Phenotype and immune function of lymph node and peripheral blood CLL cells are linked to transendothelial migration.

Marta Pasikowska1*, Elisabeth Walsby2*, Benedetta Apollonio1, Kirsty Cuthill1, Elizabeth Phillips, Eve Coulter1, Maria Serena Longhi3, Yun Ma3, Deborah Yallo1, Linda D. Barber1, Piers Patten, Chris Fegan2, Alan G. Ramsay1, Chris Pepper2**, Stephen Devereux1*** and Andrea G.S. Buggins1**.

1Department of Haemato-Oncology, Division of Cancer Studies, Faculty of Life Sciences & Medicine, King’s College London, London, United Kingdom, SE5 9NU, 2Cardiff CLL Research Group, Institute of Cancer & Genetics, School of Medicine, Cardiff University, Cardiff, UK, CF14 4XN. 3King’s College London, Institute of Liver Sciences, London, UK, SE5 9RS.

* M.P and E.W. contributed equally to the study.

** C.P., S.D., and A.G.S.B. contributed equally to the study.

Scientific section: LYMPHOID NEOPLASIA

Key Points:

1. LN-derived CLL cells have increased capacity for T-cell activation and superior immune synapse formation compared to those from PB.

2. Enhanced CLL cell immunological function is also linked to PB circulating cells with the propensity to migrate.

The authors have no conflicts of interest to disclose.
Abstract

Several lines of evidence suggest that homing of tumor cells to lymphoid tissue contributes to disease progression in chronic lymphocytic leukemia (CLL). Here we demonstrate that lymph node (LN)-derived CLL cells possess a distinct phenotype and exhibit enhanced capacity for T-cell activation and superior immune synapse formation when compared to paired peripheral blood samples (PB). LN-derived CLL cells manifest a proliferative, CXCR4$^{\text{dim}}$CD5$^{\text{bright}}$, phenotype compared to those in the PB and higher expression of T-cell activation molecules including CD80, CD86 and HLA-DR. In addition, LN-CLL cells have higher expression of $\alpha_4\beta_1$ (CD49d) which, as well as being a co-stimulatory molecule, is required for CLL cells to undergo transendothelial migration (TEM) and enter the proliferation centers of the LNs.

Using an in-vitro system that models circulation and TEM, we showed that the small population of CLL cells that migrate are CXCR4$^{\text{dim}}$CD5$^{\text{bright}}$ with higher CD49d, CD80, CD86 and HLA-DR compared to those that remain circulating; a phenotype strikingly similar to LN-derived CLL cells. Furthermore, sorted CD49d$^{\text{hi}}$ CLL cells showed enhanced capacity to activate T-cells compared to CD49d$^{\text{lo}}$ sub-populations from the same patient. Thus, although PB-CLL cells have reduced capacity to form immune synapses and activate CD4+ T-cells, this was not the case for LN-CLL cells or those with the propensity to undergo TEM. Taken together, our study suggests that CLL cell immunological function is not only modulated by microenvironmental interactions but is also a feature of a sub-population of PB-CLL cells that are primed for lymphoid tissue homing and interaction with T-cells.
Introduction

Chronic lymphocytic leukemia (CLL) is a common B-cell malignancy that follows a remarkably diverse clinical course. It is characterized by an accumulation of mature B-lymphocytes in the peripheral blood, bone marrow (BM) and secondary lymphoid organs such as the lymph nodes. Since circulating tumor cells generally have a very low proliferation rate, it was originally assumed that CLL was primarily a disease of failed apoptosis. However, in-vivo studies of tumor kinetics, using deuterated water, revealed higher than expected tumor cell turnover, with a birth rate of up to 2% per day.

The proliferative component of CLL appears to be confined to pseudofollicles or proliferation centers in secondary lymphoid tissues where interactions with non-neoplastic T-cells, and follicular dendritic cells take place and promote tumor cell growth. In contrast, very few CLL cells in the peripheral circulation show features of proliferation and those that do are believed to represent recent emigrants from the lymph node. In the peripheral circulation, CLL cells transiently interact with endothelial cells, which stimulate survival, but not proliferation. These findings suggest a two-compartment model of disease in which CLL cells traffic between the peripheral vasculature and the lymphoid tissues. In support of this, Herishanu et al. compared the gene expression of CLL cells in different compartments and identified the LN as the predominant site of CLL cell activation and proliferation. Since disease progression occurs when tumor proliferation outstrips loss, the capacity of tumor cells to migrate into tissues is an important factor in determining outcome. Transit of CLL cells to the tissues is mediated, at least in part, by their expression of CD49d and the chemokine receptors such as CXCR4 and CXCR5 and is controlled by the secretion of the chemokine ligands including CXCL12 and CXCL13.
FUNCTIONAL DIFFERENCES OF LN VERSUS PB CLL CELLS.

