Published in final edited form as:
Biol Blood Marrow Transplant. 2012 February ; 18(2): 200–209. doi:10.1016/j.bbmt.2011.10.017.

T and B Cell Immunity can be Reconstituted with Mismatched Hematopoietic Stem Cell Transplantation Without Alkylator Therapy in Artemis-Deficient Mice Using Anti-NK Antibody and Photochemically-Treated Sensitized Donor T Cells

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Abstract

Children with Artemis-deficient $T^-B^-NK^+$ SCID (SCIDA) have very high risks of graft rejection from NK cells and toxicity from increased sensitivity to alkylating agents used for mismatched hematopoietic stem cell transplantation (HSCT). We evaluated the use of a non-alkylating agent regimen prior to HSCT in Artemis-deficient (*mArt*$^{-/-}$) C57Bl/6 (B6) mice to open marrow niches and achieve long-term multilineage engraftment with full T and B cell immune reconstitution. We found that both partial depletion of recipient NK cells using anti-NK1.1 monoclonal antibody and donor T cells sensitized to recipient splenocytes were necessary. BALB/c sensitized T cells (STC) were photochemically -treated (PCT) with psoralen and UVA light to inhibit proliferation, reduce the risk of Graft-versus-host disease (GvHD) and target host hematopoietic stem cells (HSC). 4×10^5 PCT STC co-injected with 1×10^5 lineage-depleted c-kit$^+$ BALB/c HSC resulted in 43.9±3.3% CD4$^+$, 10.9±1.2% CD8$^+$ donor T cells in blood; 29±7.8% and 21.7±4.0 donor B220$^+$ IgM$^+$ in spleen and bone marrow and 15.0±3.6% donor Gran-1$^+$ cells in bone marrow at six months post transplant versus 0.02±0.0.01%, 0.13±0.10%, 0.53±0.16%, 0.49±0.09% and 0.20±0.06%, respectively, in controls that did not receive PCT STC. We found that STC target host HSC, and that PCT STC are detectable up to only 24 hours following infusion in contrast to non-photochemically treated STC which proliferate resulting in fatal GvHD. Increased mortality in the groups receiving $4\text{-}6\times10^5$ PCT-STC was associated with evidence of GvHD in particular the recipients of 6×10^5 cells. These results show that blocking NK cell mediated resistance and making niches in bone marrow are both essential to achieve multilineage engraftment of

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Contribution: T.Z.X. contribute to the design of experiments, performed experiments, generated data, analyzed, interpreted data and drafted the manuscript; K.S. performed ELISA and other experiments, helped to draft the manuscript; E.D. performed some experiments, analyzed data, helped to draft the manuscript; R.R. analyzed tissue and helped draft the manuscript; M.J.C. designed research, analyzed and interpreted data, and edited the manuscript.

Conflict-of-Interest Disclosure: The authors declare no competing financial interests.

mismatched donor cells and T and B cell reconstitution although GvHD is not completely eliminated.

Keywords

SCID; Artemis Deficiency; Hematopoietic Stem Cell Transplant (HSCT); Photochemically-Treated Sensitized T Cell

Introduction

Severe combined immunodeficiency disease (SCID) is the result of defects in over 15 known genes that cause severely abnormal T and B cell immune function [1]. With the exception of gene therapy for some types of SCID [2], allogeneic HSCT is the only curative treatment for children with SCID [3,4]. The majority of affected children are unique in that they can be treated successfully with an allogeneic graft without prior conditioning with alkylating agents. In contrast, an HSCT for all other diseases commonly requires alkylating agents in order to open up marrow niches and prevent rejection mediated by T cell immunity. However, it appears that for some children with SCID who receive an HSCT without any conditioning, there may be incomplete T and there is often limited B cell reconstitution [1,5,6,7]. For this reason, many transplant centers use high dose alkylating therapy as conditioning even for children with SCID [8]. In addition to the potential toxic effects of this approach in infants and young children, some types of SCID that result from defects in V(D)J recombination are also associated with defects in DNA repair and increased sensitivity to alkylating agents [3,5,9]. Approaches to opening marrow niches without requiring alkylating agents would be especially useful in treating these children and possibly other children with non-malignant diseases in which full 100% donor chimerism is not essential.

