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

In the era of immunotherapy, patients with diffuse large B-cell lymphoma (DLBCL) significantly benefit from the addition of CD20-targeting antibody rituximab to the standard cyclophosphamide, doxorubicin, vincristine, prednisone (R-CHOP) chemotherapy regimen.[1] However, patients with high-grade B-cell lymphoma (HGBL), characterized by *MYC*, *BCL2* and/or *BCL6* rearrangements[2] 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 into the extent to which the 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 natural killer (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 (clinicaltrials.gov identifier: 03620578), 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) DA-EPOCH-R cycles, and after the fifth DA-EPOCH-R cycle (end-of-treatment). Immune profiling was carried out using samples of the first available patients (N=70) (see *Online Supplementary Table S1* for baseline characteristics). Forty patients (57%) achieved CMR at end-of-treatment. Patients who did not achieve CMR more often had a higher World Health Organization (WHO) performance score, higher lactate dehydrogenase (LDH) levels and higher International Prognostic Index scores at baseline (*Online Supplementary Table S1*). Cryopreserved peripheral blood mononuclear cells (PBMC) were obtained through density gradient centrifugation with Ficoll-Paque Plus, and thawed and washed with IMDM medium supplemented with 20% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Next, PBMC 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 *Online Supplementary Table S2*. 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 (TIM-3, TIGIT, NKG2A or KLRG1) markers (*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 any association between CMR and various T-cell frequencies at baseline, including total $CD4^{+}$ and $CD8^{+}$ T cells, regulatory T cells (Tregs), $CD4^{-}CD8^{-}$ 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), co-stimulatory molecules (CD27, CD28), and 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

