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# TLR9 expression in Chronic Lymphocytic Leukemia identifies a pro-migratory subpopulation and novel therapeutic target.

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#### Abstract:

CLL remains incurable despite BCR-targeted inhibitors revolutionizing treatment. This suggests that other signaling molecules are involved in disease escape mechanisms and resistance. Toll-like receptor 9 (TLR9) is a promising candidate, which is activated by unmethylated CpG-DNA. Here, we show that plasma from CLL patients contains significantly more unmethylated DNA than plasma from healthy controls (p<0.0001) and that cell-free DNA levels correlate with the prognostic markers CD38, b2-microglobulin and lymphocyte doubling time. Furthermore, elevated cell-free DNA was associated with shorter time to first treatment (TTFT: p=0.003, HR=4.0). We went on to show that TLR9 expression was associated with *in-vitro* CLL cell migration (p<0.001) and intracellular endosomal TLR9 strongly correlated with aberrant surface expression ((sTLR9); r=0.9). In addition, lymph node-derived CLL cells showed increased sTLR9 (p=0.016) and RNA sequencing of paired sTLR9<sup>hi</sup> and sTLR9<sup>lo</sup> CLL cells revealed differential transcription

of genes involved in TLR signaling, adhesion, motility and inflammation in sTLR9<sup>hi</sup> cells. Mechanistically, the TLR9 agonist, ODN2006, promoted CLL cell migration (p<0.001) that was mediated, by p65 NF-kB and STAT3 transcription factor activation. Importantly, autologous plasma induced the same effects, which were reversed by a TLR9 antagonist. Furthermore, high TLR9 expression promoted engraftment and rapid disease progression in a NSG mouse xenograft model. Finally, we showed that dual targeting of TLR9 and BTK was strongly synergistic (median CI=0.2 at ED50), which highlights the distinct role for TLR9 signaling in CLL and the potential for combined targeting of TLR9 and BTK as a more effective treatment strategy in this incurable disease.

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TLR9 expression in Chronic Lymphocytic Leukemia identifies a pro-migratory subpopulation and novel therapeutic target

#### Short title: A role for TLR9 in CLL

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#### **Key Points**

- 1. TLR9 expression and its agonist, cell-free DNA, correlate with increased migration and poor prognosis in CLL.
- Dual targeting of TLR9 and BTK synergistically inhibits CLL cell migration and blocks p65 NF-κB and STAT3 activation.

#### Abstract

CLL remains incurable despite BCR-targeted inhibitors revolutionizing treatment. This suggests that other signaling molecules are involved in disease escape mechanisms and resistance. Toll-like receptor 9 (TLR9) is a promising candidate, which is activated by unmethylated CpG-DNA. Here, we show that plasma from CLL patients contains significantly more unmethylated DNA than plasma from healthy controls (p<0.0001) and that cell-free DNA levels correlate with the prognostic markers CD38,  $\beta$ 2-microglobulin and lymphocyte doubling time. Furthermore, elevated cell-free DNA was associated with shorter time to first treatment (TTFT: p=0.003, HR=4.0). We went on to show that TLR9 expression was associated with *in-vitro* CLL cell migration (p<0.001) and intracellular endosomal TLR9 strongly correlated with aberrant surface expression ((sTLR9); r=0.9). In addition, lymph node-derived CLL cells showed increased sTLR9 (p=0.016) and RNA sequencing of paired sTLR9<sup>hi</sup> and sTLR9<sup>lo</sup> CLL cells revealed differential transcription of genes involved in TLR signaling, adhesion, motility and inflammation in sTLR9<sup>hi</sup> cells. Mechanistically, the TLR9 agonist, ODN2006, promoted CLL cell migration (p<0.001) that was mediated, by p65 NF-κB and STAT3 transcription factor activation. Importantly, autologous plasma induced the same effects, which were reversed by a TLR9 antagonist. Furthermore, high TLR9 expression promoted engraftment and rapid disease progression in a NSG mouse xenograft model. Finally, we showed that dual targeting of TLR9 and BTK was strongly synergistic (median CI=0.2 at ED50), which highlights the distinct role for TLR9 signaling in CLL and the potential for combined targeting of TLR9 and BTK as a more effective treatment strategy in this incurable disease.

#### Introduction

Chronic lymphocytic leukemia (CLL) is a common B-cell malignancy with a remarkably diverse clinical course. It is characterized by the accumulation of mature B-lymphocytes in the peripheral blood (PB), bone marrow (BM) and secondary lymphoid organs such as the lymph nodes (LN). Until recently, therapeutic options for CLL patients were limited and poorly tolerated but the introduction of Bruton's tyrosine kinase (BTK) inhibitors<sup>1</sup> and drugs targeting phosphoinositide kinases (PI3Ks)<sup>2</sup> and Bcl-2<sup>3</sup> have revolutionized treatment. However, these agents are not curative and some patients have short-term responses, develop resistance or have to stop treatment because of side-effects<sup>4</sup>.

CLL has been described as a two-compartment disease as LN and PB CLL cells express distinct phenotypic and transcriptional features<sup>5,6</sup>. PB CLL cells are largely quiescent<sup>7</sup> whereas, in the LN microenvironment, disease-promoting interactions occur<sup>5,6</sup>. It appears that the pathology of CLL is influenced by the ability of tumor cells to traffic between these two compartments<sup>8</sup> and recent LN emigrants can be identified by a CXCR4<sup>dim</sup>CD5<sup>bright</sup> phenotype<sup>9</sup>. An important goal of CLL therapy is to eradicate tissue-resident CLL cells; 'trapping' them in the PB deprives them of the proliferation and survival signals afforded by protective microenvironmental niches<sup>10</sup>. Indeed, BTK and PI3Kδ inhibitors cause tumor cell tissue redistribution<sup>11-13</sup> but heterogeneity in responses and lack of curative effect show that inhibition of these individual kinases alone is not sufficient to eliminate disease. The identification of novel targets and therapeutic combinations is therefore crucial for future cures; one such potential target is toll-like receptor 9 (TLR9).

The toll-like receptor (TLR) family of pattern recognition receptors are an integral component of the innate immune system and important mediators of the adaptive immune response. Following BCR activation and co-stimulatory T-cell interactions, TLR-signaling has been coined the third essential B-cell activation signal following recognition of unmethylated CpG-DNA<sup>14,15</sup>. Each TLR shows unique ligand specificity, but most signal via the myeloid differentiation primary response 88 (MyD88) adaptor protein and Interleukin-1 receptor Kinase family (IRAK1/2/4). This culminates in the activation of the transcription factors nuclear factor kappa B (NF-κB)<sup>16</sup> and signal transducer and activator of transcription 3 (STAT3)<sup>17</sup> which in turn induce a pro-inflammatory immune response and cellular expansion.

