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Antiviral Immunity and T-Regulatory Cell Function Are Retained after Selective Alloreactive T-Cell Depletion in Both the HLA-Identical and HLA-Mismatched Settings

Jeffrey K. Davies, Mickey B. C. Koh, Mark W. Lowdell

Department of Haematology, Royal Free & University College Medical School, London, United Kingdom

Correspondence and reprint requests: J.K. Davies, MRCP, MRCPath, Department of Haematology, Royal Free Campus, Royal Free & University College Medical School, London NW3 2PF, UK (e-mail: j.davies@rfc.ucl.ac.uk).

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ABSTRACT

Nonselective T-cell depletion reduces the incidence of severe graft-versus-host disease after allogeneic hematopoietic stem cell transplantation, but the cost is delayed and disordered antigen-specific immune reconstitution and increased infection. We use a method of selective depletion of alloreactive T cells expressing the activation marker CD69 after coculture with stimulator cells in a modified or standard mixed lymphocyte reaction. The technique has been shown to reduce alloreactivity while retaining third-party responses in vitro and, in a mismatched murine model, led to donor T-cell engraftment with a virtual absence of graft-versus-host disease and increased survival. We show in a human HLA-mismatched and unrelated HLA-identical setting that this technique retains >80% of specific cellular antiviral activity by cytomegalovirus-tetramer analysis and cytomegalovirus/Epstein-Barr virus peptide-stimulated interferon-γ ELISpot assay. Furthermore, $CD4^+$ $CD25^+$ T-regulatory cells are not removed by this method of selective allodepletion and retain their function in suppressing allogeneic proliferative responses. Preservation of antiviral cytotoxic T lymphocytes in selectively allodepleted stem cell grafts would lead to improved antiviral immunity after transplantation. The retention of immunosuppressive $CD4^+$ $CD25^+$ T-regulatory cells could lead to more ordered immune reconstitution and further suppress alloreactive responses after transplantation.

KEY WORDS

T-cell depletion • Graft-versus-host disease • Allogeneic hematopoietic stem cell transplantation • T-regulatory cells

INTRODUCTION

Graft-versus-host disease (GVHD) is a cause of major morbidity and mortality in allogeneic hematopoietic stem cell (AHSC) transplantation because of the recognition by immunocompetent T cells within the AHSC graft of nonself HLA or minor histocompatibility antigens (mHags) present on recipient cells. It has long been established that nonselective T-cell depletion of the AHSC graft reduces the incidence of severe GVHD in both the HLA-matched sibling and HLA-matched unrelated donor transplant setting and even with haploidentical transplants [1-3].

Although this maneuver leads to a reduction in GVHD mortality after transplantation, the removal of pathogen-specific T cells leads to a delay in quantitative and qualitative immune reconstitution [4]. This translates into delayed recovery of antigen-specific immune responses in the recipient and an increase in the incidence and severity of a broad spectrum of infections after transplantation.

Several methods of selective depletion of alloreactive cells have been shown to be effective at reducing the allogeneic response while maintaining third-party reactivity. All rely on the stimulation of live responder cells (from the donor cell pool) by irradiated stimulator (recipient) cells in a unidirectional mixed lymphocyte reaction (MLR) or modified MLR (mMLR). Alloreactive responder cells may then be identified (and subsequently removed) by their expression of activa-

tion antigens—such as CD69 [5,6], CD25 [7-11], or both [12]—or destroyed by FAS-FAS ligand–mediated apoptosis [13] or by photodynamic purging [14]. In vitro studies and murine models have been published for all these techniques demonstrating effective reduction of alloreactivity/GVHD.

Cytomegalovirus (CMV) infection after AHSC transplantation is a significant cause of morbidity and mortality and has a considerable economic effect [15]. A significant number of adults who receive AHSC transplants have had serologically detectable past exposure to CMV. In the series of Meyer et al. [16] from 1986, as many as 70% of CMV-seropositive transplant recipients developed clinically significant CMV infection, and CMV disease was the most common cause of death in such patients. Prophylactic treatment with ganciclovir or acyclovir has only partially reduced post-AHSCT CMV infection and has the theoretical disadvantage of selecting drug-resistant viral strains [17].

The strategy of monitoring patients for viral shedding by the detection of viral genome by (real-time) polymerase chain reaction reduces CMV infection and death in the first 100 days after AHSCT but may have the effect of postponing infection to a later stage after transplantation [18]. Such preemptive treatment is associated with adverse effects, especially (reversible) neutropenia that often requires cessation of treatment. The crucial importance of T cell–mediated immunity in controlling CMV is underlined by the increased rate of CMV infection seen in nonselective T cell–depleted AHSCTs [19].

The cytotoxic T lymphocyte (CTL) response is of critical importance for control of CMV infection, and it has been estimated that 1% to 5% of CTLs in healthy CMV-seropositive individuals have specificity for CMV [20]. In controlling CMV disease, CTL responses are believed to be the main effector arms of the adaptive immune system, although they are critically dependent on $CD4^+$ T-cell help [21]. In the absence of $CD4^+$ help, there may be persistence of virus despite the presence of $CD8^+$ CMV-specific CTLs as the latter become anergic [22].

