Supplementary data

**Deciphering the complex circulating immune cell microenvironment in chronic lymphocytic leukemia using patient similarity networks**

**File summary**

File contents 6 Tables (S1-S2 Used surface markers; S3 Correlation analysis; S4-S6 Activation of immune cells in patients subgroups), 2 Figures (S1 Trend analysis for activation markers on immune cells across clusters; S2 comparative analysis), Comparison of immune cells activation during various treatment regimens, and Description of trends calculation based on network layout.

**Table S1**. List of surface markers used to characterise the immune populations.

|  |  |
| --- | --- |
| **Immune population** | **Combination of the markers** |
| CLL cells | CD5/CD19/CD20/CD27/CD38/CD49d/HLA-DR |
| Classical/intermediate/non-classical monocytes | CD11b/CD14/CD16/CD64/HLA-DR |
| Neutrophils | CD11b/CD15/CD16/CD54/CD62L/CD64 |
| CD4+ lymphocytes | CD3/CD4/HLA-DR |
| CD8+ lymphocytes | CD3/CD8/HLA-DR |
| Treg lymphocytes | CD3/CD4/CD25/CD127 |
| NK cells | CD3/CD16/CD56/CD69/HLA-DR |

**Table S2**. List of surface markers with relevant clone and fluorochrome (conjugate).

|  |  |  |
| --- | --- | --- |
| **Name** | **Clone** | **Conjugate** |
| CD3 | OKT3 | FITC |
| CD4 | RPA-T4 | APC-Cy7 |
| CD5 | UCHT2 | PerCP-Cy5.5 |
| CD8 | SK1 | PE-Cy7 |
| CD11b | ICRF44 | PerCP-Cy5.5 |
| CD14 | HCD14 | FITC |
| CD15 | W6D3 | PE-Cy7 |
| CD16 | 3G8 | PE |
| CD19 | SJ25C1 | APC-Cy7 |
| CD20 | 2H7 | PE |
| CD25 | M-A251 | PE |
| CD27 | M-T271 | FITC |
| CD38 | HB-7 | PE-Cy7 |
| CD49d | 9F10 | APC |
| CD54 | HA58 | FITC |
| CD62L | DREG-56 | APC |
| CD64 | 10.1 | APC-Cy7 |
| CD69 | FN50 | APC |
| CD86 | IT2.2 | PE |
| CD127 | A019D5 | PerCP-Cy5.5 |
| HLA-DR | L243 | APC |
| CD3/(CD16+CD56) | UCHT1/3G8+MEM-188 | FITC/PE |

**Table S3**. Correlations between absolute numbers of CLL cells and expression of activation markers on immune cell populations or percentages of immune cell subpopulations.

|  |  |  |
| --- | --- | --- |
| **Populations/markers correlated with absolute number of CLL cells** | **rs value** | ***P* value** |
| **Classical monocytes (MON)** |
| % of MON | -0.05 | 0.480 |
| HLA-DR | -0.76 | **< 0.001** |
| CD64 | -0.39 | **< 0.001** |
| CD11b | -0.11 | 0.120 |
| **Intermediate MON** |
| % of MON | -0.01 | 0.880 |
| HLA-DR | -0.74 | **< 0.001** |
| CD64 | -0.52 | **< 0.001** |
| CD11b | -0.07 | 0.330 |
| **Non-classical MON** |
| % of MON | 0.11 | 0.120 |
| HLA-DR | -0.78 | **< 0.001** |
| CD64 | -0.13 | 0.086 |
| CD11b | 0.18 | **0.014** |
| **NEU** |
| CD64 | -0.22 | **0.036** |
| CD54 | 0.10 | 0.310 |
| CD11b | -0.08 | 0.400 |
| CD62L | -0.57 | **< 0.001** |
| **NK cells** |
| HLA-DR | -0.11 | 0.270 |
| CD69 | -0.33 | **< 0.001** |
| **T cells** |
| CD4+/CD8+ ratio | 0.22 | **< 0.001** |
| HLA-DR on CD4+ cells | -0.51 | **< 0.001** |
| HLA-DR on CD8+ cells | -0.60 | **< 0.001** |
| % **Treg cells** of CD4+ cells | 0.31 | **< 0.001** |

