Program No. 292

A GemStone Workshop on Probability State Modeling of CD3+ Cells from CyTOF-Derived Data

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Abstract

Background: Human T cells in the peripheral blood, lymph nodes, and spleen can change their surface and functional markers in response to foreign antigens. These changes can be detected and quantified by cytometric phenotyping of circulating T-cell subpopulations. Unfortunately, as the number of measurements increase, traditional gating strategies can become cumbersome. Recently, a new type of analysis paradigm, probability state modeling (PSM), was successfully used to better understand circulating CD8 T-cell antigen-dependent progressions (ref 1) and also used to automate some cytometry applications (refs 2, 3). This study investigates the use of PSM for further understanding both CD3+CD4+ and CD3+CD8+ progressions with a 39 marker data set derived from the CyTOF.

Methods: See Fig. 1 for CyTOF materials & methods. See Ref 1 for details on modeling materials & methods.

Results: The markers: DNA 1, DNA 2, Cell Length, Live/Dead, CD45tot, CD33, CD14, CD8, and CD4 were used to select events of interest for modeling both CD8+ and CD4+ T-cell populations. CCR7, CD28, and CD45RA stratified events into Naïve, Central Memory (CM), Effector Memory (EM), and Terminal Effector (EF) stages. Once staged, PSM was used to explore all other markers that modulate with stage.

CD8+ population: CD161, CD56, and CCR6 identified a Natural Killer T-cell (NKT) subpopulation located mainly in the CM stage. CCR6, CD16, CD24, CD27, CD56, CD57, CD85j, CD94, CD127, CXCR3, and PD-1 all had observable stage-related changes.

CD4+ population: CCR6, CD24, CD25, CD38, CD27, CD57, CD127, CD161, CXCR3, CXCR5, HLA-DR, ICOS, and PD-1 showed stage-related modulations.

Introduction:

In January 2014, Verity held a two-day GemStone workshop in Topsham Maine. The attendees were asked to model CD8 and CD4 T-cell antigen-dependent progressions from a 39-parameter CyTOF data file kindly provided by Michael Leipold and Holden Maecker at Stanford University (**see Fig. 1** for details). The general idea was to demonstrate how to 1) select for populations of interest, 2) stratify these populations along a progression axis, and 3) examine how other markers modulate as a function of these progressions. This poster chronicles the results and conclusions drawn from that workshop.

Selections:

Figure 2A shows the gating strategy for obtaining events that were intact, non-aggregated, viable, CD3+, and either CD8+ or CD4+. Using this gating strategy as a guide, an analogous set of ten GemStone selection expression profiles (EPs) were created (**see Fig. 2B**). One of the differences between gating and modeling is shown in **Fig. 2C**. Modeling accounted for the overlap between all defined populations. Accounting for overlap was important for CyTOF data since positive marker cv's tended to be two or three times equivalent fluorescence-based cv's.

Stratifications:

Figure 3A shows the gating strategy for dividing either CD8+ or CD4+ events into the T-cell stages: **Naïve** (CD45RA+ CCR7+) , **Central Memory** (CM, CD45RA- CCR7+), **Effector Memory** (EM, CD45RA- CCR7-), and **Terminal Effector** (EF, CD45RA+ CCR7-).

Figure 3B shows the analogous GemStone stratification strategies for CD8 and CD4 T cells. Notice that for the CD8+ population (left overlay), CD45RA and CCR7 down-regulate together (**see black arrows**). A recent publication (ref 1) demonstrated that for CD8+ T cells, the down-regulation of CCR7 and CD45RA were highly correlated and occurred at the same point in the progression, suggesting that using the down-regulation of CCR7 as a CM to EM staging marker is inappropriate. Therefore, CD28 was added as an additional staging marker for the CD8+ T cells.

