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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

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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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Authorship contributions
A.V.D.J., M.E.D.C. and T.M. designed the research.
W.S.C.B. designed the flow cytometry panels.
C.D. performed computation FlowSOM analysis.
A.V.D.J., W.S.C.B., C.D., C.L.B.M.K., R.R., M.C. and M.E. performed the experiments.
A.V.D.J. analyzed the data under supervision of M.G.M.R., M.E.D.C. and T.M.
E.V.W. provided statistical support.
Y.S., R.V.R., R.F., P.M., V.V., D.I., A.B., Y.M.B. and O.V. contributed to the sample collection.

Data-sharing statement:
Data are available in the main text or the supplementary materials. Data is visualized as far as the number of figures permits. For additional data and source data, please contact t.mutis@amsterdamumc.nl.

Conflicts-of-Interests

A.V.D.J., C.D., W.S.C.B., C.L.B.M.K., R.R., M.C., M.E., Y.S., R.S.V.R., R.F., P.M., V.K.J.V., D.I., A.B., Y.M.B., O.V., E.V.W. and M.G.M.R.: nothing to disclose.
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Dear Editor,

In the era of immunotherapy, patients with diffuse large B-cell lymphoma (DLBCL) significantly benefit from the addition of CD20-targeting antibody rituximab to standard chemotherapy regimen cyclophosphamide, doxorubicin, vincristine, prednisone (R-CHOP).[1] Patients with high-grade B-cell lymphoma (HGBL), characterized by *MYC*, *BCL2* and/or *BCL6* rearrangements[2], however, have a poor prognosis upon treatment with R-CHOP. In HGBL patients, intensified DA-EPOCH-R (dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and rituximab) might improve complete metabolic remission (CMR) rates and prolong disease-free survival (DFS).[3] Yet, responses to DA-EPOCH-R are heterogeneous, relapses are frequent, and improvement of overall survival (OS) has not been demonstrated.[4] While *MYC*-driven tumor resistance to immunochemotherapy could be one of the reasons for treatment failure, little is known about the role of immune effector cells in clinical outcomes. Furthermore, a deeper insight to what extend DA-EPOCH-R regimen impacts immune effector cells might guide the development of novel immunotherapeutic approaches for refractory or relapsed patients. To address these two important issues, we have extensively profiled peripheral NK-cells and T-cells by flow-cytometry in newly diagnosed HGBL patients treated with DA-EPOCH-R in the HOVON-152 phase II trial (NCT03620578), which investigates the efficacy of nivolumab consolidation after DA-EPOCH-R induction treatment. The study protocol was approved by the local medical ethics committee of the Amsterdam UMC (NL63247.029.17) and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent prior to participating in the study.

Peripheral blood samples were collected at day 1 of the first (start) and third (mid) and after the fifth DA-EPOCH-R cycle (end-of-treatment). The immune profiling was executed using samples of the first available patients (n=70, baseline characteristics are presented in **Supplementary Table S1**). Forty patients (57%) achieved complete metabolic remission (CMR) at end-of-treatment. Patients who did not achieve CMR had more often a higher WHO performance score, higher LDH levels and higher IPI scores at baseline (**Supplementary Table S1**). Cryopreserved PBMCs, obtained through density gradient centrifugation with Ficoll-Paque Plus, were thawed and washed with IMDM-medium supplemented with 20% fetal calf serum, 100 U/mL penicillin and 100 µg/ml streptomycin. Next, PBMCs were treated with DNase (Roche, cat 10104159001), cultured for 16 hours in supplemented IMDM medium for cell and surface marker recovery, and profiled with four separate 14-15 marker panels using a 5-laser LSRFortessa™ flow cytometer (Becton Dickinson) as previously described.[5] An overview of the available samples tested is provided in **Supplementary Table 2**. In a subset of patients NK-cells were analyzed for cytotoxic activity and degranulation (n=15) and T-cells were analyzed for cytokine production (n=23) in previously-established *ex vivo* assays.[5]