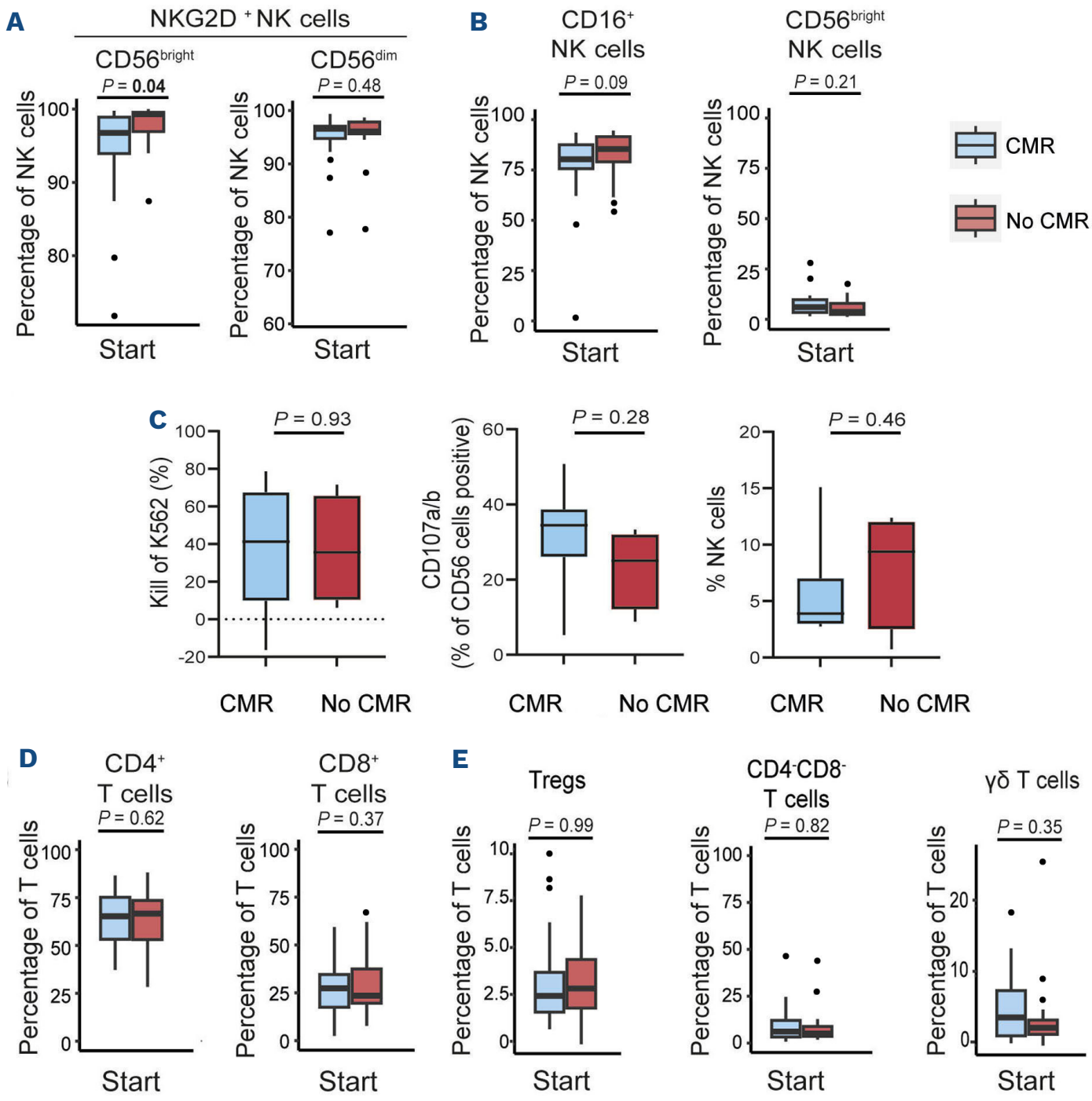


Figure 1. Natural killer cell and T-cell phenotype at baseline associated with response to DA-EPOCH-R. (A) Percentages of NKG2D$^+$ natural killer (NK) cells as percentages of CD56^{bright} and CD56^{dim} NK cells and (B) percentages of CD16$^+$ or CD56^{bright} NK cells as percentages of total NK cells 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-hour co-culture of peripheral blood mononuclear cells (PBMC) with K562 cell line at day 1 of the first DA-EPOCH-R cycle for patients who achieved CMR (blue) or did not achieve CMR (red). Cytotoxicity is calculated relative to the amount of K562 cells without PBMC. (D and 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 and 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.

DA-EPOCH-R. Previously, we have shown that one cycle of R-CHOP resulted in a reduction of ADCC-mediating CD16$^+$ NK cells and an 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 white blood cell numbers were decreased after 5 cycles of DA-EPOCH-R (*Online Supplementary Figure S1A*) in both CMR and non-CMR groups indicating a limited non-specific toxicity on all 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 in HLA-DR$^+$ NK cells (Figure