Activating mutations in MYD88 occur in about 3% of patients with CLL<sup>18</sup> and TLR signaling has been associated with CLL cell proliferation, survival, immunogenicity and resistance to the chemotherapy<sup>7,19-23</sup>. Murine knockout experiments of the TLR decoy and inhibitory receptor (TIR8) showed an increased rate of CLL cell accumulation within the secondary lymphoid tissues<sup>24</sup> and targeting IRAK4, a downstream target of MyD88, delayed CLL tumor development<sup>25</sup>. In contrast, Wang et al<sup>26</sup> showed that inhibition of TLR9 had a growth-promoting effect on splenic B-cells *in-vivo* on a MyD88 mutant background. These different findings support the rationale for further investigation of TLR9 signaling in CLL.

TLR9 is known to play a role in tumor cell trafficking, as evidenced by it's association with metastasis in solid tumors<sup>27</sup>. Furthermore, CLL cells show higher levels of endosomal TLR9 (eTLR9) when compared to healthy B-cells<sup>28</sup> and several studies have shown that CpG DNA can activate primary CLL cells<sup>7,21-23</sup>. It is worthy of note that the phenotype of TLR9-

stimulated healthy B-cells is similar to that of CLL cells<sup>29</sup>; increased expresson of CD5, CD23, CD25 and the T-cell associated ZAP70 kinase, which is characteristically expressed in poor prognosis disease. In addition to BCR-independent effects, Wagner *et al* suggested that TLR9 signaling integrates into the adaptive immune response in ZAP70+ CLL<sup>30</sup>. All of these findings implicate TLR9 signaling in the pathology of CLL.

The aims of this study were three fold: firstly, to investigate the role of TLR9 stimulation in the activation and migration of CLL cells and whether patient plasma contains levels of unmethylated cell-free DNA (cfDNA), which could trigger this. Secondly, as aberrant surface expression of TLR9 has been demonstrated in some poor prognosis solid tumors<sup>25</sup>, we investigated the expression and function of surface TLR9 (sTLR9) in CLL. Finally, we explored whether dual targeting of BTK and TLR9 has the potential to eliminate CLL cell trafficking and induce more sustained clinical responses.

#### Materials and methods

More detailed methods are provided in the supplementary methods

#### Patient samples

PB was taken from CLL patients with informed consent in accordance with the Declaration of Helsinki. Matched LN fine needle aspirate (FNA) sampling was undertaken simultaneously on patients with a palpable lymphadenopathy as previously described<sup>6</sup>.

### Plasma cfDNA quantification

Plasma cfDNA quantification was performed using real-time PCR to amplify the CCR5 gene and determining the  $C_t$  value. An assay to detect unmethylated mitochondrial (mt)DNA was designed, based on digesting DNA with the FspEI restriction endonuclease.

#### TLR9 activation and inhibition of CLL cells

CLL PBMCs were seeded at  $3x10^5$  cells/150µL of complete media (RPMI, 10% FCS, penicillin/streptomycin, L-Glutamine (Sigma) and 5µg/mL IL-4 (RayBiotech)). Cells were cultured ±1µM ODN2006 (TLR9 agonist (Invivogen)) or 20µL autologous plasma in duplicate and incubated for 24h (or 4h for p-STAT3/5 and p-p65 NF- $\kappa$ B) at 37°C/5% CO<sub>2</sub>. For the TLR9 blocking experiments, CLL cells were pre-incubated for 30 minutes with the TLR9 antagonist (5µM ODN INH-18, (Invivogen) or anti-TLR9 (eB72-1665, (ebiosciences))) at 5µM/10<sup>6</sup> PBMCs. For synergy 2µM of ODN INH-18 and 1µM ibrutinib (Selleckchem) were used. Cells were harvested for migration or phenotyping assays as described below.

#### Surface and intracellular immunophenotyping

Cells were labeled as described in the supplementary methods using antibody panels detailed in Supplementary Table 1.

#### Transwell migration assays

Transwell migration assays were performed using 5 $\mu$ m pore polycarbonate transwell inserts in 24-well plates. 600 $\mu$ L complete media+100ng/mL CXCL12 (Biolegend) was added to the basolateral chambers and PBMCs were then transferred into the apical chambers and incubated for 4h at 37°C/5% CO<sub>2</sub>. Migrated and non-migrated CLL cells were identified by CD19+CD5+ labeling and counted using an Accuri flow cytometer.

#### **Circulation System**

A hollow fiber bioreactor system (FiberCell Systems Inc) was previously adapted by our group to generate an *in vitro* model of circulating CLL<sup>6,8</sup>. PB-CLL cells were introduced into the model through the access ports in the circulating compartment and were allowed to circulate for 48 hours before samples were removed from port D (circulating) and port C (migrated). CLL cells were immunophenotyped as described above.

### Cell sorting and RNA-seq analysis

PBMCs from 5 patients with CLL were antibody-labeled (panel F, Supplementary Table 1). Viable, single CLL cells were identified by CD19+CD5+ labeling and both sTLR9<sup>hi</sup> and sTLR9<sup>lo</sup> populations sorted using a BD FACSMelody. RNA was extracted using the RNeasy Micro kit (Qiagen) as per the manufacturer's instructions and immediately frozen at -80°C. mRNA TruSeq library generation and sequencing was performed by Qiagen.

#### qPCR of TLR9

For qPCR the TLR9 (Hs00370913\_s1) and  $\beta$ -Actin (Hs99999903\_m1) Taqman gene expression assays (Applied Biosystems) were used.

#### Xenotransplantation

CLL cells from 7 different patients were xenotransplanted into NOD/Shi-*scid*/IL-2R $\gamma^{null}$  (NSG) mice as previously described<sup>31-33</sup>. Details are given in the supplementary methods.

#### Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). Data was assessed for normality and appropriate descriptive statistics and tests used. Unless otherwise stated, results are presented as mean±SD, paired t-test or wilcoxon matched-pairs signed rank and Pearson's or Spearman correlation coefficient depending on whether the data were Gaussian.

