DEVELOPMENT OF ADOPTIVE IMMUNOTHERAPY WITH ALLODEPLETED DONOR T-CELLS IMPROVES IMMUNE RECONSTITUTION AFTER HAPLOIDENTICAL STEM CELL TRANSPLANTATION

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1. SUMMARY

Poor immune reconstitution after haploidentical stem cell transplantation results in a high mortality from viral infections and relapse. One approach to overcome this problem is to infuse donor T cells from which alloreactive lymphocytes have been selectively depleted, but the immunologic benefit of this approach is unknown.

To selectively deplete the graft of alloreactive cells we used an immunotoxin directed against the activation marker CD25. However, the degree of depletion of alloreactive cells is variable following stimulation with recipient peripheral blood mononuclear cells (PBMCs), and this can result in graft versus host disease (GVHD). We have refined this approach using recipient Epstein-Barr virus (EBV)–transformed lymphoblastoid cell lines (LCLs) as stimulators to activate donor alloreactive T cells. Our studies demonstrate that allodepletion with an anti-CD25 immunotoxin following stimulation with HLA-mismatched host LCLs more consistently depleted in vitro alloreactivity than stimulation with host PBMCs, as assessed in primary mixed lymphocyte reactions (MLRs). Allodepletion using this approach specifically abrogates cytotoxic T-cell responses against host LCLs.

So we have compared immune reconstitution after allodepleted donor T cells that were infused at 2 dose levels into recipients of T-cell–depleted haploidentical SCT. Eight patients were treated at 10^4 cells/kg/dose, and eight patients received 10^5 cells/kg/dose.

Patients receiving 10^5 cells/kg/dose showed significantly improved T-cell recovery at 3, 4, and 5 months after SCT compared with those receiving 10^4 cells/kg/dose ($P < .05$). Accelerated T-cell recovery occurred as a result of expansion of the effector memory ($CD45RA^-$ $CCR\text{-}7^-$) population ($P < .05$), suggesting that protective T-cell responses are likely to be long lived. T-cell–receptor signal joint excision circles (TRECs) were not detected in reconstituting T cells in dose-level 2 patients, indicating they are likely to be derived from the infused allodepleted cells. Spectratyping of the T cells at 4 months demonstrated a polyclonal Vβ repertoire.

In interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assays, antiviral responses to adenovirus and cytomegalovirus (CMV) were preserved following allodepletion. Likewise, using HLA-A2–pp65 tetramers, we have shown that the frequency of CMV-specific T cells is unaffected by allodepletion. Moreover, the donor anti-EBV response is partially retained by recognition of EBV antigens through the nonshared haplotype.

In summary, cytomegalovirus (CMV)–and Epstein-Barr virus (EBV)–specific responses in 4 of 6 evaluable patients at dose level 2 as early as 2 to 4 months after transplantation, whereas such responses were not observed until 6 to 12 months in dose-level 1 patients. The incidence of significant acute (2 of 16) and chronic graft-versus-host disease (GVHD; 2 of 15) was low.

We studied whether allodepletion affects the response to candidate tumor antigens in myeloid malignancies. Using HLA-A2–PR1 tetramer analysis, we found that the frequency of T cells recognizing the PR1 epitope of proteinase 3 was not significantly different in allodepleted and unmanipulated PBMCs from patients with chronic myeloid leukemia (CML) undergoing transplantation. Based on these data, we have embarked on a phase 1 clinical trial of addback of allo-LCL–depleted donor T cells in the haplo-identical setting and we demonstrated that allodepleted donor T cells can be safely used to improve T-cell recovery after haploidentical SCT and may broaden the applicability of this approach.

2. RIASSUNTO

La mancanza di una piena corrispondenza dell'antigene HLA con quello del donatore È la maggior limitazione all'applicabilità del trapianto di cellule staminali emopoietiche (SCT). Comunque, quasi tutti i pazienti hanno un potenziale donatore con HLA aploidentico. Recenti studi sulla capacità di mobilitare e selezionare le cellule emopoietiche staminali hanno reso il trapianto di cellule staminali da donatori aploidentici fattibile, sia in termini di attecchimento che per un accettabile rischio di graft versus host disease (GVHD).
La ricostituzione delle cellule T è un punto determinante dell'outcome dopo SCT. La deplezione delle cellule T è necessaria per prevenire la GVHD nel caso di aploidentici portando ad una profonda immunodeficienza post-trapianto. Questo è il limite maggiore nell'applicazione ad ampio spettro del SCT aploidentico, dovuto sia all'alta morbilità che mortalità da infezioni virali in seguito alla perdita dell'immunità virale e sia per l'alta ricaduta dovuta alla riduzione della risposta graft versus leucemia (GVL). In una serie di Perugia, 27 di 101 pazienti che hanno subito un trapianto aploidentico per leucemia acuita morirono per infezione. Una semplice aggiunta di cellule T può essere efficace nel prevenire questi problemi ma causerebbe GVHD a causa dell'alta presenza di cellule T allo reattive nel sangue periferico rispetto alle cellule T antivirali e antileucemiche.
Sono stati difatti numerosi approcci nel tentativo di superare questo problema, includendo l'induzione di energia nelle cellule T del donatore, aggiunta di cellule T regolatorie (Tregs) e la traduzione dei linfociti del donatore con un gene suicida. Alla fine, cellule anergizzate e le cellule Tregs possono avere un effetto inibitore su un buon numero di risposte T mediate, e i protocolli ora in uso per un'efficiente traduzione delle cellule T possono portare ad una perdita della risposta antivirale. Un approccio alternativo È la deplezione delle cellule T allo reattive responsabili della GVHD attraverso la delezione delle cellule T che vengono attivate in risposta alle cellule presentanti l'antigene del ricevente. Questo approccio ha il vantaggio che le cellule allo reattive sono permanentemente rimosse e non possono influenzare le rimanenti cellule T. Le cellule allo reattive possono essere bersagliate a causa della loro espressione in superficie di markers di attivazione e della proliferazione in una reazione leucocitaria mista (MLR). Le cellule allo reattive possono essere eliminate usando una immunotossina, separazione immunomagnetica, agenti chemioterapici, sorting tramite il citofluorimetro.

Il gruppo di Montagna ha dimostrato che è possibile ottenere la riduzione in vitro dell'alloreattività dopo aver bersagliato le cellule T che esprimono il CD25 attivato (interleuchina 2 (IL-2) recettore della catena alfa), usando una immunotossina (RFT5-SMPT-dgA) consistente di un anticorpo murino riconosciuto dal recettore p55 dell' IL2 coniugato alla ricina A deglicosilata. Usando un approccio simile, abbiamo dimostrato che la risposta delle cellule T contro antigeni virali e potenziali antigeni tumorali è conservata anche in seguito all'allodeplezione.
Basandosi sui loro dati preclinici, il gruppo di Necker ha usato l'immunotossina CD25 per eseguire un'allodeplezione ex vivo in una fase 1-2 di uno studio clinico condotto su 15 pazienti che hanno subito un SCT aploidentico da donatore non correlato. Il gruppo di Solom ha usato un'approccio simile in 16 pazienti anziani che hanno subito un trapianto da donatore correlato HLA identico.
Mentre questi studi dimostrano che l'immunoterapia adottiva con cellule T di donatore allodeplete è fattibile ed ha una bassa incidenza di GVHD, nessuno studio si è interessato della capacità delle cellule allodeplete infuse di aumentare l'immunità cellulo-mediata.
In uno studio più recente, quattro pazienti hanno raggiunto una conta di CD3 maggiore di 500/μL in sei mesi dopo SCT, ma il follow up è stato breve ed i dati sull'immunoricostituzione sono limitati, senza nessun riferimento alla risposta antivirale. Da aggiungere che non è chiaro quante cellule allodeplete del donatore devono essere infuse per reinstaurare una risposta antivirale clinicamente rilevante. Dati derivanti dalle infusioni di linfociti del donatore in un modello HLA identico suggerisce che una dose pari a 10^6/kg potrebbe essere sufficiente per fermare una riattivazione virale da EBV e adenovirus. E' comunque critico determinare se il livello di allodeplezione raggiunto con l'immunotossina anti-CD25 sia sufficiente per permettere una infusione di cellule T sufficienti per ripristinare una risposta immunitaria utile senza causare GVHD. Per superare questo punto abbiamo comparato l'immunoricostituzione dopo l'infusione di due differenti dosi di cellule T del donatore allodeplete in un modello aploidentico.
Il principale scopo dello studio era comparare l'immunoricostituzione e la risposta immune virale specifica dopo l'infusione di due differenti dosi di cellule T del donatore allodeplete. Lo scopo secondario include il confronto dell'incidenza di GVHD acuta e cronica e l'outcome della riattivazione virale e dell'infezione virale ad ogni livello di dose.
Ciascun paziente è supposto ricevere tre infusioni di cellule T del donatore allodeplete alla stessa dose al giorno 30, 60 e 90 dopo il

trapianto, una volta accertato che non ci fosse alcuna evidenza di GVHD di grado II o maggiore, o che la conta totale delle cellule T fosse maggiore di 1000/μl. I pazienti potevano ricevere la quarta dose al giorno 120 se clinicamente indicato e la quota di cellule T circolanti fosse inferiore a 1000/μl. La dose livello 1 (10^4 cellule/Kg/dose) è stata scelta perchè è risaputo che a questa concentrazione le cellule T, anche se non allodeplete, non sono in grado di scatenare una GVHD in riceventi aploidentici. Il confronto è stato fatto con una dose di un logaritmo più alta (10^5 cellule/Kg/dose). I pazienti sono stati inizialmente arruolati alla dose livello 1, con un'escalation alla dose livello 2 quando l'incidenza di una GVHD acuta fosse inferiore a 25%. Tutti i pazienti elegibili che hanno ricevuto un trapianto aploidentico sono stati arruolati consecutivamente.

La produzione di cellule T del donatore allodeplete è stata fatta sotto le condizioni di GMP al Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX, usando procedure standard autorizzate dall'FDA. Sei settimane prima del trapianto, 30 ml di sangue periferico del ricevente vengono utilizzate per preparare cellule linfoblastoidi (LCLs) EBV-trasformate. Immediatamente prima della mobilizzazione, vengono prelevati 160 ml di sangue periferico dal donatore, isolate le cellule mononucleate (PBMCs) e messe in cocoltura con le LCLs del ricevente precedentemente irradiate a 70Gy in un mezzo libero da siero. Venivano allestite anche colture di controllo per testare la proliferazione residua. Dopo 72 ore, le cocolture venivano allodeplete tramite un trattamento overnight con l'immunotossina anti-CD25. Quindi venivano eseguiti tests di sterilità e di proliferazione residua minima (minimal residual proliferation, MLR). Criteri di accettazione per l'infusione delle cellule così preparate erano: meno dell'1% di cellule $CD3^+/CD25^+$ e meno del 10% di proliferazione residua minima contro le cellule ospite. Se le cellule trapiantate avevano attecchito con successo, le cellule T allodeplete venivano scongelate ed infuse al giorno 30, 60 e 90 post trapianto.

L'infusione delle cellule T era associata ad una bassa incidenza di tossicità. Due dei sedici pazienti hanno sviluppato una significativa GVHD acuta. P2 ha sviluppato una GVHD della cute di IV grado dopo una singola infusione di cellule allodeplete alla dose livello 1, e P12 ha sviluppato una GVHD della cute di II grado dopo due infusioni alla dose livello 2. Entrambi hanno avuto un'evoluzione verso una estesa GVHD cronica (nel primo paziente ha colpito il fegato, nell'altro la cute e la bocca). Il paziente con cronica GVHD del fegato è morto per insufficienza epatica secondaria ad adenovirus. La GVHD cronica del secondo paziente si è completamente risolta, e questo paziente è

stato senza immunosoppressione per 26 mesi a Giugno 2006. Non si è verificato nessun altro effetto attribuibile all'infusione di cellule T allodeplete del donatore durante questo studio.
Abbiamo dimostrato che l'immunoterapia adottiva con cellule T del donatore allodeplete migliora la ricostituzione delle cellule T dopo trapianto di cellule staminali aploidentico. La ricostituzione di cellule T nei pazienti infusi alla dose livello 1 è stata molto lenta, confrontata con quella osservata senza l'aggiunta di cellule T allodeplete, ed il numero normale di cellule T non si è raggiunto prima di 9 – 12 mesi dopo il trapianto aploidentico. Questo era prevedibile poiché la dose delle cellule allodeplete infuse era simile al numero di cellule T infuse con il trapianto e quindi questi pazienti costituiscono una coorte di controllo "nello-studio" per adeguare l'impatto di una infusione a dosi più alte di cellule allodeplete.
In contrasto, i pazienti alla dose livello 2 hanno dimostrato un recupero di cellule T molto più veloce, soprattutto tra 3 e 5 mesi dopo il trapianto, che è il periodo in cui i pazienti soccombono alle infezioni dopo SCT aploidentico.
CMV, EBV e le pneumocistiti raramente causano malattia nei pazienti che hanno ricevuto SCT, quando la conta delle CD4 è maggiore di 300/μl, e alcuni gruppi utilizzano questo valore come soglia per smettere la profilassi con il cotrimoxazolo e monitorare la viremia. Il tempo mediano per raggiungere questa soglia era quattro mesi nei pazienti a dose livello 2, confrontati con più di sei mesi nei pazienti a dose livello 1 senza aggiunta di cellule T allodeplete.
Inoltre, la maggior parte delle cellule T che si sono ripristinate nei pazienti alla dose livello 2 mostrano il fenotipo di cellule di memoria effettrici, implicando che la risposta protettiva derivante dalle cellule T è probabilmente destinata a rimanere per tutta la vita. Poiché è possibile che le cellule T vergini possono convertirsi in cellule di memoria effettrici, i nostri studi hanno dimostrato che nessuno dei 4 pazienti testati alla dose livello 2 ha mostrato la presenza di TRECs al tempo della ripresa delle cellule T, suggerendo come sia improbabile che le cellule T vergini derivate dalle cellule staminali trapiantate abbiano contribuito all'aumento dell'immuno-ricostituzione vista in questi pazienti. Questi dati suggeriscono fortemente che la ripresa accelerata delle cellule T vista nei pazienti a dose livello 2 sia dovuta all'infusione delle cellule T del donatore allodeplete.
Abbiamo notato che la risposta EBV-specifica è parzialmente conservata nel prodotto delle cellule T allodeplete, nonostante usassimo le LCLs come stimolatori, attraverso il riconoscimento degli epitopi EBV presentati sull'aplotipo dell'HLA. Le risposte antivirali in

questi pazienti si sono osservate precocemente tra due e quattro mesi dopo trapianto SCT, particolarmente dopo una riattivazione virale. Questo coincide con il periodo durante il quale i pazienti sono al massimo rischio di infezione virale dopo trapianto aploidentico e questo è importante perché una tale risposta non si è vista fino a 6 - 12 mesi post-SCT.

Mentre le infezioni e le riattivazioni virali erano state frequenti, l'incidenza globale di morti direttamente collegate alle infezioni era molto bassa (2 di 16 pazienti). In congiunzione con i nostri dati sulla risposta immune virale-specifica, questo dato suggerisce che mentre il numero di cellule infuse può essere insufficiente a prevenire le riattivazioni, gioca sicuramente un ruolo fondamentale nel prevenire la progressione alla malattia/morte. Presi insieme, i nostri dati suggeriscono che un'immunoterapia adottiva con cellule T allodeplete del donatore alla dose da 1 fino 3 x 10^5/kg sia sufficiente per migliorare la ricostituzione delle cellule T e l'immunità antivirale post SCT aploidentico. Questo approccio può potenzialmente ridurre la mortalità associata alle infezioni e perciò ampliare l'applicabilità del trapianto aploidentico. Chiaramente, comunque, sono necessari studi più ampi per dimostrare che tale approccio conferisce un reale beneficio terapeutico.

Il confronto del numero delle cellule T circolanti nei pazienti trattati alla dose livello più alta ed il numero di cellule infuse implica che le cellule T del donatore sono capaci di espandersi significativamente in vivo. Questo è simile a quello che è stato osservato dopo un trasferimento adottivo di cellule T citotossiche EBV- e CMV-specifiche in pazienti che dovevano subire un SCT di cellule T deplete e può essere correlato con la profonda linfopenia post trapianto.

I nostri studi confermano che l'uso dell'immunoterapia adottiva effettuata con cellule T del donatore allodeplete eseguita in pazienti riceventi un trapianto di cellule staminali emopoietiche è una tecnica sicura. Abbiamo osservato una bassa incidenza di GVHD in tutti e due i livelli di dose, confrontandolo con quello visto senza aggiunta di cellule T allodeplete. Nel caso di P12, la dose di cellule allodeplete era sensibilmente più alta della dose di cellule T infuse con il trapianto, per cui probabilmente è questa la causa della GVHD; per quanto riguarda P2 la dose cellulare era la stessa, per cui non è chiaro se le cellule T allodeplete sono state le responsabili della GVHD in questo paziente. Studi precedenti hanno osservato un'alta incidenza di GVHD acuta dopo un'infusione di linfociti del donatore non manipolati a 10^5/kg nei primi 3 mesi di SCT, anche in soggetti con HLA identico. La bassa incidenza di GVHD acuta e cronica osservata

nei nostri pazienti trattati alla dose livello 2 dimostra che la nostra strategia effettivamente elimina in maniera clinicamente rilevante le cellule alloreattive. Inoltre, la deplezione delle $CD4^+/CD25^+$ cellule T regolatorie dalle cellule infuse non sembra indurre un potenziale sviluppo della GVHD, presumibilmente a causa dell'assenza di un numero significativo di cellule effettrici alloreattive. Similarmente, non abbiamo osservato un eccesso di fenomeni autoimmuni post SCT.
Sebbene i dati preclinici dal nostro e da altri gruppi suggeriscono che le risposte antileucemiche possono essere conservate dopo l'allodeplezione, la recidiva rimane il problema maggiore nella nostre coorte di studio (7 su 16 pazienti). E' comunque evidente che nei pazienti con patologie maligne ad alto rischio, i benefici di questo approccio nel migliorare la risposta immunitaria alle infezioni possono essere controbilanciati dalla recidiva leucemica nei sopravvissuti. L'estensione di questo approccio per dimostrare la risposta antileucemica richiede uno studio più ampio, randomizzato e con una dose maggiore di cellule T allodeplete di quelle richieste per la ricostituzione di una risposta antivirale.

3. INTRODUCTION

3.1. HEMOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

As an approach to treat malignant and nonmalignant disorders, hematopoietic stem cell transplantation (HSCT), has been around for more than 50 years. The earliest work was done in animal models in the mid 1950s. In the 1960s, the first few cases of successful use of HSCT in the treatment of congenital immunodeficiency disorders and end-stage leukemia were reported. Subsequent research focused on designing conditioning regimens, decreasing transplantation-related morbidity and mortality, improving survival and our understanding of the immune mechanisms associated with both the adverse effects and the antitumoral effects of the transplanted graft (Copelan EA, 2006).

3.1.1. Types of HSCT

Until recently, the 2 major types of HSCT have been autologous and allogeneic transplantations. A third type now being used with increasing frequency is umbilical-cord blood transplantation (CBT).

a. Autologous transplantation refers to the use of the patient's own stem cells as a rescue therapy after high-dose myeloablative therapy. This is generally used in chemosensitive hematopoietic and solid tumors to eliminate all malignant cells by administering high-dose chemotherapy with subsequent rescue of the host's bone marrow with previously collected autologous stem cells. Immunosuppression is not required after autologous transplantation.
b. Allogeneic transplantation refers to the use of stem cells from a human leucocyte antigen (HLA)–matched related or unrelated donor. This is used for a variety of malignant and nonmalignant disorders to replace a defective host marrow or immune system with the normal donor marrow and immune system. The key to successful allogeneic transplantation is finding an HLA-matched donor because it decreases the risk of graft rejection and graft versus host disease (GVHD) (Socie G, 2005).
c. The 3 HLA loci critical for matching are HLA-A, HLA-B, and HLA-DR. HLA-C, and HLA-DQ were recently added to this list. A completely matched sibling donor is considered ideal. For unrelated donors, a completely matched or a single mismatch is considered acceptable for most transplantation protocols. Syngeneic transplantation is a form of allogeneic

transplantation in which the donor is an identical twin sibling of the patient. Graft rejection is less of an issue for such transplants when compared to other allogeneic transplants (Morishima Y, *et al.* 2002).

d. Umbelical-cord blood transplantation (CBT) refers to the use of hematopoietic stem cells collected from the umbilical cord and placenta. The use of CBT has rapidly increased because of several favorable factors: ease of collection, expanded and prompt availability, no risk to the donors, decreased risk of adverse effects (eg, GVHD, transmission of infections), increased tolerance to HLA-mismatch, and no risk of donor loss at the time of transplantation (Koh LP, 2004).

The traditional source of hematopoietic stem cells for use in autologous and allogeneic transplantations was bone marrow. Use of peripheral blood as a source of these cells later replaced bone marrow for all autologous and most allogeneic transplantations (Cutler C, *et al.,* 2001). Table 1 lists the differences in the cellular characteristics of these commonly used sources of stem cells, and Table 2 lists the clinical differences.

3.1.2. Indications for HSCT

More than 30,000 autologous and 15,000 allogeneic transplantation procedures are performed every year worldwide. The list of diseases for which HSCT is being used is rapidly increasing. More than half of the autologous transplantations are performed for multiple myeloma and non-Hodgkin lymphoma, and a vast majority of allogeneic transplants are performed for hematologic and lymphoid cancers.

Table 3 summarizes the common indications for HSCT. Cord-blood transplants are being used for many of the allogeneic transplant indications whenever a suitable HLA-matched donor is unavailable or whenever time for identifying, typing, and harvesting a transplant from an unrelated donor is limited.

The lack of fully HLA-matched donors is a major limitation to the applicability of hematopoietic stem cell transplantation (HSCT). However, almost all patients have potential donors who are HLA haplo-identical. Recent advances in our ability to mobilize and select HSCs have made HSCT from haplo-identical donors feasible, both in terms of reliable engraftment and acceptable rates of graft versus host disease (GVHD) (Aversa F, *et al.*, 1998; Handgretinger R, *et al.,* 2003).

Cellular Characteristics	Source		
	Bone Marrow	Peripheral Blood	Cord Blood
Stem-cell content	Adequate	Good	Low
Progenitor-cell content	Adequate	High	Low*
T-cell content	Low	High	Low, functionally immature
Risk of tumor cell contamination	High	Low	Not applicable

Table 1. Cellular Characteristics of Various Sources of Stem Cells

*Studies have shown that the cord-blood progenitor cells have greater proliferative potential than that of peripheral blood and marrow progenitor cells

Cellular Characteristics	Source		
	Peripheral Blood	Bone Marrow	Cord Blood
HLA matching	Close matching required	Close matching required	Less restrictive than others
Engraftment	Fastest	Faster than cord blood but slower than peripheral blood	Slowest
Risk of acute GVHD	Same as in bone marrow	Same as in peripheral blood	Lowest
Risk of chronic GVHD	Highest	Lower than peripheral blood	Lowest

Table 2. Clinical Characteristics With Various Sources of Stem Cells

Autologous Transplantation		Allogeneic Transplantation	
Malignant Disorders	**Nonmalignant Disorders**	**Malignant Disorders**	**Nonmalignant Disorders**
• Multiple myeloma • Neuroblastoma • Non-Hodgkin lymphoma • Hodgkin disease • Acute myeloid leukemia (AML) • Medulloblastoma • Germ-cell tumors	• Autoimmune disorders • Amyloidosis	• AML • Non-Hodgkin lymphoma • Hodgkin disease • Acute lymphoblastic leukemia (ALL) • Chronic myeloid leukemia (CML) • Myelodysplastic syndromes • Multiple myeloma • Chronic • Lymphocytic • Leukemia	• Aplastic • Anemia • Fanconi anemia • Severe combined immunodeficiency • Thalassemia major • Diamond-Blackfan anemia • Sickle cell anemia • Wiskott-Aldrich Syndrome • Osteopetrosis • Inborn errors of metabolism • Autoimmune disorders

Table 3. Common Indications for HSCT

3.2. GRAFT-VERSUS-HOST DISEASE (GVHD)

Graft-versus-host disease is a frequent complication of allogeneic BMTs. In GVHD, the donor's bone marrow attacks the patient's organs and tissues, impairing their ability to function, and increasing the patient's susceptibility to infection. Approximately 50 percent of patients undergoing an allogeneic BMT with a related HLA-matched donor develop GVHD. Fortunately, the majority of cases are mild. GVHD is not a complication of autologous BMTs. GVHD is often discussed as if it were a single disease. It is, in fact, two diseases: acute GVHD and chronic GVHD. Patients may develop one, both or neither. Acute and chronic GVHD differ in their symptoms, clinical signs and time of onset. (Clinical signs are the results of physical exams, x-rays or lab tests that confirm the existence and extent of a disease.). GVHD can be a temporary inconvenience or a serious, life-threatening disease. Older BMT patients are more likely to develop GVHD then younger patients. The incidence and severity of GVHD is also higher among patients whose bone marrow donor is unrelated or not perfectly matched. The symptoms of GVHD are many and varied, and the list may at first be overwhelming. Keep in mind however that most patients undergoing an allogeneic BMT with a related HLA-matched donor develop only a mild or moderate case of GVHD, or no

GVHD at all. Although GVHD can be life- threatening or fatal, most patients survive the disease without long-term disabling side effects.

3.2.1. Acute GVHD

Acute GVHD usually occurs during the first three months following an allogeneic BMT. T-cells present in the donor's bone marrow at the time of transplant identify the BMT patient as "non-self' and attack the patient's skin, liver, stomach, and/or intestines. The earliest sign of acute GVHD is often a skin rash that usually first appears on the patient's hands and feet (Picture 1). The rash may spread to other parts of the body and develop into a general redness similar to a sunburn, with peeling or blistering skin. Cramping, nausea, and watery or bloody diarrhea are signs of GVHD in the stomach or intestines. 9aundice (yellowing of the skin and eyes) indicates that acute GVHD has affected the liver. Physicians grade the severity of acute GVHD according to the number of organs involved and the degree to which they're affected. Acute GVHD may be mild, moderate, severe or life-threatening.

Stage 1 (mild) : a skin rash over less than 25% of the body.

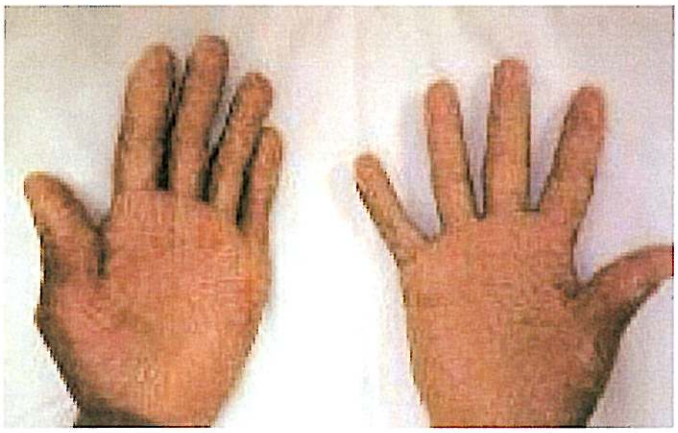
Stage 2 (moderate) : a skin rash over a more than 25% of the body accompanied by mild liver or stomach and intestinal disorders.

Stage 3 (severe) : redness of the skin, similar to a severe sunburn, and moderate liver, stomach and intestinal problems.

Stage 4 (life-threatening) : blistering, peeling skin, and severe liver, stomach, and intestinal problems.

To minimize the risk of graft rejection and GVHD, allogeneic BMT patients are given drugs to prevent GVHD before and after transplant that suppress the immune system. Use of these drugs, however, increases the risk of infection. Precautions taken to limit the patient's exposure to harmful bacteria, viruses and fungi during this period may include special air-filtering equipment in the patient's room, frequent hand- washing by visitors, use of masks, gloves and robes by the patient and/or visitors, and elimination of fresh fruits, flowers and vegetables from the patient's environment which may harbor potentially harmful bacteria. Patients over the age of 30 are more likely to develop acute GVHD than younger patients. Patients

receiving marrow from a female donor who has had two or more viable pregnancies also are more likely to develop acute GVHD (Goker H *et al.,* 2001).



Picture 1. Acute graft versus host disease involving desquamating skin lesions in a patient who underwent allogeneic bone marrow transplantation for myelodysplasia.

3.2.2. Prevention & treatment of acute GVHD

Although GVHD is not yet preventable, steps can be taken to reduce the incidence and severity of GVHD. Administration of immunosuppressive drugs such as cydosporine (alone or in combination with steroids) and methotrexate prior to the transplant have proven effective in reducing the incidence and severity of GVHD. They may be administered for several months post-transplant, particularly if acute GVHD progresses to Stage II, or if the patient develops chronic GVHD.
Cyclosporine, steroids and methotrexate weaken the ability of the donor's T cells to launch an attack against the patient's organs and tissues. These drugs, however, have potential side effects. Cyclosporine can be very toxic to the kidneys, cause increased hair growth on the body, especially facial hair on women, and on rare occasions can result in neurological problems such as seizures, confusion, anxiety, and changes in thought processes. Methotrexate may cause inflammation of the mouth, nose and/or throat. Side effects of steroids indude weight gain, fluid retention, elevated blood sugar level, mood swings and/or confused thinking. These side effects are

temporary and disappear once use of these drugs is discontinued (Iwasaki T., 2004).

3.2.3. Chronic GVHD

Chronic GVHD usually develops after the third month post-transplant. Scientists believe that new T-cells produced after the donor's bone marrow has engrafted in the patient may cause chronic GVHD. Most patients with chronic GVHD experience skin problems that may include a dry itching rash, a change in skin color, and tautness or tightening of the skin. Partial hair loss or premature graying may also occur. Liver abnormalities are seen in many patients with chronic GVHD. This is usually evidenced by jaundice and abnormal liver test results.

Chronic GVHD can also attack glands in the body that secrete mucous, saliva or other lubricants. Patients with chronic GVHD usually experience dryness or stinging in their eyes because the glands that secrete tears are impaired. Glands that secrete saliva in the mouth are often affected by chronic GVHD and, less often, those that lubricate the esophagus, making swallowing and eating difficult. It's common for patients with chronic GVHD to experience a burning sensation in their mouths when using toothpaste or eating acidic foods. Good oral hygiene is imperative to minimize the risk of infection.

Chronic GVHD may attack glands that lubricate the stomach lining and intestines, interfering with the body's ability to properly absorb nutrients. Symptoms include heartburn, stomach pain and/or weight loss.

