Highly Multiplexed, Single-Cell Functional Profiling of CAR-T Cells Enables More Predictive Product Characterization, Cell Manufacturing Analysis,and Cellular Biomarkers across Product Types

Patrick Paczkowski, Dong Liu, Colin Ng, Alaina Kaiser, Sean Mackay, Jing Zhou
IsoPlexis Corporation, 35 NE Industrial Road, Branford, CT 06405

Review Poster Previously Presented

BACKGROUND

- Collecting and using a patients' own immune cells is a rapidly emerging immunotherapy approach.
- Genetically reprograming T cells to express a chimeric antigen receptor (CAR) has already paved the way for successful immunotherapies to fight against leukemia and lymphoma, and research into solid-tumor CAR-T cells is also underway.
- A lot is still unknown in terms of exactly how these re-engineered cells will behave once reinfused into a patient, including efficacy and potential side-effects.
- We review single-cell polyfunctional profiling results from several different sets of pre-infusion CAR-T samples, including anti-CD19 CAR-T samples from both Kite Pharma (Gilead) [1] and Novartis Pharmaceuticals [2], GoCAR-T cell products from Bellicum Pharmaceuticals [3], and Bispecific CD19/22 CAR-T cells from the NIH [4]. (**Figure 3**).

IsoCode platform for analyzing highly multiplexed, single-cell secretomics

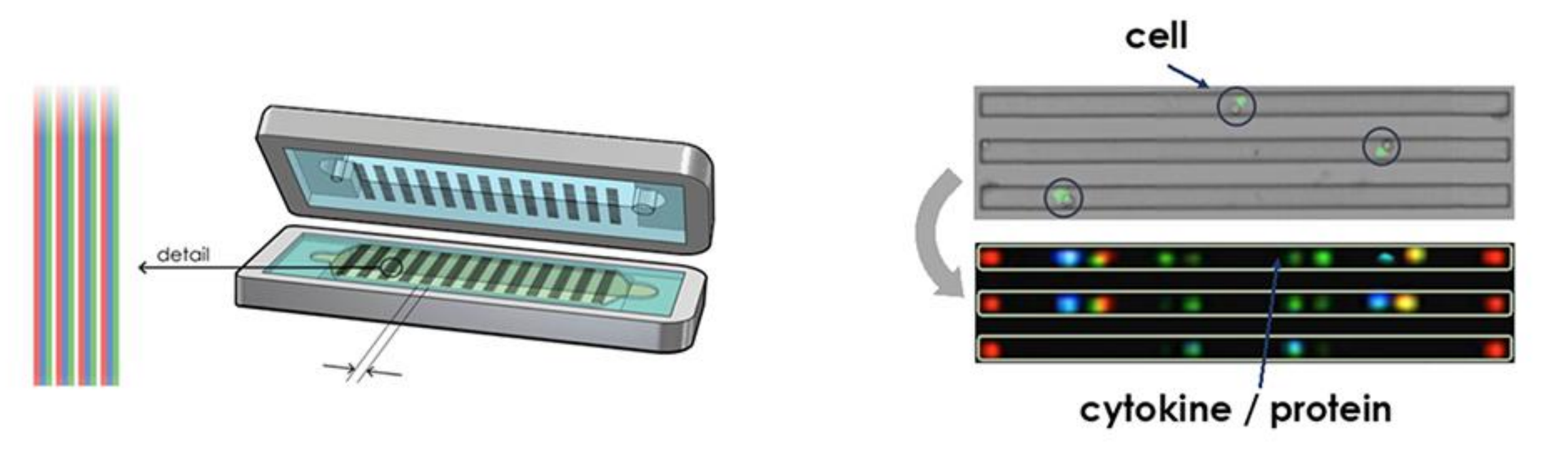

Figure 1: IsoPlexis' highly multiplexed, single-cell cytokine profiling. The IsoPlexis platform isolates thousands of single cells into individual chambers, each of which is pre-patterned with a complete copy of a 32-plex antibody array. Following a 16-hour incubation period, ELISA detection is used to determine which combinations of proteins are being secreted by each individual cell.