# Results

#### *Levels of cfDNA correlate with CD38, B2M, LDT and TTFT*

We initially investigated whether plasma from CLL patients contains cfDNA that could potentially trigger TLR9 signaling and if so, whether these levels correlated with established prognostic markers. To do this we measured the cfDNA levels in plasma from 37 patients for whom prognostic data was available. cfDNA levels were variable between patient samples but showed a positive correlation with CD38 expression (p<0.001, r=0.67; Figure 1A). In addition, patients with a lymphocyte doubling time (LDT) <12 months had a mean 8-fold higher cfDNA than those with longer LDT (p=0.030; Figure 1B) and those with  $\beta$ 2microglobulin (B2M)>3.5mg/L had a mean 3.5-fold higher cfDNA than those with B2M<3.5mg/L (p=0.002; Supplementary Figure 2A). There was a trend for raised cfDNA in IGHV unmutated cases (poor prognosis) compared to mutated cases (p=0.080, Supplementary Figure 2B) but no difference between ZAP70+ and ZAP70- cases (Supplementary Figure 2C). Notably, it was dynamic prognostic markers such as CD38<sup>34</sup> and LDT that demonstrated the strongest relationship with cfDNA levels. It is therefore possible that high leukemic burden generates raised cfDNA which in turn induces CLL proliferation via an auto-stimulation loop. In support of this, we found a positive correlation between cfDNA and white blood cell count (p<0.0001, r=0.71; Figure 1C) and a strong association between raised cfDNA and a shorter TTFT (p=0.003, HR=4.0; Figure 1D).

As only unmethylated DNA, present in eukaryotic cell mitochondria (mtDNA), can trigger TLR9 signaling, we quantified this in the plasma of CLL patients and age-matched healthy controls. Total mtDNA was 12.9-fold higher in plasma from CLL patients (p=0.001; Supplementary Figure 2D) and unmethylated mtDNA was 28.1-fold higher (p<0.0001; Figure 1E). Even when normalized to total mtDNA, the unmethylated mtDNA was 2.1-fold greater in CLL plasma (p<0.001; Figure 1F). It is well established that CLL cells proliferate when stimulated through their TLRs, but this is the first time a link between clinical CLL proliferation and the TLR9 trigger, unmethylated DNA, has been reported. We next investigated whether TLR signaling could induce a more activated and migratory phenotype in CLL cells.

#### and increases migration

We initially stimulated CLL cells using a TLR9-specific oligonucleotide B-CpG ODN2006. Following a 24-hour stimulation period, primary CLL cells were assessed for markers associated with poor prognosis and cell adhesion/migration. All CLL samples (15/15) demonstrated a marked increase in the expression of CD38 (mean 2.7-fold, p=0.020), CD49d (mean 1.9-fold, p=0.05) and CD69 (mean 2.1-fold, p=0.005; Figure 2A) following ODN2006 stimulation. As phosphorylation of p65 NF-kB and Tyrosine 705 on STAT3 are associated with migration and poor prognosis in lymphoma<sup>35</sup> and CLL,<sup>36,37</sup> and STAT5 mutations with aggressive lymphoma<sup>38</sup>, we investigated phosphorylation of these transcription factors following TLR9 stimulation. Both p-STAT3 and p-p65 NF- $\kappa$ B were upregulated in all 24 samples studied (mean 2.1-fold and 1.7-fold respectively; Figure 2B). In contrast, p-STAT5 showed a mean-fold increase of just 1.1, with 7/24 samples showing no change or reduced phosphorylation. As CLL patients have elevated levels of the physiological TLR9 ligand, unmethylated DNA (Figure 1), we next stimulated CLL cells with autologous plasma,  $\pm$  the TLR9 antagonist ODN INH-18. CLL cells were split into 4 fractions; untreated, ODN2006activated, plasma-activated and plasma-activated following pre-incubation with ODN INH-18. Autologous plasma increased both p-STAT3 and p-p65 NF-κB in all cases (1.5-fold, p<0.001 and 1.4-fold, p<0.01 respectively) which was abrogated in the presence of the TLR9 inhibitor (p<0.0001 and p<0.01; Figure 2C). Again, there was no increase in p-STAT5. These data demonstrate that CLL cfDNA can induce the activation of both STAT3 and p65 NF-κB via a TLR9-specific auto-stimulation loop.

We and others have previously demonstrated a link between CLL cell migration and expression of CD38, CD49d, and STAT3<sup>8,36,39</sup>, we therefore investigated whether TLR9 stimulation increased migration. Up to two-fold greater migration was seen in TLR9 stimulated CLL cells compared to unstimulated controls (p<0.001; Figure 2D). We have previously shown that distinct CLL sub-populations have a greater propensity to migrate<sup>6</sup>, so we investigated whether there was a link between levels of TLR9 and migration.

# Intracellular TLR9 levels in CLL cells correlate with CD38, CD49d, p-STAT3, p-p65 NF-κB and migration.

Basal levels of endosomal TLR9 (eTLR) were quantified in permeabilized unstimulated CLL cells and correlated to the expression of known CLL adhesion/migration markers; MFI values of TLR9, CD38 and CD49d were obtained by gating the viable singlet CD19+CD5+ CLL cells of 27 patients (gating strategy shown in Supplementary Figure 3A). We found no significant correlation between the expression of eTLR9 and CD49d and only a weak correlation with CD38 (Supplementary Figure 3B). However, this analysis revealed that within each patient sample, CLL cells with the highest CD38 and CD49d expression had a much greater proportion of TLR9<sup>bright</sup> cells (Figure 3A). We therefore gated on the CD19+CD5+CD38+ or CD19+CD5+CD49d+ CLL cells and showed striking correlations between the MFI of CD38 (r=0.93, p<0.001) and CD49d (r=0.73, p=0.011) with eTLR9 (Figure 3B). Furthermore, we showed that constitutive expression of p-STAT3 (Tyr 705) and p-p65 NF-κB was elevated in CD38<sup>hi</sup>CD49d<sup>hi</sup>TLR9<sup>bright</sup> CLL cells (Figure 3C). In addition, there was a correlation between CLL cell migration and basal levels of eTLR9 (r=0.72, p<0.001; Figure 3D) suggesting that, alongside the BCR, TLR9 signaling plays an important role in modulating CLL cell migration.

Although synergy between the two signaling pathways has long been established in normal B-cells<sup>40,41</sup> and suggested in CLL<sup>30</sup>, this is the first time that a link between TLR9 levels and CLL migration has been made.