In contrast, CD4+ T cells down-regulate CCR7 well after CD45RA down-regulates (**see middle overlay's red arrows**) and can therefore be used to define a CM to EM boundary. Both CD28 and CD27 were added to the CD4+ overlay (**see middle overlay**) to appreciate timing of their down-regulation with respect to CCR7's down-regulation. Since both CD28 and CD27 down-regulate after CCR7, they might be valuable to further subset the progression. The bivariate surface plots at the right of each overlay show the model's progressions against some popular marker combinations. Notice how difficult it is to interpret the timing of CD45RA, CCR7, CD28, and CD27 changes with standard dot-plots. Figure 3C again demonstrates the probabilistic nature of GemStone, where it accounts for the overlap among all the stage-related populations such as CCR7.

Exploration:

Once events were stratified along the progression axis, all the other 29 markers were examined for stage-related changes. **Figure 4A** summarizes the markers that were found to modulate with stage for CD8+ events and **Fig. 4B** for CD4+ events.

Figure 5 shows the individual CD8+ expression profiles that demonstrated significant stage-related changes. The affected markers were CCR6, CD16, CD24, CD27, CD56, CD57, CD85j, CD94, CD127, CD161, CXCR3, and PD-1. The separate population found in the CM stage for CD161 expression profile was identified as TNK events.

Figure 6 shows a TriCOM analysis for this NKT population involving CD161, CCR6, and CD56 (**see the figure legend for details**).

Figure 7 shows the CD4+ expression profiles that also changed with stage. The affected markers were CCR6, CD24, CD25, CD27, CD38, CD57, CD127, CD161, CXCR3, CXCR5, HLA-DR, ICOS, and PD-1.

Many of these CD8+ and CD4+ stage-related modulations were expected, but others were not and may deserve more study.

1

CyTOF

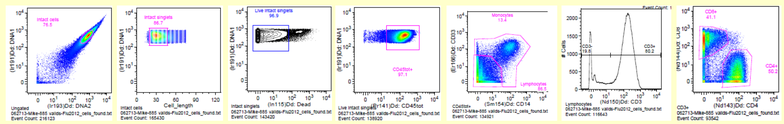
39 Correlated Measurements

Figure 1. CyTOF Immunophenotyping. This assay was performed in the Human Immune Monitoring Center at Stanford University. PBMCs were thawed in warm media, washed twice, resuspended in CyFACS buffer (PBS supplemented with 2% BSA, 2 mM EDTA, and 0.1% soium azide), and viable cells were counted by Vicell. Cells were added to a V-bottom microtiter plate at 1.5 million viable cells/well and washed once by pelleting and resuspension in fresh CyFACS buffer. The cells were stained for 60 min on ice with 50 uL of the following antibody-polymer conjugate cocktail: [insert Ab list here]. All antibodies were from purified unconjugated, carrier-protein-free stocks from BD Biosciences, Biolegend, or R&D Systems. The polymer and metal isotopes were from DVS Sciences. The cells were washed twice by pelleting and resuspension with 250 uL FACS buffer. The cells were resuspended in 100 uL PBS buffer containing 2 ug/mL Live-Dead (DOTA-maleimide (Macrocyclics) containing natural-abundance indium). The cells were washed twice by pelleting and resuspension with 250 uL PBS. The cells were resuspended in 100 uL 2% PFA in PBS and placed at 4C overnight. The next day, the cells were pelleted and washed by resuspension in fresh PBS. The cells were resuspended in 100 uL eBiosciences permeabilization buffer (1x in PBS) and placed on ice for 45 min before washing twice with 250 uL PBS. If intracellular staining was performed, the cells were resuspended in 50 uL antibody cocktail in CyFACS for 1 hour on ice before washing twice in CyFACS. The cells were resuspended in 100 uL iridium-containing DNA intercalator (1:2000 dilution in PBS; DVS Sciences) and incubated at room temperature for 20 min. The cells were washed twice in 250 uL Milli$Q$ water. The cells were diluted in a total volume of 700 uL in Milli$Q$ water before injection into the CyTOF (DVS Sciences). Data analysis was performed using FlowJo v9.3 (CyTOF settings) by gating on intact cells based on the iridium isotopes from the intercalator, then on singlets by Ir191 vs. cell length, then on live cells (Indium-LiveDead minus population), followed by cell subset-specific gating.

