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Early recovery of natural killer cells post T-cell depleted allogeneic stem cell transplantation using alemtuzumab "in the bag"

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ABSTRACT

Background: Allogeneic stem cell transplantation (SCT) is a critical therapy for haematological malignancy but may lead to acute and chronic graft *versus* host disease (GvHD). T-cell depletion with alemtuzumab, either *in vivo* or *ex vivo,* reduces the incidence of GvHD but is a risk factor for disease relapse and poor immune reconstitution. Natural killer (NK) cells are the first lymphocytes to recover. Classical NK cells make up >90% of the normal circulating population and can directly kill neoplastic or virally infected cells while the regulatory subset makes up <10%, secretes cytokines and is not cytotoxic. The recovery and balance of these subsets post SCT remains controversial, with most studies analysing patients who received unmanipulated grafts and *in vivo* immunosuppression.

Objective: The aim was to assess the early recovery of NK cells in 18 consecutive patients receiving *ex vivo* T-cell depleted SCT and to compare the results to 25 individuals receiving haploidentical non-T cell depleted grafts.

Methods: All patients received myeloablative conditioning. After stem cell collection, the stem cells of the T cell depleted group were treated "in the bag" with alemtuzumab (CAMPATH 1H) at a concentration of 1 mg/10^8 mononuclear cells and thereafter immediately infused. For those receiving non-T cell depleted grafts, GvHD prophylaxis was with post infusion therapeutic doses of cyclophosphamide. Blood samples were collected at days 21, 28 and 90. Complete blood counts were performed on an automated analyser while lymphocyte and NK subsets were examined using multiparameter flowcytometry. NK cells were defined as lymphocytes which were CD3-/CD56+. The classical subset was recognised as $CD56^{dim}$/CD16+ while the regulatory population as $CD56^{bright}$/CD16-. The results for both transplant types were compared at all time points using SPSS v8 statistical software.

Results: The recovery of lymphocytes was slow in both groups. Those receiving non-T cell depleted grafts had significantly higher T cell counts at day 21 and 28 when compared to the T cell depleted group ($P < 0.05$). In contrast, NK cells in the *ex vivo* T-cell depleted patients recovered rapidly and by day 21 was no different to normal ($p > 0.05$), while the non-T cell depleted group had significantly decreased numbers ($p < 0.001$), only recovering at day 90. Both groups had abnormal NK cell subset ratios with significantly elevated percentages of regulatory cells ($p < 0.05$). However, significant differences were observed between the two groups with those receiving T cell depleted grafts having lower percentages of regulatory cells as well as higher numbers of classical NK cells at day 21 and 28 ($p < 0.01$).

Conclusion: This study of *ex vivo* T-cell depleted SCT's demonstrates that NK cells recover quicker when compared to those receiving unfractionated grafts. These results may have implications for GvHD and the GvL effect which warrants further study.

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1. Introduction

Stem cell transplantation (SCT) has been successful in treating both malignant and non-malignant hematological disease. However, clinical outcomes are often limited by acute and chronic graft-versus-host disease (GvHD). This occurs due to the host tissue suffering from the combined effects of the conditioning regimen and cytotoxic attack by allogeneic donor T cells which are present in the infused graft [1].

Alemtuzumab, an anti-CD52 antibody, has been used both *in vivo* and *ex vivo* to effectively prevent GvHD [2]. The humanized form of the antibody (CAMPATH-1H) binds to the CD52 antigen which is present in different concentrations on the surfaces of T and B lymphocytes, monocytes, and granulocytes. CAMPATH-1H was initially registered for the treatment of chronic lymphocytic leukemia but is also effective *in vivo* to prevent GvHD, and has been successful, *ex vivo* or "in the bag", in depleting stem cell grafts of T cells [2–4]. This method is commonly used in South Africa with a study of 120 patients receiving allogeneic grafts treated with *ex vivo* CAMPATH 1G (a rat IgG2B antibody), at Groote Schuur Hospital in Cape Town, demonstrating an inverse relationship between an increasing dose of the antibody, prevention of GvHD and improved outcomes [5]. Although the *ex vivo* use of alemtuzumab in SCT has significantly reduced the prevalence of GvHD, it has led to delayed immune reconstitution, resulting in higher risks of infection and relapse of the original malignancy, particularly chronic myeloid leukemia [6].