#### A small proportion of CLL cells express surface TLR9

Sustained **surface** expression of TLR9 (sTLR9) has been previously reported and was linked to tumorigenesis and cancer progression<sup>42,25</sup>. As we have previously identified that many CLL patients have a small sub-population with a more migratory phenotype (CD38<sup>hi</sup> and CD49d<sup>hi</sup>), we gated on these cells and quantified sTLR9. sTLR9 was detected on this sub-population in all 29 CLL samples tested and, as with eTLR9, there was a strong correlation with CD38 (r=0.77, p=0.001) and CD49d (r=0.91, p<0.001; Figure 4A and 4B). For confirmation, we performed **intra-patient** analysis to compare CD38 and CD49d expression in the gated sTLR9<sup>hi</sup> and sTLR9<sup>lo</sup> sub-populations. sTLR9<sup>hi</sup> CLL cells had 2.5-fold and 3.6-fold higher expression of CD38 and CD49d respectively when compared with their matched sTLR9<sup>lo</sup> cells (p<0.001; Figure 4C). Additionally, sTLR9<sup>hi</sup> cells exhibited a CXCR4<sup>dim</sup>CD5<sup>bright</sup> phenotype; a phenotype associated with LN trafficking<sup>9</sup> (Supplementary Figure 4).

To establish whether sTLR9 is functional, we split CLL patient samples into two fractions; one was untreated and one pre-incubated with an anti-TLR9 antibody to block sTLR9. Both fractions were then stimulated with ODN2006 enabling eTLR9 to be activated as normal but not sTLR9 in the blocked fraction. As expected, both fractions showed evidence of activation, including increased CD49d, CD38 and CD69. However, in the blocked fraction the expression of these antigens was lower: 63%, 77% and 67% of the unblocked fraction, respectively (Supplementary Figure 5A). Migration assays comparing ODN2006 stimulated

CLL cells,  $\pm$  sTLR9 or total TLR9 (tTLR9) blockade, showed that blocking sTLR9 resulted in a 14% reduction in migration (p<0.01) but these CLL cells showed significantly more migration than unstimulated CLL cells indicating that sTLR9 blockade alone cannot prevent TLR9 signaling (Figure 4D). In contrast, tTLR9 blockade caused almost total abrogation of ODN2006-induced migration (p<0.01) back to that of the unstimulated fraction (Figure 4D). We went on to show that sTLR blockade reduced p-p65 NF- $\kappa$ B by 22% and p-STAT3 by 18% but tTLR9 blockade reduced both to levels below that of the unstimulated cells (Supplementary Figure 5B). Importantly, these findings were mirrored using autologous plasma stimulation; tTLR9 and sTLR9 blockade reduced p-p65 NF- $\kappa$ B in all samples but tTLR9 blockade to the resting levels or below in 7/10 and sTLR9 blockade in 0/10. Similarly, p-STAT3 was reduced by both and to the resting level or below in 8/12 samples by tTLR9 blockade but in only 1/12 following sTLR9 blockade (Figure 4E). Finally, we showed a striking correlation between levels of eTLR9 and sTLR9 implicating sTLR9 as an extracellular marker of extensive eTLR9 signaling (r = 0.9, p<0.0001, Figure 4F).

# Comparative RNA-sequencing shows a link between sTLR9<sup>hi</sup> and gene set enrichment for TLR signaling, lymphocyte activation, adhesion and migration

We next investigated whether sTLR9<sup>hi</sup> and sTLR9<sup>lo</sup> CLL cells had different transcriptional signatures. We sorted paired fractions from 5 different patients and performed RNA-sequencing. After false discovery rate correction, 653 differentially expressed genes retained significance (q<0.05):

<u>https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147561.</u> Gene ontology and gene set enrichment analysis, using WebGestalt (WEB-based GEne SeT AnaLysis Toolkit)<sup>43</sup>, confirmed that sTLR9<sup>hi</sup> CLL cells showed increased expression of genes associated with

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raised metabolic activity (glycolysis), lymphocyte activation, adhesion/migration (Rho GTPase cytoskeleton regulation and IGTB1; part of the CD49d complex) and inflammation mediated by chemokines and cytokines (Figure 5). Most importantly, in the sTLR9<sup>hi</sup> fraction, components of the TLR signaling pathway were highly over-represented with a normalization enrichment score of 2.08; the over expressed gene list included the obligate TLR signaling molecule, IRAK4<sup>44</sup>. Furthermore, there was a striking over-representation of NF-κB (NFKBIA, NFKBIE, ReIA, NFKB2, IKBKB and TRAF6 etc) and STAT3 regulated genes (JAK2 and JUNB). Finally, as shown by Wagner et al.<sup>30</sup>, there was a link between TLR9 and BCR signaling as the sTLR9<sup>hi</sup> population over expressed the BCR signaling gene, PLCγ2.

# Surface TLR9 expression is higher in LN and migrated CLL cells.

The RNA-sequencing data suggest that sTLR9<sup>hi</sup> expression represents CLL cells undergoing enhanced TLR signaling with a pro-inflammatory and pro-migratory transcriptional signature. We therefore investigated whether migrated CLL cells showed enrichment for a sTLR9<sup>hi</sup> phenotype. CLL cells from 10 patients were put through our circulation model for 48 hours<sup>8</sup> and the sTLR9 expression compared between those that migrated with those that remained circulating. sTLR9 was higher in the migrated cells from 9/10 patients tested (p=0.004; Figure 6A, Supplementary Table 2). Importantly, we were also able to demonstrate that LN-derived CLL cells were enriched for the sTLR9<sup>hi</sup> phenotype in matched FNAs and PB from CLL patients with palpable LNs. CLL cells from all 7 patients tested had higher expression of sTLR9 in the LNs compared to their PB (p=0.016; Figures 6B and C, Supplementary Table 2). To investigate the role of TLR9 in CLL cells *in-vivo*, we established CLL murine xenografts TLR9<sup>hi</sup> and TLR9<sup>lo</sup> CLL cells into NOD/Shi-*scid*/IL-2R $\gamma^{null}$  mice. Of the 7 different patient samples transplanted, 4 were TLR9<sup>hi</sup> and 3 were TLR9<sup>lo</sup>. CLL cells from the TLR9<sup>hi</sup> samples showed considerably higher levels of splenic engraftment (mean 10.2% ± 11.5%) and more rapid disease progression compared to the TLR9<sup>lo</sup> samples which all demonstrated engraftment levels <1% (mean 0.3% ± 0.3%, p=0.018; Figure 6D, Supplementary Figure 6). Furthermore, a positive correlation (p=0.01, r<sup>2</sup>= 0.23) was seen between TLR9 expression and the extent of splenic engraftment (Supplementary Figure 7). These data supports the notion that TLR9 contributes to CLL migration and engraftment *in-vivo*.