Occasionally patients with chronic GVHD experience "contractures," a tightening of the tendons in pints that makes extending or contracting their arms and legs difficult. Chronic GVHD can also affect the lungs, causing wheezing, bronchitis, or pneumonia. As is the case with acute GVHD, older patients are more likely to develop chronic GVHD than younger patients. Seventy to 80 percent of patients who develop chronic GVHD will previously have had acute GVHD. Chronic GVHD is also more common in patients whose donor is unrelated or whose marrow is not perfectly matched (Lee SJ *et al.,* 2003)

MOST COMMON	LESS COMMON
rash, itching, general redness of skin	skin scarring
dark spots, tautness of skin	partial hair loss, premature graying
jaundice (yellowing of skin and eyes)	severe liver disease
abnormal liver tests	vision impairment
dry, burning eyes	heartburn, stomach pain
dryness or sores in mouth	difficulty swallowing
burning sensation when eating acidic foods	weight loss
bacterial infections	contractures
	difficulty breathing
	bronchitus, pneumonia

Symptoms, side effects of chronic GVHD

3.2.4. Treatment of chronic GVHD

Chronic GVHD is usually treatable with steroids such as prednisone, ozothioprine and cyclosporine, which suppress the patient's immune system. Antibiotics such as Bactrim or penicillin or both are usually taken to reduce the risk of infection while chronic GVHD is being treated. In addition, patients may be required to wear face masks while around other people, stay out of crowds, and avoid fresh plants, fruits and vegetables. Patients with chronic GVHD are usually advised to avoid vaccinations with live viruses such as German measles, tetanus, polio, etc. until the GVHD problem is completely resolved and use of immunosuppressive drugs ends.
Researchers have been studying the effectiveness of using other drugs to control chronic GVHD in patients resistant to standard therapy. A recent study found thalidomide to be effective in controlling chronic GVHD in "high risk" patients (i.e., those likely to develop life-threatening GVHD) with minimal side effects (drowsiness). (Iwasaki T., 2004).

3.3. GRAFT VERSUS LEUKEMIA (GVL)

The term graft-versus-leukemia (GVL) is used here to describe the immune-mediated response which conserves a state of continued remission of a hematological malignancy following allogeneic marrow stem cell transplants. Although the evidence for a GVL effect after allogeneic bone marrow transplantation (BMT) is now well accepted, the mechanisms involved in the effect are not completely known (Barrett A.J. *et al.*, 1996) However, because graft-versus-host disease (GVHD) is intimately associated with GVL, it can be assumed that similar mechanisms control GVHD and GVL. GVHD requires the

recognition by donor T cells of antigens presented by major histocompatibility complex (MHC) molecules on the recipient cells initiating clonal expansion of responders and an effector response involving lymphocytes and cytokines. In GVHD, this leads to the clinical features of acute and chronic GVHD. In GVL reactions, the alloresponse suppresses residual leukemia. GVHD reactions are directed against a broad spectrum of tissues, including the skin, mucosa, the biliary tree, exocrine glands, synovia, lungs and bone marrow. The dominant antigens on leukemia cells driving the GVL response are not known: major or minor histocompatibility antigens coexpressed on GVHD targets (such as normal skin and gut cells) and leukemic cells could induce a nonspecific GVH/GVL alloresponse (Perreault C. *et al.,* 1990) The response against either normal or malignant bone marrow-derived cells may also overlap. Thus GVL may in part be a graft-versus-marrow effect involving lymphoid or myeloid lineages or both. Additionally, leukemia cells could induce a more specific alloresponse if they express antigens, either not present or underexpressed in cells of other tissues (Barrett AJ, *et al.*, 1996)
The mechanism whereby the GVL response confers a permanent state of remission (cure) following the transplant deserves some consideration as hematological malighancies become better understood and as their hierarchical structure becomes increasingly appreciated. Many leukemias are complex populations comprised of progenitor cells with infinite self-renewal capacity, intermediate cells (blasts) capable of limited self-renewal and end cells with no capacity for further division (Knuutila S. 1997) Clearly a GVL effect is more likely to result in leukemia eradication, if the immune attack is directed against the earlier cells in the hierarchy. However, a situation could be imagined where immune regulation does not damage dormant leukemic progenitors but prevents large-scale production of blast cells. In a steady-state, such a mechanism would be perceived as a clinical cure (with or without detectable residual disease, depending on the sensitivity of the assay). Such considerations govern whether to be curative the GVL response is required lifelong or only for a defined period. The need for a continuing GVL response has clinical implications: at any time, failure of immune surveillance could upset the balance between the GVL effectors and their targets, resulting in leukemic relapse. Furthermore, the prolonged persistence of subclinical disease could increase the chance of clonal progression and relapse due to leukemic escape from immune control (Dermime S. *et al.*, 1997).

3.3.1. T cells and GVL

The relative contribution of the $CD4^+$ and $CD8^+$ T cell subsets to GVL reactivity has been investigated extensively both in animal models and in man. Mice receiving $CD8^-$ depleted marrow, or marrow with the addition of purified $CD4^+$ T cells, had a low incidence of GVHD with high leukemia-free survival, supporting an important role of $CD4^+$ cells in GVL. $CD8^+$ T cells also mediate GVL reactivity: mice receiving CD8-depleted donor marrow had a higher leukemia relapse incidence than those receiving CD4-depleted marrow. In experimental mouse transplants the addition of purified $CD8^+$ T cells to the graft had an antitumor effect and facilitated engraftment without inducing GVHD. We can conclude that in general both $CD4^+$ and $CD8^+$ T cell subsets contribute to GVL reactions. However, the dominant mechanism is strain specific and varies with the degree of donor-recipient histocompatibility. In man, $CD4^+$ and $CD8^+$ alloreactive cells with antileukemic activity have been generated in vitro by several investigators. After BMT there is an increase of $CD4^+$ precursors recognizing leukemia demonstrated by helper T lymphocyte precursor frequency assays. We demonstrated the presence of donor-derived cytotoxic T cells in the blood of chronic myelogenous leukemia (CML) patients after BMT, showing specific reactivity to the patient's leukemia cells. Depletion of $CD4^+$ cells from the responding population significantly reduced the cytotoxic T lymphocyte precursor frequency against the recipient's leukemia cells whereas $CD8^+$ depletion had only a modest effect. These results suggest that $CD4^+$ T cells play a prominent role in the GVL response after BMT in man. Clinical trials with CD8-depleted marrow transplants or CD8-depleted peripheral blood leukocytes to treat relapsed leukemia after BMT lend support to the possibility that the residual GVL effect is mediated by $CD4^+$ cells. Nimer SD, *et al.* (1994) using CD8-depleted marrow transplants observed a significantly lower incidence of GVHD without an increase in the rate of leukemia relapse compared with T-replete transplants. Giralt S, *et al.* (1995) used CD8-depleted DLT to treat leukemia relapsing after BMT and observed a 60% response with only a 20% incidence of GVHD. Contrasting with these data are other clinical studies showing that CD4-depleted marrow transplants with an adjusted $CD8^+$ T cell content significantly reduce GVHD without affecting engraftment and immune reconstitution. These clinical observations should be interpreted with caution. It is possible that the separation of GVL and GVHD observed may simply be the result of transplanting low but nevertheless sufficient residual doses of

immunocompetent cells, since similar results have been obtained using unmanipulated T cells (Barret AJ *et al.*, 1997).

3.4. REGULATORY T-CELLS

There is accumulating evidence that regulatory T cells (Tregs), once called suppressor T cells, are present in the normal immune system as an essential cellular component for maintaining immunologic self-tolerance and immune homeostasis. Their anomaly can be a cause of aberrant or excessive immune responses, including autoimmune and immunopathological diseases.
There are several types of Tregs so far reported. Some of them are naturally generated in the immune system, others adaptively developing from naïve T cells as a result of a particular mode of immune reactions. Among them, naturally occurring $CD4^+CD25^+$ Tregs are the main focus of current research. (Taylor PA *et al.,* 2002) They have several unique features. For example, they engage not only in the sustenance of self tolerance but also in the negative control of a variety of immune responses to nonself (such as microbial antigens or allogeneic transplantation antigens) or quasi-self (such as tumor-associated antigens). The majority is produced by the normal thymus as a functionally mature T-cell subpopulation specialized for suppressive function, and persists in the periphery with stable function. In addition, it is now known that the transcription factor Foxp3 specifically determines their cell lineage fate and their manifestation of suppressive activity. Because of their natural presence in the immune system, natural Tregs are a good target for designing the ways to treat or prevent immunological diseases and to control various immune responses at the cellular and molecular level. Although physiological roles of adaptively induced Tregs secreting immunosuppressive cytokines such as IL-10 or TGF-β need to be fully established, they can still be exploited as a therapeutic tool.

3.5. DEPLETION OF ALLOREACTIVE T-CELLS

T-cells are special white blood cells that recognize foreign matter in the body. T-cells orchestrate attacks on bacteria, viruses and other substances foreign to the body. They can also distinguish "self" from "non-self"-human cells that belong in one person's body and those that do not.
On the surface of many human cells is an inherited set of genetic markers called "human leukocyte antigens" (HLA). Like a fingerprint,

no two persons' set of HLA markers are exactly alike (except for identical twins). The T- cells use these HLA markers to distinguish "self" from "non-self." If a "non- self" human cell is encountered in the body, the T- cells quickly activate the immune system to destroy it. The ability of the immune system's T-cells to distinguish "self" from "non- self" can create a serious problem after allogeneic BMTs. Unless the donor is an identical twin, his or her tissue type (those HLA markers or genetic fingerprints) will differ from that of the patient. The patient's T-cells may identify the donor's bone marrow as "non-self" and attack the donated bone marrow. This is called graft rejection.

To prevent graft rejection, total body irradiation (TBI) and/or drugs such as cyclophosphamide are used to kill the cancerous cells and to suppress a patient's immune system. The radiation and drugs disrupt the ability of T- cells to recognize the donated bone marrow as "non-self" and to launch an immune system attack. Immune system suppression is not required in autologous BMTs since the bone marrow transfused into the patient is his or her own.)

Because of the high frequency of alloreactive T cells in donor peripheral blood, rigorous T-cell depletion is necessary to prevent GVHD in the haplo-identical setting. Together with in vivo immunosuppression and the HLA disparity between host and donor, this results in profound post transplantation immunodeficiency (Haddad E, *et al.,* 1998; Eyric M *et al.,* 2001). This is the major barrier to the broader application of haplo-identical HSCT and results in high morbidity/mortality from viral infections due to the loss of antiviral immunity and high relapse rates due to the loss of the graft versus leukemia (GVL) response (Aversa F, *et al.*, 1998; Handgretinger R, *et al.,* 2003).

Simple T-cell addback is unlikely to be effective in preventing these problems because the frequency of alloreactive T cells is much higher than that of either virus-specific T cells or T cells directed against hemopoietic-specific targets on leukemic cells in the early post-SCT period. A number of approaches have evolved to circumvent this, including induction of anergy in donor alloreactive T cells, (Gribben JG *et al.,* 1996; Guinan EC *et al.,* 1999). addback of regulatory T cells (Tregs), (Taylor PA *et al.,* 2002) and transduction of donor lymphocytes with suicide genes (Bonini C *et al.,* 1997; Tiberghien P *et al.,* 2001). However, anergized cells and Tregs may have an inhibitory effect on desirable bystander T-cell responses, and current protocols for efficient transduction of T cells may lead to loss of antiviral responses (Sauce D *et al.,* 2002). An alternative approach is to deplete the graft specifically of the alloreactive T cells responsible for

GVHD by deleting T cells that are activated in response to recipient antigen-presenting cells. This approach has the advantage that alloreactive T cells are permanently removed and cannot influence the function of the remaining T cells. Alloreactive cells may be targeted by their expression of surface activation markers, proliferation in a mixed leukocyte reaction (MLR) or the preferential retention of photoactive dyes. Alloreactive cells can be eliminated using immunotoxins (Montagna D *et al.,* 1999; Mavroudis DA *et al.,* 1998), immunomagnetic separation (van Dijk AM *et al.,* 1999), chemotherapeutic agents (Gendelmen M *et al.,* 2003), flow cytometric sorting (Godfrey WR et al., 2004), or photodynamic purging (Chen BJ *et al.,* 2002). Montagna *et al* (1999) have demonstrated reduced in vitro alloreactivity after targeting activated T cells expressing the activation marker CD25 (the interleukin-2 [IL-2] receptor α chain), using an immunotoxin (RFT5-SMPT-dgA) consisting of a murine antibody moiety recognizing the IL-2 receptor p55 chain conjugated to deglycosylated ricin A. Using a similar approach, we have shown that T-cell responses against viral and potential myeloid tumor antigens are preserved following allodepletion. It has been demonstrated that this anti-CD25 immunotoxin can deplete alloreactive T cells after incubation with haplo-identical PBMCs ex vivo, while retaining proliferative responses to CMV antigens and candida (Valteau-Couanet D *et al.,* 1993) and cytotoxic T-lymphocyte precursor (CTLp) frequencies against cytomegalovirus/Epstein-Barr virus (CMV/EBV)–infected targets (Montagna D *et al.,* 1999). More recently, the same group has performed a phase 1/2 clinical study of addback of allodepleted donor T cells in 15 patients undergoing HLA-mismatched stem cell transplantation (Cavazzana-Calvo M *et al.,* 2001; Andre-Schmutz I *et al.*, 2002).

Based on their preclinical data, the Necker group have used CD25-immunotoxin (IT) based ex vivo allodepletion in a phase 1/2 clinical study in 15 patients undergoing haploidentical/unrelated donor SCT (Cavazzana-Calvo M *et al.,* 2001; Andre-Schmutz I *et al.*, 2002).

Solomon *et al* (2005) have used a similar approach in 16 elderly patients undergoing HLA-matched related donor transplantation. While these studies demonstrated that adoptive immunotherapy with allodepleted donor T cells is feasible and results in a low incidence of GVHD, neither study addressed the critical issue of whether the infused allodepleted cells improved cell-mediated immunity. In the former study, 4 patients achieved CD3 counts of more than 500/μL by 6 months after SCT, but follow-up was short and data on immune reconstitution were limited, with no formal assessment of antiviral

responses. Additionally, it is unclear how many allodepleted donor T cells must be infused to restore clinically relevant antiviral responses. Data from donor lymphocyte infusions in the HLA-matched setting suggest that doses as low as 10^6/kg may be sufficient to clear viral reactivations with EBV and adenovirus. It is therefore critical to determine whether the level of allodepletion achieved with anti-CD25 IT is sufficient to allow addback of enough T cells to restore useful immune responses without causing GVHD. To address this issue, we have compared immune reconstitution after addback of 2 different doses of allodepleted donor T cells in the haploidentical setting.

One major limitation of applying this approach to patients with leukemia and bone marrow failure concerns the source of recipient stimulator cells. For patients who are aplastic either due to disease or to chemotherapy it may be difficult to obtain sufficient PBMCs for this approach. Further, crude PBMC preparations are relatively ineffective antigen-presenting cells. In the clinical study of Andre-Schmutz I, *et al* (2002) the presence of a residual proliferative response to recipient PBMCs was strongly associated with the subsequent development of GVHD. Moreover, in leukemic patients, PBMCs may be contaminated with leukemic blasts or dendritic cells that have processed leukemic antigens, so that allodepletion results in the loss of antileukemic activity. Dendritic cells, likewise, are difficult to prepare in adequate numbers for clinical protocols and require the use of expensive cytokines. We have used recipient EBV-transformed lymphoblastoid cell lines (LCLs) as stimulators to activate alloreactive T cells. LCLs are excellent antigen-presenting cells and therefore are likely to be more effective than bulk PBMCs in activating alloreactive T cells. Additionally, LCLs are relatively cheap to prepare, are easily expanded to large numbers, and have a standard phenotype, with less variability in expression of immunostimulatory molecules than PBMCs. Finally, unlike crude PBMCs, LCLs do not express myeloid or tumor antigens that may serve as targets for the GVL effect, so that donor T cells allodepleted after stimulation with recipient LCLs would be predicted to retain their antileukemic effect, particularly in myeloid malignancies. We have developed a protocol for allodepletion utilizing an anti-CD25 immunotoxin after stimulation of donor cells with HLA-mismatched LCLs and have evaluated residual alloreactivity and antiviral and antitumor activity.

4. AIM OF THE STUDY

The primary endpoint of the study was to compare immune reconstitution and viral-specific immune responses after addback of 2 different doses of allodepleted donor T cells. Secondary endpoints included comparison of the incidence of acute and chronic GVHD and the outcome of viral infections/reactivations at each dose level.

5. PATIENTS, MATERIALS, AND METHODS

5.1. Study population

The protocol was open to all patients who were candidates for haploidentical SCT at Baylor College of Medicine and its affiliated Methodist and Texas Childrens' Hospitals (Houston, TX) and Great Ormond Street Children's Hospital (London, United Kingdom). Failure of engraftment and the presence of greater than grade I GVHD was a contraindication to infusion of allodepleted donor T cells.
Sixteen patients have been treated, 8 at each dose level. Patient characteristics and conditioning are outlined in Table 4. The median age was 9 years (range, 2-58 years) and most (12 of 16) received transplants for high-risk hematologic malignancies. Six patients were not in remission at the time of transplantation, 2 had relapsed on therapy, and 5 had relapsed after previous autologous/allogeneic SCT.

5.2. Study design

The study compared the outcomes in the previous section between 2 dose levels. Each patient was scheduled to receive 3 infusions of allodepleted donor T cells at the same dose at days 30, 60, and 90 after transplantation, provided that there was no evidence of grade II or higher GVHD, or until total T-cell numbers were greater than 1000/μL. Patients could receive a fourth dose at day 120 if clinically indicated and the circulating T-cell count was less than 1000/μL. Dose level 1 (10^4 cells/kg/dose) was chosen because this dose of T cells is known not to cause GVHD in haploidentical recipients, even when the T cells were not allodepleted. Comparison was made with a log-higher dose (10^5 cells/kg/dose). Patients were initially enrolled at dose level 1, with escalation to dose level 2 when the observed incidence of significant acute GVHD (aGVHD) at level 1 was shown to be lower than 25%. All eligible patients receiving haploidentical SCT were enrolled consecutively, but no specific methodology for ensuring equivalent patient distribution prospectively was used. The study was approved by both institutional review boards (IRBs) and by the US Food and Drug Administration (FDA) (Baylor College of Medicine [BCM]) and the Medicines and Healthcare products Regulatory Agency (MHRA) (Great Ormond Street Hospital [GOSH]). Informed consent was obtained from all patients and/or their parents.

5.3. Transplantation and engraftment

Patients underwent haploidentical SCT using routine institutional conditioning and supportive care protocols. Conditioning is outlined in Table 1. Eleven patients received myeloablative preparative regimens, which in most consisted of 90 mg/kg cyclophosphamide, 12 g/m^2 cytarabine, 1400 cGy total body irradiation in 8 fractions, and serotherapy with 12 to 40 mg total alemtuzumab (Campath 1H). Five patients received nonmyeloablative conditioning with total body irradiation at 450 cGy in a single fraction, 120 mg/m^2 fludarabine, and serotherapy with 40 mg alemtuzumab (n = 4) or 150 mg/m^2 fludarabine/10 g/m2 cytarabine and granulocyte colonystimulating factor (G-CSF) (n = 1).

CD34$^+$ peripheral blood stem cells from G-CSF–mobilized donors were selected using the Isolex 3000 (Baxter Healthcare, Deerfield, MA) or CliniMACs (Miltenyi Biotec, Bisley, United Kingdom) immunomagnetic systems. The median dose of CD34$^+$ cells infused at transplantation was 11.5 x 10^6/kg (range, 5.9-20 x 10^6/kg), and a median of 2.6 x 10^4/kg T cells were infused with the graft (range, 0.73-18 x 10^4/kg). There was no significant difference in the number of infused T cells between the 2 dose levels. FK506/cyclosporin was either not administered (n = 9) or was withdrawn prior to the first infusion of allodepleted donor T cells (n = 7). More patients at dose level 2 (5 of 8) received post-SCT immunosuppression than at dose level 1 (2 of 8). GVHD was graded using the Seattle criteria (Glucksberg H et al., 1974)

Engraftment was assayed on mononuclear and granulocyte fractions using XY fluorescence in situ hybridization (FISH) or DNA polymerase chain reaction (PCR) of short tandem repeats. All patients had primary engraftment at a median of 13 days. At 1 month, 15 of 16 were full donor chimeras, by 6 months 3 of 13 evaluable had mixed chimerism, and one had autologous reconstitution. Two of these patients have relapsed, and the other 2 have undergone successful second transplantations from alternate donors. One patient converted from a mixed chimeric state to full donor chimerism 1 month after infusion of his first dose of allodepleted donor Tcells. Data on the extent of chimerism at 6 months are shown in Table 5.

Patient no. (age, y)	Diagnosis	Conditioning	Dose level, per Kg	No. CD34/Kg	No. CD3/Kg	% $CD3^+/CD25^+$	% residual proliferation	No. Infusions
P1 (8)	Rel ALL in second CR	Cy/TBI/Ara-C/alemtuzumab	10^4	1.4×10^7	1.8×10^4	0.15	0	3
P2 (14)	Ref AML	Cy/TBI/Ara-C/alemtuzumab	10^4	1.2×10^7	0.7×10^4	0.27	0.46	1 (GVHD)
P3 (3)	Rel ALL on Rx in second CR	Cy/TBI/Ara-C/alemtuzumab	10^4	1.1×10^7	3.4×10^4	0.03	0	3
P4 (3)	AML first CR	Cy/TBI/Ara-C/alemtuzumab	10^4	6.3×10^6	1×10^5	0.1	0.71	3
P5 (12)	Ref AML	Cy/TBI/Ara-C/alemtuzumab	10^4	7.5×10^6	3.4×10^4	0.02	1.00	3
P6 (28)	Hodgking in relapse after ABMT	TBI/FDR/alemtuzumab	10^4	6.9×10^6	2.2×10^4	0.01	0.33	3
P7 (3)	HLH	Cy/TBI/Ara-C/alemtuzumab	10^4	2×10^7	4.4×10^4	0.07	3.12	3
P8 (58)	CML LBC in 2nd CP	TBI/FDR/alemtuzumab	10^4	1.3×10^7	2.6×10^4	0.11	0	1 (autologous reconstitution)
P9 (2)	Rel AML after MMUD BMT in relapse	TBI/FDR/alemtuzumab	10^5	1.2×10^7	1.3×10^4	0.11	0	1 (CD3>1000)
P10 (2)	Ref AML	Cy/TBI/Ara-C/alemtuzumab	10^5	1.8×10^7	1.8×10^5	0.19	0.04	2 (CD3>1000)
P11 (8)	HLH	Cy/TBI/Ara-C/alemtuzumab	10^5	1.4×10^7	2.4×10^4	0.13	1.18	3
P12 (12)	SAA	FDR/Cy/TBI/alemtuzumab	10^5	5.9×10^6	0.9×10^4	0.05	0	2 (GVH)
P13 (14)	MDS	FDR/Cy/TBI/ATG	10^5	7.5×10^6	2.7×10^4	0.01	2.80	4
P14 (6)	JMML/AML after haplo-SCT in third CR	FLAG	10^5	1.1×10^7	2.1×10^4	0.03	0.57	4
P15 (10)	Fanconi, rejected first haplo-SCT	FDR/Cy/TBI/ATG	10^5	2×10^7	1×10^5	0.25	0	3
P16 14)	Rel AML after syngeneic BMT in relapse	TBI/FDR/alemtuzumab	10^5	1.1×10^7	6.4×10^4	0.12	0	3

Table 4. Patient and graft characteristics: Rel indicates relapsed; ref, refractory; CR, complete remission; HLH, hemophagocytic lymphohistiocytosis; LBC, lymphoid blast crisis; MMUD, mismatched unrelated donor; SAA, severe aplastic anemia; MDS, myelodysplasia; Cy, cyclophosphamide; TBI, total body irradiation; Ara-C, cytarabine; ATG, rabbit antithymocyte globulin; FLAG, fludarabine/cytarabine/G-CSF; CPM, counts per minute in primary mixed lymphocyte reaction of day-4 donor PBMCs + host LCLs with and without immunotoxin (IT); % Resid prol, shows residual proliferation calculated as outlined in "Patients, materials, and methods"

5.4. Immunotoxin

RFT5-SMPT-dgA is an immunotoxin generated by cross-linking a murine anti-CD25 monoclonal antibody (MoAb) (immunoglobulin G1 [IgG1]) with a chemically deglycosylated ricin α chain (dgA) using an N-succinimidyloxycarbonyl-α-methyl-2 pyridyldithiol toluene (SMPT) linker. Clinical grade immunotoxin was prepared in the Good Manufacturing Practice (GMP) laboratory at the University of Texas Southwestern Medical School.

5.5. Generation of allodepleted donor T cells

Generation of allodepleted donor T cells was performed under GMP conditions at the Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, using FDA-approved standard operating procedures.
Six weeks prior to transplantation, 30 mL of blood was collected from the patient for generation of recipient EBV-transformed lymphoblastoid cell lines (LCLs). Briefly, $5x10^6$ PBMCs were infected with concentrated supernatant from B95-8 EBV-producer cell line. LCLs were cultured in RF10 medium consisting of RPMI 1640 (Biowhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).
Peripheral blood mononuclear cells (PBMCs) obtained from 160 mL of donor blood and HLA-mismatched or haplo-identical irradiated (70 Gy) recipient LCLs were each diluted 2 x 10^6/ml in AIM V serum-free medium (invitrogen, Carlsbad, CA). Donor PBMCs were then cocultures with or without irradiated recipient LCLs at a responder-to-stimulator ratio of 40:1 in T-75 or T-175 flasks for 3 days. After 72 hours cocultures were harvested and resuspended at 10^7/ml in immunidepletion medium consisting of AIM V supplemented with 20nM ammonium chloride (Sigma, St Luise, MO) to improve the bioactivity of the immunotoxin with pH adjusted to 7.75 using Na HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (Sigma).

Dose level and patient no.	% donor at 6 mo	aGVHD	cGVHD	Viral reactivation	Fungal infection	Auto-immunity	Off study, mo after SCT	Current status*
Dose level 1								
P1	100	None	None	CMZ/VZV	None	None	—	Alive in CR
P2	100	Grade 4 skin	Ext (skin/liver)	CMV/Adeno	Aspergillus (B/C)	None	8, death from liver failure	Death from liver failure (adeno/GVH)
P3	57 MCNs, 56 grans	None	None	CMV	None	None	8, 2nd SCT for mixed chimerism	Alive in CR after 2nd SCT
P4	97	None	None	None	None	None	6, relapse	Death from disease/ infection/hemorrhage
P5	Relapsed	None	None	None	None	None	5, relapse	Death from disease
P6	100	None	None	CMV/VZV/EBV	None	None	9, relapse	Death from disease/infection
P7	74 MCNs, 66 grans	None	None	CMV/EBV	None	None	5, autologous reconstitution/relapse	Death from disease/infection
P8	0 MCNs, 0 grans	None	Not evaluable	CMV	None	None	3, autologous reconstitution	Alive in CR after 2nd SCT
Dose level 2								
P9	100	Grade 1 skin	None	EBV	None	None	8, relapse	Alive in relapse
P10	Relapsed	None	None	CMV	None	None	5, death from respiratory failure	Death from interstitial pneumonitis
P11	100	Grade 1 gut	None	EBV	None	None	—	Alive in CR
P12	100	Grade 2 skin	Ext (skin/mouth)	EBV	None	None	—	Alive in CR
P13	100	None	None	CMV/EBV/JC	Pulmonary	AIHA	30, death from multiorgan failure	Death from sepsis + multiorgan failure
P14	100	None	None	None	None	None	7, relapse	Death from disease
P15	100	None	None	CMV/Adeno	Pulmonary	None	7, death from encephalopathy	Death from postinfective encephalopathy
P16	100	None	None	None	None	None	8, relapse	Alive in relapse

Table 5. Chimerism, GVHD, infection, and clinical outcome: MNCs indicates mononuclear cells; grans, granulocytes; B/C, blood cultures; and —, completed study.

* As of June 2006.

Donor PBMCs alone and donor PBMCs plus recipient LCL cocultures were each split into 2 equal aliquots and 0.22 μm filtered RFT5-SMPT-dgA anti-CD25 immunotoxin added to 1 aliquot at final concentration of 3 μg/ml. Cocultures in the presence and absence of immunotoxin were incubated overnight at 37°C. The next morning cocultures were washed twice and sampled for bacterial/fungal/*Mycoplasma* sterility and endotoxin, confirmatory tissue-typing, fluorescence-activated cell-sorting (FACS) analysis of the percentage of residual $CD3^+CD25^+$ cells, residual proliferation (MLRs) against host cells compared with control cultures, used for cultures of CTLs, and enzyme-linked immunospot (ELISPOT) assays.

The remaining allodepleted donor T-cells were cryopreserved using 1% DMSO/10% human albumin in multiple aliquots determined by the patient weight and dose level. Release criteria include less than 1% CD3+/CD25+ cells and less than 10% residual proliferation against host cells. If the patient engrafted successfully and the quality assurance/control (QA/QC) results were appropriate, thew cryopreserved, allodepleted T-cells were thawed and infused at days 30, 60 and 90 after transplantation.

Most (13 of 16) patients completed their scheduled infusions: the remainder did not because of GVHD (n = 2) or autologous reconstitution (n = 1). Two patients at dose level 2 required only one (patient 9) or 2 (patient 10) infusions to achieve a CD3 count higher than 1000/μL. The residual percentage of $CD3^+/CD25^+$ cells in the infused cells ranged from 0.01% to 0.27% (median, 0.08%), and the residual proliferation against host cells in the primary MLR ranged from 0% to 3.1% (median, 0.02%).

5.6. Proliferation and cytotoxicity assays

For proliferation assays (mixed lymphocyte reactions MLRs), 2 $x10^5$ PBMCs cultured with 5000γ-irradiated (70Gy) LCLs in 200μl AIM V medium or 2 x 10^5 day 4 cocultures were plated in triplicate U-bottom 96-well plates (Nunc, Rochester, NY). After 5 days, cultures were pulsed with 0.037 MBq (1μCi) ^{3}H-thymidine per well (Amersham Biosciences, Piscataway, NJ) and harvested onto glass fiber strips 18 hours later using a Brandel PHD cell harvested. ^{3}H-thymidine uptake was measured using a Matrix B liquid scintillation counter (Canberra Packard, Meriden,

CT) and specific proliferation calculated according to the following formula (cpm = counts per minute):

$$\frac{\text{cpm (donor PBMCs + host LCLs + IT) - cpm (donor PBMCs alone + IT)}}{\text{cpm (donor PBMCs + host LCLs no IT) - cpm (donor PBMCs alone, no IT).}}$$

The cytotoxic activity of CTL lines was evaluated in a standard 6-hour 51Cr release assay. 3 x 10^6 target LCLs were labeled with 3700 MBq (100 mCi) ^{51}Cr (Amersham Biosciences) for 2 hours, washed 3 times, and diluted to 5 x 10^4/mL (K562) in RF10 medium; 100 µL target cells were added to serial dilutions of effector cells in triplicate round-bottom, 96-well plates to obtain a total volume of 200 µL per well, giving varying effector-target (E/T) ratios. In some experiments a 20-fold excess of cold HSB-2 cells was added to cultures to assess specificity. HSB-2 cells were used because LCL targets are more sensitive to lymphokine-activate killer (LAK)– than natural killer (NK)–mediated lysis, and this cell line has previously been shown to be an excellent target for LAK cells. Assay plates were incubated for 6 hours at 37°C, 5% CO_2, and 100 µL supernatant was harvested and counted using a Packard Cobra Gamma Counter (Packard Instrument Company, Downer's Grove, IL). The percentage of specific lysis was calculated as follows: ([cpm released experimental - cpm spontaneous]/[cpm total lysis - cpm spontaneous]) x 100%.

