Toll-like receptor 9 signalling in CLL: a resistance mechanism to B-cell receptor-targeted treatments, and a potential tool for therapeutic stratification.

Background

CLL cell trafficking to secondary lymphoid tissues is fundamental to disease progression, since within these protective niches, CLL cells encounter an abundance of activating, pro-proliferative and pro-survival signals. The primary mechanism of CLL cell activation is via the B-cell receptor (BCR)-signalling pathway, and BCR-targeted treatments are extremely effective at releasing tissue-resident cells into the peripheral blood and inducing apoptosis. Despite these successes, currently available agents fail to achieve complete tumour clearance, and CLL remains incurable.

Toll-like receptor 9 (TLR9) is an intracellular pattern recognition receptor, which responds to unmethylated CpG motifs in bacterial/viral/mitochondrial DNA. We recently showed unmethylated DNA levels are 28-fold higher in CLL patient plasma relative to healthy controls, and that TLR9-ligation induces an NF-κB and STAT3-driven activation/migratory phenotype in primary CLL cells[1]. We hypothesise TLR9 signalling is a BCR-independent contributor to CLL homing and potential resistance mechanism to BCR-targeted agents.

Results

In a cohort of 74 primary CLL samples, a dichotomous migratory response was observed in response to the TLR9 agonist ODN 2006. 53% showed an ***increase*** in CLL cell migration (categorised as 'Responders'[R]), whilst 47% showed either ***no change*** or a ***decrease*** (categorised as 'Non/Reverse Responders'[NR/RR]). There was a significant (but not exclusive), correlation with mutational status, with 71% vs. 33% of M-CLL vs. U-CLL samples being categorised as R, respectively (P=0.03, n=48), and no correlation was found between CD49d+ve vs. CD49d-ve or CD38+ve vs. CD38-ve samples.

Surprisingly, there was no significant difference in total TLR9 expression between R and NR/RR samples (P=0.23, n=45), and ODN 2006 stimulation induced TLR9 signalling in **both** subgroups. Post-stimulation,

IRAK1 and IκBα were degraded in R (n=4) and NR/RR (n=3) and the activation marker CD69 was equally upregulated in each subgroup (P<0.0001, n=34 and P<0.0001, n=28, respectively). This indicates the observed dichotomous migratory response is due to downstream signalling divergence, as opposed to a complete lack of response to stimulation.

Previous studies have shown constitutive BCR activation to be heterogeneous in CLL, and generally elevated in U-CLL relative to M-CLL[2]. We hypothesised that the most basally activated CLL cells may reach their maximal activation/migratory capacity through BCR-signalling-alone, rendering them non-responsive to further external stimuli. This is supported by our finding that NR/RR have significantly lower basal CD5 (P=0.02, n=54), and significantly higher NF-κB p-p65 (P<0.01, n=12) and MCL-1 (P=0.03, n=16).

Using *in-silico* modelling, we simulated TLR9 activation in states of low/high basal BCR activity, using canonical NF-κB activity as a functional read-out. Our model showed cells with low basal activation responded strongly to TLR9 stimulation, whilst cells with high basal activation were unresponsive. These results were verified *in-vitro* as basal p-BTK and p-p65 (representative of constitutive BCR activation) showed ***negative*** correlation with the migratory response to ODN 2006 in the NR/RR subgroup (P<0.01, R=-0.91, n=8 and P=0.02, R=-0.93, n=5, respectively); these data indicate constitutive signalling impairs the ability of NR/RR samples to respond to alternative stimuli. Interestingly, we found responsiveness to ODN 2006 (migration) correlated strongly with responsiveness to IgM (p-BTK expression) (P=0.03, R^2=0.33, n=14), signifying that this trend is not TLR9-specific, but extends to other microenvironmental interactions.

Importantly, when simulating NR/RR treatment with a BTK inhibitor *in-silico*, we observed a renewed responsiveness to TLR9 activation, which was later verified *in-vitro*. In the presence of the BTK-inhibitor ibrutinib, we found a subset of previously non-responsive NR/RR samples became 'sensitised' to TLR9 activation, showing an ***increase*** in CLL cell migration in response to stimulation with ODN 2006. This suggests a switch from BCR to TLR9 signalling in ***some*** NR/RR samples and implicates TLR9 signalling

as a tumour escape mechanism following BTKi therapy. We hypothesise that TLR9 'responder' and 'sensitised' subgroups may benefit from BCR/TLR dual targeted therapy.

We are currently investigating NF-κB as a potential therapeutic target to simultaneously inhibit BCR/TLR9 signalling. Whilst pan NF-κB inhibitors have proven extremely toxic (*in-vivo*), we are opting for a more streamlined subunit-specific approach. We are working to create patient-specific NF-κB 'fingerprints' (i.e., subunit expression profiles), to ascertain whether different subgroups show targetable differences in NF-κB subunit expression. Early analyses suggest fingerprints to be distinct between R and NR/RR samples, rendering this technique a potential tool for personalising future NF-κB-targeted therapies.

References

[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.