There is a very high incidence of $T^-B^-NK^+$ SCID in Athabascan-speaking Native American children that is associated with a founder mutation in Artemis (SCIDA), a key protein in the V(D)J recombination and non-homologous DNA repair pathways [10,11]. We have previously reported our experience with transplantation in a large number of Athabascan-speaking Navajo children with this mutation [3]. Not only do these children have virtual absence of T and B cells, but their fibroblasts show increased sensitivity to ionizing radiation[12]. In addition, they appear to have clinical manifestations of increased sensitivity to alkylating agents and radiation [3]. Also, these children typically reject haplocompatible T cell depleted grafts unless some kind of immunosuppressive conditioning therapy is used to prevent NK mediated graft rejection[13,14,15]. However, because of their increased susceptibility to alkylating agents, these patients have increased morbidity and mortality when exposed to this kind of conditioning. Finally, when only immunosuppressive therapy is used, they typically only reconstitute their T cell immunity.

Studies have suggested that donor cell engraftment via HSCT can be achieved without conditioning therapy, provided an adequate number of marrow niches are available [16]. Therefore, optimal conditions for successful HSCT in patients with Artemis-deficient SCID require both overcoming NK cell mediated resistance and enhancing the availability of niches; this must be accomplished by means that do not depend on alkylating agents or ionizing radiation. We have developed a non-leaky mouse model of Artemis deficiency that mimics the immunologic phenotype seen in children with Artemis-deficient SCID [12,17]. We have previously demonstrated that allogeneic-mismatched cytotoxic donor T cells sensitized to host splenocytes and treated with psoralen and UVA light (photochemically-treated, PCT) are capable of creating marrow space in recipient wild type fetal mice [18].

The co-injection of these cells with donor hematopoietic stem cells (HSC) *in utero* resulted in durable multilineage engraftment with minimal GvHD [18]. In the current study, we demonstrate that the combined usage of anti-NK antibody and PCT sensitized cytotoxic T cells (STC) is a novel method for overcoming graft resistance and achieving multilineage engraftment in young adult Artemis-deficient SCID mice. Further, we show that STC target host HSC and that with PCT they maintain their cytotoxic capability but have a relatively short life span *in vivo*. In contrast, without PCT they are capable of expanding *in vivo* and causing fatal GvHD. Extrapolation of these methods to patients with SCID may enable effective conditioning regimens without alkylating agents or ionizing radiation for successful HSCT.

Materials and Methods

Mice

C57Bl/6 (B6, H-2^b) and BALB/c (H-2^d) wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The WT mice were mated to generate F1 haplo mice (B6 X BALB/c F1). The generation of the N10 B6 (99.9%) Artemis-deficient (*mArt*$^{-/-}$) mouse has been previously described [12,17]. All animals were maintained in the Laboratory Animal Resource Center (LARC) Rodent Barrier Facility at UCSF. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of UCSF following National Institutes of Health animal care guidelines.

Anti-NK 1.1 monoclonal antibody (mAb) and conditioning

The generation of mAb PK136, which recognizes NK1.1, has been described[19]. This cell line was kindly provided by Dr. William Seaman (Department of Medicine, UCSF). The mAb was produced as ascites in *mArt*$^{-/-}$ mice, purified by ammonium sulfate precipitation and the total protein concentration determined by UV absorption at 280 nm. Five-week-old *mArt*$^{-/-}$ mice were treated weekly with 200ug anti-NK 1.1 mAb via intraperitoneal (I.P.) injection for three weeks prior to transplantation with HSC and/or sensitized T cells.

Generation of sensitized T cells

To generate BALB/c donor T cells that were sensitized to B6 mice, 3-month-old WT BALB/c mice were injected I.P. weekly for three weeks with 10×10^6 splenocytes from WT B6 mice [18].

Isolation of sensitized CD3$^+$or CD8a$^+$ T cells and NK cells

CD3$^+$ or CD8a$^+$ T cells from sensitized mice or NK cells from unsensitized mice were enriched by negative selection from spleens using microbeads and the Midi-MACS System (Miltenyi Biotec, Auburn, CA) following the manufacturer's instructions. Purity of CD3$^+$ T cells, CD8a$^+$ T cells and CD3-NK 1.1$^+$ NK cells was determined by flow cytometry to be >99%, 96%, and 90%, respectively.

