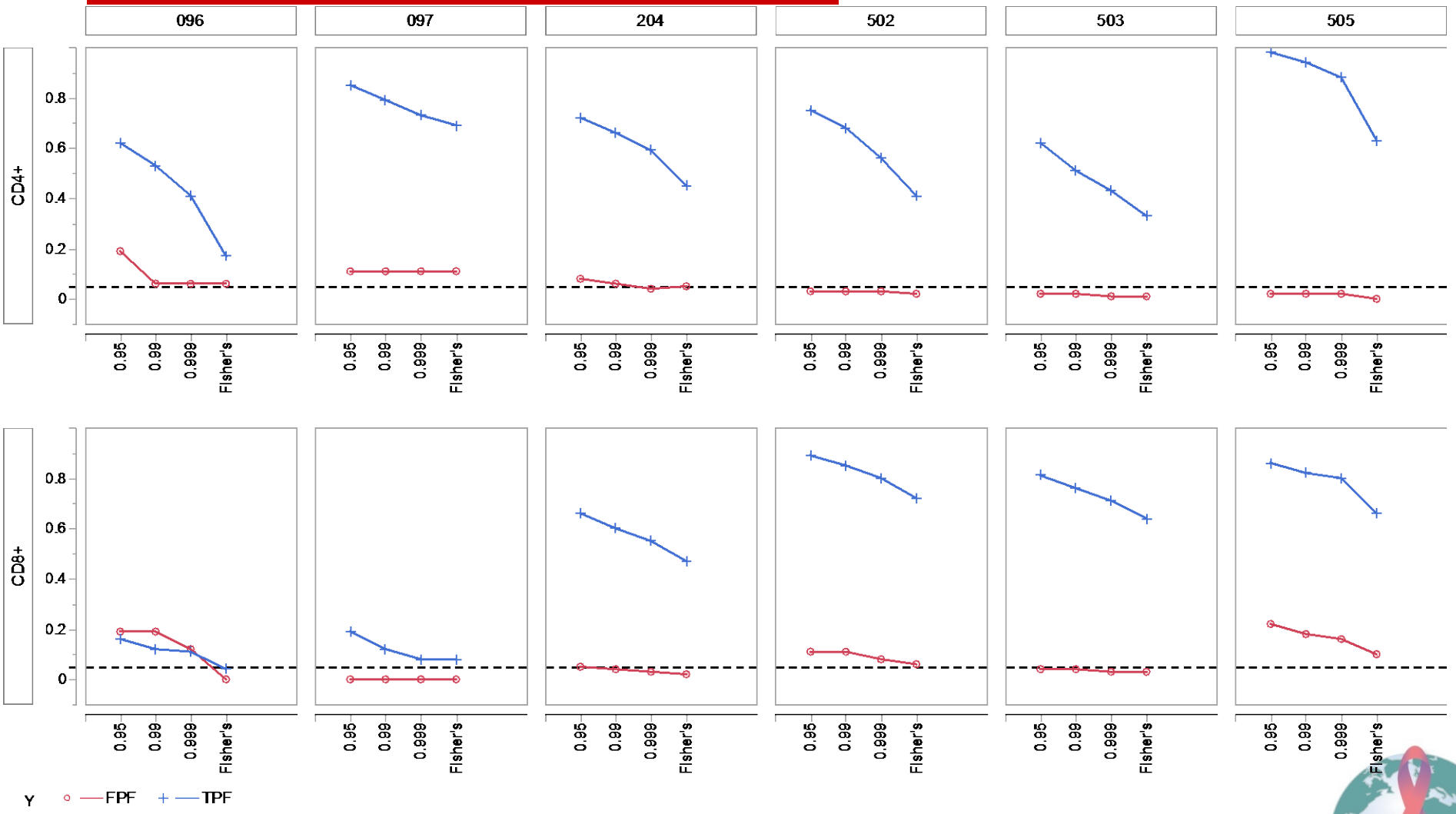
- FPF = false positive frequency, positivity among placebo recipients
- TPT = true positive frequency, positivity among vaccine recipients

FPF and TPF comparing MIMOSA at different Pr(resp) thresholds and Fisher's (Slide 1 of 2)