Work by Calissano et al.\textsuperscript{8} used \textit{in vivo} deuterium incorporation to study the phenotype and gene expression of the resting and proliferative fraction. They characterized the two compartments using differences in CD5 expression (which is upregulated following B-cell activation) and CXCR4 (which is raised in CLL cells with high sIgM and down-regulated following BCR engagement\textsuperscript{16} or binding of CXCL12). They concluded that distinct subsets of CLL cells exist within PB-CLL cells including small populations of \textit{CXCR4}\textsuperscript{dim}CD5\textsuperscript{bright} and \textit{CXCR4}\textsuperscript{bright}CD5\textsuperscript{dim} cells. They hypothesized that the former are proliferative, recent emigrants from the LN and the later ‘older’ resting cells attempting to re-enter the LN.

Several types of interaction are thought to occur in the CLL tissue microenvironment. There is good evidence that co-stimulatory signals from activated CD4\textsuperscript{+} T-cells\textsuperscript{5,17} play an important role in promoting tumor growth. Contact with activated autologous CD4\textsuperscript{+} T-cells is sufficient to induce proliferation of CLL cells \textit{in-vitro} and analysis of tissue samples from CLL patients reveals that proliferating leukemic cells frequently contact activated CD4\textsuperscript{+} T-cells\textsuperscript{6,18}.

The objective of the present study was to reconcile these findings with the extensive previous evidence that CLL cells strongly inhibit T-cell activation\textsuperscript{19-21}. Previous studies assessing the T-cell activation capabilities of CLL cells have used cells derived from the peripheral blood which it has been suggested to induce anergy\textsuperscript{22}. However, these cells are known to have different properties to those residing within the lymph nodes\textsuperscript{6,11}. Here we used fine needle aspiration to perform functional assays and determine whether matched CLL cells from lymph node (LN-CLL) and peripheral blood (PB-CLL) had a distinct compartment-specific phenotype and T-cell activation function. In addition, we utilized our novel circulation system to study migration\textsuperscript{23} in order to compare LN-derived CLL cells with CLL cells that migrated \textit{in-vitro}.
Materials and Methods

Patient samples

Matched PB and LN FNA sampling was undertaken simultaneously on 11 patients with a diagnosis of CLL and palpable lymphadenopathy. The FNA was performed by the passage of a 23-gauge needle through the skin once and sampling 6-8 times within the node. PB only was taken from another 36 patients. CLL PBMC cells were isolated from whole blood of CLL patients by density gradient centrifugation with Lymphoprep (Axis-Shield) or Histopaque (Sigma). All were taken with the patients’ informed consent in accordance with the Declaration of Helsinki. Normal T and B-cells were derived from healthy volunteers.

Circulation System

A hollow fibre bioreactor system (FiberCell Systems Inc) was adapted to generate an in-vitro model of circulating CLL previously described\(^2\) using HUVEC and HMEC endothelial cells (life technologies) at 5\% CO\(_2\) 37\(^\circ\)C. PB CLL cells were introduced into the circulating system through one of 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 subsequently immunophenotyped as described below.

Immunophenotyping

CLL cells recovered from the circulation system, following FNA or PB density gradient centrifugation were labeled using the panels shown in supplementary Table 1. For the FNA/PB staining a whole blood staining method was performed as per the manufacturer’s instructions.
FUNCTIONAL DIFFERENCES OF LN VERSUS PB CLL CELLS.

and a red cell lysis buffer (eBiosciences). For each antigen the Mean Fluorescent Intensity (MFI) of the CD19$^+$/CD5$^+$ CLL cells was recorded.

**Mixed lymphocyte reaction (MLR)**

T-cells and B-cells were purified by negative selection (Stem Cell technologies), checked for purity by flow cytometry and re-suspended to $10^6$ cells/ml in RPMI complete medium with 1% BSA (CM). Enriched (>95%) CLL B-cells from both LN and PB or CD49d$^{hi}$ and CD49d$^{lo}$ (top and bottom 20%) were sorted using a BD FACS Aria (gating strategy shown in Supplementary Figure 1), re-suspended in CM at $10^6$ cells/ml and for the thymidine-incorporation assay irradiated at 30Gy. CLL and T-cells were plated out in triplicate at 1:1 and 1:10 and incubated for 48 hours at 5% CO$_2$ 37°C then harvested as previously described$^{21}$. When Natalizumab was added, CLL cells were pre-incubated for 20 minutes at 20ug/10$^6$ cells before co-culture with T-cells and a further 20ug/10$^6$ cells added every 24 hours. For T-cell flow cytometry (antibody list: panels E & F Supplementary Table 1) CD3$^+$CD4$^+$/CD8$^+$ T-cells were tightly gated on and expression of HLA-DR, Ki67 and CD69 assessed. For the Ki67 assay, Fix and Perm (Invitrogen) was used as per the manufacturer’s instructions except that 0.5µl of 10% NP40 was added per 50µl of perm buffer. The thymidine incorporation assay was performed as previously described$^{21}$.