#### Blocking TLR9 reduces CLL cell migration and synergizes with ibrutinib.

The data presented above provides compelling evidence to support inhibition of TLR9 as a potential therapeutic strategy in order to reduce the activation of STAT3 and NF-κB and prevent CLL cell migration. Although we have shown that sTLR9 is functional in CLL, its blockade is not sufficient to prevent TLR9 signaling. Therefore, we investigated whether inhibition of tTLR9 synergizes with ibrutinib to reduce migration. To do this we used an experimentally determined fixed molar ratio of 2:1 (ODN INH-18: ibrutinib). In this model, a clinically relevant dose of 1µM of ibrutinib<sup>45</sup> was found to reduce migration of activated CLL cells in 5/6 patients when used as a single agent and the addition of ODN INH-18 (2µM) had a marked synergistic effect. In all 6 patients, migration was reduced to a level significantly below that seen with either drug alone; median combination index at the half maximal effective dose (ED50) for the combination of the two drugs was 0.2 indicating a strong synergistic effect (Figure 7A&B). The combination also synergistically reduced p-p65 NF-κB and p-STAT3 but not p-STAT5 (Figure 7C-F and Supplementary Figure 8). These data clearly

support our hypothesis that inhibition of the TLR9 pathway, alongside BCR signaling, is a potential therapeutic strategy. Even more so, given the recent report that TLR9 signaling provides a common escape mechanism for CLL cells following treatment with ibrutinib and venetoclax<sup>46</sup>.

#### Discussion

Over the last decade, targeting BCR-related signaling pathways has led to improved therapies for CLL. However, despite these advances, CLL remains incurable. The identification of MyD88 activating mutations in approximately 3% of CLL cases<sup>18</sup>, supported by the potential for TLR9 to be triggered by autoantigens/DNA<sup>47</sup>, has prompted new research into the role of TLR signaling in CLL<sup>48</sup>.

Here, we show for the first time that autologous plasma from CLL patients contains a disproportionately high level of unmethylated mtDNA, which can trigger TLR9 signaling, resulting in downstream phosphorylation of STAT3 and NF-κB. Importantly, all of these effects can be reversed by TLR9 inhibition. Furthermore, we show that patients with aggressive disease have increased levels of cfDNA which correlate strongly with the dynamic prognostic markers CD38, B2M, net proliferation (LDT), tumor burden and shorter TTFT. These strong correlations suggest that cfDNA may promote tumor proliferation. There was also a trend towards higher cfDNA and unmutated IGHV status; although mutational status itself is not dynamic, it is recognized that an unmutated IGHV correlates with raised CD38<sup>49</sup> which in turn is regulated by CLL cell interactions in the lymphoid tissue microenvironment<sup>34</sup>. It is therefore possible that raised cfDNA promotes CLL cell activation and trafficking to pro-proliferative lymphoid tissues. Although raised levels of cfDNA did not correlate with

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ZAP70 expression, it remains possible that the adapter molecule function of ZAP70<sup>50,51</sup>may enable ZAP70+ CLL cells to signal via TLR9, regardless of cfDNA levels.

CD38 and CD49d are associated with each other<sup>52,53</sup> and CLL cell homing<sup>54</sup> where it is known that the malignant clone differs from normal B-cells in that  $\alpha 4\beta 1$  (CD49d) engagement is essential for trans-endothelial migration<sup>39</sup>. Here, we demonstrate that TLR9 stimulation increases CD38, CD49d and migration. Within a patient's CLL clone, eTLR9<sup>bright</sup> cells showed higher expression of CD38, CD49d, p-STAT3 and p-p65 NF- $\kappa$ B. Furthermore, in contrast to TLR9<sup>lo</sup> CLL cells, TLR9<sup>hi</sup> CLL cells showed good engraftment in NSG mice. Taken together, these findings both support and expand on our previous study<sup>6</sup> and suggest an import role for TLR9 *in-vivo*.

TLR9 is usually triggered by pathogenic DNA which is structurally identical to self-DNA except for the presence of unmethylated CG dinucleotides. CG motifs are underrepresented within the mammalian genome and the majority exist within the heavily methylated gene promoter regions<sup>55</sup>. However, mitochondrial DNA is almost entirely unmethylated<sup>55,56</sup> and given that exposed mitochondrial DNA is a natural and extracellular-residing product of cell death, it is vital that it does not come into contact with functional TLR9. Therefore, in normal resting conditions, TLR9 is sequestered within the endoplasmic reticulum and transported to the endosomal membrane in its inactive pre-receptor form following the endocytosis of CpG DNA<sup>50,57,58</sup>. TLR9 is then transported via the golgi-dependent secretory pathway and is **temporarily** expressed at the cell surface before re-internalization. TLR9 functionality is not thought to be enabled until it is successfully integrated and cleaved within the endosomal membrane<sup>59</sup>. Such tight regulation of TLR9 is fundamental to the

distinction of self/non-self-DNA and ensures that functional TLR9 only encounters pathogen-derived nucleic acids. However, a hallmark of CLL is the large tumor turnover<sup>60</sup> and it is possible that extensive apoptosis, and the generation of excessive cfDNA, could overwhelm this regulatory system and trigger autologous TLR9 activation.

Surface TLR9 has been described in solid tumors and linked to tumorigenesis and cancer progression<sup>25</sup>. We hypothesize that the high cell turnover seen in malignancies like CLL could trigger dysregulation of the distinction of self/non-self-DNA and thus result in sustained surface expression of TLR9. In support of this hypothesis, we demonstrated sTLR9, on a small population of CLL cells within patients, which is notably higher on those that migrate or reside within the LNs. Importantly, we also showed that blocking of sTLR9 marginally reduces CLL activation and migration, demonstrating some functional properties. However, it's strong correlation with eTLR9 indicates that expression is likely a consequence of sustained eTLR9 signaling in the most aggressive CLL cell sub-population and this is supported by the striking TLR gene set enrichment seen in sorted sTLR9<sup>hi</sup> cells. Importantly, raised sTLR9 could potentially represent a biomarker for patients who would particularly benefit from TLR9 inhibition. Finally, we demonstrated that total blockade of TLR9 is highly synergistic with ibrutinib and reduces p-p65 NF-κB and p-STAT3 driven migration down below that which either drug alone can achieve.

These findings strongly support the rationale for further investigations into the role of TLR9 in CLL. It is clear that blockade of BTK or PI3K alone is not curative and these tumor cells have mechanisms for escape and clonal evolution that involve TLR9 signaling<sup>46</sup>. Therefore, simultaneous targeting of multiple signaling pathways could provide an opportunity for total

tumor eradication. In this regard, we have shown that there is a strong biological rationale for dual targeting of BTK and TLR9.