Natural killer (NK) cells normally constitute 5-15% of peripheral blood lymphocytes, form part of the innate immune response and are the first cells to recover after transplantation. Based on the surface expression of CD16 and CD56, five NK cell populations have been identified in the peripheral blood, with the two major ones being the regulatory ($CD56^{bright}$/CD16-) and the classical ($CD56^{dim}$/CD16+). Classical NK cells make up the majority (>90%), are cytotoxic and contain perforin and granzyme rich granules. This subset can directly lyse virally infected and tumour cells provided that inhibitory receptors on the NK cell surface cannot recognize MHC class I molecules on the target cell. This is known as the "missing-self hypothesis" [7,8]. The killer cell immunoglobulin like receptor (KIR) and CD94/NKG2A are inhibitory antigens, and when they bind to their corresponding MHC class I antigen, serve to suppress activation. When target cells have reduced MHC class 1 expression, the balance between activation and inhibition changes, and cytotoxic killing by NK cells is initiated [8]. In contrast, regulatory NK cells ($CD56^{bright}$/CD16-) are not cytotoxic but secrete cytokines including interferon-gamma (IFN-γ), tumour necrosis factor (TNF), interleukin (IL)-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [8,9].

Investigations of early allogeneic donor NK cell recovery have shown that NK cells could improve engraftment, promote the graft versus leukaemia (GvL) effect and enhance overall outcomes [10–12]. Furthermore, others have demonstrated that donor NK cells may assist in the prevention of GvHD by inhibiting or targeting alloreactive T cells [3]. This hypothesis has however been challenged by studies suggesting that the secretion of inflammatory cytokines by regulatory NK cells could activate T cells and promote GvHD [13]. Most studies were performed on patients receiving non-T-cell depleted grafts with the addition of *in vivo* immunosuppression such as cyclosporin [13]. In addition, for haploidentical donor transplants, post-transplant cyclophosphamide may further delay recovery. The variation in GvHD prevention methods could account for the conflicting results.

Although it is evident that the early recovery of donor NK cells may play a significant role in the success of SCT, the effects of *ex vivo* alemtuzumab "in the bag" on the recovery of NK cells remains unclear.

2. Hypothesis and objective

We hypothesised that by using alemtuzumab to T-cell deplete the graft prior to infusion, the early recovery of NK cells could be enhanced. The objective of this study was therefore to examine the early recovery of NK cell subsets at days 21, 28 and 90 post graft infusion in eighteen patients receiving *ex vivo* T-cell depleted allogeneic stem cell grafts and to compare these results to healthy controls and twenty-five patients receiving haploidentical non-T cell depleted grafts.

3. Research design and methods

3.1. Ethical statement

The study was approved by the Research Ethics Committee of the University of Cape Town and Groote Schuur Hospital (871/2015) and was carried out according to the principles of the Declaration of Helsinki. All participants were required to provide informed consent after the study and procedures were fully explained in their language of choice.

3.2. Study design

This was a prospective laboratory-based study that took place between 2015 and 2018. Eighteen consecutive patients who received *ex vivo* alemtuzumab-treated allogeneic stem cell grafts from HLA- identical sibling donors were compared to seven healthy controls and twenty-five individuals receiving haploidentical non-T cell depleted stem cell grafts from related donors. The groups were age matched with the mean age of those receiving T cell depleted grafts being 27 years (SD +/-17), the non-T-cell depleted group 32 years (SD +/-21) and the healthy controls 32 years (SD+/- 16).

Patients received fractionated myeloablative conditioning which consisted of a combination of total body irradiation (TBI, 12 Gy) and fludarabine. After myeloablative conditioning and infusion of the alemtuzumab treated stem cell graft, lymphocyte subsets and NK cell recovery were analysed and compared at 21, 28 and 90 days.

3.3. Stem cell collection and GvHD prevention

Donor CD34+ cells were mobilised with 5-10μg/kg of G-CSF for five consecutive days. Thereafter the peripheral blood stem cells were collected using a Cobe Spectra or Optia apheresis machine. The number of CD34+ peripheral blood stem cells was measured on a Facs Canto flow cytometer (Becton Dickinson New Jersey, United States) using standardised flow cytometric techniques as recommended by the International Society for Hematotherapy and Graft Engineering (ISHAGE) [14]. A minimum of 2×10^6/kg CD34+ stem cells were collected.

For those patients receiving T-cell depleted grafts, the peripheral blood stem cell bag was treated "in the bag" by adding alemtuzumab (CAMPATH 1H) at a concentration of 1 mg/10^8 mononuclear cells [5,15]. The stem cell bag was incubated at room temperature for 30 minutes and thereafter immediately infused into the patient without further manipulation.