Because rituximab mediates its primary effects via antibody-dependent cellular cytotoxicity (ADCC) through the Fc-γ receptor IIIa (CD16) on NK-cells, we first investigated whether the composition of the NK-cell compartment at baseline was associated with therapy outcome. Achievement of CMR was associated with a higher frequency of $CD56^{bright}$ NK-cells expressing the activating receptor NKG2D (**Figure 1A**). This association was, however, not significant after correction for LDH and WHO performance score (generalized multivariable linear model, P=0.27). Achievement of CMR was not associated with any other NK-cell subsets at the start of therapy, including highly cytotoxic $CD16^{+}$ NK-cells and poorly cytotoxic $CD56^{bright}$ NK-cells (**Figure 1B**) and NK-cells expressing various maturation-related (CD57, NGK2C), activating (DNAM-1, HLA-DR) or inhibition markers (TIM-3, TIGIT, NKG2A or KLRG1, data not shown). The overall killing capacity and degranulation of NK-cells at start were also comparable between patients who did or did not achieve CMR (**Figure 1C**).

In the T-cell compartment, we also did not find an association between CMR and various T-cell frequencies at baseline, including total $CD4^{+}$ and $CD8^{+}$ T-cells, regulatory T-cells, $CD4^{-}CD8^{-}$ and innate-like γδ-T-cells (**Figure 1D-E**). In addition, frequencies of $CD4^{+}$ and $CD8^{+}$ T-cells expressing activation markers (HLA-DR, CD38 or CD25), costimulatory molecules (CD27, CD28), markers associated with T-cell senescence (CD57, KLRG1) or immune checkpoints (PD-1, TIM-3, LAG-3 or TIGIT) were comparable between patients who did or did not achieve CMR (data not shown). These results suggested that outcome to DA-EPOCH-R therapy could not be predicted by composition of NK-cell or T-cell subsets at baseline. In this study, patients had received one cycle of R-CHOP prior to start of DA-EPOCH-R. Previously, we have shown that cycle of R-CHOP resulted in a reduction of ADCC-mediating $CD16^{+}$ NK-cells and increase in $CD56^{bright}$ NK-cells.[5] Therefore, we next investigated whether DA-EPOCH-R could induce additional changes in the composition of immune cell subsets and whether these alterations would correlate with the therapy outcome.

As expected, total WBC numbers were decreased after 5 cycles of DA-EPOCH-R (**Supplementary Figure 1A**) in both CMR and non CMR groups indicating a limited non-specific toxicity on al lymphocyte subsets. Nonetheless, specific effects on immune cell subsets were observed. Similar to what has been observed after one cycle of R-CHOP, DA-EPOCH-R treatment was associated with a decrease in $CD16^{+}$ NK-cell frequencies. Interestingly, however, the decrease was only significant and progressive in patients who did not achieve CMR (**Figure 2A**). A progressive increase in non-cytotoxic $CD56^{bright}$ NK-cells was detected in both patient groups who did or did not achieve CMR (**Figure 2A**). Further analyses revealed that DA-EPOCH-R treatment was in both groups associated with a progressive increase of $HLA\text{-}DR^{+}$ NK-cells (**Figure 2B**), which are described as effective killers associated with antigen presentation[6], and progressive decrease in partly overlapping NK-cells expressing CD57, KLRG1 and TIGIT and the increase of NK-cells expressing the inhibitory receptor

NKG2A (**Figure 2C-D**). UMAP analysis indicated that NK-cells may exhibit co-expression of CD57, KLRG1 and TIGIT (**Supplementary Figure 1B**). While NK-cells expressing the activating receptors NKG2C and NKG2D and the inhibitory checkpoint TIM-3 did not change (data not shown), there was a transient drop in the frequency of NK-cells expressing DNAM-1 in patients who did not achieve CMR (**Figure 2E**).

