Poster #: 1010

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Toll-like receptor 9 signalling is a potential tumour escape mechanism following B-cell receptor targeted treatments in subsets of patients with Chronic Lymphocytic Leukaemia.

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OBJECTIVES

- **Investigate the heterogeneous migratory responses of primary CLL cells to TLR9-activation.**
- **Determine whether TLR9-induced CLL cell migration is dependent upon BCR-signalling.**
- **Investigate whether TLR9 signalling is a potential mechanism of resistance to BCR-inhibition.**

CONCLUSIONS

- TLR9 ACTIVATION INDUCES HETEROGENEOUS MIGRATORY CHANGES IN PRIMARY CLL CELLS.
- TLR9-INDUCED CLL CELL MIGRATION CORRELATES WITH TLR9-INDUCED CANONICAL NFKB-ACTIVATION.
- IN SILICO MODELLING PREDICTS TLR9-INDUCED NFKB ACTIVATION TO BE STRONGEST IN SAMPLES WITH LOWER BASAL BCR-ACTIVATION.
- RESPONDER SAMPLES HAVE LOWER BASAL LEVELS OF BCR AND NFKB-ACTIVATION, RELATIVE TO REVERSE RESPONDER SAMPLES.
- BTK REPRESSION RESULTED IN A SIGNIFICANT INCREASE IN MIGRATION IN TLR9-ACTIVATED REVERSE RESPONDER SAMPLES.
- CLINICAL RESPONSE DATA SUGGEST MIGRATORY RESPONSE GROUPS MAY BETTER PREDICT CLINICAL OUTCOMES OF PATIENTS ON IBRUTINIB-THERAPY THAN IGHV-STATUS.

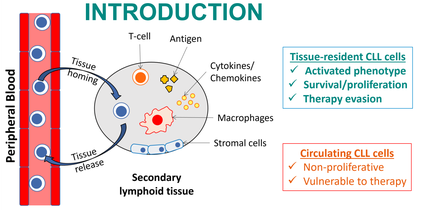
ACKNOWLEDGMENTS

Crawley Leukaemia Research

Thank you to all the CLL patients who donated blood for our research ☺

CONTACT DETAILS

INTRODUCTION



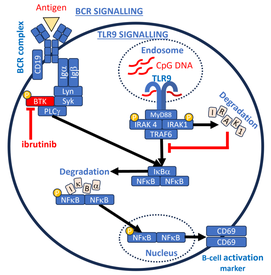
CLL cell trafficking to secondary lymphoid tissues is fundamental to disease progression. Within the protective niche of the lymph nodes and bone marrow, CLL cells encounter a multitude of activating and pro-survival signals including activation of both the B-cell receptor (BCR) signalling pathway (BCR) and the Toll-like receptor (TLR) signalling pathway.

BCR and TLR9 signalling in CLL.

BCR signalling:

Current B-cell receptor (BCR)-targeted therapies promote CLL cell egress from secondary lymphoid tissues yet often fail to achieve a complete systemic response.

Acquired resistance to BTK inhibitors (BTKi) cannot be solely attributed to acquired BTK mutations[1] implicating the involvement of other signalling pathways.



TLR9 signalling:

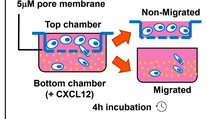
TLR9 is an intracellular pattern recognition receptor, which recognises unmethylated CpG motifs in bacterial, viral, and mitochondrial DNA.

Since unmethylated DNA levels are up to 28-fold higher in CLL patient plasma relative to healthy controls[2], we investigated TLR9-signaling as a potential mechanism of resistance to BTKi therapy.

We explored the effects of TLR9-activation upon primary CLL cell migration and identified subsets of patient samples with a heightened capacity for TLR9 signalling.

METHODS

TLR9-Activation/BCR-Inhibition: Primary PBMCs were incubated overnight ± 1μM TLR9 agonist ODN 2006 ± 1μM BTK-inhibitor ibrutinib.



Transwell Migration Assays: Pre-stimulated PBMCs were transferred into transwell migration plates. Migrated PBMCs were collected and counted volumetrically by flow cytometry. CLL cells were identified as CD5+/ CD19+/ CD3-.

