Evaluation of antigen-specific T-cell immunity at the single cell level using large panels of DNA barcoded MHC multimers

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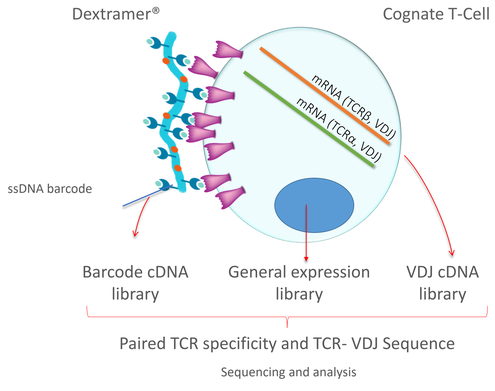
1) Introduction:

Identification of disease-specific T-cell epitopes is key to the development of many novel vaccines and immunotherapies. Profiling disease-specific T cells, emerging during a cellular immune response e.g. in tumor development or destruction, is an important aspect of personalized immunotherapy.
We have developed dCODE™ Dextramer® technology, for detection of antigen specific T-cells through DNA barcode using next generation sequencing. In combination with 10x Genomics feature Barcode protocol the technology allows coupling of TCR-specificity and –sequence of antigen-specific T cells (ASTC), in combination with transcriptional analysis.
Here we show data from sample analysis with a panel of 50 MHC dCODE™Dextramer® specificities, including 6 negative controls, spanning 8 alleles.



2) dCODE™Dextramer®panel design and staining

CD8-enriched HPBMC from a healthy donor (HLA-Alleles: A*0201, A*1101, B*3501) were stained with a panel of 50 dCODE Dextramer reagents according to established protocol. The panel consisted of 44 dCODE™Dextramer®, comprising different viral and cancer epitopes, and 4 negative control dCODE™Dextramer® reagents (Table 1). Cells were phenotyped using 11 TotalSeq™C antibodies (BioLegend); CD3, CD19, CD45Ra, CD4, CD8a, CD14, CD45Ro, CD279(PD-1), CD127(IL7Ra), CD197(CCR7), HLA-DR.

The stained sample was FACSorted for CD8+ MHC dCODE™Dextramer®+ cells and loaded onto a 10x Chromium controller. Using the Chromium single cell V(D)J Reagent kit with feature barcoding technology, 3 libraries were generated: Feature barcode (dCODE and TotalSeq), VDJ and RNA expression libraries. Next generation Sequencing (NGS) of each library were performed using Illumina sequencing platform.