These results demonstrated that DA-EPOCH-R therapy dynamically changes the composition of NK-cell subsets. Some changes in NK-cell compositions were more pronounced in patients who did or did not achieve CMR, suggesting an association with therapy outcome. To gain more insight into this association, we compared frequencies of NK-cell subsets amongst patients who did or did not achieve CMR at midterm and at end-of-treatment. Notably, progressive patients went off-study prematurely, resulting in a reduced sample size for analysis at these time points (**Supplementary Table 2**). The progressive drop of $CD16^{+}$ NK-cells in non-CMR patients did not result in different NK-cell frequencies between patients who did or did not achieve CMR, and neither were the $CD56^{bright}$ frequencies (data not shown). Patients who achieved CMR appeared to have lower frequencies of $TIM\text{-}3^{+}$, but higher frequencies of $KLRG1^{+}$ NK-cells at midterm and at the end-of-treatment (data not shown). In terms of NK-cell effector functions, we found no differences at start (n=12) or at end-of-treatment (n=6) in cytotoxic activity and degranulation (**Supplementary Figure 1C**), supporting the rational for larger studies to better address this issue.

The most interesting and somewhat unexpected findings of this study were observed in the T-cell compartment. While DA-EPOCH-R treatment did not alter the frequency of several T-cell subsets ($CD4^{+}$ and $CD8^{+}$ T-cells, Tregs, $CD4^{-}CD8^{-}$ T-cells and innate-like γδ-T-cells, **Supplementary Figure 1D**), DA-EPOCH-R treatment was significantly associated with a progressive increase in the frequencies of T-cells expressing activation markers HLA-DR or CD38 (**Figure 3A**), but not CD25 (data not shown). More interestingly, there was a progressive decrease in $PD\text{-}1^{+}CD8^{+}$ T-cells, only in patients achieving CMR (**Figure 3B**). UMAP analysis indicated that T-cells may exhibit co-expression of CD38, HLA-DR or PD-1 (**Supplementary Figure 1E**). The frequencies of T-cells expressing other immune checkpoints (LAG-3 or TIGIT) or senescence markers (loss of CD27 or CD28, or gain of CD57 or KLRG1) did not change (**Supplementary Figure 1F-G**).

Supporting the idea of T-cell activation and differentiation, the frequency of $CD4^{+}$ naïve T-cells decreased and the frequencies of central memory and effector T-cells increased (data not shown). The differentiation states of $CD8^{+}$ T-cells did not alter during DA-EPOCH-R treatment (data not shown). Increased production of IFN-γ and TNF-α by $CD4^{+}$ T-cells at the end of DA-EPOCH-R therapy further supports the idea of T-cell activation and differentiation during DA-EPOCH-R (**Figure 3C**).

Finally, when we compared frequencies of T-cell subsets during and at end-of-treatment between patients who did or did not achieve CMR, we observed that patients who achieved CMR exhibited

lower frequencies of $CD8^+$ T-cells expressing PD-1 at end-of-treatment as compared to patients who did not achieve CMR (**Figure 3D**). $CD4^+$ and $CD8^+$ T-cells expressing TIM-3 showed lower frequencies at midterm only in patients who achieved CMR (P=0.04 and P=0.08, respectively, data not shown). Other subsets were not different between patients who did or did not achieve CMR (**Supplementary Figure 1H**).

All these results support the idea that the response to DA-EPOCH-R treatment was associated with the activation of "fitter", less exhausted cytotoxic T-cells. These findings are in line with a previous study showing increased frequencies of $HLA\text{-}DR^+$ and $IFN\text{-}\gamma^+$ T-cells over the course of R-CHOP therapy in DLBCL patients.[7] Thus, therapies inducing tumor cell kill could ultimately activate T-cell compartment for long-term effects. With this in mind, it is noteworthy that cyclophosphamide, a standard component of EPOCH, has been described to have immunomodulatory mechanisms of action, including Treg modulation and increased IFN-γ secretion *in vivo*.[8,9] On the other hand, when dosed too high, doxorubicin and cyclophosphamide can have detrimental effects on T-cell metabolism.[10] Therefore, for full benefit of DA-EPOCH-R therapy, future studies may further investigate the maximal effective dose and best time intervals of therapy by taking its effects on immune effector cells in account. While it is too early to make firm conclusions, the increase in activated T-cells during DA-EPOCH-R therapy holds promise for second-line treatment with T-cell based immunotherapeutic approaches for patients who do not achieve CMR to DA-EPOCH-R.