Response to ANY antigen; Sub-pools by protein summed first (e.g., PTE_g Env1, Env2, Env3)

FPF and TPF comparing MIMOSA at different Pr(resp) thresholds and Fisher's (Slide 2 of 2)



Response to ANY antigen; Sub-pools by protein summed first (e.g., PTE_g Env1, Env2, Env3)

Conclusions

- Compared with the Fisher's method, MIMOSA at the most stringent threshold of 0.999 increases false positivity substantially only for 055 CD4, 086 CD8, and 096 CD8, yet increases sensitivity substantially (note especially 096 CD4, 505 CD4 and CD8).
- Lowering threshold to 0.99 increases false positivity further for 065 CD4, 087 CD8, 096 CD8, 502 CD8. Although sensitivity improves further at 0.99 from 0.999, the largest increase in sensitivity is between Fisher's and 0.999.
- Threshold of 0.999 may be the optimal conservative choice to control false positivity.

COMPASS: Combinatorial Polyfunctionality Analysis of Single-cell Subsets

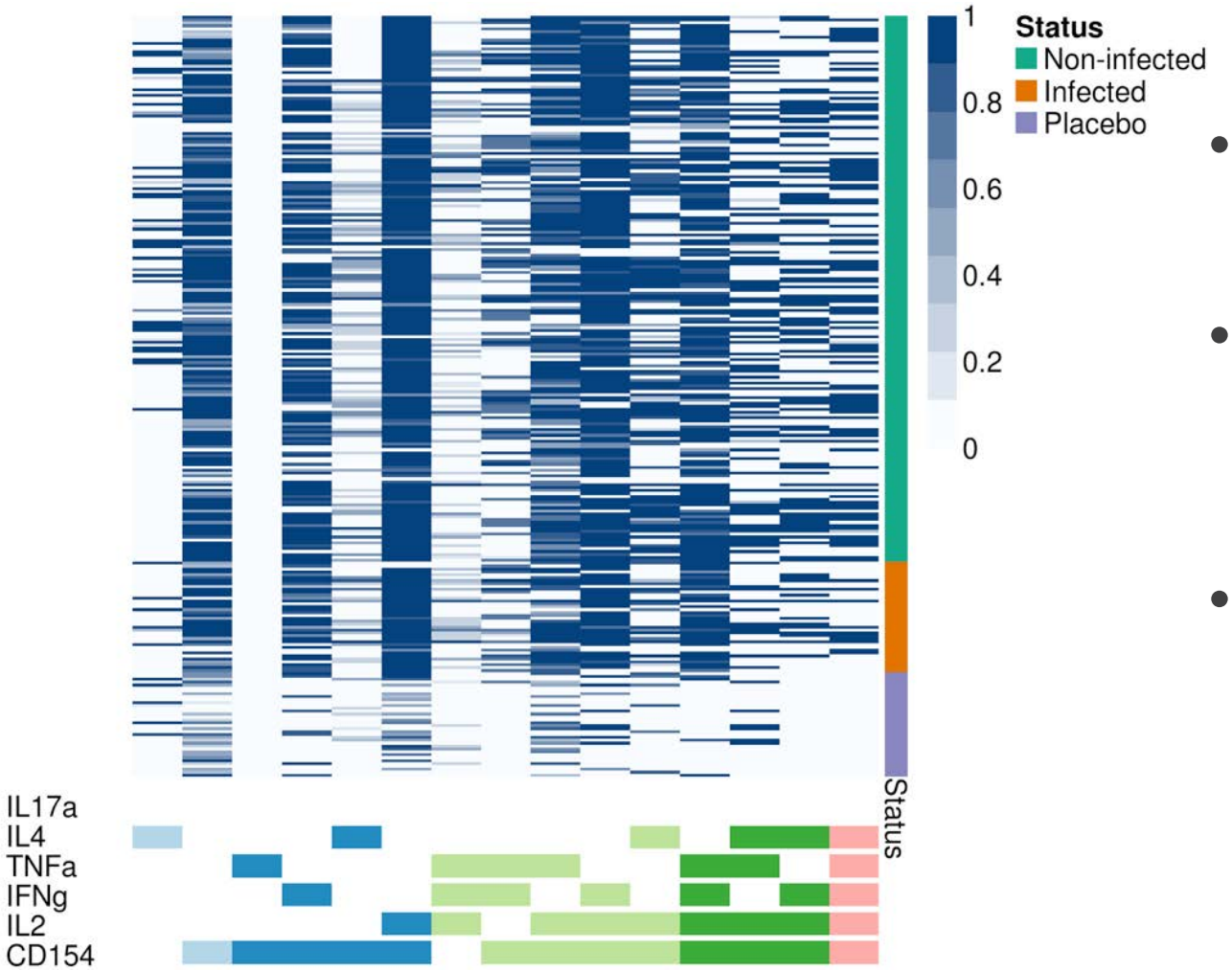
- Goals:
  - Characterize the **complete Ag-specific T-cell profile** using ICS
  - Quantify response **for each cell subset in each subject**
- COMPASS extends MIMOSA in several ways:
  - Jointly models **all combinatorial subsets** using a **multivariate** model (multinomial/Dirichlet)
  - Identifies Ag-specific responses in **specific cell-subsets** in **each subject** (via a variable selection prior)
- Output: a **probability of Ag-specific response for each cell subset/subject**