Nonselective T-cell depletion to reduce GVHD, especially in the unrelated donor setting or in nonidentical transplants, removes both $CD8^+$ CMV-specific CTLs and $CD4^+$ CMV-specific T-helper cells and slows the recovery of CMV-specific immunity. A major advantage of selective alloreactive cell depletion is that it leaves the donor $CD8^+$ and $CD4^+$ T-cell pool intact, and it has been shown to retain functional anti-CMV activity in the HLA-mismatched setting [11]. Epstein-Barr virus (EBV)–driven posttransplantation lymphoproliferative disorder (PTLD) is another cause of significant morbidity and mortality after AHSCT. EBV-specific CTL responses are of paramount importance in PTLD, and infusion of very small numbers of donor-derived EBV-specific CTLs are very effective at suppressing PTLD. Selective alloreactive cell depletion may leave donor EBV-specific CTLs intact in the donor cell pool and confer more active EBV immunity after AHSC transplantation.

$CD4^+$ $CD25^+$ T-regulatory cells, which form fewer than 10% of resting human $CD4^+$ lymphocytes, are now recognized as playing an important role in the suppression of immune responses [23]. These cells are required for the ex vivo induction of tolerance to alloantigen via co-stimulatory blockade and for the inhibition of allogeneic skin graft rejection. There is also evidence that these cells can suppress the alloreactive response and limit the development of GVHD. Depletion of $CD4^+$ $CD25^+$ cells from the donor T-cell inoculum or in vivo CD25 depletion of the recipient before transplantation resulted in increased GVHD mediated by $CD4^+$ or whole T cells in mice. The infusion of freshly purified donor $CD4^+$ $CD25^+$ cells modestly inhibited GVHD when administered in equal numbers with whole $CD4^+$ cells but at higher levels significantly inhibited rapidly lethal GVHD [24].

The kinetics of upregulation of CD69 expression on alloantigen-activated lymphocytes have been determined, and in a unidirectional MLR, the maximal expression of CD69 occurs after 72 to 96 hours—somewhat earlier than the maximal expression of CD25 [25]. CD69 is thus a suitable antigen to use to selectively deplete alloantigen-activated lymphocytes while maintaining maximal cell viability. CD69 has been used to identify and immunomagnetically deplete alloreactive cells after mixed lymphocyte culture with irradiated recipient cells. An mMLR [26] with cytokine pretreatment of stimulator cells has been used to increase their alloimmunogenicity and thus maximize the activation of alloreactive cells in HLA-matched donor/recipient pairs in vitro. After depletion of alloreactive cells, the nonalloreactive cell fractions showed significantly reduced proliferative responses against the original matched stimulators and had preservation of responses to third-party stimulator cells [5].

This technique has also been tested in a completely mismatched nonobese diabetic/severe combined immunodeficient murine model of aggressive GVHD. Murine recipients of infusions of nonmanipulated major histocompatability complex class I and class II mismatched donor T cells experienced a rapid onset of acute (and generally fatal) GVHD. This model is akin to aggressive transfusion-associated GVHD. Recipients of lymphocyte infusions depleted of $CD69^+$ alloreactive donor cells ex vivo and monitored for 10 weeks after infusion demonstrated significantly improved survival (71.4% compared with 12.5% in the nonmanipulated group) and the absence

of clinical or histologic evidence of GVHD despite the presence of circulating donor lymphocytes demonstrated by flow cytometry [6].

We have used the CD69-based selective allodepletion technique to remove alloreactive cells from both HLA-identical unrelated donor/recipient pairs and totally HLA-mismatched donor/recipient pairs in which the donor was known to be CMV immunoglobulin (Ig)G positive. CMV-reactive T cells were enumerated before and after selective allodepletion by CMV-peptide specific interferon (IFN)-γ ELISpot assay and by CMV-peptide HLA A0201 tetramer analysis. EBV-reactive T cells by IFN-γ ELISpot were also enumerated before and after selective alloreactive cell depletion in the HLA-mismatched setting. $CD4^+$ $CD25^+$ T-regulatory cell depletion should not occur with our CD69-based strategy, and to confirm this, we enumerated and tested the functionality of $CD4^+$ $CD25^+$ T-regulatory cells after CD69-based selective allodepletion.

Table 1. *HLA Typing of CMV IgG-Positive Responders in Sample Pairs Used for Selective Depletion of Alloreactive Cells and Peptides Used in CMV Peptide–Stimulated IFN-γ ELISpot Assays before and after Selective Depletion of Alloreactive Cells*

Pair	Stimulator (Recipient)	Responder (Donor) HLA Typing		CMV Peptide Used in IFN-γ ELISpot
1	HLA mismatched	A3	B0702	TPRVTGGGAM
		A26	B0801	
2	HLA mismatched	A0201	B0702	NLVPMVATV
		A2402	B1401	
3	HLA mismatched	A0101	B0702	TPRVTGGGAM
		A0101	B37	
4	HLA mismatched	A0201	B14	NLVPMVATV
		A25	B58	
5	HLA mismatched	A0201	B44	NLVPMVATV
		A74	B38	
6	HLA mismatched	A0201	N/A	NLVPMVATV
7	HLA mismatched	A0201	N/A	NLVPMVATV
8	Matched at HLA-A, -B, and -DR	A0101	B0801	DANDIYRIF
		A0101	B0801	
9	Matched at HLA-A, -B, and -DR	A0201	B3501	NLVPMVATV
		A0301	B3701	
10	Matched at HLA-A, -B, and -DR	A0101	B0702	TPRVTGGGAM
		A0101	B37	
11	Matched at HLA-A, -B, and -DR	A0201	B0702	NLVPMVATV
		A2402	B1401	
12	Matched at HLA-A, -B, and -DR	A0201	B4402	NLVPMVATV
		A3101	B3503	

N/A indicates not assessed.