References

1. Coiffier B, Lepage E, Briere J, et al. CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large B-cell lymphoma. N Engl J Med. 2002;346(4):235-242.
2. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood. 2016;127(20):2375-2390.
3. Dunleavy K, Fanale MA, Abramson JS, et al. Dose-adjusted EPOCH-R (etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, and rituximab) in untreated aggressive diffuse large B-cell lymphoma with MYC rearrangement: a prospective, multicentre, single-arm phase 2 study. Lancet Haematol. 2018;5(12):e609-e617.
4. Howlett C, Snedecor SJ, Landsburg DJ, et al. Front-line, dose-escalated immunochemotherapy is associated with a significant progression-free survival advantage in patients with double-hit lymphomas: a systematic review and meta-analysis. Br J Haematol. 2015;170(4):504-514.
5. de Jonge AV, Duetz C, Bruins WSC, et al. Distinct Peripheral T-cell and NK-cell Profiles In HGBL-MYC/BCL2 versus DLBCL NOS Patients. Blood Adv. 2024;8(5):1094-1104.
6. Erokhina SA, Streltsova MA, Kanevskiy LM, et al. HLA-DR-expressing NK cells: Effective killers suspected for antigen presentation. J Leukoc Biol. 2021;109(2):327-337.
7. Hou H, Luo Y, Tang G, et al. Dynamic changes in peripheral blood lymphocyte subset counts and functions in patients with diffuse large B cell lymphoma during chemotherapy. Cancer Cell Int. 2021;21(1):282.
8. Binotto G, Trentin L, Semenzato G. Ifosfamide and cyclophosphamide: effects on immunosurveillance. Oncology. 2003;65 Suppl 2:17-20.
9. Matar P, Rozados VR, Gervasoni SI, et al. Th2/Th1 switch induced by a single low dose of cyclophosphamide in a rat metastatic lymphoma model. Cancer Immunol Immunother. 2002;50(11):588-596.
10. Das RK, O'Connor RS, Grupp SA, et al. Lingering effects of chemotherapy on mature T cells impair proliferation. Blood Adv. 2020;4(19):4653-4664.

Figure legends

Figure 1. NK-cell and T-cell phenotype at baseline associated with response to DA-EPOCH-R.
(**A**) Percentages of NKG2D^{+} NK-cells as percentages of CD56^{bright} and CD56^{dim} NK-cells, (**B**) percentages of CD16^{+} or CD56^{bright} NK-cells as percentages of total NK-cells for patients who at the end of DA-EPOCH-R achieved complete metabolic remission (CMR, blue) or did not achieve CMR (red). (**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 DA-EPOCH-R cycle for patients who achieved (blue) or did not achieve CMR (red). Cytotoxicity is calculated relative to the amount of K562 cells without PBMCs. (**D-E**) Percentages of CD4^{+}, CD8^{+} T-cells (D) and regulatory T-cells (Tregs), CD4^{-}CD8^{-}, innate-like γδ-T-cells (E). Details as in A-B.
For all box plots, the lower upper hinges correspond to the 25th and 75th 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. Non-parametric Mann-Whitney U test between two groups was used for statistical analysis in which $P < 0.05$ was considered significant.