**Synapse assays**

Quantitative CLL:T-cell synapse assays were performed and analyzed as previously described$^{24}$. Blinded confocal images were analyzed and CD4$^+$T-cell/APC conjugates were identified only when T-cells were in direct contact interaction with CLL APCs (blue fluorescent channel). The area analysis tool was then used to measure the total area (µm$^2$) of F-actin (red
fluorescent channel) accumulation at all T-cell contact sites and synapses with APCs.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 4.0 and 7.0 (GraphPad Software, San Diego, CA). Data was assessed for Gaussian distribution using the D'Agostino-Pearson normality test and appropriate tests applied.
Results

Comparison of the phenotype of PB and LN CLL cells

In order to directly compare the phenotype of CLL cells derived from lymph nodes and the peripheral circulation, we performed matched FNA and PB sampling on 11 CLL patients with lymphadenopathy. Only patients with lymphadenopathy were selected and these patients typically have high-risk disease and manifest other markers of poor prognosis (Supplementary Table 2). Using multi-color flow cytometry, CD5+/CD19+ CLL cells were gated and MFI of HLA-DR, CD5, CD80, CD86 and CD69 established. In all cases, LN-CLL cells had significantly higher expression of the markers associated with antigen presentation, co-stimulation and activation: HLA-DR (p<0.0001), CD5 (p=0.0036), CD80 (p=0.0002), CD86 (p=0.0079) and CD69 (p=0.0037; Figure 1A). These findings are in keeping with previous work showing activation of CLL cells in the LN\textsuperscript{11} and support our hypothesis that LN-CLL cells have better T-cell activation potential than those from the PB. Interestingly, compared to PB-CLL cells, these LN-CLL cells also had a phenotype associated with adhesion and migration namely increased CD49d (p=0.0021) and CD38 (p=0.0083) and decreased CXCR4 expression (p=0.0003) (Figure 1B). This may be because BCR activation occurs with in the LN and is associated with downregulation of CXCR4. In addition, although raised CXCR4 expression is associated with the propensity to migrate, following migration the CLL cells encounter high local concentrations of CXCL12 which promotes rapid down regulation of this chemokine receptor.

Immunosuppressive markers were also analyzed, but expression of PD-L1 and PD-L2 was absent in both PB and LN-CLL cells in 5/8 patients and the remaining 3 patients showed very low levels with no difference between PB and LN-CLL cells. CD200 expression was high in both LN-CLL and PB-CLL (data not shown).
Enhanced capacity of LN derived CLL cells to activate T-cells

Since LN-CLL cells have increased expression of molecules associated with T-cell activation compared to their PB counterparts, we next sought to determine whether they also had an increased capacity to stimulate normal allogeneic T-cells. Paired LN and PB CLL cells plus normal B-cells were mixed with purified CD3+ T-cells from a healthy donor in a MLR and the proliferation and activation status of the T-cells was assessed by measuring $^3$H thymidine incorporation and the expression of Ki67, CD69 and HLA-DR by flow cytometry. LN-CLL cells induced greater activation of both CD4+ and CD8+ T-cells as measured by their higher expression of Ki67, CD69 and HLA-DR when compared with PB-CLL cells and this was equivalent to that induced by normal B-cells (Figure 2A and Supplementary Figure 2A). Furthermore, enhanced T-cell proliferation was confirmed in the presence of irradiated LN-CLL cells, again equivalent to that induced by normal B-cells, as evidenced by significantly increased thymidine incorporation (Figure 2A). The CLL cells were purified by negative selection to avoid modification of properties by antibody binding; the resultant cells were >95% CD5+CD19+. To control for the potential that the small residual non-CLL cell pool contained different numbers of APCs, we evaluated the proportion of CD5+CD19+HLA-DR+ cells in the LN and the PB residual populations and showed there was no difference: LN 1.4% ±1.2% and the PB 1.9% ±1.8 (p=0.52; data not shown).