MATERIALS AND METHODS

HLA-A–, -B–, and -DR–identical unrelated donor/recipient pairs and complete HLA-A–, -B–, and -DR–mismatched donor/recipient pairs were chosen with donors seropositive for CMV IgG or EBV nuclear antigen IgG (Table 1). Written consent was obtained for the use of all blood samples. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation (Lymphoprep; Nycomed-Amersham, Buckinghamshire, UK), and cells were used fresh or after storage in liquid nitrogen.

Alloreactivity was assessed by a primary unidirectional MLR in an unmodified form in the HLA-mismatched pairs. Irradiated (30-Gy) stimulators were cocultured in RPMI 1640 supplemented with 10% human AB serum and 1% penicillin/streptomycin (complete media; all Gibco, Paisley, UK) with live responder cells in 96-well plates (Nunc, Rochester, NY) with a stimulator-responder ratio of 1:1 at 37°C, 5% CO_2, and 60% humidity. After 108 hours, 1 μCi of tritiated thymidine (Amersham, Buckinghamshire, UK) was added per 10^5 responder cells. The plates were harvested at 120 hours on a harvester, and proliferative alloreactive responses were measured as tritiated thymidine incorporation in responder cells by using a beta-scintillation counter (Wallac, Turku, Finland).

In the HLA-matched unrelated donor/recipient samples, the primary MLR was modified by pretreatment of the stimulator cells with OKT3 or cytokines. OKT3 was obtained from a fermentation system by using a murine hybridoma–secreting human mitogenic OKT3 (American Type Culture Collection cell line CRL-8001). The concentration of OKT3 in hybridoma supernatant was quantified by murine IgG enzyme-linked immunosorbent assay, and 500 ng of OKT3 per well was immobilized on a 24-well plate by overnight incubation at 4°C. Wells were then washed twice, and 10^6 stimulator PBMCs were added per well in complete media. The plates were incubated for 48 hours as described previously, and the cells washed twice before use as stimulators in the MLR. No additional cytokines were added to the MLR in this strategy.

In the HLA-matched unrelated donor/recipient samples, the primary MLR was alternately modified by pretreatment of stimulator cells with recombinant human IFN-γ 1000 IU/10^6 cells and recombinant human tumor necrosis factor-α 1000 IU/10^6 cells for 24 hours (R&D Systems, Abingdon, UK). Stimulator cells were then diluted in complete medium and washed twice before use as stimulators in the MLR. Recombinant human interleukin-4 (R&D Systems) was added at a dose of 1000 IU/10^6 responder cells to the MLR with this strategy. All experiments were performed in triplicate.

Selective allodepletion was achieved by performing a bulk MLR in the HLA-mismatched setting or a bulk OKT3 and cytokine mMLR in the HLA-matched setting by following the protocols outlined previously. However, after 72 hours of coculture, the cells were sorted to deplete the responder cell fraction of $CD69^+$ alloreactive cells. Depletion was achieved by using CD69/fluorescein isothiocyanate (FITC) an-

tibody (Becton Dickinson, Oxford, UK) and anti-FITC microbeads with single use depletion columns and a magnetically activated cell sorter (Miltenyi Biotech, Cologne, Germany). The depleted cell fraction was used as the responder cell fraction in a secondary MLR to confirm reduction in first-party alloreactivity and preservation of third-party alloreactivity. The depletion efficiency (%) of $CD69^+$ cells was defined as

$$\frac{(\%\ CD69^+ \text{ cells before depletion} - \%\ CD69^+ \text{ cells after depletion}) \times 100}{\%\ CD69^+ \text{ cells before depletion}}$$

CMV tetramer analysis was performed on unmanipulated PBMCs and on selectively allodepleted PBMCs on HLA-A0201–positive donors by using a phycoerythrin (PE)–conjugated HLA-A0201–restricted human CMV AE42 peptide tetrameric construct (a gift from the Anthony Nolan Research Institute, London, UK). A total of 1 μg of tetramer was incubated with 10^6 cells for 30 minutes and then counterstained with CD3-allophycocyanin and CD8-FITC (Becton Dickinson), and 10^4 events were acquired on a FACSCalibur flow cytometer (Becton Dickinson). Data were subsequently analyzed with CellQuest Pro software (BD Biosciences).

A selection of HLA class I–restricted immunogenic human CMV virus peptides were used as stimulatory peptides in an IFN-γ ELISpot assay (MabTech, Nacka, Sweden) by using polyvinylidene difluoride–lined 96-well plates (Millipore, Watford, UK). Peptides used were the A0201-restricted pp65 tegmental-derived nonamer AE42 (NLVPMVATV), the B0702-restricted decamer TPRVTGGGAM, and the B0801-restricted nonamer DANDIYRIF (Proimmune, Oxford UK) [27-29]. Peptides were stored at a high concentration in dimethyl sulfoxide at −70°C and diluted to 10 μg/mL in complete media before use.

The HLA specificity of the AE42 peptide was confirmed before use in a T2-binding assay and was also shown to prevent the binding of PE-conjugated HLA-A0201–restricted human CMV AE42 peptide tetramer to the $CD8^+$ CTLs of a CMV IgG-positive HLA-A0201 subject (data not shown). Stimulatory peptides were also tested in the IFN-γ ELISpot assay on healthy CMV IgG-positive individuals of suitable HLA class I type. Briefly, polyvinylidene difluoride–lined 96-well plated were washed with 70% ethanol, and a capture antibody was added at 1.5 μg per well and incubated overnight at 4°C. After washing and blocking with RPMI 1640 containing 10% fetal calf serum (FCS) (150 μL per well) for 1 hour, cells were washed and live PBMCs were added (10^5 to 2×10^5 per well).