Lin *et al.* Combinatorial Polyfunctionality Analysis of Antigen-Specific T-cell Subsets Identifies Novel Cellular Subsets Correlated with Clinical Outcomes (Submitted and under review)

COMPASS analysis of RV144

- **262 subjects**
  - 226 vaccinees (38 infected, 188 non-infected), 36 placebos
  - 2 stimulation conditions per subject (stimulated with ENV, unstimulated)
- **CD4+ T-cells**
  - 6 functional markers: CD154, IL2, IFNγ, TNFα, IL17, IL4
  - 2^6=64 possible combinations / cell subsets
  - Many empty combinations → 15 considered

COMPASS permits the unbiased characterization of polyfunctional subsets

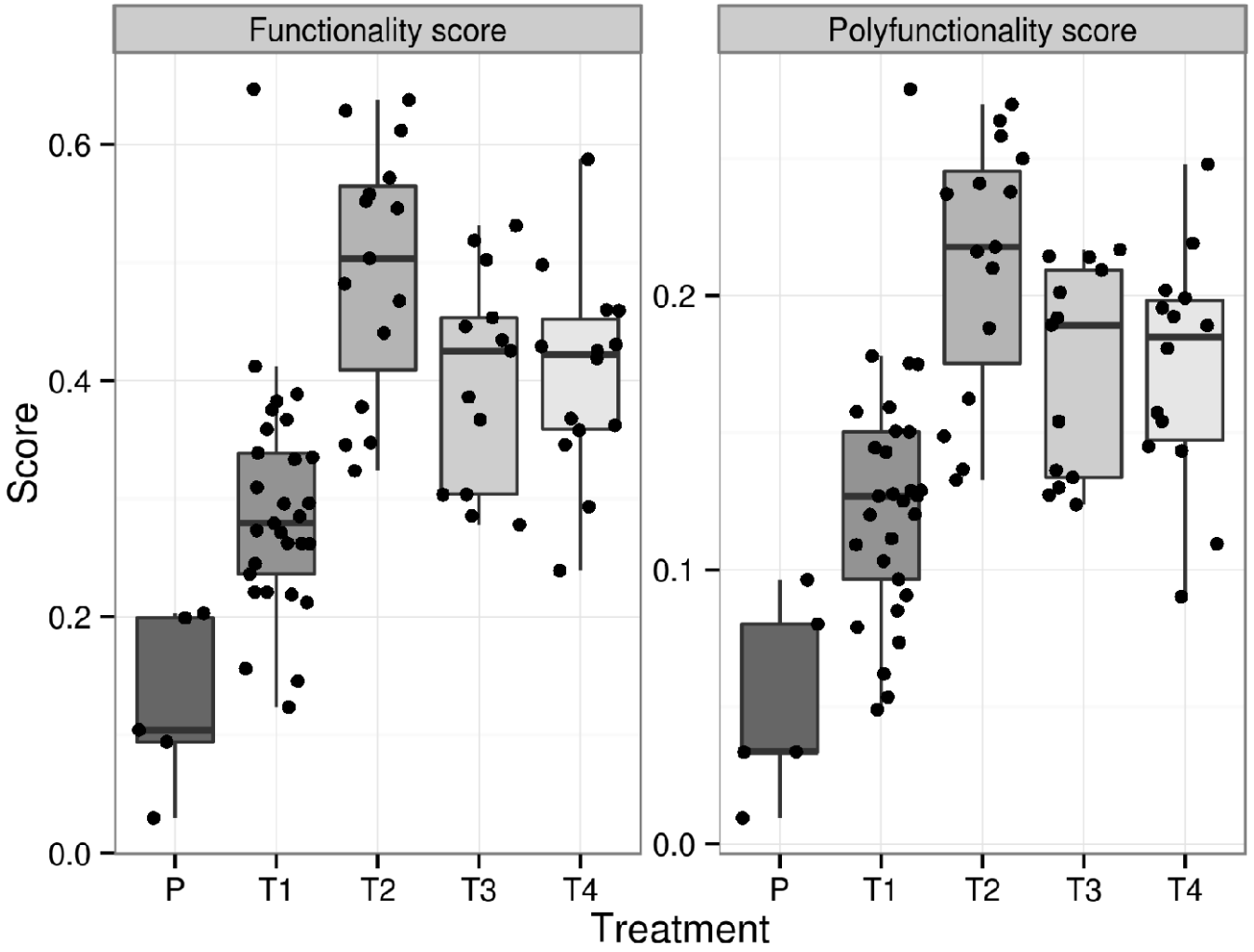


- **Immunogenic vaccine**
- **Highly polyfunctional response**
- **Summarize subject's response profile using a *score***

Two new scores

- Summarize an individual's entire Ag-specific polyfunctional profile into a single numerical value
- Functionality score: proportion of Ag-specific subsets detected among all possible ones
- Polyfunctionality score: similar, but weighs the different subsets by their degree of functionality, favoring subsets with higher degrees of functionality

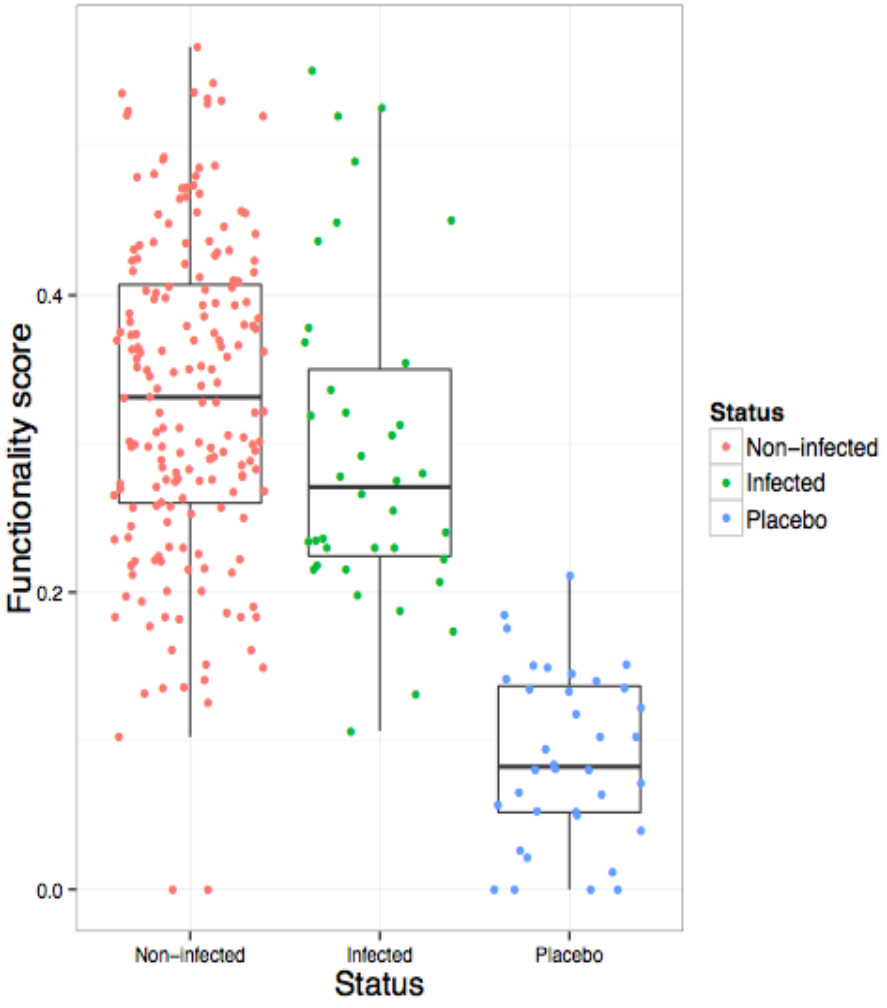
Functionality scores differ by treatment group in HVTN 078