Photochemically-treated (PCT) STC

Sensitized BALB/c CD3$^+$ or CD8a$^+$ T cells were pretreated with Uvadex (methoxsalen, Therakos, Inc, Exton, PA) at 20ng/ml in RPMI 1640 (5% FBS) or indicated concentrations and exposed to UVA light for 2 (PCT-2) or 4 (PCT-4) minutes (equivalent to 1J or 2J, respectively) by using a UVA irradiator (Cole-Parmer, Inc, Chicago, IL)[18]. PCT-4 was used for most of the experiments. Cells were then washed 3× with RPMI 1640 (10% FBS) media and were injected with or without donor HSC into recipient mice as described in the

Results. Aliquots were evaluated for proliferative response to anti-CD3, expression of CD25 and CD69, and ^{51}Cr release cytotoxicity.

^{51}Cr release assay

^{51}Cr release was measured to assess NK cell-mediated cytotoxicity against Yac-1 tumor cells as previously described[19]. Sensitized BALB/c CD3$^+$ or CD8a$^+$ T cells were isolated from sensitized BALB/c mouse spleens (see above) and used as effector cells against ^{51}Cr-labeled B6 splenocyte or lin$^-$c-kit$^+$ HSC targets in an overnight ^{51}Cr-release assay (RPMI 1640 medium, 10% FBS, 1× HEPES buffer, 1× non-essential amino acid, 1× sodium pyruvate, 1× glutamine, UCSF Cell Culture Facility)[18].

Hematopoietic stem cell transplantation

BALB/c WT mice (2-4-month-old) were used as donors for *mArt*$^{-/-}$ B6 (CD45.2) recipients. The donor bone marrow lin$^-$ c-kit$^+$ HSC preparations were followed by the manufacturer's instructions and >95% c-kit$^+$ (CD117$^+$) by flow cytometry. 1×10^5 lin$^-$c-kit$^+$ allogeneic mismatched BALB/c HSC with or without STC (±PCT) were injected into recipient *mArt*$^{-/-}$ B6 mice at 8 weeks of age via the tail vein. The *mArt*$^{-/-}$ B6 mice received prior anti-NK1.1 mAb injections as indicated. The transplanted mice were maintained in the LARC barrier facility with antibiotic-supplemented water. Controls included age-matched B6 *mArt*$^{-/-}$, B6 WT, or heterozygotes that received a sham injection or HSC.

Assessment of GvHD

The severity of GvHD was assessed by the percent weight change [20,21]. Weights were recorded on day+l and weekly until euthanasia. Also, GvHD was assessed by a scoring system that incorporates five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity. Mice were graded from 0 to 2 for each criterion. A clinical index was generated by summation of the five scores [22].

Secondary HSCT

Lin$^-$c-kit$^+$ bone marrow HSC were isolated 3-6 months post-transplant from *mArt*$^{-/-}$B6 mice previously transplanted with allogeneic BALB/c HSC. 1×10^5 HSCs were injected via the tail vein into lethally irradiated (9 Gy) B6 X BALB/c F1 mice (10-12 weeks old) using a ^{137}cesium animal irradiator (520A, Xetex Inc, Sunnyvale, CA)[16].

Multilineage and chimerism analyses

T cell, B cell and granulocyte phenotyping and chimerism analyses were done on blood from the lateral saphenous vein (at 4 week intervals) and single cell suspensions (at >5 months) of spleen, thymus, lymph node and bone marrow by flow cytometry using a four-color FACSCalibur, and data were processed by Cell Quest Pro software (BectonDickinson, San Jose, CA). Fluorescently conjugated antibodies (BD Pharmingen, San Diego, CA or e-Bioscience, San Diego, CA) to BALB/c (anti-H-2^d) donor, B6 (anti-H-2^b) recipient, CD45, CD3, CD4, CD8, CD45r/B220, CD25, CD69, and Ly6G/C (Gran-1) were used. In addition, anti-CD45r/B220 and anti-IgM antibodies were used to examine B cell maturation in bone marrow and peripheral blood.