2A

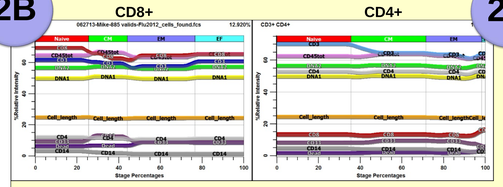
Strategies for Population Selections

Gating



2B

GemStone Modeling



2C

Population Overlap

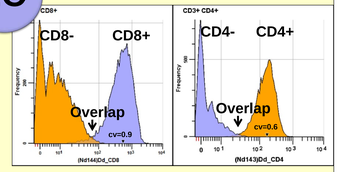
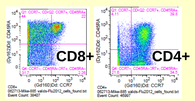


Figure 2. Strategies for population selections. Panel A shows the gating strategy for selecting intact, non-aggregated, live, CD3+, and either CD4+ or CD8+ populations. Panel B shows the analogous GemStone modeling strategy using ten expression profiles (CD3, CD4, CD8, CD14, CD33, CD45tot, Cell_length, Dead, DNA1, and DNA2). Panel C demonstrates the probabilistic nature of GemStone that accounts for any overlap between the populations such as CD8 and CD4 positive and negative populations. Accounting for overlap was important for CyTOF data since positive marker cv's tended to be two or three times equivalent fluorescence-based cv's.

3A

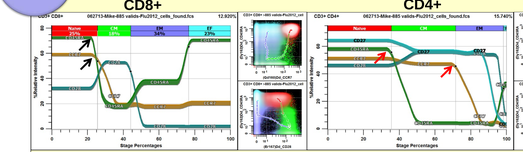
Strategies for Analyzing Progressions

Gating



3B

GemStone Modeling



3C

Population Overlap

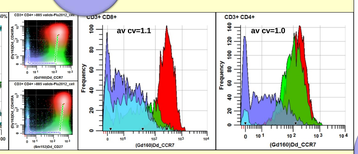


Figure 3. Strategies for analyzing progressions. Panel A shows the gating strategy for dividing either CD8+ or CD4+ events into the T-cell stages: Naïve (CD45RA+ CCR7+) , Central Memory (CM, CD45RA- CCR7-), Effector Memory (EM, CD45RA- CCR7-), and Terminal Effector (EF, CD45RA+ CCR7-). Panel B shows the analogous GemStone stratification strategies for CD8 and CD4 T cells. Notice that for the CD8+ population (left-most overlay), CD45RA and CCR7 down-regulate together (see black arrows). A recent publication (ref 1) demonstrated that for CD8+ T cells, the down-regulation of CCR7 and CD45RA were highly correlated and occurred at the same point in the progression, suggesting that using the down-regulation of CCR7 as a CM to EM staging marker is probably inappropriate. Therefore, CD28 was added as an additional staging marker for the CD8+ T cells.

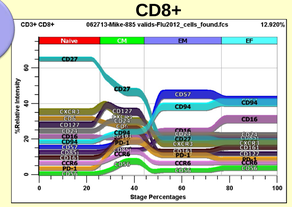
In contrast, CD4+ T cells down-regulate CCR7 well after CD45RA down-regulates (see middle overlay's red arrows) and can therefore be used to define a CM to EM boundary. Both CD28 and CD27 were added to the CD4+ overlay (see middle overlay) to appreciate timing of their down-regulation with respect to CCR7's down-regulation. Since both CD28 and CD27 down-regulate after CCR7, they might be valuable to further subset the progression. The bivariate surface plots at the right of the overlays show the model's progressions against some popular marker combinations. Notice how difficult it is to interpret the timing of CD45RA, CCR7, CD28, and CD27 changes with standard dot-plots.

Panel C again demonstrates the probabilistic nature of GemStone, where it accounts for the overlap among all the stage-related populations such as CCR7.

