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Selected memory T cells infused post haploidentical hematopoietic stem cell transplantation persist and hyper-expand

Tracking no: ADV-2022-007735R1

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Abstract:
Haploidentical hematopoietic stem cell transplantation (haplo-HSCT) with post-transplant cyclophosphamide is a curative treatment for many hematological malignancies, yet a majority of patients still suffers from recurrent infections. Post-transplant infusion of memory T cells could potentially enhance immunological protection without increasing the risk of eliciting acute graft-versus-host disease, which is mainly induced by naïve T cells. Here, we performed longitudinal analysis of the lymphocyte compartment in 19 haplo-HSCT patients previously enrolled in a phase II prospective clinical trial (ClinicalTrials.gov Identifier: NCT04687982), in which they received post-transplant CD45RA-depleted donor lymphocyte infusions (DLI). T cell receptor sequencing analysis showed that, surprisingly, CD45RA-depleted DLI do not increase T cell clonal diversity, but lead to prominent expansion of a selected number of infused memory T cell clones, suggestive of recruitment of these cells in the immune response. Pathogen-specific memory T cells, including cytomegalovirus (CMV)-specific cells, engrafted and were able to persist for at least one month. Deep immunophenotyping revealed strong polyfunctional effector CMV-specific T cell responses in the majority of patients, with their expansion correlating with the frequency of CMV-specific cells in the donor. These findings provide a rationale behind the suggested improved protection against viral infections for patients receiving CD45RA-depleted DLI.

Conflict of interest: COI declared - see note

COI notes: EL received honoraria and in-kind reagents from BD Biosciences Italy, preclinical funding from Bristol-Myers Squibb on topics unrelated to the content of this manuscript and royalties for a patent that describes methods for generating and isolating TSCM cells. JJPvB is currently employed at Janssen. EZ is currently employed at Sanofi. AS has received honoraria as advisory board member from Bristol-Myers Squibb, Servier, Gilead, Pfizer, Eisai, Bayer and Merck Sharp & Dhome; as speaker's bureau member from Takeda, Roche, Abb-vie, Amgen, Celgene, Astrazeneca, Lilly, Sandoz, Novartis, Bristol-Myers Squibb, Servier, Gilead, Pfizer, Arqule, Eisai; and for consultancy from Arqule.

Preprint server: No;

Author contributions and disclosures: JJPvB and EL conceived the study. CDP, BS, IT, RC, DM, RM, JM, RC, AS, LC and SB provided clinical samples and clinical information. JJPvB, FDP, EZ, MC and AS performed the experiments. CP, GB and JC sequenced the TCRs. JJPvB, SP and CDV analyzed the data. JJPvB and EL wrote the manuscript. LC, SB, DM and EL supervised the study. All authors contributed to and approved the final manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: For original data, please contact enrico.lugli@humanitasresearch.it.

Clinical trial registration information (if any): ClinicalTrials.gov Identifier: NCT04687982

Selected memory T cells infused post haploidentical hematopoietic stem cell transplantation persist and hyper-expand

Short title: Memory T cell infusion post haplo-HSCT

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Word count text: 3963; Word count abstract: 197; Figure count: 5, Reference count: 34

Key Points

- Infusions of donor memory T cells following haplo-HSCT lead to engraftment, persistence and prominent expansion of selected T cell clones.
- Infused patients harbor highly functional CMV-specific T cells and their expansion correlates with their frequency in the donor.

Abstract

Haploidentical hematopoietic stem cell transplantation (haplo-HSCT) with post-transplant cyclophosphamide is a curative treatment for many hematological malignancies, yet a majority of patients still suffers from recurrent infections. Post-transplant infusion of memory T cells could potentially enhance immunological protection without increasing the risk of eliciting acute graft-versus-host disease, which is mainly induced by naïve T cells. Here, we performed longitudinal analysis of the lymphocyte compartment in 19 haplo-HSCT patients previously enrolled in a phase II prospective clinical trial (ClinicalTrials.gov Identifier: NCT04687982), in which they received post-transplant CD45RA-depleted donor lymphocyte infusions (DLI). T cell receptor sequencing analysis showed that, surprisingly, CD45RA-depleted DLI do not increase T cell clonal diversity, but lead to prominent expansion of a selected number of infused memory T cell clones, suggestive of recruitment of these cells in the immune response. Pathogen-specific memory T cells, including cytomegalovirus (CMV)-specific cells, engrafted and were able to persist for at least one month. Deep immunophenotyping revealed strong polyfunctional effector CMV-specific T cell responses in the majority of patients, with their expansion correlating with the frequency of CMV-specific cells in the donor. These findings provide a rationale behind the suggested improved protection against viral infections for patients receiving CD45RA-depleted DLI.

Introduction

Allogeneic hematological stem cell transplantation (HSCT) has curative potential for multiple hematological malignancies. The development of HSCT from HLA-haploidentical related donors (haplo-HSCT) has greatly

increased the accessibility of patients to allogeneic HSCT, and use of post-transplant cyclophosphamide (PT-Cy) even allows for the transfer of T-replete grafts in this setting, which enhances immune reconstitution.[1,2] PT-Cy decreases the risk of acute graft-versus-host disease (aGvHD) by preferentially targeting highly proliferating cells, thereby depleting[3] or functionally inhibiting[4] alloreactive T cells transferred with the graft. However, the procedure may deplete alloreactive memory T cells that are otherwise pathogen-specific.[5] The loss of antigen-specific memory T cell precursors might explain why a majority of patients still experiences post-transplant infections, in particular reactivation of latent viruses such as cytomegalovirus (CMV), that are a major cause of non-relapse morbidity and mortality.[6]

Studies in mice have shown that aGvHD is mainly caused by alloreactive naïve T cells.[7–10] On the other hand, memory T cells harbor a poised epigenetic state that, amongst other factors, allows faster and superior protection against pathogens in comparison with naïve T cells.[11,12] Based on these differences, we hypothesize that add-back of donor memory T cells can enhance immune reconstitution and immunological protection without eliciting aGvHD.