Proliferation assays

4×10^5 post-Ficoll splenocytes were stimulated with either lipopolysaccharide or anti-CD3 as previously described[17].

TCRVβ quantitative immunoscope

RNA was obtained from T cells isolated from thymus or spleen using a pan T cell negative selection kit (Miltenyi Biotec, Auburn, CA). TCR Vβ repertoire analysis was modified and performed as previously described[17].

ELISA for serum IgM and IgG antibody

To measure antibody responses, mice were injected I.P. with 100 μg of 4-Hydroxy-3-nitrophenylacetyl hapten 24-keyhole limpet hemocyanin (NP24-KLH, Biosearch Technologies, Novato, CA) and boosted with an additional 100 μg of NP24-KLH five weeks following the initial injection. Post-immune serum was collected one week following the boost via saphenous vein blood draw and stored at -20°C. NP-specific antibody response was measured by sandwich ELISA [17].

Carboxyfluorescein succinimidyl ester (CFSE) staining

CFSE is an intracellular fluorescent dye that is retained in daughter cells during cell division. 6×10^5STC or PCT-4 STC were washed twice in PBS and labeled with 10uM CFSE binding dye (Invitrogen, Carlsbad, CA), incubated at 37°C for 10 minutes, cooled on wet ice and re-suspended in culture medium, then injected intravenously into *mArt*$^{-/-}$B6 mice and assayed for proliferating cells after 2h, 24h, 48h and 72h. PE-conjugated anti-CD3 monoclonal antibody (BD Pharmingen, San Diego, CA) was used to identify the T cell population, and CFSE intensity was analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer. A population of T cells that had proliferated extensively in different tissues was identified based on CFSE dye dilution [23,24].

Competitive repopulation experiment

To assess the marrow cell targets of STC, 10-12 week-old (B6 X BALB/c) F1 recipients were irradiated with a single fraction of 9Gy using a ^{137}cesiumanimal irradiator (520A, Xetex Inc, Sunnyvale, CA) which was uniformly sub-lethal in the absence of donor cells. Competitive repopulation experiments were performed by co-injecting into the dorsal tail vein of the irradiated recipients equal numbers of unmanupulated *mArt*$^{-/-}$ B6 HSC or *mArt*$^{-/-}$ B6 HSC from animals which had been previously injected with 4×10^5 BALB/c PCT-4 STC 24 hours earlier, combined with naïve WT BALB/c bone marrow cells [25]. At 2 and 4 months post transplant, peripheral blood was obtained from the lateral saphenous vein of the recipients and stained by using fluorescently conjugated antibodies to BALB/c (anti-H-2^d) and B6 (anti-H-2^b)[16].

Histology

The tissues (liver, gut and skin) removed from euthanized animals were placed into cold PBS to remove all blood and then placed into 70% ethanol fixative overnight at 4°C. All tissues were processed following the standard H&E staining protocol at the UCSF Immunohistochemistry (IHC) facility.

Statistical analysis

Either the independent samples t-test or the nonparametric unpaired Mann-Whitney test was used to test goodness of fit and independence. In the Kaplan-Meier survival analysis, the overall comparison used either the Log Rank or Generalized Wilcoxin tests to determine differences between the various PCT-4 STC doses.

Results

Long-term, multilineage engraftment post anti-NK1.1 mAb treatment and co-injection of PCT-4 STC with allogeneic HSCT