A total of 1 μg of stimulatory peptide was used per 10^5 live responder cells incubated for 20 hours as described previously. Cells were then removed and wells washed before the addition of 100 μL of diluted biotinylated antibody in filtered phosphate-buffered saline (PBS) with 0.5% FCS to each well and incubated for 90 minutes at room temperature. Wells were then washed 6 times with sterile, filtered PBS. Then 100 μL of streptavidin/alkaline phosphatase antibody (diluted 1:1000 in filtered PBS/0.5% FCS) was added to each well and incubated for 1 hour at room temperature. Wells were washed 6 times with sterile filtered PBS, and 100 μL of color change substrate (Bio-Rad, Hemel Hempsted, UK) was added per well. Plates were agitated gently at room temperature for 10 to 20 minutes until spots appeared. Color development was stopped by washing in tap water 200 μL per well 3 times. Plates were left to air-dry at room temperature overnight, and wells were photographed with an Olympus (Tokyo, Japan) digital camera and dissection microscope.

Negative controls were cells plus no peptide and cells plus dummy (HLA-mismatched) peptide. Positive controls were cells stimulated with phytohemagglutinin (10 ng/10^6cells; Sigma-Aldrich, Dorset, UK). All experiments were performed in triplicate. ELISpot-responding cells were expressed as

$$\frac{\text{Number of dots/well in test} - \text{number of dots/well in controls}}{\text{Number of cells/well}}$$

The frequency of $CD3^+CD8^+$ CMV-responding cells was expressed as (% $CD3^+CD8^+$ PBMCs) × number of responding cells. The frequency of CMV-reactive $CD3^+$ $CD8^+$ CTLs was determined in unmanipulated donor samples and in selectively allodepleted donor samples in both the HLA-matched and HLA-mismatched donor/recipient pairs.

A variety of EBV-derived peptides stimulate EBV-CTL responses. We chose to use the SVRDRLARL peptide derived from the latent EBV nuclear antigen 3A, which demonstrates immunodominance in HLA-A0201 individuals [30]. EBV-CTL activity was measured by peptide-stimulated IFN-γ ELISpot by using the reagents and methodology for CMV peptide ELISpot outlined previously.

The frequency of $CD4^+$ $CD25^+$ T-regulatory cells was measured before and after selective allodepletion by using CD25-PE and CD4-peridinin chlorophyll-a protein antibodies (Becton Dickinson), and a FACSCalibur flow cytometer was used for analysis with CellQuest Pro software. After selective allodepletion, the CD69-depleted fraction was sorted by flow cytometry to select the $CD4^+$ $CD25^+$ T-regulatory cell fraction. These sorted T-regulatory cells were added at a ratio of 1:1 to an allogeneic mismatched MLR with first-party stimulators and responders autologous to the T-regulatory cells to assess the immunosuppressive capacity of the T-regulatory cells after selective allodepletion. The T-regulatory negative fraction from the sort was also added to a

Table 2. *CD69 Expression on Alloreactive Responder Cells and Depletion Efficiency of CD69$^+$ Cells in HLA-Mismatched Standard MLR and HLA-Matched OKT3 and Cytokine-Stimulated MLRs: Mean (SD)*

	HLA-Mismatched Pairs: Standard MLR			HLA-Matched Pairs					
				OKT3 MLR			Cytokine MLR		
Variable	CD3$^+$	CD3$^+$ CD4$^+$	CD3$^+$ CD8$^+$	CD3$^+$	CD3$^+$ CD4$^+$	CD3$^+$ CD8$^+$	CD3$^+$	CD3$^+$ CD4$^+$	CD3$^+$ CD8$^+$
% Responder CD69$^+$*	5.9 (5)	4.9 (2)	4.8 (4)	20 (11)	24 (15)	15 (7)	7.2 (9)	8.3 (10)	5.0 (6)
CD69$^+$ depletion efficiency	92 (7)	91 (8)	85 (10)	92 (6)	91 (8)	83 (14)	86 (16)	86 (15)	88 (12)

*Above autologous control.

first-party allogeneic MLR with responders autologous to the T-regulatory negative fraction as a negative control.

The unpaired *t* test with the Welch correction in the presence of unequal variance and the paired *t* test were used as tests of statistical significance, and a *P* value of $<.05$ was chosen to reject the null hypothesis.

RESULTS

Allostimulation Was Effective and Allodepletion Was Efficient

In the HLA-mismatched pairs, CD69 was upregulated on 5.9% ± 5% (mean ± SD) of responder CD3$^+$ cells, 4.9% ± 2.3% of CD3$^+$ CD4$^+$ cells, and 4.8% ± 4.2% of CD3$^+$ CD8$^+$ cells after allostimulation in the standard MLR at 72 hours. The depletion efficiency of these cells was 92% ± 7%, 91% ± 8%, and 85% ± 10%, respectively.

In the HLA-matched pairs, CD69 expression at 72 hours was not increased above autologous control in the standard MLR (data not shown; $P > .05$). In contrast, allostimulation in the OKT3 mMLR led to CD69 expression on 20% ± 11% of CD3$^+$ responder cells, 24% ± 15% of CD3$^+$ CD4$^+$ cells, and 15% ± 7% of CD3$^+$ CD8$^+$ cells at 72 hours. The time scale of maximal responder CD69 expression was similar to that seen in HLA-mismatched pairs, although upregulation of CD69 occurred somewhat earlier (data not shown). The depletion efficiency of these cells was 92% ± 6%, 91% ± 8%, and 83% ± 14%, respectively. In the same donor/recipient pairs, allostimulation in the cytokine mMLR led to CD69 expression on 7.2% ± 9% of CD3$^+$ responder cells, 8.3% ± 9% of CD3$^+$ CD4$^+$ cells, and 5% ± 6% of CD3$^+$ CD8$^+$ cells at 72 hours. The time scale of maximal responder CD69 expression was similar to that seen in HLA-mismatched pairs (data not shown). The depletion efficiency of these cells was 86% ± 16%, 86% ± 15%, and 88% ± 12%, respectively. These results are summarized in Table 2.