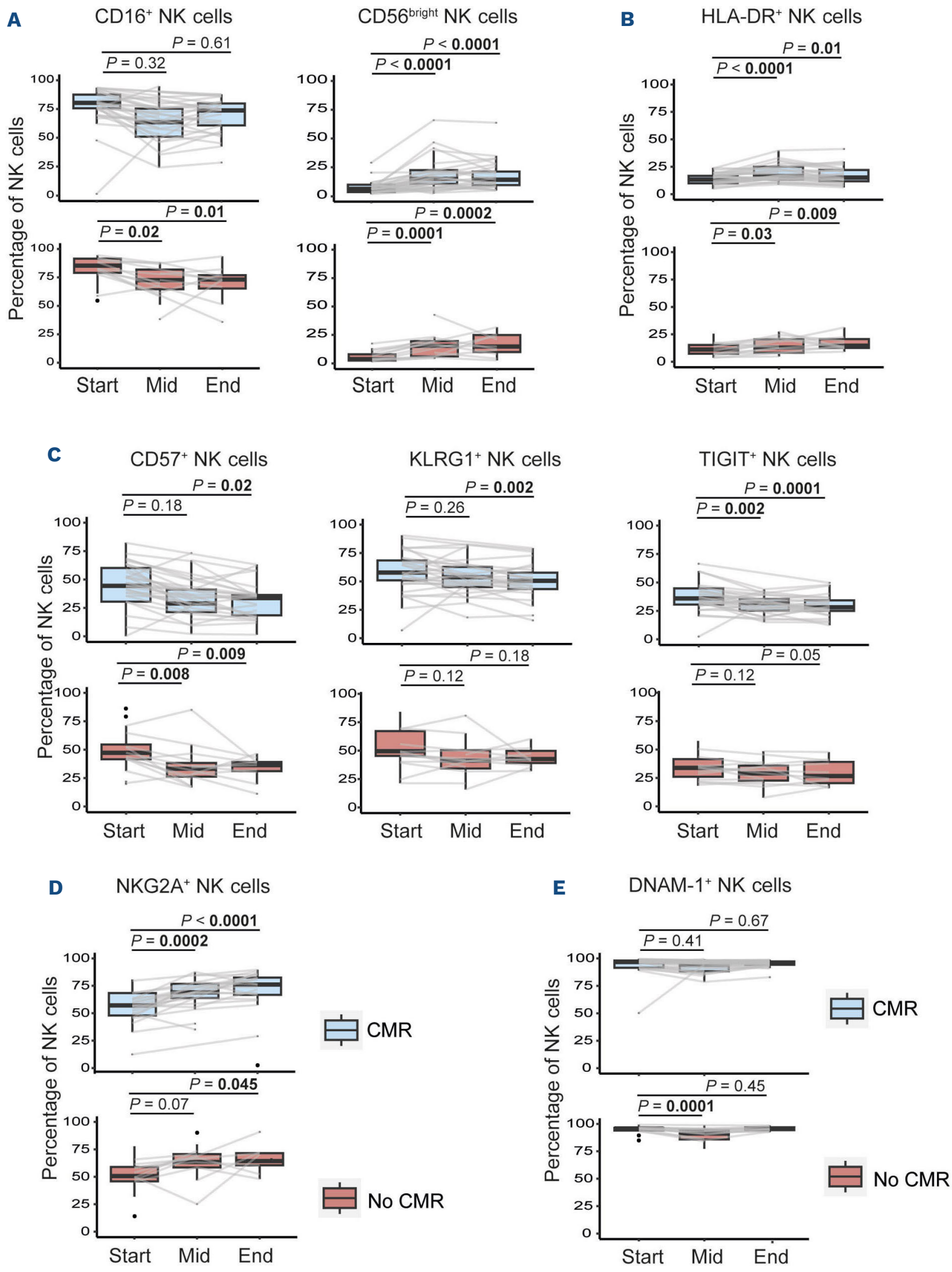
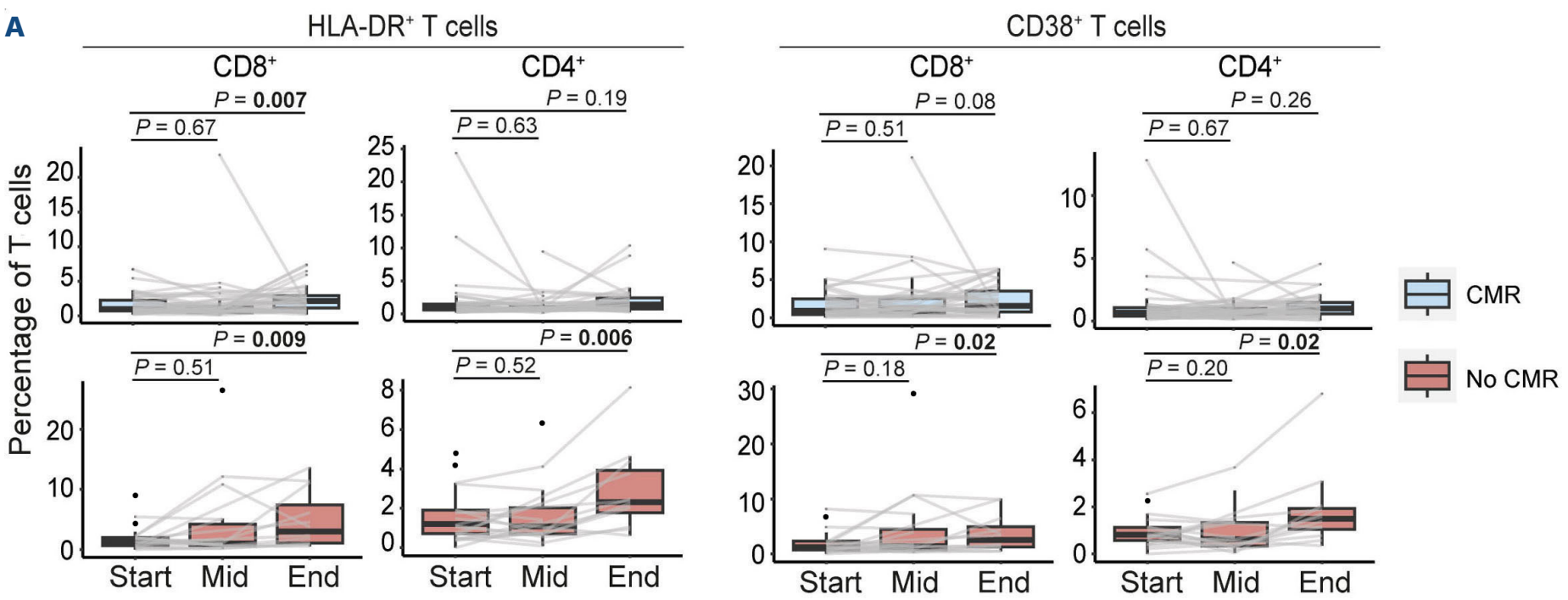


