

ORIGINAL ARTICLE

Clinical-scale single-step CD4⁺ and CD8⁺ cell depletion for donor innate lymphocyte infusion (DLI)

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The ability to selectively deplete or enrich cells of specific phenotype by immunomagnetic selection to reduce the risk of GVHD holds significant promise for application in adoptive immunotherapy. Current clinical-scale approaches for T-cell depletion (e.g., CD34⁺ selection, CD3⁺ depletion), usually deplete $\gamma\delta$ T cells, which may be advantageous in mediating graft-versus-tumor (GVT) effects and augmenting the innate immune response against infections. Here, we present a new method for depletion of T cells with potential GVHD reactivity by using a single-step immunomagnetic protocol, which efficiently depletes CD4⁺ and CD8⁺ $\alpha\beta$ T cells under good manufacturing practice (GMP) conditions. Depletion from unstimulated leukapheresis products ($n = 6$) containing up to 2.0×10^{10} cells showed high efficiency (mean log depletion of CD4⁺ cells: 4.12, CD8⁺ cells: 3.77). In addition, immunomagnetic CD4/CD8 depletion resulted in passive enrichment of innate lymphocytes (mean recovery of natural killer (NK) cells: 38%, $\gamma\delta$ T cells: 50%). We demonstrated that $\gamma\delta$ /NK cells preserved their proliferative and cytotoxic capacity and conclude that simultaneous large-scale depletion of CD4⁺/CD8⁺ T cells is feasible and can be performed under GMP conditions with high-depletion efficacy for $\alpha\beta$ T cells and recovery of functionally intact innate effector lymphocytes for potential use in adoptive immunotherapy studies.

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Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) has been shown to provide a potential curative option for a variety of hematological and nonhematological diseases.

However, the use of HSCT is limited by the availability of a suitable matched related or unrelated donor and significant transplant-associated complications such as GVHD, infections and conditioning regimen-related toxicities. Nevertheless, it is now generally accepted that HSCT is associated with a graft-versus-tumor (GVT) reaction mediated by donor lymphocytes and the successful application of donor lymphocyte infusions (DLI) for relapsed leukemia provided the first direct and indisputable demonstration of a GVT reaction in the clinical setting.^{1–4} Acute and chronic GVHD have been the major direct complications from DLI. Therefore, investigators have been using modified strategies such as T-cell depleted DLI⁵ or graded incremental DLI doses, since lower T-cell doses may be beneficial due to less GVHD.^{6,7} Despite these attempts, the risk of GVHD remains an obstacle to successful DLI therapy, prompting a substantial amount of investigative work that has focused on the search for allogeneic cellular immunotherapy strategies that would minimize the risk of GVHD while providing effective GVT effects.

Innate lymphocytes such as $\gamma\delta$ T cells and natural killer (NK) cells may provide an ideal source of allogeneic cellular immunotherapy as they respond to malignancy without recognition of alloantigens that could result in GVHD. Unlike $\alpha\beta$ T cells, which recognize specific processed peptide antigens presented on major histocompatibility complex (MHC) molecules by antigen-presenting cells, $\gamma\delta$ T cells and NK cells directly recognize and respond to a variety of MHC-like stress-induced self-antigens (MICA/B, ULBPs) and to less well-characterized ligands expressed on malignant cells via the $\gamma\delta$ T-cell receptor (TCR) or the natural cytotoxicity receptors.^{8,9}

With increasing knowledge regarding their antigen specificity and function, human $\gamma\delta$ T cells have attracted more and more interest in clinical immunotherapy approaches. V γ 9V δ 2T cells which represent the vast majority of human circulating $\gamma\delta$ T cells (50–70% of $\gamma\delta$ T cells and 0.5–7% of CD3⁺ T cells, respectively) recognize phosphorylated, nonpeptide compounds (so-called phosphoantigens), which naturally occur in the microbial nonmevalonate pathway of isoprenoid biosynthesis (Rohrer pathway). The activation of V γ 9V δ 2T cells by these naturally occurring phosphoantigens facilitates a specific immune response to numerous microbial pathogens via