5.7. Flow cytometry and tetramer analysis

A total of 2 x 10^5 cultured cells were washed, stained for 30 minutes at 4°C with antibody, and analyzed by flow cytometry (FACSCalibur and CellQuest software; Becton Dickinson, San Jose, CA). A total of 10 000 events were analyzed. Cells were stained with fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, or peridinin chlorophyll protein (PerCP)–conjugated monoclonal antibodies to CD3 (clone UCHT1), CD4 (L200), CD8 (RPAT8), CD25 (M-A251), CD40 (5C3), CD54 (HA-58), CD80 (L307.4), CD86 (FUN-1), HLA-ABC (G46-2.6), and HLA-DR (L243) (BD Biosciences, San Jose, CA). For tetramer analysis, 10^6 PBMCs or allodepleted T cells from donors known to have tetramer-positive populations were costained with CD8 FITC, CD3 PerCP, and either isotype PE control antibody or PE-conjugated HLA-A2–CMV pp65

(NLVPMVATV) or HLA-A2–LMP2 (CLGGLLTMV), titrated for optimal specific staining. For the HLA-A2–PR1 (VLQELNVTV) tetramer, which stains with lower fluorescence intensity, CD8 FITC clone 3B5 (Caltag, Burlingame, CA) and a "dump" channel consisting of CD4/CD14/CD16 and CD19 PerCP were used to reduce background. The percentage of tetramer-positive cells in the $CD3^+/CD8^+$ lymphocyte gate was expressed as a proportion of the $CD8^+$ cells.

5.8. Optimization of allodepletion following stimulation with recipient LCLs

As a prelude to subsequent clinical studies we optimized a variety of parameters in our allodepletion protocol using recipient LCLs as stimulators. A donor PBMC/recipient LCL ratio of 40:1 was found to result in the lowest percentage of residual $CD3^+/CD25^+$ and residual proliferation. An immunotoxin concentration of 3 μg/mL was found to give effective depletion of secondary proliferative responses to host without significant diminution of responses to third party, and this concentration was therefore used in further studies. Based on sequential FACS analysis showing peak levels of CD25 expression at 3 to 4 days of coculture, allodepletion was performed at this time. To adapt our allodepletion protocol for a clinical trial, we scaled up our experiments using serum-free medium and clinical grade reagents. The residual percentage $CD3^+/CD25^+$ cells and residual proliferation following allodepletion in serum-free AIM V medium was equivalent to that in medium containing fetal calf serum. Assuming a 70kg recipient, multiple doses of 10^5/kg or 10^6/kg allodepleted donor T cells could routinely be generated from 150 mL or 500 mL donor peripheral blood.

5.9. Generation of EBV-specific and alloreactive CTLs

Polyclonal EBV-specific and alloreactive T-cell lines were generated. Allodepleted donor T-cell cultures (2×10^6 per well of a 24-well plate) were stimulated with 40 Gy–irradiated LCLs from the initial stimulator, an HLA-mismatched third-party donor (alloreactive CTL cultures), or autologous donor LCLs at a responder-stimulator ratio of 4:1. After 9 days, cultures were restimulated with irradiated LCLs (at 4:1 R/S ratio) and after 24 and 96 hours supplemented with 20 U/mL recombinant

human interleukin-2 (rhIL-2; Proleukin, Chiron, Emeryville, CA). Cultures were restimulated in a similar fashion weekly and cytotoxicity assayed 5 to 6 days following the third round of stimulation.

5.10. ELISPOT assay

ELISPOT assays were used to determine the frequency of adenovirus-, CMV-, and EBV-specific T precursors in allodepleted donor T-cell cultures producing interferon-γ (IFN-γ) in response to stimulation with autologous PBMCs transduced with vaccinia or adenoviral vectors carrying green fluorescent protein (Vacc-GFP, Ad5f35-GFP) or CMV pp65 transgenes (Vacc-pp65, Ad5f35-pp65-GFP) or autologous LCLs. To generate pp65-expressing stimulator cells, 106 thawed autologous PBMCs were resuspended in 200 μL AIM V and transduced with either Ad5f35-pp65-GFP or an identical control vector lacking the pp65 transgene (Ad5f35-GFP) at a multiplicity of infection (MOI) of 100 for 2 hours, washed twice, resuspended at 10^6/mL in AIM V, and irradiated (30 Gy). For assessment of response to EBV, irradiated (70 Gy) autologous LCLs diluted to 10^6/mL in AIM V were used as stimulators. MAHAS4510 plates (Millipore, Billerica, MA) were coated with anti–IFN-γ catcher MAB91 DIK (Mabtech, Cincinnati, OH) overnight and blocked with RF10 medium for 1 hour at 37°C. Serial dilutions starting at 2 x 10^5 allodepleted donor T cells or thawed donor PBMCs per well were plated in the presence of 10^5 stimulators in duplicate or triplicate wells for 18 to 24 hours at 37°C. Controls consisting of 2 x 10^5 responder alone, 10^5 stimulator alone, and 2 x 10^5 responder plus 10^5 untransduced autologous PBMCs cells were also plated. Plates were washed the next day and then incubated for 2 hours at 37°C with biotin–anti-IFN-γ detection antibody 7-B6-1 (Mabtech). Avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) was added for 1 hour at room temperature and spots developed with 3-amino-9-ethylcarbazole (AEC, Sigma) substrate mix. The numbers of spots were counted (Zellnet Consulting, New York, NY), the means of duplicate/triplicate wells calculated and expressed spot-forming cells per 10^6 cells. The mean number of specific spot-forming cells was calculated by subtracting the mean number of spots produced by responder alone and stimulator-alone wells from the mean number of spots in test wells for each dilution. Linear regression

analysis was then used to determine the number of specific spot-forming cells per 10^6 cells.

5.11. Measurement of alemtuzumab levels, HAMA, and HARA antibody responses

Plasma alemtuzumab (Schering Health Care, Burgess Hill, United Kingdom) levels were measured in 10 patients at the time of the first infusion of allodepleted donor T cells. Serial dilutions of patient/control plasma or standard dilutions of alemtuzumab were incubated with 6.25 x 10^5 normal donor phytohemagglutinin (PHA) blasts in 96-well plates on ice for 30 minutes, washed, and then secondary-stained with FITC anti-human IgG (BD Pharmingen, San Diego, CA). CD52 expression was analyzed flow cytometrically and compared with the standard curve. Values of less than 150 ng/mL are equivalent to the background for this assay. Alemtuzumab was undetectable in 6 patients and was detected at low levels in the remaining 4 patients (P1, 6, 8 and 11). Antibody responses against mouse IgG1 and dgA were assayed in the patients prior to each infusion and at 6 months and 1 year after SCT. Triplicate wells of 96-well plates were coated with either mouse RFB4 or with dgA, washed with phosphate-buffered saline (PBS), blocked with 10% fetal calf serum (FCS), and dilutions of the test serum or a known standard added. The plates were incubated for 6 hours at room temperature and washed, and radiolabeled goat anti–human Ig was added. Plates were incubated for 4 to 6 hours at 4°C and washed. Individual wells were cut out and counted on a gamma counter. Standard curves were plotted and human anti–mouse Ig (HAMA) and human antiricin (HARA) levels in the experimental sera were calculated from the curves.

5.12. Monitoring viral reactivation and immune reconstitution

Blood samples from the patients were screened weekly for CMV antigenemia (BCM) or by DNA PCR (GOSH), and patients with significant viremia were treated with ganciclovir. All patients were also screened weekly for EBV viremia using the real-time PCR method of Wagner et al. Screening for adenoviremia was performed when clinically indicated and adenoviremia was treated with intravenous cidofovir. Immune reconstitution was studied monthly for 9 months and then at a year after

SCT by flow cytometric analysis of PBMCs using FITC/PE/PercP labeled antibodies against CD3, CD4, CD8, CD16, CD19, CD56, CD45RA/RO, and CCR-7 antibodies (Becton Dickinson, San Jose, CA). Proliferative/ATP responses to mitogenic stimulation with PHA were measured using ^{3}H-thymidine uptake and luciferase (Cylex, Columbia, MD) assays.

Antiviral responses were assayed using tetramer and ELISPOT assays. Tetramer analysis was performed on cells from 10 patients with HLA-A2–,HLA-A24–, HLA-B7– or HLA-B8–positive donors. PBMCs (10^{6}) from the patient after SCT were costained with CD8 FITC, CD3 PerCP, and either isotype PE control antibody or PE-conjugated tetramers (obtained from M.C. or Proimmune [Oxford, United Kingdom]), previously titrated for optimal specific staining. Donor PBMCs and PBMCs from donors with known positive populations served as positive controls, and PBMCs from healthy donors negative for the restricting HLA type were used as additional negative controls. The following tetramers were used: CMV (HLA-A2 NLVPMVATV [pp65], HLA-B7 TPRVTGGGAM [pp65], HLA-B7 RPHERNGFTVL [pp65], and HLA-B8 QIKVRVDMV[IE1]; and EBV (HLA-A2 CLGGLLTMV [LMP2], HLA-A2 GLCTLVAML [BMLF- 1], HLA-B7 RPPIFIRRL [EBNA3a], and HLA-B8 RAKFKQLL [BZLF-1]). A total of 200.000 events in the lymphocyte gate were analyzed where possible and the percentage of tetramer-positive cells in the $CD3^{+}/CD8^{+}$ lymphocyte gate was expressed as a proportion of the $CD8^{+}$ cells with the isotype control subtracted. For a population to be labeled as positive, at least 50 $CD3^{+}CD8^{+}$ tetramer-positive cells with the staining characteristics of the positive control population had to be acquired. ELISPOT assays were used to determine the frequency of CMV- and EBV-specific T cells in patient PBMCs producing interferon gamma (IFN-γ) in response to stimulation with autologous donor PBMCs transduced with adenoviral vectors carrying the green fluorescent protein (GFP; Ad5f35GFP) or GFP and CMV pp65 transgenes (Ad5f35pp65GFP) or autologous LCLs (Amrolia P *et al.,* 2003). Thawed PBMCs (2×10^{5}) from the patient at varying time-points after SCT were plated in the presence of 2×10^{5} stimulator cells in triplicate wells for 18 to 24 hours at 37°C on MAHA S45 plates (Millipore, Billerica, MA) coated with anti–IFN-γ capture antibody 1 DIK (Mabtech, Mariemont, OH). Controls consisted of 2×10^{5} responder cells alone, 2×10^{5} stimulator cells alone, and 2×10^{5} unirradiated donor PBMCs plus 2×10^{5} stimulator cells. Plates were

developed and counted (Amrolia P *et al.*, 2003). The mean number of specific spot-forming cells (SFCs) was calculated by subtracting the mean number of spots from responder cells alone and stimulator cells alone from the mean number of spots in test wells. Responses against CMVpp65 were calculated by subtracting the mean number of spots after stimulation with PBMCs transduced with Ad5f35-GFP (always <100 SFC/10^6 cells) from the mean number of spots after stimulation with PBMCs transduced with Ad5f35-pp65-GFP.

T-cell receptor signal joint excision circle (TREC) levels were analyzed by real-time quantitative PCR (qPCR) assay. PBMCs were lysed using proteinase K solution and 5 μL duplicate samples were used as a template for qPCR using primers and probes (Trasher AJ *et al.*, 2005) on an ABI PRISM 7000 Sequence Detection (PE Applied Biosystems, Warrington, United Kingdom). For each run, a standard curve was generated from duplicate samples of 5-fold serially diluted known copies of plasmid DNA containing a human TREC fragment. A threshold cycle (Ct) value for each duplicate was calculated by determining the point at which the fluorescence exceeded the threshold limit. We used the mean Ct value of the 2 duplicates plotted against the standard curve to calculate the TREC number in the sample. To normalize for cell equivalents, the β-actin gene was quantified by qPCR. Results were expressed as TREC copies/10^6 cells. T-cell receptor (TCR) spectratypes were analyzed. (Trasher AJ *et al.*, 2005) RNA was extracted and cDNA prepared from frozen PBMCs. A number of variable region (Vβ)–specific primers (Cavazzana-Calvo M *et al.*, 2001) were used with a fluorescentlabeled constant region (Cβ)–specific primer to reverse transcriptase (RT)–PCR amplify the CDR3 region of the TCR β chain. Products were run on a megaBACE 500 genetic analyser (Amersham Biosciences, Buckingham, United Kingdom) and analyzed using Genetic Profiler software (Amersham Biosciences). Each Vβ family was scored for the number of detectable bands and size distribution to assess the TCR repertoire. A normal spectratype has been shown to consist of 5 to 8 bands per family with a Gaussian size distribution (Gorski J *et al.*, 1994)

5.13. Statistical analysis

The proportion of patients achieving normal T- and B-cell numbers at each dose level at given time points was compared using the Fisher

exact test. Univariate comparisons of immune reconstitution between the 2 dose levels were performed at each month of follow-up using the Wilcoxon rank-sum nonparametric test. To summarize the overall kinetics of immune reconstitution, the area under the curve (AUC) for each lymphocyte subset was calculated using the trapezoidal rule, and the mean AUC at 4 months and 6 months was compared between dose levels 1 and 2 using the 2 sample t test. Multivariate analysis was designed primarily to determine if differences in immune reconstitution observed between dose levels were significant after adjustment for other factors, rather than to determine the significance of these other factors.

6. RESULTS

6.1. GVHD and toxicity

Infusion of allodepleted T cells was associated with a low incidence of toxicity. Two of 16 patients developed significant acute GVHD. P2 developed grade IV skin GVHD after a single infusion of allodepleted donor T cells at dose level 1, and P12 developed grade II skin GVHD after 2 infusions at dose level 2. Both subsequently evolved to extensive chronic GVHD (1 affecting the liver, the other affecting skin and mouth). The patient with chronic GVHD of the liver died of liver failure secondary to adenovirus. Chronic GVHD in the other patient fully resolved, and this patient has been off immunosuppression for 26 months as of June 2006. No other severe adverse effects attributable to the infused allodepleted donor T cells were observed during the study. No patients developed HAMA, and one of 16 (P8) developed a transient HARA.

6.2. Comparison of allodepletion following stimulation with HLA-mismatched PBMCs and LCLs

We initially compared the expression of a variety of immunostimulatory molecules on PBMCs and LCLs from 5 individuals using FACS analysis. Table 6 shows that although the expression of HLA class 1 was similar in PBMCs and LCLs, the latter expressed much higher and less variable levels of HLA class 2, the costimulatory molecules CD40, CD80, and CD86 and the adhesion molecule CD54. We then investigated the capacity of recipient LCLs to activate alloreactive T cells from HLA-mismatched donors.

Marker	PBMCs, % positive	PBMCs, MFI	LCLs, % positive	LCLs, MFI
HLA class1	96.6 ± 4.2	185.5 ± 159.5	97.4 ± 2.8	334.4 ± 146.3
HLA-DR	31.5 ± 4.0	181.4 ± 127.4	99.7 ± 0.2	1904 ± 545
CD40	3.9 ±5.0	3.3 ±1.4	42.4 ± 8.0	36 ± 18.6
CD54	25.4 ± 12.3	28.8 ± 28.5	95.4 ± 2.3	393.6 ± 203.7
CD80	1.0 ±0.8	2.2 ± 0.5	95.1 ± 3.6	206.8 ± 135
CD86	10.4 ± 4.0	8.3 ± 2.2	81.3 ± 5.5	184.1 ± 132

Table 6. LCLs express higher levels of immunostimulatory molecules than PBMCs. Data are the results of FACS analysis of PBMCs or LCLs from the same individuals. The results are the means ± SDs of 5 healthy donors. MFI indicates mean fluorescence intensity.

As shown in Figure 1A, coculture with recipient LCLs results in a marked increase in expression of CD25 in both $CD4^+$ and $CD8^+$ T cells. Overnight treatment of such cocultures with the anti-CD25 immunotoxin very effectively depletes $CD3^+/CD25^+$ in both $CD4^+$ and $CD8^+$ subsets. As seen in Figure 1B, in 5 different haplo-identical patient-donor pairs, allodepletion with the anti-CD25 immunotoxin after stimulation with recipient LCLs resulted in an average 15-fold decrease in proliferation in primary mixed lymphocyte reactions (MLRs) compared with untreated cocultures. To compensate for the effect of overnight culture in the immunodepletion medium, we included controls that were mock-treated with immunodepletion medium but in the absence of immunotoxin.

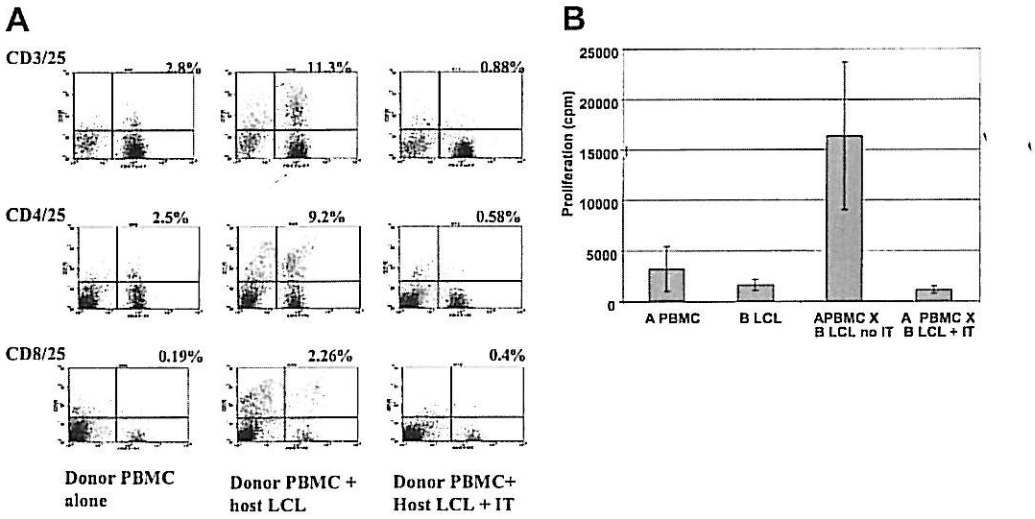


Figure 1. Depletion of alloreactive T cells by anti-CD25 immunotoxin. (**A**) FACS analysis showing increased expression of CD25 (y axis) on $CD3/4/8^+$ T cells after activation with HLA-mismatched LCLs and effective depletion of $CD3^+/CD25^+$, $CD4^+/CD25^+$, and $CD8^+/CD25^+$ cells following treatment with anti-CD25 immunotoxin. The figure shows a representative FACS analysis from 6 different donor-recipient pairs. The percentage of double-positive cells is indicated. (**B**) Primary mixed lymphocyte reaction showing a mean 15-fold decrease in proliferation in response to HLA-mismatched LCL stimulators after treatment with anti-CD25 immunotoxin (IT). Results are the mean ± SD of 5 haplo-identical donor-patient pairs each assayed in triplicate.

Because the absolute counts per minute in primary MLRs is highly variable depending on the degree and nature of the HLA mismatch, we standardized these results by calculating the residual proliferation according to the formula previously descrived. In 5 independent experiments with haplo-identical patient donor pairs, the residual proliferation after stimulation with recipent LCLs followed by treatment with anti-CD25 immunotoxin was less than 0.5%.

Based on these results, we compared T-cell activation and the efficacy of depletion of alloreactive donor T cells following stimulation with HLA-mismatched recipient PBMCs or LCLs. Normal donor PBMCs were stimulated for 4 days with irradiated HLA-mismatched PBMCs at a ratio of 1:1 or LCLs from the same recipients at a ratio of 40:1.

As illustrated in Figure 2A, in 5 donor-patient pairs, the percentage of activated $CD3^+/CD25^+$ cells was similar after stimulation with PBMCs or LCLs, although the mean fluorescence intensity for CD25 was generally higher after stimulation with LCLs. Likewise, after depletion of alloreactive cells with the $CD25^-$ immunotoxin the percentage of residual $CD3^+/CD25^+$ cells was equivalent after stimulation with PBMCs or LCLs. However, as seen in Figure 2B, the specific proliferation of PBMCs from 6 different donors stimulated with PBMCs or LCLs from the same HLA-mismatched recipients was significantly higher after stimulation with LCLs, despite the much lower number of stimulators used ($P<.05$). More importantly, the residual proliferation after treatment of these cocultures with anti-CD25 immunotoxin was more variable after stimulation with HLA mismatched recipient PBMCs (mean, 8.5% ± 11.3%).

In contrast, the residual proliferation after stimulation with LCLs from the same recipients was lower and consistently less than 5% (mean, 0.8% ± 1.8%) and was statistically significantly lower than after stimulation with PBMCs from the same donors ($P<.05$) (Table 7).

Thus, allodepletion with anti-CD25 immunotoxin following stimulation with host LCLs appears more consistently effective in depleting in vitro alloreactivity than after stimulation with host PBMCs.

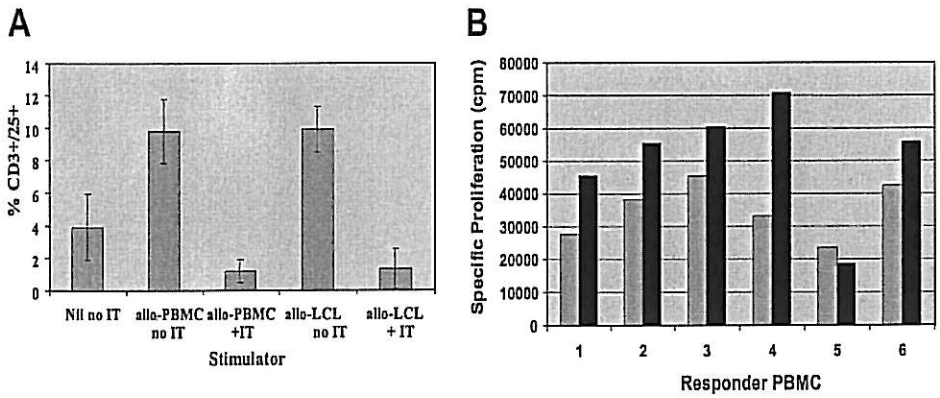


Figure 2. Comparison of activation and allodepletion following stimulation with HLA-mismatched PBMCs or LCLs. (**A**) FACS analysis showing percent $CD3^+/CD25^+$ cells after stimulation with HLA-mismatched PBMCs or LCLs in the presence or absence of immunotoxin. Results are the mean ± SD of 5 HLA-mismatched donor-patient pairs. (**B**) Primary mixed lymphocyte reactions showing proliferation of 2 x 10^5 donor PBMCs by HLA-mismatched PBMCs (R/S 1:1; ▨) or LCLs (R/S 40:1; ■) from the same recipient. The results are the mean specific proliferation of triplicate wells for 6 HLA-mismatched donor-recipient pairs.

	Responder							
Stimulator	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Mean ± SD**
Allo-PBMCs	0	14.8	0	27.4	0	17.4	0	8.5 ± 11.3
Allo-LCLs	4.8	0	0.7	0	0	0	0	0.8 ± 1.8

Table 7. Residual proliferation in 7 donor-patient pairs Residual proliferation in 7 donor-patient pairs after allodepletion with anti-CD25 immunotoxin following stimulation of donor PBMCs with PBMCs (R/S 1:1) or LCLs (R/S 40:1) from the same HLA-mismatched recipient. Residual proliferation was calculated using the formula in "Results" and was significantly higher after stimulation with PBMCs ($P < .05$).

6.3. Allodepletion specifically abrogates the ability to generate alloreactive CTLs

To determine the effect of allodepletion with anti-CD25 immunotoxin on alloreactive and bystander cytotoxic T-cell precursors, we restimulated allodepleted donor T cells from 3 different donors with either LCLs from the initial recipient or from an HLAmismatched third party and then performed cytotoxicity assays. Cold-target inhibition with a 20-fold excess

of unlabeled HSB-2 cells was used to assess specificity. The results of a representative experiment are shown in Figure 3. As seen in Figure 3A, in the absence of immunotoxin treatment, donor cells restimulated with LCLs from the initial recipient efficiently lysed recipient LCLs and most of the observed cytotoxicity was specific, because it was largely preserved in the presence of cold HSB-2 targets. In contrast, allodepleted donor T cells showed much lower cytolytic activity against the recipient, and much of the residual cytotoxicity seen at high E/T ratios was nonspecific, because it was lost in the presence of cold HSB-2 targets (Figure 3B). When donor T cells exposed to recipient LCLs were restimulated with third-party LCLs (Figure 3C-D), the cytolytic activity against third-party LCLs was somewhat reduced with immunotoxin treatment, but allodepleted donor T cells nonetheless showed good killing of third-party targets, which appeared specific, in that it was largely preserved in the presence of cold target inhibitors. In 3 different HLA-mismatched donor recipient pairs, at an E/T ratio of 20:1, the mean specific cytotoxicity of cocultures against recipient LCLs in the presence of excess cold HSB-2 was reduced from 46.3% ± 1.8% in the absence of immunotoxin to 13.6% ± 1.8% in its presence. In contrast, the cytotoxicity against third-party LCLs was largely preserved (68.3% ± 11.5% versus 44.9% ± 5.9%).

6.4. Antiviral T-cell responses are preserved following allodepletion

To determine the specificity of allodepletion, we studied whether antiviral T-cell responses were retained following immunotoxin treatment. PBMCs from 4 HLA-A2–positive donors known to have significant populations of CMV-specific $CD8^+$ cells detectable by HLA-A2–pp65 peptide tetramers were cocultured with HLAmismatched (HLA-A2–negative) LCLs for 3 days and then treated overnight with anti-CD25 immunotoxin. As shown in Figure 4, there was no significant difference in the frequency of HLA-A2-pp65–specific $CD8^+$ T cells in allodepleted donor T-cell cultures and unmanipulated PBMCs. These results suggested that virus specific T cells are retained following allodepletion. To study the functionality of antiviral T cells, we then performed ELISPOT analyses to determine the frequency of T cells secreting IFN-γ in response to adenoviral antigens and CMV pp65. Unmanipulated or allodepleted donor T cells were stimulated with irradiated autologous PBMCs transduced with vaccinia

vectors carrying GFP (Vacc-*GFP*) or CMV*pp65* (Vacc-*pp65*) transgenes or with adenoviral vector carrying the *GFP* transgene (Ad5f35-*GFP*) or *GFP* and CMVpp65 (Ad5f35-*pp65-GFP*).

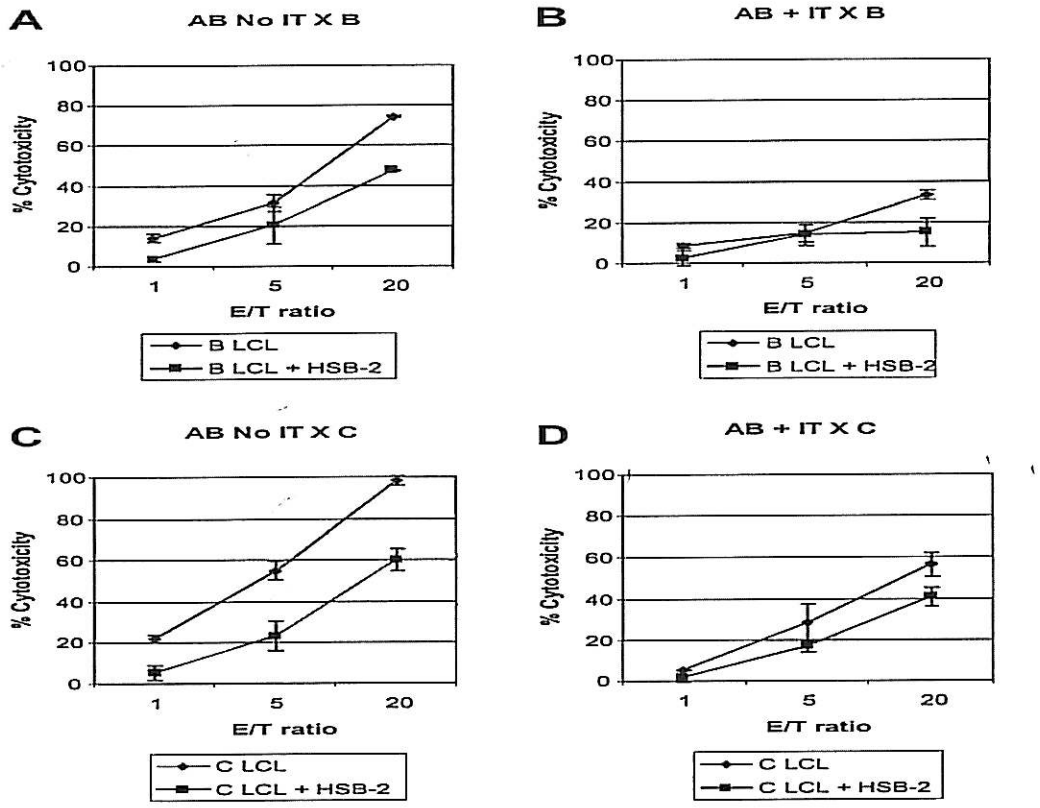


Figure 3. Allodepletion specifically abrogates the generation of alloreactive CTLs. Cytotoxicity assay showing lysis of LCL targets by cocultures of donor A PBMCs with recipient B LCLs in the presence or absence of anti-CD25 immunotoxin after secondary stimulation with B LCLs (A-B) or with third-party LCLs (C-D). In each case cytotoxicity was also assayed in the presence of a 20-fold excess of cold HSB-2 targets to assess specificity. Results are the mean specific cytotoxicity ± SD of triplicate wells in an assay representative of 3 experiments with different patient-donor pairs.