Figure 2. NK-cell phenotype during DA-EPOCH-R.
(**A-D**) Percentages of CD16^{+} and CD56^{bright} NK-cells (A), HLA-DR^{+} NK-cells (B), CD57^{+}, KLRG1^{+}, TIGIT^{+} NK-cells (C), NKG2A^{+} NK-cells (D) 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 CMR (blue) or did not achieve CMR (red). (**E**) DNAM-1^{+} NK-cells, details as in A. Linear mixed effect models were used for statistical analysis. For all box plots, the lower upper hinges correspond to the 25th and 75th 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. $P < 0.05$ was considered significant.

Figure 3. T-cell phenotype during DA-EPOCH-R.
(**A-B**) Percentages of HLA-DR^{+} and CD38^{+} CD4^{+} and CD8^{+} T-cells (A) and PD-1^{+} CD4^{+}, CD8^{+}, innate-like γδ-T-cells and regulatory T-cells (Tregs) (B) as percentages of total T-cells 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).
(**C**) Percentages of CD4^{+} T-cells positive for interferon-γ and TNF-α after 4 hour stimulation with PMA/ionomysin 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 CMR (blue) or did not achieve CMR (red).
(**D**) Percentages PD-1^{+} CD8^{+} (E) as percentage of total T-cells at the 3rd (mid) and 5th (end) DA-EPOCH-R cycle for patients who at the end of DA-EPOCH-R achieved complete metabolic remission (CMR, blue) or did not achieve CMR (red).