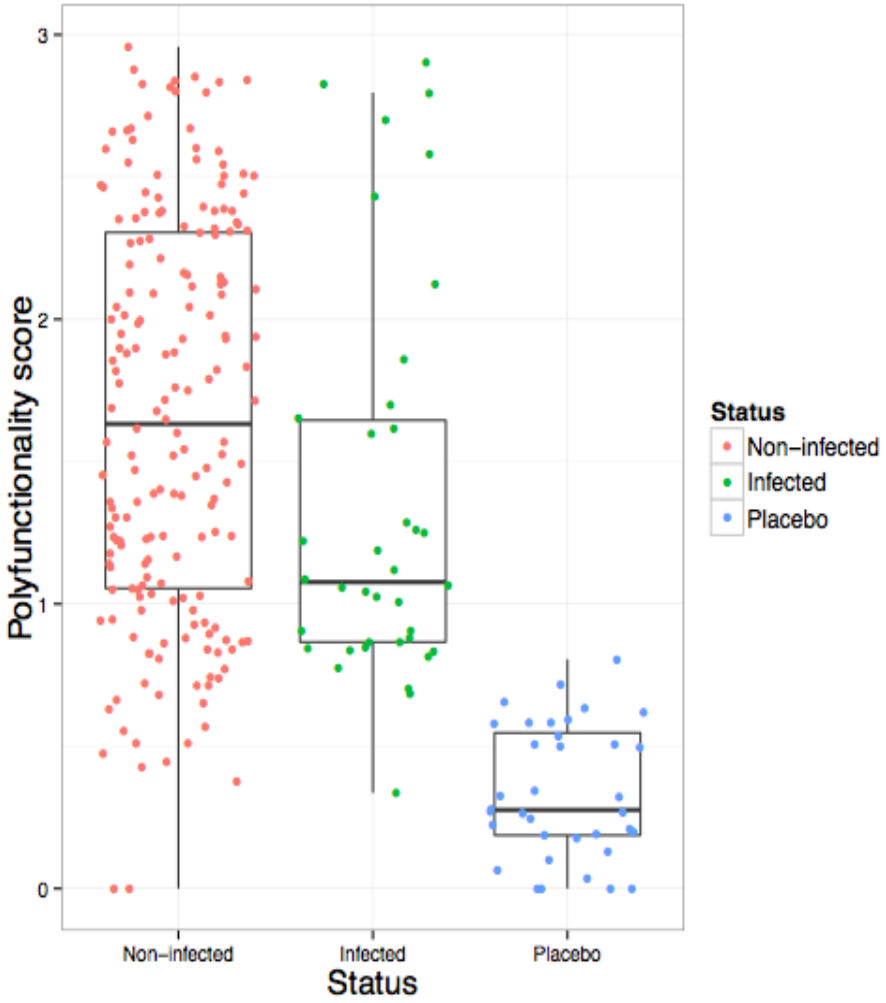
T1=NyNyAd5 10^{10}, T2=Ad5 10^{8}NyNy, T3=Ad5 10^{9}NyNy, T4=Ad5 10^{10}NyNy