Table 1: dCODE™Dextramer®panel

Cat.no	Alelle	Peptide	Antigen	Cat.no	Alelle	Peptide	Antigen
WA2131-PfBC	A*0101	VTEHDTLLV	CMV/Viral	WB2177-PfBC	A*0201	RMFPNAPYL	WT-1/cancer
WB2660-PfBC	A*0201	KTWGQYWQV	gp100/Cancer	WB2191-PfBC	A*0201	YLNDHLEPWI	BCL-X/Cancer
WB2162-PfBC	A*0201	ELAGIGILTV	MART-1/Cancer	WB2652-PfBC	A*0201	MLDLQPETT	HPV/Viral
WB3697-PfBC	A*0201	CLLWSFQTSA	Tyrosinase/Cancer	WC2197-PfBC	A*0301	KLGGALQAK	IE-1/CMV
WB2158-PfBC	A*0201	IMDQVPFSV	gp100/Cancer	WC2656-PfBC	A*0301	RLRAEAQVK	EBV/Viral
WB3247-PfBC	A*0201	SLLMWITQV	NY-ESO-1/Cancer	WC2632-PfBC	A*0301	RIAAWMATY	BCL-2L1/Cancer/
WB3497-PfBC	A*0201	KVAELVHFL	MAGE A3/Cancer	WD2175-PfBC	A*1101	IVTDFSVIK	EBV/Viral
WB3474-PfBC	A*0201	KVLEYVIKV	MAGE-A1/Cancer	WD2149-PfBC	A*1101	AVFDRKSDAK	EBV/Viral
WB5066-PfBC	A*0201	CLLGTYTQDV	Kana. B dioxyg.	WK2138-PfBC	B*3501	IPSINVHHY	CMV/Viral
WB2143-PfBC	A*0201	LLDFVRFMGV	EBV/Viral	WF2196-PfBC	A*2402	AYAQKIFKI	CMV/Viral
WB3307-PfBC	A*0201	LLMGTLGIVC	HPV/Viral	WF2133-PfBC	A*2402	QYDPVAALF	CMV/Viral
WB2144-PfBC	A*0201	CLGGLLTMV	EBV/Viral	WH2165-PfBC	B*0702	QPRAPIRPI	EBV/Viral
WB3531-PfBC	A*0201	YLLEMLWRL	EBV/Viral	WH2136-PfBC	B*0702	TPRVTGGGAM	CMV/Viral
WB3529-PfBC	A*0201	FLYALALLL	EBV/Viral	WH2166-PfBC	B*0702	RPPIFIRRL	EBV/Viral
WB2161-PfBC	A*0201	GILGFVFTL	Flu/Viral	WH2135-PfBC	B*0702	RPHERNGFTVL	CMV/Viral
WB2130-PfBC	A*0201	GLCTLVAML	EBV/Viral	WI2148-PfBC	B*0801	RAKFKQLL	EBV/Viral
WB2132-PfBC	A*0201	NLVPMVATV	CMV/Viral	WI2137-PfBC	B*0801	ELRRKMMYM	CMV/Viral
WB2139-PfBC	A*0201	ILKEPVHGV	HIV/Viral	WI2147-PfBC	B*0801	FLRGRAYGL	EBV/Viral
WB5335-PfBC	A*0201	FLASKIGRLV	P-lipase	Negative controls			
WF2639-PfBC	A*2402	CYTWNQMNL	WT1/Cancer				
WB2646-PfBC	A*0201	RTLNAWVKV	HIV/Viral	WA3580-PfBC	A*0101	SLEGGGLGY	NC
WB2157-PfBC	A*0201	KLQCVDLHV	PSA/Cancer	WA3579-PfBC	A*0101	STEGGGLAY	NC
WB2141-PfBC	A*0201	LLFGYPVYV	HTLV-1/Viral	WB2666-PfBC	A*0201	ALIAPVHAV	NC
WB3338-PfBC	A*0201	SLFNTVATL	HIV/Viral	WF3231-PfBC	A*2402	AYSSAGASI	NC
WB3339-PfBC	A*0201	SLYNTVATLY	HIV/Viral	WH3397-PfBC	B*0702	GPAESAAGL	NC
WB3340-PfBC	A*0201	SLFNTVATLY	HIV/Viral	NI3233-PfBC	NR	AAKGRGAAL	NC

3) NGS Data analysis:

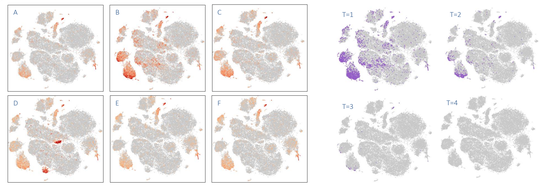
Analysis of the sequencing data were performed using Cell Ranger, by dimensional reduction, clustering, and t-SNE calculation. Loupe Cell Browser, and Loupe V(D)J Browser were used for visual analysis (10x Genomics software package).

Analysis method:

The following stringent rules were set up for the targeted analysis:

- The "background" threshold was defined using the negative controls
- The dCODE positive ASTC populations were defined as having a signal over the said threshold "signals" defined by the negative control reagents
- Only MHC matching the alleles of the donor were considered positive
- ASTC's must be CD8+
- ASTC's binding more than one dCODE Dextramer was excluded

Fig. 1: Setting dCODE Dextramer threshold



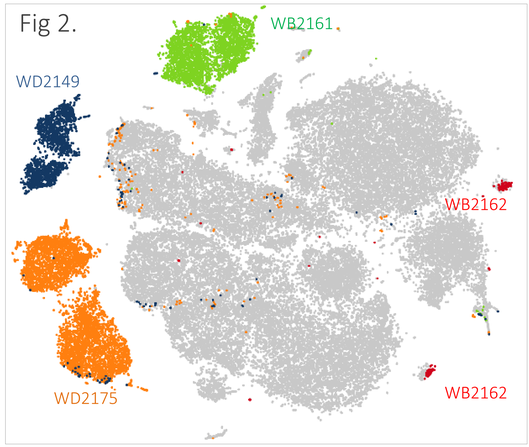
(Fig 1, left A-F,) Staining observed for the 6 negative control dCODE Dextramer reagents.
(Fig 1, T=1 to T=4), shows the effect of increasing the threshold on the negative control. The ***Threshold = log$_2$(reads+1) = 4*** *was used to analyze each of the 44 dCODE Dextramer specificities, in the assay. Same approach was used for the Antibody staining, using iso type control antibodies.*

4) Antigen-specific T-cell populations detected

Each of the 44 MHC dCODE reagent data were analyzed for identification of positive cells using a threshold=4 and visualized by t-SNE plot