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### Author contributions

EK, EC, EH, NP-P, EW, BC, EHP, TAB, SM, RS, MS, AA CO, ND, TS, LL and CJ performed experiments, analysed data and revised the manuscript. SD, RJ and TC provided vital reagents and revised the manuscript. AGSP, CDF and CP conceived the study, analysed data and drafted the manuscript.

The authors have no relevant conflicts of interest to disclose.

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Figure legends

Figure 1. CLL patients have raised levels of both total and unmethylated cfDNA, which correlate with high CD38, short LDT, high leukemic burden and reduced TTFT. Levels of cfDNA were measured in the plasma of 37 patients with a confirmed diagnosis of CLL. (A) There was a positive correlation between the levels of cfDNA and percentage CD38 positive CLL cells. (B) Using a cut-off of 12 months, patients with a LDT <12 months had higher plasma cfDNA than those with a LDT >12 months. (C) There was also a strong positive correlation between the levels of cfDNA and leukemic burden (white blood cell count x 10<sup>9</sup>/L). (D) Patients were segregated into high (red) or low (blue) plasma DNA depending whether their levels were higher or lower than the median. Those with higher levels of plasma cfDNA had a much shorter TTFT. (E) Unmethylated DNA was quantified in the plasma of patients with CLL (n=15) and healthy age-matched controls (n=27) and presented here as the fold difference compared to the mean of the controls. Patients with CLL had a mean of 28.1-fold higher unmethylated DNA. (F) The fold difference in unmethylated DNA was normalized to the total DNA concentration and was still 2.1-fold higher in plasma of patients with CLL compared to that from healthy controls.

**Figure 2. Stimulating CLL cells through TLR9 causes an increase in CD38, CD49d, CD69, p-STAT3,** p-p65 NF-κB **and migration. (A)** PBMCs from 15 different CLL patients were incubated alone or in the presence of ODN2006 for 24 hours. Cells were then harvested and labeled with antibodies against CD5, CD19, CD38, CD49d, CD69 and a viability dye. Fluorescence minus one (FMO) control tubes using stimulated CLL cells defined the thresholds for positivity. The MFI of CD38, CD49d and CD69 were assessed by flow

cytometry; all were significantly increased in viable CD5+CD19+ CLL cells following activation with ODN2006. (B) Primary PBMCs were incubated for 4 hours with or without ODN2006. Cells were harvested and labeled for CD5, CD19 and intracellular p-STAT3, p-p65 NF-κB, p-STAT5 or an isotype matched control (IMC) and then assessed by flow cytometry. (i) Representative overlaid histograms showing that gated CD5+CD19+ cells have some constitutive p-STAT3 (red histogram), which is increased following stimulation with ODN2006 (blue histogram). (ii) This figure shows the MFI fold change compared to resting cells. There is an increase in both p-STAT3 and p-p65 NF- $\kappa$ B for all 24 cases following ODN2006 stimulation but not p-STAT5. (C) Primary PBMCs were incubated for 4 hours with or without ODN2006, autologous plasma or autologous plasma with the TLR9 inhibitor ODN-INH-18. Cells were harvested and stained for CD5, CD19 and intracellular p-STAT3, p-p65 NFκB or p-STAT5 and then assessed by flow cytometry. Autologous plasma increased both (i) p-STAT3, (ii) p-p65 NF- $\kappa$ B, but not (iii) p-STAT5, and this was abrogated in the presence of a TLR9 inhibitor. (D) Primary PBMCs from 24 different patients were incubated alone or in the presence of ODN2006 for 24 hours. Cells were then harvested and then transferred into 5µm pore polycarbonate transwell migration chambers and incubated for 4 hours; cells migrated towards a CXCL12 (100ng/mL) gradient. The migrated and non-migrated cells were collected, stained with CD5 and CD19 for CLL cell identification and then quantitated volumetrically. The ODN2006 pre-stimulated CLL cells had greater levels of migration compared to the unstimulated fraction. Due to between patient variance in levels of migration, the results are normalized to the unstimulated.

**Figure 3. CLL TLR9 levels correlate with CD38, CD49d, p-STAT3,** p-p65 NF-κB **and ability to migrate**. Unstimulated primary PBMCs from 11 CLL patients were stained with antibodies to CD5, CD19, CD38, CD49d and a viability dye and then permeabilized and stained for eTLR9 or an IMC. Cell were assessed by flow cytometry and positivity determined by FMO for surface antigens or IMC for eTLR9. (A) A representative figure showing that all viable CD5+CD19+ gated CLL cells have eTLR9 but, within a patient, a much larger proportion of the CD38+ and CD49d+ populations have exceptionally high TLR9 (TLR9<sup>bright</sup>) compared to their negative counterparts. (B) (i) CD5+CD19+CD38+ and (ii) CD5+CD19+CD49d+ CLL cells from 11 different patients were gated on and the MFI of CD38, CD49d and TLR9 established. There is a clear positive correlation between the MFIs of both CD38 and CD49d with TLR9. (C) Basal intra-nuclear p-STAT3 (Tyr 705) and p-p65 NF-KB levels were established on CLL cells from 12 patients using the same panel as above and including an anti-p-STAT3 or p-p65 NF- $\kappa$ B antibody. (Ci) This representative figure shows higher expression of p-STAT3 in CLL cells in a small population of CLL cells (shaded green) which also have higher expression of CD38, CD49d and TLR9. (Cii) The MFIs for both p-STAT3 and p-p65 NF-κB were quantified on both the CD38<sup>hi</sup> CD49d<sup>hi</sup> TLR9<sup>bright</sup> and the CD38<sup>lo</sup> CD49d<sup>lo</sup> TLR9<sup>dim</sup> population. The MFI of both p-STAT3 and p-p65 NF-κB was significantly higher in the CD38<sup>hi</sup> CD49d<sup>hi</sup> TLR9<sup>bright</sup> CLL cells. (D) Basal eTLR9 levels of CLL cells from 20 patients were compared with their level of migration (as described in Figure 2C but in the absence of any stimulation). There is a clear correlation between the percentage of CLL cells that migrate and the MFI of their eTLR9.