Profiled CAR-T samples in each reviewed study

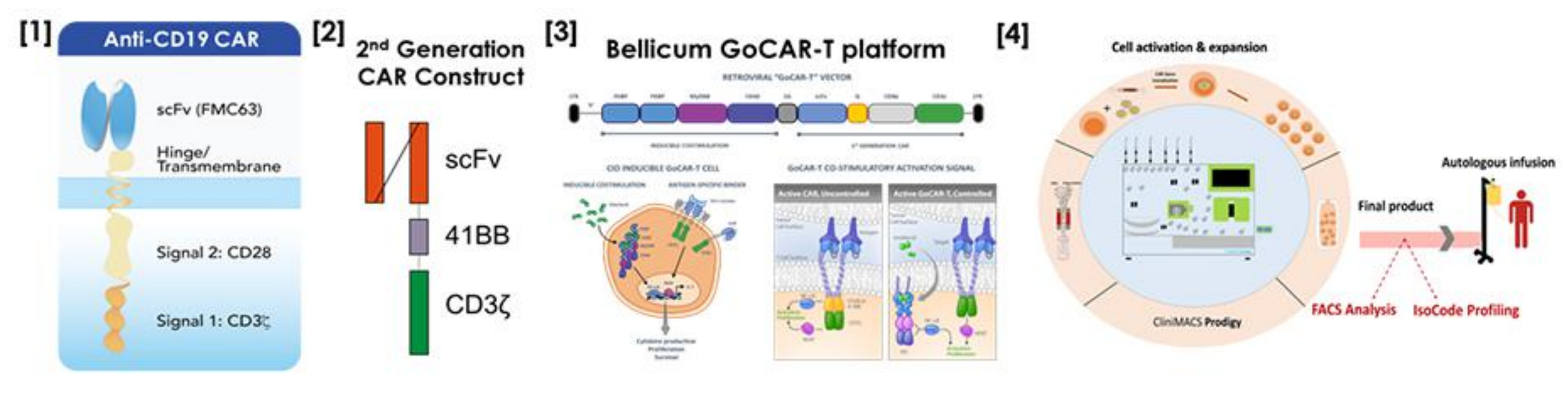

Figure 3: Profiled CAR-T samples in each reviewed study. [1] 22 NHL patients (19 DLBCL, 2 FL and 1 MCL) were treated with CAR-T therapy -- T cells transduced with an anti-CD19 CAR composed of CD28 and CD3ζ signaling domains. Of these, 20 pre-infusion CAR-T products were evaluated on a single-cell level and stimulated with target CD19-K562 cells. 14 of 20 patients showed objective response to therapy. **[2]** CAR-T cells were manufactured from human PBMCs transfected with the lentivirus encoding the CD19-BB-z transgene and expanded with anti-CD3/anti-CD28 coated beads. The enriched CAR-T cells of four healthy donors were stimulated with anti-CAR or control IgG beads. **[3]** GoCAR-T cells were manufactured from human PBMCs of healthy donors transduced with a rimiducid-inducible costimulatory unit, MyD88/CD40 (iMC), and a first-generation CAR targeting PSCA. CD4+ and CD8+ GoCAR-T cells were enriched by microbeads and stimulated with the PSCA+ human pancreatic adenocarcinoma cell line (HPAC) in the presence or absence of CID. **[4]** Bispecific CD19/CD22 CAR-T cell product purity and potency compared across two manufacturing methods. For potency analysis, antigen-specific polyfunctional cytokine upregulation in CD4+ and CD8+ CAR-T cell products was measured on 5 samples.

CD4+ CAR-T cells had a stronger measured association with clinical outcome of NHL CAR-T therapy (p = 0.0117) [1]

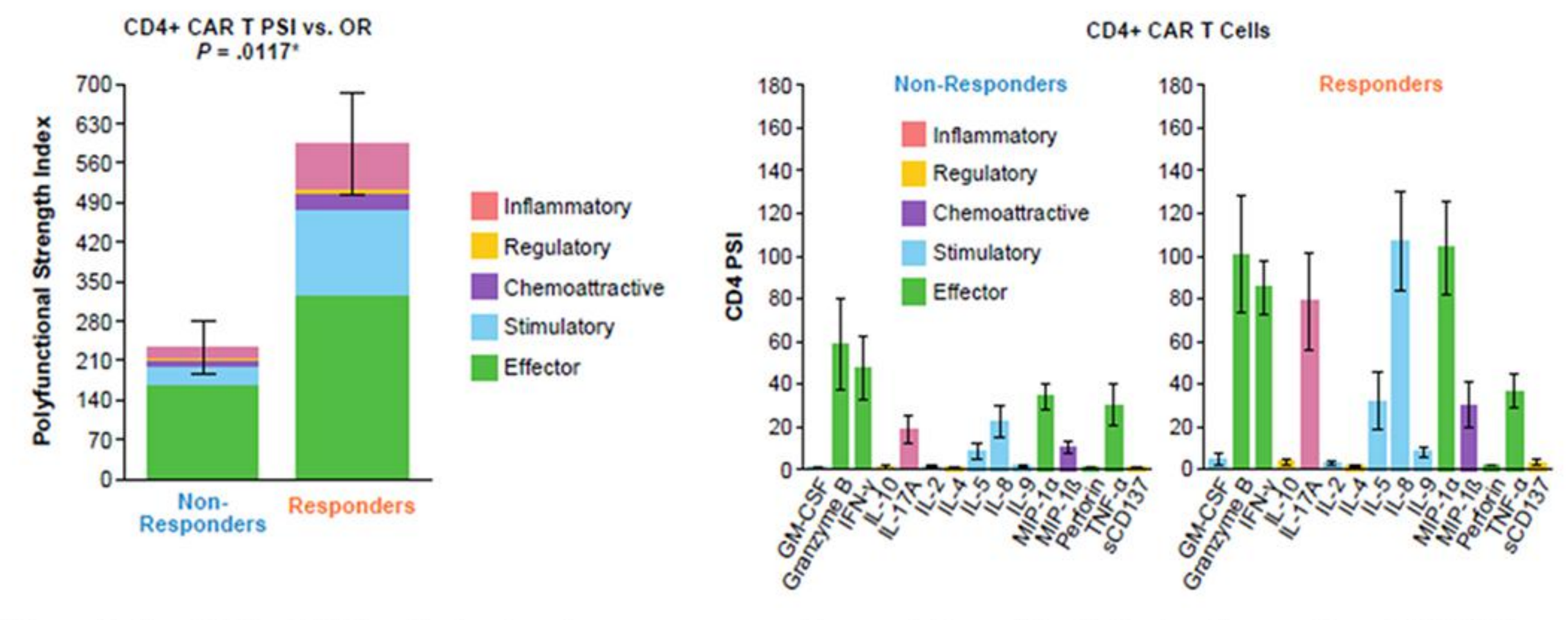

Figure 5: The CD4+ CAR-T cells had a stronger measured association with clinical outcome (p = 0.0117) than CD8+ CAR-T cells (p = 0.1528). Product CD4+ and CD8+ T-cell PSI profiles were broken down per cytokine, between patient groups with no response and objective response to CAR T-cell therapy. On average, CD4 PSI was around 220 for non-responders and 600 for responders. The dominant contributors to PSI are Granzyme B, IFN-g, IL-17A, IL-8 and MIP-1a. Smaller contributions were also visible from IL-5, MIP-1b, and TNF-a.