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targeting a distinctive and essential metabolic route of these organisms. Besides these natural phosphoantigens, several synthetic phosphoantigens have been identified which selectively stimulate V γ 9V δ 2T cells either by mimicking the highly potent natural phosphoantigens (for example, bromohydrin pyrophosphate) or by inducing accumulation of cross-reactive endogenous mevalonate metabolites (for example, isopentenyl pyrophosphate) via inhibition of the mevalonate pathway (aminobisphosphonates such as pamidronate or zoledronate). $\gamma\delta$ T cells do not only recognize microbial antigens, but are also capable of exerting significant MHC-unrestricted antitumor activity against a broad spectrum of tumor cells *in vitro*.^{10,11} This has been demonstrated by potent lytic activity against hematopoietic^{12,13} and solid tumor cell lines *in vitro*^{14,15} and by the fact that $\gamma\delta$ T cells *in vivo* are overrepresented in reactive lymphatic regions surrounding neoplastic lesions, for example, in patients with melanoma,¹⁶ lung,¹⁷ renal,¹⁵ esophageal,¹⁸ or colorectal cancer.¹⁹ Previous studies of our group have provided additional evidence that selective stimulation of $\gamma\delta$ T cells by phosphoantigens can induce antilymphoma/myeloma activity *in vivo*.^{20,21}

Here, we present a rapid, automated and efficient good manufacturing practice (GMP)-conforming method for depletion of CD4⁺ and CD8⁺ $\alpha\beta$ T cells from standard leukapheresis products by using a single-step immunomagnetic protocol with anti-CD4 and anti-CD8-coated microbeads on a CliniMACS device, which results in a target cell fraction enriched for major cellular components of the innate immune system (NK cells and $\gamma\delta$ T cells) with potent antitumor activity while not inducing GVHD.

Materials and methods

Collection of PBMCs

Six large-scale experiments were performed. PBMCs were collected from six different healthy volunteer donors. A single unstimulated leukapheresis was performed using a Cobe Spectra (Gambro BCT, Planegg-Martinsried, Germany). All donors gave written informed consent.

Depletion of CD4⁺ and CD8⁺ cells

PBMCs were stored overnight at 4°C. The cells were washed once with CliniMACS phosphate-buffered saline (PBS)/EDTA buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 0.5% human albumin (ZLB Behring, Marburg, Germany) and incubated with both the anti-CD4 Ab and anti-CD8 Ab conjugated to paramagnetic particles (approximately 50 nm in size, Miltenyi Biotec). One vial (7.5 ml) of CD4 beads per 12×10^9 positive cells, and one vial (7.5 ml) of CD8 beads per 4×10^9 positive cells were used, according to the manufacturer's recommendations. Cells were incubated under continuous agitation at room temperature for 30 min, washed once with CliniMACS buffer, resuspended in 100 ml buffer and then processed with the fully automated device CliniMACS Plus (Miltenyi Biotec) equipped with tubing set LS (162-01) using the program 'Depletion 2.1' according to the manufacturer's instructions. The depletion

procedure was performed within 2–4 h depending on the numbers of CD4⁺ and CD8⁺ cells within the leukapheresis product.

Flow cytometric analysis

Prior to and after CD4/CD8 T cell depletion, aliquots were analyzed for CD3, CD4, CD8, CD14, CD15, CD16 + CD56, CD19, TCR $\alpha\beta$ and TCR $\gamma\delta$ expression by 4-colour FACS analysis. All of these Abs were fluorochrome-labeled (Becton Dickinson, Heidelberg, Germany or Beckman Coulter, Krefeld, Germany). Viability was tested by flow cytometry using propidium iodide. Flow cytometric analysis was performed using a flow cytometer (either FACS-Calibur, Becton Dickinson or Epics XL, Beckman Coulter). To determine the depletion efficacy up to 500 000 events were acquired after CD4/CD8 T cell depletion.

Proliferation assay

In round-bottom microtiter wells, 1×10^5 depleted PBMCs were cultured with zoledronate (0.1 and 1 μ M/l, Novartis, Basel, Switzerland) in presence of low-dose IL-2 (10 U/ml), high-dose IL-2 (300 U/ml) or medium alone for 8 days in a 5% CO₂ humidified atmosphere. Medium consisted of RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% pooled human serum, L-glutamine (2 mmol/l, Biochrom, Berlin, Germany), and 1% penicillin-streptomycin and IL-2 (10 U/ml, Novartis, Basel, Switzerland). Cells were periodically restimulated with IL-2 (10 or 300 U/ml) from day 2 to 6. Relative counts of $\gamma\delta$ TCR⁺, CD3⁺, CD56⁺ and HLA-DR⁺ cells were determined by flow cytometric analysis at the beginning and the end of culture.