**Figure 4. A small proportion of CLL cells with an aggressive phenotype and high levels of eTLR9 express functional surface TLR9 (sTLR9).** Unstimulated primary PBMCs from 29 CLL patients were stained with antibodies to CD5, CD19, CD38, CD49d, surface TLR9 and a viability dye. Cell were assessed by flow cytometry and positivity determined using FMO. (A) This is a representative figure showing CLL cells from a patient with very high levels of sTLR9 expression. Viable CD5+CD19+ CLL cells were gated on and sTLR9 plotted against either CD38 or CD49d. The majority of sTLR9 positive CLL cells are positive for CD49d and many for CD38 as well. (B) (i) CD5+CD19+CD38+ and (ii) CD5+CD19+CD49d+ CLL cells from all 29 patients were gated on and the MFI of CD38, CD49d and sTLR9 established. There is a clear positive correlation between the MFIs of both CD38 and CD49d with that of sTLR9. (C) Using a different 29 patient cohort, viable CD5+CD19+CLL cells were gated on and the sTLR9 positive and negative populations were further gated on. The MFI of CD38 and CD49d established and within every patient, both CD38 and CD49d are much more highly expressed in the sTLR positive population compared to their negative counterparts. (D) PBMCs from 8 different CLL patients were split into four fractions. One fraction was preincubated with an antibody to block surface TLR9 and one with ODN INH-18 to block tTLR9. Both these fractions of cells, and an untreated fraction, were then stimulated with ODN2006 overnight. The fourth fraction remained unstimulated. Following overnight incubation, cells were harvested and then transferred into transwell migration chambers and incubated for 4 hours; cells migrated towards a CXCL12 (100ng/mL) gradient. The migrated and non-migrated cells were collected, stained with CD5 and CD19 for CLL cell identification and then quantitated volumetrically. The fold change compared to the normalized unstimulated fraction was then assessed. The ODN2006 pre-stimulated CLL cells had greater levels of migration compared to the unstimulated fraction. This was marginally reduced following the blocking of sTLR9 but abrogated to the resting level in the presence of tTLR9 inhibition. (E) The same experiment was repeated using autologous plasma stimulation for a 4-hour period and then cells collected, stained with CD5, CD19 and either an IMC or (i) p-p65 NF-kB or (ii) p-STAT3. p-p65 NF-kB and p-STAT3 MFIs were quantified and the fold change compared to the normalized unstimulated fraction assessed. Both were

upregulated in the presence of plasma and this was very marginally reduced with sTLR9 blocking but much more with tTLR9 blockade. **(F)** eTLR9 and sTLR9 levels were compared on CLL cells from 30 different patients. Samples were split into two fractions and one stained with CD5, CD19 and permeabilised for eTLR9 staining and the other with CD5, CD19 and surface TLR9. For each the CD5+CD19+ CLL cells were gated on and the MFI of the TLR9 quantified. There is a very strong correlation between the levels of eTLR9 and expression of sTLR9.

Figure 5. RNA sequencing of paired sTLR9+ and sTLR9- samples revealed a striking gene set enrichment for lymphocyte activation, adhesion/migration, TLR signaling and inflammation. (A) Shows the top 10 over-represented pathways in the differentially expressed gene list between the sTLR9+ and sTLR9- samples. (B) Shows the differentially expressed genes in the toll-like receptor signaling pathway and the inflammation mediated by cytokine and chemokine signaling pathway.

Figure 6. Higher TLR9 expression is seen in LN and migrated CLL cells and is associated with preferential engraftment in a CLL xenograft model. (A) Primary PBMCs from 10 different CLL patients were pumped through the circulating system for 48 hours and then those that had migrated out of the circulating compartment harvested. CLL cells that remained circulating were harvested at the same time and both stained for expression of sTLR9 alongside CD5 and CD19 for CLL cell identification. CD5+CD19+ CLL cells were gated on and the sTLR9 MFI positivity determined by FMO. Due to the variation in MFIs the circulating compartment results were normalized to 1 and the migrated results presented as fold difference. Migrated CLL cells from 9 of the 10 cases had higher sTLR9 MFI compared

with those that remained circulating. **(B)** FNA and matching PB samples were taken from 7 CLL patients with palpable LNs. PBMCs were stained as above and CD5+CD19+ CLL cells gated on. Due to the variation in MFIs the PB results were normalized to 1 and the LN results presented as fold difference. For all 7 cases, sTLR9 MFI was higher in the LN derived CLL cells compared to those from the PB. The scatter plots in **(C)** are representative examples showing matched PB and LN-derived gated CLL cells from the same patient. **(D)** CLL cells from 4 patients with raised TLR9 and 3 patients with low TLR9 (relative transcription normalized to  $\beta$ -actin) were xenotransplanted into NOD/Shi-*scid*/IL-2R $\gamma^{null}$ mice. Mice were euthanised according to the criteria described in the supplementary methods and the percentage of CLL cells in the spleen determined using the flow cytometry panel G in Supplementary Table 1. Mice engrafted with TLR9<sup>hi</sup> CLL cells had considerably higher numbers in their spleen compared with those engrafted with TLR9<sup>lo</sup> CLL cells.

#### Figure 7. Blocking TLR9 reduces CLL cell migration and is synergistic with ibrutinib.

(A) PBMCs from 6 different patients were split into 4 fractions. One fraction was stimulated with ODN2006 alone, one fraction stimulated in the presence of ibrutinib, one fraction stimulated in the presence of the TLR9 inhibitor ODN INH-18 and one fraction stimulated in the presence of both (2:1 fixed molar ratio of ODN INH-18 to ibrutinib). Following overnight incubation, cells were harvested and then transferred into transwell migration chambers and incubated under the conditions described above. The migrated and non-migrated cells were collected, stained with CD5 and CD19 for CLL cell identification and then quantitated volumetrically. The percentage change compared to the normalized stimulated fraction was then assessed. The combination of ODN INH-18 and ibrutinib gave maximum CLL cell migration inhibition. **(B)** Combination Index (CI) analysis demonstrated that the two drugs

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were synergistic in all 6 samples using 1μM ibrutinib + 2μM ODN INH-18 (i.e. CI<1). The mean CI at the half maximal effective dose (ED50) for the combination of the two drugs was 0.2 indicating a strong synergistic effect **(C & E)** CLL cells from 11 or 12 different patients were split and treated as above but stimulated for 4 hours. Following activation, cells were harvested and stained for CD5, CD19 and intracellular p-STAT3, p-p65 NF- $\kappa$ B or an IMC and then assessed by flow cytometry and the MFI recorded. The combination of ODN INH-18 and ibrutinib gave the maximum inhibition of both p-STAT3 and p-p65 NF- $\kappa$ B. Combination Index (CI) analysis demonstrated that the two drugs were synergistic at reducing **(D)** p-STAT3 in 10/11 patients and **(F)** p-p65 NF- $\kappa$ B in all 12 patients using 1μM ibrutinib + 2μM ODN INH-18 (i.e. CI<1). The mean CI at the half maximal effective dose (ED50) for the combination of the two drugs was 0.5 for p-STAT3 and 0.4 for p-p65 NF- $\kappa$ B indicating a strong synergistic effect.