Flow Cytometry: Intracellular staining was performed using Cyto-Fast™ Fix/Perm (p-BTK) and True-Phos™ Perm Buffer (p-p65).

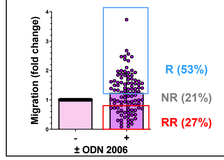
Western Blotting: Proteins were transferred using the iblot™2 dry blotting system and membranes were probed for IRAK1 and IRAK4. Blots were normalised to Total Protein (TPN).

RNA Sequencing: Sequencing performed by Active Motif and data analysed using Biojupies.

***In Silico* Modelling:** TLR9 activation was simulated in cells with low vs high basal BCR signalling (n=25 cells). The model was run as described in Jayawant et al (2023).

RESULTS

Figure 1: TLR9 activation induces a heterogenous migratory response in primary CLL cells.

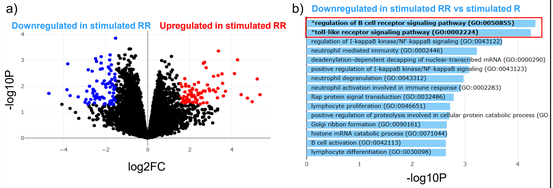


We firstly investigated the effects of TLR9 activation on CLL cell migration. Following stimulation with TLR9 agonist ODN 2006, migratory responses of 102 primary CLL samples ranged from 3.7 to 0.09-fold.

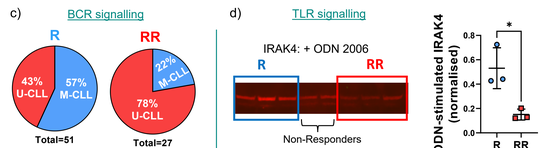
Responders (R) (migration >1.2-fold)
Reverse Responders (RR) (migration <0.8-fold)
Non-Responders (NR) (migration 1,2-0.8-fold)

Figure 2: R and RR samples may preferentially signal through distinct signalling pathways.

We performed RNA sequencing on samples from either end of the migratory spectrum to investigate transcriptional differences between TLR9-activated R vs RR samples.



a) Volcano plot of differentially expressed genes in ODN 2006-stimulated RR (n-8) vs R samples (n=5). b) Gene ontology enrichment analysis identified a downregulation of genes involved in the 'Regulation of B-cell receptor signalling pathway' and 'Toll-like receptor signalling pathway' in RR samples, relative to R samples. This included FCRL3 and PTPN22 (negative regulators of BCR signalling) and IRAK4 (downstream TLR-signalling molecule). **This suggests R samples may be better primed for TLR-signalling, whilst RR may be better primed for BCR-signalling.**

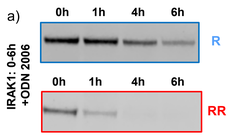


In further support, (c) 78% of RR samples were IGHV-unmutated: a subgroup of CLL, known to signal strongly and constitutively though the BCR[4], and (d) protein expression of IRAK4 was confirmed to be significantly higher in ODN 2006-stimulated R vs RR samples.

Figure 3: TLR9 stimulation induces stronger activation of NFκB in R vs RR samples.

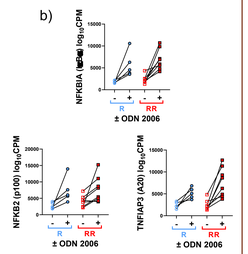
We then wanted to assess whether TLR9 signalling was inducible following TLR9 activation in both R and RR samples.

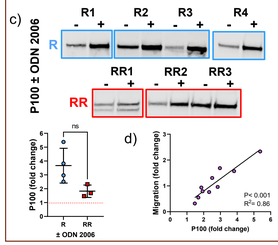
Following activation of the TLR-signalling pathway, IRAK1 is degraded as a negative feedback mechanism. Here we used IRAK1 degradation as a marker of TLR9 signal induction.



a) Representative examples of IRAK1 expression following a 0-6h stimulation with ODN 2006, in R vs RR samples. IRAK1 degradation is evident in both R and RR samples.