Cell lines

$\gamma\delta$ T-cell lines were established by culturing 1×10^5 depleted PBMCs in round-bottom microtiter wells in standard medium consisting of RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% pooled human serum, L-glutamine (2 mmol/l, Biochrom, Berlin, Germany), 1% penicillin-streptomycin and IL-2 (100 U/ml, Novartis, Basel, Switzerland) with a single dose of aminobisphosphonate (1 μ M/l zoledronate). After 9 days, up to 90% of the cells expressed the V γ 9V δ 2 TCR, as confirmed by flow cytometry.

Daudi, K-562 and THP-1 cell lines were obtained from the DSMZ, Braunschweig, Germany. All cell lines were grown in RPMI 1640 media supplemented with 10% fetal calf serum and 2 mmol/l L-glutamine, and 1% antibiotics.

Cytotoxicity assay

A standard 4-h chromium release assay was performed. In brief, target cell lines (Daudi, K-562 and THP-1) were labeled with 100 μ Ci ⁵¹Cr, and 10 000 cells per well were incubated in round-bottom triplicate wells with the zoledronate-reactive V γ 9/V δ 2 T cell line at the indicated effector/target (E/T) ratios. After 4 h, the amount of ⁵¹Cr released into supernatant was measured as counts per minute (c.p.m.) and expressed as specific lysis according to the following formula: % specific lysis = % specific ⁵¹Cr

release = (effector-induced c.p.m. – spontaneous c.p.m.) / maximum c.p.m. – spontaneous c.p.m.) × 100. Spontaneous c.p.m. represents the amount of ^{51}Cr released by target cells incubated without effector cells, and maximum c.p.m. was obtained by lysis with 1% sodium dodecyl sulfate.

Results

Depletion of CD4⁺ and CD8⁺ cells by magnetic cell separation under GMP conditions

A total of six large-scale experiments were evaluated. The total nucleated cell count prior to CD4/CD8 depletion was 13.4×10^9 (range $4.4\text{--}20.6 \times 10^9$) and 2.9×10^9 (range $1.2\text{--}7.4 \times 10^9$) after depletion. The mean absolute number of CD4⁺ cells prior to depletion was 5.5×10^9 (range $0.9\text{--}12.3 \times 10^9$) and 0.5×10^6 (range $0.08\text{--}0.8 \times 10^6$) after depletion. The mean log₁₀ CD4⁺ cell depletion was therefore 4.1 (range 3.2–4.9).

The mean absolute number of CD8⁺ cells prior to depletion was 2.2×10^9 (range $0.7\text{--}4.1 \times 10^9$) and 0.7×10^6 (range $0.08\text{--}2.9 \times 10^6$) after depletion. The mean log₁₀ CD8⁺ cell depletion was therefore 3.8 (range 3.0–4.5). In fact, CD4⁺ and CD8⁺ αβ T cells were almost undetectable after CD4/CD8 depletion and the low percentage of CD4⁺ and CD8⁺ cells still present in the target cell fraction mainly consisted of CD4^{dim} and CD8^{dim} cells, respectively, and did not coexpress T-cell markers such as CD3 or the αβ TCR (Figure 1). Importantly, the majority of remaining αβ T cells after CD4/CD8 depletion were of the CD4[–]CD8[–] (DN) phenotype. In addition to efficient depletion of CD4⁺ and CD8⁺ cells, immunomagnetic CD4/CD8 depletion resulted in passive enrichment of NK cells and γδ T cells. After depletion, the mean number of retained γδ T cells was 1.6×10^8 (range $1\text{--}3 \times 10^8$), which reflects a mean recovery of 50% (range 34–79%). The mean number of NK cells after depletion was 7.1×10^8 (range $3\text{--}16 \times 10^8$), which reflects a mean recovery of 38% (range 19–73%). The viability of cells before and after processing was consistently greater than 90%. Summarized results from all six depletions are shown in Tables 1 and 2. Representative cytometric analysis illustrating the data before and after CD4/CD8 depletion is depicted in Figure 1.