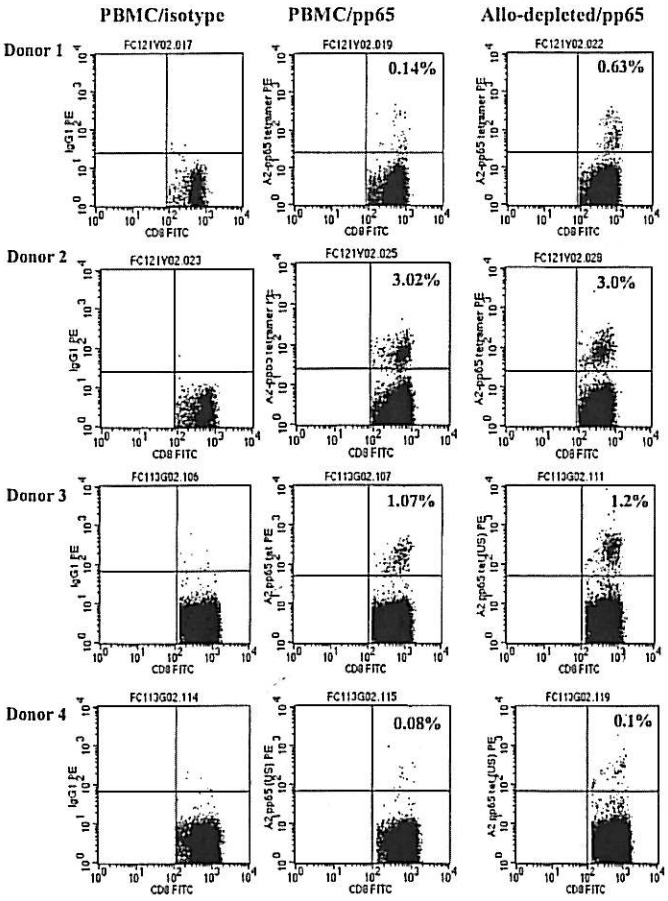


Figure 4. CMV-specific CD8$^+$ T cells are not deleted by allodepletion. The figure shows FACS analysis following staining of either unmanipulated PBMCs (right and center columns) or allodepleted cells (left column) from 4 HLA-A2–positive, CMV-seropositive donors with IgG PE (left column) or an HLA-A2–CMV pp65 tetramer (center and right columns). The percentages of tetramer-positive cells as a proportion of CD8$^+$ cells with isotype subtracted are shown.

As can be seen in Figure 5, in 5 different CMV-seropositive donors, there was no statistically significant difference in frequency of IFN-γ–secreting cells after stimulation with Ad5f35-*GFP*– (Figure 5A, P = .22) or Vacc*pp65*– (Figure 5B, P = .95) transduced PBMCs in allodepleted T-cell cocultures and unmanipulated PBMCs, implying that allodepletion does not affect the function of adenovirus- or CMVspecific T cells. IFN-γ responses to PBMCs transduced with the control vector Vacc-*GFP* were always less than 30 spot-forming cells per 10^6 cells, demonstrating the specificity of these responses. Likewise, when responder cells were stimulated with antigens from both adenovirus and CMV, using the Ad5f35pp65GFP vector, the frequency of IFN-γ–secreting cells was similar in allodepleted donor T cells and unmanipulated PBMCs (Figure

5C, P = .37). In some donors the response to Ad5f35-pp65-GFP was greater than the sum of the responses to Ad5f35-GFP and to Vacc-pp65. This may reflect the known inhibitory effect of vaccinia on IFN-γ secretion by T cells. One potential concern about the use of donor T cells allodepleted after stimulation with recipient LCLs is that, in the haplo-identical setting, T-cell responses to EBV would be abrogated, thereby increasing the risk of lymphoproliferative disease after transplantation. We reasoned that because T cells recognizing EBV antigens in the context of HLA molecules from the nonshared donor haplotype should not be activated in response to recipient LCLs, some anti-EBV activity should be retained following allodepletion. To investigate this, we stimulated PBMCs from EBV-seropositive donors with haplo-identical recipient LCLs for 3 days followed by allodepletion with anti-CD25 immunotoxin. We then used ELISPOT assays to determine the frequency of IFN-γ–secreting cells after secondary stimulation with autologous donor LCLs to assess anti-EBV responses.

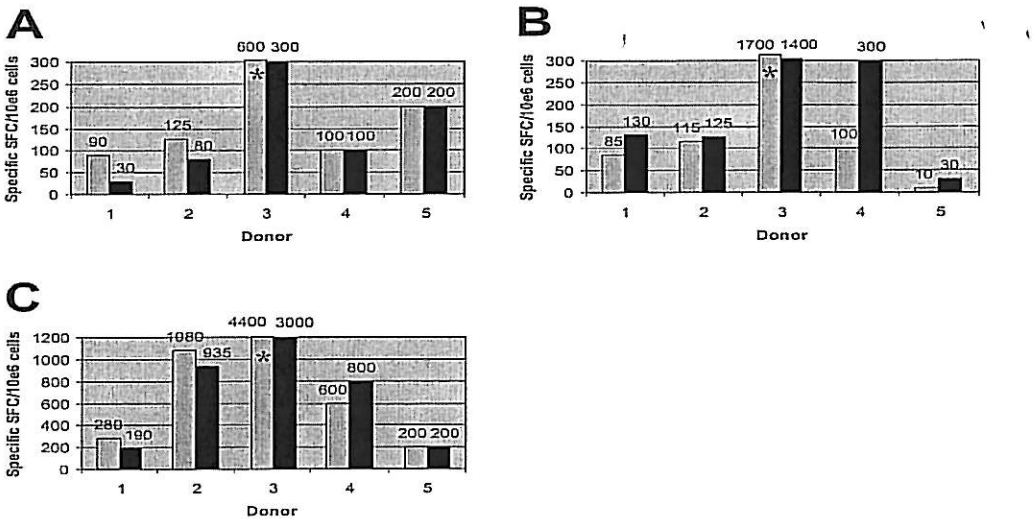


Figure 5. T-cell responses to adenoviral and CMV antigens are preserved after allodepletion. The figure shows the frequency of cells secreting IFN-γ as determined by ELISPOT assays. Unmanipulated PBMCs from 5 different seropositive donors (▤) or donor PBMCs allodepleted after stimulation with HLA-mismatched LCLs (■) were stimulated with irradiated autologous PBMCs transduced with an adenoviral vector carrying the GFP gene (A), vaccinia vectors carrying the GFP gene with (B) or without (not shown) the CMV pp65 gene, or an adenoviral vector carrying the CMV pp65 and GFP genes (C). Results are shown numerically above columns.

*The result falls above the axis limit. Results are the mean number of specific spot-forming cells calculated by linear regression of duplicate wells assayed at 3 dilutions.

As shown in Figure 6A, the frequency of cells secreting IFN-γ after stimulation with autologous LCLs in allodepleted donor T-cell cultures was partially retained in all 3 haplo-identical donor-patient pairs, demonstrating that significant anti-EBV responses persist following allodepletion. In contrast, control cultures depleted with immunotoxin after initial stimulation with autologous LCLs showed a much more marked loss of T cells secreting IFN-γ in response to EBV antigens than observed in the haplo-identical setting (Figure 6B). These results suggest that the residual responses to EBV following allodepletion are likely to be mediated through recognition of EBV antigens in the context of the nonshared haplotype.

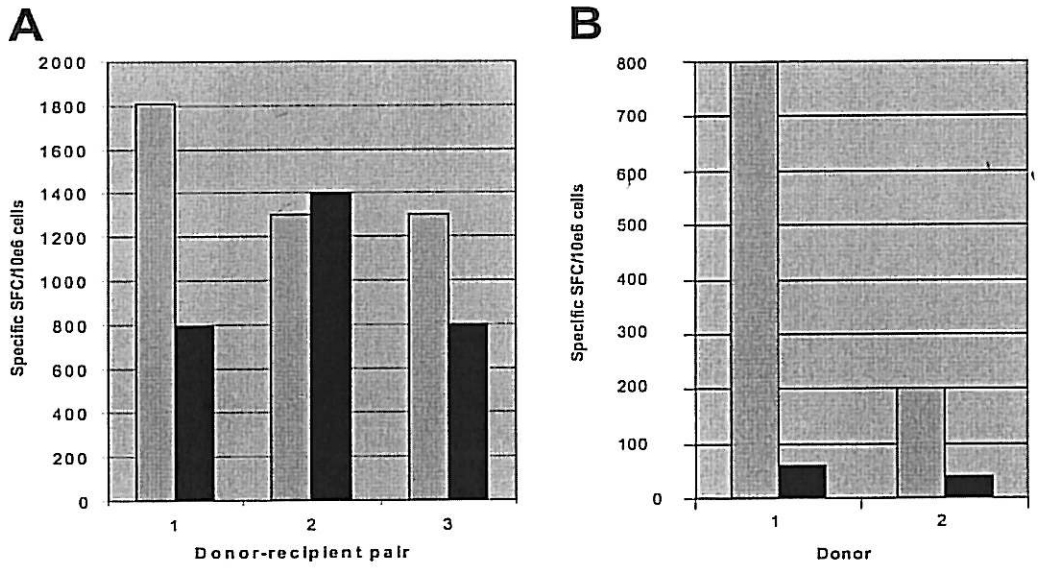


Figure 6. T-cell responses to EBV are partially preserved following allodepletion with haplo-identical LCLs. The figure shows the frequency of cells secreting IFN-γ as determined by ELISPOT assays. (A) Unmanipulated donor PBMCs (▨) or donor PBMCs allodepleted after stimulation with recipient LCLs from 3 different haplo-identical donor-recipient pairs (■) were stimulated with irradiated autologous donor LCLs. (B) Unmanipulated donor PBMCs (▨) or donor PBMCs depleted with immunotoxin after stimulation with autologous LCLs (■) were restimulated with irradiated autologous LCLs. Results are the mean number of specific spot-forming cells calculated by linear regression of duplicate wells assayed at 3 dilutions.

6.5. T cells recognizing myeloid tumor antigens are retained following allodepletion

We then investigated whether allodepletion after stimulation with recipient LCLs affected the response to candidate tumor antigens in myeloid malignancies. As our model, we studied T cells recognizing the PR1 epitope from proteinase 3, which have been shown to have potent cytolytic activity against primary leukemic cells in chronic myeloid leukemia (CML) and acute myeloid leukaemia (AML) and to preferentially inhibit growth of leukemic granulocytemacrophage colony-forming units (CFU-GMs) from patients with CML (Molldrem JJ *et al.,* 1996; 1997). Using HLA-A2–PR1 tetramer analysis in 3 patients with CML undergoing transplantation and known to have detectable circulating PR1-specific CTLs, we compared the frequency of T cells recognizing the PR1 epitope in unmanipulated PBMCs and after stimulation with HLA-mismatched stimulators and allodepletion with anti-CD25 immunotoxin. As shown in Figure 7A, after stimulation with allogeneic, mismatched HLA-A2–positive CML PBMCs, allodepletion resulted in the complete loss of PR1-specific T cells. By contrast, when LCLs were used as stimulators, PR1-specific $CD8^+$ cells were largely preserved following allodepletion, regardless of whether this was done following stimulation with HLA-A2–positive or HLA-A2–negative LCLs (Figure 7B).

6.6. Immune reconstitution

Thirteen patients with more than 3 months of follow-up at June 2006 were evaluable for immune reconstitution. P8 was excluded because of autologous reconstitution after a single dose of allodepleted donor T cells. Figure 8 shows the kinetics of T-, B-, and natural killer (NK)–cell recovery after SCT in the evaluable patients. T-cell reconstitution was slow in dose-level 1 patients, comparable to that reported in published series without T-cell infusions.4 In contrast, our data show that T-cell reconstitution in both in the CD4 and CD8 compartments was significantly accelerated in patients receiving allodepleted donor T cells at dose level 2 (Figure 8A-C). Univariate comparison of CD3 levels between dose levels 1 and 2 (data not show) showed significantly improved T-cell numbers in dose-level 2 patients at 3, 4 and 5 months after SCT (P =

.016, .017, and .04, respectively). There was a trend toward higher CD4 and CD8 levels in patients at dose level 2 compared with those at dose level 1 (data not show) at 3, 4, and 5 months after SCT, but the difference only reached significance at 4 months (P = .017 and .03, respectively). B- and NK-cell reconstitution was not statistically different between patients at the 2 dose levels (Figure 8D-E).

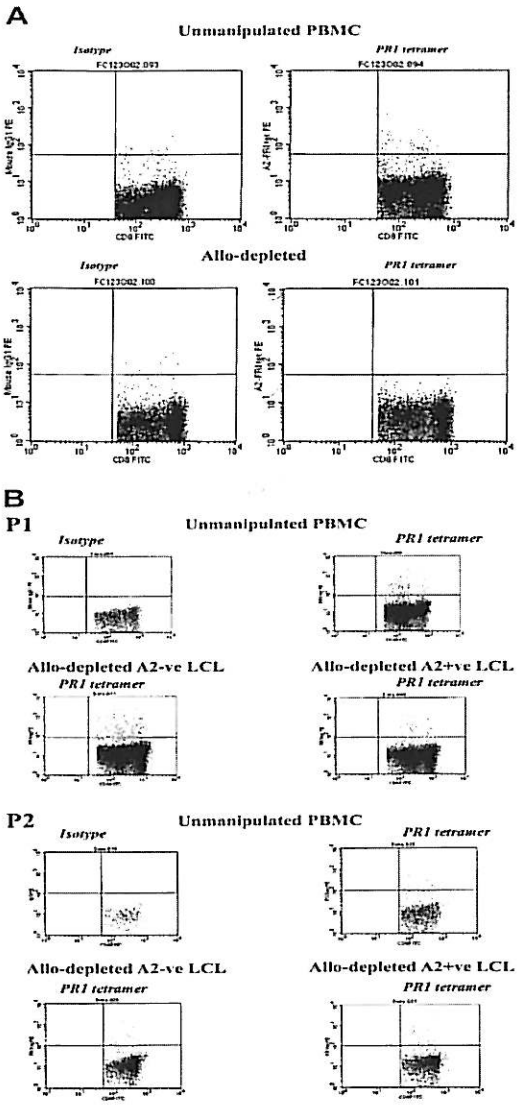


Figure 7. CD8$^+$ T cells specific for the myeloid tumor epitope PR1 are retained after allodepletion after stimulation with mismatched LCLs but not CML PBMCs. (**A**) FACS analysis following staining with HLA-A2–PR1 tetramer of unmanipulated PBMCs (top row) or allodepleted PBMCs (bottom row) from a patient with CML. In each case isotype controls are shown on the left and tetramer-stained cells on the right. Allodepletion was performed after stimulation with allogeneic HLA-A2–positive PBMCs from a mismatched donor with CML. (**B**) FACS analysis following staining with HLA-A2–PR1 tetramer of unmanipulated PBMCs or allodepleted T cells from 2 HLA-A2–positive patients with CML. Allodepletion was performed after stimulation with either HLA-A2–positive or –negative LCLs. The percentages of tetramer-positive cells as a proportion of CD8$^+$ cells (isotype subtracted) are shown.

To evaluate the kinetics of T-cell reconstitution over the entire first 4 or 6 months after SCT, AUC analyses were performed. In patients with at least 4 months of follow-up, there was a significantly higher AUC in patients at dose level 2 for $CD3^+$ (P = .048), $CD4^+$ (P = .032), and $CD8^+$ (P = .046) cells than for patients at dose level 1.

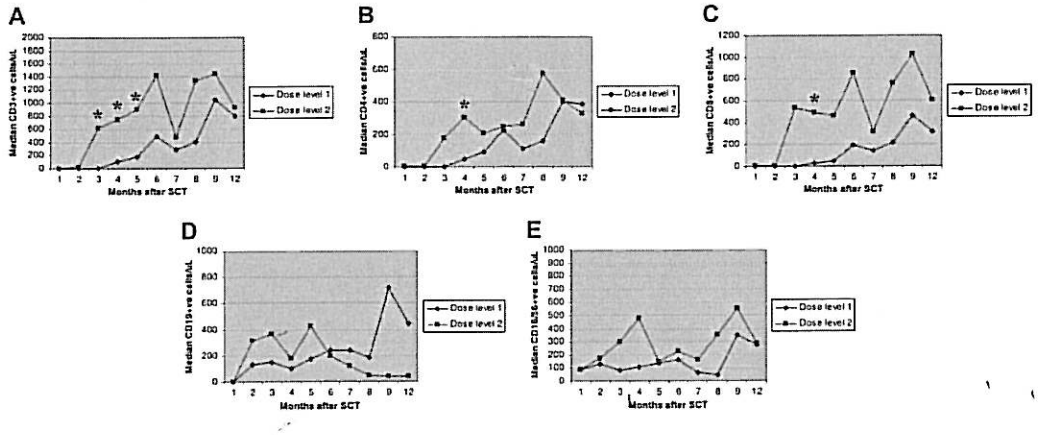


Figure 8. Kinetics of recovery of $CD3^+$, $CD4^+$, and $CD8^+$ T, B, and NK cells after transplantation. Panel A shows $CD3^+$ cells; **panel B**, $CD4^+$ cells; **panel C**, $CD8^+$ cells; **panel D**, B cells; and **panel E**, NK cells. Median circulating cell counts at each time point are compared between patients treated at dose levels 1 and 2. Time points at which level 2 patients had statistically significant increased counts are indicated by an asterisk.

A significantly higher mean AUC was also observed for $CD3^+$ (P = .034) and $CD4^+$ (P = .037) at 6 months after SCT. At 4 months after transplantation, 0 of 6 evaluable patients at dose level 1 had achieved normal T-cell, CD4, or CD8 numbers, whereas the respective figures for dose level 2 were 3 of 6, 2 of 6, and 5 of 6 (Figure 9A). By 6 months after SCT, only one of 5 evaluable patients at dose level 1 had normal T-cell counts, and none had normal CD4 counts. By contrast, all 5 evaluable patients at dose level 2 had normal T-cell and CD8 counts, and 3 of 5 had normal CD4 counts (Figure 9B). The proportion of patients achieving normal CD8 counts at 4 months and normal CD3 counts at 6 months was significantly greater in patients treated at the higher dose level (P =.05). To assess T-cell function, we assayed responses to PHA. As shown in Figure 9, at 6 months after SCT, 0 of 5 evaluable patients at dose level 1

had achieved normal PHA responses, whereas 2 of 5 patients at dose level 2 had normal responses. There was no significant difference in recovery of CD3, CD4, or CD8 cells between patients who received nonmyeloablative or conventional intensity conditioning, and patients with detectable alemtuzumab levels at the time of first infusion did not have slower immune reconstitution.

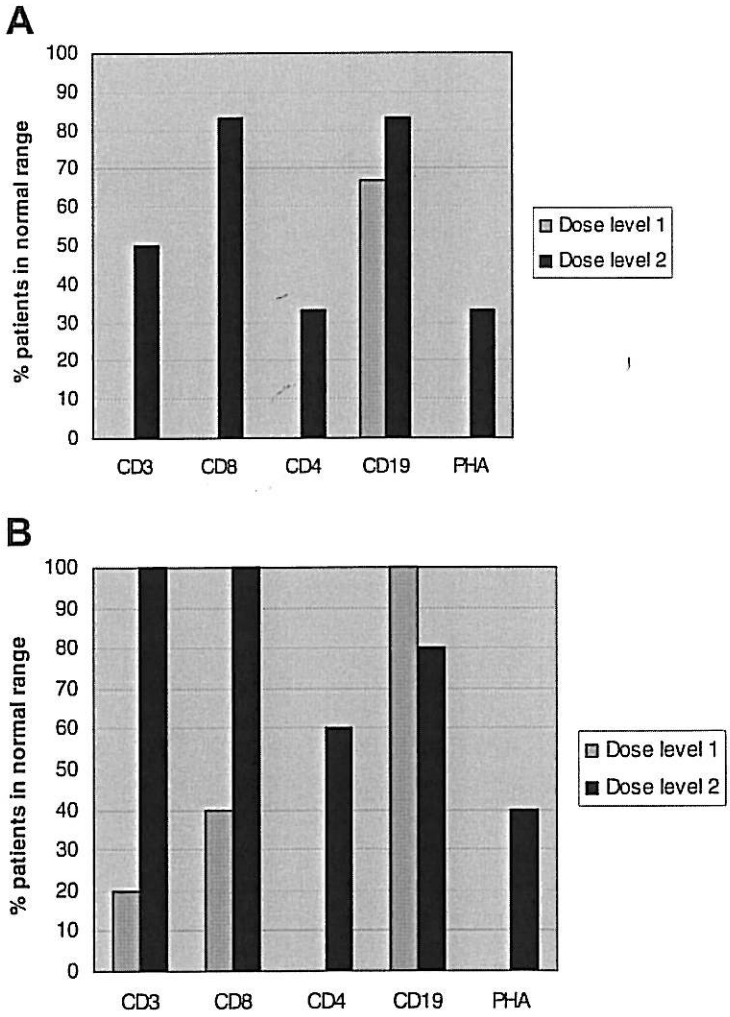


Figure 9. Percentage of patients achieving normal CD3, CD8, CD4, and CD19 counts, and responses to PHA stimulation. Data at 4 months are shown in panel **A** and 6 months in panel **B**.

6.7. Memory phenotype

As shown in Figure 10, most of the T cells recovering in patients at dose level 1 had a naive $CD45RA^{+}CCR\text{-}7^{+}$ phenotype. By contrast, the increase in T-cell reconstitution observed in patients at dose level 2 primarily reflects an expansion in T cells with an effector memory ($CD45RA^{+}CCR\text{-}7^{+}$) phenotype (Figure 10C). Univariate comparisons between dose levels 1 and 2 showed a significant ($P < .05$) increase in the levels of effector memory (but not central memory or naive) cells in patients at dose level 2 at 3, 4, and 5 months after SCT. Similarly, AUC analyses demonstrated a significantly higher mean AUC for effector memory cells in patients treated at dose level 2 at both 4 and 6 months ($P = .022$ and $P = .048$, respectively).

6.8. TRECs and spectratyping

TRECs were assayed in 9 patients (5 treated at dose level 1, 4 at dose level 2) at 4 and 6 months after SCT. Six of 9 patients showed undetectable TREC levels at both time points. TRECs were detectable at low levels in P1 (6962 TRECs/10^6 PBMCs at 4 months, 5387 TRECs/10^6 PBMCs at 6 months), P4 (8447 TRECs/10^6 PBMCs at 6 months), and P6 (1472 TRECs/10^6 PBMCs at 6 months), all of whom were treated at dose level 1. The normal range for TRECs in healthy donors is 1200 to 155000 TRECs/10^6 PBMCs (Chen X *et al.*, 2005).

Spectratyping of TCR Vβ genes was performed on PBMCs from 9 patients (5 treated at dose level 1, 4 treated at dose level 2). The distribution of TCR Vβ receptors at 4 months after SCT in patients at both dose levels was polyclonal with a median of 6.8 peaks (range, 5.3-7.8 peaks), with a shift to a more oligoclonal Vβ repertoire by 6 months (median, 4.2 peaks; range, 3.5-5.9 peaks), and normalization of TCR diversity by 12 months after SCT. The median number of peaks was similar between the 2 dose levels at either 4 or 6 months after SCT (data not show)

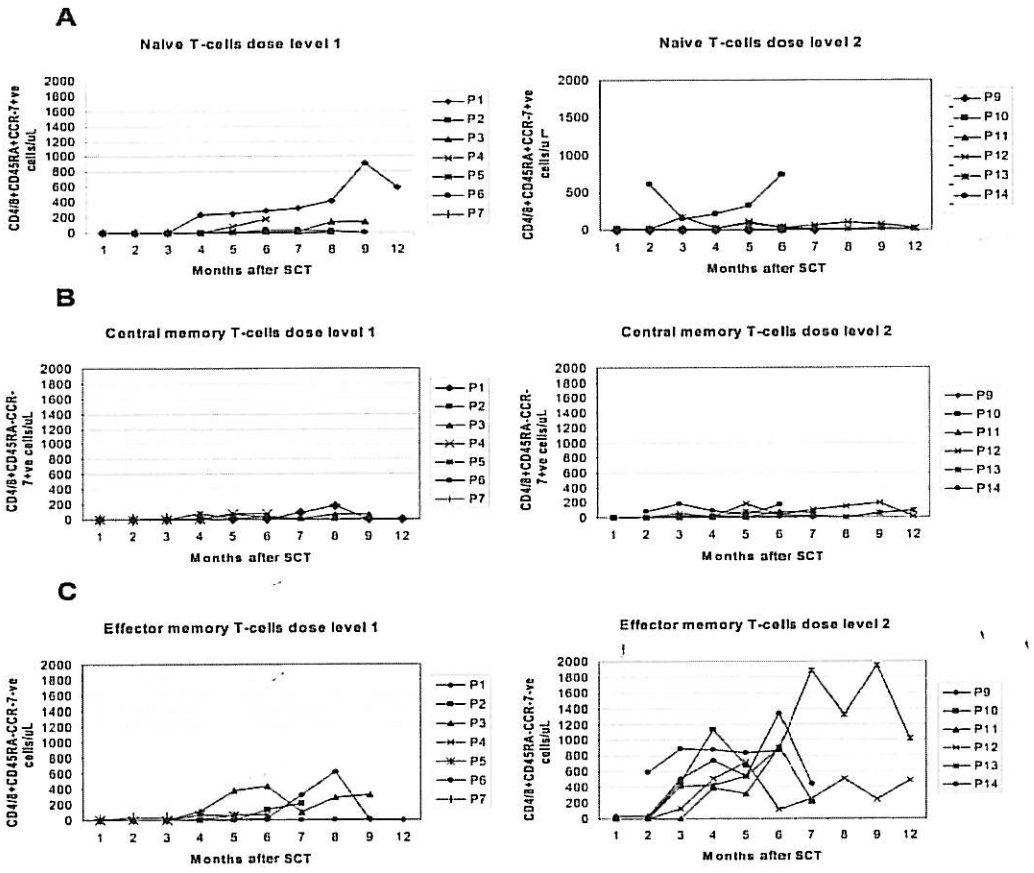


Figure 10. Kinetics of naive, central memory, and effector memory reconstitution after transplantation. Naive cells ($CD45RA^+CCR\text{-}7^+$) are shown in **panel A**; central memory ($CD45RA^-CCR\text{-}7^-$) in **panel B**; and effector memory ($CD45RA^-CCR\text{-}7^-$) in **panel C**. Dose-level 1 patients (left column) are compared with dose-level 2 patients (right column).

6.9. Viral-specific immunity

In order to study the recovery of viral-specific immunity, we used flow cytometric analysis of $CD3^+CD8^+$ cells from 10 evaluable patients with HLA-A2–, HLA-A24–, HLA-B7–, or HLA-B8– positive donors, at varying time points after SCT, after staining with HLA peptide tetramers. Peripheral blood from the donor premobilization was used as a control. As shown in Figure 11A, none of the 6 evaluable patients at dose level 1

had significant tetramer-positive populations recognizing EBV epitopes up to 9 to 12 months after SCT, despite the fact that 2 had viral reactivation. Tetramer-positive cells were detected in 3 of 4 evaluable patients at dose level 2 at 4 to 6 months after SCT, in each case shortly after viral reactivation. Tetramer-positive cells were directed predominantly against epitopes from lytic (eg, BMLF-1), but also in some case latent (eg, EBNA3a) antigens. Similarly, for CMV (Figure 11B), none of the 6 evaluable patients at dose level 1 had tetramer-positive populations detectable before 9 months after SCT, despite the fact that 5 of these patients had viral reactivations. In contrast, tetramer-positive cells were detected in 2 of 4 evaluable patients at dose level 2 as early as 2 and 4 months after SCT. In P13, this correlated with CMV reactivation, while in P9 no reactivation was apparent. Taking the data from CMV and EBV together, in all but 2 cases, the tetramer-positive cells detected in the patient recognized the same epitopes as those detected in the donor, but there was often significant expansion of the tetramer-positive population in the patient compared with the donor, particularly after viral reactivation. In 2 cases, tetramer-positive cells were observed in the patient that were not detected in the donor.

Figure 11C shows an example of both the expansion of pre-existing tetramer-positive cells (A2-NLV) and an apparent de novo tetramerpositive population (B8-ELR) directed against CMV antigens in P13, a patient at dose level 2, after CMV reactivation. To determine whether the antiviral responses we observed were functional, we performed IFN-γ ELISPOT analyses on PBMCs isolated from patients at varying time points after SCT. All 13 patients with more than 3 months follow-up were evaluable.

As shown in Figure 12A, 2 of 7 patients at dose level 1 (P6 and P7), both of whom had prior EBV reactivations, showed significant (> 200 cells/10^6 PBMCs) IFN-γ responses at 4 and 6 months after SCT. IFN-γ responses were detected in 4 of 6 patients at dose level 2 and occurred earlier (2-4 months) and were of greater magnitude than those seen in patients at dose level 1. In general, these responses correlated with viral reactivation, but one patient (P12) showed no IFN-γ response despite viral reactivation. For CMV (Figure 12B), only one of 7 evaluable patients at dose level 1 showed significant IFN-γ secretion in response to CMV pp65 from 6 months after SCT, despite the fact that 5 of these patients had CMV reactivations. In contrast, 3 of 6 evaluable patients at dose

level 2 had significant responses that were detectable as early as 2 months after SCT. The correlation between viral reactivation and responses was less consistent than with EBV. One patient at dose level 2 (P10) who had CMV reactivation did not have detectable ELISPOT responses. Data from tetramer and ELISPOT analyses were largely concordant, although in 3 cases (P3 for CMV, P6 and P7 for EBV) IFN-γ responses were detectable when no tetramer-positive cells were detected, presumably reflecting secretion from either $CD4^+$ cells or $CD8^+$ cells recognizing epitopes other than those on the tetramers.

6.10. Infections and outcome

These are summarized in Table 5. Nine patients (of 16 at risk) had CMV reactivation detectable in the peripheral blood. All 9 patients were treated with ganciclovir with or without foscarnet, and none (table 4) developed CMV-related disease. Six patients have had EBV reactivations, of whom 1 had a viral load greater than 4000 copies/μg PBMC DNA, which has previously been shown to be predictive of lymphoproliferative disease (LPD). (Wagner HJ *et al.,* 2004) None of these patients were treated preemptively with rituximab, and none developed LPD. Three patients developed proven/probable fungal infections and all resolved on liposomal amphotericin B with or without caspofungin therapy. Two patients developed adenoviremia. In P2 (dose level 1), this progressed to fatal acute liver failure in association with chronic GVHD affecting the liver. P15 had persistent adenoviremia with fever from 2 weeks after SCT, despite multiple courses of cidofovir and ribavirin and 3 doses of allodepleted donor T cells at dose level 2. She was treated off-study with a single dose of 2.5 x 10^6/kg allodepleted donor T cells on a compassionate basis, with clearance of viremia, but died subsequently from encephalopathy. P13 developed severe progressive multifocal leucoencephalopathy, which progressed despite cidofovir and intravenous immunoglobulin therapy at 2 months after transplantation, at a time when he was profoundly lymphopenic. This patient made a remarkable clinical and radiologic recovery following infusion of allodepleted donor T cells at dose level 2, with full recovery of motor function and continence and marked improvement in cognitive skills, associated with T-cell recovery. He later died from presumed sepsis with multiorgan failure while on immunosuppression for autoimmune

hemolysis. Outcome is shown in Table 2. Overall, at a median follow-up of 33 months, 7 patients have relapsed and 5 are alive and disease free at the time of writing (June 2006).

