**Supplementary Methods**

**Description of the biomarkers used to determine the immune cells in the microenvironment of HL:**

To study the type and proportion of the cells in the microenvironment of HL and to compare the cellular component of HL between EBV-related and EBV-unrelated cases, we used a panel of immunomarkers. TMA sections were stained with antibodies against: CD20 (B-cell marker), CD3 (T-cell marker), CD4 (non-cytotoxic cell marker), CD8 (cytotoxic T-cell marker), FOXP3 (Treg), T-bet (Th-1), CMAF (Th-2) and PD1. The immune-stained cells were then quantified using HALO image analysis software and the proportion of each cell in the microenvironment of HL cells were estimated.

**Quantification of IHC stained immune cells in the microenvironment of HL**

The IHC stained immune cells were counted on TMA. Four cores of 1 mm2 were prepared for each sample. We added two cores of two reactive tonsils for quality control at each batch of TMA. The tissue material (whole sections and TMA sections) was assessed by two researchers and one hematopathologist with consultation from a senior hematopathologist. We selected valid cores with high number of tumor cells for quantification, areas with fibrosis were excluded, as well as cases with poor quality cores. The automated quantification of positive immune cells was performed by HALO 2.3, a platform for image analysis from Indica Lab Inc©.

For the HALO platform analysis, the images of the stained TMA were prepared using high quality Philips image scanning technique at a magnification of x20. The scanned images were loaded in a computer with HALO software at the pathology department at Skane University Hospital in Malmo, Sweden. The cytonuclear v1.6 algorithm was used to count the membranous and nuclear markers. The software was trained to differentiate between the membrane and nuclear staining of the cells in the TME, and adjusted for the cellular parameters and annotation of the area to be scored 1. The threshold for each core was set based on the pixel´s value. The quantified data by HALO software was extracted in CSV format containing raw data for each core, then converted into XL format, containing data on total cells/mm2, biomarker positive cells/mm2, percentage of biomarker positive cells/mm2, average cell area/µm², average cytoplasmic area/µm² and average nuclear area/µm² of the quantified cells/mm2 for each core.

The mean of cells count/mm2 along with their Std. Errors, Std. Deviations and ranges are shown in Table SII. In addition, the median, range, and percentile of the cell count and percentage/mm2 of the immune cells expressing the tested biomarkers in the TME of cHL cases are shown in Tables SII A & B. The mean of the average cell area/ µm² for the scored immune cells using the HALO image analysis platform was 53 µm², and the std. Error and std. Deviation of the mean were 0.47 and 4.1, respectively, and a range of (42-67).

**Supplementary Tables**

**Table S1 EBV association with clinicopathological characteristics of HL in Ethiopia**

|  |  |  |
| --- | --- | --- |
| **Characteristics** | **LMP1/EBER in tumor cells N (%)** | ***P\**** |
| **EBV infection** |  | 0.013 |
| Positive cases | 77 (61.1) |  |
| Negative cases | 49 (38.9) |  |
| **Age groups (years)** |  | 0.020 |
| 0-14 (N = 42) | 34 (44.2) |  |
| 15-24 (N = 41) | 21 (27.3) |  |
| 25-34 (N = 17) | 10 (13) |  |
| 35-44 (N = 7) | 4 (5.2) |  |
| ≥45 (N = 18) | 8 (10.4) |  |
| **Sex** |  | 0.012 |
| Male (N = 91) | 64 (83.1) |  |
| Female (N = 31) | 13 (16.9) |  |
| **Stage of the disease** |  | 0.261 |
| Stage I (N = 32) | 24 (75) |  |
| Stage II (N = 48) | 29 (60.4) |  |
| Stage III (N = 25) | 14 (56) |  |
| Stage IV (N = 15) | 8 (53.3) |  |
| **HL subtypes** |  | <0.001 |
| MCCHL (65) | 48 (62.3) |  |
| NSCHL (40) | 23 (29.9) |  |
| Others (11) | 6 (7.8) |  |
| NLPHL (10) | 0 |  |

*\**Chi-Squar (*x2*) or Fisher exact test as appropriate

**Table SII The Description of the quantified immune cells biomarker in the tumor microenvironment of HL by HALO image analysis software**