Functional characteristics of innate lymphocytes after magnetic cell separation under GMP conditions

In order to rule out that this CD4/CD8 depletion method has any negative impact on the biological function of the innate effector lymphocytes, *in vitro* proliferation and cytotoxicity assays were performed. For the proliferation assays, PBMCs prior to and after CD4/CD8 depletion were stimulated with zoledronate and IL-2 and analysed after 8 days. There was no nonspecific loss of proliferation and activation capacities. On the contrary, relative counts of the tested lymphocyte subsets were higher after depletion, due to the passive enrichment of NK cells and γδ T cells (Figure 2). Therefore, γδ T cells retained their proliferative capacity in presence of phosphoantigens and IL-2 after CD4/CD8 depletion. Similar results were observed for IL-2-induced proliferation of NK cells (data not shown).

In addition, we tested the cytotoxic potential of cell lines derived from zoledronate and low-dose IL-2 stimulated PBMCs after CD4/CD8 depletion (to induce preferential expansion of γδ T cells). To stimulate preferential NK cells high-dose IL-2 alone was used. Therefore, effector cells were incubated with the sensitive cell lines (erythroleukemic cell line K562 and the Burkitt lymphoma cell line Daudi) and the resistant acute monocytic leukemia cell line THP-1 as negative control at different effector-to-target ratios. Figure 3 shows a representative cytotoxicity profile of a γδ T-cell line (consisting of 90% γδ T cells). As illustrated in Figure 3, γδ T-cell lines derived from CD4/CD8 depleted PBMCs exhibited a preserved cytotoxic activity against the γδ T-cell sensitive Daudi cell line and intermediate lytic activity against K562 targets, whereas no significant lytic activity against THP-1 cells was observed. NK cell lines derived from CD4/CD8 depleted PBMCs in presence of high-dose IL-2 (consisting of 70% NK cells) showed strong cytotoxic activity against the NK cell sensitive K562 cell line and intermediate lytic activity against Daudi cells (data not shown). In summary, innate lymphocytes retained their functional characteristics after the immunomagnetic CD4/CD8 depletion procedure.

Discussion

The therapeutic application of DLI is limited by the potential development of GVHD. Several strategies have been proposed to control the incidence and severity of GVHD associated with DLI. These have included incremental infusions of limited numbers of lymphocytes, selective depletion of lymphocyte subsets and genetic manipulation of cells to regulate their sensitivity to agents such as ganciclovir.^{22–25} If DLI is to become established as a valuable clinical modality for either the treatment/prevention of relapse post transplantation or other adoptive immunotherapy approaches with allogeneic lymphocytes, it is critical to minimize the risk of GVHD while preserving effective GVT effects.

Recent studies suggest that not all T-cell depletion methods are equivalent and that selective depletion of T-cell subsets is superior to ‘broad specificity’ depletion techniques.²⁶ Selective depletion of CD8⁺ cells has emerged as being particularly interesting, based on marrow transplant and DLI data which suggest that CD8⁺ αβ T cells may preferentially mediate GVHD, while CD4⁺ αβ T cells might be responsible for GVT.^{5,22,27} However, purified MHC-unrestricted (CD4[–]CD8[–]) innate lymphocytes such as γδ T cells and NK cells offer an alternative approach. Several *in vitro* and *in vivo* studies have shown that γδ T cells and NK cells induce potent antitumor effects without causing GVHD.^{28–31} In addition, these innate lymphocytes may facilitate engraftment of human stem cells^{32–34} and are even able to prevent or mitigate GVHD.³⁵ These findings are consistent with a recent analysis by Godder *et al.*,³⁶ who demonstrated a long-term survival advantage without increased GVHD incidence in a group of high-risk acute leukemia patients recovering with increased γδ T cells after partially mismatched related donor marrow transplantation. Thus, MHC-unrestricted