Those receiving haploidentical non-T cell depleted transplants obtained stem cells from haploidentical related donors as they were mostly non-Caucasian and a matched unrelated donor (MUD) from international registries was unavailable. The preconditioning regimen consisted of cyclophosphamide, fludarabine and myeloablative TBI of 8-10Gy. After infusion of the unmanipulated stem cells, GvHD prevention followed a protocol described by Luznik et al (2008). Briefly, this comprised of 50mg/kg cyclophosphamide, together with Mesna, which was administered at day 3-4 post infusion of the graft [16].

3.4. Full blood count and flow cytometry of lymphocyte subsets

Five millilitres of peripheral blood, from both the T cell depleted and unfractionated transplant patients, were collected into EDTA at days 21, 28 and 90 post infusion of the stem cell graft. A full blood count (FBC) and differential white cell count was performed in an ISO 15189 accredited laboratory on an automated full blood count analyser

(Sysmex XN-9000; Sysmex Corporation, Kobe, Japan). To measure the immune recovery of T cells, B cells and NK cells, the following fluorescent conjugated antibodies were used; CD3-FITC; CD16-PC7, CD45-APC (Beckman Coulter, California, United States) and CD4-PerCP, CD8-PerCP, CD19-FITC and CD56-PE (Becton Dickinson). Briefly, 100μl of peripheral blood was incubated with the relevant concentration of antibody at room temperature for 20 minutes. Following incubation, the red cells were lysed using FACslyse solution (Becton Dickinson) and thereafter the remaining white cells were washed twice at 2000 rpm and reconstituted with 0.5 ml phosphate buffered saline (PBS) before analysing on a FacsCalibur flow cytometer (Becton Dickinson) using a standard four colour protocol. CD45 APC was used as a backbone marker to gate the lymphocytes ($CD45^{bright}$, low SS) and a relevant isotypic control was included to identify non-specific binding of the antibodies. Analysis was performed using CellQuest Pro software (Becton Dickinson) and the absolute values for each subset was calculated using the automated lymphocyte count.

3.5. Analysis and gating of natural killer cells

In both the T-cell depleted and haploidentical non-T-cell depleted groups, NK cells were further analysed for expression of CD56 and CD16 using a sequential gating strategy. NK cells were identified as lymphocytes ($CD45^{bright}$/low SS) which were CD3-/CD56+. After isolating the NK cells the proportion of classical and regulatory NK cells were analysed. Classical NK cells were defined as $CD56^{dim}$/CD16+ while the regulatory or immature NK population was defined as $CD56^{bright}$/CD16-. Fig. 1 depicts the gating strategy used to differentiate the two NK cell populations.

3.6. Statistics

All results were captured on an excel spreadsheet (Microsoft 2013, version 15.0.4420.1017). For the statistical analysis the software SPSS v.28 (IBM Corp., 2021) was used. The data was tested for normality using the normality Q-Q plot. A p-value of >0.05 was used to determine a normally distributed variable. Continuous variables were presented as mean (standard deviation) for normally distributed variables, while median (25th and 75th percentiles) was used for skewed values. For comparison, the analysis of variance test (ANOVA) or Kruskal-Wallis test was used as appropriate. A p value < 0.05 was used to characterize statistically significant results.

4. Results

4.1. Basic characteristics of the study subjects

There was no significant difference in the age of the participants between the two transplant types (p> 0.05). The mean age of those receiving T cell depleted grafts was 27 years (SD +/-17) while the non-T-cell depleted group had a mean age of 32 years (SD +/-21). Fourteen patients were diagnosed with a haematological malignancy (7 Acute Lymphoblastic leukaemia, 6 Acute Myeloid leukaemia and 1 Follicular lymphoma) while the remaining three had non-malignant disorders (2 Aplastic anaemia, 1 Fanconi syndrome). Ten patients (56%) receiving T cell depleted grafts were female compared to 14 (56%) in the non-T cell depleted group while the healthy controls comprised of 2 females and 5 males. Patients who had a donor of the opposite sex (11 T cell depleted and 14 unfractionated) underwent chimerism testing on day 28 by fluorescence in vitro hybridization (FISH) for the presence or absence of the Y chromosome (VYSIS CEP XY probe, Abbott Molecular probe, Illinois, United States) and were found to have 100% chimerism indicating successful engraftment. In addition, the recovery of neutrophils was measured in all patients and once this had reached 500 cells/μl, it indicated successful engraftment.

4.2. Lymphocyte subset recovery

As expected, in both groups, the median absolute white cell count (WCC) and total lymphocyte (Ly) count was slow to recover and remained below the normal range at day 90. The rate of total lymphocyte recovery was similar between the two transplant types, with no significant difference being detected at all three time points (p>0.05).