WB2161 A*0201/GILGFVFTL (Flu MP, Influenza)
WD2175 A*1101/IVTDFSVIK (EBNA 3B, EBV)
WD2149 A*1101/AVFDRKSDAK (EBNA 3B, EBV)
WB2162 A*0201/ELAGIGILTV (MART-1)

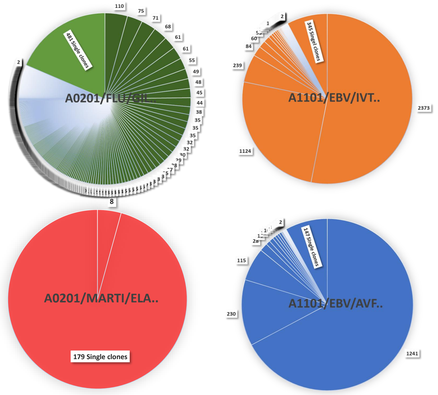
Fig 2.



- 4 distinct dCODE Dextramer positive ASTC populations were found (Fig 2). Each population was bisected, indicating different phenotypes within each ASTC populations (see why in 6)

5) Clonal distribution of ASTC's

Table 2:	Cat.no.	WB2161	WD2175	WD2149	WB2162
Specificity (pMHC)		FLU	EBV1	EBV2	MART 1
Number of positive cells		2594	4472	1846	187
Number of specific clones		732	389	166	180
No. cell in highest freq. clone		110	2373	1241	8*
Frequency of highest clone		4%	53%	67%	4%
Clones of > 1 cell		251	44	19	1
Clones of 1 cell		481	345	147	179
Frequency of Clones with 1 cell		66%	89%	89%	99%
Frequency of Clones > 1 cell		34%	11%	11%	1%*



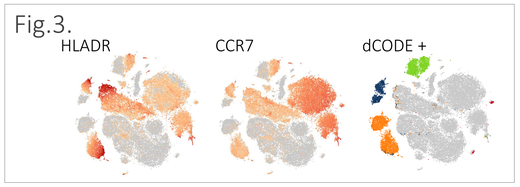
The distribution of unique TCR clones were evaluated for each of the 4 positive antigens.

- The influenza epitope (Flu), detected a high number of different TCR clones. *This may reflect that influenza is a reoccurring infection where some TCR clones falls away in between the infective periods, and new clones are generated at next infection, resulting in a high diversity of expanded clones.*
- The two EBV epitopes is quite alike and detected fewer and larger TCR clones. *This may reflect, that EBV is a persistent infection, where TCR clones are selected over time, decreasing the clonal distribution, and increasing the number of same clones.*
- The MART1 epitope detected low number of TCR clones with a high distribution. *Only one clonal TCR with more than 1 cell was observed, the rest was single different clonotypes. This may reflect, that MART 1 is an endogenous cancer antigen, which is unexpanded in healthy donors..*

6) Phenotyping the bisected ASTC populations:

The found ASTC populations were further phenotyped using TotalSeq™C antibody data.
Two markers, HLADR, and CCR7 (Fig 3), was shown to stain one of each of the bisected ASTC clusters.

Fig.3.



HLADR and CCR7, both have increased labeling on the same half of each of the dCODE positive populations, indicating that the bisecting of the ASTC populations in the t-SNE plots was driven by these two activations makers, HLADR and CCR7 (Fig 3).

Conclusion

High plex staining using MHC dCODE™ Dextramer®, in combination with TotalSeqC™ antibodies (Biolegend), in the 10x Chromium 5' feature barcoding workflow gives a powerful tool for deep phenotyping of immune relevant cells.

- ✓ We identified four different antigen specific T cell populations, in a CD8 sorted HPBMC samples, using a dCODE Dextramer panel of 44 specificities.
- ✓ Paired clonal TCR sequence and TCR specificity (MHC dCODE Dextramer positive) were directly obtained, and quantified, by overlaying the VDJ expression of each cell with the dCODE Positive cell clusters
- ✓ The antigen specific T cell populations were further phenotyped using TotalSeq™ antibody data, identifying HLADR and CCR7 subpopulations within each of the ASTC clusters
- ✓ This targeted analysis method revealed immunological answers, however, is restrained by the t-SNE cluster analysis. Cells that does not cluster well in t-SNE space, such as cells of low frequency, or dCODE positive cells with very diverse TCR's, might be overseen.
- ✓ In waste dataset the information is merely unlimited. To get relevant biological information filtered out, both unbiased bioinformatics and "targeted" analysis (drilling though deep phenotyping) will be important.