1. **Description of the biomarker in the TME of all cases (NLPHL & CHL cases)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Biomarker (N) | Total cells count/mm2 | | Positive cells count/mm2 | |
| Mean (SE) | Std deviation (range) | Mean (SE) | Std. deviation (Range) |
| CD20+ (126) | 7961 (390) | 4386 (141-17135) | 2319 (214) | 2406 (1-11984) |
| CD3+ (120) | 12019 (264) | 2895 (4613-21078) | 9163 (338) | 3707 (747-16310) |
| CD4+ (120) | 12635 (308) | 3381 (4460-22671) | 6654 (431) | 4725 (17-19809) |
| CD8+ (120) | 11742 (286) | 3134 (4121-22043) | 3738 (263) | 2882 (109-12870) |
| FOXP3+ (120) | 12458 (270) | 2962 (3723-20801) | 1181 (90) | 988 (27-4792) |
| T-bet+ (119) | 12501 (277) | 3029 (4868-19966) | 2669 (200) | 2190 (14-9295) |
| C-maf + (118) | 12162 (273) | 2971 (3734-19460) | 3227 (171) | 1863 (228-10891) |
| PD1+ (113) | 12731 (296) | 3156 (2906-19976) | 2051 (269) | 1. (0-15872) |

1. **Description of the biomarkers in the TME of CHL**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Biomarker (N) | Positive cells count /mm2 | | Percentile of cells count/mm2 | | |
| Median | Range | 25% | 50% | 75% |
| CD20+ (N = 112) | 1447 | 1-9721 | 311 | 1447 | 3730 |
| CD3+ (N = 107) | 9031 | 747-15486 | 6714 | 9031 | 11606 |
| CD4+ (N = 107) | 5343 | 17-18683 | 2230 | 5343 | 10357 |
| CD8+ (N = 107) | 3119 | 109-12870 | 1366 | 3119 | 5030 |
| FOXP3+ (N = 107) | 941 | 66-4792 | 479 | 941 | 1938 |
| T-bet+ (N=106) | 2289 | 14-9295 | 827 | 2289 | 4175 |
| C-maf +(N=105) | 2778 | 228-10891 | 1818 | 2778 | 4496 |
| PD1+ (N=102) | 621 | 0-9337 | 208 | 621 | 2254 |

1. **Description of the expressed biomarkers in the TME of CHL**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Biomarker (N) | Percent of positive cells /mm2 | | Percentile | | |
| Median | Range | 25% | 50% | 75% |
| CD20+ (112) | 29 | 0-99 | 5.6 | 28 | 72.9 |
| CD3+ (107) | 79.6 | 15-99 | 63.7 | 79.6 | 90 |
| CD4+ (107) | 52 | 0-99 | 20.2 | 52.6 | 75 |
| CD8+ (107) | 27.9 | 1-95 | 11.9 | 27.9 | 46.5 |
| FOXP3+ (107) | 8.8 | 1-35 | 3.8 | 8.8 | 15 |
| T-bet+ (106) | 18.7 | 0-77 | 8 | 18.7 | 34.7 |
| C-maf + (105) | 24.4 | 1-78 | 16.6 | 24.4 | 37 |
| PD1+ (102) | 5 | 0-64 | 1.6 | 5 | 16.6 |

**Table SIII the difference between the proportion/mm2 of biomarkers expression in the TME of HL cases (all cases)**

|  |  |  |
| --- | --- | --- |
| Biomarker | Cell count/mm2 | Percent/mm2 |
|  | P\* | P |
| CD3 vs CD20 | <0.001 | 0.036 |
| CD4 vs CD8 | <0.001 | <0.001 |
| C-maf vs T-bet | 0.029 | 0.004 |

\*Wilcoxon Signed Rank test

**Table SIV Correlation of biomarkers expressed in the cells of the TME of the HL cases**

1. **Correlations between the inhibitory checkpoint marker expression and the T-cell biomarkers in the TME of HL (all cases)**

|  |  |  |  |
| --- | --- | --- | --- |
| Biomarkers | Spearman’s rho | *P* value | 95% CI |
| PD1 with CD4 | 0.497 | <0.001 | 0.34-0.62 |
| PD1 with CD8 | -0.032 | 0.735 | -0.22-0.16 |
| PD1 with FOXP3 | -0.289 | 0.002 | -0.45—0.1 |
| PD1 with T-bet | -0.038 | 0.876 | -0.23-.015 |
| PD1 with C-maf | 0.195 | 0.039 | 0.005-0.37 |