We also compared the ability of normal PB-B-cells, LN-CLL cells and matched PB-CLL cells to form immune synapses with autologous CD4+ T-cells. Previous work has shown that PB-CLL cells exhibit impaired T-cell synapse formation\(^20\). Here, we measured synapse assembly (CLL:CD4+ T-cell conjugates) in four patient samples and demonstrated for the first time that LN-CLL cells showed enhanced autologous T-cell synapse formation, equivalent to that induced by
healthy B-cells, when compared to their matched PB-CLL cells (patient 1-4 = p < 0.0001, p = 0.03, p = 0.05 and p< 0.0001 respectively; Figures 2B-D).

**PB-CLL cells that migrate in our in-vitro model possess a strikingly similar phenotype to LN-CLL cells**

We investigated whether the phenotype manifested by LN-CLL cells was dependent on their residence in the LN microenvironment or could be identified in a subset of PB-CLL cells with a propensity to migrate. We used a physiologically relevant in-vitro circulation system of CLL (Figure 3A) into which PBMC from 36 CLL patients were then individually introduced for 48 hours before samples were harvested. Matched samples were obtained from port D (CLL cells remaining in circulation) and port C (those that had migrated through the endothelial cell coated fibers into the extravascular space (EVS)). In keeping with our previous report, a small percentage of CLL cells migrated into the EVS after 48h (1.37% ± 2.32%). Compared to CLL cells remaining in the circulating compartment, migrated cells showed lower expression of CXCR4 (p=0.0058) as well as increased expression of CD49d (p<0.0002), CD38 (p<0.0001), HLA-DR (p=0.0002), CD5 (p=0.0044), CD80 (p<0.0001), CD86 (p=0.0006) and CD69 (p=0.0007; Figure 3B). This phenotype was strikingly similar to that of LN-derived CLL cells. It is worthy of note that when we repeated these experiments without endothelial cells lining the hollow fibers there was significantly reduced migration but the tiny number that migrated manifested higher levels of CXCR4 (p=0.055; Figure 3C) and no difference in CD5. The other phenotypic markers showed similar increases in the absence of endothelial cells (data not shown). These results confirm our previous observation that PB-CLL cells with high CXCR4 and CD49d have an increased propensity to migrate, but implies that following transendothelial migration, the CXCR4 expression is reduced and CD5 is increased.
FUNCTIONAL DIFFERENCES OF LN VERSUS PB CLL CELLS.

**Migrated and LN-derived CLL cells are CXCR4<sup>dim</sup> CD5<sup>bright</sup> compared with their circulating and PB-derived counterparts**

The work by Calissano et al<sup>8</sup> identified small intra-clonal sub-populations of CLL cells with different proliferative characteristics. A small number of CXCR4<sup>dim</sup> CD5<sup>bright</sup> CLL cells were identified as being the proliferative sub-population and it was hypothesized that these had recently emigrated from the LN. Here we demonstrate that the small number of CLL cells that have migrated our *in-vitro* model were enriched for a CXCR4<sup>dim</sup> CD5<sup>bright</sup> phenotype when compared to those that remained circulating. In addition, we showed a clear negative correlation between the expression of CD5 and CXCR4; (p=0.0095; Figure 4A and 4C). Whether these cells are recent emigrants from the LN that are better primed to migrate in this system due to their enhanced activation status, or whether they are a small population with an increased migratory potential remains unknown. However, we repeated this analysis on LN-CLL cells and clearly showed that, compared with matched PB-CLL cells, LN-CLL cells also had this CXCR4<sup>dim</sup> CD5<sup>bright</sup> phenotype (p=0.0051; Figure 4B and 4D).

**CD49d expression is associated with expression of activation and costimulatory molecules and an increased capacity to activate T-cells**

The phenotype of migrated CLL cells suggests that as well as having a greater propensity to migrate their increased expression of co-stimulatory molecules could potentially have a greater affect on T-cell activation. It has been previously shown that CD49d expression identifies CLL cells that have an increased capacity to undergo transendothelial migration<sup>12,23</sup> and interestingly it is also a co-stimulatory molecule<sup>26</sup>. In order to investigate whether there is a link between migration and co-stimulation in CLL, we correlated the expression of CD49d with a variety of activation and costimulatory molecules in the LN and PB of patients with CLL and in the circulating and extravascular compartment of the *in-vitro* circulation system. We found that CD49d expression positively correlated with CD5 expression in both the circulating and EVS.
Functional differences of LN versus PB CLL cells. 

Compartments of the in-vitro system and in the matched LN/PB ex-vivo samples (p=0.016 and p=0.0252 respectively; Figure 4E). There was also a strong and statistically significant correlation between CD49d levels and CD80, CD86, HLA-DR, CD69 and CD38 in both in-vitro and in-vivo systems (Figure 5) and once again the correlation patterns from the circulating model system and the matched LN-CLL and PB-CLL cells are strikingly similar.