Consistently more robust polyfunctional response of CD19/CD22 stimulated CAR-T cell products made with modified manufacturing method. [4]

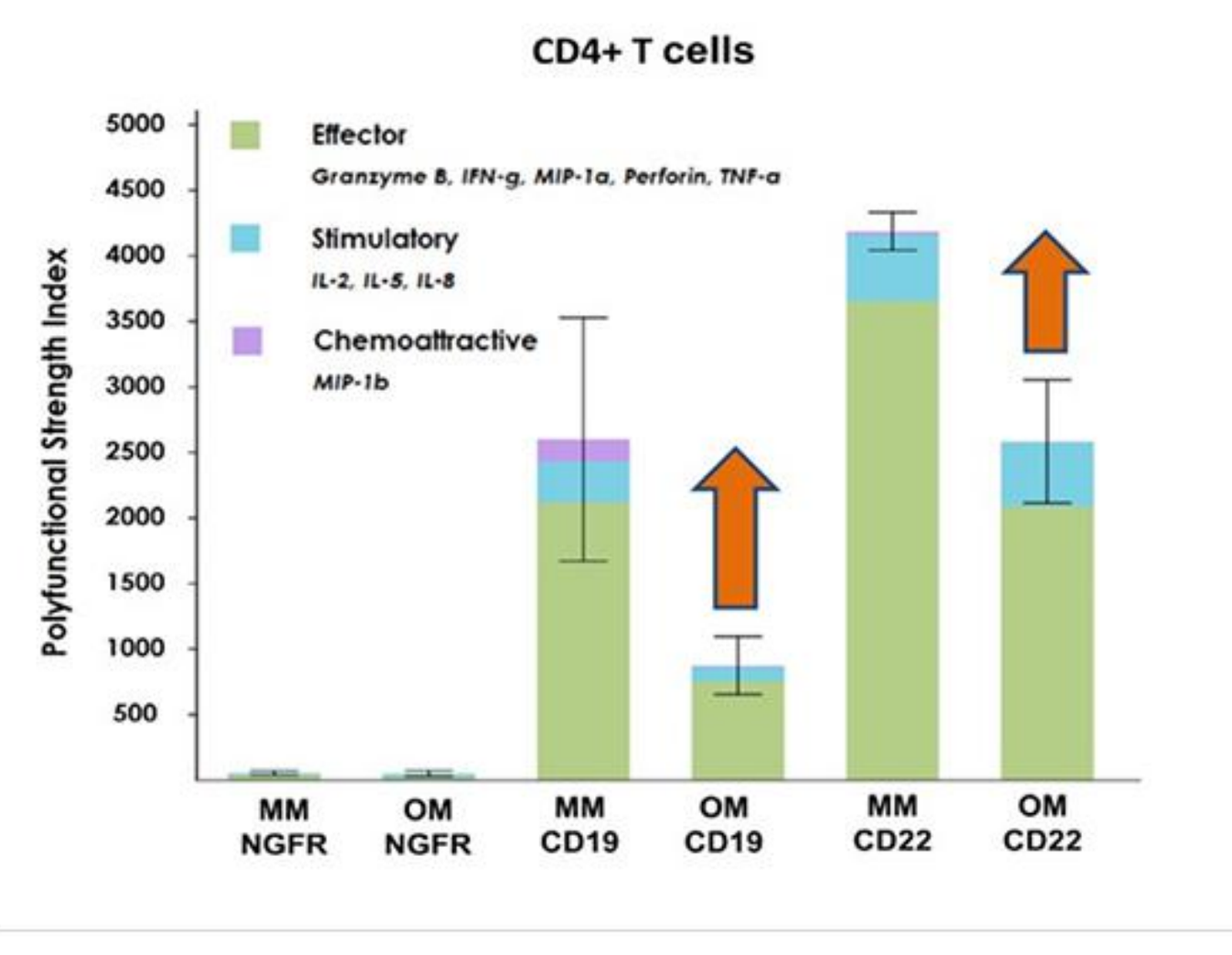

Figure 7: A more robust polyfunctional response of CD4+ (left) and CD8+ (not shown) CAR-T cell products was seen in response to stimulation with CD19 or CD22 with the modified manufacturing method (MM) compared to the original manufacturing method (OM). In particular, the effector contributions to the PSI of the MM CAR-T samples were 2x higher, and the stimulatory and chemoattractive secretions increased in response to CD19 stimulation. This data demonstrates that the modified CD19/CD22 bispecific CAR-T cell manufacturing method (MM), which terminated T cell activation/transduction by culture day 3, resulted in reproducible and robust CAR-T cell production, even in the relatively more sensitive patient cells.