CD19+ B cells remained significantly reduced at days 21 and 28, with similar values being observed between the two transplant types (p>0.05). By day 90 however, the T cell depleted transplants, although still decreased compared to normal, had significantly higher numbers of B cells (p=0.008). In contrast, although all T cell subsets remained below normal levels throughout, those receiving unmanipulated non-T cell depleted grafts recovered at a quicker pace, with total numbers of T cells as well as CD4+ T helper cells (Th) and CD8+ cytotoxic T cells (Tc), being significantly higher compared to those receiving T cell depleted transplants (p <0.05). At day 90, although the CD4+Th subset remained significantly below normal, the absolute counts were similar between the two transplant types (p 0.08) (Table 1).

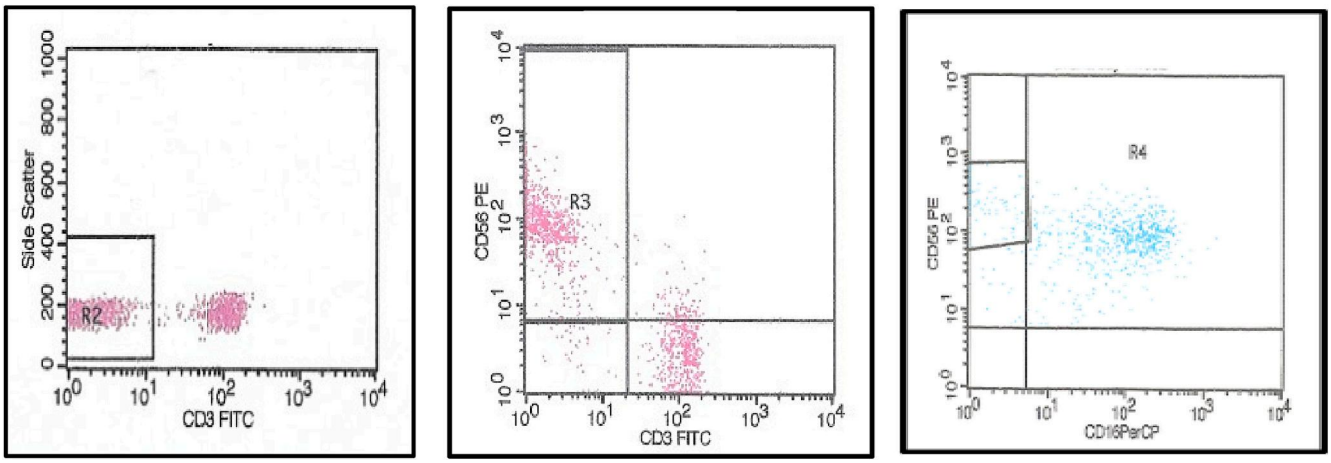

Fig. 1. Gating strategy: After selecting the mature lymphoid population (CD45++/lowSS), all CD3+ T cells were excluded (R2). Natural killer cells were identified as those cells which were CD56+ but CD3- (R3). The cells in R3 were further analysed by creating a dot plot with CD16 (x axis) and CD56 (y axis). The % of classical NK cells ($CD56^{dim}$/ CD16+) and immature or regulatory NK cells ($CD56^{bright}$/CD16-) could then be measured.

Table 1
Lymphocyte subset recovery post stem cell infusion in patients receiving T-cell depleted grafts treated with alemtuzumab "in the bag" and haploidentical non-T cell depleted grafts.