4A

Exploration of Stage-Related Marker Changes

CD8+



4B

CD4+

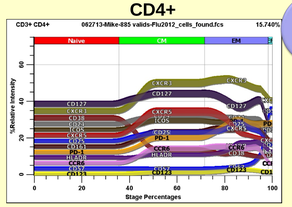


Figure 4. Exploration of Stage-Related Marker Changes. Panel A summarizes the 12 expression profiles that were also found to modulate with the CD8+ stages (CCR6, CD16, CD24, CD27, CD56, CD57, CD85j, CD94, CD127, CD161, CXCR3, and PD-1) and Panel B summarizes the 13 expression profiles that also modulated with CD4+ stages (CCR6, CD24, CD25, CD27, CD38, CD57, CD127, CD161, CXCR3, CXCR5, PD-1, HLA-DR, and ICOS).

5

CD8+ Stage-related Changes

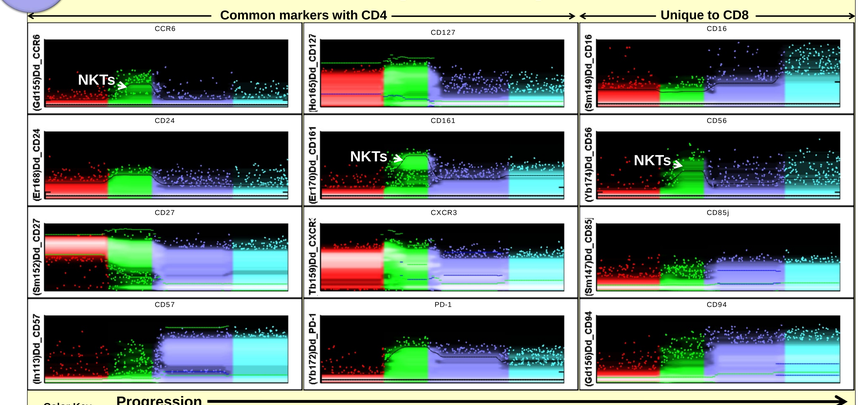


Figure 5. Stage-related changes for CD8+ events. The affected markers were CCR6, CD16, CD24, CD27, CD56, CD57, CD85j, CD94, CD127, CD161, CXCR3, and PD-1. The separate population shown best in the CD161 expression profile was identified as NKT cells. Both CCR6 and CD56 were also found to be mostly positive for this population (see Fig. 6 for details). Many of these stage-related changes are well known (CCR6, CD27, CD57, CD127, PD-1); however, many others were not and therefore may deserve further study. The first two columns of markers also modulated with stage for the CD4+ population (see Fig. 7) and the last column only significantly changed with stage for the CD8+ population.

6

NKT TriCOM Analysis

Key for CM Combinations

CD161 A	CD56 B	CCR6 C	%
+	+	+	1.53
+	-	+	0.96
+	+	-	0.68
-	+	+	0.0
+	-	-	1.07
-	+	-	0.85
-	-	+	0.60

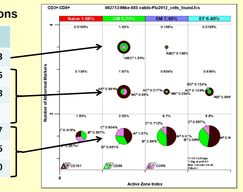


Figure 6. NKT TriCOM Analysis. Pie charts on the bottom row of the TriCOM graph (right panel) are positive for just one of the three markers: CD161, CD56, and CCR6. The next row up shows the distribution of events that are positive for two markers. This sequence continues for however many markers are being subjected to the analysis. The area of each pie chart is proportional to its percentage.

The segments of each pie chart show the individual marker combinations in descending order, moving clock-wise around the pie chart. This analysis is done on each of the stages or progression zones, which divides up the chart into four columns for this particular case. Each marker is assigned a color and the combination key is shown at the bottom of the TriCOM panel. For the higher rows, the pie charts are divided into concentric circles showing the specific combinations with the marker colors. Not shown in this case is the capability of showing lower than normal expression using hatched fills. The boundaries between low-to-normal as well as normal-to-high are determined statistically. The key at the left shows the same data for just the CM stage in a more familiar format.

In this sample, 1.53% of the CD8+ events were CD161+ CD56+ CCR6+ and 1.67% were either CD161+ CD56+ CCR6- or CD161+ CD56- CCR6+. Most of the NKTs were found in the CD8+ CM stage.