Novel PAT PCA visualization reveals distinct CD4+ CAR-T cell profiles across healthy donors [2]

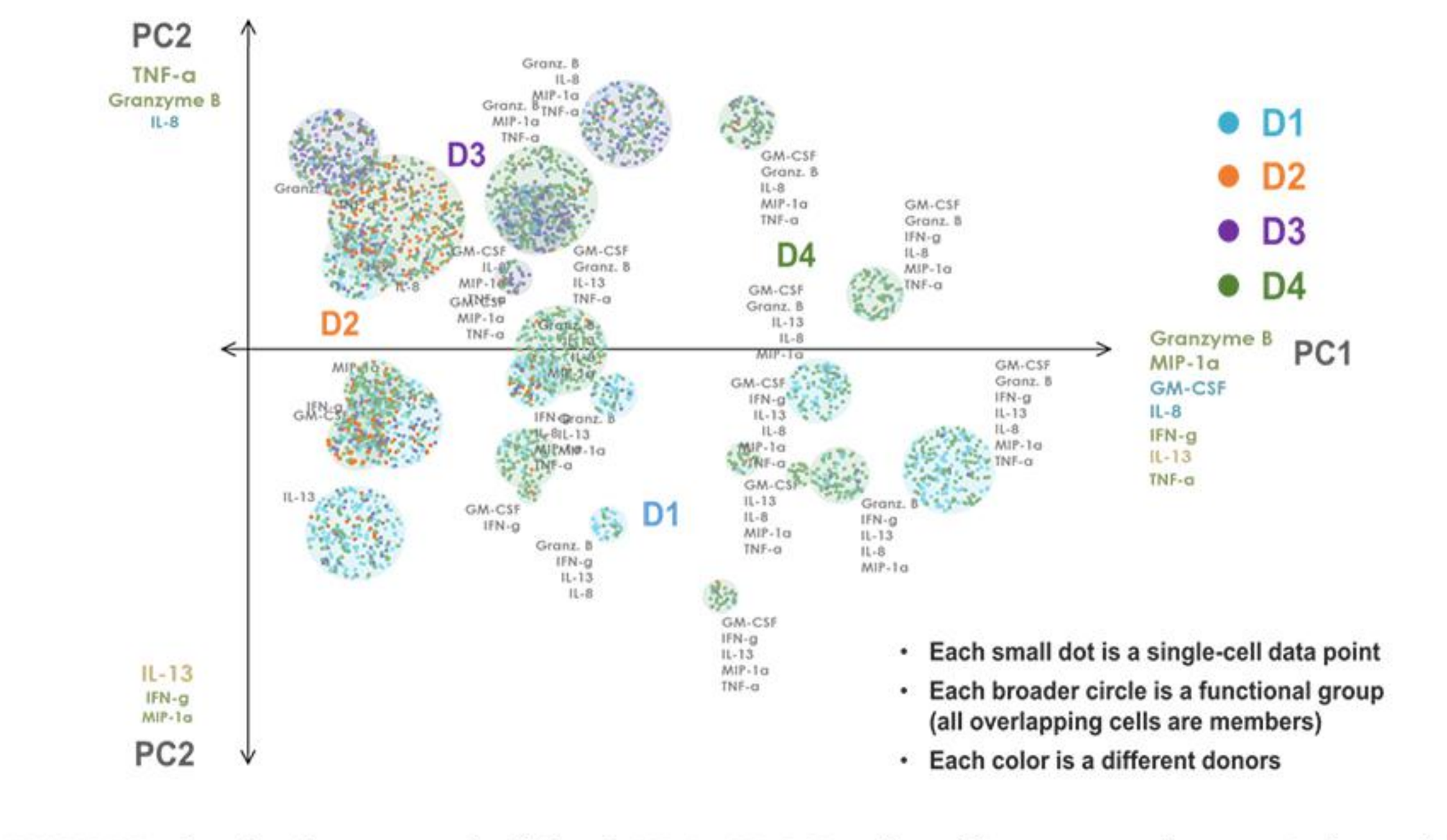

Figure 9: PAT PCA visualization reveals distinct CD4+ CAR-T cell profiles across donors. Data points are color-coded based on donor; circles represent functional groups with size proportional to frequency. The principal components are labeled according to their correlation with specific cytokines. The lack of donor 2 (orange) subsets indicates the lower polyfunctionality of this sample, while the presence of numerous donor 1 (blue) and 4 (green) groups in the right area of the graph indicates the highly-polyfunctional makeup of these two samples. Donor 3 has generally less polyfunctional subsets, typically including combinations of Granzyme B, MIP-1a, IL-8, and TNF-a but lacking IFN-γ, IL-13, and GM-CSF. Donor 4 largely spans the polyfunctional profiles of both donors 1 and 3.

Cytokine breakdown of enhanced PSI of single GoCAR-T cells stimulated by CID + HPAC [3]