1. **Correlations between the T-reg marker (FOXP3) expression with the expression of CD4+ T-subsets and CD8 biomarkers on the cells of the TME of HL**

|  |  |  |  |
| --- | --- | --- | --- |
| Biomarkers | Spearman’s rho | P value | 95% CI |
| FOXP3 with CD8 | 0.225 | 0.014 | 0.04-0.39 |
| FOXP3 with T-bet | 0.235 | 0.01 | 0.052-0.403 |
| FOXP3 with C-maf | 0.177 | 0.056 | -0.01-0.35 |

1. **Correlations between the CD8-expression with the expression of T-bet biomarker on the cells of the TME of HL**

|  |  |  |  |
| --- | --- | --- | --- |
| Biomarker | Spearman’s rho | P value | 95% CI |
| C8 with T-bet | 0.396 | <0.001 | 0.22-0.54 |

**Table SV The association of biomarkers with the two subtypes of HL**

1. **The mean differences of immune cells biomarkers count/mm2 between HL types**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Immune marker (N) | Mean (Std. error) | *t* (95% CI), *P\** | *P\*\** |  |
| **CD20** |  | 0.9 (-892-2250), 0.4 | 0.5 |  |
| NLPHL (10) | 2644 (1129) |  |  |  |
| CHL (116) | 2265 (212) |  |  |  |
| **CD3** |  | 2.4 (517-5507), 0.018 | 0.02 |  |
| NLPHL (9) | 11950 (1127) |  |  |  |
| CHL (111) | 8937 (346) |  |  |  |
| **CD4** |  | 2 (148-6547), 0.04 | 0.045 |  |
| NLPHL (9) | 9761 (1644) |  |  |  |
| CHL (111) | 6413 (440) |  |  |  |
| **CD8** |  | 0.6 (-1372-2595), 0.5 | 0.7 |  |
| NLPHL (9) | 3142 (700) |  |  |  |
| CHL (111) | 3754 (278) |  |  |  |
| **FOXP3** |  | 6.2 (618-1235), <0.001 | <0.001 |  |
| NLPHL (9) | 324 (114) |  |  |  |
| CHL (111) | 1251 (94) |  |  |  |
| **T-bet** |  | 1.2 (-619-2383), 0.25 | 0.28 |  |
| NLPHL (9) | 1853 (513) |  |  |  |
| CHL (110) | 2735 (212) |  |  |  |
| **C-maf** |  | 0.5 (-952-1615), 0.6 | 0.5 |  |
| NLPHL (9) | 3534 (672) |  |  |  |
| CHL (109) | 3202 (177) |  |  |  |
| **PD1** |  | 4 (2848-11042), 0.006 | <0.001 |  |
| NLPHL (7) | 8567 (1673) |  |  |  |
| CHL (106) | 1621 (208) |  |  |  |

\*The mean differences analyzed using independent t-test

\*\* Non-parametric Mann-Whitney test

1. The mean differences of immune cells biomarkers percentage/mm2 between HL types

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Immune marker (N) | Mean (Std. error) | *t* (95% CI), *P\** | *P\*\** |  |
| **CD20** |  | 1.8 (-2-42), 0.07 | 0.07 |  |
| NLPHL (10) | 57 (10) |  |  |  |
| CHL (116) | 37 (3) |  |  |  |
| **CD3** |  | 1.3 (-4-23), 0.19 | 0.18 |  |
| NLPHL (9) | 82 |  |  |  |
| CHL (111) | 73 (2) |  |  |  |
| **CD4** |  | 1.3 (-6-35), 0.17 | 0.19 |  |
| NLPHL (9) | 64 (7) |  |  |  |
| CHL (111) | 49 (2) |  |  |  |
| **CD8** |  | 1.2 (-6-24), 0.2 | 0.2 |  |
| NLPHL (9) | 22 (5) |  |  |  |
| CHL (111) | 32 (2) |  |  |  |
| **FOXP3** |  | 6.8 (5-10), <0.001 | <0.001 |  |
| NLPHL (9) | 2 (0.9) |  |  |  |
| CHL (111) | 10 (0.7) |  |  |  |
| **T-bet** |  | 1.6 (-1-21), 0.1 | 0.09 |  |
| NLPHL (9) | 12 (3) |  |  |  |
| CHL (110) | 22 (1) |  |  |  |
| **C-maf** |  | 0.8 (-5-13), 0.4 | 0.5 |  |
| NLPHL (9) | 23 |  |  |  |
| CHL (109) | 27 (1) |  |  |  |
| **PD1** |  | 6.5 (29-54), <0.001 | <0.001 |  |
| NLPHL (7) | 54 (8) |  |  |  |
| CHL (106) | 12 (1) |  |  |  |