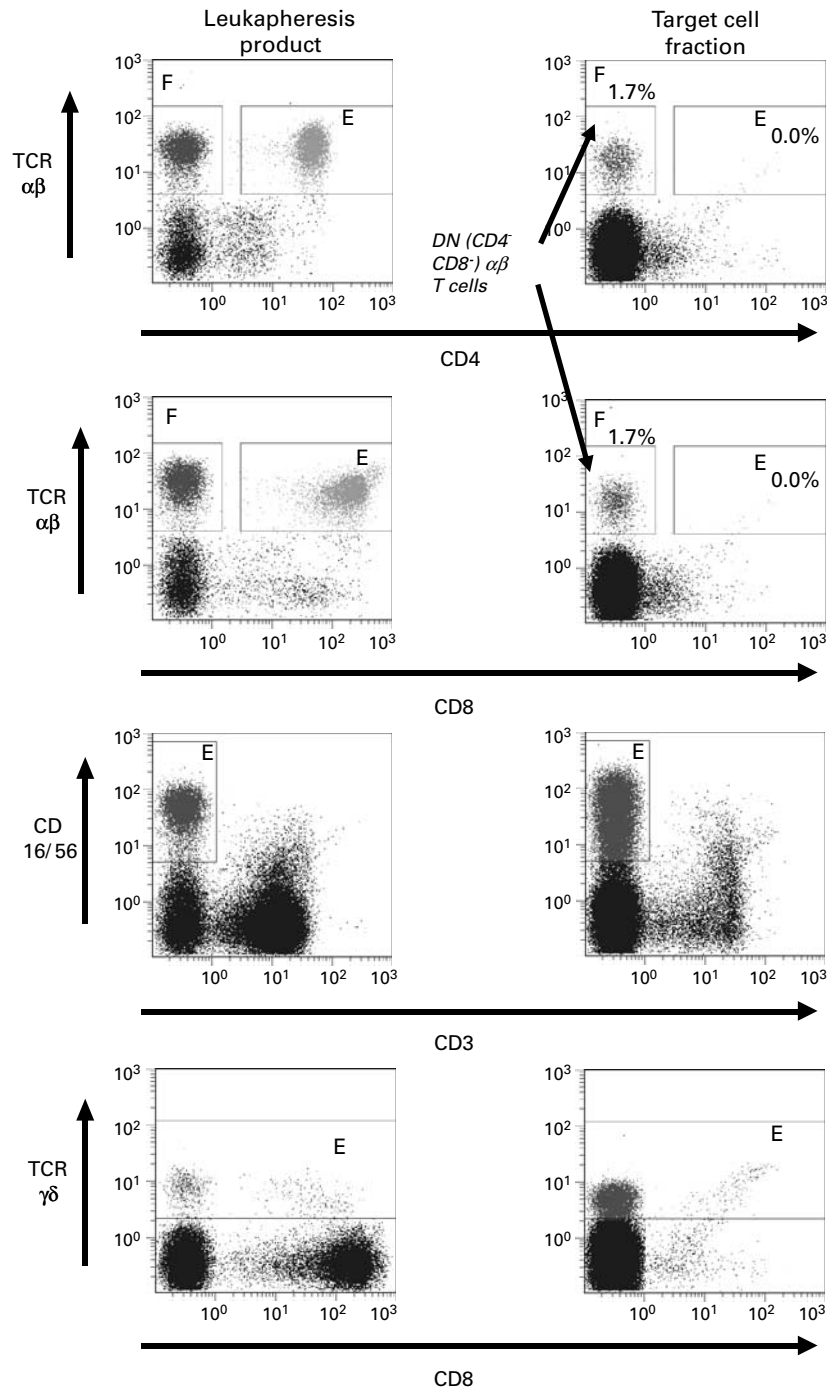


Figure 1 A representative flow cytometric analysis of the proportion of $\alpha\beta$ T cells, NK cells and $\gamma\delta$ T cells before and after CD4/8 depletion.

innate lymphocytes are excellent candidates for use in adoptive immunotherapy approaches or may be useful for graft modulation in HLA-mismatched/haploidentical transplantation settings. The absence of CD4 and CD8 expression on the majority of $\gamma\delta$ T cells and NK cells is in line with the lack of MHC-restriction of these lymphocytes. Although $\gamma\delta$ T cell and NK cell killing is MHC-unrestricted, they possess a number of activating and inhibitory receptors (KARs and KIRs) that bind to classical and nonclassical MHC molecules to modulate their response.

Therefore, the overall lytic activity against a target cell will be the result of a balance between all activating and inhibitory signals. This notion is supported by recent clinical data from MHC-mismatched or haploidentical transplantation and adoptive transfer studies with haploidentical NK cells, which suggested that a KIR mismatch may lead to greater tumor kill.^{31,37}