**Table S4**. Comparison of immune subset percentages, cell counts and activation markers expressed on immune cells in untreated CLL patients and CLL patients after the immunochemotherapy treatment with the same CLL cell counts (20-80x109/L).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Populations/markers**  | **Untreated** ***Mean (CI)*** | **Chemotherapy *Mean (CI)*** | ***FC*** | ***P value*** |
| **CLL cell count [****x109/L]** | 44.0 (37.5-50.6) | 42.2 (32.3-51.9) | -0.07 | 0.683 |
| **Classical monocytes (MON)** |
| % of MON | 85.3 (82.6-88.0) | 82.6 (77.9-87.3) | -0.04 | 0.257 |
| Cell count [x109/L] | 0.46 (0.38-0.54) | 0.45 (0.30-0.60) | -0.02 | 0.880 |
| HLA-DR | 122 (101-142) | 172 (130-215) | 0.57 | **0.032** |
| CD64 | 229 (209-249) | 277 (221-333) | 0.20 | **0.044** |
| CD11b | 544 (462-625) | 604 (513-694) | 0.10 | 0.228 |
| **Intermediate MON** |
| % of MON | 5.32 (4.23-6.41) | 6.61 (5.14-8.08) | 0.36 | 0.130 |
| Cell count [x109/L] | 0.03 (0.02-0.03) | 0.03 (0.02-0.05) | 0.31 | 0.103 |
| HLA-DR | 792 (554-1031) | 1034 (645-1423) | 0.24 | 0.133 |
| CD64 | 200 (174-227) | 258 (191-326) | 0.19 | 0.061 |
| CD11b | 444 (373-515) | 573 (485-661) | 0.35 | 0.050 |
| **Non-classical MON** |
| % of MON | 9.38 (7.36-11.4) | 10.8 (6.32-15.2) | 0.21 | 0.645 |
| Cell count [x109/L] | 0.04 (0.03-0.05) | 0.05 (0.03-0.06) | 0.17 | 0.250 |
| HLA-DR | 285 (222-348) | 519 (304-734) | 0.38 | **0.030** |
| CD64 | 146 (135-158) | 170 (133-208) | 0.01 | 0.615 |
| CD11b | 89.9 (77.9-102) | 116 (94.6-138) | 0.19 | **0.041** |
| **NEU** |
| Cell count [x109/L] | 5.38 (4.33-6.43) | 4.26 (3.47-5.05) | -0.21 | 0.335 |
| CD64 | 69.5 (58.9-80.1) | 80.9 (74.0-87.8) | 0.04 | 0.162 |
| CD54 | 20.4 (17.4-23.5) | 19.0 (16.8-21.2) | -0.10 | 0.708 |
| CD11b | 178 (124-232) | 159 (123-196) | -0.18 | 0.615 |
| CD62 | 275 (201-350) | 306 (139-472) | 0.03 | 0.965 |
| **NK cells** |
| Cell count [x109/L] | 0.48 (0.33-0.63) | 0.34 (0.24-0.43) | -0.30 | 0.254 |
| HLA-DR | 83.8 (5.17-173) | 42.7 (6.55-78.8) | -0.15 | 0.958 |
| CD69 | 29.1 (23.5-34.7) | 25.1 (21.9-28.3) | -0.02 | 0.792 |
| **T cells** |
| Cell count [x109/L] | 2.89 (2.41-3.36) | 2.75 (1.90-3.60) | -0.05 | 0.660 |
| CD4+/CD8+ ratio | 2.02 (1.57-2.47) | 1.04 (0.75-1.34) | -0.31 | **0.008** |
| HLA-DR on CD4+ cells | 19.2 (17.7-20.7) | 27.8 (22.5-33.1) | 0.25 | **< 0.001** |
| HLA-DR on CD8+ cells | 25.2 (22.0-28.4) | 36.1 (27.2-45.1) | 0.12 | **0.013** |
| **% Treg** **cells** of CD4+ cells | 10.7 (9.19-12.2) | 10.7 (8.39-13.0) | 0.01 | 0.949 |
| Cell count [x109/L] | 0.14 (0.10-0.18) | 0.09 (0.07-0.12) | -0.33 | 0.278 |

