LETTER TO THE EDITOR

Response to DA-EPOCH-R is associated with activation of ‘fitter’ cytotoxic T cells in patients with newly diagnosed double and triple hit high-grade B-cell lymphoma

Authors

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https://doi.org/10.3324/haematol.2024.285170

SUPPLEMENTS

Response to DA-EPOCH-R is associated with activation of 'fitter' cytotoxic T-cells in patients with newly diagnosed double and triple hit high-grade B-cell lymphoma

Running title: Immune effector cells during treatment of HGBL

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Supplementary files:

- Supplementary Table 1 & 2
- Legend Supplementary Figure 1
- Supplementary Figure 1

Supplementary Table S1

Baseline characteristics of newly diagnosed high grade B-cell lymphoma patients stratified for achieving complete metabolic remission (CMR) after treatment with DA-EPOCH-R (left columns) and all patients (right column). Statistical tests used for group comparisons include the Pearson $\chi 2$ test and the Kruskall–Wallis test for categorical and continuous variables, respectively. P-values < 0.05 are considered statistically significant.

	CMR (N=40)	**No CMR** (N=30)	**P-value**	**All patients** (N=70)
Age (years)				
Mean (SD)	59.3 (10.5)	61.5 (9.16)	0.35	60.2 (9.93)
Median [Min, Max]	60.0 [35.0, 79.0]	62.5 [36.0, 78.0]		62.0 [35.0, 79.0]
Sex				
Male	27 (67.5%)	19 (63.3%)	0.91	46 (65.7%)
Female	13 (32.5%)	11 (36.7%)		24 (34.3%)
WHO Performance score				
0	29 (72.5%)	15 (50.0%)	0.03	44 (62.9%)
1	11 (27.5%)	11 (36.7%)		22 (31.4%)
2	0 (0%)	4 (13.3%)		4 (5.7%)
3	0 (0%)	0 (0%)		0 (0%)
LDH				
Within reference range	17 (42.5%)	6 (20.0%)	0.002	23 (32.9%)
Elevated	23 (57.5%)	24 (80.0%)		47 (67.1%)
Extranodal localisations (no.)				
None	17 (42.5%)	12 (40.0%)	0.62	29 (41.4%)
1	9 (22.5%)	5 (16.7%)		14 (20.0%)
2 or more	14 (35.0%)	12 (40.0%)		26 (37.1%)
Unknown	0 (0%)	1 (3.3%)		1 (1.4%)
IPI-score				
Low	7 (17.5%)	3 (10.0%)	0.02	10 (14.3%)
Low-intermediate	21 (52.5%)	8 (26.7%)		29 (41.4%)
High-intermediate	8 (20.0%)	17 (56.7%)		25 (35.7%)
High	4 (10.0%)	2 (6.7%)		6 (8.6%)
DH/TH status				
BCL2 DH	31 (77.5%)	18 (60.0%)	0.18	49 (70.0%)
BCL6 DH	4 (10.0%)	3 (10.0%)		7 (10.0%)
TH	5 (12.5%)	9 (30.0%)		14 (20.0%)
COO classification (Hans)				
GCB	31 (77.5%)	28 (93.5%)		59 (84.3%)
Non-GCB	4 (10%)	2 (6.7%)		4 (5.7%)
Unknown (poor morphology)	5 (12.5%)	0		7 (10%)

Supplementary Table S2.
Flow-cytometric phenotyping of cryopreserved PBMCs and the number of patient samples measured for each flow-cytometry panel per time point.

Human normal immunoglobulins (final concentration 0.1 mg/ml, Nanogram, Sanquin Plasma Products B.V.) were added before staining to reduce unwanted non-specific FC-receptor binding of fluorochrome-conjugated antibodies. Spectral overlap was automatically calculated and compensated using compensation beads and FACSDiva™ software. Flow cytometer performance and standardization were monitored daily with fluorescent-labeled CS&T beads (BD, 655051). Laser voltages were optimized within a <2% deviation using BD OneFlow™ Setup Beads (BD, 658620) and Alignflow™ Flow cytometry Alignment Beads (Thermo Fisher Scientific, A16502) on a daily setting.
Raw flow cytometry data (fcs 3.1 files) were manually analyzed using FCS Express Flow Cytometry Software (version 6) to identify, gate and export single T-cell and NK-cell populations. Pre-processing included data cleaning (PeacoQC18), hyperbolic arcsin transformation, approximated min-max scaling and batch correction (quantile normalization) in R and R studio (version 4.0.3). Computational data analysis was performed using UMAP and FlowSOM on 10.000 cells per fcs file.