CD69 responses seen after allostimulation in HLA-matched pairs with both the OKT3 mMLR and the cytokine mMLR were not significantly different from those seen after stimulation with HLA-mismatched third-party controls (data not shown). The yield in all depletion procedures was >85% of viable nonalloreactive CD3$^+$ cells.

Allodepletion Selectively Reduced First-Party Proliferation in MLRs

In HLA-mismatched donor/recipient pairs, the first-party alloreactivity in the secondary MLR assay was reduced to 3.98% ± 4.5% of that seen in the primary MLR with retention of third-party proliferative responses (73.2% ± 24.3% of the predepletion value). In HLA-matched donor/recipient pairs, the first-party alloreactivity was reduced to 0.8% ± 0.9% of the predepletion value after selective allodepletion with OKT3-pretreated stimulator cells, with retention of third-party proliferative responses (58% of predepletion values). In HLA-matched donor/recipient pairs, the first-party alloreactivity was reduced to 2.6% ± 4.4% of the predepletion value after selective allodepletion with cytokine-pretreated stimulator cells, with retention of third-party proliferative responses (133% of predepletion values).

CMV Tetramer-Positive Cells Were Retained after Selective Alloreactive Cell Depletion

HLA-A0201 restriction of the tetramer was demonstrated by tetramer binding to CD3$^+$ CD8$^+$ cells in an HLA-A0201 CMV IgG-positive subject but not in an HLA-B0701 CMV IgG-positive subject. The AE42 peptide specificity was demonstrated by blocking of tetramer binding to CD3$^+$ CD8$^+$ cells in an HLA-A0201 CMV IgG-positive subject by treatment of PBMCs with exogenous AE42 peptide in excess.

In HLA-mismatched donor/recipient pairs after selective allodepletion, 93% ± 9% of CMV-AE42 peptide tetramer-positive CTLs were retained. In HLA-matched donor/recipient pairs after OKT3-stimulated MLR selective allodepletion, 88.5% ± 36% of CMV-AE42 peptide tetramer-positive CTLs were retained, and after cytokine-stimulated MLR-selective allodepletion, 87.0% ± 18.4% of CMV-AE42 peptide tetramer-positive CTLs were retained. An example of AE42 peptide tetramer-positive CTL preservation after HLA-matched selective allodepletion is shown in Figure 1.

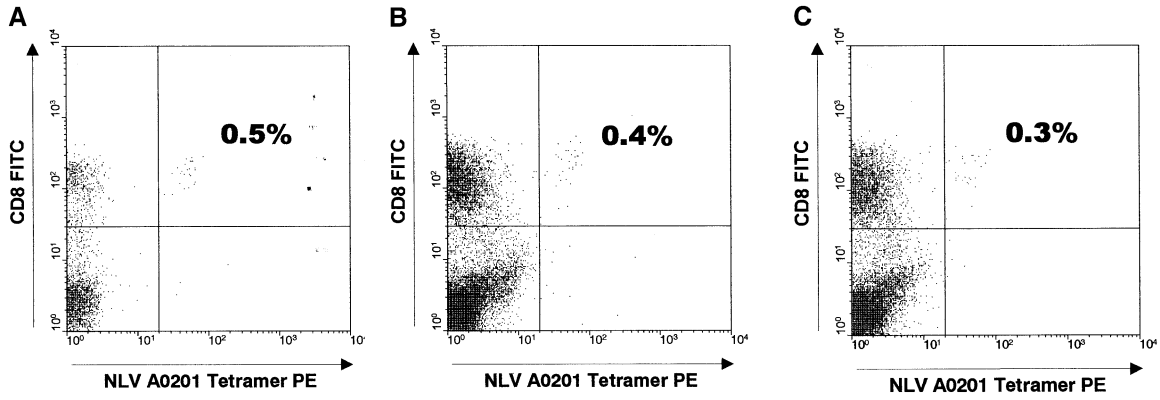

Figure 1. CMV CTLs are retained after CD69$^+$-based selective allodepletion. a, HLA-A2 CMV AE42 (NLV) peptide tetramer-binding CD3$^+$ cells in an unmanipulated donor cell pool. b, HLA-A2 CMV AE42 (NLV) peptide tetramer-binding CD3$^+$ cells in an HLA-matched selectively allodepleted fraction with OKT3-stimulated MLR. c, HLA-A2 CMV AE42 (NLV) peptide tetramer-binding CD3$^+$ cells in an HLA-matched selectively allodepleted fraction with cytokine-stimulated MLR. (This figure show dot plots from a representative experiment.)

There was no significant difference in paired 2-tailed *t* test results between the frequency of CMV-AE42 peptide tetramer-binding CTLs before and after selective allodepletion in the HLA-mismatched donor/recipient pairs or in the HLA-matched pairs selectively allodepleted with either the OKT3-stimulated MLR or the cytokine mMLR.

Functional CMV-Specific CTLs Were Retained after Selective Alloreactive Cell Depletion

The CMV peptides and ELISpot assays were optimized and validated in healthy CMV IgG-positive individuals of known HLA class I types. The AE42 peptide was further validated in a T2-binding assay in which the addition of HLA-A0201–restricted peptide to transporter-associated protein-deficient HLA-A0201 T2 cells in culture resulted in stabilization and an increase of expression of HLA-A0201 on the surface of T2 cells of 100% (whereas the addition of an HLA B0702-restricted CMV-peptide did not alter the expression of HLA-0201). The AE42 peptide also increased CMV-specific CTL-mediated killing in a cytotoxicity assay with T2 target cells (data not shown).