We conducted a phase II clinical trial, in which patients treated with haplo-HSCT with PT-Cy for hematological malignancies received post-transplant infusions of memory T cells.[13] These donor lymphocyte infusions (DLI) consisted of CD45RA-depleted lymphocytes, based on the principle that naïve T cells express the CD45RA isoform of the CD45 gene, whereas memory T cells preferentially express the CD45RO isoform. The treatment was deemed safe, with only 1 out of 19 treated patients developing grade 2 aGvHD that was successfully treated by steroids. Comparison with a historical cohort of haplo-HSCT patients treated with PT-Cy suggested a lower incidence of infections for the patients receiving CD45RA-depleted DLI.[13]

Here, we performed a prospective immunological study on the patients enrolled in the trial to better understand the impact of CD45RA-depleted DLI on immune reconstitution and responses. DLI administration led to a prominent expansion of selected T cell clones, suggestive of recruitment of these

cells in the immune response. Pathogen-specific memory T cells were able to engraft and persist for at least one month, potentially explaining the tendency to a lower incidence of viral infections observed for the CD45RA-depleted DLI-treated patients.

Methods

Patients

The results presented here are the immunological analysis of samples from patients enrolled in a phase II single-center prospective study (ClinicalTrials.gov Identifier: NCT04687982).[13] Inclusion criteria for the study were age ≥18 years and HSCT with PT-Cy for hematological disease, with myeloablative condition regimens, reduced intensity conditioning regimens or nonmyeloablative condition regimens. Exclusion criteria were active grade 2 to 4 aGvHD, uncontrolled infection, severe cytopenia and progressive disease at time of enrollment. Nineteen patients received CD45RA-depleted DLI, prepared from donor apheresis using CliniMACS and the CD45RA-depletion product line (Miltenyi Biotec). Patient characteristics are summarized in Supplemental Table 1. Patients received up to three CD45RA-depleted DLI, each 4 to 6 weeks apart and with dose escalation. The first DLI consisted of fresh cells and a dose of $5x10^5$ CD3/kg bodyweight, the second and third dose used thawed, cryopreserved aliquots with a dose of $1x10^6$ and $5x10^6$ CD3/kg bodyweight, respectively. Patients were monitored for CMV reactivation using polymerase chain reaction, twice a week from day +15 to day +100 and then weekly until day +180. 12 patients (63%) experienced CMV reactivation prior to receiving the first DLI. DLI-infused patients were compared to control haplo-HSCT with PT-Cy patients analyzed in previous studies,[14,15] using only patients that would have hypothetically matched the inclusion criteria for the clinical study. Blood samples from 10 additional patients treated with haplo-HSCT with PT-Cy at our institute were used as controls for TCR-seq analysis. The trial was approved by the local independent Ethic committee (approval no. ONC-2016-002) and the clinical work was approved by the internal review board of the IRCCS Humanitas Research Hospital (protocols 8/2/2013 and 1397) and conducted in accordance with the Declaration of Helsinki. Signed written informed consent was obtained from all patients and donors.

TCR-seq

RNA was extracted from $3x10^5$ cells per sample using the Qiagen RNeasy Micro kit. For DLI samples not containing a sufficient number of cells, PBMCs from the donor were manually depleted of $CD45RA^+$ cells using CD45RA MicroBeads (Milteny Biotec). Two DLI samples were excluded due to insufficient RNA integrity as determined by Tape Station. Of the remaining 48 samples, libraries were constructed using the SMARTer Human TCR $\alpha\beta$ Profiling Kit v2 (Takara Bio USA), from which the CDR3 region of the TCR β chain was sequenced in a single run on the Illumina NextSeq. The frequency of TCR β chain clonotypes was determined using MiXCR (version 3.0.14)[16,17] with the following parameters: "--starting-material rna --receptor-type trb --region-of-interest CDR3 --5-end no-v-primers --3-end j-primers --only-productive". Libraries contained 2,334 to 44,388 functional CDR3β clonotypes. Exploratory analysis and diversity estimation of the TCRβ repertoire was performed with the Immunarch package (version 0.6.6)[18] for R, while repertoire overlap analysis was performed using VDJtools version 1.2.1[19] with the following parameters: "--intersect-type aa --top 10". The clonotype scatter plot was produced using a custom script based on the Matplotlib package (version 3.4.3). Annotation of clonotypes was performed with the Immunarch package, using the immune receptor databases VDJDB, McPAS-TCR and PIRD TBAdb.

Cell culture

Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed and stained directly, or rested for 6 hours at 37°C 5% CO_2 and then stimulated for 16 hours at 37°C 5% CO_2, in presence of GolgiPlug and GolgiStop (both BD Biosciences), 1 μg/mL soluble anti-CD28 (BD Biosciences) and either a pool of 138 peptides (15mers with 11 aa overlap) derived from CMV pp65 protein (1 μg/mL; JPT Technologies) or peptide solvent (DMSO) for unstimulated samples. Culture medium consisted of RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1% Ultra-glutamine and 1% penicillin-streptomycin (both Lonza).

Flow cytometry

Detailed flow cytometry procedures have been previously described.[20] Briefly, a fraction of the freshly thawed PBMCs were labeled with Zombie Aqua Fixable Viability dye and a flow cytometry panel designed to analyze NK cell, γδ T cell and ILC phenotypes (Supplementary Table 2). The cells cultured overnight were labeled with Zombie Aqua Fixable Viability dye and a flow cytometry panel designed to analyze T cell phenotypes (Supplementary Table 3). Fixation and staining for intracellular markers was performed with the FoxP3 transcription factor buffer kit (eBioscience). Cells were acquired on a BD FACSymphony A5.

Manual flow cytometric analysis

Flow cytometric compensation and gating was performed using FlowJo version 9. The gating strategy is shown in Supplemental Figure 1A-B. Frequencies of CMV-specific T cells were determined by the sum of IFN-γ-, TNF- and IL-2-expressing cells (Figure 5A). Boolean gating was used to define expression of combinations of cytokines. Background correction was applied using the unstimulated samples, and negative values were set to 0. Effector function analysis was performed with SPICE (version 5)[21].

Unsupervised high-dimensional analysis

Flow cytometric compensation and gating was performed using FlowJo version 9. After removing aggregates and $CD14^+$ cells and gating for live $CD3^+$ lymphocytes, a maximum of 1000 $CD8^+$ events and $CD4^+$ events from unstimulated samples were exported per for bulk T cell profiling. Within the $CD8^+$ and $CD4^+$ gates, Boolean gating was applied for 4-1BB, IFN-γ, TNF and IL-2. A maximum of 1000 activation marker-positive cells from peptide-stimulated and unstimulated samples were exported for CMV-specific T cell analysis. For NK cell profiling, aggregates, $CD14^+$, $CD3^+$ and $Lineage^+$ cells were removed, NK cells were identified based on expression of CD56 and/or CD16, and a maximum of 2000 events were exported per sample. Exported events were biexponentially transformed in FlowJo version 10 and clustered with PhenoGraph (version 1.5.3) as previously described,[22] using a pipeline in Python available at http://github.com/luglilab/Cytophenograph. The following *K* number of nearest neighbors were used: 50, 100, 50, 50 and 30 for bulk $CD8^+$ T cell, bulk $CD4^+$ T cell, NK cell, CMV-specific $CD8^+$ T cell and CMV-specific $CD4^+$ T cell analysis, respectively. A fixed seed of 123456 was used for all analyses. Clusters representing <

1% of the total events were excluded from downstream analysis. Balloon plots and heatmaps were generated using the ggplot2 package for R. To calculate background-corrected CMV-specific T cell cluster frequencies, cluster frequencies were first expressed as frequencies among total $CD8^+$ or $CD4^+$ T cells. Background correction was then applied by subtracting the frequency of a given cluster found in an unstimulated sample from the frequency of the same cluster found in the same sample stimulated with CMV peptides, and negative values were set to 0.