However, current clinical-scale approaches to reduce the risk of GVHD in MHC-mismatched or haploidentical settings usually use either broad specificity antibodies (for

example, ATG, anti-CD52/Campath, anti-CD3, anti-CD2) or CD34-enrichment for T-cell depletion or purification of innate lymphocytes from apheresis products focused on NK cells by depletion of CD3⁺ T cells,^{31,38} in several attempts followed by a second step of positive NK cell selection using anti-CD56 antibodies.^{39,40} All these strategies result in the depletion of $\gamma\delta$ T cells, an innate

lymphocyte subset with powerful capabilities in mediating GVT effects and augmenting innate immune response against infections. In addition, positive selection of CD56^{high} NK cells by immunomagnetic methods significantly reduces the number of CD56^{dim} NK cells, which are more cytotoxic against NK sensitive targets than CD56^{high} NK cells and are exclusively capable of mediating ADCC by expressing the Fc γ RIII.⁸

Direct depletion of $\alpha\beta$ T cells with anti-TCR $\alpha\beta$ antibodies would be an attractive approach, but its clinical use has been limited due to a lack of GMP grade anti-TCR $\alpha\beta$ antibodies and difficulties in direct conjugating available anti-TCR $\alpha\beta$ antibodies (T10B9-IgM and BMA031-IgG) to paramagnetic particles.⁴¹ Due to the lack of exclusive surface markers for $\gamma\delta$ T cells, antibodies against the $\gamma\delta$ TCR were used for positive enrichment purposes of this rare T-cell subset. Otto *et al.*⁴² described a large-scale immunomagnetic positive enrichment approach from stimulated leukapheresis products using an anti-pan TCR $\gamma\delta$ Hapten antibody. Although this method was

Table 1 Composition of the leukapheresis products before and after CliniMACS CD4/CD8 depletion (range, mean and s.d. of six experiments)

(n = 6)	Range	Mean	s.d.
<i>Before CliniMACS depletion</i>			
Viability (%)	92–99	98	3
TNC ($\times 10^9$)	4.4–20.6	13.41	6.35
CD4 ⁺ ($\times 10^9$)	0.9–12.3	5.46	4.06
CD8 ⁺ ($\times 10^9$)	0.7–4.1	2.20	1.31
CD56 ⁺ ($\times 10^9$)	0.5–2.1	1.38	0.68
TCR $\gamma\delta$ ⁺ ($\times 10^9$)	0.2–0.5	0.31	0.12
<i>After CliniMACS depletion</i>			
Viability (%)	91–98	97	3
TNC ($\times 10^9$)	1.2–7.4	2.85	2.26
CD4 ⁺ ($\times 10^6$)	0.08–0.82	0.49	0.28
CD8 ⁺ ($\times 10^6$)	0.08–2.89	0.71	1.10
CD56 ⁺ ($\times 10^9$)	0.3–1.6	0.71	0.47
TCR $\gamma\delta$ ⁺ ($\times 10^9$)	0.1–0.3	0.16	0.09

Abbreviations: TCR = T-cell receptor; TNC = total nucleated cells.

Table 2 Analysis of the CliniMACS depletion procedures (range, mean and s.d. of six experiments)

(n = 6)	Range	Mean	s.d.
CD4 ⁺ purity (%)	99.95–100.00	99.98	0.02
CD4 ⁺ recovery (%)	21.4–58.1	34.21	13.99
CD4 ⁺ log ₁₀ depletion	3.2–4.9	4.12	0.61
CD8 ⁺ purity (%)	99.96–99.99	99.98	0.02
CD8 ⁺ recovery (%)	10.5–41.7	26.36	11.28
CD8 ⁺ log ₁₀ depletion	3.0–4.5	3.77	0.56
CD56 ⁺ purity (%)	10.3–44.4	24.34	13.26
CD56 ⁺ recovery (%)	18.7–72.9	38.03	19.94
TCR $\gamma\delta$ ⁺ purity (%)	3.4–11.3	6.02	3.21
TCR $\gamma\delta$ ⁺ recovery (%)	34.3–78.9	50.23	17.54

Abbreviation: TCR = T-cell receptor.

