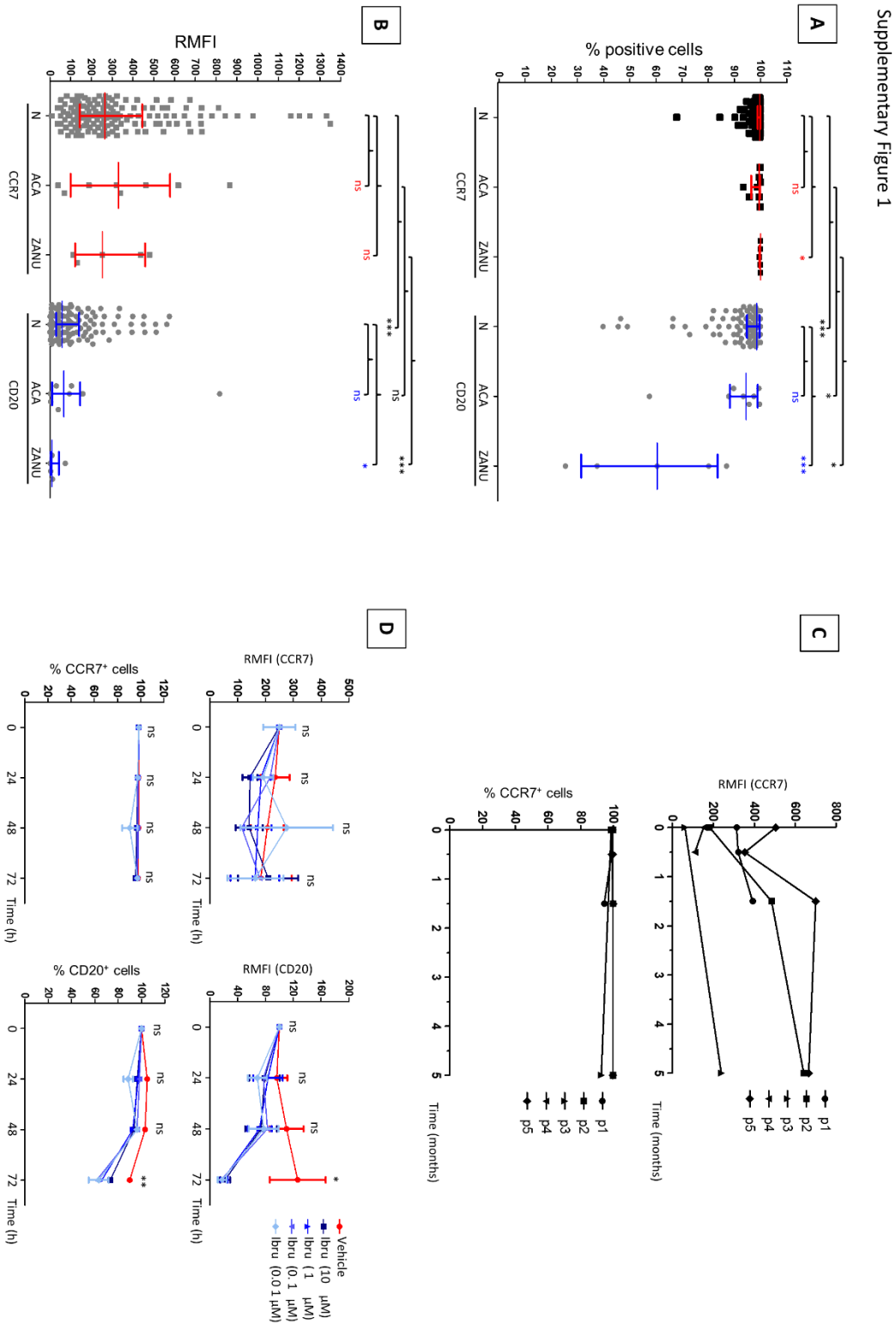
SUPPLEMENTARY MATERIAL

1- MATERIAL AND METHODS

Table S1. Reagents

Material	Supplier	Catalogue nº	Remarks
Biocoll Separating Solution (isotonic solution)	Merck Millipore	Ref:L6115	Cell isolation
Bovine Serum Albumin FV	Roche Diagnostics	Ref:10735086001	For cell culture
Fetal Bovine Serum	HyClone	Ref:SV30160.03	For cell culture
L-Glutamine	Lonza	Ref:BE17-605E	For cell culture
RPMI medium 1640(1X)+GlutaMAX	Gibco/Life technologies	Ref:72400-021	For cell culture
Mouse anti-human CD3-FITC (clone:SK7)	Becton-Dickinson	Ref:345763	FACS assays
Mouse anti-human CD5-APC clone:L17F12)	Becton-Dickinson	Ref:345783	FACS assays
Mouse anti-human CD19-APC-H7 (clone:SJ25C1)	Becton-Dickinson	Ref:641395	FACS assays
Mouse anti-Human CCR7-PE (clone:150503-IgG2A)	R&D systems	Ref:FAB197P	FACS assays
Mouse anti-Human CD20-PE (clone:369444)	R&D systems	Ref:FAB4225P	FACS assays
Mouse IgG2a-PE	Becton-Dickinson	Ref:349053	Irrelevant isotype control/FACS
BD Lysing solution	Becton-Dickinson	Ref:349202	Irrelevant isotype control/FACS