Statistics

GraphPad Prism software (version 7) and the R stats package were used for statistical analysis. Statistical significance was tested with Wilcoxon, Mann-Whitney or Kruskal-Wallis test. *P* values < 0.05 were considered significant.

Data Sharing Statement

For original data, please contact enrico.lugli@humanitasresearch.it.

Results

Limited contribution of CD45RA-depleted DLI on lymphocyte counts following haplo-HSCT.

19 recipients of haplo-HSCT with PT-Cy were enrolled in a phase II clinical study around day +50 after transplant, in which they received DLI with the aim of enhancing protection against opportunistic pathogens. The DLI product was depleted of $CD45RA^+$ cells, thus consisting almost exclusively of memory T cells (Figure 1A). Patients received up to 3 DLI with increasing cell number, each about one month apart. Blood samples were collected just before DLI administration, one week after DLI administration, and one month after the third DLI (Figure 1B). Blood samples of the patients, their donors, and of healthy controls harboring CMV-specific T cells were analyzed through high-dimensional flow cytometry and bulk TCR-sequencing (TCR-seq), to reveal the dynamics and immunological phenotypes of NK cells, and total and CMV-specific T cells (Figure 1C).

From day +50 (baseline) to day +150 (T7), counts of $CD8^+$, $CD4^+$ and γδ T cells gradually increased, while counts of NK cells and ILCs remained stable (Supplemental Figure 2). The number of $CD8^+$ T cells significantly increased one week after administration of the third DLI. Considering that the number of infused T cells was low compared to the total T cell pool, and no immediate increase in $CD4^+$ T cell counts was observed following DLI administration, the increase in $CD8^+$ T cell counts suggests that infused $CD8^+$ T cells had expanded.

We then compared cell counts of major lymphocyte subsets of the DLI-infused patients with that of control haplo-HSCT patients analyzed in a previous study.[14,15] Counts of $CD8^+$ T cells, $CD4^+$ T cells and NK cells were similar between the two groups, but rare ILC numbers were slightly decreased in the DLI-infused patients compared to the controls (Figure 1D). Manual gating of flow data revealed that, beside minor alterations in rare NK cell and ILC subsets, $CD8^+$ and $CD4^+$ naïve and memory subsets were remarkably similar (Figure 1E).

High-dimensional profiling reveals the T and NK cell dynamics following DLI infusion.

We analyzed T and NK cell phenotypes of DLI-infused patients in greater detail through uniform manifold approximation and projection (UMAP; Supplemental Figure 3) and unsupervised clustering using the PhenoGraph algorithm (Figure 2A-C and Supplemental Figure 4),[22] thereby taking advantage of all markers included in the high-dimensional flow panels. Transplanted patients showed a strong defect in naïve T cells (CD8 clusters 5, 13 and CD4 clusters 5, 10), an increased frequency of regulatory T cells (CD4 cluster 11) and HLA-$DR^{hi}CD95^{hi}$ activated phenotypes (CD8 clusters 7, 8, 12 and CD4 clusters 7, 9) that waned over time, and an accumulation of T-$bet^{hi}CX_3CR1^{hi}$ effector (CD8 cluster 3) and terminal effector clusters also expressing CD45RA and/or CD57 (CD8 clusters 1, 2, 4 and CD4 cluster 2), corroborating previous analyses.[14] Interestingly, PD-1 expression was increased on $CD4^+$ effector memory cells (cluster 8), while the patients also uniquely harbored a PD-$1^{hi}TIGIT^{hi}CD27^{hi}CD127^{lo}CCR7^{lo}$ $CD4^+$ transitional memory subset (cluster 4). Within the NK cell compartment, the frequency of cluster 1 of $CD56^{bright}$ cells and cluster 3 of $CD56^{dim}$ cells, both featuring high levels of NKG2A, CD94 and NKp30, was greatly increased at baseline, but diminished during follow-up. Instead, $CD56^{dim/lo}NKG2A^{lo}CD94^{lo}NKp30^{lo}$ cells (clusters 6, 7, 9) increased over time, as

previously reported.[15] Taken together, CD45RA-depleted DLI appear to have only a limited impact on lymphocyte reconstitution at the phenotypic, bulk level.

DLI-derived pathogen-specific memory T cells engraft and are recruited in the immune response.

Memory T cells detected in the patients could be derived from the DLI or the initial graft. To gain more insight in the specific contribution of DLI-derived clonotypes on immune reconstitution, we performed TCR-seq on blood samples of 10 patients receiving DLI and 10 control patients. We analyzed the clonal diversity by computing both the Chao1 estimator, a measure of TCR richness, and the Gini coefficient, a measure of the inequality among clonotype size within the repertoire. Even though the DLI lacked naïve T cells, the clonal diversity of the patient samples was even lower, indicated by a low Chao1 estimator and high Gini coefficient (Figure 3A), and consistent with a previous report on the very clonal nature of the T cell pool in HSCT patients.[23] Nevertheless, TCR richness significantly increased for control haplo-HSCT patients from day +50 to day +150, while that for the patients receiving the DLI did not. Instead, the inequality among clonotypes increased during follow-up for the DLI-infused patients, suggesting expansion of certain clones. The increased clonal diversity at T7 for control haplo-HSCT patients was attributed to a modest though significant increase in small clones, at the expense of medium clones (Figure 3B). For patients receiving DLI, no change was observed for small clones. Instead, there was a strong increase in 'hyper-expanded' clones (constituting > 1% of all TCR β sequences[24]), at the expense of medium and large clones. This suggests that CD45RA-depleted DLI do not increase clonal diversity of the T cell pool, but may nonetheless significantly contribute to T cell reconstitution through recruitment of infused clones in the immune response. Reactivation of CMV may have a strong impact on the clonality of the T cell pool through the process of memory inflation of CMV-specific T cells.[25] Yet, the difference in hyper-expanded clones between the two patient groups could not be solely attributed to a difference in the incidence of CMV reactivation (Supplemental Figure 5A), suggesting that this is a DLI-dependent phenomenon.