We demonstrated that administration of anti-NK1.1 mAb suppresses NK cytotoxic function comparable to what has been previously reported (Figure S1A) [19,26]. Also, we showed that PCT inhibits proliferation of STC while maintaining cytotoxic activity and expression of activation markers CD25 and CD69, and that the optimal UVA dose was PCT-4 (equivalent to 2 joule) (Figure S1B, C, D). To determine whether administration of pre-transplant anti-NK1.1 mAb and co-injection of PCT STC might be required for effective allogeneic HSCT, and to address whether PCT STC promote multilineage engraftment in a dose dependent manner, we transplanted 1×10^5 $lin^-c\text{-}kit^+$ BALB/c HSC in combination with various numbers (1, 2, 4 and 6×10^5) of BALB/c PCT-4 STC into 8-week-old B6 *mArt*$^{-/-}$ mice pretreated with anti-NK1.1 mAb. At two months post HSCT, neither anti-NK antibody nor PCT-4 STC alone resulted in significant donor engraftment while the optimal engraftment of both donor T and B cells was seen in anti-NK treated mice that received 4×10^5 PCT-4 STC plus HSC (Figure S2A). Multilineage engraftment at 2 months was also optimized at that cell dose (Figure S2B).

Long term (6 months) evidence of recovery of both T- and B-cell numbers and function was only seen in the 4×10^5 PTC-4 STC treated group (Figure 1). Normal numbers of splenic lymphocytes were observed (Figure 1A) in all long term surviving mice (n=7) in this group. These mice achieved normal to near-normal numbers of mature $CD4^+$ (43.9±3.3%, 21.1±2.6%, 39.6±2.1%, 8.3±0.1%) and $CD8^+$ (10.9±1.2%, 6.0±1.0%, 13.7±0.8%, 2.1±0.1%) cells in peripheral blood, spleen, lymph node, and thymus, respectively (Figure 1C, 1D). Likewise, phenotype analysis using antibodies to B220 and IgM cell surface markers showed the presence of mature B cells at significant levels in spleen and bone marrow (29.0±7.8%, 21.7±4.0%) (Figure 1B). The flow cytometry analysis is shown in Figure S3A. Consistent with peripheral blood granulocyte chimerism at 8 weeks post transplant (Figure S2B), granulocyte engraftment in bone marrow (Figure 1E) was also achieved in long-term surviving animals that received 4×10^5 PCT-4 STC plus 1×10^5 HSC (15%±3.6%) in comparison with recipients of 1×10^5(0.2±0.1%, $p<0.01$), 2×10^5(1.5±1.2%, $p<0.05$) and 6×10^5 (0.7±0.3%, $p<0.01$) PCT-4 STC and 1×10^5 HSC.

Lymphocyte proliferative responses to anti-CD3 and LPS were also tested post transplant. Splenocytes from surviving mice that received 4 or 6 $\times10^5$ PCT-4 STC had significantly improved *in vitro* proliferative responses to T and B cell mitogens compared with B6 *mArt*$^{-/-}$ ($p<0.01$) (Figure 2A). Splenocytes from surviving mice that received 1 or 2×10^5 PCT-4 STC did not have improved *in vitro* proliferative responses when compared to control B6 *mArt*$^{-/-}$ mice ($p>0.05$). Although some recipients of 2×10^5 PCT-4 STC did have positive proliferative responses to mitogens the response was very variable.

To evaluate B lymphocyte function post HSCT, we immunized recipient mice with a T cell dependent antigen, NP24-KLH. IgM responses were relatively lower in the 4×10^5 PCT-4 STC group (Figure 2B), while no significant difference in IgG antibodies was seen in the 4×10^5 PCT-4 STC group when compared with B6 WT mice (Figure 2C). Specific but relatively lower levels of IgM (Figure 2B) and IgG (Figure 2C) antibodies to NP24-KLH also were detected in the recipients of 6×10^5 PCT-4 STC.

To assess T cell diversity in the transplanted mice, we determined the TCR Vβ repertoire in T cells. In the group of mice receiving allogeneic HSC with 2×10^5 or 4×10^5 PCT-4 STC, near-normal patterns were observed in all four of the regions analyzed when compared to the normal Gaussian distribution of the different CDR3 lengths in WT mice (Figure 2D). In

the 6×10^5 PCT-4 STC group, the T cell repertoire had skewed profiles or monoclonal patterns in the immunoscope analysis, indicating preferential and restricted clonal expansion of the engrafted T cells. The comparative TCR Vβ repertoire analysis for other transplanted mice (1×10^5 PCT-4 STC) was not possible due to the absence or limited numbers of T cells.

To determine if donor repopulating stem cells had engrafted in recipient mice, two secondary transplant experiments (n=3 recipients per experiment) were performed, both of which resulted in multilineage BALB/c donor engraftment (Figure S3B).