In HLA-mismatched donor/recipient pairs after selective allodepletion, 89.2% ± 26.9% of the functional CMV-reactive CTLs by IFN-γ ELISpot were retained. In HLA-matched donor/recipient pairs after OKT3-stimulated MLR-selective allodepletion, 72.3% ± 39.5% of the functional CMV-reactive CTLs by IFN-γ ELISpot were retained. In HLA-matched donor/recipient pairs after cytokine-stimulated MLR-selective allodepletion, 80.7% ± 17.9% of the functional CMV-reactive CTLs by IFN-γ ELISpot were retained.

There was no significant difference in a paired 2-tailed *t* test between the frequency of functional CMV-reactive CTLs by IFN-γ ELISpot before and after selective allodepletion in the HLA-mismatched donor/recipient pairs or in the HLA-matched pairs selectively allodepleted with either the OKT3-stimulated MLR or the cytokine mMLR. Preservation of both the AE42 peptide tetramer-positive CTLs and CMV-reactive CTLs by IFN-γ ELISpot in both the HLA-mismatched and -matched setting is shown in Figure 2.

Functional EBV-Specific CTLs Were Retained after Selective Alloreactive Cell Depletion

Functional EBV-specific CTLs were measured by IFN-γ ELISpot in the HLA-mismatched setting. After selective allodepletion, there was no significant difference in the number of EBV-reactive CTLs compared with the predepletion samples (data not shown).

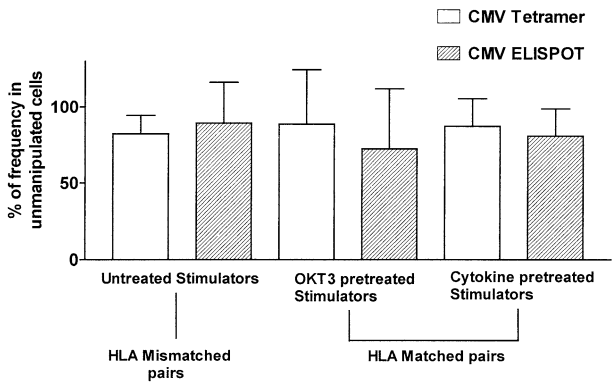

Figure 2. Functional CMV CTLs are retained after CD69$^+$-based selective allodepletion. Retention of CMV CTLs by HLA-A2 CMV AE42 (NLV) peptide tetramer and retention of functional CMV CTLs by CMV peptide–stimulated IFN-γ ELISpot after selective allodepletion expressed as a percentage of the of value seen in unmanipulated cells.

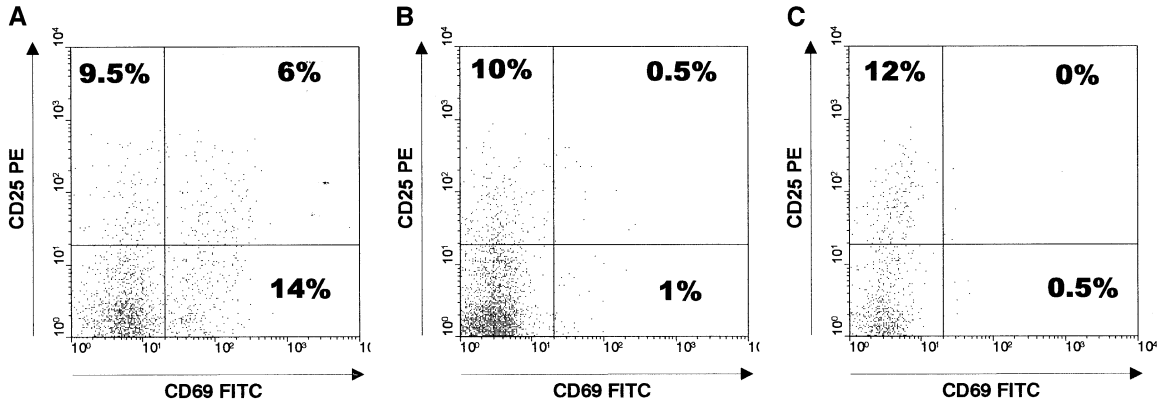

Figure 3. $CD4^+$ $CD25^+$ T-regulatory cells are not removed by $CD69^+$ selective allodepletion. Allostimulation in the OKT3-stimulated MLR in HLA-matched samples leads to an appearance of both $CD69^+$ $CD25^-$ and $CD69^+$ $CD25^+$ alloreactive $CD3^+$ $CD4^+$ T cells, which are removed by selective allodepletion based on CD69 expression. T-regulatory cells ($CD4^+$ $CD25^+$ $CD69^-$) are not removed by this process. A, Allostimulated responder cells; B, untreated responder cells; C, selectively allodepleted responder cells.

$CD4^+$ $CD25^+$ T-Regulatory Cells Were Retained after Selective Allodepletion

The level of $CD4^+$ $CD25^+$ T-regulatory cells present in the responder cell population after allostimulation at 72 hours was not significantly altered from the baseline value in unmanipulated cells ($CD4^+$ $CD25^+$ after allostimulation, 14.6% ± 7.9% versus the baseline value, 17.9% ± 1.0%; $P = .6$) or from the level seen after autologous cell culture ($CD4^+$ $CD25^+$ after allostimulation, 14.6% ± 7.9% versus autologous cell culture, 15.8% ± 9.7%; $P = .9$).