Figure 1

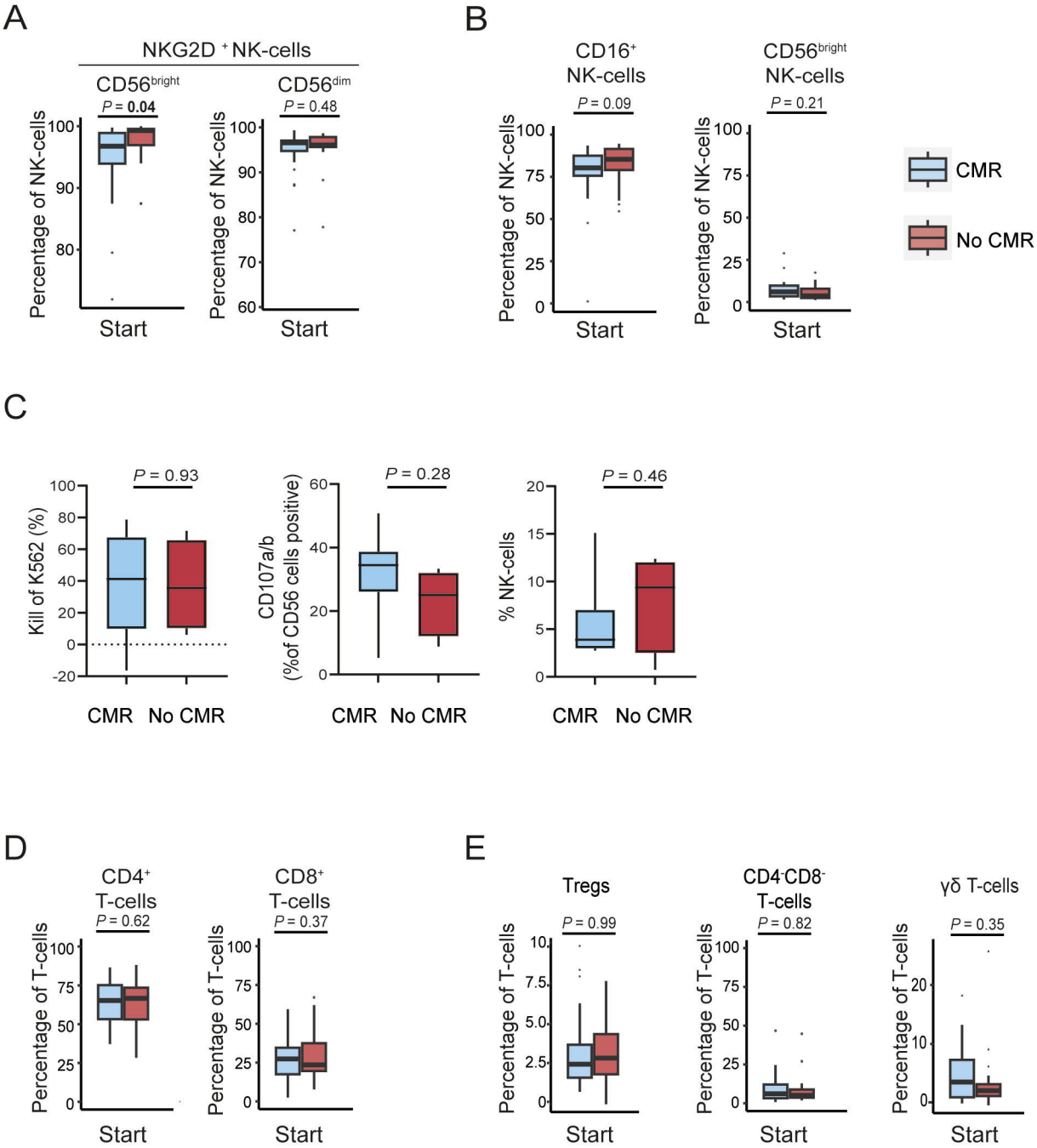

A
NKG2D + NK-cells
CD56bright
P = 0.04
CD56dim
P = 0.48
Percentage of NK-cells
Start
B
CD16+ NK-cells
P = 0.09
CD56bright NK-cells
P = 0.21
CMR
No CMR
C
P = 0.93
Kill of K562 (%)
P = 0.28
CD107a/b (%of CD56 cells positive)
P = 0.46
% NK-cells
CMR
No CMR
D
CD4+ T-cells
P = 0.62
CD8+ T-cells
P = 0.37
Percentage of T-cells
E
Tregs
P = 0.99
CD4-CD8- T-cells
P = 0.82
γδ T-cells
P = 0.35