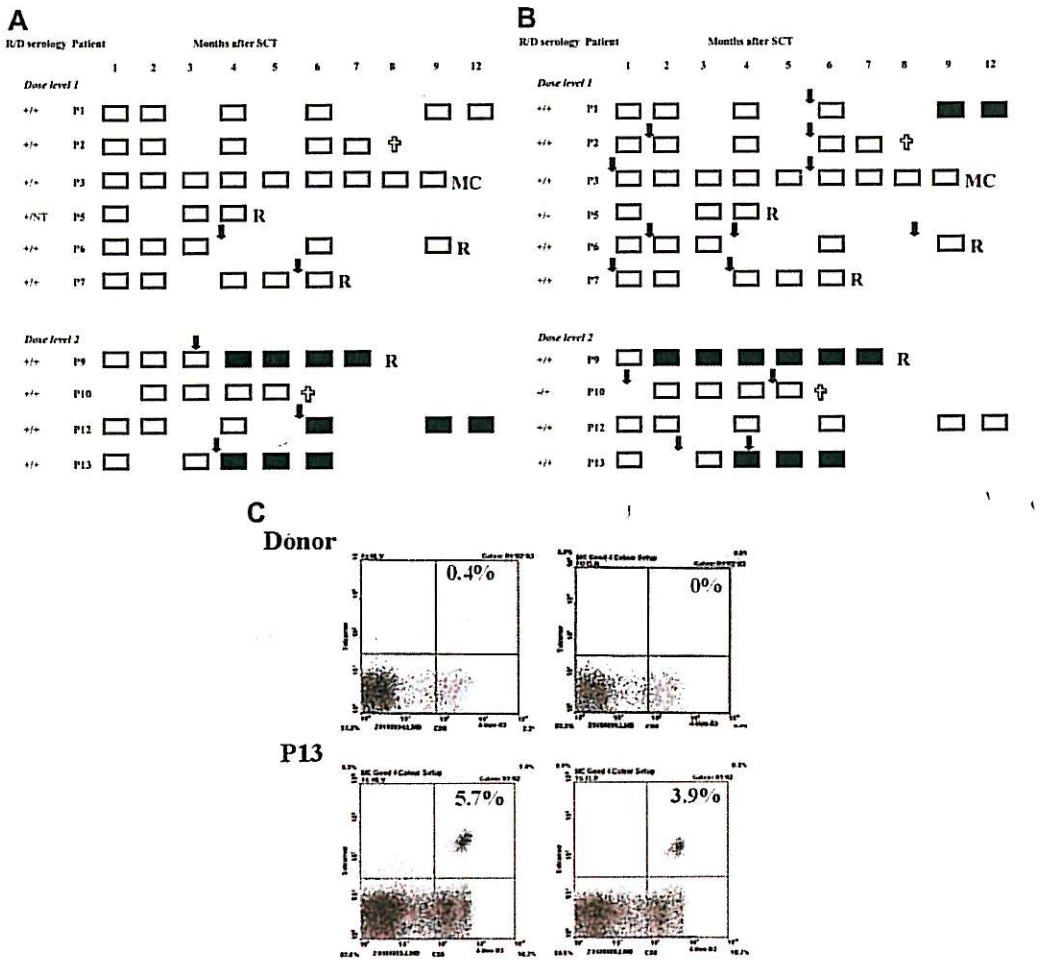


Figure 11. Recovery of CD8 responses to viruses. (A) Recovery of CD8 responses against EBV. ■ represents time points at which a significant (> 0.1% above isotype) tetramer-positive population was identified in the peripheral blood of recipients; □, time points at which no tetramer-positive cells were observed. The arrows indicate EBV viremia. Time points at which patient went off study for relapse (R) or mixed chimerism (MC) are shown. Crosses indicate time points at which patients died. (B) CD8 responses against CMV. Schema as in panel A. (C) Flow cytometric analysis of peripheral blood from patient P13 at 6 months after transplantation (bottom 2 panels) and his donor (top 2 panels) using CD8-FITC/tetramer-PE staining. The left panels show samples stained with the HLA-A2-NLV tetramer (pp65), and the right panels show samples stained with the HLA-B8-ELR tetramer (IE1). The percentage of $CD3^+8^+$ T cells that were tetramer positive is shown.

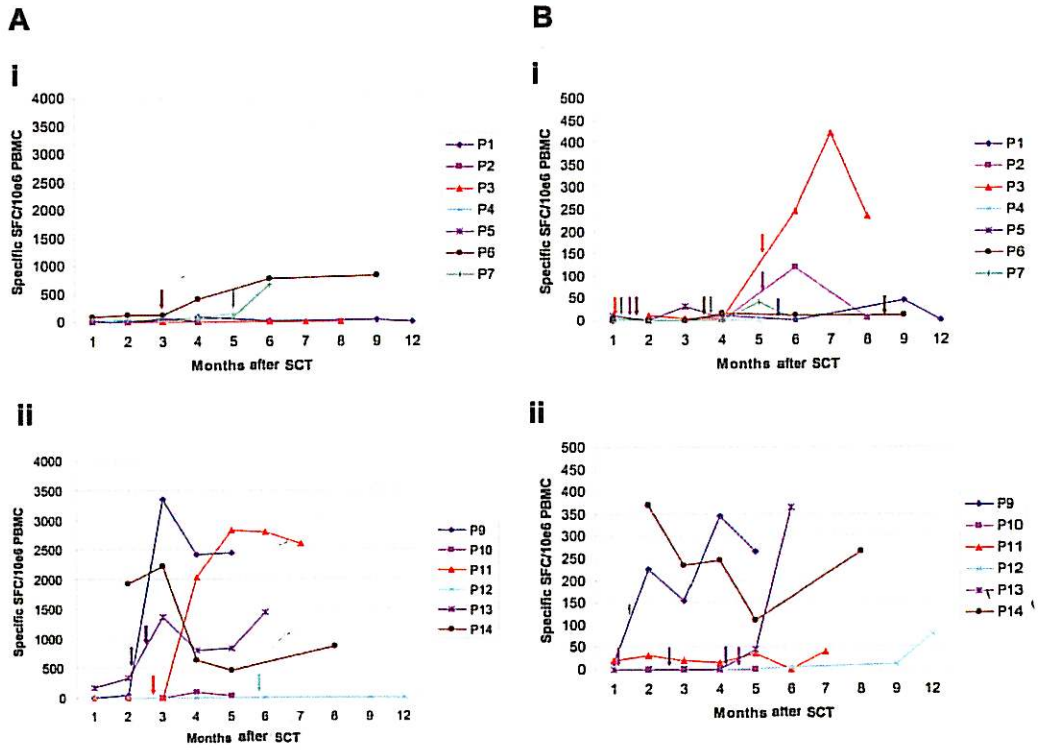


Figure 12. Recovery of functional antiviral responses. (**A**) Functional response to EBV. The number of cells secreting interferon-ϒ in response to stimulation with donor EBV-LCL in ELISPOT assays are shown at varying time points after SCT. Dose-level 1 patients are shown in the top panel and level 2 patients in the bottom panel. (**B**) Functional response to CMV. The number of cells secreting interferon-ϒ in response to stimulation with donor PBMCs expressing a pp65 transgene in ELISPOT assays are shown at varying time points after SCT. Patients at dose-level 1 are shown in panel **i** and dose-level 2 patients in panel **ii**. Viral reactivations are indicated by the arrows.

7. DISCUSSION

Previous studies have demonstrated that after activation with recipient PBMCs, alloreactive donor T cells can be effectively depleted by targeting activation markers such as CD25 and CD69 (Valteau-Couanet D *et al.,* 1993; Montagna D *et al.,* 1999; Cavazzana-Calvo M *et al.,* 1990; Koh MB *et al.,* 2002). We have attempted to refine this approach to make it more reproducible in a clinical setting, particularly for patients with malignant or aplastic disorders. A major limitation of current approaches to allodepletion is the source of recipient stimulator cells. In patients who are aplastic either due to disease or to therapy it may be difficult to obtain adequate numbers of recipient PBMCs or dendritic cells to stimulate donor T cells prior to allodepletion. In contrast, even in these patients LCLs are readily generated and expanded to large numbers. Even when sufficient PBMCs are available, these cells are inefficient at antigen presentation and have a variable phenotype, resulting in inconsistent allo-activation and hence allodepletion. Indeed, in the only clinical study of addback of allodepleted donor T cells to date,11 the 4 patients who developed GVHD all had significant residual proliferation to recipient stimulators in primary mixed lymphocyte reactions, demonstrating ineffective allodepletion. GVHD was significantly correlated with this residual proliferation. In the current study we have shown that LCLs activate alloreactive T cells from HLA-mismatched donors to proliferate more effectively than PBMCs, even at much lower responder/stimulator ratios. Further, we have demonstrated that allodepletion with an anti-CD25 immunotoxin following stimulation with HLA mismatched LCLs is more consistently effective at removing alloreactive cells, as assessed by the residual proliferation, than following stimulation with PBMCs. This may reflect the heterogeneity of crude PBMC preparations as antigen-presenting cells compared with LCLs, which have a more standard phenotype and more uniformly express high levels of immunostimulatory molecules. If adoptive immunotherapy with allodepleted donor T cells is to be useful in restoring antiviral and antileukemic T-cell responses clinically, it is critical to demonstrate the specificity of depletion. T-cell receptors on alloreactive T cells may recognize limited sets of cross-reactive peptides presented by foreign major histocompatibility complex (MHC) molecules (Moris A *et al.,* 2001) and, conversely, T cells specific

for environmental antigens may cross-react with foreign MHC (Ashwell JD *et al.,* 2001; Lombardi G *et al.,* 1991; Matzinger P, 1994). Potentially then, allodepletion could be associated with a reduction in T cells specific for viral antigens, limiting the clinical usefulness of this approach. Likewise, the CD25 immunotoxin could bind to bystander T cells non-specifically activated by paracrine secretion of cytokines, and its ricin moiety could exert negative effects on bystander T cells. Previous in vitro studies on allodepletion have not adequately addressed the issue of whether antiviral responses are preserved following allodepletion. In general they have relied on demonstration of preserved proliferative or CTLp responses to third party as surrogate markers for antiviral T-cell responses in allodepleted donor T cells (Cavazzana-Calvo M *et al.*, 1990; Koh MB *et al.*, 1999). In our hands, however, secondary proliferative responses to third party are highly dependent on the timing of secondary stimulation in relation to immunodepletion. Similarly, the preservation of proliferative responses to candidin and CMV lysates after allodepletion shown by Valteau-Couanet *et al* (1993) may not truly reflect preservation of donor T-cell responses to infectious agents because of the supraphysiological levels of antigen used in these assays.

We have used ELISPOT and tetramer assays to compare the frequency of antiviral T cells before and after allodepletion: These assays have the advantage that they enable direct quantification of the frequency of viral-specific T cells. Using these assays, we have clearly shown that the frequency of T cells recognizing CMV and adenoviral antigens is unaffected by allodepletion with our protocol. Thus, adoptive immunotherapy with allodepleted donor T cells should result in improved T-cell responses to these viruses. Likewise, we have shown that the donor anti-EBV response is partially retained following allodepletion with recipient LCL stimulators, through recognition of EBV antigens on the nonshared haplotype, so that addback of allodepleted donor T cells after haplo-identical SCT would be predicted to confer some degree of immunity against EBV. Given the high relapse rates after haplo-identical SCT (Aversa F *et al.,* 1998; Handgretinger R *et al.,* 2003), the issue of whether antileukemic responses are preserved following allodepletion is also critical. If allodepletion is performed after stimulation with host PBMCs, it would be predicted that T-cell responses to myeloid antigens overexpressed in AML and CML, such as proteinase 3 and WT-1, which may be important targets for the GVL effect (Molldrem J *et al.,* 1996;

MoldremJJ *et al.,* 1997; Gao L *et al.,* 2000; Scheibenbogen C *et al.,* 2002), would be lost. Our data suggest that this may indeed be the case. Similarly, recipient PBMCs are frequently contaminated with leukemic cells, so that the response to leukemiaspecific antigens may be lost. In contrast, using our approach with recipient LCLs as stimulators, these responses should be preserved. The studies of Montagna *et al* (1999) and Mavroudis *et al* (1998) suggest that CTLp and HTLp frequencies against leukemic cells may be maintained after allodepletion. These data, however, are complicated by the indirect nature of the CTLp and HTLp assays, which involve in vitro restimulation, as well as by the wide confidence intervals for these assays, which may obscure significant changes. We have instead directly studied the T-cell response to a defined candidate tumor antigen, the PR1 epitope of proteinase 3. PR1-specific CTLs lyse primary leukemic cells from patients with AML, (Molldrem J *et al.,* 1996) preferentially inhibit leukemic CFU-GM colony formation, (MoldremJJ *et al.,* 1997) and have been correlated with clinical responses to IFN-α and allogeneic HSCT in patients with CML. (MoldremJJ *et al.,* 2000) We have demonstrated that, after stimulation with HLA-mismatched LCLs, T-cell responses to PR1 are preserved following allodepletion.

Donor NK cell alloreactivity, mediated through absent expression of the killer cell inhibitory receptor (KIR) ligand on recipient cells, may play a major role in the GVL response in haplo-identical SCT, particularly in AML (Ruggeri L *et al.,* 2002). The percentage of NK cells and cytotoxicity against K562 targets is unaffected by allodepletion using our protocol (data not shown). Alloreactive NK cells express CD25 upon activation,(Trinchieri G *et al.,* 1995) and these $CD56^{+}/CD25^{+}$ cells are depleted by anti-CD25 immunotoxin (data not shown), so that adoptive immunotherapy with allodepleted donor cells would not be predicted to enhance NK-mediated GVL reactions. However, because the recovery of donor NK cells is rapid following haplo-identical SCT,(Eyrich M *et al.,* 2001) this should not adversely impact on the efficacy of such a strategy. The pace of immune reconstitution has been directly correlated with the number of T cells in the infused graft (Lowdell MW *et al.,* 1998). Likewise, it is known that doses of unmanipulated donor T cells as low as 3 x 10^4/kg can be associated with severe GVHD in the haploidentical setting. We can routinely generate multiple doses of 10^6 allodepleted donor T cells per kilogram from 500 mL donor peripheral blood. It remains

unclear, however, how many allodepleted donor T cells can safely be given in the haploidentical setting and whether infusion of this number would be sufficient to confer useful antiviral and antileukemic activity. To address this question we have initiated a phase 1 clinical study of addback of escalating doses of allodepleted donor T cells after haploidentical SCT.

We have demonstrated that adoptive immunotherapy with allodepleted donor T cells improves T-cell reconstitution after haploidentical SCT. T-cell reconstitution in the patients at dose level 1 was slow, comparable with that observed without allodepleted T-cell addback, (Eyrich M *et al.*, 2001) where normal T-cell numbers were not observed until 9 to 12 months after haploidentical SCT. This was predictable, since the dose of allodepleted cells infused is similar to the numbers of T cells infused with the graft, and thus, these patients form an in-study control cohort for assessing the impact of infusing higher doses of allodepleted cells. In contrast, patients at dose level 2 exhibited significantly more rapid recovery of T cells, particularly at 3 to 5 months after SCT, which is frequently the time period at which patients succumb to infection after haploidentical SCT. CMV, EBV, and pneumocystis rarely cause disease in SCT recipients when the CD4 count is greater than 300/μL, and some groups use this as a threshold to stop cotrimoxazole prophylaxis and monitoring of viremia. The median time to reach this threshold was 4 months in patients at dose level 2, compared with more than 6 months in patients at dose level 1 and 8 months in the series of Eyrich et al (2001) without allodepleted T-cell addback. Further, most T cells that did recover in patients at dose level 2 exhibited an effector memory phenotype, implying that protective T-cell responses are likely to be long lived. While it is possible that naive T cells may also have shifted to an effector memory phenotype, our finding that none of the 4 dose-level 2 patients tested had detectable circulating TRECs at the time of T-cell recovery argues that naive T cells derived from the stem cell graft are unlikely to have contributed to the improved immune reconstitution seen in these patients. These data strongly suggest that the accelerated T-cell recovery seen in dose-level 2 patients is due to the infused allodepleted donor T cells.

Multivariate analysis demonstrated that the improved T-cell reconstitution in dose-level 2 patients was independent of intensity of conditioning, malignant diagnosis, and post-SCT immunosuppression. Since most of

our patients were children, our findings are primarily relevant to pediatric patients and further studies will be needed to determine the effect of recipient age. In view of progressive thymic involution with age, it is critical that strategies to improve T-cell immunity in the early post-SCT period are independent of thymic maturation. Our data on the absence of TRECs in dose-level 2 patients with accelerated T-cell recovery support such a thymus-independent mechanism for immune reconstitution, but this will be an important issue to confirm in adults. Previous studies have shown 2 pathways that contribute to reconstitution of the T-cell compartment. In the initial months after SCT, the T-cell repertoire depends on peripheral expansion of mature T cells in the graft, due to thymic damage associated with conditioning (Heitger A *et al.,* 2000). Because of the rigorous T-cell depletion of the graft in haploidentical SCT, the T-cell repertoire is very restricted, with severe skewing of T-cell–receptor complexity for the first 6 to 9 months after SCT. Subsequently, de novo maturation of naive T cells derived from bone marrow emigrants passaged through the thymus has been shown to occur later than 6 months after SCT and may play a part in normalization of the T-cell repertoire (Small TN *et al.,* 1999). Our TREC data demonstrate that new thymic emigrants play a limited role in T-cell reconstitution for the first 6 months after SCT in most of our patients and are consistent with published data on heavily pretreated patients (Chen X *et al.,* 2005). Our spectratyping data show a significantly more polyclonal distribution of Vβ receptor gene usage at 4 months after SCT in patients at both dose levels than has been reported in published series of pediatric haploidentical SCT without allodepleted T-cell addback. In the study by Eyrich et al (2001) in a similar pediatric patient cohort, all patients had a markedly skewed repertoire with a median of 3 to 4 bands per Vβ family for the first 6 months after SCT, whereas our patients had a median of 7 bands per Vβ family at 4 months. This may reflect the polyclonal pattern of Vβ usage in the infused T cells. The absence of viral-specific responses in dose-level 1 patients despite polyclonal Vβ usage may be due to the higher sensitivity of the PCR-based spectratyping assay compared with tetramer/ELISPOT analyses. In most patients, the spectratyping pattern became more oligoclonal at 6 months, and this may reflect preferential expansion of T-cell clones that have been stimulated by their cognate antigens, with subsequent normalization of TCR diversity by 12 months after SCT.

Similarly, published data on pathogen-specific responses (eg, against fungal antigens) suggest these are generally absent until 9 months after SCT (Volpi I *et al.,* 2001). Our data on T-cell responses to CMV and EBV in patients at dose level 1 are in line with these findings. In contrast, we have observed accelerated recovery of CMV- and EBV-specific immunity in patients treated at the higher dose level, using both flow cytometric and functional assays. EBV-specific responses are partially retained in the allodepleted donor T-cell product, despite using LCLs as stimulators, through recognition of EBV epitopes presented on the non-shared HLA haplotype. Antiviral responses were observed as early as 2 to 4 months after SCT in these patients (ie, after a single infusion in some cases), particularly after viral reactivation. This coincides with the period during which patients are at maximum risk of viral infections after haploidentical SCT and is remarkable because such responses are not seen until 6 to 12 months after SCT even after the less rigorous T-cell depletion used in nonmyeloablative unrelated donor SCT (Chakrabarti S *et al.,* 2003)

Our study was not designed with sufficient power to demonstrate clinical efficacy. While infections and viral reactivations were frequent, the overall incidence of directly infectious deaths was low (2 of 16). In conjunction with our data on viral-specific immune responses, this suggests that while the number of cells infused may be insufficient to prevent such reactivations, they may have played a role in preventing progression to disease/death. Taken together, our data suggest that adoptive immunotherapy with allodepleted donor T cells at doses of 1 to 3 x 10^5/kg is sufficient to improve T-cell reconstitution and antiviral immunity after haploidentical SCT. This approach may potentially reduce infection associated mortality and thereby substantially broaden the applicability of haploidentical SCT. Clearly, however, larger studies will be needed to demonstrate if such an approach confers a real therapeutic benefit.

Comparison of the numbers of circulating T cells in patients treated at the higher dose level and the number of cells infused implies that allodepleted donor T cells are able to expand significantly in vivo, particularly in the face of viral reactivation. This is similar to what has been observed after adoptive transfer of EBV and CMV-specific cytotoxic T cells in patients undergoing T-cell–depleted SCT (Rooney CM *et al.,* 1998; Peggs KS *et al.,* 2003), and may relate to the profound lymphopenia after haploidentical SCT. There is growing evidence for lymphoid homeostatic mechanisms (Ge Q *et al.,* 2002; Mackall CL *et al.,*

1997), which in the lymphopenic environment favours rapid repopulation of the peripheral T-cell compartment through expansion of relatively small numbers of infused memory T cells. In both murine models and a human study, lymphodepletion may have a marked effect on the efficacy of adoptive T-cell transfer (Rosenberg SA *et al.,* 1986; Dudley ME *et al.,* 2002). Such homeostatic mechanisms may explain why the infusion of allodepleted donor T cells at doses as low as 3 x 10^5/kg may be sufficient to confer significant viralspecific immunity in the context of the profoundly lymphodepleted host after haploidentical SCT.

Our study confirms the safety of adoptive immunotherapy with allodepleted donor T cells in haploidentical stem cell transplant recipients. We have observed a low incidence of GVHD at both dose levels, comparable to that seen without the addback of allodepleted donor T cells (Aversa F *et al.,* 1998; 2005). In the case of P12, the dose of allodepleted donor T cells was substantially higher than the T-cell dose infused with the graft, so it is likely the former contributed to GVHD, but in P2, cell doses were similar, so it is not clear whether the allodepleted donor T cells were responsible for GVHD in this patient. Previous studies have observed a high incidence of acute GVHD after infusion of unmanipulated donor lymphocyte infusions at 10^5/kg within the first 3 months of SCT, even in the HLA-matched setting (Chakrabarti S *et al.,* 2003). The low incidence of acute and chronic GVHD observed in our patients treated at dose level 2 demonstrates that our strategy effectively depletes clinically relevant alloreactive cells. Further, the depletion of $CD4^+CD25^+$ regulatory T cells from the infused cells does not appear to enhance the potential for GVHD, presumably reflecting the absence of significant numbers of alloreactive effector cells. Similarly, we have not observed an excess of post-SCT autoimmune phenomena. In the study by Andre-Schmutz et al (2002), the 4 patients who developed acute GVHD were those with high residual proliferative responses to host cells. Our preclinical data have demonstrated the importance of strong activation of alloreactive cells for this strategy to work reproducibly, and we believe the choice of host antigen-presenting cell is critical in this regard (Amrolia PJ *et al.,* 2003). We have demonstrated that allodepletion with this IT following stimulation with HLA-mismatched LCLs may be more consistently effective at removing alloreactive cells than following stimulation with PBMCs, (Amrolia PJ *et al.,* 2003). so we used recipient LCL as stimulators in our clinical study. Using our approach, we

did not observe a correlation between residual proliferation in the primary MLR or residual $CD3^+/CD25^+$ cells in the infused product and the development of GVHD. While, as noted, our study was not designed to demonstrate clinical efficacy, given the accelerated T-cell recovery in dose-level 2 patients, disease-free survival was disappointing (only 5 of 16 patients), with relapse being the major cause of treatment failure. Although preclinical data from our group and others (Montagna D *et al.,* 1999; Amrolia PJ *et al.,* 2003). suggest that antileukemic responses may be preserved after allodepletion, relapse clearly remains a major problem in our cohort (7 of 16 patients). It is thus evident that in patients with high-risk malignancies, the benefits of this approach in improved immune responses to infection may be offset by leukemic relapse in survivors. Extension of this approach to demonstrate antileukemic responses will require larger, randomized studies, and is likely to need larger doses of allodepleted donor T cells than are required for reconstitution of antiviral responses (Papadopulos EB *et al.,* 1994; Hromas R *et al.,* 1994; Dazzi F *et al.,* 2000).

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Plenary paper

Adoptive immunotherapy with allodepleted donor T-cells improves immune reconstitution after haploidentical stem cell transplantation

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Poor T lymphocyte reconstitution limits the use of haploidentical stem cell transplantation (SCT) because it results in a high mortality from viral infections. One approach to overcome this problem is to infuse donor T cells from which alloreactive lymphocytes have been selectively depleted, but the immunologic benefit of this approach is unknown. We have used an anti-CD25 immunotoxin to deplete alloreactive lymphocytes and have compared immune reconstitution after allodepleted donor T cells were infused at 2 dose levels into recipients of T-cell–depleted haploidentical SCT. Eight patients were treated at 10^4 cells/kg/dose, and 8 patients received 10^5 cells/kg/dose. Patients receiving 10^5 cells/kg/dose showed significantly improved T-cell recovery at 3, 4, and 5 months after SCT compared with those receiving 10^4 cells/kg/dose ($P < .05$). Accelerated T-cell recovery occurred as a result of expansion of the effector memory ($CD45RA^-CCR\text{-}7^-$) population ($P < .05$), suggesting that protective T-cell responses are likely to be long lived. T-cell–receptor signal joint excision circles (TRECs) were not detected in reconstituting T cells in dose-level 2 patients, indicating they are likely to be derived from the infused allodepleted cells. Spectratyping of the T cells at 4 months demonstrated a polyclonal Vβ repertoire. Using tetramer and enzyme-linked immunospot (ELISPOT) assays, we have observed cytomegalovirus (CMV)– and Epstein-Barr virus (EBV)–specific responses in 4 of 6 evaluable patients at dose level 2 as early as 2 to 4 months after transplantation, whereas such responses were not observed until 6 to 12 months in dose-level 1 patients. The incidence of significant acute (2 of 16) and chronic graft-versus-host disease (GVHD; 2 of 15) was low. These data demonstrate that allodepleted donor T cells can be safely used to improve T-cell recovery after haploidentical SCT and may broaden the applicability of this approach. (Blood. 2006; 108:1797-1808)

Introduction

The lack of fully human leukocyte antigen [(HLA)–matched donors is a major limitation to the applicability of hematopoietic stem cell transplantation (SCT). However, almost all patients have potential donors who are HLA haploidentical. Recent advances in our ability to mobilize and select hematopoietic stem cells (HSCs) have made SCT from haploidentical donors feasible, both in terms of reliable engraftment and acceptable rates of graft-versus-host disease (GVHD).[1,2]

T-cell reconstitution is a key determinant of outcome after SCT. The rigorous T-cell depletion necessary to prevent GVHD in the haploidentical setting results in profound posttransplantation immunodeficiency.[3,4] This is the major barrier to the broader application of haploidentical SCT, because it results in high morbidity and mortality from viral infections due to the loss of antiviral immunity and high relapse rates due to the reduction in the graft-versus-leukemia (GVL) response.[1,2,5] In the Perugia series, 27 of 101 patients who underwent haploidentical SCT for acute leukemia died from infection.[5] Simple T-cell addback is unlikely to be effective in preventing these problems without causing GVHD, because the frequency of alloreactive T cells in peripheral blood is higher than that of either antiviral or antileukemic T cells. A number of approaches have evolved to circumvent this, including induction of anergy in donor alloreactive T cells,[6,7] addback of regulatory T cells (Tregs),[8] and transduction of donor lymphocytes with suicide genes.[9,10] However, anergized cells and Tregs may have an inhibitory effect on desirable bystander T-cell responses, and current protocols for efficient transduction of T cells may lead to loss of antiviral responses.[11] An alternative approach is to deplete the graft specifically of the alloreactive T cells responsible for GVHD by deleting T cells that are activated in response to recipient antigen-presenting cells. This approach has the advantage that alloreactive T cells are permanently removed and cannot influence the function of the remaining T cells. Alloreactive cells may be targeted by their expression of surface activation markers, proliferation in a mixed leukocyte reaction (MLR) or the preferential

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retention of photoactive dyes. Alloreactive cells can be eliminated using immunotoxins,[12,13] immunomagnetic separation,[14-16] chemotherapeutic agents,[17,18] flow cytometric sorting,[19,20] or photodynamic purging.[21,22] Montagna et al[12] have demonstrated reduced in vitro alloreactivity after targeting activated T cells expressing the activation marker CD25 (the interleukin-2 [IL-2] receptor α chain), using an immunotoxin (RFT5-SMPT-dgA) consisting of a murine antibody moiety recognizing the IL-2 receptor p55 chain conjugated to deglycosylated ricin A. Using a similar approach, we have shown that T-cell responses against viral and potential myeloid tumor antigens are preserved following allodepletion.[23]

Based on their preclinical data, the Necker group have used CD25-immunotoxin (IT) based ex vivo allodepletion in a phase 1/2 clinical study in 15 patients undergoing haploidentical/unrelated donor SCT.[24,25] Solomon et al[26] have used a similar approach in 16 elderly patients undergoing HLA-matched related donor transplantation. While these studies demonstrated that adoptive immunotherapy with allodepleted donor T cells is feasible and results in a low incidence of GVHD, neither study addressed the critical issue of whether the infused allodepleted cells improved cell-mediated immunity. In the former study, 4 patients achieved CD3 counts of more than 500/μL by 6 months after SCT, but follow-up was short and data on immune reconstitution were limited, with no formal assessment of antiviral responses. Additionally, it is unclear how many allodepleted donor T cells must be infused to restore clinically relevant antiviral responses. Data from donor lymphocyte infusions in the HLA-matched setting suggest that doses as low as 10^6/kg may be sufficient to clear viral reactivations with EBV[27] and adenovirus.[28] It is therefore critical to determine whether the level of allodepletion achieved with anti-CD25 IT is sufficient to allow addback of enough T cells to restore useful immune responses without causing GVHD. To address this issue, we have compared immune reconstitution after addback of 2 different doses of allodepleted donor T cells in the haploidentical setting.

Patients, materials, and methods

Study objectives

The primary endpoint of the study was to compare immune reconstitution and viral-specific immune responses after addback of 2 different doses of allodepleted donor T cells. Secondary endpoints included comparison of the incidence of acute and chronic GVHD and the outcome of viral infections/reactivations at each dose level.

Study population

The protocol was open to all patients who were candidates for haploidentical SCT at Baylor College of Medicine and its affiliated Methodist and Texas Childrens' Hospitals (Houston, TX) and Great Ormond Street Children's Hospital (London, United Kingdom). Failure of engraftment and the presence of greater than grade I GVHD was a contraindication to infusion of allodepleted donor T cells.

Sixteen patients have been treated, 8 at each dose level. Patient characteristics and conditioning are outlined in Table 1. The median age was 9 years (range, 2-58 years) and most (12 of 16) received transplants for high-risk hematologic malignancies. Six patients were not in remission at the time of transplantation, 2 had relapsed on therapy, and 5 had relapsed after previous autologous/allogeneic SCT.