The level of $CD4^+$ $CD25^+$ T-regulatory cells present in the responder cell population after selective allodepletion at 72 hours was not significantly altered from the predepletion value ($CD4^+$ $CD25^+$ after allostimulation, 14.6% ± 7.9% versus after depletion, 11.4% ± 2.2%; $P = .54$). In HLA-matched donor/recipient pairs, no difference in the levels of $CD4^+$ $CD25^+$ T-regulatory cells was found either after allostimulation or after selective allodepletion in either the OKT3-stimulated MLR or the cytokine mMLR depletion strategies (data not shown). In both the HLA-mismatched and -matched samples, $CD69^+$ selective allodepletion removed a small fraction of $CD25^+$ cells that also expressed CD25; most of the $CD4^+$ $CD25^+$ cells remained $CD69^-$ and were retained in the selectively allodepleted fraction (Figure 3).

$CD4^+$ $CD25^+$ T-Regulatory Cells Present in Selectively Allodepleted Cell Fractions Retain Immunosuppressive Function

$CD4^+$ $CD25^+$ T-regulatory cells sorted after selective allodepletion retained their immunosuppressive function by suppressing allogeneic MLR responses to first-party stimulators. In the mismatched setting, the mean first-party allogeneic MLR response was significantly reduced to 18.9% ± 9.4% of the control value by the addition of sorted selectively allodepleted $CD4^+$ $CD25^+$ T-regulatory cells autologous to and at equal numbers to the MLR responders ($P = .005$). Addition of autologous non–T-regulatory cells from selectively allodepleted cell fractions in similar quantities did not significantly alter the first-party allogeneic MLR response in this setting (Figure 4).

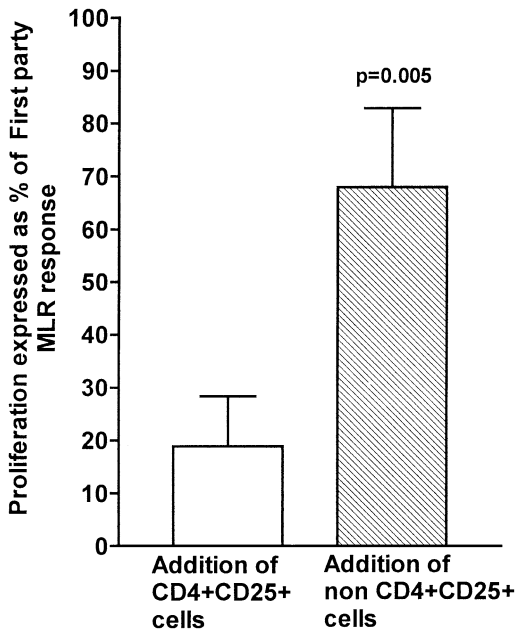

Figure 4. $CD4^+$ $CD25^+$ T-regulatory cells present in selectively allodepleted cells retain immunosuppressive function. Proliferative responses to first-party stimulators in HLA-mismatched allogeneic MLRs is significantly reduced after the addition of $CD4^+$ $CD25^+$ T-regulatory cells (autologous to the responders) from selectively allodepleted cell fractions, whereas proliferation is not significantly affected after the addition of the $CD4^+$ $CD25^+$ T-regulatory cell negative fraction from selectively allodepleted cell fractions.

DISCUSSION

All modalities of selective alloreactive cell depletion rely on the principle of stimulation of alloreactive cells in a live responder (donor) cell population by irradiated stimulator (recipient) cells in a unidirectional mixed lymphocyte culture. In the HLA-A–, -B–, and -DR–identical setting, we used a technique of pretreatment of stimulator cells with mitogenic OKT3 to increase the capacity of the stimulator cell pool (mixed PBMCs) to present alloantigens. Human T-cell clones have been shown to be capable of presenting antigens [31]. Although the stimulation of T cells with mitogenic OKT3 has been reported to lead to anergy in responder cells when such T cells alone are used as nonprofessional antigen-presenting cells in the MLR [32], here we show that the HLA-matched unrelated donor/recipient pairs tested demonstrated a significant upregulation of CD69 on responder cells and a significant proliferative response in the OKT3-stimulated MLR with mixed PBMCs as stimulators (akin to that seen with the established cytokine mMLR). The stimulation and expansion of T-cell stimulator cells with mitogenic OKT3 has been described recently as part of the protocol for clinical scale application of a selective allodepletion technique [33]. Both this work and other work within our group has shown that OKT3-treated/expanded T cells significantly upregulate molecules involved in alloantigen presentation, such as CD80 and CD86 [34]. Although it could be argued that important alloantigens presented only by monocytes would be lost by the exclusive use of T cells as APCs, our 48-hour pretreatment of PBMCs with OKT3 retains a proportion (5%-10%) of monocytes within the APC pool. Moreover, the use of predominantly T cells as APCs might lead to retention of identified myeloid or B-cell–restricted tumor-associated alloantigens [35-37] with preservation of a graft-versus-leukemia effect.