Figure 2. Natural killer cell phenotype during DA-EPOCH-R. (A-D) Percentages of CD16^{+} and CD56^{bright} natural killer (NK) cells (A), HLA-DR^{+} NK cells (B), CD57^{+}, KLRG1^{+}, TIGIT^{+} NK cells (C), NKG2A^{+} NK cells (D) collected at day 1 of the first (Start) and third (Mid) DA-EPOCH-R cycles, and after the fifth DA-EPOCH-R cycle (End; end-of-treatment) for patients who at the end of DA-EPOCH-R achieved complete metabolic remission (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*inter-quartile range. Outliers are plotted individually. *P*<0.05 was considered significant.

2B), which are described as effective killers associated with antigen presentation,[6] and a progressive decrease in partly overlapping NK cells expressing CD57, KLRG1 and TIGIT, and an increase in NK cells expressing the inhibitory receptor NKG2A (Figure 2C, D). Uniform Manifold Approximation and Projection (UMAP) analysis indicated that NK cells may exhibit co-expression of CD57, KLRG1 and TIGIT (*Online Supplementary Figure S1B*). 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 (*Online Supplementary Table S2*). The progressive drop in the number 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 affected (*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 end-of-treatment (*data not shown*). In terms of NK-cell effector function, we found no differences at start (N=12) or at end-of-treatment (N=6) in cytotoxic activity and degranulation (*Online Supplementary Figure S1C*), supporting the rationale 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) (*Online Supplementary Figure S1D*), 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 (*Online Supplementary Figure S1E*). There was no change in 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) (*Online Supplementary Figure S1F, 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 status 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*). There was no difference in other subsets between patients who did or did not achieve CMR (*Online Supplementary Figure S1H*). All these results support the idea that the response to