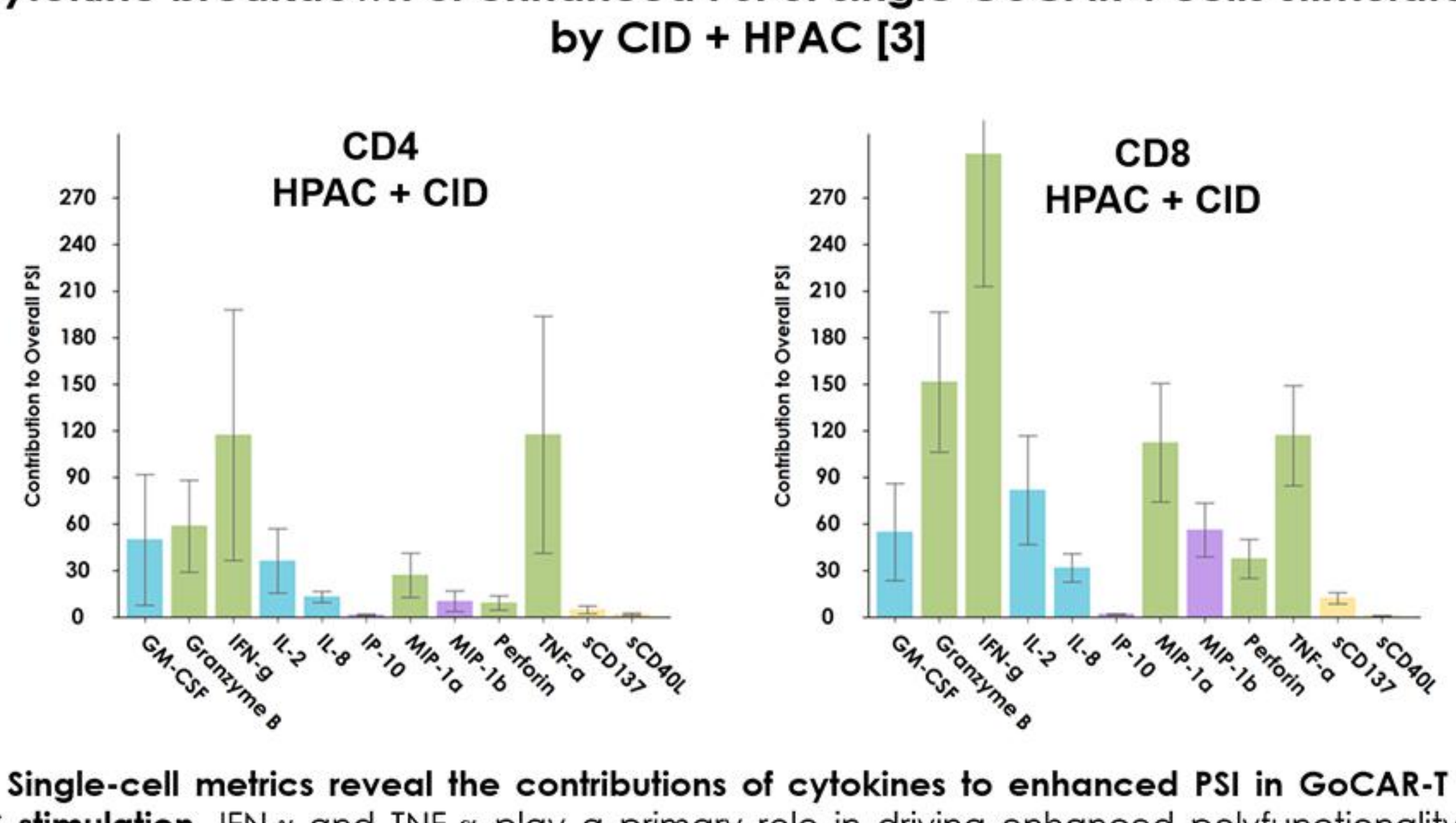

Figure 11: Single-cell metrics reveal the contributions of cytokines to enhanced PSI in GoCAR-T cells by CID+HPAC stimulation. IFN-γ and TNF-α play a primary role in driving enhanced polyfunctionality of CD4 CAR-T cells, followed by Granzyme B, GM-CSF, IL-2 and MIP-1α, and to a small degree by IL-8, MIP-1α, Perforin and sCD137. In contrast, IFN-γ followed by Granzyme B, MIP-1α, TNF-α and IL-2 are the major contributors to the enhanced polyfunctional CD8 CAR-T cells, and to a lesser extent GM-CSF, IL-8, Perforin and sCD137.

METHODS

- In each case, CD4+ and CD8+ CAR-T cells were stimulated (details in [1], [2], and [3]) and subsequently analyzed at a single-cell level using IsoPlexis' IsoCode chip. A 17-plex cytokine panel was used in study [2], while studies [1] and [3] used a 32-plex cytokine panel.
- Stimulated CAR-T cells were loaded onto IsoPlexis IsoCode chips containing ~12000 microchambers pre-patterned with a complete, 17-plex, antibody array (see **Figure 1**).
- Cells on the IsoCode chips were imaged to identify single-cell locations and incubated for 16 hours at 37°C, 5% CO_2; single-cell cytokine signals were then captured and digitized with a microarray scanner.
- The polyfunctional expression (2+ cytokines per cell, see **Figure 2**) of single CAR-T cells was evaluated using IsoPlexis' software across the panel of profiled cytokines comprising effector, stimulatory, regulatory, inflammatory, and chemoattractive functional groups.

Measuring a sample's single-cell Polyfunctional Strength Index (PSI)

