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

MRC Medical Research Council

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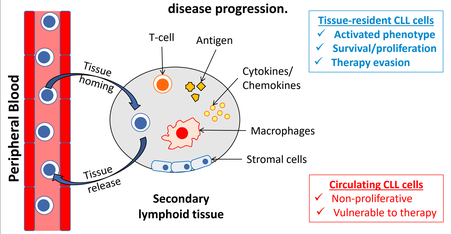
BACKGROUND

CLL cell trafficking to secondary lymphoid tissues is fundamental to disease progression.

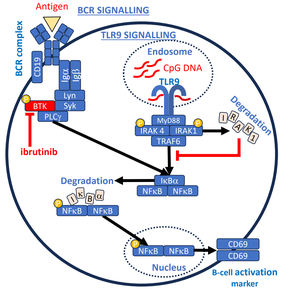


Tissue-resident CLL cells
- ✓ Activated phenotype
- ✓ Survival/proliferation
- ✓ Therapy evasion

Circulating CLL cells
- ✓ Non-proliferative
- ✓ Vulnerable to therapy

Circulating and tissue-resident CLL cells are phenotypically distinct. Within the protective niche of the lymph nodes and bone marrow, CLL cells encounter a multitude of activating and pro-survival signals.

B-cell receptor (BCR) and Toll-like receptor 9 (TLR9) signalling in CLL.



BCR signalling:
- The primary mechanism of B-cell activation
- **Activates NFκB** to drive CLL proliferation and migration

BCR receptor-targeted treatments (such as the BTK inhibitor (BTKi) ibrutinib are:

✓ Extremely effective at releasing tissue-resident CLL cells and inducing apoptosis

X Unable to achieve complete tumour clearance

X Prone to acquired resistance

TLR9: an intracellular pattern recognition receptor, that recognises **unmethylated CpG DNA** in bacterial/ viral/ mitochondrial DNA.

TLR9 signalling:
- An alternative mechanism of B-cell activation and potential therapeutic target
- **Activates NFκB** independently of BCR activation

✓ **Unmethylated CpG DNA levels are** 28-fold higher in CLL patients than healthy controls[1]

✓ TLR9-ligation induces an NFκB and STAT3-driven **migratory phenotype** in primary CLL cells[1]

AIMS

Aim 1: **To investigate the different migratory responses seen in CLL patient samples stimulated through TLR9.**

Aim 2: **To identify patients with the ability to signal via TLR9 as a resistance mechanism to BTKi therapies.**

Aim 3: **To investigate TLR9 driven migration patterns, and NFκB fingerprinting, as potential tools to identify patients who would benefit from NFκB-targeted therapies.**

HYPOTHESIS

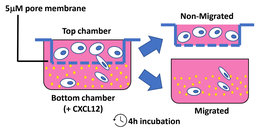
TLR9 signalling is a BCR-independent contributor to CLL homing and potential resistance mechanism to BCR-receptor targeted agents.

RESULTS

1. TLR9 activation induces a dichotomous migratory response in CLL patient samples.

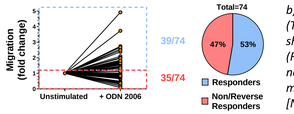
a) Transwell migration assays

a) Primary PBMCs were transferred to the apical chambers of 24-well transwell migration plates and incubated for 4h at 37°C/5%Co_2.



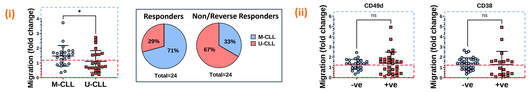
At 4h migration, migrated PBMCs were collected, stained for the CLL identification markers CD5/CD19/CD3 and counted volumetrically by flow cytometry. CLL cells were identified as CD5+/CD19+/CD3-.

b) Migratory response to TLR9 stimulation with ODN 2006



b) Following stimulation with ODN 2006 (TLR9 agonist), 39/74 patient samples showed an increase in CLL cell migration (Responders [R]) and 35/74 showed either no change or a decrease in CLL cell migration (Non/Reverse Responders [NR/RR]).