PCT limited but did not completely abrogate GvHD

To assess the severity of GvHD, the percent weight change was recorded post-transplant. Weight loss of greater than 10% was considered indicative of significant GvHD [14,15,16]. None of the recipients of up to 4×10^5 PCT-4 STC lost more than 10% of their initial weight even at 1 week post transplant. When control mice that received 1×10^5 allogeneic donor HSC plus 6×10^5 STC not treated with PCT (6×10^5 (Non-PCT) in Figure 3A) were evaluated, nearly 100% of the animals showed weight loss of more than 10% (most were over 30%), a threshold described for clinically important GvHD 5 weeks post transplant (Figure 3A). The percentage of weight change at 5 weeks for this control group (-28.8±0.9%) was significant ($p<0.05$) only in this group compared with mice that received 1×10^5 allogeneic donor HSC plus1×10^5 (2.6±0.8%), 2×10^5(-2±0.2%), 4×10^5(-1±0.2%) or 6×10^5 (-8.8±0.3%) PCT-4 STC, respectively (Figure 3A). However, the weight change at 1, 2, 4 and 5 weeks in the 6×10^5 recipient group does appear to be different from that in the other experimental groups and could be consistent with GvHD. To further assess for GvHD, a clinical scoring system was employed (see details in Materials and Methods). Nearly 100% of mice receiving 6×10^5 non-PCT-4 STC failed to gain weight, lost their fur, and displayed abnormal posture and limited activity, all signs of GvHD [14,15,16]. As shown in Figure 3B, this semi-quantitative assessment demonstrated that clinical scores of mice receiving 6×10^5 PCT-4 STC (7.2±0.9) did not differ significantly from scores of non-PCT-4 control mice (9.3±0.5). However, there was a significant difference between GvHD scores of allogeneic HSCT recipient mice in the non-PCT-4 STC control group and the recipient mice treated with 1×10^5 (1.4±0.2), 2×10^5 (1.4±0.4), and 4×10^5 (3.1±0.9) PCT-4 STC, respectively ($p<0.05$). There was also a significant difference between scores of allogeneic HSCT recipient mice treated with 4×10^5 and 6×10^5 PCT-4 STC ($p<0.05$); however, there was no significant difference in GvHD scores between the 1×10^5 and 4×10^5PCT-4 STC recipient groups ($p>0.05$). Histologic analysis of transplanted mice with various doses of STC (±PCT) further supported the fact that PCT significantly limits GvHD in all recipients of PCT-4 STC although to a lesser extent in recipients of 6×10^5 PCT-4 STC (Figure S4).

Survival of mice treated with PCT-4 STC and allogeneic mismatched donor HSC

Without PCT-4 four of four mice that were treated with 6×10^5 STC died within 6 weeks (Figure 3C). With PCT-4 STC, long term (180 day) estimated survival rates (±SE) were 100±0%, 80±18%, 60±15% and 50±16% and mean (±SE) survival days were 180±0, 171±8, 129±20, and 131±18 (days) respectively for 1×10^5 (n=5), 2×10^5 (n=5), 4×10^5 (n=10) and 6×10^5 (n=10) PCT-4 STC doses.

PCT-4 STC are capable of making niches in bone marrow

To determine if PCT-4 STC actually target repopulating stem cells, sensitized BALB/c $CD8a^+$ T cells were generated and assayed in an overnight culture with ^{51}Cr- labeled B6 WT $lin^-c\text{-}kit^+$ HSC. At a 50:1 effector to target (E:T) ratio, 70±15.1% cytotoxicity was seen with STC alone, and 63±12% when PCT-4 STC were used. Comparable levels of ^{51}Cr release between the two T cell populations were also achieved at other E:T ratios (Figure 4A).

