**Investigating Toll-Like Receptor 9 as a mechanism of resistance to B-cell receptor-targeted therapies in Chronic Lymphocytic Leukaemia.**

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**Objectives:**

B-cell receptor (BCR)-targeted therapies are extremely effective at releasing tissue-resident Chronic Lymphocytic Leukaemia (CLL) cells and inducing apoptosis, however, CLL remains an incurable disease. Toll-Like Receptor 9 (TLR9) is a pattern recognition receptor of the innate immune system, and a potential candidate for a dual targeted approach to CLL treatment. TLR9 recognises unmethylated CpG DNA motifs (present in bacterial/viral and mitochondrial DNA) and TLR9 ligation induces an NFB and STAT3-driven activation/migratory phenotype in primary CLL cells. We have recently shown that unmethylated DNA is up to 28-fold higher in CLL patient plasma, relative to healthy controls1, and have therefore investigated TLR9 signalling as a potential mechanism of resistance to BCR-targeted agents.

**Results:**

In response to the TLR9 agonist ODN 2006, 25/42 (60%) patient samples showed an ***increase*** in CLL cell migration, whilst the remaining 17/42 (40%) showed either ***no change*** or a ***decrease*** in CLL cell migration; we therefore identified a dichotomous migratory response to TLR9 signalling and categorised our cohort into two subgroups: ‘Responders’ and ‘Non/Reverse Responders’. Interestingly, the observed dichotomy was associated with IGHV mutational status; 14/17 (82%) IGHV-mutated (M-CLL) samples were categorised as Responders, whilst IGHV-unmutated (U-CLL) samples showed a heterogeneous response (i.e., 11/25 (44%) Responders and 14/25 (56%) Non/Reverse Responders).

Previous studies have shown that U-CLL cells exhibit higher levels of constitutive BCR activation2, relative to M-CLL cells. In this study, U-CLL samples showed a significantly higher basal expression of the B-cell activation marker CD69, and the migratory response to ODN 2006 **negatively** correlated with both CD19 expression and basal migration. We therefore hypothesised that the most activated U-CLL cells had reached their maximal migratory capacity through BCR-signalling-alone and were consequently unable to respond to TLR9 activation.

Using mathematical modelling, we simulated BCR and TLR9 stimulation in M-CLL and U-CLL cells. Consistent with our laboratory findings, our simulations showed M-CLL cells to be responsive to TLR9 activation and U-CLL cells to be unresponsive. Strikingly, introducing a Bruton’s Tyrosine Kinase inhibitor (BTKi) into the simulation (to repress BCR-signalling), resulted in a renewed responsiveness of U-CLL cells to TLR9 activation. In support of this model, we have since identified a subset of U-CLL Non/Reverse Responder samples that become ‘Sensitised’ to TLR9 activation. In these samples, the TLR9 agonist, ODN 2006, induced an ***increase*** in CLL cell migration **in the presence of the BTKi, ibrutinib**. This suggests a potential switch from BCR to TLR9 signalling in some U-CLL patients, and implicates TLR9 signalling as a tumour escape mechanism following BTKi therapy. Additionally, in the Responder subgroup, dual inhibition of BTK and TLR9 induced a strong and synergistic reduction in CLL cell migration.

**Conclusions:**

CLL patient plasma contains high levels of unmethylated DNA1, which has the potential to promote tumour escape in TLR9-responsive patients. We therefore hypothesise that a BCR/TLR9 dual targeted treatment approach may benefit both TLR9 ‘Responder’ and BTKi ‘Sensitised’ patients. Since both the BCR and TLR9 signalling pathways culminate in NFB activation, we are currently investigating components of NFB signalling as potential novel therapeutic targets.

1 Kennedy E, Coulter E, Halliwell E, Profitos-Peleja N, Walsby E, Clark B, Phillips EH, Burley TA, Mitchell S, Devereux S, Fegan CD, Jones CI, Johnston R, Chevassut T, Schulz R, Seiffert M, Agathanggelou A, Oldreive C, Davies N, Stankovic T, Liloglou T, Pepper C, Pepper AGS. TLR9 expression in chronic lymphocytic leukemia identifies a promigratory subpopulation and novel therapeutic target. Blood. 2021 Jun 3;137(22):3064-3078. doi: 10.1182/blood.2020005964. PMID: 33512408.

2 Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. Blood. 2011 Oct 20;118(16):4313-20. doi: 10.1182/blood-2011-06-338855. Epub 2011 Aug 3. PMID: 21816833.