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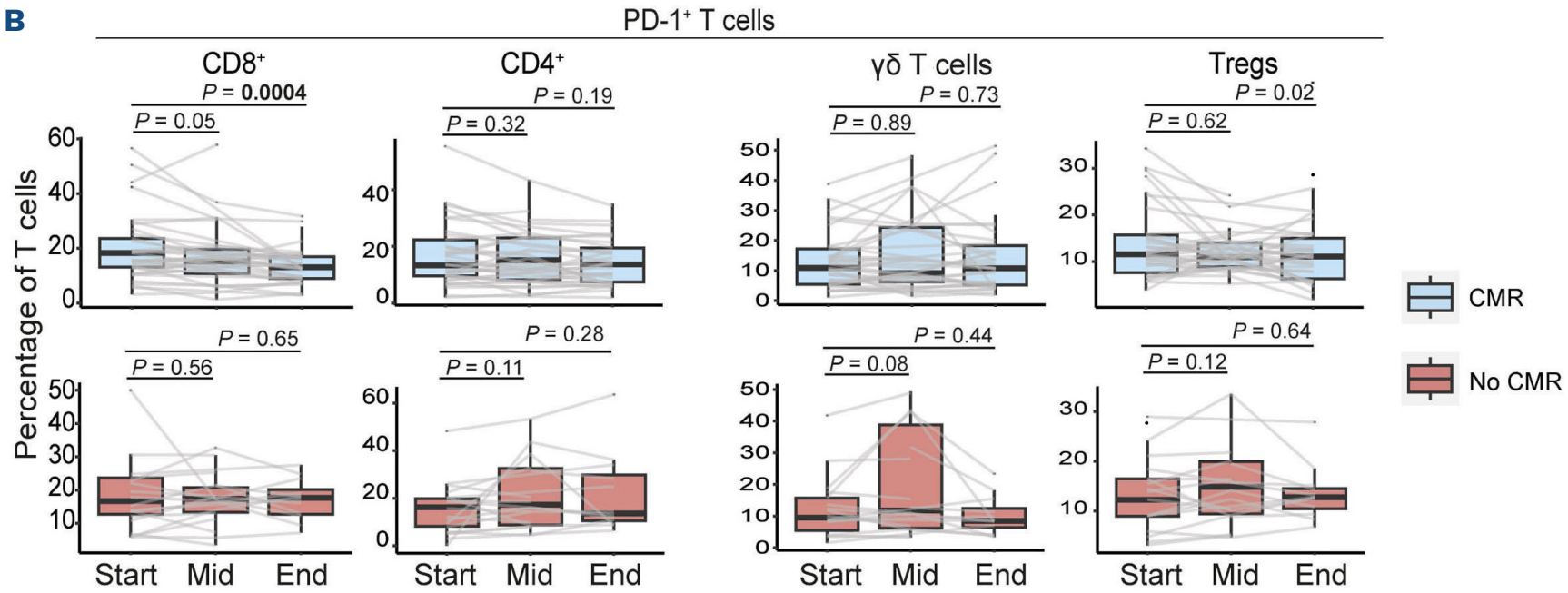