Transwell Permeable Supports (6.5-mm diameter, 10-mm thickness, 5-mm diameter pore size).	Costar / Corning Incorporated	Ref:3421	Cell migration assays /
Human CCL19	Peprotech	Ref:300-29	Cell migration
Human CCL21	Peprotech	Ref:300-35	Cell migration
Human IL-2	StemCell Technologies	Ref:78036.2	NK cell activation
Herceptin (trastuzumab)	Roche Registration Ltd	NDC: 50242-0134-68	mAb targeting Her-2 not binding blood cells/ Used as an irrelevant human IgG1 in FACS
Mabthera (rituximab)	Roche Registration Ltd	3281574E (E6)111	mAb targeting CD20
Imbruvica (ibrutinib)	Janssen		TKI targeting BTK Cys481
7-AAD	Becton-Dickinson	Ref:51-6898-1E	FACS assays
CellTrace Calcein Violet, AM	Invitrogen	Ref :C34858	ADCC assays / Effector cells labeling

2- RESULTS


Supplementary Figure 1
A
% positive cells
CCR7
CD20
N
ACA
ZANU
ns
*
***
B
RMFI
C
RMFI (CCR7)
% CCR7⁺ cells
Time (months)
p1
p2
p3
p4
p5
D
RMFI (CCR7)
% CCR7⁺ cells
RMFI (CD20)
% CD20⁺ cells
Time (h)
Vehicle
Ibru (10 µM)
Ibru (1 µM)
Ibru (0.1 µM)
Ibru (0.01 µM)

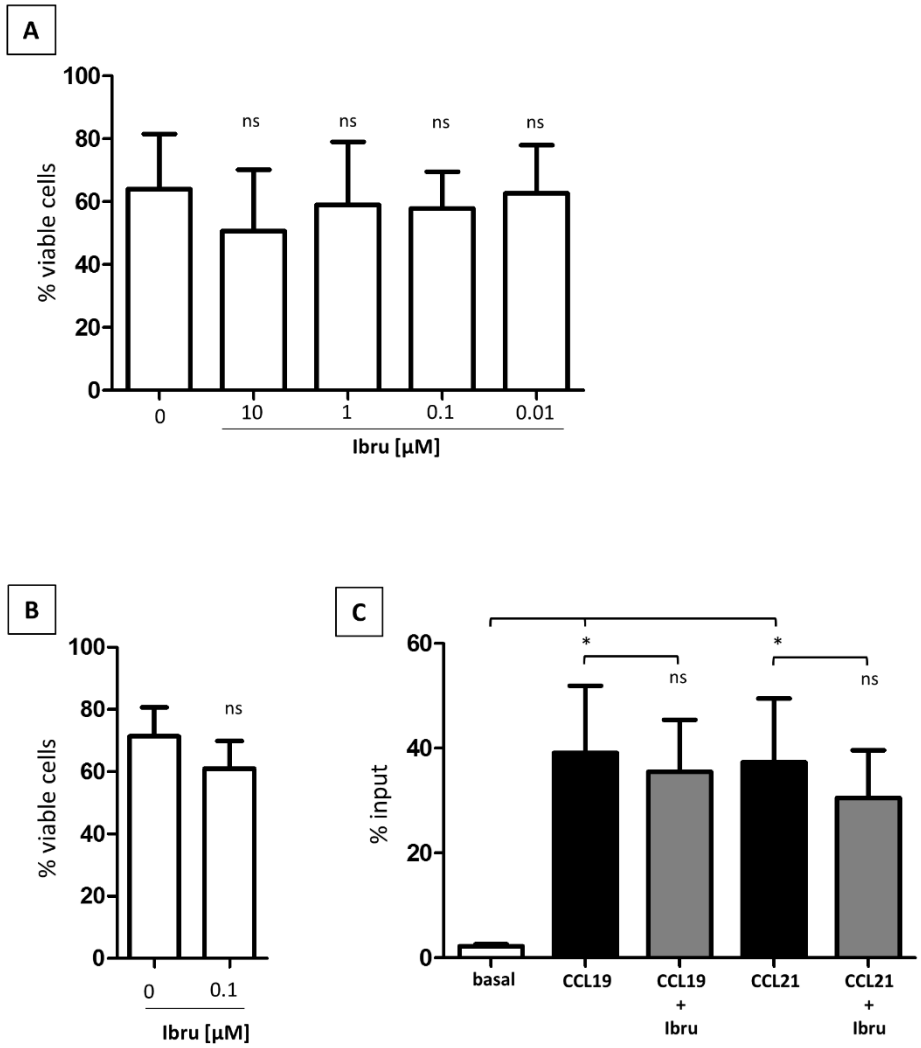
Supplementary Figure 1.