	Non-T dep ($N = 25$)	T dep ($N = 18$)	p-value (Non-T dep *vs* T dep)	p-value (Healthy Control *vs* Non-T dep)	*p*-value (Healthy Control *vs* T dep)
	Median (25th–75th percentile)	Median (25th–75th percentile)			
		Day 21			
Lymphocytes x10^9/l	0.35 (0.11; 0.8)	0.425 (0.125; 0.843)	0.825	<0.001	<0.001
CD19x10^9/l	0.004 (0.001; 0.01)	0.003 (0.001; 0.02)	0.469	<0.001	<0.001
CD3 × 10^9/l	0.315 (0.162; 0.805)	0.02 (0.007; 0.11)	0.003	<0.001	<0.001
CD4 × 10^9/l	0.1 (0.025; 0.248)	0.03 (0.01; 0.04)	0.032	<0.001	<0.001
CD8×10^9/l	0.225 (0.033; 0.338)	0.04 (0.01; 0.08)	0.028	0.002	<0.001
NK cellsx10^9/l	0.02 (0.003; 0.11)	0.19 (0.065; 0.385)	<0.001	<0.001	0.567
CD56 bright %	43.6 (29.185; 58.75)	15.135 (5.935; 32.193)	0.005	<0.001	0.017
CD56 bright ×10^9/l	0.01 (0.002; 0.045)	0.035 (0.009; 0.078)	0.121	0.774	0.071
CD56 dim%	48.5 (23.75; 66.55)	85.37 (67.815; 93.438)	0.003	<0.001	0.025
CD56dim × 10^9/l	0.006 (0.002; 0.11)	0.195 (0.06; 0.328)	0.004	0.002	0.37
WCC ×10^9/l	1.96 (0.67; 5.715)	2.87 (1.3; 5.04)	0.546	0.002	0.002
NK cells% of Ly	5.47 (1.445; 12.365)	61.91 (24.155; 78.05)	<0.001	0.156	0.002
		Day 28			
Lymphocytes ×10^9/l	0.66 (0.385; 1.035)	0.415 (0.155; 0.783)	0.155	<0.001	<0.001
CD19×10^9/l	0.004 (0.001; 0.02)	0.006 (0.002; 0.009)	0.534	<0.001	<0.001
CD3 × 10^9/l	0.57 (0.375; 1)	0.08 (0.03; 0.188)	<0.001	<0.001	<0.001
CD4 × 10^9/l	0.13 (0.04; 0.32)	0.03 (0.01; 0.075)	0.002	<0.001	<0.001
CD8×10^9/l	0.42 (0.2; 0.6)	0.03 (0.015; 0.13)	<0.001	0.006	<0.001
NK cells×10^9/l	0.02 (0.002; 0.08)	0.235 (0.07; 0.518)	<0.001	0.002	0.739
CD56 bright %	66.6 (35.5; 82.7)	14.49 (4.568; 32.318)	0.002	0.002	0.025
CD56 bright ×10^9/l	0.018 (0.001; 0.045)	0.035 (0.01; 0.07)	0.12	0.540	0.027
CD56 dim%	33 (16.75; 55.25)	85.51 (58.088; 95.185)	<0.001	<0.001	0.030
CD56dim × 10^9/l	0.005 (0.001; 0.045)	0.19 (0.1; 0.45)	<0.001	0.003	0.626
WCC ×10^9/l	4.75 (3.26; 7.625)	3.64 (2.098; 6.383)	0.446	0.053	0.046
NK cells% of Ly	4.295 (1.428; 8.158)	59.72 (41.473; 81.275)	<0.001	0.089	<0.001
		Day 90			
Lymphocytes ×10^9/l	1.09 (0.66; 1.45)	0.8 (0.695; 1.235)	0.330	<0.001	0.003
CD19×10^9/l	0.055 (0.008; 0.133)	0.205 (0.06; 0.355)	0.008	<0.001	0.063
CD3 × 10^9/l	0.76 (0.558; 1.185)	0.49 (0.34; 0.715)	0.013	0.001	<0.001
CD4 × 10^9/l	0.24 (0.158; 0.388)	0.15 (0.09; 0.263)	0.082	<0.001	<0.001
CD8×10^9/l	0.615 (0.342; 0.825)	0.35 (0.183; 0.545)	0.017	0.060	0.001
NK cells×10^9/l	0.165 (0.068; 0.263)	0.1 (0.065; 0.37)	0.733	0.042	0.113
CD56 bright %	45.5 (32.4; 71.3)	15.84 (3.38; 23.89)	0.001	<0.001	0.033
CD56 bright ×10^9/l	0.07 (0.02; 0.13)	0.015 (0.01; 0.05)	0.058	0.006	0.169
CD56 dim%	54.4 (28.6; 67.5)	83.49 (76.11; 95.2)	0.001	0.001	0.042
CD56dim × 10^9/l	0.095 (0.03; 0.16)	0.065 (0.048; 0.32)	0.597	0.006	0.096
WCC × 10^9/l	3.91 (3.33; 8.1)	3.565 (2.143; 6.15)	0.240	0.040	0.009
NK cells% of Ly	11.58 (8.135; 20.083)	13.71 (9.258; 30.593)	0.446	0.304	0.128

Abbreviations: T-dep: T cell depleted; non-T dep: non-T cell depleted; WCC: white cell count; Ly: lymphocytes; Th; T helper cells; Tc: Cytotoxic T cells; NK cells: Natural killer cells.