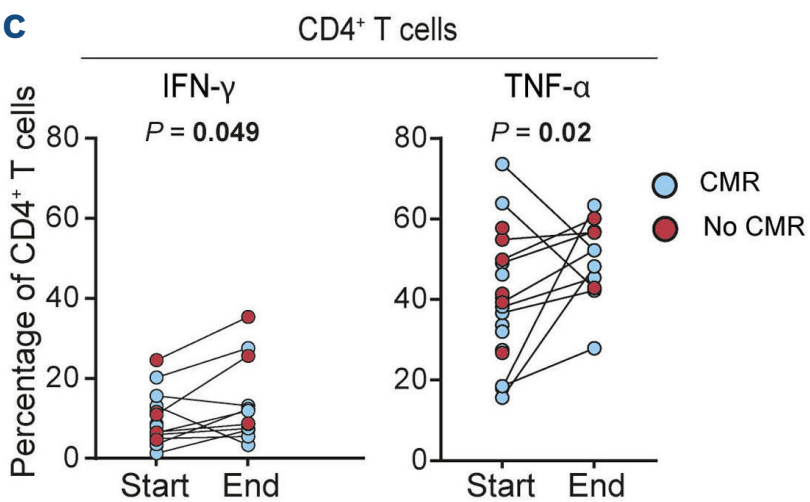

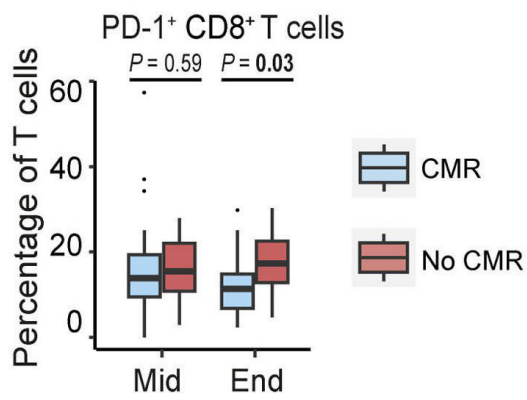

Figure 3. T-cell phenotype during DA-EPOCH-R. (A and B) Percentages of HLA-DR$^+$ and CD38$^+$ CD4$^+$ and CD8$^+$ T cells (A) and PD-1$^+$ CD4$^+$, CD8$^+$, innate-like γδ-T cells and regulatory T cells (Tregs) (B) as percentages of total T cells collected at day 1 of the first (Start) and third (Mid) DA-EPOCH-R cycles, and after the fifth DA-EPOCH-R cycle (End; end-of-treatment) 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 (End) DA-EPOCH-R cycles for patients who at the end of DA-EPOCH-R achieved CMR (blue) or did not achieve CMR (red). (D) Percentages of PD-1$^+$ CD8$^+$ (E) as percentage of total T cells at the 3rd (Mid) and 5th (End) DA-EPOCH-R cycles for patients who at the end of DA-EPOCH-R achieved CMR (blue) or did not achieve CMR (red). P<0.05 was considered significant.

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-DR$^+$ and IFN-γ$^+$ T cells over the course of R-CHOP therapy in DLBCL patients.[7] Thus, therapies inducing tumor cell kill could ultimately activate the 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 dosage is too high, doxorubicin and cyclophosphamide can have detrimental effects on T-cell metabolism.[10] Therefore, in order to achieve the full benefit of DA-EPOCH-R therapy, future studies could further investigate the maximal effective dose and best time intervals of therapy by considering its effects on immune effector cells. While it is too early to come to any 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 with DA-EPOCH-R.

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

Received: February 11, 2024.
Accepted: June 26, 2024.
Early view: July 4, 2024.

Disclosures

MJK received research support from BMS/Celgene, Galapagos, Kite (a Gilead Company), Roche, and Takeda, and had an advisory role at Adicet Bio, Miltenyi Biotech, Novartis, and Roche. MEDC received research support from AbbVie, Genmab, BMS, and Gilead, and had an advisory role at AbbVie, Novartis, and Incyte. TM received research funding from Takeda, Janssen, and Genmab. All other authors have no conflicts of interest to disclose.

Contributions

AVDJ, MEDC and TM designed the research. WSCB designed the flow cytometry panels. CD performed computation FlowSOM analysis. AVDJ, WSCB, CD, CLBMK, RR, MC and ME performed the experiments. AVDJ analyzed the data under the supervision of MGMR, MEDC and TM. EVW provided statistical support. YS, RVR, RF, PM, VV, DI, AB, YMB and OV contributed to the sample collection.

Funding

This study was financially supported by Bristol Myers Squibb.

Data-sharing statement

Data are available in the main text or the *Online Supplementary Appendix*. Data are visualized as far as the number of figures permits. For additional data and source data, please contact t.mutis@amsterdamumc.nl.

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