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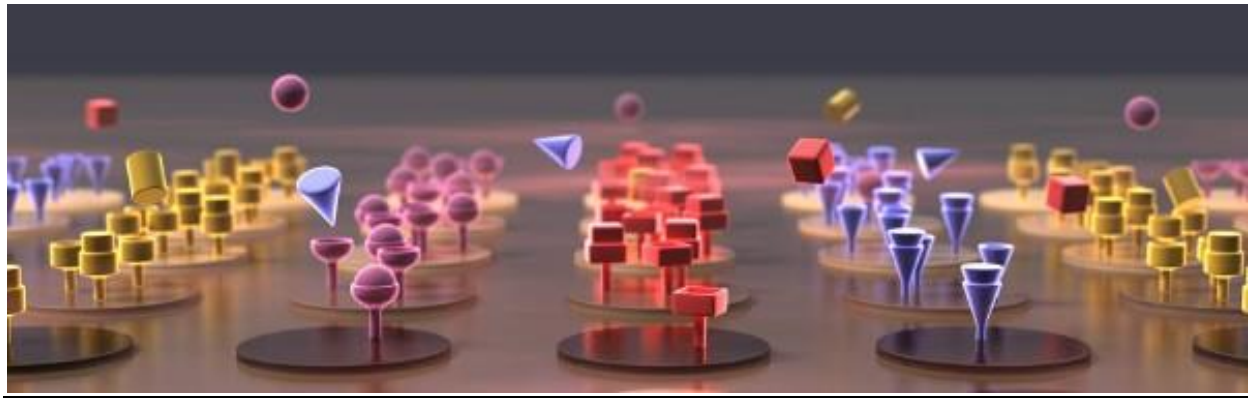


IBIS White paper

X-scan TCR HLA-peptide interactions

 # 11.141210

1. Abstract

Immunocore prepares therapeutics that can re-direct the immune response to recognise and kill diseased cells. Immunocore's "ImmTAC" technology requires production of high affinity monoclonal T-Cell receptors (mTCR) that bind to cancer-specific peptides presented on the surface of diseased cells as a peptide-human leukocyte antigen (pHLA) complex. Since these mTCRs have high (picomolar) affinities and long binding half-lives it is imperative that they retain antigen specificity. The mTCR specificity is currently investigated in a cellular X-scan experiment whereby cells are pulsed with peptides similar in sequence to the wild-type peptide. Using this method a specificity motif can be elucidated when membrane-bound mTCR binds to target pHLA containing a single amino acid exchange at each position along the cognate 9-mer peptide antigen.

In this white paper Immunocore utilises surface plasmon resonance (SPR) (IBIS MX96, IBIS Technologies) to compare the specificity of two soluble mTCRs binding to 1-substituted peptides. A key feature of the IBIS MX96 instrument is that the affinity of up to 96 HLA-peptide interactions can be determined for each TCR with only a single injection of only 100 µl of) highly concentrated TCR in serial diluted concentrations. Issues due to HLA-peptide precipitates were observed and definitely it is an advantage that the precipitates can be visually inspected by SPR imaging after the spotting process of the biotinylated HLA peptide complexes.

It is concluded that the multiplexing nature of the IBIS MX96 and CFM printer (IBIS Technologies) would allow full X-scan motifs to be elucidated for several low affinity mTCRs on a single pHLA-loaded SensEye® sensor.

2. Introduction

The fight against cancer follows many paths Immunocore Ltd, Abingdon, UK have created a novel platform of reagents termed ImmTACs (immune mobilising monocloncal TCRs against cancer) for targeted killing of cancer cells. ImmTACs combine TCR-based recognition of peptide-HLA (pHLA) complexes with potent T cell redirection though anti-CD3 as shown in Figure 1.