**Table S5**. Comparison of immune subset percentages and activation markers expressed on immune cells in CLL patients after the immunochemotherapy treatment and patients on novel therapy with the equal CLL cell counts (<10.0x109/L).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Populations/markers**  | **Chemotherapy *Mean (CI)*** | **Novel drugs *Mean (CI)*** | ***FC*** | ***P value*** |
| **CLL cell count [x109/L]** | 2.21 (0.96-3.46) | 1.37 (0.85-1.89) | -0.32 | 0.389 |
| **Classical monocytes (MON)** |
| % of MON | 77.4 (71.8-83.0) | 82.2 (79.1-85.3) | 0.07 | **0.038** |
| Absolute count [x109/L] | 0.27 (0.19-0.35) | 0.33 (0.28-0.39) | 0.23 | 0.212 |
| HLA-DR | 274 (211-337) | 357 (302-411) | 0.32 | 0.099 |
| CD64 | 338 (287-390) | 352 (315-389) | -0.06 | 0.914 |
| CD11b | 689 (557-820) | 744 (656-833) | 0.11 | 0.503 |
| **Intermediate MON** |
| % of MON | 10.4 (7.94-12.9) | 8.84 (7.37-10.3) | -0.34 | 0.207 |
| Cell count [x109/L] | 0.03 (0.02-0.04) | 0.04 (0.03-0.04) | 0.23 | 0.666 |
| HLA-DR | 1517 (1042-1992) | 1998 (1756-2240) | 0.50 | **0.012** |
| CD64 | 313 (253-373) | 329 (296-632) | 0.12 | 0.379 |
| CD11b | 580 (468-692) | 597 (536-658) | 0.11 | 0.532 |
| **Non-classical MON** |
| % of MON | 12.2 (8.25-16.1) | 8.03 (6.36-9.70) | -0.23 | **0.046** |
| Cell count [x109/L] | 0.03 (0.02-0.04) | 0.03 (0.02-0.03) | -0.17 | 0.419 |
| HLA-DR | 778 (510-1045) | 1011 (823-1198) | 0.53 | **0.028** |
| CD64 | 162 (144-180) | 157 (147-167) | -0.04 | 0.416 |
| CD11b | 108 (77.3-139) | 116 (103-129) | 0.33 | 0.093 |
| **NEU** |
| Cell count [x109/L] | 3.02 (2.24-3.79) | 3.05 (2.59-3.51) | 0.01 | 0.907 |
| CD64 | 89.1 (77.0-101) | 95.6 (84.3-107) | 0.02 | 0.439 |
| CD54 | 18.5 (15.9-21.2) | 21.0 (18.3-23.8) | 0.10 | 0.225 |
| CD11b | 145 (96.1-194) | 179 (144-214) | 0.12 | 0.247 |
| CD62L | 452 (278-626) | 548 (449-648) | 0.01 | 0.288 |
| **NK cells** |
| Cell count [x109/L] | 0.17 (0.12-0.21) | 0.17 (0.12-0.22) | 0.03 | 0.577 |
| HLA-DR | 62.4 (34.2-90.5) | 10.8 (31.6-110) | -0.27 | 0.267 |
| CD69 | 46.8 (30.9-62.0) | 35.3 (30.3-40.3) | -0.25 | 0.538 |
| **T cells** |
| Cell count [x109/L] | 1.34 (0.95-1.76) | 1.64 (1.27-2.00) | 0.21 | 0.496 |
| CD4+/CD8+ ratio | 1.32 (0.79-1.85) | 1.05 (0.80-1.30) | -0.23 | 0.317 |
| HLA-DR on CD4+ cells | 29.6 (24.5-34.6) | 31.4 (26.5-36.3) | -0.06 | 0.701 |
| HLA-DR on CD8+ cells | 45.2 (36.9-53.4) | 48.1 (41.9-54.3) | 0.13 | 0.629 |
| % **Treg cells** of CD4+ cells | 12.0 (9.23-14.8) | 6.70 (5.50-7.89) | -0.45 | **< 0.001** |
| Cell count [x109/L] | 0.07 (0.04-0.11) | 0.04 (0.03-0.05) | -0.43 | 0.062 |