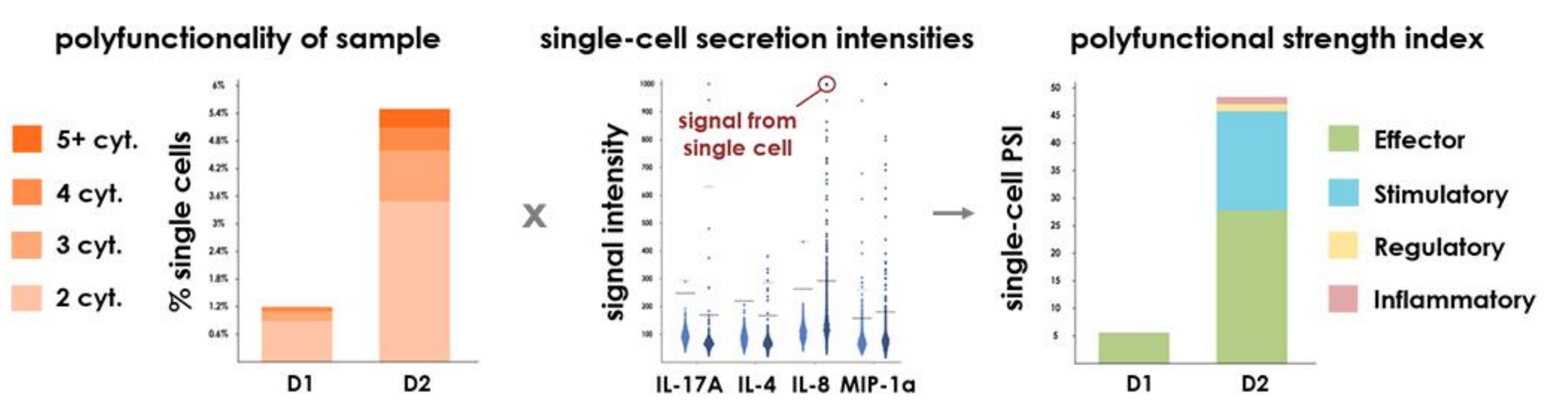

Figure 2: Measuring single-cell Polyfunctional Strength Index (PSI). A published IsoPlexis metric that quantifies the overall activity of a sample; the percentage of polyfunctional cells (secreting two or more cytokines) in a sample multiplied by the average signal intensity of the secreted cytokines. In each study, PSI was used to characterize the profiled CAR-T product, and also showed an association with clinical outcome in study [1].

Pre-infusion single-cell CAR PSI associated significantly with objective response (OR) of NHL patients [1]

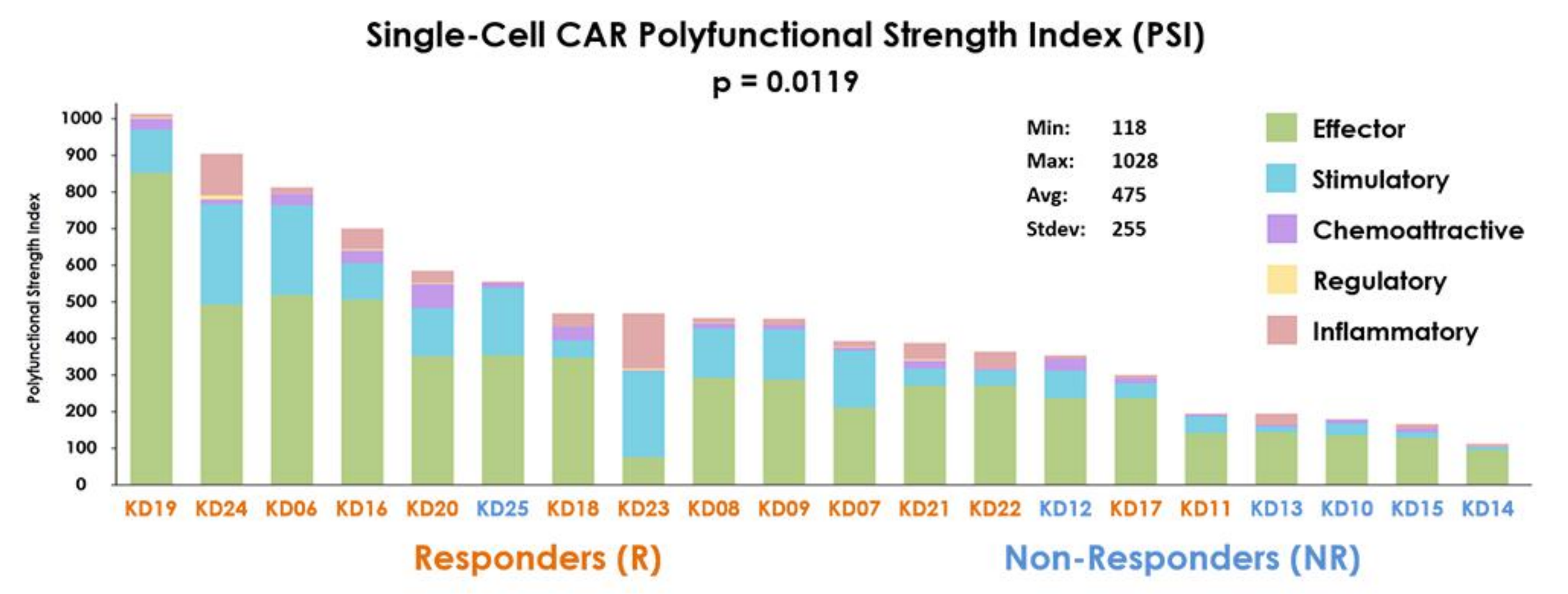

Figure 4: Pre-Infusion single-cell CAR PSI associated significantly with objective response (OR) of NHL patients (p = 0.0119). Patients are rank ordered by PSI, with responders shown in orange and non-responders shown in blue. The majority of non-responding patients had low PSI (under 200), whereas the majority of responding patients had higher PSI (350-1000). Overall, the average PSI of all patients was 475, with a standard deviation of 255, i.e. there was high heterogeneity in each patient's single-cell CAR-T product response.

CD4+ pre-infusion CAR-T drivers of association with objective response [1]