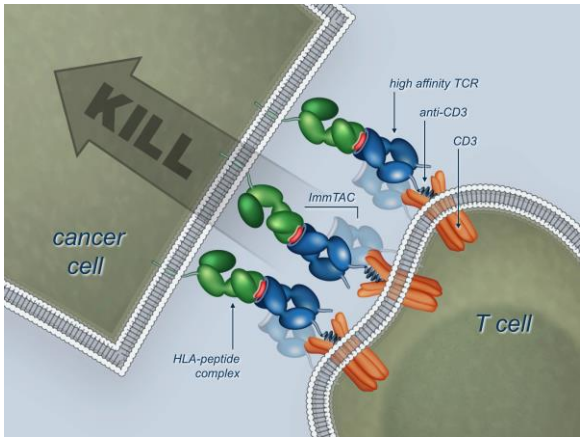


Figure 1. *ImmTAC-mediated killing of a cancer cell*

A key step in the preparation of ImmTACs is the production of soluble TCRs known as monoclonal, or 'm' TCRs that incorporate a non-native disulphide bond. The mTCRs are then further engineered to have enhanced affinity for cognate pHLA (Boulter et al., 2003; Li et al., 2005), before being fused to an anti-CD3 antibody fragment (Liddy et al 2012). In this way, ImmTACs overcome both the inherent low affinity of TCRs for cancer antigens imposed by thymic selection and down regulation of HLA on the cancer cell surface.

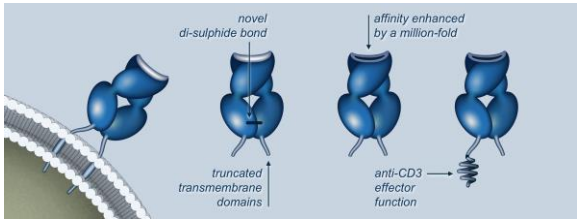


Figure 2. *The steps involved in ImmTAC construction.*

To ensure that a given mTCR has a high degree of specificity for target pHLA, its ability to recognise alternative peptides is investigated using an approach termed X-scan. This paper shows for the first time the capability of the IBIS MX96 for measuring multiplex TCR – pHLA interactions in an X-scan experiment, and demonstrates the power of the MX96 array.

3. Materials and methods

pHLA preparation

X-scan peptides were synthesized such that each amino acid along the nonameric (9-mer) native peptide was substituted by each of the other 19 amino acids. Therefore a full set of X-scan peptides contains 171 peptides (9 positions x 19 amino acids). pHLA solutions were prepared using a cleavable peptide displacement methodology and added to 96-well plates in a volume of 200 µl.

Sensor spotting

SensEye® sensors were prepared by immobilizing the X-scan biotinylated pHLA complexes to a Strep G-COOH SensEye® (Fig. 3)

Figure 3. *SensEye® biosensor that will be inserted in the slider*

An array of 48 biotinylated pHLA spots was printed on the sensor using the continuous flow micro-spotter (CFM) and a 6 x 8 printhead. The 48 pHLA solutions included 19 x 1-substituted peptides, 19 x 2-substituted peptides, 6 x 3-substituted peptides, 3 x control pHLA samples and 1 x PBS blank. Four sensors would be required to print all 171 X-scan peptides using a single 6 x 8 printhead (48 spots per sensor); alternatively only 2 sensors would be required with a 4x12 double printhead (96 spots per sensor).

Binding measurements

After creating the array, the sensor was inserted in the IBIS MX96. TCR samples (100 µl) were injected in the following sequence (1) 1 µM TCR1 (2) 10 µM TCR1 (3) 100 µM TCR1 (4) 0.6 µM TCR2 (5) 6 µM TCR2 (6) 60 µM TCR2. This 2 log concentration range was chosen to span the K_D for the target native peptide.

Controls

Three pHLA controls were prepared to evaluate both the success of the peptide displacement assay and quality of the TCR sample. A pHLA positive control containing wild-type peptide (i.e. not X-scan mutated) prepared by standard refold method (WTr); a pHLA positive control containing wild-type peptide prepared by peptide displacement (WTdis); and a negative control containing the HLA bound to the cleavable peptide only (No disp).

4. Results

Sensor spotting

pHLA solutions were printed on the sensor surface by the CFM printer. Immobilisation of the biotinylated pHLA solutions was visualised using the MX96 imaging feature (Figure 4).

Some spots were clear while others contained varying amounts of a white precipitate. This could be due to precipitation of the pHLA protein-peptide complex or may reflect the solubility of the excess peptide present during pHLA preparation.