Functional assays were not possible with cells harvested from the EVS due to the limited number of cells that could be obtained. We instead exploited the observation that CD49d$^{hi}$ CLL cells that have the highest migratory potential. We sorted the top and bottom 20% CD49d-expressing CLL cells (based on MFI) from the PB of 5 CLL patients and compared their ability to activate alloreactive T-cells in an MLR. CD49d$^{hi}$ and CD49d$^{lo}$ CLL cells were mixed with purified CD3$^{+}$ T-cells from a healthy donor and the proliferation and activation status of the T-cells assessed by measuring their expression of Ki67, CD69, HLA-DR by flow cytometry and incorporation of $^{3}$H thymidine as described previously. CD49d$^{hi}$ CLL cells induced greater activation of CD4$^{+}$ and CD8$^{+}$ T-cells as evidenced by higher expression of Ki67 (p=0.05 and p <0.001), CD69 (p=0.05 and p=0.04) and HLA-DR (p=0.03 and p=0.006) when compared to T-cells co-cultured with CD49d$^{lo}$ CLL cells (Figure 6A and Supplementary Figure 2B). Furthermore, enhanced T-cell proliferation was confirmed in the presence of irradiated CD49d$^{hi}$ cells by significantly increased thymidine incorporation (p=0.013; Figure 6A). These results show that, for CLL cells, there is a clear relationship between the propensity to migrate and the ability to activate T-cells. We have previously demonstrated that blocking CD49d using Natalizumab prevented CLL migration in our circulating system$^{23}$. Here, we investigated whether Natalizumab could also inhibit the ability of CLL cells to activate allo-reactive T-cells in an MLR. CLL cells from 6 CD49d$^{hi}$ CLL patients were pre-incubated with Natalizumab prior to irradiation and co-culture with purified CD3$^{+}$ T-cells from a healthy donor. Following a 5-day MLR there was no difference in the thymidine incorporation of the T-cells incubated with CLL cells in the
presence or absence of Natalizumab. This suggests that the functional role of CD49d on these cells relates to migratory potential rather than T-cell activation.
Discussion

In this study we set out to compare the phenotypic and functional properties of PB CLL cells with those that have undergone transendothelial migration into LNs. Traditionally, investigations into the pathophysiology of CLL were largely restricted to peripheral blood-derived cells, but recent data demonstrating the key role of the lymph node microenvironment in this disease has highlighted the importance of understanding the differences in cells residing within the lymph nodes compared to those in the peripheral blood.

In this study we utilized fine needle aspiration to access simultaneous lymph node and peripheral blood CLL cells and used this material to answer some important questions regarding differences in phenotype and function between the two compartments. Unfortunately no matched BM samples were available for this study but previous work by Herishanu et al\(^\text{11}\) suggests that this microenvironment is not as pro-proliferative or activation-inducing as the LN. Previous comparisons have used disaggregated LN biopsies from cases when there is diagnostic doubt or atypical disease behavior. Our study recruited typical cases of CLL and the LN sample was fresh suspension cells. Using these samples we clearly demonstrated that LN-CLL cells had enhanced expression of markers that would induce T-cell activation. In contrast to previous studies which suggest that PB-CLL cells are poor APCs\(^\text{22,27}\), induce T-cell anergy and inhibit T-cell activation\(^\text{20,21,28}\), we showed that LN-CLL cells can induce T-cell activation and proliferation. We also showed that, in contrast to PB CLL cells, those from the LN were capable of forming immune synapses with autologous CD4\(^+\)T-cells that were comparable to those formed by healthy B and T-cells. These results provide a plausible explanation for the observation that CLL lymph nodes contain significant numbers of activated T-cells, despite the known inhibitory effects of the tumor\(^\text{21}\). It has been suggested that CLL cells might present antigen to T-cells\(^\text{29}\) and thereby initiate a self-sustaining stimulatory loop in which CLL cells cause T-cell activation which in turn leads to activation and proliferation of the tumor. The
FUNCTIONAL DIFFERENCES OF LN VERSUS PB CLL CELLS.

observation that CLL cells are capable of presenting red cell-derived rhesus antigen, causing the expansion of auto-reactive T-cell clones in patients with autoimmune hemolytic anemia\(^{30}\), provides further \textit{in-vivo} evidence in support of this theory. Whether the interactions promote an anti-tumor response or a self-stimulatory tumor survival loop remains unknown, but the nature of the disease suggests it is the latter.