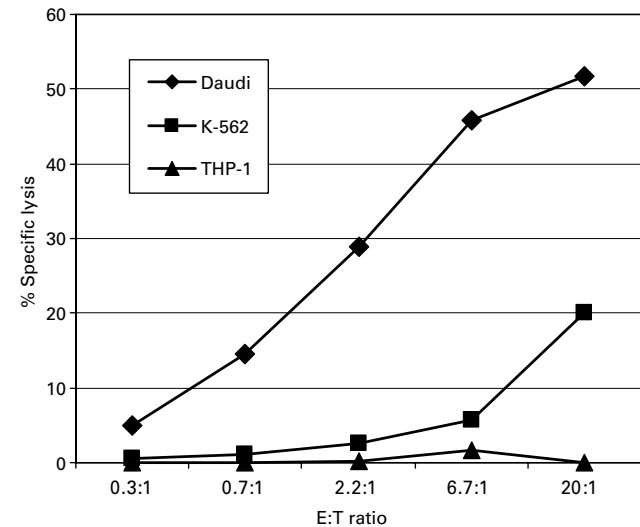


Figure 3 Cytotoxic capacity of CD4/CD8 depleted mononuclear cells against $\gamma\delta$ T/NK-sensitive cell lines and a negative control (THP-1) after stimulation with zoledronate (1 μ M) and IL-2 for 9 days. Results represent mean values of triplicate experiments of one representative PBMC donor. Similar cytotoxic profiles were observed in two additional PBMC donors.

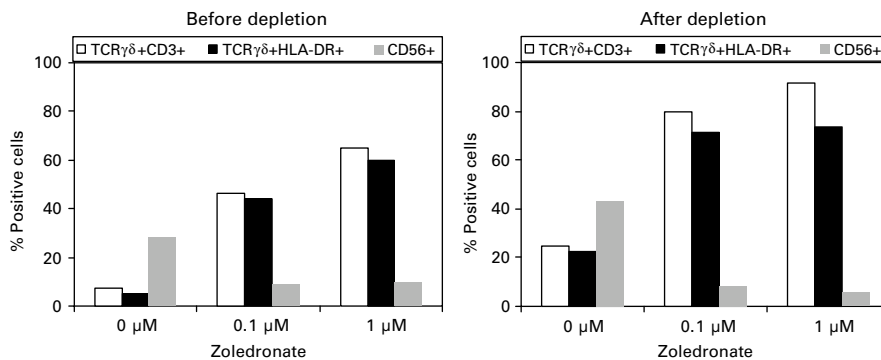


Figure 2 Proliferative capacity of $\gamma\delta$ T cells stimulated for 8 days with zoledronate and IL-2 before and after CD4/8 depletion. Results represent mean values of triplicate cultures of one representative PBMC donor. Similar proliferation profiles were observed in the other five PBMC donors.

reported to yield sufficient recovery of $\gamma\delta$ T cells, the engagement of the TCR by using an anti-TCR $\gamma\delta$ antibody contributed to significant activation of this T-cells subset. Our own data indicate that anti-TCR $\gamma\delta$ antibodies affect the viability and functional properties of $\gamma\delta$ T cells after positive selection procedures and therefore might limit its use in the clinical setting. (V Kunzmann *et al.*, unpublished data).

To this end, we thought to develop a strategy which ensures large-scale reliable enrichment of both $\gamma\delta$ T cells and NK cells and simultaneous depletion of $\alpha\beta$ T cells from leukapheresis products under GMP conditions with a minimal number of reagents and selection steps. This pilot study presented here allowed to setup technical conditions to generate a clinical-scale product with reliable recovery of functionally intact $\gamma\delta$ T cells and NK cells and efficient depletion of (potential GVHD inducing) CD4⁺ and CD8⁺ $\alpha\beta$ T cells by using a single step of T-cell depletion with anti-human CD4 and CD8 microbeads on a CliniMACS device (median log₁₀ depletion 3.0–4.9). This T-cell depletion efficacy is comparable to strategies using anti-CD3 antibodies on the CliniMACS device.^{38,43} For CD3 depletion, a low incidence of GVHD has been reported in the clinical setting (that is, haploidentical transplantation) even without GVD prophylaxis unless T-cell dose exceeded 5×10^4 CD3⁺ cells per kg.^{38,43} The median processing time for this large-scale CD4/CD8 depletion protocol was only 2–4 h. Similar clinical-scale techniques for depletion of CD4⁺ cells and (partial) depletion of CD8⁺ cells from bone marrow grafts using a different immunomagnetic method have been described previously.^{44–46} However, all these approaches reported a significant lower T-cell depletion efficacy with a significant number of residual CD8⁺ T cells in the graft (mean log₁₀ CD4⁺/8⁺ cell depletions ranged around 2) and despite GVHD prophylaxis, acute GVHD was observed after transplantation of these (partially) CD4/CD8 depleted marrow grafts. Graft composition concerning remaining NK cells and $\gamma\delta$ T cells after CD4/CD8 depletion was not reported in these studies.