DA-EPOCH-R Flow cytometry panels	**Day 1 first cycle** **START**	**Day 1 third cycle** **MID**	**After fifth cycle** **END**
I. T-cell phenotype Differentiation, senescence, exhaustion (PD-1, TIM3, TIGIT, CD27, CD28)	CMR N=40	CMR N=35	CMR N=37
	No CMR N=30	No CMR N=23	No CMR N=12
II. T-cell phenotype Tregs, gd-T-cells Exhaustion (PD-1, LAG3) Activation (CD38, HLA-DR)	CMR N=40	CMR N=30	CMR N=34
	No CMR N=26	No CMR N=18	No CMR N=12
III. NK-cell phenotype NK-cell subsets, senescence, activation (DNAM-1, HLA-DR)	CMR N=35	CMR N=23	CMR N=31
	No CMR N=23	No CMR N=15	No CMR N=11
IV. NK-cell phenotype NK-cell subsets, receptors (NKG2A, NKG2C, NKG2D and TIM-3)	CMR N=31	CMR N=18	CMR N=26
	No CMR N=18	No CMR N=13	No CMR N=9

Legend

Supplementary Figure 1. NK-cell and T-cell phenotypes associated with response to DA-EPOCH-R. (**A**) Total white blood cell (WBC) count as $*10^9$/L collected at day 1 of the first (start) and third (mid) and after the fifth DA-EPOCH-R cycle (end) for patients who at the end of DA-EPOCH-R achieved complete metabolic remission (CMR, blue) or did not achieve CMR (red). (**B**) Dimensionality reduction by UMAP of the flow cytometry data of NK-cells. Color overlays for CD16, CD56, CD57, KLRG1 and TIGIT. (**C**) Percentages of kill of K562 and degranulation as measured by CD107a/b surface expression on NK-cells after 4 hours co-culture of peripheral blood mononuclear cells (PBMCs) with K562 cell line at day 1 of the first (start) and after the fifth DA-EPOCH-R cycle (end) for patients who at the end of DA-EPOCH-R achieved complete metabolic remission (CMR, blue) or did not achieve CMR (red). Cytotoxicity is calculated relative to the amount of K562 cells without PBMCs.
(**D**) Percentages of $CD4^+$ T-cells, $CD8^+$ T-cells, regulatory T-cells (Tregs), $CD4^-CD8^-$ and innate-like γδ-T-cells as percentages of total T-cells at day 1 of the first (start) and third (mid) and after the fifth DA-EPOCH-R cycle (end) for patients who at the end of DA-EPOCH-R achieved complete metabolic remission (CMR, blue) or did not achieve CMR (red).
(**E**) Dimensionality reduction by UMAP of the flow cytometry data of T-cells. Color overlays for CD8, CD38, HLA-DR and PD-1.
(**F-G**) $LAG3^+$ (left panels) or $TIGIT^+$ (right panels) (F) and $CD4^+$ (left panels) and $CD8^+$ (right panels) expressing markers associated with T-cell senescence (loss of CD27 and CD28, and gain of KLRG1 and CD57) (G) as percentages of total T-cells at day 1 of the first (start) and third (mid) and after the fifth DA-EPOCH-R cycle (end) for patients who at the end of DA-EPOCH-R achieved complete metabolic remission (CMR, blue) or did not achieve CMR (red). (**H**) Percentages of $CD4^+$ (upper panels) $CD8^+$ (lower panels) T-cells expressing markers associated with T-cell activation (CD38, HLA-DR and CD25), immune checkpoints (LAG3 and TIGIT), and markers associated with T-cell senescence (loss of CD27 and CD28, and gain of KLRG1 and CD57) at third (mid) and after the fifth DA-EPOCH-R cycle (end) for patients who at the end of DA-EPOCH-R achieved complete metabolic remission (CMR, blue) or did not achieve CMR (red).

For all box plots, the lower upper hinges correspond to the 25^{th} and 75^{th} percentiles. The middle hinge corresponds to the median. The whiskers extend from the largest to smallest value +/- 1.58 * interquartile range. Outliers are plotted individually.
Linear mixed effect models (A,D, F, G) and non-parametric Mann-Whitney U test between two groups (C, H) were used for statistical analysis in which $P < 0.05$ was considered significant. No multiple correction was applied.

Supplementary figure 1