\*The mean differences analyzed using independent t-test

\*\* Non-parametric Mann-Whitney test

**Table SVI Association of the clinical characteristics of HL with the expression of the biomarkers in the TME of HL cases (both NLPHL and CHL cases)**

|  |  |  |
| --- | --- | --- |
| **Biomarker/clinical characteristic** | **Adj OR (95% CI), *P* \*** | |
| **Biomarker count/mm2** | **Biomarker percent/mm2** |
| **CD3 proportion** |  |  |
| HL classes\*\* | 2.4 (503-5649), 0.02 | 1.4 (-4-24), 0.16 |
| Age | 0.13 (-44-50), 0.9 | -0.5 (-0.3-0.2),0.6 |
| Sex | 0.6 (-1090-2135), 0.5 | -0.4 (-11-7), 0.7 |
| **CD4 proportion** |  |  |
| HL classes | 2 (46-6601), 0.047 | 1.4 (-6-36), 0.16 |
| Age | 0.6 (-43-77), 0.6 | 0.2 (-0.35-0.42), 0.8 |
| Sex | 1.2 (-856-3302), 0.2 | 1.01 (-6-20), 0.3 |
| **CD8 proportion** |  |  |
| HL classes | -0.5 (-2488-1458), 0.6 | -1.1 (-24-6), 0.3 |
| Age | -0.3 (-42-30), 0.8 | -0.3 (-0.3-0.23), 0.7 |
| Sex | 1.7 (-2264-203), 0.1 | 2 (0.08-19), 0.05 |
| **FOXP3 proportion** |  |  |
| HL classes | -2.9 (-1642-(-304)), 0.005 | -3.2 (-13-(-3)), 0.002 |
| Age | 1.09 (-5-19), 0.2 | 1.3 (-0.03-0.16), 0.2 |
| Sex | 1.5 (-93-743), 0.1 | 1.5 (-0.78-5), 0.13 |
| **T-bet proportion** |  |  |
| HL classes | -1.3 (-2576-510), 0.18 | -1.9 (-22-0.75), 0.07 |
| Age | 1.3 (-10-46),0.2 | 1.4 (-0.06-0.37), 0.16 |
| Sex | 0.8 (-581-1377), 0.4 | 1.16 (-3-11), 0.2 |
| **C-maf proportion** |  |  |
| HL classes | 0.12 (-1221-1380), 0.9 | -1.2 (-16-3), 0.2 |
| Age | 2.2 (2-50), 0.03 | 2.09 (0.01-0.38), 0.04 |
| Sex | 0.08 (-791-860), 0.9 | 0.37 (-5-7), 0.7 |
| **PD1 proportion** |  |  |
| HL cases | 7.3 (4918-8588), <0.001 | 6.2 (27-54), <0.001 |
| Age | 1.5 (-7-55), 0.13 | 1.3 (-0.08-0.37), 0.2 |
| Sex | 0.12 (-1012-1145), 0.9 | -0.7 (-10-4), 0.5 |

\*Linear regression

\*\*HL classes are the CHL and NLPHL

**Table SVII The association of biomarkers expression on the TME immune cells with EBV-related and EBV-unrelated CHL cases,**