**Table S6**. Comparison of immune subset percentages and activation markers expressed on immune cells in CLL patients treated with ibrutinib or idelalisib.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Populations/markers**  | **ibrutinib*****Mean (CI)*** | **Idelalisib** ***Mean (CI)*** | ***FC*** | ***P value*** |
| **CLL cell count [x109/L]** | 4.98 (2.23-7.73) | 2.52 (0.46-4.58) | -0.12 | 0.324 |
| **Classical monocytes (MON)** |
| % of MON | 83.1 (79.1-87.0) | 80.5 (76.6-84.4) | -0.04 | 0.065 |
| Cell count [x109/L] | 0.35 (0.27-0.44) | 0.29 (0.20-0.38) | -0.18 | 0.402 |
| HLA-DR | 339 (270-408) | 274 (289-459) | 0.08 | 0.287 |
| CD64 | 339 (300-379) | 408 (331-486) | 0.33 | 0.118 |
| CD11b | 753 (674-832) | 762 (551-974) | 0.03 | 0.936 |
| **Intermediate MON** |
| % of MON | 7.79 (6.35-9.23) | 11.3 (8.71-13.9) | 0.92 | **0.002** |
| Cell count [x109/L] | 0.03 (0.02-0.04) | 0.04 (0.02-0.07) | 0.29 | 0.568 |
| HLA-DR | 1676 (1372-1980) | 2163 (1788-2537) | 0.51 | **0.007** |
| CD64 | 308 (269-348) | 368 (306-430) | 0.29 | **0.018** |
| CD11b | 600 (533-667) | 590 (471-709) | -0.02 | 0.857 |
| **Non-classical MON** |
| % of MON | 7.10 (5.43-8.77) | 8.24 (5.71-10.8) | -0.01 | 0.375 |
| Cell count [x109/L] | 0.03 (0.02-0.03) | 0.03 (0.02-0.04) | 0.01 | 0.878 |
| HLA-DR | 949 (698-1201) | 931 (803-1059) | 0.12 | 0.364 |
| CD64 | 153 (141-165) | 168 (149-187) | 0.10 | 0.087 |
| CD11b | 125 (109-141) | 99.7 (86.1-113) | -0.14 | 0.075 |
| **NEU** |
| Cell count [x109/L] | 3.19 (2.65-3.73) | 2.50 (1.58-3.43) | -0.21 | 0.069 |
| CD64 | 96.0 (82.6-109) | 95.7 (73.7-118) | -0.06 | 0.871 |
| CD54 | 21.5 (17.2-25.9) | 21.3 (18.2-24.3) | 0.15 | 0.258 |
| CD11b | 163 (132-193) | 179 (94.7-263) | 0.17 | 0.910 |
| CD62L | 519 (390-648) | 547 (372-721) | 0.41 | 0.377 |
| **NK cells** |
| Cell count [x109/L] | 0.14 (0.11-0.18) | 0.20 (0.11-0.29) | 0.38 | 0.444 |
| HLA-DR | 47.2 (27.6-66.8) | 82.2 (0.79-165) | 0.05 | 0.685 |
| CD69 | 38.0 (28.7-47.3) | 45.4 (16.8-74.0) | -0.09 | 0.904 |
| **T cells** |
| Cell count [x109/L] | 1.61 (1.13-2.09) | 1.97 (1.12-2.82) | 0.22 | 0.299 |
| CD4+/CD8+ ratio | 0.92 (0.75-1.09) | 0.96 (0.37-1.55) | -0.36 | 0.255 |
| HLA-DR on CD4+ cells | 30.7 (25.7-35.7) | 30.8 (21.0-40.6) | -0.04 | 0.618 |
| HLA-DR on CD8+ cells | 45.8 (37.8-53.9) | 47.7 (37.9-57.5) | 0.15 | 0.488 |
| % **Treg cells** of CD4+ cells | 7.50 (6.07-8.93) | 6.80 (4.12-9.48) | -0.22 | 0.256 |
| Cell count [x109/L] | 0.05 (0.03-0.06) | 0.03 (0.02-0.05) | -0.27 | 0.146 |

**Description of trends based on network layout**

Network layouts in Fig. 3 can also be interpreted as the ordering of individual vertices (patients) in vertical (from bottom to top on the y-axis) and horizontal (from left to right on the x-axis) direction. We utilised the horizontal ordering to create scatter plots (Fig. S1) for each attribute. Subsequently, we interpolated the relationship between normalised attribute values and the order of individual patients from left to right in the network layout by a linear trend. The trend illustrates a correlation between patient placement in the network and a selected attribute.



**Figure S1**. Trend analysis of HLA-DR expression on monocyte subsets (MON), CD4+ lymphocytes, CD8+ lymphocytes, and NK cells in CLL patients across clusters in the patient similarity network layout (Figure 3). Each dot represents the value of an individual patient. Values of all parameters were normalised to a maximum of 1. Individual lines then show the linear trend of each parameter.

***Regardless of CLL cell count, the activation of circulating immune cells is dependent on the treatment regimen***

To recognise the differences in circulating cells between the groups of patients with different treatment regimens, we compared studied parameters in patients with the comparable levels of CLL cells to reduce the impact of “CLL cell number” on studied parameters. We compared untreated patients (n=36) with treated patients with chemotherapy in the past (n=18) with CLL cell count from 20.0 to 80.0x109 CLL cells/L and previously treated patients with immunochemotherapy (n=22) with patients treated with the novel drugs (n=53) with CLL cell count less than 10.0x109 CLL cells /L. The untreated patient group was not compared with the group of patients treated with novel drugs because CLL cell number in both groups was mostly incomparable.

Comparison of treatment-naïve patients with the patients after chemotherapy (Table S4) revealed higher activation of immune cells in patients after chemotherapy. Particularly, the expression of HLA-DR on CD4+ (*P<*0.001) and CD8+ lymphocytes (*P*=0.013), classical (*P*=0.032) and non-classical (*P*=0.030) subsets of monocytes, as well as expression of CD64 on classical monocytes (*P*=0.044) and CD11b on non-classical monocytes (*P*=0.041) were lower in the untreated group. CD4+/CD8+ ratio was found to be higher in untreated patients (*P*=0.008).

When we compared the patients with passed chemotherapy treatment with the patients on novel drug therapy, higher activation of immune cells was observed in patients treated with novel drugs (Table S5).



**Figure S2**. Difference in HLA-DR expression on intermediate monocytes (MON) between CLL patients with lower and higher numbers of intermediate monocytes (cut-off 5.4%).