To further validate this finding, we explored the clonal overlap between baseline, T7 and DLI samples of 8 DLI-infused patients. Clonotypes overlapping between T7 and DLI, but not between T7 and baseline, were present in all 8 patients (Figure 4A, Supplemental Figure 5B). These DLI-derived clonotypes constituted up

to 11.9% (mean 6.5%) of all unique clonotypes at T7. When looking at their relative abundance, the contribution of DLI-derived clonotypes was even greater (mean 16.0%; Figure 4B, Supplemental Figure 5C). In patient #1, DLI-derived clonotypes made up 49.5% of the entire repertoire in terms of abundance one month after the final infusion. The 10 most abundant clones in this fraction contributed to 22.7% of the entire repertoire, underlining the expanded nature of these clones. Since CMV can strongly impact T cell reconstitution following haplo-HSCT with PT-Cy,[14] we asked whether CMV-specific T cells could be detected within the DLI-derived clones. Indeed, public clonotypes associated with CMV-specificity were detected in all patients, as were EBV- and influenza-specific clonotypes (Figure 4C). These DLI-derived pathogen-specific cells could have been administered with any of the three DLI and so their ability to persistent is at least one month.

Magnitude of the CMV-specific T cell response in DLI-infused patients correlates with the abundance of CMV-specific T cells in the donor.

CMV is one of the major pathogens causing infections early after HSCT, even when using T-replete grafts of a CMV^+ donor,[6] and despite the transfer of CMV-specific T cells with haplo-HSCT and PT-Cy.[5] DLI of enriched CMV-specific T cells from a donor have been reported to help resolve CMV infection.[26] Since CMV-specific memory T cells were transferred with the DLI, engrafted and persisted, we analyzed their number and phenotype in an *in vitro* CMV-pp65 peptide library stimulation assay. Comparing DLI-infused and control transplant patients revealed no significant difference in absolute numbers of CMV-specific T cells during follow-up, but a large inter-patient variability was apparent (Figure 5A). We next explored the quality of the CMV-specific T cell response by examining specific combinations of effector functions. Early after transplant, practically all CMV-specific $CD8^+$ T cells expressed IFN-γ (Figure 5B, Supplemental Figure 6A). During follow-up, the contribution of $CD8^+$ T cells producing solely IFN-γ decreased, and that of cells with three effector functions (CD107a, IFN-γ and TNF) increased, as previously reported.[14] IL-2 production was rare, and by T7 even significantly decreased in both patient cohorts compared to healthy controls. In contrast, IL-2 production was relatively common in the CMV-specific $CD4^+$ T cell compartment, with the two patient groups showing again a very similar spectrum of effector functions (Supplemental Figure 6B, 7A).

PhenoGraph clustering analysis revealed that in the DLI-infused patients, CMV-specific $CD8^+$ T cells predominantly display an effector phenotype (i.e. most abundant clusters 1-4, 6, 7 and 9) featuring high expression of T-bet, CX_3CR1 and CD57, and low expression of CCR7, CD27 and CD127 (Figure 5C, Supplemental Figure 8). 4-1BB and IFN-γ were the most commonly expressed activation markers. Likewise, the most prevalent CMV-specific $CD4^+$ T cell clusters (2, 3, 7 and 9) displayed an effector phenotype expressing T-bet, CX_3CR1 and CD57, although also several memory phenotypes were visible, though at low frequencies (Supplemental Figure 7B, 8). Of interest was the existence of multifunctional cluster 12 which expressed all three cytokines IFN-γ, TNF and IL-2, together with CD40L and 4-1BB, a phenotype that was previously associated with CMV viremia control following haplo-HSCT with PT-Cy.[14] Overall, the observed phenotypes and effector functions are very similar to those of haplo-HSCT patients who did not receive post-transplant DLI,[14] suggesting that antigen-specific cells infused with the DLI are not qualitatively different from those initially infused with the graft.

Overlaying the total frequency of CMV-specific T cell clusters on CMV viremia revealed that 13 out of 19 patients controlled CMV viremia following the development of a strong CMV-specific T cell response (Supplemental Figure 9). Nevertheless, 3 patients (#4, #7 and #8) did not develop a CMV-specific T cell response, despite the presence of multiple CMV viremia blips. Of note, patient #4 received the graft and DLI from a CMV^+ donor, suggesting that not all patients might benefit from adoptive transfer of donor-derived CMV-specific T cells in this setting. Finally, patients #2 and #14 developed neither CMV-specific T cells nor CMV viremia, indicating that the virus did not reactivate in these patients. For those patients that were able to mount a strong CMV-specific T cell response, the maximum expansion of CMV-specific cells positively correlated with the frequency of such cells in the peripheral blood of the donor (Figure 5D), suggesting that besides the quality also the quantity of transferred pathogen-specific memory T cells is of importance.

Discussion

T-replete haplo-HSCT with PT-Cy results in low rates of GvHD and non-relapse mortality and favorable immune reconstitution,[27] but nonetheless patients often still suffer from opportunistic pathogens, in

particular CMV reactivation.[6] In a phase II clinical trial, we investigated whether post-transplant infusions of memory T cells could enhance immune protection without increasing the risk of aGvHD. Cumulative incidence of viral infection from day +43 onwards was lower in the DLI cohort compared to a cohort of haplo-HSCT patients without post-transplant DLI (32% vs 53%, respectively).[13] Here, we report the in-depth immunological analysis and show that infused pathogen-specific memory T cells persist and clonally expand, potentially explaining the lower incidence of viral infections for patients receiving CD45RA-depleted DLI.