Recovery of $\gamma\delta$ T cells and NK cells after our CD4/CD8 depletion protocol from leukapheresis products was on average 50% (range 34–79%) and 38% (range 19–73%), respectively. Thus, the target cell products contained clinically relevant numbers of $\gamma\delta$ T cells and NK cells for adoptive transfer studies (on average 1.6×10^8 $\gamma\delta$ T cells and 7.1×10^8 NK cells). *Ex vivo* expansion of $\gamma\delta$ T cells and NK cells from peripheral blood samples to yield comparable number of innate lymphocytes would take several weeks and holds the risk of contamination during an expensive GMP-grade *in vitro* culture process. The mechanisms responsible for the variable loss of $\gamma\delta$ T and NK cells after CD4/CD8 depletion remain unknown, but besides the general risk of cell loss during *ex vivo* cell processing (for example, washing steps) the fact that varying subsets of $\gamma\delta$ T and NK cells express CD8 might have contributed to this heterogeneity. Although the majority of resting human peripheral blood NK cells and $\gamma\delta$ T cells are negative for CD4 and CD8, small subsets of human NK cells (20–30%, predominantly the CD56^{dim}CD161⁺ CD16⁺ subset) and $\gamma\delta$ T cells (<10%, predominantly the V δ 1⁺ subset which is not sensitive to phosphoantigens) have been shown to

express the CD8 molecule.^{8,47} However, only the CD8 α/α homodimer is expressed on these innate lymphocytes and the density of expression is approximately a log-fold lower than that of CD8⁺ $\alpha\beta$ T cells (which usually express the CD8 α/β heterodimer).^{47–49} In our protocol, the anti-human CD8 beads resulted in almost complete depletion of cells with high CD8 expression, while cells with intermediate expression of CD8 (majority of NK cells and $\gamma\delta$ T cells) were mostly retained. Therefore, expression of CD8 on subsets of $\gamma\delta$ T cells and NK cells cannot alone explain the cell loss after CD4/CD8 depletion. In addition, $\gamma\delta$ T cells and NK cells retain their proliferative and cytotoxic characteristics after the immunomagnetic CD4/CD8 depletion procedure, indicating that the functionally relevant subsets of these innate lymphocytes are not depleted.

Remarkably, the CD4/8 depletion procedure leaves CD4[–]CD8[–] double negative regulatory $\alpha\beta$ T cells (DN $\alpha\beta$ T cells) in the separation product. Regulatory T cells play an important role in the maintenance of immune tolerance to self and foreign antigens,^{50,51} and are involved in regulating the immune responses in transplant rejection,⁵¹ tumor immunity,^{52,53} infectious diseases⁵⁴ and GVHD.^{55,56} DN $\alpha\beta$ T cells⁵⁷ have also been shown to possess the ability to regulate immune responses. They express a unique set of cell surface markers and have a unique cytokine profile.^{57,58} In addition to their proposed role in regulating syngeneic CD8⁺ T cell-mediated immune responses to self-antigens,⁵⁹ DN $\alpha\beta$ T cells have been shown to downregulate immune responses to allo- and xenogeneic antigens.^{57,60,61} Recently, a study with murine DN $\alpha\beta$ T cells had revealed additional antitumor properties of this subset by directly preventing tumor growth.⁵³ When examined *ex vivo*, these DN $\alpha\beta$ T cells showed strong cytolytic activity toward A20 lymphoma cells with a robust ability to suppress autologous CD8⁺ effector T cells responsible for GVHD.^{53,56,62} Therefore, DN $\alpha\beta$ T cells will supplement a separation product engineered by CD4/CD8 depletion with further interesting capabilities regarding prevention of GVHD and tumor growth.

In conclusion, this study demonstrates an efficient new method for rapid *ex vivo* simultaneous large-scale depletion of CD4⁺ and CD8⁺ cells from leukapheresis products under GMP conditions which preserves adequate numbers of innate effector cells ($\gamma\delta$ T cells and NK cells) as well as regulatory DN $\alpha\beta$ T cells in the product. The recovery of functionally intact innate effector lymphocytes opens up a new depletion strategy for potential use of Donor Innate Lymphocyte Infusion in adoptive immunotherapy studies. In the HLA-mismatched or haploidentical setting after allogeneic HSCT, this depletion strategy may be useful in graft modulation, as well as for tailoring DLI.

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