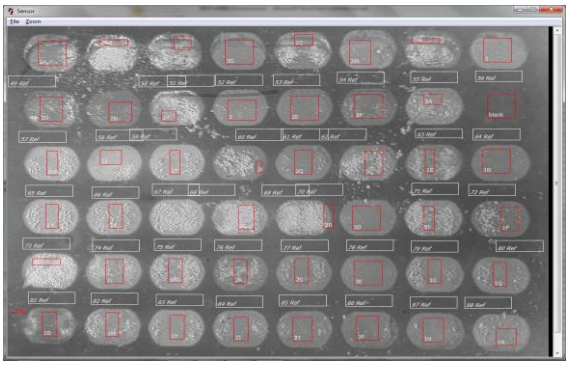


Figure 4. *Surface plasmon resonance image of one of the SensEye sensors from the X-scan experiment with spotted HLA-peptide complexes. Clearly some HLA-peptides formed a precipitate. Signal RoI (red boxes) and reference RoI's (white boxes) were repositioned. This SPR-imaging feature is an additional check for the quality of the generated data.*

Sensorgrams

Sensorgrams were generated at 3 TCR concentrations for each of the 48 pHLA spots (Figure 5). These sensorgrams were overlaid in individual tiles giving rise to a x48 tile plot for each TCR.

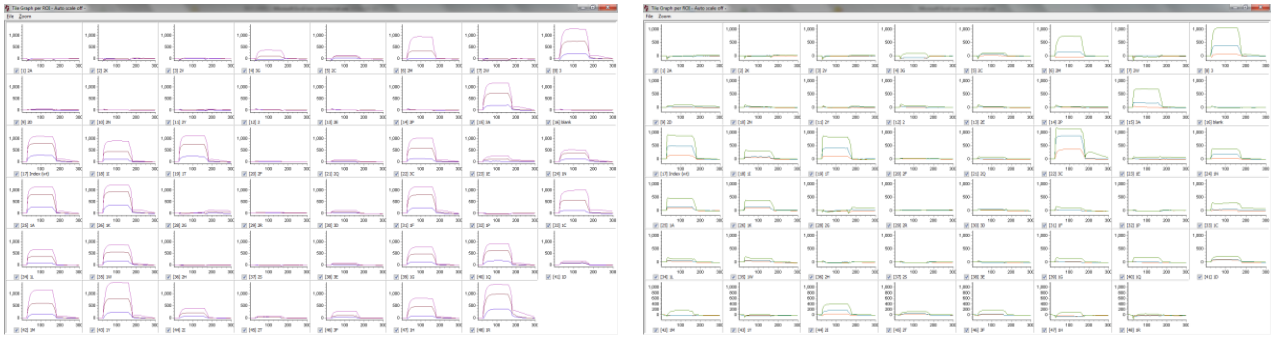
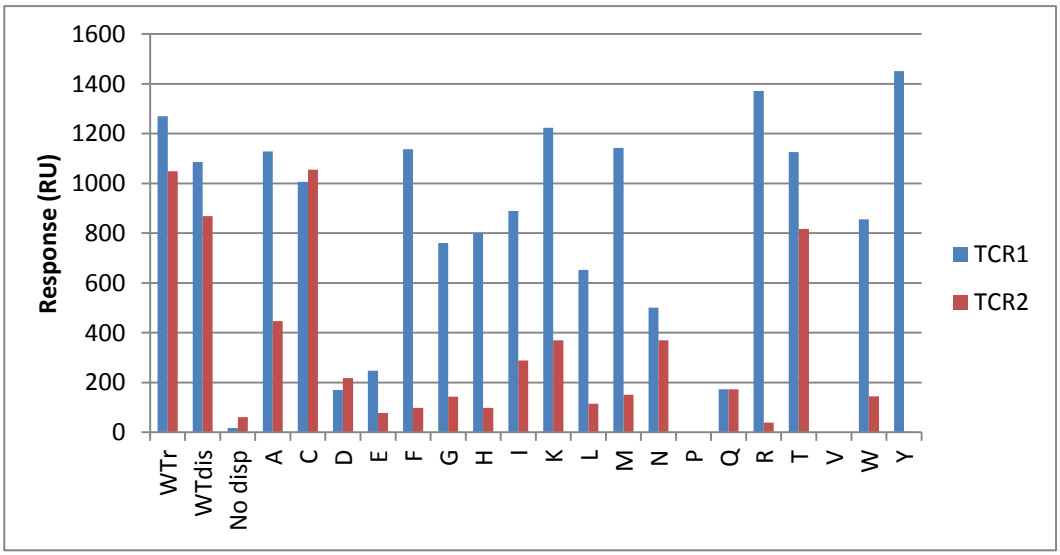