A-B) CCR7 expression in CLL cells remains high in patients treated with alternative BTK inhibitors. Expression of surface CCR7 (or CD20) was analyzed in terms of proportion of malignant cells expressing the receptor (**A**) or of relative median fluorescence intensity (RMFI, relative to an irrelevant isotype control, arbitrary units) (**B**). Expression of CCR7 and CD20 was determined in CLL samples obtained from naïve patients (N, n=144) or receiving current treatment with acalabrutinib (ACA, n=8) or zanubrutinib (ZANU, n=5). In A and B, the graphs show the median ± interquartile range. Man-Whitney-U was used to test statistical differences: ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

C) Ibrutinib discontinuation leads to re-expression of surface CCR7. The graph shows a 5 months follow-up on surface CCR7 expression in five relapsed/refractory patients (p) who discontinued ibrutinib. For each patient, the expression of CCR7 (determined as RMFI or proportion of positive cells) is shown.

D) CCR7 expression is maintained in CLL cells after *in vitro* incubation with ibrutinib. CLL cells obtained from naïve patients (n=7) were incubated for 72 h in the presence of ibrutinib at different final concentrations [0 (vehicle, DMSO); 0.01; 0.1; 1; 10 µM]. At time 0 and every 24 h CCR7 and CD20 surface expression was determined by flow cytometry and analyzed in terms of proportion of malignant cells expressing the receptor or of relative median intensity of fluorescence (RMIF, relative to an irrelevant isotype control, arbitrary units). In both cases, relative values to time 0 are shown. ns, not significant.

Supplementary Figure 2



Supplementary Figure 2.

A) Exposure to ibrutinib does not reduce CLL cell viability during the *in vitro* migration assays. The graph shows CLL cells viability after ibrutinib treatment *in vitro*. Freshly obtained primary CLL cells were isolated and seeded ($5x10^5$ cells/100 µl) in the upper chamber of transwell inserts. Then, cells were exposed to ibrutinib (range dose: 0/vehicle; 0.01; 0.1; 1; 10 µM) for 3 hours right before performing migration assays. Cells were also exposed to ibrutinib during the migration assay (4 hours). At the conclusion, the percentage of viable cells was determined by gating on 7-AAD-negative CLL cells. The graph shows the mean ± SD for a total of 7 samples. Ns, not significant.

B-C) Treatment (*in vitro*) with ibrutinib does not impair migration mediated by CCR7 in CLL cells. To discard that the lack of ibrutinib inhibition could be associated to short incubation times we conducted migration assays where naïve CLL cells were incubated with or without ibrutinib at 0.1 µM for 24 h before testing chemotaxis. After 24 hours, the proportion of viable CLL cells was measured in both groups by gating on 7-AAD-negative cells (**B**). In these settings ibrutinib induced a slight though not significant reduction in viability (mean ± SD control vs 0.1 µM ibrutinib: 71.34 ± 26.56 vs 63.29 ± 24.82; p= 0.5). Again, no effect of ibrutinib was seen in CCR7-mediated migration of CLL cells towards CCL19 or CCL21 (**C**). The graph shows a comparative analysis of migration indices (% of input) in CLL cells obtained from naïve untreated patients (n=6) that where incubated for 24 h with ibrutinib at a final concentration of 0 (vehicle/DMSO) or 0.1 µM prior to exposure to CCR7 ligands CCL19 or CCL21 (1 µg/ml). Spontaneous migration, not mediated by a chemotactic stimulus, was considered as basal migration (in this point, no chemokine was added). As positive controls, cells without ibrutinib exposure where used (black bars). Bars represent mean ± standard error of the mean (SEM). ns, not significant; *, $p<0.05$.