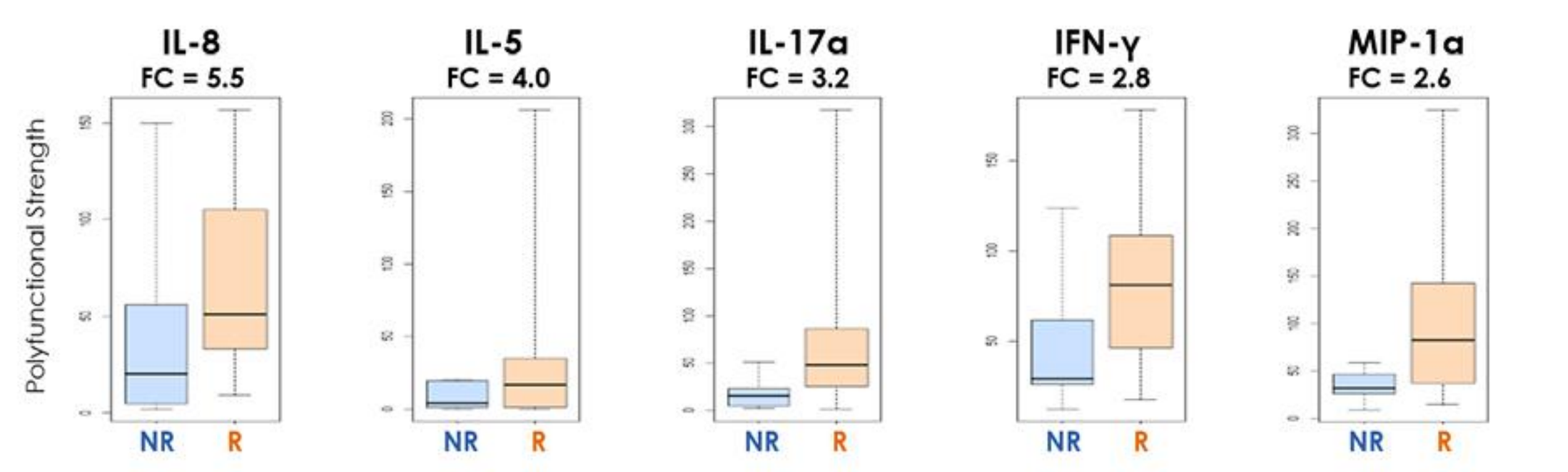

Figure 6: Drivers of CD4+ polyfunctionality in responders. Numerous pre-infusion CD4+ cytokine secretions showed an association with clinical patient outcome of CAR-T therapy, including IL-8, IL-5, IL-17A, IFN-g and MIP-1a. The PSI contributions of all the cytokines was 2-5 times higher in responding patients' CAR-T products versus non-responding patients' CAR-Ts.

Polyfunctional heterogeneity of anti-CD19 CAR products across healthy donors [2]

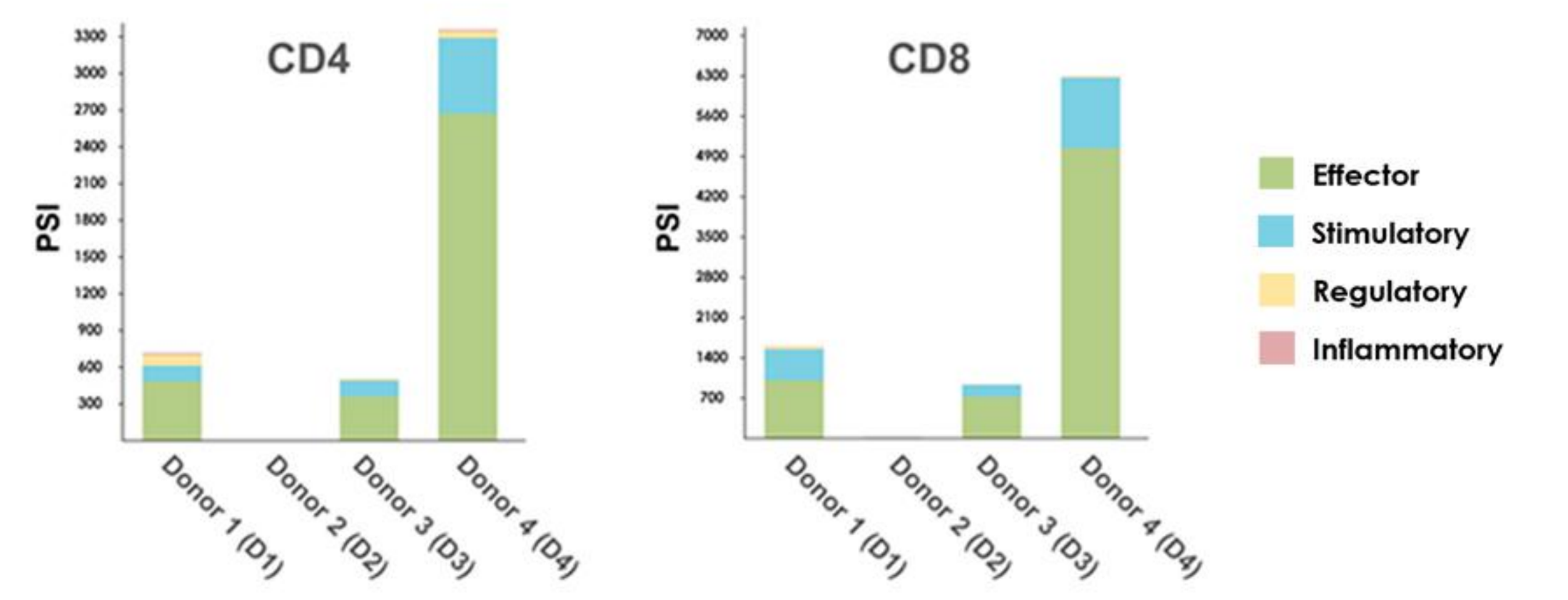

Figure 8: Polyfunctional heterogeneity of anti-CD19 CAR products across healthy donors. The CD4+ and CD8+ PSI of each donor is shown. The polyfunctional profiles of donors 1, 3 and 4 is dominated by effector and stimulatory cytokine subsets. High heterogeneity observed across donors: donor 2 had a PSI close to 0, donors 1 and 3 had PSI in the 1000-2000 range, while donor 4's PSI was several fold higher.