4.3. Natural killer cells

The pace of CD56+ NK cell recovery in those receiving stem cell grafts depleted of T cells was significantly quicker in the early stages post infusion compared to those receiving unmanipulated grafts ($p<0.001$) and, by day 21, their values were not significantly different to normal (0.19 vs 0.28; $p>0.05$). By day 90 there was no significant difference in the total NK cells numbers between the two transplant types (Fig. 2).

4.4. Recovery of classical $CD56^{dim}$ and regulatory $CD56^{bright}$ natural killer cells

After gating the CD56+/CD3- population and excluding all T cells, the percentage of classical $CD56^{dim}$ and regulatory $CD56^{bright}$ NK cells was measured at each time point. Although the results showed a large variation, there was a significantly reduced percentage of classical $CD56^{dim}$ NK cells in the non-T cell depleted transplants compared to those who had received T cell depleted grafts at day 21 ($p = 0.003$), day 28 ($p < 0.001$) and day 90 ($p < 0.001$). Conversely, although increased

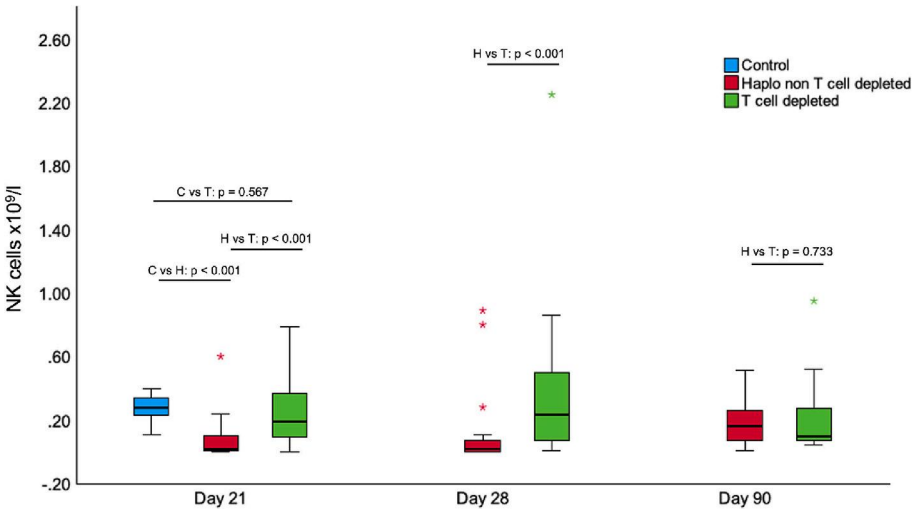

Fig. 2. The absolute number of NK cells in 18 patients receiving T cell depleted grafts compared to 25 receiving unmanipulated grafts and post infusion immunosuppression at day 21, 28 and 90. The T cell depleted group recovered quickly and achieved normal levels by day 21. By day 90, however, there was no significant difference between the two transplant types ($P = 0.733$).

compared to normal, the percentage of NK cells which were regulatory ($CD56^{bright}$) were significantly lower in the T cell depleted transplants at days 21 (p=0.005), 28 (p=0.002) and 90 (p=0.001) post infusion of the graft when compared to the non-T cell depleted group (Figs. 3 and 4).

5. Discussion

In this prospective laboratory study of eighteen consecutive patients undergoing HLA-matched allogeneic SCT depleted of T cells using alemtuzumab *ex vivo*, the authors measured the post-transplant rate of lymphoid and NK cell recovery at three time points post graft infusion and compared it to twenty-five non-T cell depleted haplo-identical related donor transplants and seven normal healthy controls. While T-cells and their CD4 and CD8 subsets expanded quicker in the non-T cell depleted group, NK cells in the alemtuzumab treated grafts recovered faster, had lower percentages of regulatory NK cells and higher percentages of the classical NK subtype. Despite the post-transplant cyclophosphamide treatment of patients who received unmanipulated grafts, the rate of T cell recovery was quicker while the reconstitution of B-cells was similar.

The speed of recovery of NK cells post stem cell transplantation has been previously described [13,17–19]. These studies have been performed on patients receiving non-T cell depleted grafts with the additional use of post-transplant immunosuppression such as cyclosporin [7,13,20] or using different methods of T-cell depletion such as immunomagnetic beads [20]. As expected, the results of these reports often vary and are dependent on the method used to prevent GvHD.

Alemtuzumab or CAMPATH 1H effectively kills CD52 expressing T cells and other cells of the immune system in a dose dependent manner via complement –dependent cytotoxicity (CDCC). Treating patients with 120mg of alemtuzumab intravenously, prior to transplantation, has been shown to improve engraftment and significantly reduce GvHD. However, as alemtuzumab has an estimated half-life of 15 to 23 days [21], the residual presence of alemtuzumab results in slow immune reconstitution and the *in vivo* continued destruction of newly developing T cells and NK cells [3,6].