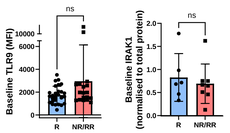
c) The migratory response to ODN 2006 is associated with IGHV mutational status.



c) There was a significant difference between the migratory response to ODN 2006 in IGHV-mutated (M-CLL) vs IGHV-unmutated (U-CLL) samples; M-CLL were significantly more responsive than U-CLL samples (P=0.03), but there was no difference between CD49 +ve vs –ve (P=0.17) or CD38 +ve vs –ve samples (P=0.94).

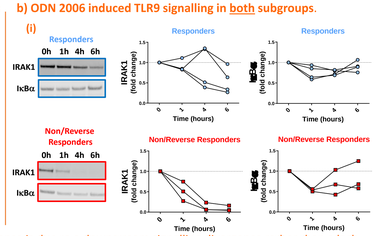
2. ODN 2006 induces TLR9 signalling in both the Responder and Non/Reverse Responder subgroups.

a) TLR9 and IRAK1 expression: R vs NR samples



a) There was no significant difference in the expression of TLR9 (P=0.23) or the downstream signalling kinase IRAK1 (P=0.66) in R vs NR/RR samples.

b) ODN 2006 induced TLR9 signalling in both subgroups.

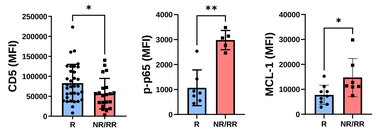


b) Following stimulation with ODN 2006 (i) IRAK1 (downstream kinase) and IκBα (NFκB inhibitor), were degraded in both R (n=4) and NR/RR (n=3) patient samples and (ii) CD69 (a B-cell activation marker) was equally upregulated in each subgroup (R [P<0.01]; NR/RR [P<0.01]).

This indicates the dichotomous migratory response is due to a downstream signalling divergence rather than a lack of response to stimulation.

3. HYPOTHESIS: The most basally activated CLL cells may have reached their maximal migratory capacity through BCR signalling-alone.

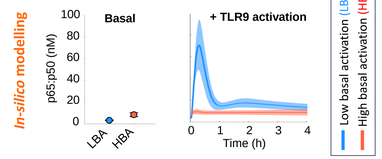
a) NR/RR samples are more basally active than R samples.



~70% of NR/RR were U-CLL (Figure 1b). U-CLL cells are known to exhibit higher levels of constitutive basal BCR signalling.

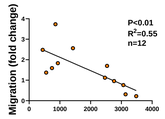
a) NR/RR samples showed significantly lower basal expression of CD5 (repressor of BCR signalling) (P=0.02), and significantly higher basal expression of p-p65 (canonical NFκB subunit) (P<0.01) and MCL-1 (NFκB driven anti-apoptotic protein) (P=0.03).

b) *In-silico* modelling predicts highly basally active cells to be unresponsive to TLR9 stimulation.



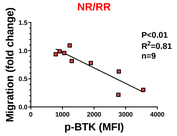
b) In silico: TLR9 activation was simulated in states of low/high basal BCR activity. Here, p65:p50 dimerisation represents canonical NFκB activity. Our model shows cells with low basal activity (LBA) responded strongly to TLR9 activation (i.e., increased p65:p50), whilst cells with high basal activity (HBA) were unresponsive to the stimulation (i.e., no change in p65:p50).

c) *In-vitro* data supports modelling predictions



c) In vitro: basal p-65 activation (p-p65) showed negative correlation with the migratory response to ODN 2006 (P<0.01, R²=0.55). This supports the modelling prediction that the most basally activated CLL cells, respond the least to TLR9 activation.

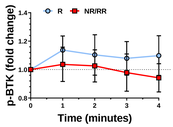
d) Constitutive (basal) BCR signalling shows negative correlation with the migratory response to ODN 2006 in NR/RR samples.



d) Basal p-BTK also showed negative correlation with the migratory response to ODN 2006 in NR/RR samples (P<0.01,R²=0.81).

This suggests CLL cells with high basal levels of BCR activation are the least responsive to TLR9 activation.

e) (i) Constitutive basal signalling impairs the ability of CLL cells to respond to alternative microenvironmental stimuli

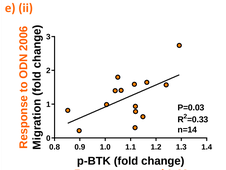


(i) To identify whether CLL cells were unresponsive to TLR9 activation specifically, or all external stimuli, R (n=7) and NR/RR (n=7) samples were stimulated with anti-IgM (BCR agonist) for 1-5 minutes; p-BTK was analysed as a measure of responsiveness. The R subgroup were more responsive to anti-IgM stimulation than NR/RR samples.