b) RNA sequencing data identified significant upregulation of the NFκB-target genes: NFKBIA, NFKB2 and TNFAIP3 (which code for IκBα, P100 and A20 respectively) in both R and RR samples. Individual read counts are shown as log_{10} counts per million (log_{10}CPM). **Together this suggests canonical NFκB signalling was inducible following TLR9-activation, independently of the migratory outcome.**

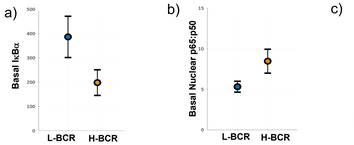




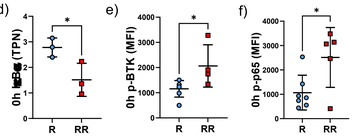
c) P100 (an inactive pre-cursor of the NFκB subunit p52) was confirmed to upregulate at the protein level. d) P100 fold change correlated with the migratory response to ODN 2006. **This suggests that the strength of TLR9-induced NFκB-signalling may influence migratory outcomes.**

Figure 4: *In Silico* modelling predicts basal BCR signalling to determine the responsiveness to TLR9-activation.

We hypothesised that TLR9-induced signalling may differ between patient samples due to heterogenous levels of basal BCR-signalling. Using *in-silico* modelling, we simulated TLR9 activation with low/high levels of basal BCR activation (L-BCR/H-BCR).



Our model predicted L-BCR cells to express (a) higher basal levels of the NFκB-inhibitor IκBα, and (b) lower basal levels of nuclear (i.e., activated) p65:p50 (canonical NFκB subunits), indicating lower basal NFκB activation in L-BCR vs H-BCR samples. Following TLR9 activation, L-BCR cells showed a dramatically greater increase in p100, relative to H-BCR cells. In keeping with our hypothesis, therefore, the simulated responses of L-BCR and H-BCR CLL mirror those of R and RR samples, respectively. *(Simulated plots show mean expression (+SD) of n=25 cells).*



In further support of our modelling data: d) protein expression of IκBα was significantly higher in R vs RR samples and e-f) p-BTK and p-p65 were significantly lower.

Figure 5: TLR9-activated RR CLL cells show increased migration when BTK is repressed.

We next investigated the effects of inhibiting BCR-signalling in RR samples.

a) In unstimulated RR CLL cells, CLL migration remained unchanged in the presence of the BTK-inhibitor ibrutinib (p=ns, Wilcoxon matched-pairs signed rank test). b) In TLR9-activated RR CLL cells, BTK repression resulted in a significant increase in migration (relative to stimulation with ODN 2006-alone) (p<0.001, Wilcoxon matched-pairs signed rank test).

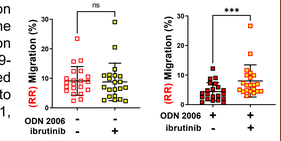
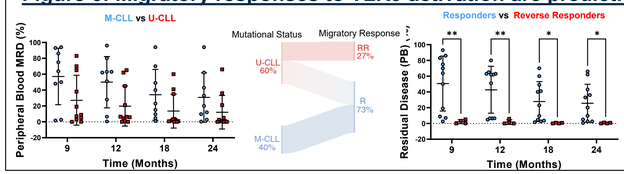


Figure 6: Migratory responses to TLR9 activation are predictive of clinical responses to ibrutinib.



Clinical response data from 15 patients receiving ibrutinib-monotherapy on the FLAIR clinical trial. a) There was no significant difference in the percentage of Minimal Residual Disease (MRD) in the peripheral blood (PB) of M-CLL vs U-CLL samples (Two-way Anova). (b) When reclassified into R vs RR migratory subgroups, PB-MRD was significantly higher in R vs RR samples at 9- (p<0.01), 12- (p<0.01), 18- (p=0.02) and 24- (p=0.03) months (Two-way Anova, Multiple Comparisons). **These data suggest migratory response groups may better predict the clinical outcomes of patients on ibrutinib-therapy than the conventionally used IGHV-status.**

REFERENCES: [1]Bonfiglio et al (2023) DOI: 10.1182/bloodadvances.2022008821; [2]Kennedy et al (2021) DOI: 10.1182/blood.2020005964; [3]Jayawant et al (2023) DOI: 10.3389/fonc.2023.1181660; [4]Stevenson et al (2011) DOI: 10.1182/blood-2011-06-338855.