In contrast, using an ELISA based assay, it has been shown that if the optimum amount of alemtuzumab is added directly to the stem cell bag

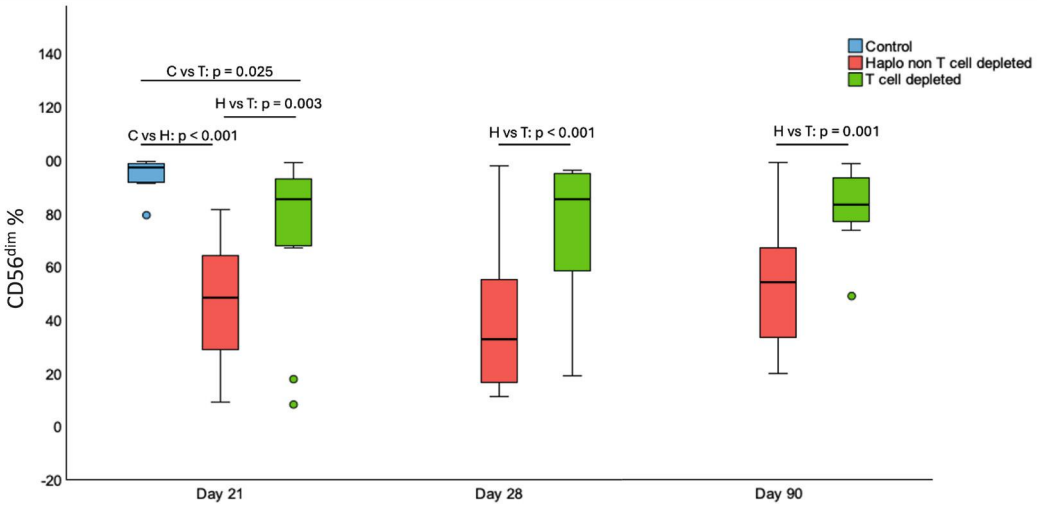

Fig. 3. Classical Natural killer cells: The percentage of total natural killer cells which were classical ($CD56^{dim}$/CD16+) were compared between 18 patients receiving T cell depleted transplants and 25 who received unmanipulated grafts at days 21, 28 and 90. The results were compared to healthy controls (blue) and were significantly reduced in both transplant types. However, in the T-cell depleted group, the % was significantly higher and closer to normal at all time points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