In both the HLA-mismatched and -matched unrelated donor/recipient pairs, immunomagnetic depletion of $CD69^+$ responder cells was efficient and led to a reduction in proliferation to the original stimulators to $<5\%$ of predepletion values and preservation of proliferative responses to third-party controls. Yields of viable nonalloreactive T cells were commonly 85% to 90%, meaning that the nonalloreactive donor T-cell pool remains quantitatively intact with this strategy. Depletion efficiency of alloreactive T cells was greater than 90%, and residual $CD69^+$ cells left in responder cell pools only weakly expressed the antigen. If infused into the recipient, such cells might still be capable of mounting an alloreactive response and causing GVHD, although if still bound to CD69 antibody they might be easily destroyed by the recipient's reticuloendothelial system. In the published nonobese diabetic/severe combined immunodeficient mouse model of CD69-selective allodepletion, the depletion efficiency of 93% was sufficient to completely abrogate GVHD in sublethally irradiated major histocompatibility complex nonidentical mice [6]. However, the ability of small numbers of alloreactive cells infused to recipients in the context of the cytokine release that accompanies pretransplantation conditioning remains a potential concern.

Responses to highly immunogenic HLA-disparate antigens might mask responses against minor histocompatability antigens, which might stimulate responders more weakly or more slowly in the MLR. The cytokine mMLR has been shown to be predictive of clinical GVHD in HLA-identical AHSC transplant recipients after 120 hours of coculture, indicating that alloresponders can proliferate to mHags in this time scale [26]. Both the cytokine and the OKT3 pretreatment strategy in the HLA-identical pairs we used led to a maximal CD69 responder expression by 72 hours: thus, alloresponses to mHags do not seem to be delayed with respect to HLA-disparate antigens.

Greater than 80% of HLA-A0201 CMV AE42 peptide tetramer-reactive CTLs were retained in the allodepleted fraction in both the HLA-mismatched and HLA-matched unrelated donor pairs. This is in keeping with a recent study of CD25-based selective allodepletion in HLA-mismatched pairs [11].

However, tetramer analysis gives no indication of functionality; tetramer-binding cells have been reported without cytolytic function, especially in the absence of CD4 cell help [22]. Baseline levels of CMV tetramer-binding CTLs were 3- to 5-fold higher than baseline CMV-reactive CTLs by IFN-γ ELISpot assay, which is in keeping with published data [38].

Thus, the IFN-γ ELISpot assay, a functional test of CMV reactivity, gives us important additional information. We have shown that the great majority of CMV-reactive CTLs by IFN-γ ELISpot assay are retained after selective allodepletion in the HLA-matched setting with HLA-A0201, -B0702, and -B0801 responders, adding to published data using mismatched pairs with HLA-A0201 responders [11].

The dominant CMV peptide response in every individual can vary, even between individuals of the same HLA class I type [39], and thus additional CMV responses may have been retained that cannot be identified by a single HLA-restricted peptide approach.

In the HLA-mismatched setting, we have shown that functional EBV immunity is preserved after selective depletion of alloreactive cells. EBV and other viruses that are controlled primarily by a T cell–mediated immune response are important pathogens after AHSCT; these include respiratory syncytial virus and adenovirus [40-42]. Life-threatening complications arising from infection from all of these viruses after AHSCT have been treated successfully with donor lymphocyte infusions, with presumed transfer of

antiviral CTLs [43-45]. Specific cellular antiviral immunity to such viruses might also be preserved by selective alloreactive T-cell depletion of the donor T-cell pool.

Our data also demonstrate the quantitative preservation of $CD4^+$ $CD25^+$ T-regulatory cells after selective allodepletion in both HLA-mismatched donor/recipient and matched unrelated donor/recipient pairs. Moreover, the functionality of such $CD4^+$ $CD25^+$ T-regulatory cells was preserved after selective allodepletion, as demonstrated by retention of their immunosuppressive capability when added to autologous live responder cells in an allogeneic MLR.

In common with CD69, expression of CD25 (the low-affinity interleukin-2 receptor) is upregulated on activated T cells. Alloreactive cells that upregulate CD69 after allostimulation come from both the CD8 and CD4 subsets: some alloreactive cells express only CD69, some express only CD25, and some express both. It is possible that the $CD25^+$ cell population in the allodepleted cell fractions contains some alloreactive cells that have upregulated CD25 after presentation of alloantigens in the MLR. However, reduction of first-party proliferative responses after selective allodepletion to $<5\%$ of their predepletion values indicates that any $CD69^-$ $CD25^+$ cells that have upregulated CD25 in response to alloantigens and remain after depletion are unable to make a significant proliferative response to first-party stimulators and are thus unlikely to contribute significantly to in vivo allogeneic responses. Purified $CD4^+$ $CD25^+$ cells from allodepleted cell fractions maintained the phenotype in vitro of T-regulatory cells [23] by reducing proliferative responses to first-party stimulators, and it thus seems unlikely that any significant population of alloreactive cells had appeared within the $CD4^+$ $CD25^+$ cell fraction or, if they had, that their responses were overwhelmed by the suppressive effects of the true T-regulatory cells. Other markers of T-regulatory cells are being identified (intracellular CD152 and CD150) [46] and might be useful in further distinction between T-regulatory cells and alloreactive cells in the donor cell pool.

The role of $CD4^+$ $CD25^+$ T-regulatory cells in suppressing alloreactivity is increasingly being recognized, and the loss of such cells from the donor pool might result in increased GVHD. Loss of T-regulatory function is seen in individuals with autoimmune and atopic conditions [47]. Nonselective T-cell depletion leads to disordered immune reconstitution and the loss of hierarchical control, which may be manifested as autoimmune disorders such as autoimmune hemolytic anemia [48].

Selective allodepletion based on the upregulation of CD25 will remove or destroy not only alloreactive cells that have upregulated CD25 on allostimulation, but also any cells that express CD25 constitutively, including $CD4^+$ $CD25^+$ T-regulatory cells: such techniques will therefore deplete the donor T-cell pool of the T-regulatory compartment with the potential of increased alloreactivity and disordered immune reconstitution.

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