1. **count of cells expressing biomarkers/mm2**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Immune marker (N)** | **Mean (Std. error)** | ***t* (95% CI), *P\**** | ***P\*\**** |  |
| **CD20** |  |  |  |  |
| **LMP1/EBER expression** |  | 1.3 (-297-1521), 0.2 | 0.07 |  |
| LMP1 positive (73) | 2493 (274) |  |  |  |
| LMP1 negative (39) | 1881 (359) |  |  |  |
| **CD3** |  |  |  |  |
| **LMP1/EBER expression** |  | 0.8 (-844-2072), 0.4 | 0.5 |  |
| LMP1 positive (70) | 9145 (414) |  |  |  |
| LMP1 negative (37) | 8531 (639) |  |  |  |
| **CD4** |  |  |  |  |
| **LMP1/EBER expression** |  | 0.5 (-1462-2323), 0.7 | 0.7 |  |
| LMP1 positive (71) | 6234 (538) |  |  |  |
| LMP1 negative (36) | 6656 (819) |  |  |  |
| **CD4:CD8** |  |  |  |  |
| **LMP1/EBER** |  | 3.4 (2-7), 0.001 | 0.002 |  |
| LMP1 positive (70) | 2.2 (0.3) |  |  |  |
| LMP1 negative (36) | 7.2 (1.9) |  |  |  |
| **CD8** |  |  |  |  |
| **LMP1/EBER expression** |  | 3.1 (623-2923), 0.003 | <0.001 |  |
| LMP1 positive (70) | 4334 (350) |  |  |  |
| LMP1 negative (37) | 2560 (444) |  |  |  |
| **FOXP3** |  |  |  |  |
| **LMP1/EBER expression** |  | 2.5 (100-899), 0.015 | 0.018 |  |
| LMP1 positive (71) | 1391 (126) |  |  |  |
| LMP1 negative (36) | 997 (136) |  |  |  |
| **T-bet** |  |  |  |  |
| **LMP1/EBER expression** |  | 2.9 (451-2182), 0.003 | <0.001 |  |
| LMP1 positive (71) | 3164 (265) |  |  |  |
| LMP1 negative (35) | 1847 (343) |  |  |  |
| **C-maf** |  |  |  |  |
| **LMP1/EBER expression** |  | 1.5 (-197-1339), 0.1 | 0.1 |  |
| LMP1 positive (70) | 3024 (204) |  |  |  |
| LMP1 negative (35) | 3595 (364) |  |  |  |
| **PD1** |  |  |  |  |
| **LMP1/EBER expression** |  | 3.08 (354-2375), 0.001 | 0.001 |  |
| LMP1 positive (70) | 1199 (210) |  |  |  |
| LMP1 negative (32) | 2564 (596) |  |  |  |

\*The mean differences analyzed using independent t-test

\*\* Non-parametric Mann-Whitney test

1. **percent of cells expressing biomarkers out of the total cells/mm2**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Immune marker (N)** | **Mean (Std. error)** | ***t* (95% CI), *P\**** | ***P\*\**** |  |
| **CD20** |  |  |  |  |
| **LMP1/EBER expression** |  | 0.6 (-6-17), 0.6 | 0.4 |  |
| LMP1 positive (73) | 39 (3) |  |  |  |
| LMP1 negative (39) | 35 (5) |  |  |  |
| **CD3** |  |  |  |  |
| **LMP1/EBER expression** |  | 0.7 (-5-12), 0.4 | 0.8 |  |
| LMP1 positive (70) | 74 (2) |  |  |  |
| LMP1 negative (37) | 71 |  |  |  |
| **CD4** |  |  |  |  |
| **LMP1/EBER expression** |  | 0.5 (-9-15), 0.6 | 0.5 |  |
| LMP1 positive (71) | 48 (3) |  |  |  |
| LMP1 negative (36) | 51 (5) |  |  |  |
| **CD8** |  |  |  |  |
| **LMP1/EBER expression** |  | 3.3 (6-23), 0.001 | <0.001 |  |
| LMP1 positive (70) | 37 (2) |  |  |  |
| LMP1 negative (37) | 22 (3) |  |  |  |
| **FOXP3** |  |  |  |  |
| **LMP1/EBER expression** |  | 2.2 (0.3-6), 0.03 | 0.006 |  |
| LMP1 positive (75) | 11 (0.9) |  |  |  |
| LMP1 negative (45) | 8 (1) |  |  |  |
| **T-bet** |  |  |  |  |
| **LMP1/EBER expression** |  | 4 (6-17), <0.001 | <0.001 |  |
| LMP1 positive (75) | 25 (1) |  |  |  |
| LMP1 negative (44) | 14 (2) |  |  |  |
| **C-maf** |  |  |  |  |
| **LMP1/EBER expression** |  | 1.7 (-0.7-9), 0.08 | 0.1 |  |
| LMP1 positive (74) | 25 (1) |  |  |  |
| LMP1 negative (44) | 30 (2) |  |  |  |
| **PD1** |  |  |  |  |
| **LMP1/EBER expression** |  | 3.9 (7-24), <0.001 | <0.001 |  |
| LMP1 positive (74) | 9 (1) |  |  |  |
| LMP1 negative (39) | 25 (3) |  |  |  |

\*The mean differences analyzed using independent t-test

\*\* Non-parametric Mann-Whitney test

References

1. Koelzer VH, Sirinukunwattana K, Rittscher J, et al: Precision immunoprofiling by image analysis and artificial intelligence. Virchows Archiv 474:511-522, 2019