7

CD4+ Stage-Related Changes

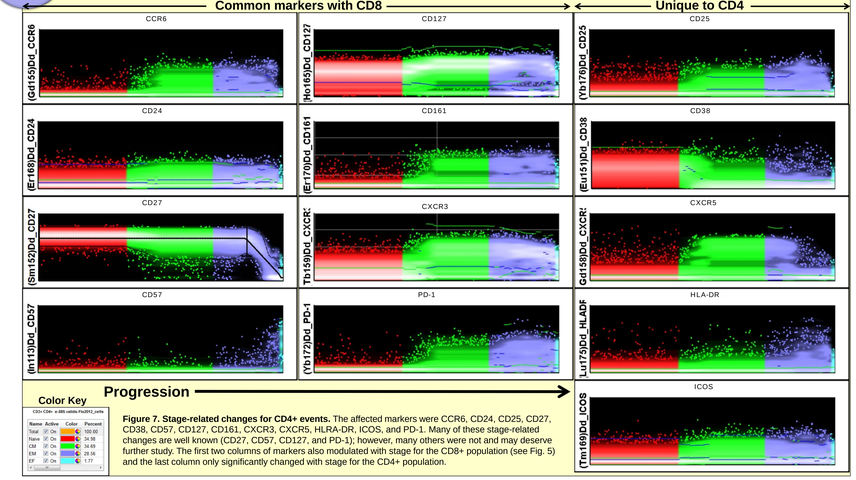


Figure 7. Stage-related changes for CD4+ events. The affected markers were CCR6, CD24, CD25, CD27, CD38, CD57, CD127, CD161, CXCR3, CXCR5, HLRA-DR, ICOS, and PD-1. Many of these stage-related changes are well known (CD27, CD57, CD127, and PD-1); however, many others were not and may deserve further study. The first two columns of markers also modulated with stage for the CD8+ population (see Fig. 5) and the last column only significantly changed with stage for the CD4+ population.

Conclusions

- Selecting cells of interest for CyTOF data is basically the same as for fluorescence-based cytometric data except that CyTOF positive populations generally have wider cv's and possibly more overlap between these populations.
- For CD8+ T cells, both CCR7 and CD45RA down-regulate together and CD28 is necessary to properly stage the events.
- For CD4+ T cells, CCR7 down-regulates well after CD45RA and therefore just those two markers can adequately stage the events.
- For CD4+ T cells, CD28 and CD27 down-regulate after CCR7 and may potentially be used to further stage the events.
- A NKT subset of events (CD161+ CD57+/-, CCR6+/-) was found primarily in the CD8+ CM stage.
- The markers; CCR6, CD16, CD24, CD27, CD56, CD57, CD85j, CD94, CD127, CD161, CXCR3, and PD-1 were found to modulate with CD8+ stage in addition to the staging markers CCR7, CD45RA, and CD28.
- The markers; CCR6, CD24, CD25, CD27, CD38, CD57, CD127, CD161, CXCR3, CXCR5, HLRA-DR, ICOS, and PD-1 were found to modulate with CD4+ stage in addition to the staging markers CCR7, CD45RA, and CD28.
- Markers CCR7, CD28, CD45RA, CCR6, CD24, CD27, CD57, CD127, CD161, CXCR3, and PD-1 modulate with stage for both CD8+ and CD4+ T cells.
- Markers CD16, CD56, CD85j, and CD94 seem to modulate with only CD8+ T cells.
- Markers CD25, CD38, CXCR5, HLA-DR, and ICOS seem to modulate only with CD4+ T cells.
- Probability state modeling with GemStone can select and stage events based on numerous correlated measurements.
- Once staged, it is very easy to screen addition markers for stage-related changes and thereby better understand T-cell biology.

References

1. Inokuma et al. JIM 397 (2013) 8-17.

2. Miller et al. Cytometry Part B 82B (2012) 319-324.

3. Herbert et al. Cytometry Part B 82B (2012) 313-318.

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Final dimensions: 44 in high, 66 in wide.