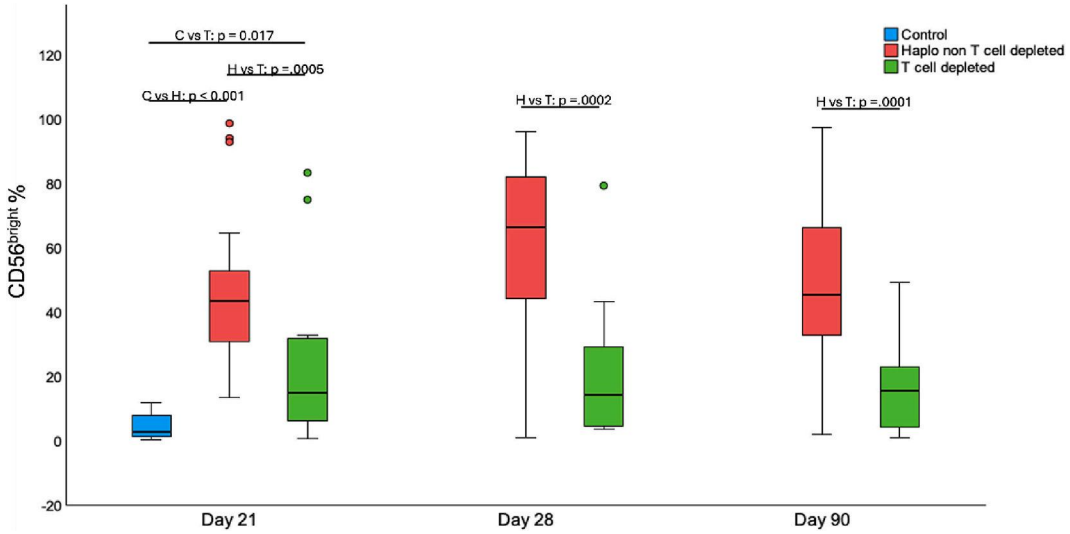

Fig. 4. Regulatory natural killer cells: The percentage of total NK cells identified as regulatory (CD56^{bright} /CD16-) in 18 T cell depleted transplants were compared to normal (blue) and 25 Non- T cell depleted transplants at days 21, 28 and day 90 post infusion. Compared to the normal control both groups were elevated. However, in the T-cell depleted group, the % was significantly lower and closer to normal at all time points ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ex vivo, levels of residual free antibody are significantly reduced after cell binding has taken place, with little being transferred into the patient [22]. Therefore, a feasible alternative to intravenous immunosuppression is to deplete the stem cell graft of allogeneic T-cells *ex vivo* with "alemtuzumab in the bag" before infusion. This is a simple and cost-effective way of preventing GvHD and currently utilised in South Africa [5]. In this present study, those who underwent non-T cell depleted related haplo- identical transplants, despite receiving 50mg/kg cyclophosphamide *in vivo* at day 3-4 post SCT infusion, had good T cell reconstitution but slower recovery of NK cells. The early recovery of NK cells has the potential of playing an important role in the initial stages post SCT by not only enhancing the GvL effect, but also targeting virally infected cells and preventing infection.

Natural killer cells as part of the innate immune system are responsible for killing tumour and virally infected cells. The value of elevated NK cells in the initial stages post SCT is well described, with reports suggesting lower disease relapse and increased survival in both allogeneic and autologous SCT [13,23–28]. Further studies have shown that in those receiving KIR-ligand mismatched haploidentical transplants, disease free survival can be significantly increased compared to those in which KIR and the corresponding MHC class I molecule are fully matched [29]. In this setting, donor NK cells expressing KIRs will not recognise the corresponding MHC class I molecule as self, leading to activation and cytotoxic killing of any residual leukemic cells [30]. Allogeneic NK cells can therefore increase the GvL effect, while not damaging normal tissue, as killing only takes place if target cells express antigens indicating viral infection or malignant transformation [31]. The importance of NK cells has led to investigators exploring the use of adoptive NK cell therapy post stem cell transplantation. In these studies, *ex vivo* expansion of NK cells with cytokines, anti-CD3 antibodies and feeder cells have resulted in promising results and future studies in this area are warranted [25,26].

Studies have demonstrated that NK cells have a lower density of CD52 antigen expression and consequently are less sensitive to cell lysis by alemtuzumab [[22,27]]. In this present study, all NK cells recovered quickly in the alemtuzumab T-cell depleted patients and although the percentage of regulatory CD56^{bright}/CD16-cells increased compared to healthy controls, it was significantly lower than those receiving non-T cell depleted transplants. Regulatory NK cells do not express KIRs nor CD16, have minimal cytotoxic activity and instead secrete inflammatory cytokines. These differences have led to the conclusion that they have little anti-tumour function [[9]]. Recent evidence, however, has suggested that after IL-15 priming and signalling, the CD56^{bright} NK subset is capable of cytotoxic killing and is able to kill malignant cells [[32]]. Furthermore, the secretion of IL-10 inhibits the inflammatory response and can control the development of GvHD [33]. Therefore, increasing evidence suggests that all NK cells can participate in the prevention of GvHD and the anti-tumour effect and the role of CD56^{bright}CD16- NK cells, requires further investigation.

This study had limitations which included the low number of patients and, since Groote Schuur is a national reference hospital for stem cell transplantation, the lack of long-term follow up clinical data. This prevented the authors from correlating the NK cell numbers and subtypes with overall clinical outcomes and the development of GvHD. In addition, it is acknowledged that NK cells are complex with several sub populations. These were not all analysed and should be included in future studies to fully understand the dynamics of NK cell reconstitution. However, despite these limitations, the value of this study is that it provides valuable information on the early reconstitution of NK cells following *ex vivo* T cell depletion using alemtuzumab which is not widely reported.

Our results are important as NK cells play a significant role in the prevention of relapse and successful engraftment. As the value of NK cells in various transplant settings is further defined, therapies may be developed to boost NK proliferation in the initial stages post-transplant, leading to improved clinical outcomes and prevention of malignant relapse.

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CRediT authorship contribution statement

Glenda M. Davison: Writing – review & editing, Writing – original draft, Conceptualization, Data curation, Mthodology, Formal analysis. **Jessica J. Opie:** Writing – review & editing, Data curation. **Saarah F.G. Davids:** Writing – review & editing, Formal analysis. **Rygana**

Mohammed: Investigation, Data curation, Writing – review & editing. **Nicolas Novitzky:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors have no conflict of interests to declare.

Data availability

Data will be made available on request.

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