Figure 5 *Tile plot showing binding of (5a) TCR1 and (5b) TCR2 to the X-scan peptides on the spotted sensor from Figure 4. Each tile consists of overlaid sensorgrams for three injections of TCR. TCR1 concentrations were 1 µM, 10 µM and 100 µM (TCR1 $K_{D(WT)}$ = 10 µM). TCR2 concentrations were 0.6 µM, 6 µM and 60 µM (TCR2 $K_{D(WT)}$ = 16 µM).*

Figure 6. *Bar chart showing SPR responses for 1-substituted peptides at the top concentration of TCR1 (blue bars) and TCR2 (red bars). The letter on the x-axis represents the amino acid substitution at position 1 of the target peptide. WTr, WTdis and No disp refer to the control pHLA samples (see Materials and Methods).*



Comparison of the tiles clearly shows TCR binding to some pHLA but not to others (Figure 5a). An increase in the steady state response was observed when the concentration of TCR was increased, reflecting the K_D for that peptide. When comparing the tile plot for TCR1 (Figure 5a) with TCR2 (Figure 5b) it is clear that some peptides were bound by TCR1 but not TCR2. This difference in TCR specificity becomes even more obvious by comparing plots showing responses for each of the 1-substituted peptides at the top TCR concentration (Figure 6).

The data indicate TCR1 is very promiscuous at position 1 and can tolerate almost all amino acid substitutions (Figure 6 blue bars), while TCR2 can tolerate only a few substitutions at the same position in the peptide (Figure 6 red bars). This is in keeping with previous cellular X-scan data that clearly showed TCR2 had a much more restricted specificity than TCR1 at the N-terminal residue of the target peptide (data not shown).

Analysis of the TCR responses generated towards the control pHLA samples indicated that the peptide displacement assay was successful (Figure 6). The TCR1 responses towards WTr and WTdis were very similar at 1270 RU and 1086 RU respectively. The ~10% difference in the values may reflect either the efficiency of the displacement assay or a difference in HLA loading between the two spots. A value of 1270 RU at 100 µM TCR1 reflects maximal binding to the wild-type peptide since for TCR1 $K_{D(WT)}$ = 10 µM. Reassuringly several of the responses to the 1-substituted peptides were ~1200 RU indicating that TCR1 binds to these peptides with a similar affinity to wild-type peptide and that a maximal response had been reached.

The negative response towards 'No disp' shows that the TCR does not bind to the low affinity cleavable peptide when bound to HLA.

5. Discussion

It has been shown that the multiplexing IBIS MX96 in conjunction with CFM printer is an ideal instrument to investigate specificity of soluble mTCRs. The main advantage of the IBIS MX96 is that binding by mTCRs to 48 pHLA (single printhead) or 96 pHLA (double printhead) can be monitored simultaneously. For low affinity mTCRs with relatively short dissociation times ($t_{1/2}$) sensorgrams can be generated on a single sensor chip at several mTCR concentrations so that a binding constant (K_D) can be calculated for each peptide in the array. Ensuring the last mTCR injection has fully dissociated then the same sensor chip can be re-used to investigate more mTCRs. In this way specificity of several mTCRs can be directly compared by using the same pHLA solutions and identical conditions.

A key feature of the IBIS MX96 instrument is that the affinity of up to 96 HLA-peptide interactions can be determined for each TCR with only a single injection of only 100µl of highly concentrated TCR and serial diluted concentrations. Software is available to analyse the data with unrivalled speed.

6. Future improvements

Active pHLA was measured by ILT-2 titration. Cleaved pHLA samples containing excess displacing peptide should yield positive ILT-2 responses whereas a negative control containing cleaved pHLA but no excess peptide should yield a zero ILT-2 titration. ILT-2 cannot distinguish between a non-cleaved low affinity pHLA and a successfully displaced X-scan pHLA. To distinguish between these two pHLAs, a TCR specific to the cleavable peptide has been generated for future experiments.

7. Conclusion

The IBIS MX96 showed high multiplex power to perform a full X-scan in 180-plex at the highest speed and the lowest cost. During only 1.5 days of experiments more than 2500 sensorgrams were generated. The effect of peptide displacement to the affinity has clearly been demonstrated.

It has been shown that with further efforts and streamlining of the experimental set-up, the IBIS MX96 can generate reliable data that complement the data from cell assays.

8. References

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