Considering the clonal nature of the T cell pool in haplo-HSCT patients, we initially hypothesized that DLI could increase clonal diversity. We did not find evidence for this, instead finding hyper-expansion of a small number of clones, suggesting recruitment of infused clones in the immune response. These results are in line with findings from murine studies demonstrating antigen-driven expansion of T cells following lymphocyte depletion.[28] Through such clonal expansion, DLI can have a profound effect on the T cell repertoire, exemplified by patient #1 having DLI-derived T cells constitute almost 50% of the entire clonal repertoire one month after the third DLI. Besides expansion of directly infused clones, DLI might also affect the dynamics of T cells transplanted with the initial graft.[29]

We detected public TCRs associated with the response against CMV, EBV and influenza in the fraction of engrafted clones that were present in the infusion product, but absent at baseline. These cells likely derive from the DLI, although we cannot exclude the possibility that they were also present at baseline at very low frequency and thereby not detected. Furthermore, these clonotypes could have been administered with any of the three DLI, and so their ability to persistent is at least one month. The DLI-derived clones that showed the greatest expansion did not feature public TCRs associated with the CMV, EBV or influenza response, but this does not rule out that these cells were in fact responding to such antigens. Indeed, CMV reactivation may have a profound effect on posttransplant T cell reconstitution.[30] A future study should couple antigen-specificity of DLI T cells with clonality to provide a definitive answer to this matter. An alternative explanation for the expansion of certain infused T cell clones is that these clones react to allo-

antigens. Even though adoptive transfer of memory T cells do not strongly induce aGvHD, they are still able to proliferate in response to allogeneic PBMCs *in vitro*.[5] In this regard, it would be interesting to follow a larger cohort of patients long-term to determine if CD45RA-depleted DLI lowers the relapse rate due to an improved graft-versus-tumor response.

Despite expansion of infused memory T cell clones, no major effects were found on the global phenotype of the T cell compartment, which early after transplant is composed mainly of activated and effector phenotypes, with low frequencies of naïve and early memory cells probably due to the highly inflammatory milieu and delayed thymic output.[5,31] It can be expected that even though DLI-derived memory T cells could be of an early memory phenotype initially, the clones that did not expand meant they contributed little to the overall T cell pool, while clones that hyper-expanded likely adopted an activated and effector phenotype, thus resulting in no net change of overall phenotype of the T cell compartment. No major changes in the effector functions and phenotypes of CMV-specific T cells were observed for DLI-treated patients. Still, a protective CMV-specific T cell response might be a numbers game, as there was a positive correlation between the maximum expansion of CMV-specific cells in the recipient and the frequency of CMV-specific cells in the donor. With this in mind, and considering that all infusions even up to the highest number was deemed safe,[13] we hypothesize that a single administration with a maximum number of memory T cells should be applied.

The use of CD45RA-depleted T cells has also been studied in other transplantation protocols.[32–34] In a randomized trial of TCR$\alpha\beta$-depleted allogeneic HSCT, post-transplant CD45RA-depleted DLI were associated with improved recovery of CMV-specific T cells in CMV-seropositive recipients, though CMV viremia incidence did not differ.[34] A study comparing CD45RA-depleted versus CD3-depleted haplo-HSCT found improved T cell reconstitution and reduced CMV and adenovirus viremia for the former method.[32] These studies further underline the potential protective effect of adoptive transfer of CD45RA-depleted T cells in the allogeneic transplant setting.

Collectively, our data show that post-transplant CD45RA-depleted DLI facilitate engraftment of pathogen-specific memory T cells in recipients of haplo-HSCT. These cells persist and can greatly expand, and thereby have a substantial impact on the clonal repertoire. In the case of CMV-specific T cells, these cells are highly functional. As such, CD45RA-depleted DLI-derived T cells are expected to contribute to control of opportunistic pathogens without increasing the risk of aGvHD.

Acknowledgements

This work was funded by the European Research Council (ERC-StG-2014 PERSYST #640511 to EL) and the Italian Ministry of Health (Grant Giovani Ricercatori GR-2013-02359185 to EL and RC). EL is a CRI Lloyd J. Old STAR (CRI award 3914). The purchase of a FACSymphony A5 was defrayed in part by a grant from the Italian Ministry of Health (agreement 82/2015).

Authorship Contributions

JJPvB and EL conceived the study. CDP, BS, IT, RC, DM, RM, JM, RC, AS, LC and SB provided clinical samples and clinical information. JJPvB, FDP, EZ, MC and AS performed the experiments. CP, GB and JC sequenced the TCRs. JJPvB, SP and CDV analyzed the data. JJPvB and EL wrote the manuscript. LC, SB, DM and EL supervised the study. All authors contributed to and approved the final manuscript.

Conflict of Interest Disclosure

EL received honoraria and in-kind reagents from BD Biosciences Italy, preclinical funding from Bristol-Myers Squibb on topics unrelated to the content of this manuscript and royalties for a patent that describes methods for generating and isolating T_{SCM} cells. JJPvB is currently employed at Janssen. EZ is currently employed at Sanofi. AS has received honoraria as advisory board member from Bristol-Myers Squibb, Servier, Gilead, Pfizer, Eisai, Bayer and Merck Sharp & Dhome; as speaker's bureau member from Takeda, Roche, Abb-vie, Amgen, Celgene, Astrazeneca, Lilly, Sandoz, Novartis, Bristol-Myers Squibb, Servier, Gilead, Pfizer, Arqule, Eisai; and for consultancy from Arqule.

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Figure Legends

Figure 1. Immune reconstitution following haplo-HSCT with CD45RA-depleted DLI. (**A**) CD45RA expression by lymphocytes in the DLI product before and after depletion of $CD45RA^+$ cells. A representative DLI product is shown. (**B**) Schematic overview of DLI administration and blood sample collection. Median days post-transplant are indicated. (**C**) Experimental approach. Blood samples from DLI-infused patients, their donors and unrelated healthy controls were stained with a flow cytometry panel allowing the detection of innate lymphocytes. The remaining cells of each sample were cultured overnight in presence or absence of a CMV-peptide library, and stained with T cell-focused flow cytometry panel. Unstimulated samples were used for bulk T cell analysis, while CMV-specific cells were identified through Boolean gating for activation markers in response to CMV peptide stimulation. NK cells, bulk and CMV-specific T cell phenotypes were analyzed in more detail with unsupervised clustering. In addition, TCR sequencing of blood and DLI samples from DLI-infused and control patients was performed to assess the impact of the DLI at the clonal level. (**D**) Median lymphocyte counts with interquartile range in haplo-HSCT patients receiving CD45RA-depleted DLI or not (*n* = 7–17/group). Statistical significance was determined with Mann-Whitney test. (**E**) Median lymphocyte counts with interquartile range at T7 in haplo-HSCT patients receiving CD45RA-depleted DLI or not (*n* = 10–14/group). Statistical significance was determined with Mann-Whitney test. ILC1, group 1 innate lymphoid cell; ILC2, group 2 innate lymphoid cell; ILC3, group 3 innate lymphoid cell; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{EMRA}, effector memory T cell re-expressing CD45RA; T_N, naïve T cell.