Functionality scores are inversely correlated with infection in RV144

OR: 0.63; p=0.02

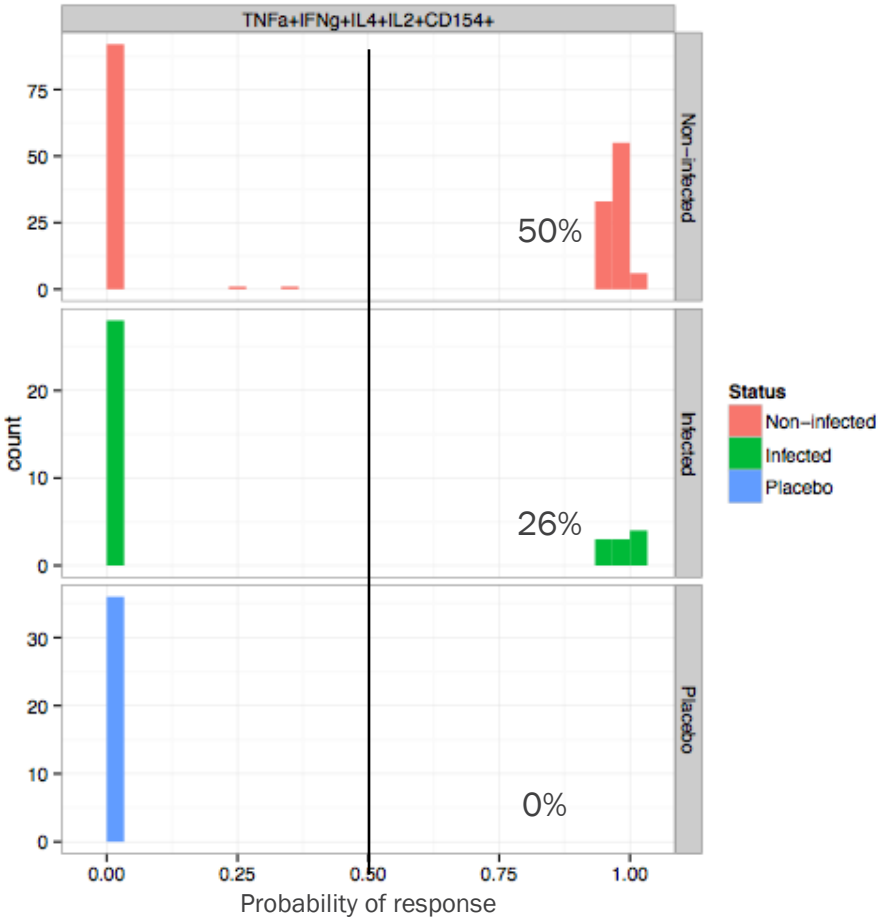


OR: 0.58; p= 0.006



High order polyfunctional subsets are driving the correlation

OR: 0.57; p=0.0062



- **5 function subset IFNγ/TNFα/IL4/IL2/CD154**
- **Some other subsets show some correlation**
  - IL4/IL2/CD154
  - IFNγ/IL4/IL2/CD154
- **Very rare subset: ~10 cells out of about 50,000 (.02%)**

Polyfunctionality appears to be an independent correlate of risk

- The five-function subset remains significant (OR=0.59, p=0.010) when both it and the V1V2 and IgA correlates are included in the model
- The effects of the V2 correlate and the five-function subset are *additive* (no evidence of interaction)

Variable	OR	Confidence Interval	P-value
5-function subset	0.59	0.40-0.89	0.011
V1V2 primary	0.62	0.42-0.94	0.022
IgA antibody primary	1.76	1.2-2.6	0.003

Why was this subset not detected in the primary analysis?

- IL4 was excluded
- Subset is rare – magnitude is very small
  - Signal drowned out by looking at cells expressing ANY cytokine
- We model the cohort as a whole rather than each subject independently
  - e.g., borrow strength
  - Small but consistent vaccine induced response

Summary

- What is a possible interpretation?
  - Ab is likely the primary correlate of protection – B-cell driven
  - B-cells require T-cell help – expect T cells producing IL4, IFNγ
  - Polyfunctionality is believed to be a good feature for CD4+ and CD8+ T cells, supported by other studies
  - Surprising that the polyfunctional correlate has Th1 and Th2 like qualities (IL4 and IFNγ simultaneously)
    - Important to be unbiased
- We will be looking at the polyfunctionality score for evaluating future vaccine candidates

Acknowledgments

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Study Volunteers!