Study design

The study compared the outcomes in the previous section between 2 dose levels. Each patient was scheduled to receive 3 infusions of allodepleted donor T cells at the same dose at days 30, 60, and 90 after transplantation, provided that there was no evidence of grade II or higher GVHD, or until total T-cell numbers were greater than 1000/μL. Patients could receive a fourth dose at day 120 if clinically indicated and the circulating T-cell count was less than 1000/μL. Dose level 1 (10^4 cells/kg/dose) was chosen because this dose of T cells is known not to cause GVHD in haploidentical recipients, even when the T cells were not allodepleted. Comparison was made with a log-higher dose (10^5 cells/kg/dose). Patients were initially enrolled at dose level 1, with escalation to dose level 2 when the observed incidence of significant acute GVHD (aGVHD) at level 1 was shown to be lower than 25%. All eligible patients receiving haploidentical SCT were enrolled consecutively, but no specific methodology for ensuring equivalent patient distribution prospectively was used. The study was approved by both institutional review boards (IRBs) and by the US Food and Drug Administration (FDA) (Baylor College of Medicine [BCM]) and the Medicines and Healthcare products Regulatory Agency (MHRA) (Great Ormond Street Hospital [GOSH]). Informed consent was obtained from all patients and/or their parents.

Transplantation and engraftment

Patients underwent haploidentical SCT using routine institutional conditioning and supportive care protocols. Conditioning is outlined in Table 1. Eleven patients received myeloablative preparative regimens, which in most consisted of 90 mg/kg cyclophosphamide, 12 g/m^2 cytarabine, 1400 cGy total body irradiation in 8 fractions, and serotherapy with 12 to 40 mg total alemtuzumab (Campath 1H). Five patients received nonmyeloablative conditioning with total body irradiation at 450 cGy in a single fraction, 120 mg/m^2 fludarabine, and serotherapy with 40 mg alemtuzumab (n = 4) or 150 mg/m^2 fludarabine/10 g/m^2 cytarabine and granulocyte colony-stimulating factor (G-CSF) (n = 1). CD34$^+$ peripheral blood stem cells from G-CSF–mobilized donors were selected using the Isolex 3000 (Baxter Healthcare, Deerfield, MA) or CliniMACs (Miltenyi Biotec, Bisley, United Kingdom) immunomagnetic systems. The median dose of CD34$^+$ cells infused at transplantation was 11.5 × 10^6/kg (range, 5.9-20 × 10^6/kg), and a median of 2.6 × 10^4/kg T cells were infused with the graft (range, 0.73-18 × 10^4/kg). There was no significant difference in the number of infused T cells between the 2 dose levels. FK506/cyclosporin was either not administered (n = 9) or was withdrawn prior to the first infusion of allodepleted donor T cells (n = 7). More patients at dose level 2 (5 of 8) received post-SCT immunosuppression than at dose level 1 (2 of 8). GVHD was graded using the Seattle criteria.[29]

Engraftment was assayed on mononuclear and granulocyte fractions using XY fluorescence in situ hybridization (FISH) or DNA polymerase chain reaction (PCR) of short tandem repeats. All patients had primary engraftment at a median of 13 days. At 1 month, 15 of 16 were full donor chimeras, by 6 months 3 of 13 evaluable had mixed chimerism, and one had autologous reconstitution. Two of these patients have relapsed, and the other 2 have undergone successful second transplantations from alternate donors. One patient converted from a mixed chimeric state to full donor chimerism 1 month after infusion of his first dose of allodepleted donor T cells. Data on the extent of chimerism at 6 months are shown in Table 2.

Immunotoxin

RFT5-SMPT-dgA immunotoxin was generated by linking a murine anti-CD25 monoclonal antibody (MoAb) to a deglycosylated ricin α chain (dgA) as described.[30] Clinical grade IT was prepared in the Good Manufacturing Practice (GMP) laboratory at the University of Texas Southwestern Medical School.

Generation and infusion of allodepleted donor T cells

Generation of allodepleted donor T cells was performed under GMP conditions at the Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, using FDA-approved standard operating procedures. Six weeks prior to transplantation, 30 mL of blood was collected from the patient for generation of recipient EBV-transformed lymphoblastoid cell lines (LCLs). Immediately prior to mobilization, peripheral blood mononuclear cells (PBMCs) from 160 mL of donor blood were cocultured with irradiated (70 Gy) recipient LCLs in serum-free medium as described.[23]

Table 1. Patient and graft characteristics

Patient no. (age, y)	Diagnosis	Conditioning	Dose level, per kg	No. CD34/kg	No. CD3/kg	% $CD3^+/CD25^+$	CPM, no.		% residual proliferation	Alemtuzumab level at first infusion, ng/mL	No. infusions
							No.IT	IT			
P1 (8)	Rel ALL in second CR	Cy/TBI/Ara-C/alemtuzumab	10^4	1.4×10^7	1.8×10^4	0.15	4 014	765	0	1180	3
P2 (14)	Rel AML	Cy/TBI/Ara-C/alemtuzumab	10^4	1.2×10^7	0.7×10^4	0.27	21 033	1005	0.46	Undetectable	1 (GVH)
P3 (3)	Rel ALL on Rx in second CR	Cy/TBI/Ara-C/alemtuzumab	10^4	1.1×10^7	3.4×10^4	0.03	1 770	510	0	Undetectable	3
P4 (3)	AML first CR	Cy/TBI/Ara-C/alemtuzumab	10^4	6.3×10^6	1×10^5	0.1	41 980	578	0.71	Undetectable	3
P5 (12)	Ref AML	Cy/TBI/Ara-C/alemtuzumab	10^4	7.5×10^6	3.4×10^4	0.02	20 633	1071	1.00	NE	3
P6 (28)	Hodgkins in relapse after ABMT	TBI/FDR/alemtuzumab	10^4	6.9×10^6	2.2×10^4	0.01	28 256	866	0.33	350	3
P7 (3)	HLH	Cy/TBI/Ara-C/alemtuzumab	10^4	2.0×10^7	4.4×10^4	0.07	14 068	601	3.12	NE	3
P8 (58)	CML LBC in 2nd CP	TBI/FDR/alemtuzumab	10^4	1.3×10^7	2.6×10^4	0.11	4 160	367	0	303	1 (autologous reconstitution)
P9 (2)	Rel AML after MMUD BMT in relapse	TBI/FDR/alemtuzumab	10^5	1.2×10^7	1.3×10^4	0.11	2 569	513	0	Undetectable	1 (CD3 > 1000)
P10 (2)	Ref AML	Cy/TBI/Ara-C/alemtuzumab	10^5	1.8×10^7	1.8×10^5	0.19	22 252	1423	0.04	Undetectable	2 (CD3 > 1000)
P11 (8)	HLH	Cy/TBI/Ara-C/alemtuzumab	10^5	1.4×10^7	2.4×10^4	0.13	72 204	1366	1.18	426	3
P12 (12)	SAA	FDR/Cy/TBI/alemtuzumab	10^5	5.9×10^6	0.9×10^4	0.05	2 103	1369	0	NE	2 (GVH)
P13 (14)	MDS	FDR/Cy/TBI/ATG	10^5	7.5×10^6	2.7×10^4	0.01	4 695	340	2.80	—	4
P14 (6)	JMML/AML after haplo-SCT in third CR	FLAG	10^5	1.1×10^7	2.1×10^4	0.03	21 719	1346	0.57	—	4
P15 (10)	Fanconi, rejected first haplo-SCT	FDR/Cy/TBI/ATG	10^5	2×10^7	1×10^5	0.25	6 879	717	0	—	3
P16 (14)	Rel AML after syngeneic BMT in relapse	TBI/FDR/alemtuzumab	10^5	1.1×10^7	6.4×10^4	0.12	39 601	604	0	Undetectable	3

Rel indicates relapsed; ref, refractory; CR, complete remission; HLH, hemophagocytic lymphohistiocytosis; LBC, lymphoid blast crisis; MMUD, mismatched unrelated donor; SAA, severe aplastic anemia; MDS, myelodysplasia; Cy, cyclophosphamide; TBI, total body irradiation; Ara-C, cytarabine; ATG, rabbit antithymocyte globulin; FLAG, fludarabine/cytarabine/G-CSF; CPM, counts per minute in primary mixed lymphocyte reaction of day-4 donor PBMCs + host LCLs with and without immunotoxin (IT); % Resid prol, shows residual proliferation calculated as outlined in "Patients, materials, and methods"; NE, not evaluated; and —, not given alemtuzumab.

Table 2. Chimerism, GVHD, infection, and clinical outcome

Dose level and patient no.	% donor at 6 mo	aGVHD	cGVHD	Viral reactivations	Fungal infection	Auto-immunity	Off study, mo after SCT	Current status*
Dose level 1								
P1	100	None	None	CMV/VZV	None	None	—	Alive in CR
P2	100	Grade 4 skin	Ext (skin/liver)	CMV/Adeno	Aspergillus (B/C)	None	8, death from liver failure	Death from liver failure (adeno/GVH)
P3	57 MNCs, 56 grans	None	None	CMV	None	None	8, 2nd SCT for mixed chimerism	Alive in CR after 2nd SCT
P4	97	None	None	None	None	None	6, relapse	Death from disease/ infection/hemorrhage
P5	Relapsed	None	None	None	None	None	5, relapse	Death from disease
P6	100	None	None	CMV/VZV/EBV	None	None	9, relapse	Death from disease/infection
P7	74 MNCs, 66 grans	None	None	CMV/EBV	None	None	5, autologous reconstitution/relapse	Death from disease/infection
P8	0 MNCs, 0 grans	None	Not evaluable	CMV	None	None	3, autologous reconstitution	Alive in CR after 2nd SCT
Dose level 2								
P9	100	Grade 1 skin	None	EBV	None	None	8, relapse	Alive in relapse
P10	Relapsed	None	None	CMV	None	None	5, death from respiratory failure	Death from interstitial pneumonitis
P11	100	Grade 1 gut	None	EBV	None	None	—	Alive in CR
P12	100	Grade 2 skin	Ext (skin/mouth)	EBV	None	None	—	Alive in CR
P13	100	None	None	CMV/EBV/JC	Pulmonary	AIHA	30, death from multiorgan failure	Death from sepsis + multiorgan failure
P14	100	None	None	None	None	None	7, relapse	Death from disease
P15	100	None	None	CMV/Adeno	Pulmonary	None	7, death from encephalopathy	Death from postinfective encephalopathy
P16	100	None	None	None	None	None	8, relapse	Alive in relapse

MNCs indicates mononuclear cells; grans, granulocytes; B/C, blood cultures; and —, completed study.
*As of June 2006.

Control cultures were also set up to assess residual proliferation. After 72 hours, cocultures were allodepleted by overnight treatment with IT as described.[23] Cocultures were washed twice and sampled for bacterial/fungal/*Mycoplasma* sterility and endotoxin, confirmatory tissue-typing, fluorescence-activated cell-sorting (FACS) analysis of the percentage of residual $CD3^+CD25^+$ cells, and residual proliferation against host cells compared with control cultures. Residual proliferation in primary MLRs was calculated according to the following formula: counts per minute (cpm) (donor PBMCs ± host LCLs ± IT) − cpm (donor PBMCs alone ± IT] / cpm (donor PBMCs + host LCLs no IT) − cpm (donor PBMCs alone, no IT).

The remaining allodepleted donor T-cells were cryopreserved using 10% DMSO/10% human albumin in multiple aliquots determined by patient weight and dose level. Release criteria include less than 1% $CD3^+/CD25^+$ cells and less than 10% residual proliferation against host cells. If the patient engrafted successfully and the quality assurance/control (QA/QC) results were appropriate, the cryopreserved, allodepleted T cells were thawed and infused at days 30, 60 and 90 after transplantation.

Most (13 of 16) patients completed their scheduled infusions: the remainder did not because of GVHD (n = 2) or autologous reconstitution (n = 1). Two patients at dose level 2 required only one (patient 9) or 2 (P10) infusions to achieve a CD3 count higher than 1000/μL. The residual percentage of $CD3^+/CD25^+$ cells in the infused cells ranged from 0.01% to 0.27% (median, 0.08%), and the residual proliferation against host cells in the primary MLR ranged from 0% to 3.1% (median, 0.02%).

Measurement of alemtuzumab levels, HAMA, and HARA antibody responses

Plasma alemtuzumab (Schering Health Care, Burgess Hill, United Kingdom) levels were measured in 10 patients at the time of the first infusion of allodepleted donor T cells. Serial dilutions of patient/control plasma or standard dilutions of alemtuzumab were incubated with 6.25×10^5 normal donor phytohemagglutinin (PHA) blasts in 96-well plates on ice for 30 minutes, washed, and then secondary-stained with FITC anti-human IgG (BD Pharmingen, San Diego, CA). CD52 expression was analyzed flow cytometrically and compared with the standard curve. Values of less than 150 ng/mL are equivalent to the background for this assay. Alemtuzumab was undetectable in 6 patients and was detected at low levels in the remaining 4 patients (P1, 6, 8 and 11).

Antibody responses against mouse IgG1 and dgA were assayed in the patients prior to each infusion and at 6 months and 1 year after SCT. Triplicate wells of 96-well plates were coated with either mouse RFB4 or with dgA, washed with phosphate-buffered saline (PBS), blocked with 10% fetal calf serum (FCS), and dilutions of the test serum or a known standard added. The plates were incubated for 6 hours at room temperature and washed, and radiolabeled goat anti–human Ig was added. Plates were incubated for 4 to 6 hours at 4°C and washed. Individual wells were cut out and counted on a gamma counter. Standard curves were plotted and human anti–mouse Ig (HAMA) and human antiricin (HARA) levels in the experimental sera were calculated from the curves.

Monitoring viral reactivation and immune reconstitution

Blood samples from the patients were screened weekly for CMV antigenemia (BCM) or by DNA PCR (GOSH), and patients with significant viremia were treated with ganciclovir. All patients were also screened weekly for EBV viremia using the real-time PCR method of Wagner et al.[31] Screening for adenoviremia was performed when clinically indicated and adenoviremia was treated with intravenous cidofovir.

Immune reconstitution was studied monthly for 9 months and then at a year after SCT by flow cytometric analysis of PBMCs using FITC/PE/PercP-labeled antibodies against CD3, CD4, CD8, CD16, CD19, CD56, CD45RA/RO, and CCR-7 antibodies (Becton Dickinson, San Jose, CA). Proliferative/ATP responses to mitogenic stimulation with PHA were measured using ^{3}H-thymidine uptake and luciferase (Cylex, Columbia, MD) assays.

Antiviral responses were assayed using tetramer and ELISPOT assays. Tetramer analysis was performed on cells from 10 patients with HLA-A2–, HLA-A24–, HLA-B7– or HLA-B8–positive donors. PBMCs (10^6) from the patient after SCT were costained with CD8 FITC, CD3 PerCP, and either isotype PE control antibody or PE-conjugated tetramers (obtained from M.C. or Proimmune [Oxford, United Kingdom]), previously titrated for optimal specific staining. Donor PBMCs and PBMCs from donors with known positive populations served as positive controls, and PBMCs from healthy donors negative for the restricting HLA type were used as additional negative controls. The following tetramers were used: CMV (HLA-A2 NLVPMVATV [pp65], HLA-B7 TPRVTGGGAM [pp65], HLA-B7 RPHERNGFTVL [pp65], and HLA-B8 QIKVRVDMV [IE1]; and EBV (HLA-A2 CLGGLLTMV [LMP2], HLA-A2 GLCTLVAML [BMLF-1], HLA-B7 RPPIFIRRL [EBNA3a], and HLA-B8 RAKFKQLL [BZLF-1]). A total of 200 000 events in the lymphocyte gate were analyzed where possible and the percentage of tetramer-positive cells in the $CD3^+/CD8^+$ lymphocyte gate was expressed as a proportion of the $CD8^+$ cells with the isotype control subtracted. For a population to be labeled as positive, at least 50 $CD3^+CD8^+$ tetramer-positive cells with the staining characteristics of the positive control population had to be acquired.

ELISPOT assays were used to determine the frequency of CMV- and EBV-specific T cells in patient PBMCs producing interferon gamma (IFN-γ) in response to stimulation with autologous donor PBMCs transduced with adenoviral vectors carrying the green fluorescent protein (GFP; Ad5f35GFP) or GFP and CMV pp65 transgenes (Ad5f35pp65GFP) or autologous LCLs, as reported previously.[23] Thawed PBMCs (2×10^5) from the patient at varying time-points after SCT were plated in the presence of 2×10^5 stimulator cells in triplicate wells for 18 to 24 hours at 37°C on MAHA S45 plates (Millipore, Billerica, MA) coated with anti–IFN-γ capture antibody 1 DIK (Mabtech, Mariemont, OH). Controls consisted of 2×10^5 responder cells alone, 2×10^5 stimulator cells alone, and 2×10^5 unirradiated donor PBMCs plus 2×10^5 stimulator cells. Plates were developed and counted as described.[23] The mean number of specific spot-forming cells (SFCs) was calculated by subtracting the mean number of spots from responder cells alone and stimulator cells alone from the mean number of spots in test wells. Responses against CMV pp65 were calculated by subtracting the mean number of spots after stimulation with PBMCs transduced with Ad5f35-GFP (always < 100 SFC/10^6 cells) from the mean number of spots after stimulation with PBMCs transduced with Ad5f35-pp65-GFP.

T-cell receptor signal joint excision circle (TREC) levels were analyzed by real-time quantitative PCR (qPCR) assay. PBMCs were lysed using proteinase K solution and 5 μL duplicate samples were used as a template for qPCR using primers and probes previously described[32] on an ABI PRISM 7000 Sequence Detection (PE Applied Biosystems, Warrington, United Kingdom). For each run, a standard curve was generated from duplicate samples of 5-fold serially diluted known copies of plasmid DNA containing a human TREC fragment. A threshold cycle (Ct) value for each duplicate was calculated by determining the point at which the fluorescence exceeded the threshold limit. We used the mean Ct value of the 2 duplicates plotted against the standard curve to calculate the TREC number in the sample. To normalize for cell equivalents, the β-actin gene was quantified by qPCR. Results were expressed as TREC copies/10^6 cells. T-cell receptor (TCR) spectratypes were analyzed as previously described.[32] Briefly, RNA was extracted and cDNA prepared from frozen PBMCs. A number of variable region (Vβ)–specific primers (24) were used with a fluorescent-labeled constant region (Cβ)–specific primer to reverse transcriptase (RT)–PCR amplify the CDR3 region of the TCR β chain. Products were run on a megaBACE 500 genetic analyser (Amersham Biosciences, Buckingham, United Kingdom) and analyzed using Genetic Profiler software (Amersham Biosciences). Each Vβ family was scored for the number of detectable bands and size distribution to assess the TCR repertoire. A normal spectratype has been shown to consist of 5 to 8 bands per family with a Gaussian size distribution.[33]

Statistical analysis

The proportion of patients achieving normal T- and B-cell numbers at each dose level at given time points was compared using the Fisher exact test. Univariate comparisons of immune reconstitution between the 2 dose levels were performed at each month of follow-up using the Wilcoxon rank-sum nonparametric test. To summarize the overall kinetics of immune reconstitution, the area under the curve (AUC) for each lymphocyte subset was calculated using the trapezoidal rule, and the mean AUC at 4 months and 6 months was compared between dose levels 1 and 2 using the 2 sample *t* test. Multivariate analysis was designed primarily to determine if differences in immune reconstitution observed between dose levels were significant after adjustment for other factors, rather than to determine the significance of these other factors.

Results

GVHD and toxicity

Infusion of allodepleted T cells was associated with a low incidence of toxicity. Two of 16 patients developed significant acute GVHD. P2 developed grade IV skin GVHD after a single infusion of allodepleted donor T cells at dose level 1, and P12 developed grade II skin GVHD after 2 infusions at dose level 2. Both subsequently evolved to extensive chronic GVHD (1 affecting the liver, the other affecting skin and mouth). The patient with chronic GVHD of the liver died of liver failure secondary to adenovirus. Chronic GVHD in the other patient fully resolved, and this patient has been off immunosuppression for 26 months as of June 2006. No other severe adverse effects attributable to the infused allodepleted donor T cells were observed during the study. No patients developed HAMA, and one of 16 (P8) developed a transient HARA.

Immune reconstitution

Thirteen patients with more than 3 months of follow-up at June 2005 were evaluable for immune reconstitution. P8 was excluded because of autologous reconstitution after a single dose of allodepleted donor T cells. Figure 1 shows the kinetics of T-, B-, and natural killer (NK)–cell recovery after SCT in the evaluable patients. T-cell reconstitution was slow in dose-level 1 patients, comparable to that reported in published series without T-cell infusions.[4] In contrast, our data show that T-cell reconstitution in both in the CD4 and CD8 compartments was significantly accelerated in patients receiving allodepleted donor T cells at dose level 2 (Figure 1A-C). Univariate comparison of CD3 levels between dose levels 1 and 2 (Table 3) showed significantly improved T-cell numbers in dose-level 2 patients at 3, 4 and 5 months after SCT ($P = .016$, .017, and .04, respectively). There was a trend toward higher CD4 and CD8 levels in patients at dose level 2 compared with those at dose level 1 (Table 3) at 3, 4, and 5 months after SCT, but the difference only reached significance at 4 months ($P = .017$ and .03, respectively). B- and NK-cell reconstitution was not statistically different between patients at the 2 dose levels (Figure 1D-E). To evaluate the kinetics of T-cell reconstitution over the entire first 4 or 6 months after SCT, AUC analyses were performed. In patients with at least 4 months of follow-up, there was a significantly higher AUC in patients at dose level 2 for $CD3^+$ ($P = .048$), $CD4^+$ ($P = .032$), and $CD8^+$ ($P = .046$) cells than for patients at dose level 1. A significantly higher mean AUC was also observed for CD3 ($P = .034$) and CD4 ($P = .037$) at 6 months after SCT. At 4 months after transplantation, 0 of 6 evaluable patients at dose level 1 had achieved normal T-cell, CD4, or CD8 numbers, whereas the respective figures for dose level 2 were 3 of 6, 2 of 6, and 5 of 6 (Figure 2A). By 6 months after SCT, only one of 5 evaluable patients at dose level 1 had normal T-cell counts, and none had normal CD4 counts. By contrast, all 5 evaluable patients

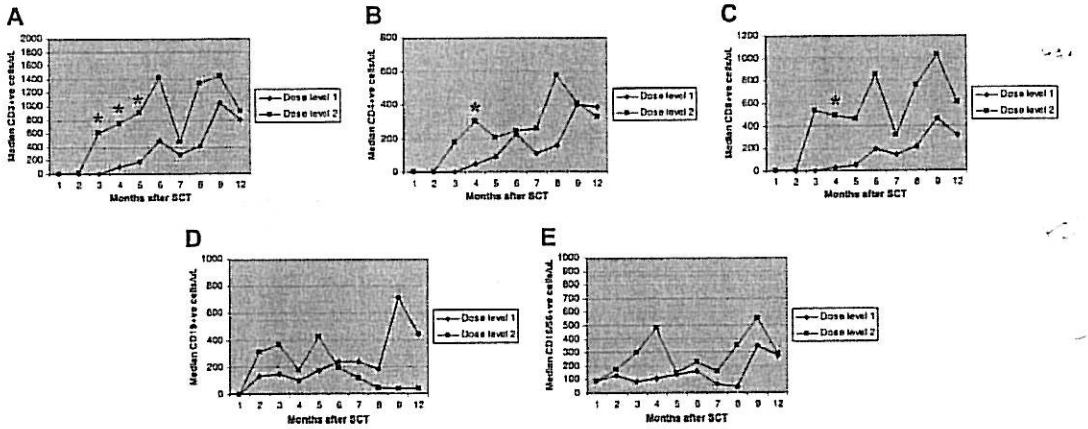

Figure 1. Kinetics of recovery of CD3+, CD4+, and CD8+ T, B, and NK cells after transplantation. Panel A shows CD3+ cells; panel B, CD4+ cells; panel C, CD8+ cells; panel D, B cells; and panel E, NK cells. Median circulating cell counts at each time point are compared between patients treated at dose levels 1 and 2. Time points at which level 2 patients had statistically significant increased counts are indicated by an asterisk.

at dose level 2 had normal T-cell and CD8 counts, and 3 of 5 had normal CD4 counts (Figure 2B). The proportion of patients achieving normal CD8 counts at 4 months and normal CD3 counts at 6 months was significantly greater in patients treated at the higher dose level ($P < .05$).

To assess T-cell function, we assayed responses to PHA. As shown in Figure 2, at 6 months after SCT, 0 of 5 evaluable patients at dose level 1 had achieved normal PHA responses, whereas 2 of 5 patients at dose level 2 had normal responses.

There was no significant difference in recovery of CD3, CD4, or CD8 cells between patients who received nonmyeloablative or conventional intensity conditioning, and patients with detectable alemtuzumab levels at the time of first infusion did not have slower immune reconstitution. Multivariate analysis (Table 4) showed that the accelerated reconstitution of $CD3^+$, $CD4^+$, and $CD8^+$ cells (as assessed by AUC at 4 and 6 months) seen in dose-level 2 patients was independent of whether patients had a malignant or nonmalignant disease, conditioning regimen, or treatment with post-SCT immunosuppression.

Memory phenotype

As shown in Figure 3, most of the T cells recovering in patients at dose level 1 had a naive $CD45RA^+CCR\text{-}7^-$ phenotype. By contrast, the increase in T-cell reconstitution observed in patients at dose level 2 primarily reflects an expansion in T cells with an effector memory ($CD45RA^-CCR\text{-}7^-$) phenotype (Figure 3C). Univariate comparisons between dose levels 1 and 2 showed a significant ($P < .05$) increase in the levels of effector memory (but not central memory or naive) cells in patients at dose level 2 at 3, 4, and 5 months after SCT (Table 3). Similarly, AUC analyses demonstrated a significantly higher mean AUC for effector memory cells in patients treated at dose level 2 at both 4 and 6 months ($P = .022$ and $P = .048$, respectively).

TRECs and spectratyping

TRECs were assayed in 9 patients (5 treated at dose level 1, 4 at dose level 2) at 4 and 6 months after SCT. Six of 9 patients showed undetectable TREC levels at both time points. TRECs were detectable at low levels in P1 (6962 TRECs/10^6 PBMCs at 4 months, 5387 TRECs/10^6 PBMCs at 6 months), P4 (8447 TRECs/10^6 PBMCs at 6 months), and P6 (1472 TRECs/10^6 PBMCs at 6 months), all of whom were treated at dose level 1. The normal range for TRECs in healthy donors is 1200 to 155 000 TRECs/10^6 PBMCs.[34]

Spectratyping of TCR Vβ genes was performed on PBMCs from 9 patients (5 treated at dose level 1, 4 treated at dose level 2).

Table 3. Comparison of $CD3^+$, $CD4^+$, $CD8^+$, and $CD3^+CD45RA^-CCR\text{-}7^-$ T-cell recovery between dose levels by month after SCT

	CD3+					CD4+					CD8+					Effector memory cells				
	Dose level 1		Dose level 2			Dose level 1		Dose level 2			Dose level 1		Dose level 2			Dose level 1		Dose level 2		
Mo	No. pts	Median, cells/μL	No. pts	Median, cells/μL	P	No. pts	Median, cells/μL	No. pts	Median, cells/μL	P	No. pts	Median, cells/μL	No. pts	Median, cells/μL	P	No. pts	Median, cells/μL	No. pts	Median, cells/μL	P
1	7	0	6	4	.215	7	0	6	3.5	.086	7	0	6	6	.113	7	0	5	0	.807
2	7	2	6	19.5	.216	7	0	6	5	.276	7	1	6	5.5	.519	7	0	6	0	.402
3	7	2	6	615.5	.016*	7	1	6	177	.059	7	1	6	533	.065	7	0	6	438	.025*
4	6	111.5	6	747	.017*	6	47	6	303.5	.017*	6	26	6	494.5	.031*	6	0	6	622.5	.016*
5	6	175	6	900	.040*	6	92	6	206.5	.093	6	52.5	6	460.5	.054	6	26.5	6	610	.022*
6	5	488	5	1421	.129	5	226	5	244	.425	5	195	5	850	.178	5	68	5	897	.066
7	4	284.5	4	479	.678	4	110	4	259.5	.346	4	144	4	319.5	.494	4	155.5	4	353	.156
8	4	410.5	2	1342.5	.299	4	158	2	572.5	.299	4	213.5	2	761.5	.518	3	287	2	915	.435
9	3	1047	2	1450	.787	3	399	2	410	.787	3	462	2	1030	>.999	3	10	2	1094.5	.435

Pts indicates patients.
*Statistically significant increases in dose level 2 compared with dose level 1.

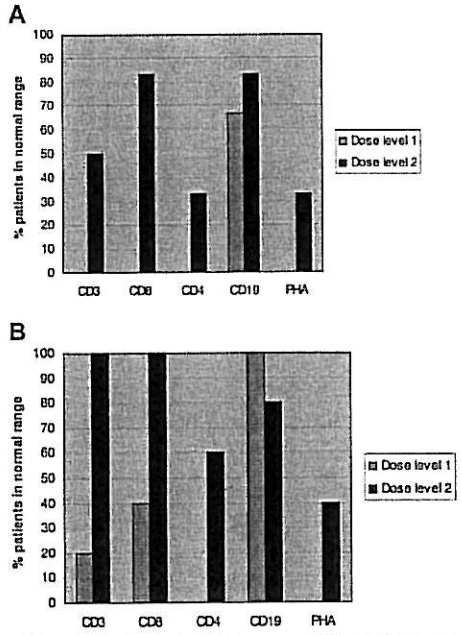

Figure 2. Percentage of patients achieving normal CD3, CD8, CD4, and CD19 counts, and responses to PHA stimulation. Data at 4 months are shown in panel A and 6 months in panel B.

As shown in Figure 4A, the distribution of TCR Vβ receptors at 4 months after SCT in patients at both dose levels was polyclonal with a median of 6.8 peaks (range, 5.3-7.8 peaks), with a shift to a more oligoclonal Vβ repertoire by 6 months (median, 4.2 peaks; range, 3.5-5.9 peaks), and normalization of TCR diversity by 12 months after SCT. The median number of peaks was similar between the 2 dose levels at either 4 or 6 months after SCT. Representative plots from a patient at dose level 2 are shown in Figure 4B.

Viral-specific immunity

In order to study the recovery of viral-specific immunity, we used flow cytometric analysis of $CD3^+CD8^+$ cells from 10 evaluable patients with HLA-A2–, HLA-A24–, HLA-B7–, or HLA-B8–positive donors, at varying time points after SCT, after staining with HLA peptide tetramers. Peripheral blood from the donor premobilization was used as a control. As shown in Figure 5A, none of the 6 evaluable patients at dose level 1 had significant tetramer-positive populations recognizing EBV epitopes up to 9 to 12 months after SCT, despite the fact that 2 had viral reactivation. Tetramer-positive cells were detected in 3 of 4 evaluable patients at dose level 2 at 4 to 6 months after SCT, in each case shortly after viral reactivation. Tetramer-positive cells were directed predominantly against epitopes from lytic (eg, BMLF-1), but also in some case latent (eg, EBNA3a) antigens. Similarly, for CMV (Figure 5B), none of the 6 evaluable patients at dose level 1 had tetramer-positive populations detectable before 9 months after SCT, despite the fact that 5 of these patients had viral reactivations. In contrast, tetramer-positive cells were detected in 2 of 4 evaluable patients at dose level 2 as early as 2 and 4 months after SCT. In P13, this correlated with CMV reactivation, while in P9 no reactivation was apparent. Taking the data from CMV and EBV together, in all but 2 cases, the tetramer-positive cells detected in the patient recognized the same epitopes as those detected in the donor, but there was often significant expansion of the tetramer-positive population in the patient compared with the donor, particularly after viral reactivation. In 2 cases, tetramer-positive cells were observed in the patient that were not detected in the donor. Figure 5C shows an example of both the expansion of preexisting tetramer-positive cells (A2-NLV) and an apparent de novo tetramer-positive population (B8-ELR) directed against CMV antigens in P13, a patient at dose level 2, after CMV reactivation.