In addition, LN-CLL cells also expressed higher levels of CD49d and CD38, which are both involved in transendothelial migration. This suggests there is a link between the enhanced ability to activate T-cells and the capacity to migrate. Tissue invasion of CLL requires transendothelial migration (TEM) of the malignant cells but a full understanding of the mechanisms behind this is not yet available. However, it has been established that CLL clones differ from normal B-cells in that they require \(\alpha 4\beta 1\) (CD49d) engagement to undergo TEM and enter the proliferation centers of lymph nodes\(^{12}\). In addition, CD38 is associated with CD49d\(^{31,32}\) and homing from the blood to the lymphoid organs\(^{33}\). In this study we showed that both of these poor prognostic markers were expressed at higher levels in LN-CLL cells. In much the same way that we now know that CD38 expression is temporal\(^{6,34}\), it seems likely that CD49d, CXCR4 and CD5 are also temporally regulated and this could give rise to intra-clonal subsets with a higher and lower predisposition to migrate depending on their phenotype. In support of this theory, we demonstrated that CD38\(^{hi}\)/CD49d\(^{hi}\) LN-CLL cells were CXCR4\(^{dim}\) CD5\(^{bright}\) compared to PB-CLL cells which supports the Calissano model of them being the proliferative ‘robust’ fraction\(^8\).

In order to investigate whether these cells could be identified as a subset in PB-CLL and, if so, whether they have an increased propensity to migrate, we utilized our novel in-vitro circulation system. We demonstrated that compared to the majority of cells that remained circulating, the small population of CLL cells that migrated had significantly higher expression of CD38, CD49d,
HLA-DR, CD80, and CD86. In addition, following migration through endothelium, they also possessed a CXCR4\textsuperscript{dim} CD5\textsuperscript{bright} phenotype. Importantly, this CXCR4\textsuperscript{dim} CD5\textsuperscript{bright} phenotype was not seen in CLL cells that migrated in the absence of endothelial cells. We therefore hypothesized that CXCR4 and CD5 are modulated, at least in part, by endothelial cell contact, their secretion of CXCL12 and the process of transendothelial migration. Although the data presented here does not completely validate the Calissano model, it does support the concept that there is a small and distinct population of CLL cells with a propensity to migrate in the peripheral circulation and these cells could well be the recent LN emigrants (Supplementary Figure 3).

Importantly, we demonstrated a strong positive correlation between the expression of CD49d and that of CD5, HLA-DR, CD80 and CD86 further supporting a link between migration and the potential for increased contact with, and activation of, T-cells. These correlations were confirmed in matched LN-CLL and PB-CLL cells adding weight to the argument that these phenotypes are physiologically relevant.

The limited numbers of cells that migrated in our \textit{in-vitro} model prevented functional assays from being performed but, as it is CLL cells with the highest expression of CD49d that migrate, we compared the T-cell stimulatory properties of CD49d\textsuperscript{hi} with CD49d\textsuperscript{lo} CLL cells derived from the same patient. These assays demonstrated that CD49d\textsuperscript{hi} CLL cells induce superior T-cell activation. We have previously shown that blocking CD49d with Natalizumab prevents CLL cell migration but, interestingly, here we have established that CD49d itself is not directly responsible for T-cell activation. This supports the hypothesis that the poor prognosis associated with CD49d expression in CLL is predominately caused by its ability to modulate tumor cell migration rather than directly induce T-cell activation.
CLL is a disease characterized by immune suppression that is exemplified by poor responses to vaccination. However, a number of studies have shown that this suppression is not irreversible, PD-1 is not always a marker of terminal exhaustion and immune responses can be reinvigorated by antibody blockade. Previous contradictory literature indicated that T-cell activation in CLL is in equilibrium between pro- and anti-activation signals but here we show that there appears to be a balance shift towards pro-activation in the LNs. In support of this, Herishanu et al. demonstrated that LN-CLL cells had the signature of BCR activation and Buhman et al. showed that CD40L expression by CLL cells up regulated T-cell stimulatory activity.

Our results add to this by suggesting a role for transendothelial migration in the T-cell activation capabilities of the LN-CLL cells. Previously, we have shown that the interaction of CLL cells with endothelium in static culture activated NF-κB resulting in enhanced transcription and protein expression of NF-κB-regulated genes such as CD38 and CD49d. In our novel circulation system, which more closely simulates the situation in-vivo, a much larger effect was seen in migrated cells and as CD49d is also a co-stimulatory molecule, this further supports the link between migration and T-cell activation by CLL cells.

In conclusion, LN-CLL cells manifest a distinct phenotype to those in the PB and demonstrate an enhanced capacity for T-cell activation and immunological synapse formation. Data from our in-vitro circulation model implies that there is a link between the process of migration and these phenotypic and functional differences. Clearly the microenvironment plays a vital role in the pathology of CLL but it would appear that, within a patient, a subset of CLL cells with a distinct phenotype are inherently more capable of migrating and are primed for interaction with T-cells. Although the striking reduction in tumor bulk observed with drugs like Ibrutinib and Idelalisib is only partially due to tissue redistribution, it seems likely that their clinical effect is, at least in
part, elicited by inhibiting CLL cell lymphoid tissue homing, which consequently prevents antigen presentation, T-cell activation and tumor proliferation.