Figure 2. T and NK cell dynamic changes following haplo-HSCT with CD45RA-depleted DLI. (**A**-**C**) Total $CD8^+$ T cells (A), $CD4^+$ T cells (B) and NK cells (C) were clustered by PhenoGraph. Cluster identities are revealed by balloon plots, while the heatmaps show median cluster dynamics in DLI-infused patients (*n* = 19) at different time points, their donors (*n* = 18), and unrelated CMV^+ healthy controls (*n* = 12). Samples containing < 50 total $CD8^+$ T cells, $CD4^+$ T cells or NK cells were excluded from temporal analysis. Act, activated; HC, healthy control; T_{CM}, central memory T cell; T_{EF}, effector T cell; T_{EM}, effector memory T cell; T_{EMRA}, effector memory T cell re-expressing CD45RA; T_M, memory T cell; T_N, naïve T cell; T_{REG}, regulatory T cell; T_{TE}, terminal effector T cell; T_{TM}, transitional memory T cell.

Figure 3. CD45RA-depleted DLI induce hyper-expansion of selected T cell clones. TCR-seq was performed on samples of DLI-infused patients collected at baseline and T7 (*n* = 10 patients), the DLI (*n* = 8), and samples of control haplo-HSCT patients (*n* = 10 patients) collected around day +50 and day +150. (**A**) Left graph shows the Chao1 diversity index, right graph shows the Gini diversity index. Medians are indicated. Statistical significance was determined by Wilcoxon test. (**B**) Clonotypes were categorized into small, medium, large, and hyper-expanded clones which constituted < 0.01%, 0.01% – 0.1%, 0.1% – 1%, and > 1% of the repertoire, respectively. Medians are indicated. Statistical significance was determined by Wilcoxon test.

Figure 4. DLI-derived memory T cells engraft and are recruited in the immune response. (**A**) Venn diagram shows the clonal overlap in number of unique clonotypes of patient #1. The bar graph shows the clonal overlap at T7 with the DLI separately for DLI-infused patients as frequency of the total number of unique clonotypes at T7, while their average is shown in the pie chart. (**B**) Left graph shows the relative abundance of clonotypes overlapping between the DLI and T7 of patient #1. Roman numerals indicate the clonotype fractions shown in (A). The top 10 most abundant clones of fraction III are highlighted in color. The bar graph shows the abundance of overlapping clonotypes at T7 separately for DLI-infused patients, while their average is shown in the pie chart. (**C**) DLI-derived engrafted clonotypes (fraction III) of each patient were

analyzed for the presence of public TCR sequences known to harbor specificity for CMV-, EBV- or influenza-derived epitopes.

Figure 5. Magnitude of recipient CMV-specific T cell responses correlates with the abundance of CMV-specific T cells in the donor. (**A**) Counts of CMV-specific T cells in CMV-reactivating haplo-HSCT patients receiving CD45RA-depleted DLI or not (*n* = 9–16/group). Medians with interquartile range are shown. Statistical significance was determined with Mann-Whitney test. (**B**) Effector functions of CMV-specific $CD8^+$ T cells in haplo-HSCT patients receiving CD45RA-depleted DLI or not, and in CMV^+ healthy controls (*n* = 6–20/group in pie charts, *n* = 10–20/group in bar graphs). Samples containing < 35 CMV-specific $CD8^+$ T cells were excluded from analysis. Medians are shown. Statistical significance was determined by Kruskal-Wallis and post-hoc Dunn's test with Bonferroni correction. (**C**) CMV-specific $CD8^+$ T cell PhenoGraph cluster identities are revealed by balloon plot, while the heatmap shows median cluster dynamics in CMV-reactivating, DLI-infused patients (*n* = 17), their donors (*n* = 16), and CMV^+ healthy controls (*n* = 12). Samples containing < 50 total $CD8^+$ T cells were excluded from temporal analysis. (**D**) Linear regression with 95% confidence interval bands on the maximum measured abundance of CMV-specific T cells in the DLI-infused recipient versus the frequency of CMV-specific T cells in the peripheral blood of the donor. Only patients with a clear CMV-specific T cell response are included in the analysis (*n* = 12; one patient was excluded because of missing donor sample). Count values were log-transformed. HC, healthy control; Multif, multifunctional; T_{CM}, central memory T cell; T_{EF}, effector T cell; T_{EM}, effector memory T cell; T_{EMRA}, effector memory T cell re-expressing CD45RA.