To determine whether the antiviral responses we observed were functional, we performed IFN-γ ELISPOT analyses on PBMCs isolated from patients at varying time points after SCT. All 13 patients with more than 3 months follow-up were evaluable. As shown in Figure 6A, 2 of 7 patients at dose level 1 (P6 and P7), both of whom had prior EBV reactivations, showed significant (> 200 cells/10^6 PBMCs) IFN-γ responses at 4 and 6 months after SCT. IFN-γ responses were detected in 4 of 6 patients at dose level 2 and occurred earlier (2-4 months) and were of greater magnitude than those seen in patients at dose level 1. In general, these responses correlated with viral reactivation, but one patient (P12) showed no IFN-γ response despite viral reactivation.

For CMV (Figure 6B), only one of 7 evaluable patients at dose level 1 showed significant IFN-γ secretion in response to CMV pp65 from 6 months after SCT, despite the fact that 5 of these patients had CMV reactivations. In contrast, 3 of 6 evaluable patients at dose level 2 had significant responses that were detectable as early as 2 months after SCT. The correlation between viral reactivation and responses was less consistent than with EBV. One patient at dose level 2 (P10) who had CMV reactivation did not have detectable ELISPOT responses.

Data from tetramer and ELISPOT analyses were largely concordant, although in 3 cases (P3 for CMV, P6 and P7 for EBV) IFN-γ responses were detectable when no tetramer-positive cells were detected, presumably reflecting secretion from either $CD4^+$ cells or $CD8^+$ cells recognizing epitopes other than those on the tetramers.

Infections and outcome

These are summarized in Table 2. Nine patients (of 16 at risk) had CMV reactivation detectable in the peripheral blood. All 9 patients were treated with ganciclovir with or without foscarnet, and none

Table 4. Multivariate analysis of factors influencing AUC for T-cell recovery at 4 and 6 months

	P values					
	CD3		CD8		CD4	
	AUC to 4 mo	AUC to 6 mo	AUC to 4 mo	AUC to 6 mo	AUC to 4 mo	AUC to 6 mo
Dose level 1 vs 2	.002*	.043*	.005*	.035*	.012*	.136
Malignant vs nonmalignant	.311	.569	.165	.249	.771	.821
Conventional-intensity vs nonmyeloablative conditioning	.734	.742	.597	.695	.769	.6
Post-SCT immunosuppression vs none	.497	.528	.509	.409	.821	.774

*Statistically significant.

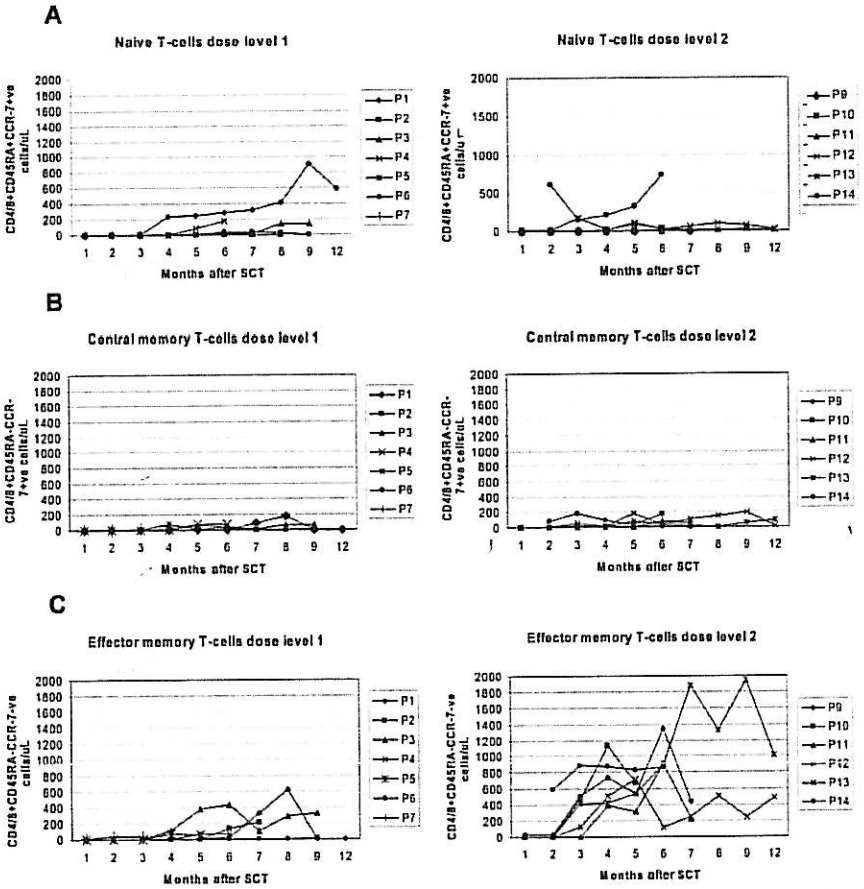

Figure 3. Kinetics of naive, central memory, and effector memory reconstitution after transplantation. Naive cells ($CD45RA^+CCR\text{-}7^+$) are shown in panel A; central memory ($CD45RA^-CCR\text{-}7\text{-}ve$) in panel B; and effector memory ($CD45RA^-CCR\text{-}7^-$) in panel C. Dose-level 1 patients (left column) are compared with dose-level 2 patients (right column).

developed CMV-related disease. Six patients have had EBV reactivations, of whom 1 had a viral load greater than 4000 copies/μg PBMC DNA, which has previously been shown to be predictive of lymphoproliferative disease (LPD).[31] None of these patients were treated preemptively with rituximab, and none developed LPD. Three patients developed proven/probable fungal infections and all resolved on liposomal amphotericin B with or without caspofungin therapy. Two patients developed adenoviremia. In P2 (dose level 1), this progressed to fatal acute liver failure in association with chronic GVHD affecting the liver. P15 had persistent adenoviremia with fever from 2 weeks after SCT, despite multiple courses of cidofovir and ribavirin and 3 doses of allodepleted donor T cells at dose level 2. She was treated off-study with a single dose of 2.5×10^6/kg allodepleted donor T cells on a compassionate basis, with clearance of viremia, but died subsequently from encephalopathy. P13 developed severe progressive multifocal leucoencephalopathy, which progressed despite cidofovir and intravenous immunoglobulin therapy at 2 months after transplantation, at a time when he was profoundly lymphopenic. This patient made a remarkable clinical and radiologic recovery following infusion of allodepleted donor T cells at dose level 2, with full recovery of motor function and continence and marked improvement in cognitive skills, associated with T-cell recovery. He later died from presumed sepsis with multiorgan failure while on immunosuppression for autoimmune hemolysis.

Outcome is shown in Table 2. Overall, at a median follow-up of 33 months, 7 patients have relapsed and 5 are alive and disease free at the time of writing (June 2006).

Discussion

We have demonstrated that adoptive immunotherapy with allodepleted donor T cells improves T-cell reconstitution after haploidentical SCT. T-cell reconstitution in the patients at dose level 1 was slow, comparable with that observed without allodepleted T-cell addback,[4] where normal T-cell numbers were not observed until 9 to 12 months after haploidentical SCT. This was predictable, since the dose of allodepleted cells infused is similar to the numbers of T cells infused with the graft, and thus, these patients form an in-study control cohort for assessing the impact of infusing higher doses of allodepleted cells. In contrast, patients at dose level 2 exhibited significantly more rapid recovery of T cells, particularly at 3 to 5 months after SCT, which is frequently the time period at which patients succumb to infection after haploidentical SCT. CMV, EBV, and pneumocystis rarely cause disease in SCT

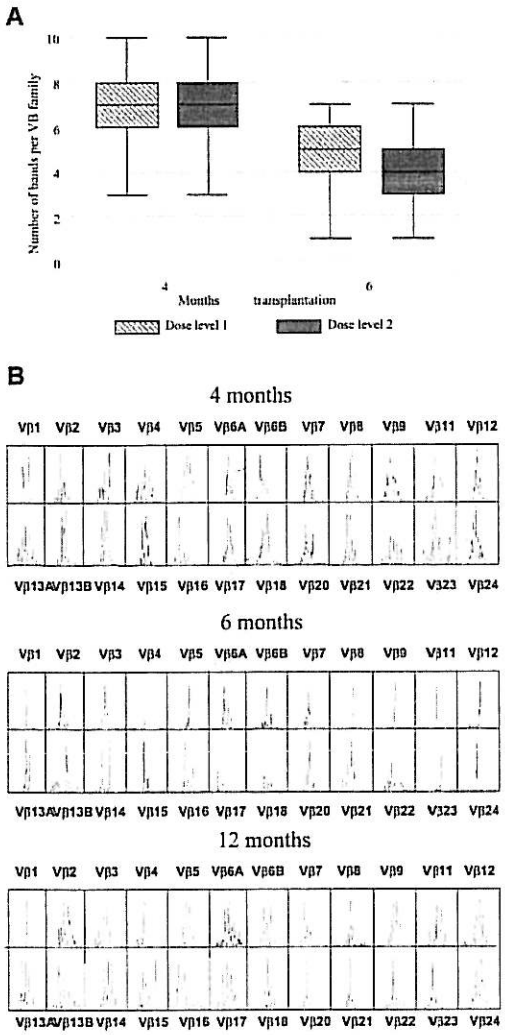

Figure 4. T-cell receptor spectratyping. (A) Boxplot of number of bands per Vβ family (1-24) at 4 and 6 months after SCT for evaluable dose levels 1 and 2 patients. Line indicates mean; box indicates 25th to 75th centile; and error bars indicate 90th and 10th percentile. (B) Representative plots of TCR Vβ repertoire in P11 (dose level 2) at 4, 6, and 12 months after SCT.

recipients when the CD4 count is greater than 300/μL, and some groups use this as a threshold to stop cotrimoxazole prophylaxis and monitoring of viremia. The median time to reach this threshold was 4 months in patients at dose level 2, compared with more than 6 months in patients at dose level 1 and 8 months in the series of Eyrich et al[4] without allodepleted T-cell addback. Further, most T cells that did recover in patients at dose level 2 exhibited an effector memory phenotype, implying that protective T-cell responses are likely to be long lived. While it is possible that naive T cells may also have shifted to an effector memory phenotype, our finding that none of the 4 dose-level 2 patients tested had detectable circulating TRECs at the time of T-cell recovery argues that naive T cells derived from the stem cell graft are unlikely to have contributed to the improved immune reconstitution seen in these patients. These data strongly suggest that the accelerated T-cell recovery seen in dose-level 2 patients is due to the infused allodepleted donor T cells.

Multivariate analysis demonstrated that the improved T-cell reconstitution in dose-level 2 patients was independent of intensity of conditioning, malignant diagnosis, and post-SCT immunosuppression. Since most of our patients were children, our findings are primarily relevant to pediatric patients and further studies will be needed to determine the effect of recipient age. In view of progressive thymic involution with age, it is critical that strategies to improve T-cell immunity in the early post-SCT period are independent of thymic maturation. Our data on the absence of TRECs in dose-level 2 patients with accelerated T-cell recovery support such a thymus-independent mechanism for immune reconstitution, but this will be an important issue to confirm in adults.

Previous studies have shown 2 pathways that contribute to reconstitution of the T-cell compartment. In the initial months after SCT, the T-cell repertoire depends on peripheral expansion of mature T cells in the graft, due to thymic damage associated with conditioning.[35] Because of the rigorous T-cell depletion of the graft in haploidentical SCT, the T-cell repertoire is very restricted, with severe skewing of T-cell–receptor complexity for the first 6 to 9 months after SCT.[4] Subsequently, de novo maturation of naive T cells derived from bone marrow emigrants passaged through the thymus has been shown to occur later than 6 months after SCT and may play a part in normalization of the T-cell repertoire.[36] Our TREC data demonstrate that new thymic emigrants play a limited role in T-cell reconstitution for the first 6 months after SCT in most of our patients and are consistent with published data on heavily pretreated patients.[34] Our spectratyping data show a significantly more polyclonal distribution of Vβ receptor gene usage at 4 months after SCT in patients at both dose levels than has been reported in published series of pediatric haploidentical SCT without allodepleted T-cell addback. In the study by Eyrich et al[4] in a similar pediatric patient cohort, all patients had a markedly skewed repertoire with a median of 3 to 4 bands per Vβ family for the first 6 months after SCT, whereas our patients had a median of 7 bands per Vβ family at 4 months. This may reflect the polyclonal pattern of Vβ usage in the infused T cells. The absence of viral-specific responses in dose-level 1 patients despite polyclonal Vβ usage may be due to the higher sensitivity of the PCR-based spectratyping assay compared with tetramer/ELISPOT analyses. In most patients, the spectratyping pattern became more oligoclonal at 6 months, and this may reflect preferential expansion of T-cell clones that have been stimulated by their cognate antigens, with subsequent normalization of TCR diversity by 12 months after SCT.

Similarly, published data on pathogen-specific responses (eg, against fungal antigens) suggest these are generally absent until 9 months after SCT.[37] Our data on T-cell responses to CMV and EBV in patients at dose level 1 are in line with these findings. In contrast, we have observed accelerated recovery of CMV- and EBV-specific immunity in patients treated at the higher dose level, using both flow cytometric and functional assays. We have previously shown[23] that EBV-specific responses are partially retained in the allodepleted donor T-cell product, despite using LCLs as stimulators, through recognition of EBV epitopes presented on the non-shared HLA haplotype. Antiviral responses were observed as early as 2 to 4 months after SCT in these patients (ie, after a single infusion in some cases), particularly after viral reactivation. This coincides with the period during which patients are at maximum risk of viral infections after haploidentical SCT and is remarkable because such responses are not seen until 6 to 12 months after SCT even after the

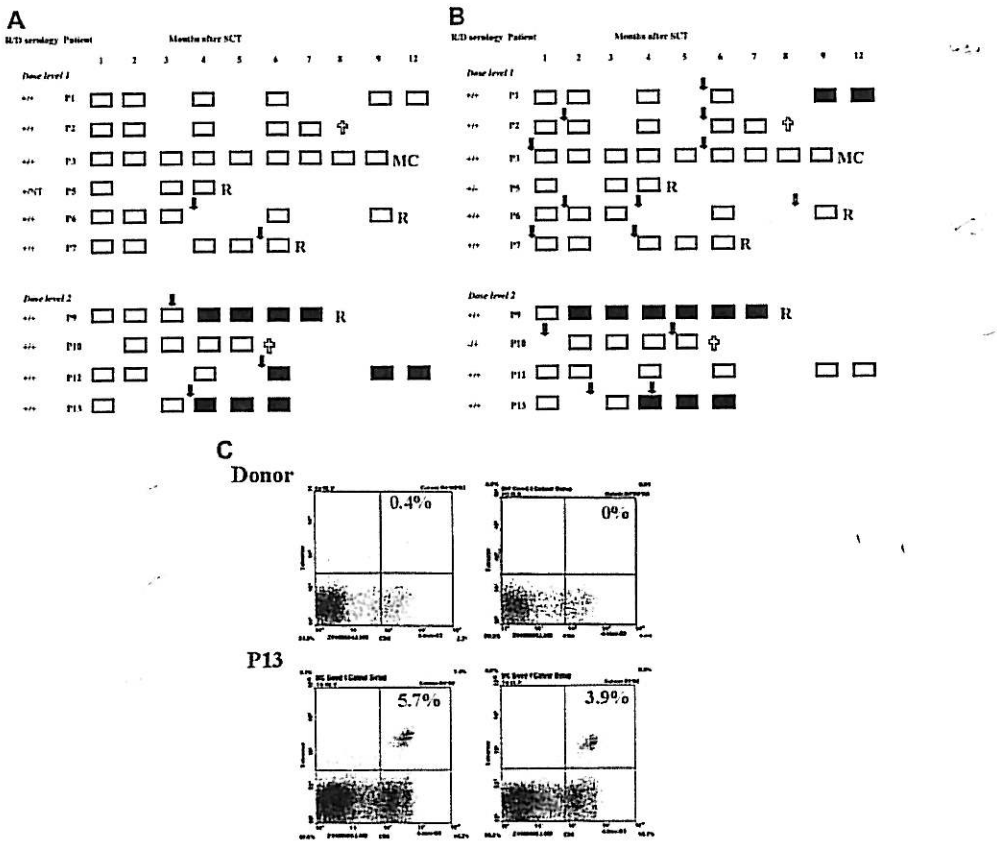

Figure 5. Recovery of CD8 responses to viruses. (A) Recovery of CD8 responses against EBV. ■ represents time points at which a significant (> 0.1% above isotype) tetramer-positive population was identified in the peripheral blood of recipients; □, time points at which no tetramer-positive cells were observed. The arrows indicate EBV viremia. Time points at which patient went off study for relapse (R) or mixed chimerism (MC) are shown. Crosses indicate time points at which patients died. (B) CD8 responses against CMV. Schema as in panel A. (C) Flow cytometric analysis of peripheral blood from patient P13 at 6 months after transplantation (bottom 2 panels) and his donor (top 2 panels) using CD8-FITC/tetramer-PE staining. The left panels show samples stained with the HLA-A2-NLV tetramer (pp65), and the right panels show samples stained with the HLA-B8-ELR tetramer (IE1). The percentage of $CD3^+8^+$ T cells that were tetramer positive is shown.

less rigorous T-cell depletion used in nonmyeloablative unrelated donor SCT.[38]

Our study was not designed with sufficient power to demonstrate clinical efficacy. While infections and viral reactivations were frequent, the overall incidence of directly infectious deaths was low (2 of 16). In conjunction with our data on viral-specific immune responses, this suggests that while the number of cells infused may be insufficient to prevent such reactivations, they may have played a role in preventing progression to disease/death. Taken together, our data suggest that adoptive immunotherapy with allodepleted donor T cells at doses of 1 to 3×10^5/kg is sufficient to improve T-cell reconstitution and antiviral immunity after haploidentical SCT. This approach may potentially reduce infection-associated mortality and thereby substantially broaden the applicability of haploidentical SCT. Clearly, however, larger studies will be needed to demonstrate if such an approach confers a real therapeutic benefit.

Comparison of the numbers of circulating T cells in patients treated at the higher dose level and the number of cells infused implies that allodepleted donor T cells are able to expand significantly in vivo, particularly in the face of viral reactivation. This is similar to what has been observed after adoptive transfer of EBV- and CMV-specific cytotoxic T cells in patients undergoing T-cell–depleted SCT,[39,40] and may relate to the profound lymphopenia after haploidentical SCT. There is growing evidence for lymphoid homeostatic mechanisms,[41,42] which in the lymphopenic environment favors rapid repopulation of the peripheral T-cell compartment through expansion of relatively small numbers of infused memory T cells. In both murine models and a human study, lymphodepletion may have a marked effect on the efficacy of adoptive T-cell transfer.[43,44] Such homeostatic mechanisms may explain why the infusion of allodepleted donor T cells at doses as low as 3×10^5/kg may be sufficient to confer significant viral-specific immunity in the context of the profoundly lymphodepleted host after haploidentical SCT.

Our study confirms the safety of adoptive immunotherapy with allodepleted donor T cells in haploidentical stem cell transplant recipients. We have observed a low incidence of GVHD at both dose levels, comparable to that seen without the addback of allodepleted donor T cells.[1,5] In the case of P12, the dose of allodepleted donor T cells was substantially higher than the T-cell dose infused with the graft, so it is likely the former contributed to GVHD, but in P2, cell doses were similar, so it is not clear whether the allodepleted donor T cells were responsible for GVHD in this

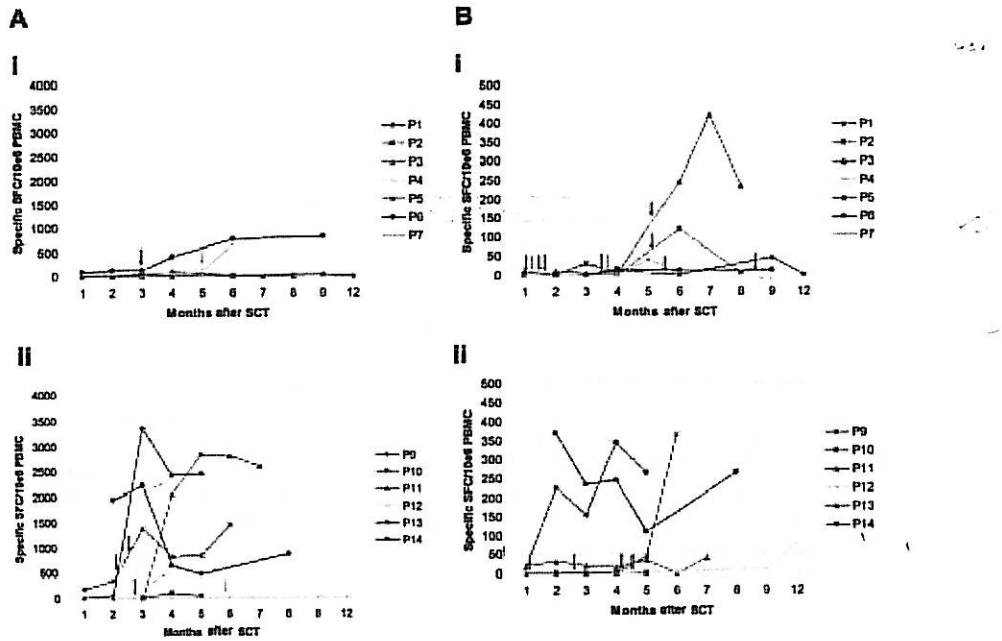

Figure 6. Recovery of functional antiviral responses. (A) Functional response to EBV. The number of cells secreting interferon-γ in response to stimulation with donor EBV-LCL in ELISPOT assays are shown at varying time points after SCT. Dose-level 1 patients are shown in the top panel and level 2 patients in the bottom panel. (B) Functional response to CMV. The number of cells secreting interferon-γ in response to stimulation with donor PBMCs expressing a pp65 transgene in ELISPOT assays are shown at varying time points after SCT. Patients at dose-level 1 are shown in panel I and dose-level 2 patients in panel II. Viral reactivations are indicated by the arrows.

patient. Previous studies have observed a high incidence of acute GVHD after infusion of unmanipulated donor lymphocyte infusions at 10^5/kg within the first 3 months of SCT, even in the HLA-matched setting.[4,36] The low incidence of acute and chronic GVHD observed in our patients treated at dose level 2 demonstrates that our strategy effectively depletes clinically relevant alloreactive cells. Further, the depletion of $CD4^+CD25^+$ regulatory T cells from the infused cells does not appear to enhance the potential for GVHD, presumably reflecting the absence of significant numbers of alloreactive effector cells. Similarly, we have not observed an excess of post-SCT autoimmune phenomena.

In the study by Andre-Schmutz et al,[25] the 4 patients who developed acute GVHD were those with high residual proliferative responses to host cells. Our preclinical data have demonstrated the importance of strong activation of alloreactive cells for this strategy to work reproducibly, and we believe the choice of host antigen-presenting cell is critical in this regard.[23] We have demonstrated that allodepletion with this IT following stimulation with HLA-mismatched LCLs may be more consistently effective at removing alloreactive cells than following stimulation with PBMCs,[23] so we used recipient LCL as stimulators in our clinical study. Using our approach, we did not observe a correlation between residual proliferation in the primary MLR or residual $CD3^+/CD25^+$ cells in the infused product and the development of GVHD.

While, as noted, our study was not designed to demonstrate clinical efficacy, given the accelerated T-cell recovery in dose-level 2 patients, disease-free survival was disappointing (only 5 of 16 patients), with relapse being the major cause of treatment failure. Although preclinical data from our group and others[12,23] suggest that antileukemic responses may be preserved after allodepletion, relapse clearly remains a major problem in our cohort (7 of 16 patients). It is thus evident that in patients with high-risk malignancies, the benefits of this approach in improved immune responses to infection may be offset by leukemic relapse in survivors. Extension of this approach to demonstrate antileukemic responses will require larger, randomized studies, and is likely to need larger doses of allodepleted donor T cells than are required for reconstitution of antiviral responses.[27,28,45]

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Add-back of allodepleted donor T cells to improve immune reconstitution after haplo-identical stem cell transplantation

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Poor immune reconstitution after haplo-identical stem cell transplantation results in high mortality from viral infections and relapse. One approach to overcome this problem is to deplete alloreactive cells selectively by deleting T cells activated by recipient stimulators, using an immunotoxin directed against the activation marker CD25. However, the degree of depletion of alloreactive cells is variable following stimulation with recipient PBMC, and this can result in GvHD. We have previously shown that using recipient EBV-transformed LCL as stimulators to activate donor alloreactive T cells results in more consistent depletion of in vitro *alloreactivity while preserving T-cell responses to viral and potential myeloid tumor Ag. Based on these data, we have embarked on a phase I clinical dose escalation study of add-back of allo-LCL-depleted donor T cells in the haplo-identical setting, to determine if the allodepletion we achieve is sufficient to allow infusion of sufficient T cells to restore useful antiviral/anti-leukemic responses without causing GvHD. Fifteen patients have so far been treated. The incidence of significant acute or chronic GvHD has been low (2/15), as has mortality from infection (1/15). Preliminary data show accelerated immune reconstitution in dose level 2 patients. Infused allodepleted donor T cells appear able to expand significantly in the face of viral reactivations, and doses as low as 3×10^5/kg may be sufficient to confer useful antiviral immunity in this setting. At a median follow-up of 19.5 months, nine of 15 patients are alive and disease-free. Five patients have relapsed, all of whom have died. Strategies to augment the degree of depletion of alloreactive cells will probably be necessary to enable adoptive transfer of enough T cells for useful anti-leukemic activity.*

Keywords

Haplo-identical, allodepletion, immune reconstitution, adaptive immunotherapy.

Introduction

The lack of fully HLA-matched donors is a major limitation to the applicability of hematopoietic stem cell transplantation (HSCT) to patients with leukemia, aplastic anemia and inborn errors of metabolism. However, almost all patients have potential donors who are HLA haplo-identical. Recent advances in our ability to mobilize and select HSC have made HSCT from haplo-identical donors feasible, both in terms of reliable engraftment and acceptable rates of GvHD [1,2].

The rigorous T-cell depletion necessary to prevent GvHD in the haplo-identical setting results in profound post-transplant immunodeficiency [3,4]. This is the major barrier to the broader application of haplo-identical HSCT, and results in high morbidity/mortality from viral infections because of the loss of antiviral immunity and high relapse rates as a result of the loss of the GvL response [1,2]. Simple T-cell add-back is unlikely to be effective in preventing these problems because the frequency of alloreactive T cells is far higher than that of either antiviral or anti-leukemic T cells.

Two major approaches have evolved to circumvent this difficulty. Induction of anergy in alloreactive donor T cells can be achieved by co-culture with host PBMC in the

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presence of CTLA4-Ig [5,6]. However, the anergized cells may have an inhibitory effect on bystander T cells and anergy may be overcome in the context of high levels of IL-2. An alternative is to deplete the graft specifically of the alloreactive T cells responsible for graft-versus-host reactions. This can be accomplished by deleting T cells that become activated in response to recipient APC. This approach has the advantage that alloreactive T cells are permanently eliminated and cannot influence the function of the remaining T cells. As discussed elsewhere in this issue of *Cytotherapy*, the Necker group has targeted activated T cells expressing the activation marker CD25 (IL-2 receptor) using an immunotoxin (RFT5-SMPT-dgA) consisting of a murine Ab moiety recognizing the IL-2 receptor p55 chain conjugated to deglycosylated ricin A. They have used this approach in a phase I/II clinical study of add-back of allodepleted donor T cells in 15 patients undergoing HLA-mismatched stem cell transplantation [7,8].

One major limitation of this approach in patients with leukemia and BM failure concerns the source of recipient stimulator cells. For patients who are aplastic as a result of disease or chemotherapy, it may be difficult to obtain sufficient PBMC for this approach. Further, crude PBMC preparations are relatively ineffective APC. In the clinical study of Andre-Schmutz *et al.* [8], the presence of a residual proliferative response to recipient PBMC was strongly associated with subsequent development of GvHD. Moreover, in leukemic patients PBMC may be contaminated with leukemic blasts or DC that have processed leukemic Ag, so that allodepletion results in the loss of anti-leukemic activity.

We have used recipient EBV-transformed lymphoblastoid cell lines (LCL) as stimulators to activate alloreactive T cells. LCL are excellent APC and therefore are likely to be more effective than bulk PBMC in activating alloreactive T cells. Additionally, LCL are relatively cheap to prepare, easily expanded to large numbers and have a standard phenotype, with less variability in expression of immunostimulatory molecules than PBMC. Finally, unlike crude PBMC, LCL do not express myeloid or tumor Ag that may serve as targets for the GvL effect. Hence the allodepletion of donor T cells after stimulation with recipient LCL should allow them to retain their anti-leukemic activity, particularly in myeloid malignancies. We have developed a protocol for allodepletion using an anti-CD25 immunotoxin after stimulation of donor cells with HLA-mismatched LCL. We have evaluated residual alloreactivity, antiviral and anti-tumor activity *ex vivo* and subsequently assessed the function of these cells in 15 patients *in vivo*.

Pre-clinical studies

Immunotoxin

RFT5-SMPT-dgA is an immunotoxin generated by cross-linking a murine anti-CD25 MAb (IgG1) with a chemically deglycosylated ricin α chain (dgA) using a N-succinimidyloxycarbonyl-α-methyl-2 pyridyldithiol toluene (SMPT) linker according to published methods [9]. Clinical grade immunotoxin was prepared in the GMP laboratory of Prof. E. Vitetta (University of Texas Southwestern Medical Center, Dallas, TX, USA) and has been approved for human use by the FDA in the United States.