Figure 2

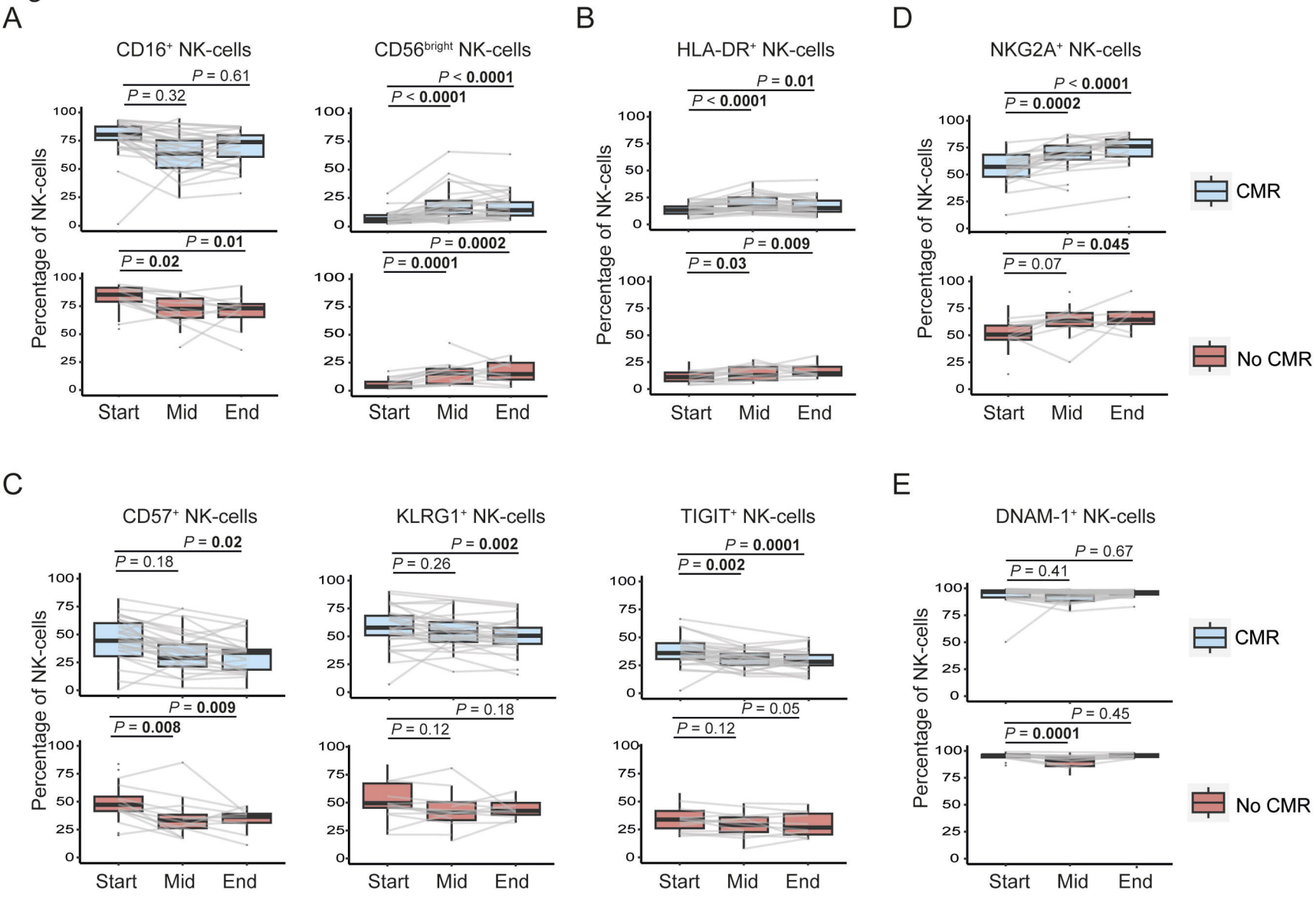
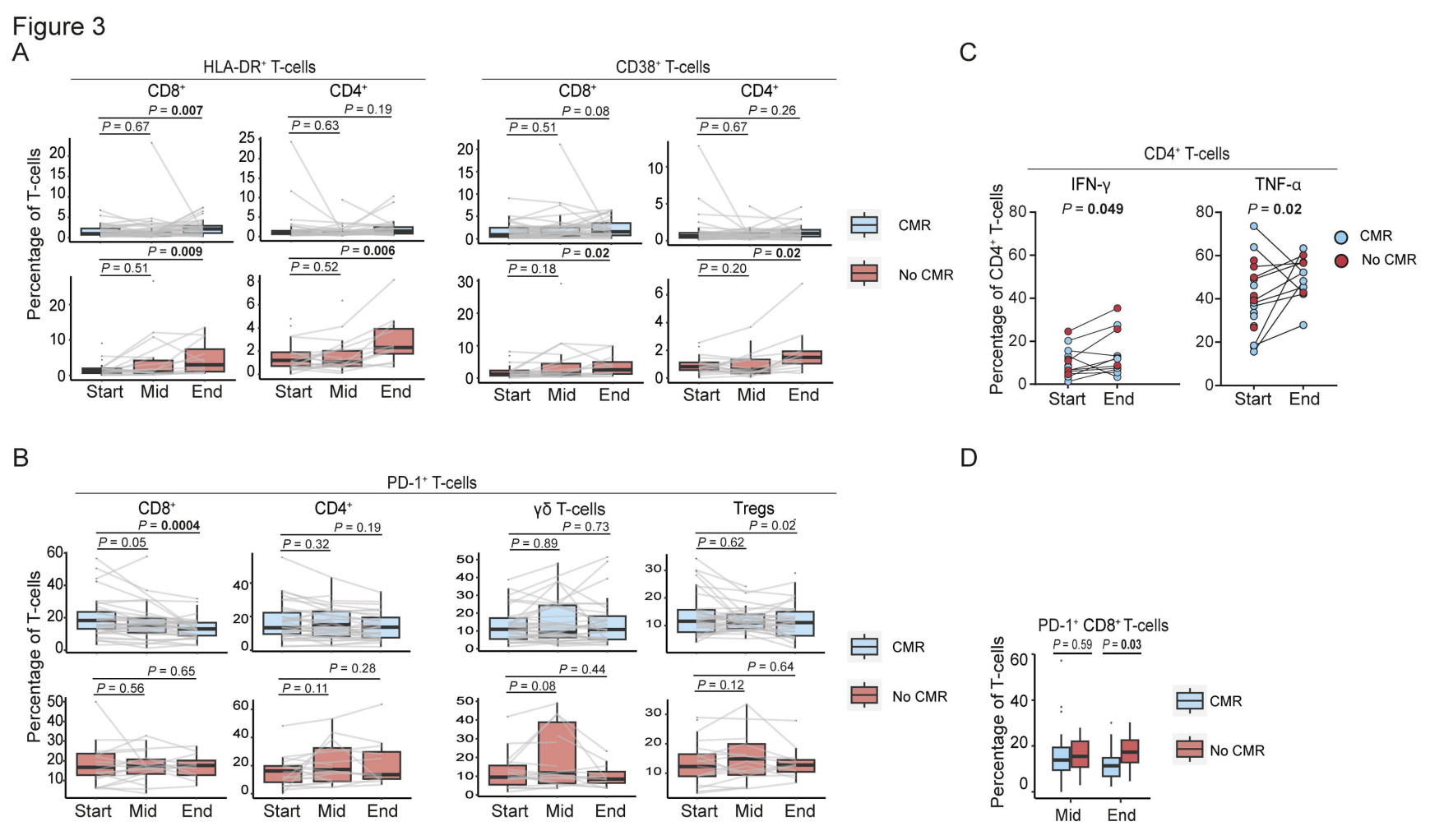