Figure 1

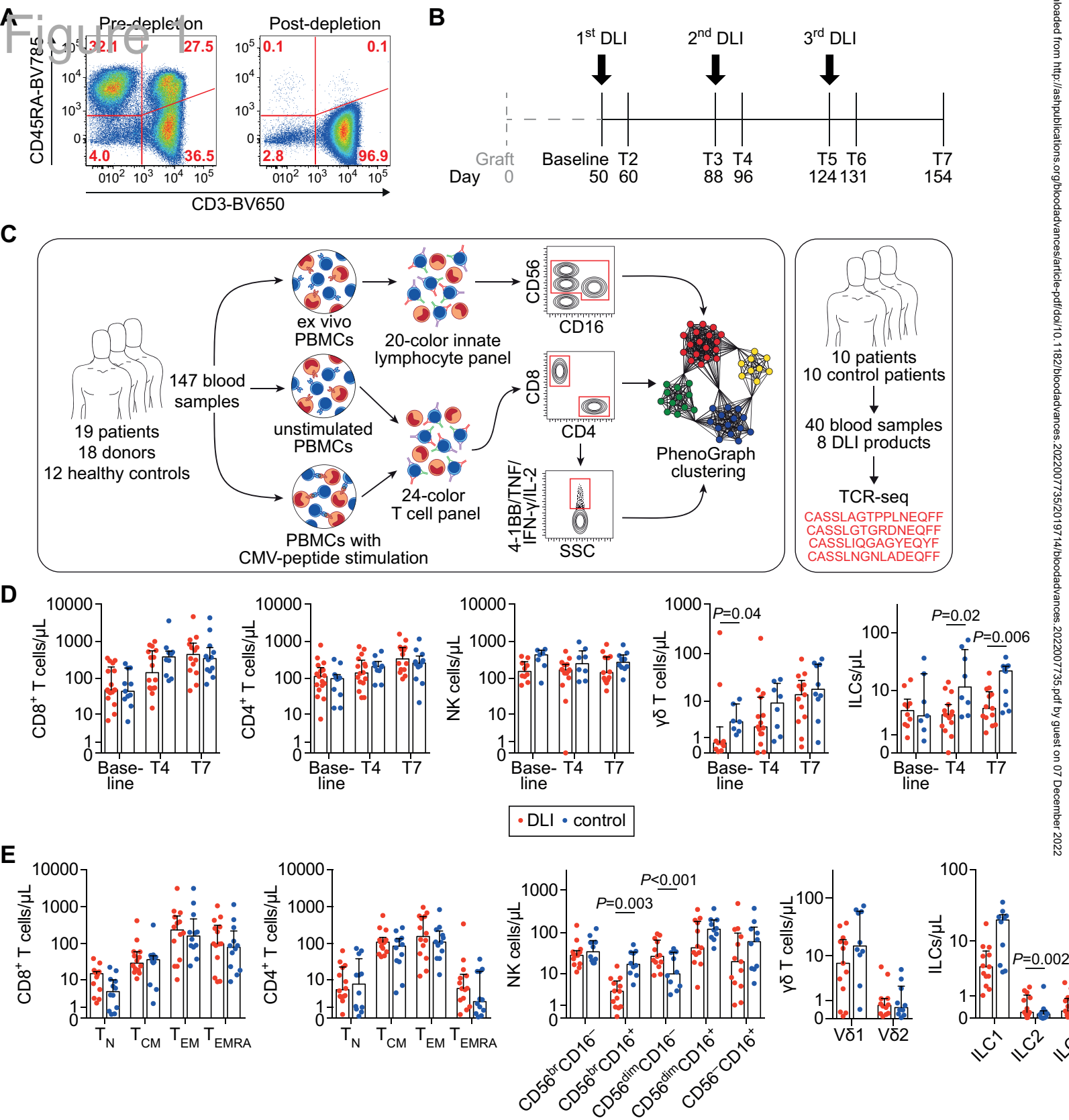


Figure 2

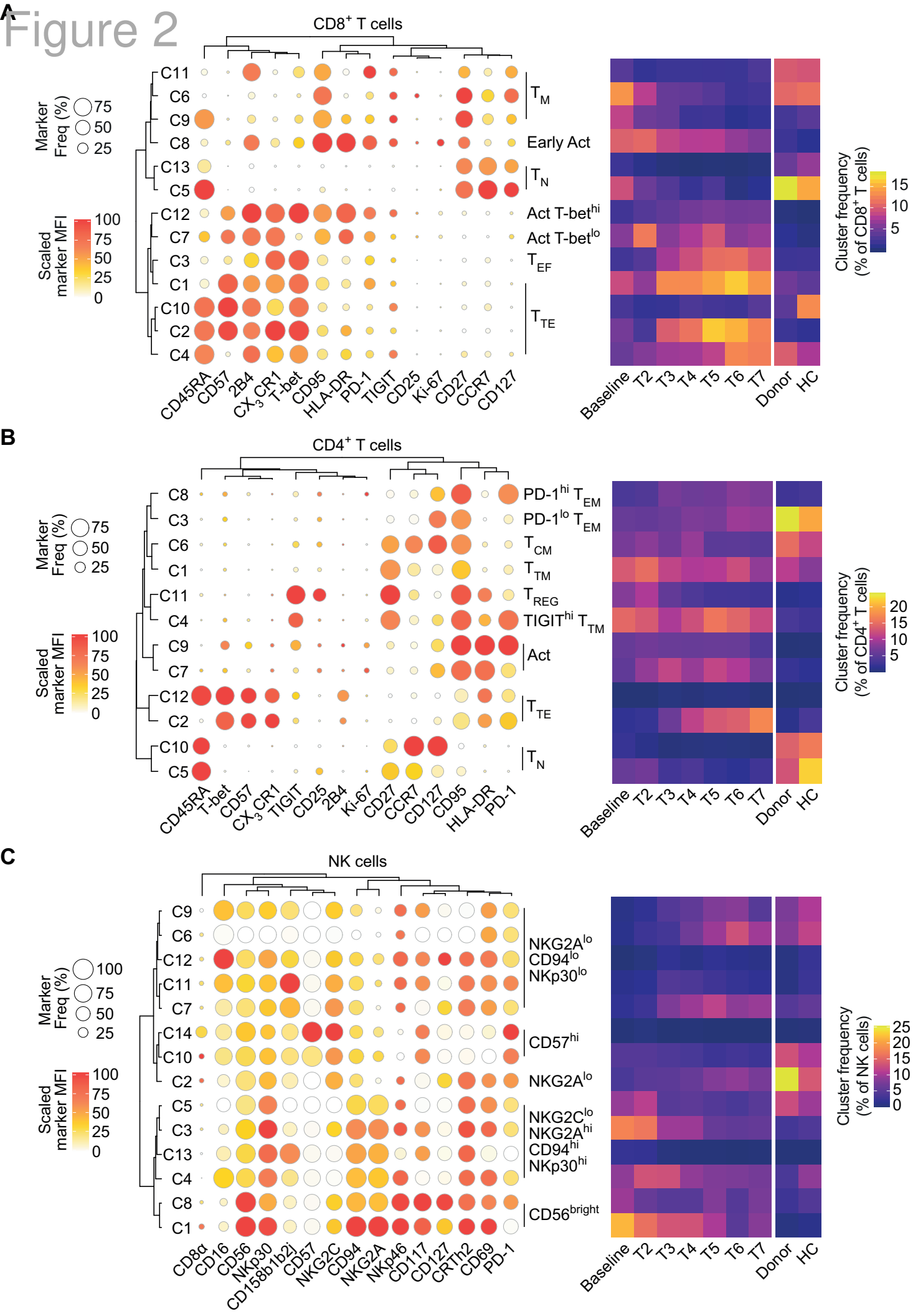
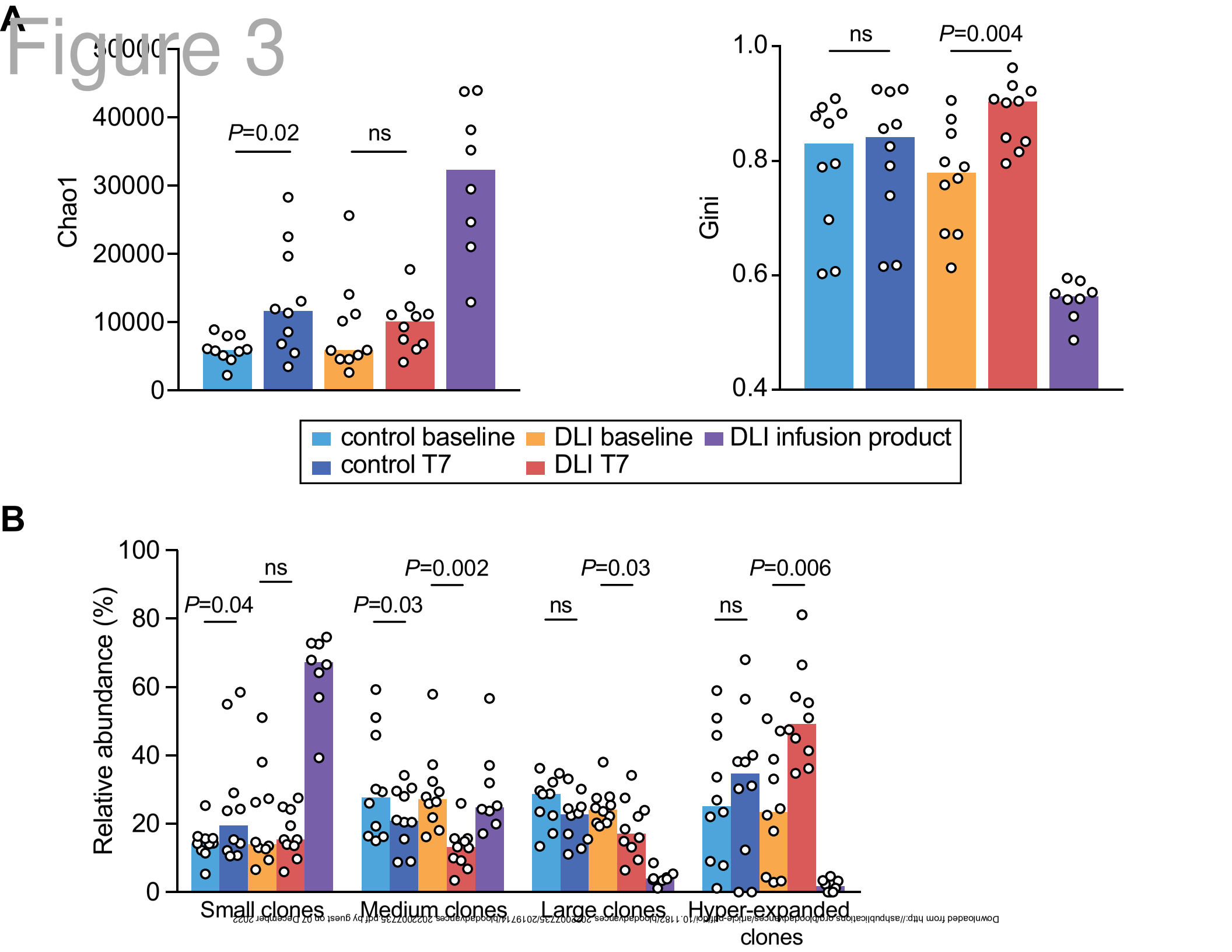