Acknowledgements
This work was funded in part by Bloodwise (MP, EW & KC) Kay Kendall Leukaemia Fund (EC) and British Society of Haematology (BA).

Authorship Contributions
A.G.S.B. designed the research, performed experiments and co-wrote the paper, M.P., E.W, DY, E.C, B.A, K.C. P.P, E.P. and M.S.L. performed practical work, L.B., C.D.F and A.G.R provided analytical tools and C.P and S.D. designed research and co-wrote the paper.

Conflicts-of-interest disclosure
The authors declare no competing financial interests.
References


Figure 1. LN-derived CLL cells have a phenotype associated with T-cell activation and migration.

Matched LN and PB samples from 11 CLL patients were analyzed using multicolor flow cytometry and the % change between the LN and PB MFI calculated.

A. Compared to PB-derived CLL cells, LN-derived CD19+CD5+ CLL cells showed higher expression of the markers associated with antigen-presentation, co-stimulation and activation: HLA-DR, CD5, CD80, CD86 and CD69.

B. In addition, they have a phenotype associated with recent migration: raised CD49d and CD38 and reduced CXCR4.

Figure 2. LN-CLL cells are functionally better at T-cell activation and induce superior CLL:T-cell synapses.

A. Paired LN and PB-CLL cells (irradiated for thymidine-incorporation assays) from 6 patients and B-cells from 5 normal donors were mixed at a 1:1 or 1:10 ratio with allogeneic T-cells in triplicate. Compared to PB-CLL cells, LN-CLL cells and normal B-cells have an increased ability to stimulate T-cell activation and proliferation, as shown by increased expression of Ki67 on gated CD4+ cells and CD8+ cells after a 48 hour coculture and increased thymidine-incorporation by CD3+ T-cells in a 5 day MLR.

B. To investigate the ability of the LN-derived CLL cells to induce autologous T-cell synapse formation compared to PB-CLL cells and normal B-cells,
we measured the area of F-actin polymerization in 90 CLL or B-cell:CD4+T-cell conjugates in four paired patient samples and one paired normal sample. This representative figure from one patient shows the increased synapse formation induced by the LN-derived CLL cells compared to matched PB-CLL cells.

C. Representative result from one patient showing that synapse area was significantly greater when LN-derived CLL cells were used (mean synapse area induced by LN-CLL 6.534 µm$^2$ ±2.7 vs PB-CLL 3.594 µm$^2$ ±2.3 p<0.0001).

D. Representative result from another patient demonstrating the synapse area generated by LN-CLL cells is comparable to that of normal B-cells.

Figure 3. CLL cells that migrate have a strikingly similar phenotype to those derived from the LN.

A. CLL PBMC were introduced in to the circulating model system coated with human endothelial cells (HUVEC$^{23}$ or HMEC-$^{19,10}$) and samples were collected from port C (migrated) and port D (circulating) after 48 hours. Matched CD5+/CD19+ CLL cells from each compartment were analyzed using multi-color flow cytometry.

B. Compared to CLL cells that remained circulating, migrated CLL cells had a phenotypic pattern strikingly similar to LN-CLL cells: higher HLA-DR (n=7), CD5 (n=12), CD80 (n=19), CD86 (n=7), CD69 (n=26), CD49d (n=36), CD38 (n=32) and reduced CXCR4 (n=34). CLL cells that remained circulating had a pattern reflective of PB-CLL cells.
C. PBMCs from 11 patients were introduced into the circulating model system in the absence of endothelial cell coating. The numbers of CLL cells migrating was much lower than in the presence of endothelial cells and only sufficient for analysis migrated from 8 patients. In these 8 cases, the migrated CLL cells had increased CXCR4 expression compared to those that remained circulating.

Figure 4. Both models show negative correlation between CD5 and CXCR4 but a positive correlation between CD5 and CD49d.

A. A representative figure showing matched circulating and migrated CD19⁺CD5⁺ CLL cells from a single patient harvested after 48 hours in the circulation system. Compared to each other, the migrated CLL cells are CD5brightCXCR4dim and the circulating cells CD5dimCXCR4bright.

B. A representative figure showing matched PB and LN CD19⁺CD5⁺ CLL cells from a single patient. The LN CLL cells are CD5brightCXCR4dim and the PB cells CD5dimCXCR4bright.