Cont...

3e. cont... Constitutive basal signalling impairs the ability of CLL cells to respond to alternative microenvironmental stimuli.

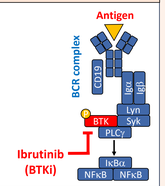
e) (ii)



(ii) Responsiveness to ODN 2006 [fold change in migration] positively correlates with responsiveness to IgM [fold change in p-BTK @ 1 min post-stimulation].

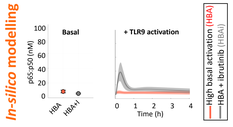
This signifies that the most basally activated CLL cells have an impaired ability to respond to any alternative external stimuli.

We next investigated how BTKi inhibition may affect CLL cell responsiveness to TLR9 activation. BTKi treatment would be expected to reduce basal activation and may therefore re-sensitise previously non-responsive CLL cells to microenvironmental stimuli.



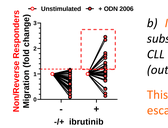
4. Non/Reverse Responders can 'signal switch' in the presence of ibrutinib (BTK inhibitor).

a) In-silico modelling predicts BTK-inhibition to renew responsiveness to TLR9 activation in HBA cells.



a) In-silico: When simulating treatment of HBA cells with a BTK inhibitor, basal p65:p50 was reduced, and TLR9 activation stimulated an increase in p65:p50.

b) A subset of NR/RR samples become 'sensitised' to TLR9 activation, in the presence of ibrutinib (In-vitro).

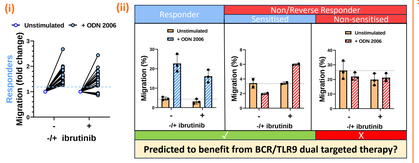


b) In-vitro: in the presence of ibrutinib, a subset of NR/RR samples show an increase in CLL cell migration in response to ODN 2006 (outlined in red box).

This implicates TLR9 as a potential tumour escape mechanism following BTKi therapy.

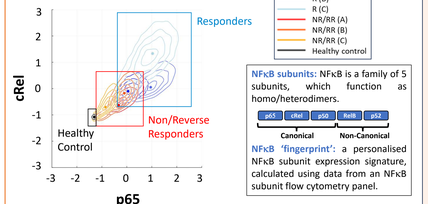
5. HYPOTHESIS: TLR9 Responder and TLR9 'sensitised' subgroups may benefit from BCR/TLR9 dual targeted therapy.

a) Patient samples can be divided into R, NR/RR 'sensitised' and NR/RR 'non-sensitised' subgroups.



(i) In the presence of ibrutinib, ODN 2006 stimulated a significant increase in CLL cell migration in the Responder subgroup, again implicating TLR9 as a tumour escape mechanism following BTKi therapy. (ii) Representative transwell migration data of R, NR/RR 'sensitised' and NR/RR 'non-sensitised' patient samples: (individual points = technical repeats). We predict the R and NR/RR 'sensitised' subgroups but not the NR/RR 'non-sensitised' subgroup would benefit from BCR/TLR9 dual targeted therapy.

b) As both BCR and TLR9 signalling activate NFκB, we are currently investigating NFκB subunits as potential therapeutic targets to simultaneously inhibit BCR /TLR9 signalling.



b) Early analyses suggest fingerprints to be distinct between R and NR/RR samples, rendering this technique a potential tool for personalising future NFκB-targeted therapies.

CONCLUSIONS

1: TLR9 activation may **promote CLL cell trafficking and BTKi resistance** in select subgroups of patients.

2: Targeting **downstream NFκB signalling components** has the potential to **simultaneously inhibit both BCR and TLR9 signalling**, and to increase treatment efficacy in TLR9 'Responder' and BTKi 'Sensitised' patients.

3: Individual CLL patients may have **distinct NFκB 'fingerprints'** (i.e., NFκB subunit expression profiles), inspiring a **personalised and subunit-specific approach to NFκB-inhibition**.

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