Figure 3



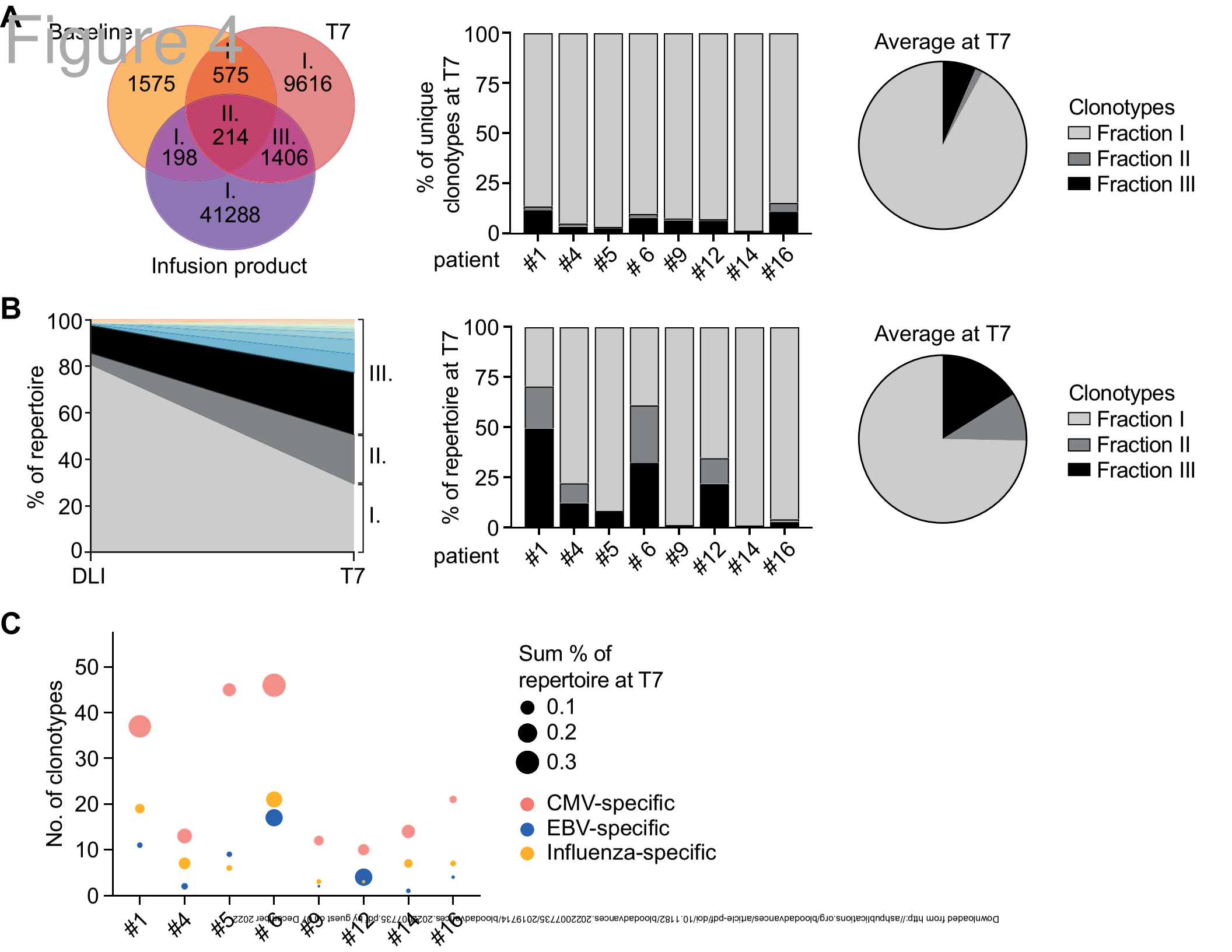


Figure 5