C. For both the migrated and circulating CD19⁺CD5⁺ CLL cells, the MFI of the CD5 and CXCR4 were established and show a negative correlation. This was done on 8 patients and a color assigned to each patient. For each case the dots surrounded by a colored box is the result for the migrated and that surrounded by an oval in the same color the matched circulating result. In each case the migrated cells have higher CD5 and lower CXCR4 than the circulating ones.
D. The same was done for the LN and PB CD19^CD5^ CLL cells from 11 patients. In each case the LN-derived CLL cells have higher CD5 and lower CXCR4 (box) than those from the PB (oval).

E. In both the circulating/migrated model and the LN/PB model CD5 and CD49d positively correlate.

Figure 5. Both models show strikingly similar positive correlation between CD49d and markers associated with antigen presentation and activation.

A. PBMCs were introduced into the circulating model system for 48 hours and the MFI values for both the migrated and circulating CLL cells recorded. There is a positive correlation between expression of CD49d and CD80, CD86, HLA-DR, CD69 and CD38.

B. MFI data from matched LN and PB CLL cells from 11 patients were analyzed for correlation. There is a positive correlation between expression of CD49d and CD80, CD86, HLA-DR, CD69 and CD38.
Figure 6. CD49d<sup>hi</sup> CLL cells are superior stimulators of T-cell activation.

Paired CD49d<sup>hi</sup> and CD49d<sup>lo</sup> CLL cells (irradiated for thymidine-incorporation assays) from 5 patients were mixed at a 1:1 or 1:10 ratio with allogeneic T-cells in triplicate.

A. Compared to CD49d<sup>lo</sup> cells, CD49d<sup>hi</sup> cells have an increased ability to stimulate T-cell activation and proliferation as shown by increased expression of Ki67 on gated CD4<sup>+</sup> and CD8<sup>+</sup> cells after 48 hours and increased thymidine-incorporation in a 5 day MLR.

B. Irradiated CLL cells from 6 patients known to express CD49d were mixed at a 1:1 ratio with allogeneic T-cells in triplicate and in the absence or presence of Natalizumab and proliferation measured by thymidine-incorporation in a 5 day MLR. Blocking of CD49d had no effect on the ability of CLL cells to induce T-cell proliferation.
Figure 1

A.

- % change in HLA-DR MFI
  - PB vs. LN: p < 0.0001
- % change in CD5 MFI
  - PB vs. LN: p = 0.0030
- % change in CD80 MFI
  - PB vs. LN: p = 0.0002

B.

- % change in CD94 MFI
  - PB vs. LN: p = 0.0021
- % change in CD38 MFI
  - PB vs. LN: p = 0.0063
- % change in CXCR4 MFI
  - PB vs. LN: p = 0.0003
Figure 2

A. CMAC PHALLOIDIN pTyr

B. PB LN

C. CD4+ T cell F-actin

D. Immune synapse area (µm²)
Figure 3

A. Diagram showing the process of migrating and non-migrating circulating CLL cells through a cartridge with hollow fibers coated with endothelial cells.

B. Graphs showing the percentage change in HLA-DR MFI, CD5 MFI, CD20 MFI, CD23 MFI, and CXCRO MFI for both migrating and non-migrating cells at 48 hours.

C. Graph showing the percentage change in CXCRO MFI for cells migrating to the extravascular space after 48 hours.
Figure 4

A. Combined circulating and migrated

B. Combined LN and PB

C. Full Gating Path
   - Lymphocytes/Migrated CLL cells
   - Lymphocytes/Circulating CLL cells

D. Full Gating Path
   - Lymphocytes/LN CL cells
   - Lymphocytes/PB CLL cells

E. Scatter plots showing correlation between CD5 MFI and CXCR4 MFI
   - Circulating: r = -0.6256, r² = 0.3913, p = 0.0095
   - Migrated: r = -0.5757, r² = 0.3314, p = 0.0051

Scatter plots showing correlation between CD5 MFI and CD49d MFI
   - Circulating: r = 0.5907, r² = 0.3489, p = 0.016
   - Migrated: r = 0.4759, r² = 0.2265, p = 0.0252

Figure 4
A. Correlation of combined circulating and migrated cell MFI's with CD49d

B. Correlation of combined LN and PB MFI's with CD49d
Figure 6

A.

B.
Phenotype and immune function of lymph node and peripheral blood CLL cells are linked to transendothelial migration

Marta Pasikowska, Elisabeth Walsby, Benedetta Apollonio, Kirsty Cuthill, Elizabeth Phillips, Eve Coulter, Maria Serena Longhi, Yun Ma, Deborah Yallop, Linda D. Barber, Piers Patten, Chris Fegan, Alan G. Ramsay, Chris Pepper, Stephen Devereux and Andrea G.S. Buggins