A
CD16+ NK-cells
P = 0.61
P = 0.32
P = 0.01
P = 0.02
CD56bright NK-cells
P < 0.0001
P < 0.0001
P = 0.0002
P = 0.0001
B
HLA-DR+ NK-cells
P = 0.01
P < 0.0001
P = 0.009
P = 0.03
D
NKG2A+ NK-cells
P < 0.0001
P = 0.0002
P = 0.045
P = 0.07
CMR
No CMR
C
CD57+ NK-cells
P = 0.02
P = 0.18
P = 0.009
P = 0.008
KLRG1+ NK-cells
P = 0.002
P = 0.26
P = 0.18
P = 0.12
TIGIT+ NK-cells
P = 0.0001
P = 0.002
P = 0.05
P = 0.12
E
DNAM-1+ NK-cells
P = 0.67
P = 0.41
P = 0.45
P = 0.0001
Percentage of NK-cells
Start
Mid
End

Figure 3


A
HLA-DR+ T-cells
CD38+ T-cells
CD8+
CD4+
Percentage of T-cells
P = 0.007
P = 0.67
P = 0.19
P = 0.63
P = 0.08
P = 0.51
P = 0.26
P = 0.67
P = 0.009
P = 0.51
P = 0.006
P = 0.52
P = 0.02
P = 0.18
P = 0.02
P = 0.20
Start
Mid
End
CMR
No CMR
B
PD-1+ T-cells
CD8+
CD4+
γδ T-cells
Tregs
P = 0.0004
P = 0.05
P = 0.19
P = 0.32
P = 0.73
P = 0.89
P = 0.02
P = 0.62
P = 0.65
P = 0.56
P = 0.28
P = 0.11
P = 0.44
P = 0.08
P = 0.64
P = 0.12
C
CD4+ T-cells
IFN-γ
TNF-α
Percentage of CD4+ T-cells
P = 0.049
P = 0.02
D
PD-1+ CD8+ T-cells
P = 0.59
P = 0.03

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