Synergistic impacts of Rimiducid (CID) and antigen on polyfunctional upregulation of anti-PSCA GoCAR-T cell products [3]

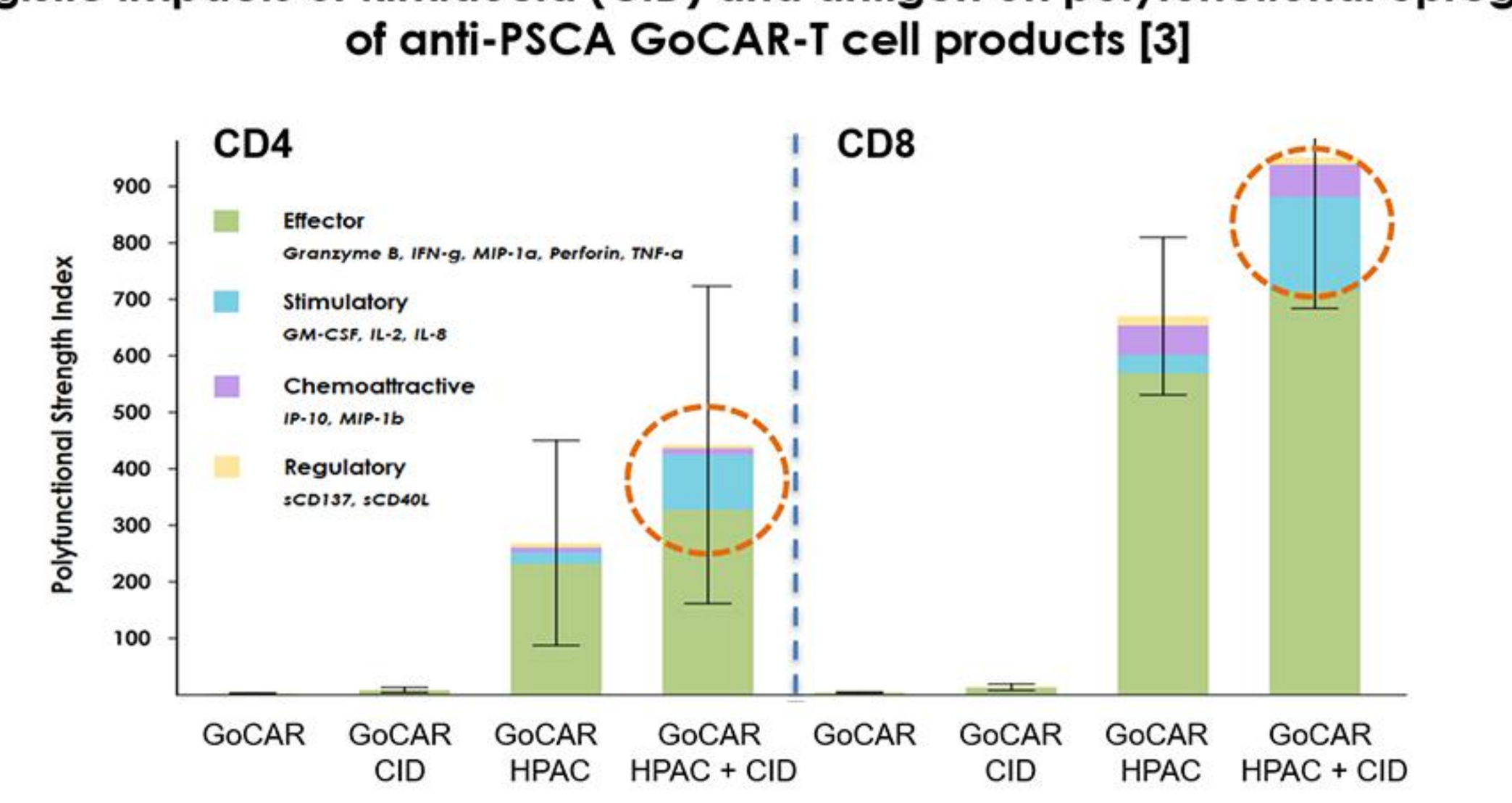

Figure 10: Synergistic impacts of Rimiducid (CID) and antigen on polyfunctional upregulation of anti-PSCA GoCAR-T cell products. Predominantly effector function PSI mixed with small amounts of stimulatory, chemoattractive and regulatory functions was induced by HPAC ± CID in both CD4+ and CD8+ GoCAR-T cells from 5 healthy donors relative to the controls of GoCAR-T cells ± CID. The average PSI across the 5 donors is shown for each of the four stimulation conditions.

RESULTS

- Single-cell profiling revealed highly polyfunctional and heterogeneous responses across each patient cohort.
- In each case, the CAR-T cells secreted a wide range of effector, stimulatory, regulatory, and inflammatory (CD4 samples only) cytokines. The secretions were highly specific to antigen-stimulation, and a significant portion of the cells were polyfunctional (secreted multiple cytokines).
- In study [3], an association was determined between the polyfunctional profiles of the CAR-T samples, and the clinical outcome of the NHL patients.
- Study [4] showed significantly higher potency of CAR-T cells manufactured using a new manufacturing method.
- See **Figures 4-11** for a review of detailed results from these three studies. Further results may also be found in the associated references [1-4].

CONCLUSIONS

- The results of these studies demonstrate the potential benefits of single-cell profiling as a way to better understand how these CAR-T products behave in response to antigen-specific stimulation.
- Analyzing the single-cell polyfunctionality of CAR-T profiles may provide a valuable quality check of the pre-infusion product, and may also help in developing biomarkers to predict patient outcome in response to the therapy.

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