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| Gene Symbol | Gene Name  | Entrez Gene   |
|-------------|--|---|
| TLR7        | toll like receptor 7   | 51284   |
| JUN         | Jun proto-oncogene, AP-1 transcription factor subunit  | 3725  |
| TLR10       | toll like receptor 10  | 81793   |
| NFKBIA      | NFKB inhibitor alpha   | 4792  |
| MAP2K3      | mitogen-activated protein kinase kinase 3  | 5606  |
| UBE2N       | ubiquitin conjugating enzyme E2 N  | 7334  |
| NFKB2       | nuclear factor kappa B subunit 2   | 4791  |
| MAP2K2      | mitogen-activated protein kinase kinase 2  | 5605  |
| IRAK4       | interleukin 1 receptor associated kinase 4   | 51135   |
| TLR6        | toll like receptor 6   | 10333   |
| МАРКЗ       | mitogen-activated protein kinase 3   | 5595  |
| TBK1        | TANK binding kinase 1  | 29110   |
| TNFAIP3     | TNF alpha induced protein 3  | 7128  |
| TANK        | TRAF family member associated NFKB activator   | 10010   |
| TOLLIP      | toll interacting protein   | 54472   |
| NFKBIE      | NFKB inhibitor epsilon   | 4794  |
| MAP3K8      | mitogen-activated protein kinase kinase kinase 8   | 1326  |
| TICAM2      | toll like receptor adaptor molecule 2  | 353376  |
| RELA        | RELA proto-oncogene, NF-kB subunit   | 5970  |
| MAPK9       | mitogen-activated protein kinase 9   | 5601  |
| IKBKB       | inhibitor of nuclear factor kappa B kinase subunit beta  | 3551  |
| TRAF6       | TNF receptor associated factor 6   | 7189  |
| MAP2K1      | mitogen-activated protein kinase kinase 1  | 5604  |
| TICAM1      | toll like receptor adaptor molecule 1  | 148022  |
|             | Gene Symbol<br>TLR7<br>JUN<br>TLR10<br>NFKBIA<br>MAP2K3<br>UBE2N<br>NFKB2<br>MAP2K2<br>IRAK4<br>TLR6<br>MAP2K2<br>IRAK4<br>TLR6<br>MAPK3<br>TBK1<br>TNFAIP3<br>TANK<br>TOLLIP<br>NFKBIE<br>MAP3K8<br>TICAM2<br>RELA<br>MAPK9<br>IKBKB<br>TRAF6<br>MAP2K1<br>TICAM1 | Gene Symbol     Gene Name       TLR7     toll like receptor 7       JUN     Jun proto-oncogene, AP-1 transcription factor subunit       TLR10     toll like receptor 10       NFKBIA     NFKB inhibitor alpha       MAP2K3     mitogen-activated protein kinase kinase 3       UBE2N     ubiquitin conjugating erzyme E2 N       NFKB2     nuclear factor kappa B subunit 2       MAP2K2     mitogen-activated protein kinase kinase 4       TLR6     toll like receptor associated kinase 4       TLR6     toll like receptor 6       MAPK3     mitogen-activated protein kinase 3       TBK1     TANK binding kinase 1       TNFAIP3     TNF alpha induced protein 3       TANK     TRAF family member associated NFKB activator       TOLLP     toll like receptor adaptor molecule 2       RELA     NFKB inhibitor epsilon       MAP3K8     mitogen-activated protein kinase kinase kinase 8       TICAM2     toll like receptor adaptor molecule 2       RELA     RELA proto-oncogene, NF-KB subunit       MAP3K8     mitogen-activated protein kinase 9       IKEKB     inhibitor of nuclear factor kappa B kinase subunit beta |

| ID: P00031; Name: Inflammation mediated by chemokine and cytokine signaling<br>pathway  | 3           |   |             |
|---|-------------|---|-------------|
| size=196; overlap=26; expect=15.66; enrichmentRatio=1.66; PValue=1.610e-3; FDR=5.002e-2 |             |   |             |
| User ID   | Gene Symbol | Gene Name   | Entrez Gene |
| RAC2  | RAC2        | Rac family small GTPase 2                                 | 5880        |
| JUNB  | JUNB        | JunB proto-oncogene, AP-1 transcription factor<br>subunit | 3726        |
| CXCR3   | CXCR3       | C-X-C motif chemokine receptor 3                          | 2833        |
| ARPC1B  | ARPC1B      | actin related protein 2/3 complex subunit 1B              | 10095       |
| ITGB7   | ITGB7       | integrin subunit beta 7                                   | 3695        |
| GNAI2   | GNAI2       | G protein subunit alpha i2                                | 2771        |
| JUND  | JUND        | JunD proto-oncogene, AP-1 transcription factor<br>subunit | 3727        |
| RHOC  | RHOC        | ras homolog family member C                               | 389         |
| NRAS  | NRAS        | NRAS proto-oncogene, GTPase                               | 4893        |
| CXCR5   | CXCR5       | C-X-C motif chemokine receptor 5                          | 643         |
| MYH10   | MYH10       | myosin heavy chain 10                                     | 4628        |
| ITGAL   | ITGAL       | integrin subunit alpha L                                  | 3683        |
| JUN   | JUN         | Jun proto-oncogene, AP-1 transcription factor subunit     | 3725        |
| ARPC2   | ARPC2       | actin related protein 2/3 complex subunit 2               | 10109       |
| GNB3  | GNB3        | G protein subunit beta 3                                  | 2784        |
| PLCB2   | PLCB2       | phospholipase C beta 2                                    | 5330        |
| COL6A2  | COL6A2      | collagen type VI alpha 2 chain                            | 1292        |
| ARPC3   | ARPC3       | actin related protein 2/3 complex subunit 3               | 10094       |
| ACTG1   | ACTG1       | actin gamma 1   | 71          |
| PLCG2   | PLCG2       | phospholipase C gamma 2                                   | 5336        |
| JAK2  | JAK2        | Janus kinase 2  | 3717        |
| муоза   | мүөза       | myosin IIIA   | 53904       |
| PTEN  | PTEN        | phosphatase and tensin homolog                            | 5728        |
| МҮН9  | MYH9        | myosin heavy chain 9                                      | 4627        |
| NFKB2   | NFKB2       | nuclear factor kappa B subunit 2                          | 4791        |
| ITGB1   | ITGB1       | integrin subunit beta 1                                   | 3688        |



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