Generation of allodepleted donor T cells

Allodepleted donor T cells were generated as outlined previously [10]. Normal donor PBMC and HLA-mismatched or haplo-identical recipient LCL that had received 70 Gy γ-irradiation were each diluted to 2×10^6/mL in AIM V serum-free medium (Invitrogen, Carlshad, CA, USA). Donor PBMC were then co-cultured with or without irradiated recipient LCL at a ratio of 40:1 in T-75 or T-175 flasks for 3 days. After 72 h co-cultures were harvested and resuspended at 10^7/mL in immunodepletion medium consisting of AIM V supplemented with 20 mm ammonium chloride (Sigma, St Louis, MO, USA) to improve the bioactivity of the immunotoxin, with pH adjusted to 7.75 using sodium HEPES (Sigma). Donor PBMC alone and donor PBMC+recipient LCL co-cultures were each split into two equal aliquots, and 0.22 μm filtered RFT5-SMPT-dgA anti-CD25 immunotoxin added to one aliquot at a final concentration of 3 μg/mL. Co-cultures in the presence and absence of immunotoxin were incubated overnight at 37°. The next morning co-cultures were washed twice in 10 mL AIM V medium and resuspended at 2×10^6/mL in AIM V. From each day 4 co-culture, 100 μl (2×10^5 cells) was sampled in triplicate for primary MLR, and 300 μl for FACS analysis. For secondary MLR, culture of CTL and ELISPOT assays the remaining cells were rested in 24-well plates at 2×10^6/well for 3 days prior to secondary stimulation.

Optimization of the allodepletion procedure following stimulation with recipient LCL

As a prelude to subsequent clinical studies we optimized a variety of parameters in our allodepletion protocol using recipient LCL as stimulators. We initially studied the effect of varying the ratio of donor PBMC:recipient LCL in the HLA-mismatched setting. When increasing numbers of recipient LCL were used to stimulate a fixed number of donor PBMC for 4 days, the percentage of residual $CD3^+$ $CD25^+$ T cells after treatment with anti-CD25 immunotoxin was higher with increasing numbers of recipient LCL. Likewise, we observed that residual proliferation in primary MLR was higher with increasing numbers of recipient LCL. Based on these results, a donor PBMC:recipient LCL ratio of 40:1 was used in subsequent experiments.

We next studied the effects of varying concentrations of anti-CD25 immunotoxin during allodepletion. In order to determine the effect of increasing immunotoxin concentration on bystander T cells, we assayed proliferative responses of co-cultures following allodepletion with varying immunotoxin doses to secondary stimulation with either the original recipient LCL or HLA-mismatched third-party LCL. At immunotoxin doses between 2 and 3.5 μg/mL, the secondary response to the recipient LCL was low (approximately 10% of untreated co-cultures) and did not change with increasing immunotoxin concentration. In contrast, the proliferative response to third-party LCL was preserved at immunotoxin concentrations between 2 and 3 μg/mL, but decreased at higher concentrations, suggesting that at concentrations above 3 μg/mL non-specific inhibition of the proliferation of bystander T cells was occurring. In view of this, an immunotoxin concentration of 3 μg/mL was used in further studies.

Additionally, we determined the optimum time of stimulation with recipient LCL before immunodepletion. Sequential FACS analysis demonstrated that, while the percentage of $CD3^+$ $CD25^+$ cells in co-cultures increased with time, peak levels of CD25 expression occurred between 3 and 4 days of co-culture. To avoid depletion of bystander T cells, we therefore performed allodepletion at this time.

To adapt our allodepletion protocol for a clinical trial, we scaled-up our experiments using serum-free medium and clinical grade reagents. The residual percentage of $CD3^+$ $CD25^+$ cells and residual proliferation following allodepletion in serum-free AIM V medium was equivalent to, or less than, that obtained in medium containing FCS. Assuming a 70-kg recipient, multiple doses of 10^5/kg or 10^6/kg allodepleted donor T cells could routinely be generated from 150 mL or 500 mL donor peripheral blood.

Comparison of allodepletion following stimulation with HLA-mismatched PBMC and LCL

We initially compared T-cell activation and the efficacy of depletion of alloreactive donor T cells following stimulation with HLA-mismatched recipient PBMC or LCL. Normal donor PBMC were stimulated for 4 days with irradiated HLA-mismatched PBMC at a ratio of 1:1, or LCL from the same recipients at a ratio of 40:1. To compensate for the effect of overnight culture in the immunodepletion medium, we included controls that were mock-treated with immunodepletion medium but in the absence of immunotoxin, standardizing the results by calculating the residual proliferation according to the formula:

$$\frac{\text{cpm [donor PBMC + host LCL + immunotoxin]} - \text{cpm [donor PBMC alone + immunotoxin]}}{\text{cpm[donor PBMC + host LCL no immunotoxin]} - \text{cpm[donor PBMC alone no immunotoxin]}}$$

As illustrated in Table 1, the residual proliferation after treatment of these co-cultures with anti-CD25 immunotoxin was more variable after stimulation with HLA-mismatched recipient PBMC (mean $8.5 \pm 11.3\%$). In contrast, the residual proliferation after stimulation with LCL from the same recipients was lower and consistently $< 5\%$ (mean $0.8 \pm 1.8\%$), and was statistically significantly lower than after stimulation with PBMC from the same donors ($P < 0.05$). Thus allodepletion with anti-CD25 immunotoxin following stimulation with host LCL appeared more consistently effective in depleting *in vitro* alloreactivity than after stimulation with host PBMC.

Antiviral T-cell responses are preserved following allodepletion

To determine the specificity of allodepletion, we studied whether antiviral T-cell responses were retained following immunotoxin treatment. PBMC from four $HLA\text{-}A2^+$

Table 1. Comparison of allodepletion following stimulation with HLA-mismatched PBMC or LCL. Residual proliferation in seven donor–patient pairs after allodepletion with anti-CD25 immunotoxin following stimulation of donor PBMC with PBMC (R:S 1:1) or LCL (R:S 40:1) from the same HLA-mismatched recipient. Residual proliferation was calculated using the formula in the text, and was significantly higher after stimulation with PBMC ($P < 0.05$)

	Responder							
Stimulator	1	2	3	4	5	6	7	Mean ± SD
Allo-PBMC	0	14.8	0	27.4	0	17.4	0	8.5 ± 11.3
Allo-LCL	4.8	0	0.7	0	0	0	0	0.8 ± 1.8

donors known to have significant populations of CMV-specific $CD8^+$ cells detectable by HLA-A2-pp65 peptide tetramers were co-cultured with HLA-mismatched (HLA-$A2^-$) LCL for 3 days and then treated overnight with anti-CD25 immunotoxin. As shown in Figure 1, there was no significant difference in the frequency of HLA-A2-pp65 specific $CD8^+$ T cells in allodepleted donor T-cell cultures and unmanipulated PBMC. These results suggested that virus-specific T cells are retained following allodepletion. To study the functionality of antiviral T cells, we then performed ELISPOT analyses to determine the frequency of T cells secreting IFN-γ in response to adenoviral Ag and CMV pp65. Unmanipulated or allodepleted donor T cells were stimulated with irradiated autologous PBMC transduced with Vaccinia vectors carrying the GFP (Vacc-GFP) or CMVpp65 (Vacc-pp65) transgenes or with adenoviral vector carrying the GFP transgene (Ad5f35-GFP) or GFP and CMVpp65 (Ad5f35-pp65-GFP). As can be seen in Figure 2, in five different CMV-seropositive donors, there was no statistically significant difference in frequency of IFN-γ-secreting cells after stimulation with Ad5f35-GFP- (Figure 2a, $P = 0.22$) or Vacc-pp65- (Figure 2b, $P = 0.95$) transduced PBMC in allodepleted T-cell co-cultures and unmanipulated PBMC, implying that allodepletion does not affect the function of adenovirus- or CMV-specific T cells. Similarly, when responder cells were stimulated with Ag from both adenovirus and CMV, using the Ad5f35pp65GFP vector, the frequency of IFN-γ-secreting cells was equivalent in allodepleted donor T cells and unmanipulated PBMC (Figure 2c, $P = 0.37$).

One potential concern about the use of donor T cells allodepleted after stimulation with recipient LCL is that, in the haplo-identical setting, T-cell responses to EBV would be abrogated, thereby increasing the risk of lymphoproliferative disease after transplantation. We reasoned T cells recognizing EBV Ag in the context of HLA molecules from the non-shared donor haplotype should not be activated in response to recipient LCL. Consequently, significant anti-EBV activity to peptides presented by the non-shared ('non-depleting') haplotype should be retained following allodepletion. To investigate this, we stimulated PBMC from EBV-seropositive donors with haplo-identical recipient LCL for 3 days followed by allodepletion with anti-CD25 immunotoxin. We then used ELISPOT assays to determine the frequency of IFN-γ-secreting cells after secondary stimulation with autologous donor LCL to assess anti-EBV responses. As shown in Figure 3, the frequency of cells secreting IFN-γ after stimulation with autologous LCL in allodepleted donor T-cell cultures was partially retained in all three haplo-identical donor–patient pairs, demonstrating that significant anti-EBV responses persist following allodepletion. Depletion with immunotoxin after stimulation with autologous LCL resulted in a much more marked loss of T cells secreting IFN-γ in response to EBV Ag than observed in the haplo-identical setting, suggesting that the residual responses to EBV following allodepletion are likely to be mediated through recognition of EBV Ag in the context of the non-shared haplotype.

T cells recognizing myeloid tumor Ag are retained following allodepletion

We then investigated whether allodepletion after stimulation with recipient LCL affected the response to candidate tumor Ag in myeloid malignancies. As our model, we studied T cells recognizing the PR1 epitope from proteinase 3, which have been shown to have potent cytolytic activity against primary leukemic cells in CML and AML and to inhibit preferentially growth of leukemic

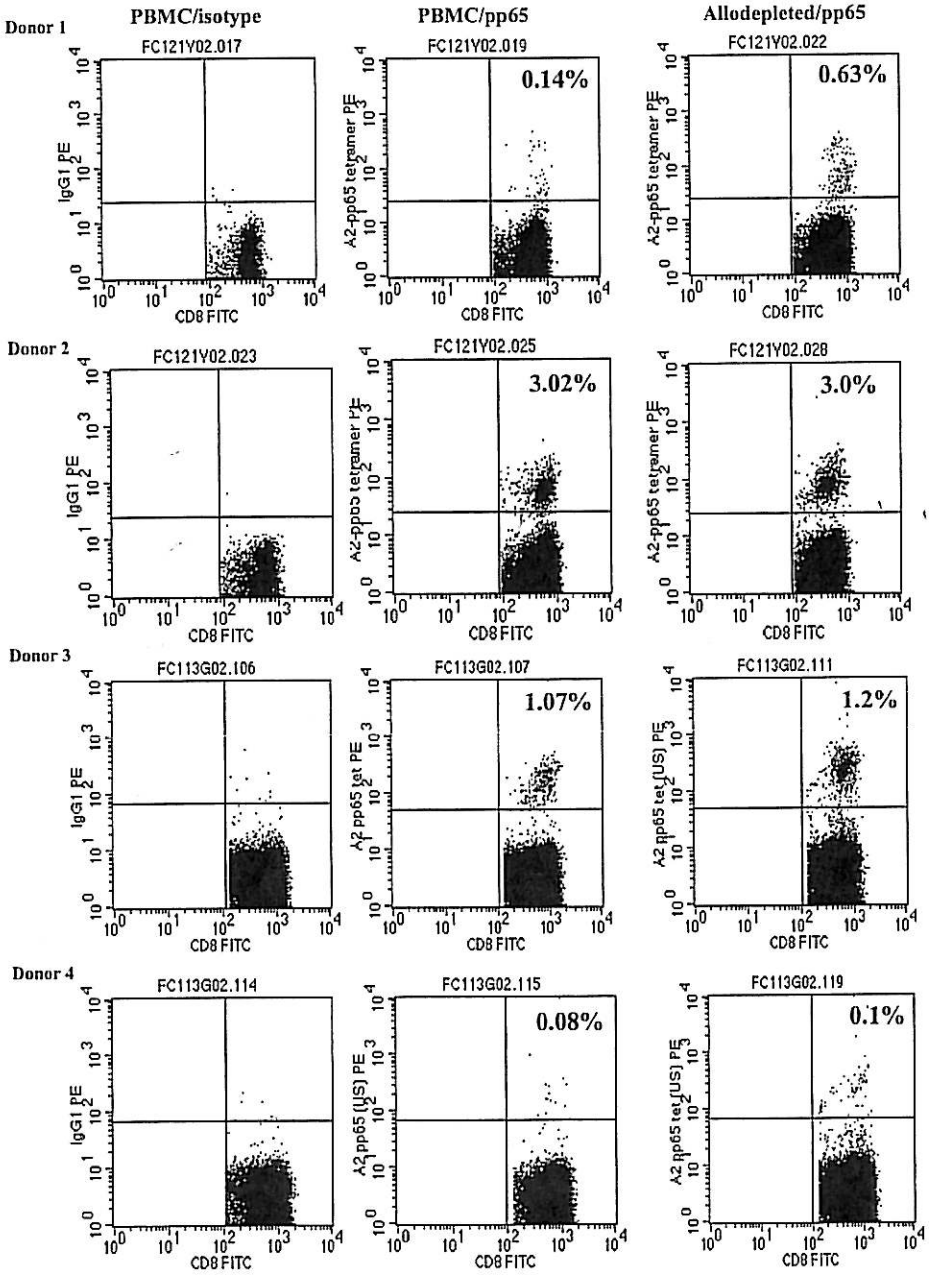

Figure 1. CMV-specific CD8$^+$ T cells are not deleted by allodepletion. The figure shows FACS analysis following staining of either unmanipulated PBMC (left and central panels) or allodepleted cells (right) from four HLA-A2$^+$, CMV-seropositive donors with IgG–PE (left) or an HLA-A2-CMV pp65 tetramer (central and left panels). The percentages of tetramer-positive cells as a proportion of CD8$^+$ cells with isotype subtracted is shown.

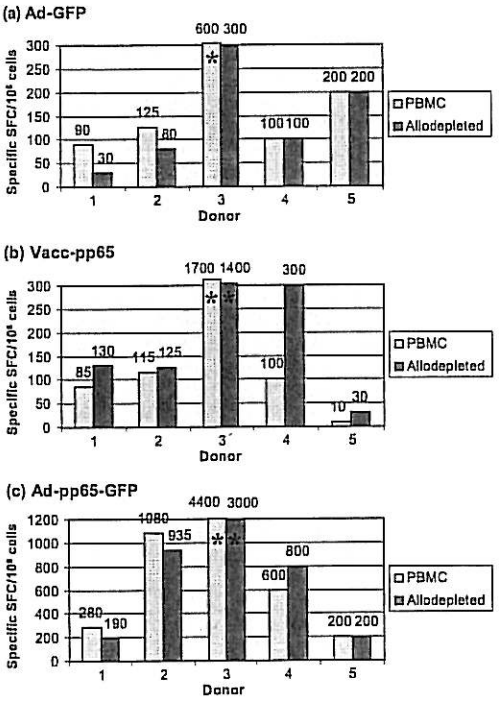

*Figure 2. T-cell responses to adenoviral and CMV Ag are preserved after allodepletion. The figure shows the frequency of cells secreting IFN-γ as determined by ELISPOT assays. Unmanipulated PBMC from five different seropositive donors or donor PBMC allodepleted after stimulation with HLA-mismatched LCL were stimulated with irradiated autologous PBMC transduced with an adenoviral vector carrying the GFP gene (upper panel), Vaccinia vectors carrying the GFP gene with (middle panel) or without (not shown) the CMV pp65 gene, or an adenoviral vector carrying the CMV pp65 and GFP genes (lower panel). *The result falls above the axis limit and the result is shown numerically above the column. Results are the mean number of specific spot-forming cells calculated by linear regression of duplicate wells assayed at three dilutions.*

CFU-GM from patients with CML [11,12]. Using HLA-A2-PR1 tetramer analysis in transplanted patients with CML known to have detectable circulating PR1-specific CTL, we compared the frequency of T cells recognizing the PR1 epitope in unmanipulated PBMC and after stimulation with HLA-mismatched stimulators and allo-depletion with anti-CD25 immunotoxin. We demonstrated that, after stimulation with allogeneic, mismatched HLA-$A2^+$ CML PBMC, allodepletion resulted in the complete loss of PR1-specific T cells. In contrast, when LCL were

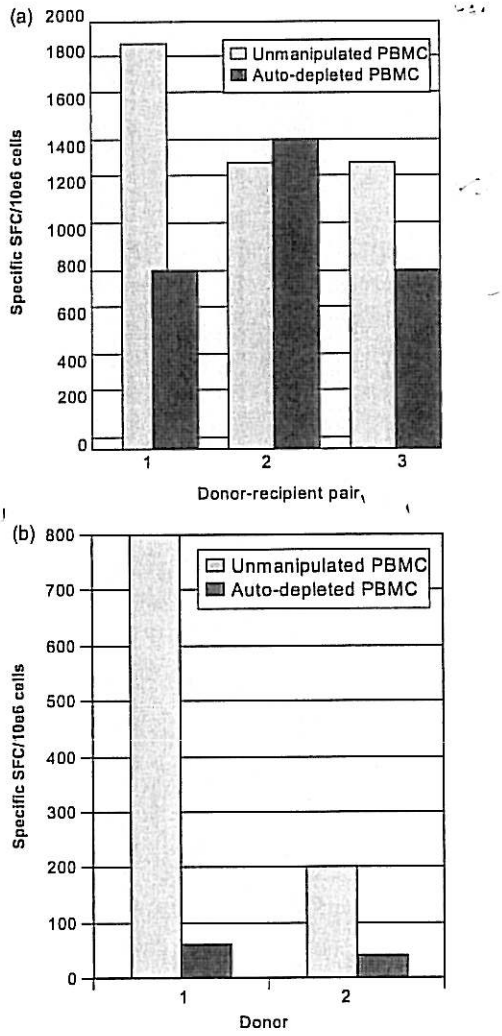

Figure 3. T-cell responses to EBV are partially preserved following allodepletion with haplo-identical LCL. The figure shows the frequency of cells secreting IFN-γ as determined by ELISPOT assays. (a) Unmanipulated donor PBMC and donor PBMC allodepleted after stimulation with recipient LCL from three different haplo-identical donor–recipient pairs were stimulated with irradiated autologous donor LCL. (b) Unmanipulated donor PBMC or donor PBMC depleted with immunotoxin after stimulation with autologous donor LCL were restimulated with irradiated autologous LCL. Results are the mean number of specific spot-forming cells calculated by linear regression of duplicate wells assayed at three dilutions.

used as stimulators PR1-specific $CD8^+$ cells were largely preserved following allodepletion [10].

Conclusions from the pre-clinical studies

We believe that the choice of recipient APC is critical for the efficient activation, and hence successful depletion, of alloreactive cells using CD25-based strategies. Our data demonstrate that allodepletion with an anti-CD25 immunotoxin following stimulation with HLA-mismatched LCL is more consistently effective at removing alloreactive cells than after stimulation with PBMC. This may reflect the heterogeneity of crude PBMC preparations as APC compared with LCL, which have a more standard phenotype and more uniformly express high levels of immunostimulatory molecules. We have shown that T-cell responses to viral and potential myeloid tumor Ag are preserved following allodepletion using our protocol. We have determined optimal conditions for allodepletion and scaled-up our protocol using clinical grade reagents under good manufacturing practice (GMP) conditions.

We have now tested our approach clinically, to determine if the degree of depletion of alloreactive cells achieved is sufficient to enable add-back of enough allodepleted T cells to confer useful antiviral and antileukemic activity without causing GvHD. This study began at Baylor College of Medicine, Houston, TX, USA, and was then extended to Hospital for Sick Children, Great Ormond St., London, UK.

Phase I clinical study of adoptive immunotherapy with allodepleted donor T cells after haplo-identical SCT

Aims

The primary end-point of the study was to determine the number of donor lymphocytes that can be given to recipients of haplo-identical SCT after depletion of recipient-reactive T lymphocytes by *ex vivo* treatment with RFT5-SMPT-dgA immunotoxin, and result in a rate of grade III/IV GvHD of $\leq 25\%$. Secondary end-points were to analyze immune reconstitution and the frequency/outcome of viral infections in these patients, and to determine survival at 100 days and 1 year.

Eligibility criteria and transplantation

The protocol is open to all patients at the Baylor College of Medicine and Great Ormond St. Children's Hospital who are candidates for SCT but lack a 5/6 or 6/6 HLA Ag-matched donor. Patients with a life expectancy <6 weeks and severe lung and liver disease are excluded, and the presence of $>$ grade 1 GvHD is a contraindication to infusion of allodepleted donor T cells. Patients undergo haplo-identical stem cell transplantation using routine institutional conditioning and supportive care protocols. Grafts are CD34 selected using the Sysmex (Sysmex, Mundelein, IL, USA) or CliniMACs (Miltenyi Biotec, Bisley, UK) systems. CYA is withdrawn prior to the first infusion of allodepleted donor T cells.

Generation of allodepleted donor T cells

Generation of allodepleted donor T cells is performed under GMP conditions at the Center for Cell and Gene Therapy, Baylor College of Medicine, in compliace with FDA-approved SOP. Six weeks prior to transplant, 30 mL of blood is taken from the patient for generation of recipient LCL. Immediately prior to mobilization, 160 mL of blood is taken from the donor, mononuclear cells are isolated, and these are co-cultured with irradiated recipient LCL in serum-free medium. Control cultures are also set up to assess residual proliferation. After 72 h, co-cultures are incubated overnight with clinical grade RFT5-SMPT-dgA anti-CD25 immunotoxin (generated by Professor E. Vitetta, University of Texas Southwestern Medical Center) at a final concentration of 3 μg/mL. The next day, cells are washed and sampled for bacterial/fungal/mycoplasma sterility and endotoxin, confirmatory tissue-typing, FACS analysis of the percentage of residual $CD3^+$ $CD25^+$ cells, and residual proliferation to host compared with control cultures. The remaining cells are frozen in aliquots determined by patient weight and dose level. Release criteria include $<1\%$ $CD3^+$ $CD25^+$ cells and $<10\%$ residual proliferation to host. If the test results are appropriate and the patient has engrafted, the cryopreserved, allodepleted T cells are thawed and infused at days 30, 60 and 90 post-transplant.

Study design

This study was initiated with a dose of T cells (10^4/kg) known not to cause GvHD in haplo-identical recipients, even when the T cells administered have not first been allodepleted. Initially, the study was designed to have three patients at dose level 1, with subsequent dose escalation using the continual reassessment method. Each patient receives three infusions of allodepleted donor T cells at the same dose at days 30, 60 and 90 post-transplant, provided there is no evidence of grade II or higher GvHD, until total T-cell numbers are $>1000/\mu L$. If there is no grade III or IV GvHD by day 40 after T-cell infusion at dose

level 1, then patients may be treated at dose level 2. This process continues until at least one grade III or IV GvHD event is observed. Planned dose escalation is as follows: dose level 1, 10^4/kg; dose level 2, 10^5/kg; dose level 3, 10^6/kg. The maximum tolerated dose (MTD) is defined to be that dose that causes GvHD >grade II in 25% of treated patients.

Patients

To date 15 patients have been entered onto the study between the two centers. Patient characteristics and conditioning are outlined in Table 2. The median age is 8 years (range 2–58 years) and the majority (11/15) were transplanted for high risk hematologic malignancies. Four patients received reduced intensity conditioning regimens. The median CD34 dose infused at transplant was 11.6×10^6/kg (range 5.9–20), and a median of 3.9×10^4/kg T cells was infused with the graft (0.73–18). Nine patients were treated with allodepleted donor T cells at dose level 1 and so far six have been treated at dose level 2. Twelve of 15 have completed their scheduled infusions; the remainder have not because of GvHD ($n = 2$) or relapse ($n = 1$). The residual percentage $CD3^+$ $CD25^+$ cells in the infused product has ranged from 0.01% to 0.27% (median 0.09%), and the residual proliferation against host cells in the primary MLR from 0% to 3.1% (median 0.68%).

Engraftment

All patients had primary engraftment. At 1 month 13/15 were full donor chimeras, as assessed by VNTR analysis/XY FISH; by 6 months 4/13 evaluable patients had mixed chimerism, and one patient had autologous reconstitution. Interestingly one patient converted from a mixed chimeric state to full donor chimerism after infusion of his first dose of allodepleted donor T cells.

GvHD and viral infections

The observed incidence of GvHD has so far been low: 2/15 patients have developed significant acute GvHD (one grade II skin and one grade IV skin); both subsequently evolved to extensive chronic GvHD (one affecting skin and mouth, the other affecting the liver). Viral reactivations/infections have predictably been frequent, including CMV ($n = 8$) and EBV ($n = 5$) reactivations, adenoviremia ($n = 1$) and progressive multifocal leucoencephalopathy (n = 1), but only one patient has died of viral causes.

Immune reconstitution

Immune reconstitution has been slow in dose level 1 patients, comparable to that seen in a published series [4]. This was predictable, as the dose of allodepleted cells infused is similar to the numbers of T cells infused with the graft and thus the dose level 1 patients form a convenient in-study control cohort for assessing the impact of infusing higher doses of allodepleted cells. In contrast, our data suggest that T-cell reconstitution is significantly accelerated in patients receiving allodepleted donor T cells at dose level 2. By 6 months post-transplant, only 1/7 patients at dose level 1 had achieved normal T-cell numbers, whereas 4/4 evaluable dose level 2 patients had. Moreover, T-cell recovery was qualitatively different between the two cohorts. Whereas virtually all the T cells that do eventually appear in dose level 1 patients have a naive $CD45RA^+$ $CCR\text{-}7^+$ phenotype, the majority of T cells that appear in the first 6 months post-transplant in dose level 2 patients have either a central ($CD45RA^-$ $CCR\text{-}7^+$) or effector memory ($CD45RA^-$ $CCR\text{-}7^-$) phenotype, suggesting that they are derived from the infused allodepleted cells. Reconstitution of B-cell numbers has been equivalent in dose level 1 and 2 patients.

Additionally, we have studied recovery of viral-specific immunity, using tetramer and ELISPOT assays. We have observed rapid recovery of CMV- and EBV-specific immunity by both techniques as early as 2–4 months post-SCT in patients who have received allodepleted donor T cells at dose level 2 and experienced viral reactivation. In contrast, in dose level 1 patients no viral-specific immunity was detectable until 6–9 months post-SCT, even in patients who had viral reactivations. These data suggest the infused allodepleted donor T cells are able to expand significantly in the face of viral reactivation, and that doses as low as 3×10^5/kg may be sufficient to confer useful viral-specific immunity in this setting.

Current status

At a median follow-up of 19.5 months, nine of 15 patients are alive and disease-free. One dose level 1 patient died of disseminated adenoviremia in the context of liver GvHD, and five others relapsed at 4–9 months post-transplant, all of whom have since died. One patient has had autologous reconstitution but remains in CR.

Table 2. Patient characteristics: diagnoses, presence/absence of GvHD and viral infections and outcomes are shown

Patient	Age (years)	Diagnosis	Conditioning	% RP	Dose level	Number of infusions	aGvHD	cGvHD	Viral reactivations	Outcome
UPN1	8	rel ALL	Cy/TBI/Ara-C/ATG	0	10^4/kg	3	None	None	CMV/VZV	Alive in CR
UPN2	14	ref AML	Cy/TBI/Ara-C/ATG	0.46	10^4/kg	1 (GvH)	Grade 4 skin	Ext (skin/liver)	CMV/Adeno	Died liver failure (adeno/ GVH)
UPN3	12	ref AML	Cy/TBI/Ara-C/ATG	1	10^4/kg	3	None	None	None	Died relapse
UPN4	3	rel ALL	Cy/TBI/Ara-C/ATG	0	10^4/kg	3	None	None	CMV	Alive in CR post 2nd SCT
UPN5	3	rel AML	Cy/TBI/Ara-C/ATG	0.71	10^4/kg	3	None	None	None	Died relapse
UPN6	3	HLH	Cy/TBI/Ara-C/ATG	3.12	10^4/kg	3	None	None	CMV/EBV	Died relapse
UPN7	8	Fanconi	FDR/Campath/ anti-CD45	0	10^4/kg	3	None	None	EBV	Alive in CR
UPN8	28	Hodgkin's	TBI/FDR/Campath	0.33	10^4/kg	3	None	None	CMV/VZV	Died relapse
UPN9	58	CML LBC	TBI/FDR/Campath	0	10^4/kg	1 (AR)	None	None	CMV	Alive in CR AR
UPN10	2	ref AML	Cy/TBI/Ara-C/ATG	0.04	10^5/kg	3	None	None	CMV	Alive in CR
UPN11	8	HLH	Cy/TBI/Ara-C/ATG	1.18	10^5/kg	2	None	None	None	Alive in CR
UPN12	2	rel AML	TBI/FDR/Campath	0	10^5/kg	1 (CD3 > 1000)	Grade I skin	None	EBV	Alive in CR
UPN13	12	SAA	FDR/Cy/TBI/Campath	0	10^5/kg	2 (GVH)	Grade I skin	Ext (skin/ mouth)	EBV	Alive in CR
UPN14	6	rel JMML	FLAG	0.57	10^5/kg	4	None	None	None	Died relapse
UPN15	14	MDS	FDR/Cy/TBI/ATG	2.8	10^5/kg	4	None	None	CMV/EBV/JC	Alive in CR

Rel: relapsed, Ref: refractory, LBC: lymphoid blast crisis, RP: residual proliferation, AR: autologous reconstitution.

Conclusions

Our study confirms the feasibility and safety of adoptive immunotherapy with donor T cells allodepleted using a CD25 immunotoxin-based strategy after haplo-identical SCT. Our pre-clinical data underline the importance of strong activation of alloreactive cells for this strategy to work reproducibly, and we believe the choice of APC is critical. This may also limit the efficacy of this approach in the MHC-matched setting. In our clinical study, we have observed a low incidence of GvHD at the dose levels given to date, suggesting that our strategy does effectively deplete clinically relevant alloreactive cells. The depletion of $CD4^+$ $CD25^+$ regulatory T cells from the infused product does not therefore seem to enhance the potential for GvHD, presumably reflecting the absence of significant numbers of alloreactive effector cells. Similarly, we have not observed an excess of post-SCT autoimmune phenomena.

While the numbers of patients evaluated are small, a number of lines of evidence suggest that the infused allodepleted donor T cells contribute to immune reconstitution at dose level 2. These include the more rapid recovery of T-cell numbers and the memory phenotype of the T cells that do recover at this dose level, the accelerated recovery of CMV- and EBV-specific immunity and the impressively low incidence of infectious deaths in our study to date. It would seem, therefore, that in the context of a profoundly lymphopenic host post-haploSCT, the infusion of allodepleted donor T cells at doses as low as 3×10^5/kg may be sufficient to confer useful viral-specific immunity when patients experience viral reactivations.

Clearly, relapse remains a major problem. Our *in vitro* assays suggest a 1–2 log depletion of alloreactive T cells using the CD25 immunotoxin strategy (median 17-fold by ELISPOT, 150-fold by MLR). Based on the numbers of T cells that are required to exert a GvL effect with donor lymphocyte infusions (10^7–10^8/kg in the MHC-matched setting), it is possible that we will not be able to infuse sufficient allodepleted donor T cells to achieve clinically useful GvL without causing GvHD in the haplo-identical setting. Hence subsequent studies, we will attempt to enhance the degree of depletion of alloreactive donor T cells.

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