To further confirm depletion of functional HSC, we infused PCT-4 STC into anti-NK1.1 mAb-treated B6 *mArt*$^{-/-}$ mice. Twenty-four hours later we co-transplanted treated 1×10^5 lin$^-$c-kit$^+$HSC isolated from the bone marrows of these mice with an equal number of HSC from BALB/c WT into lethally irradiated B6 X BALB/c F1 recipients. The F1 recipient mice were evaluated for engraftment at 2 months post transplant. We found 0.4±0.1% B6 versus 55.7±6.8% BALB/c granulocyte engraftment in these F1 recipients compared to 11.3±2.6% B6 versus 64.7±9.9% BALB/c granulocyte engraftment in F1 recipients of equal numbers of HSC from bone marrows of untreated B6 *mArt*$^{-/-}$ and BALB/c WT mice (Figure 4B). This represented a significant difference in B6 granulocyte chimerism ($p<0.05$) and it reflects an approximately 30-fold decrease in the number of transplanted functional donor HSC in the PCT-4 STC donor mice. There was no difference in BALB/c granulocyte chimerism ($p>0.05$).

Persistence of PCT-4 STC in NK-depleted *mArt*$^{-/-}$ mice

While engraftment increased with increasing numbers of PCT-4 STC co-injected with a fixed number of HSC, so did mortality. We first asked whether PCT-4 STC could survive *in vivo*. We infused 6×10^5 STC with or without PCT-4 that had been labeled with CFSE into anti-NK1.1 mAb treated B6 *mArt*$^{-/-}$ mice and tested for the presence of these cells in different tissues over time. We found that PCT-4 STC could be found in the bone marrow at 2 hours but not at 24 and 48 hours post injection (Figure 4C) In contrast, STC alone were still detectable 48 hours after injection and appeared to be increasing in number (Figure 4C). Similar results were obtained in the blood and spleen (data not shown). Next, we wanted to determine if the 4-5% of STC that were still capable of proliferating after PCT-4 of 6×10^5 STC could cause GvHD. We injected 25,000 STC (~4% of 6×10^5 which is the number we estimate would have survived PCT-4 based on the proliferation assay) into anti-NK1.1 mAb-treated B6 *mArt*$^{-/-}$ mice (n=4). No significant engraftment was found (data not shown) and none of four injected mice died, suggesting that a small number of proliferating STC remaining after PCT-4 could neither explain the multilineage engraftment seen with PCT-4 nor cause GvHD.

Discussion

We previously demonstrated that the C57Bl/6 *mArt*$^{-/-}$ mouse model accurately reflects Artemis-deficient SCID in humans in that it is T and B cell deficient, non-leaky, has normal NK cell numbers and function, and is sensitive to ionizing radiation [17]. It also compares to Artemis-deficient children in that the T cell but not the B cell defect can be corrected with a congenic HSCT, while allogeneic mismatched grafts (using BALB/c donors) are rejected when conditioning therapy is not used [17].

In this study we found that when allogeneic mismatched HSC were co-injected with donor T cells that previously had been sensitized to recipient antigens and treated with psoralen and UVA light to prevent proliferation significant multilineage engraftment was achieved with full T and B cell reconstitution. Furthermore, in secondary transplants we showed that lethally irradiated wild type recipients could be reconstituted with marrow from primary engrafted mice, and we confirmed that multilineage engraftment was due to engraftment of donor hematopoietic stem cells.

We found that when PCT-4 STC or anti-NK1.1 mAb was employed independent of each other, there was limited engraftment of allogeneic mismatched HSC. The reason for the lack of efficacy of anti-NK1.1 mAb alone may be explained by the fact that there are sufficient niches for no more than a limited number of donor cells to engraft, even when NK-mediated graft resistance is reduced. It is also possible that because only about 60% of NK function was eliminated by anti-NK1.1 mAb treatment alone, graft resistance was not suppressed

sufficiently. Interestingly, injecting higher doses of anti-NK1.1 mAb into control animals resulted in significant mortality but only modest additional reduction in cytotoxic function (data not shown). We speculate that PCT-4 STC has some anti-recipient NK cell activity, and when it is combined with anti-NK1.1 mAb treatment, there is sufficient suppression of graft resistance to allow robust engraftment.

Our *in vitro* experiments showed that the PCT-4 sensitized BALB/c $CD8a^+$ T cells directly target B6 HSC. However, when we tried $CD8a^+$ PCT-4 STC *in vivo*, there was no effect on engraftment (data not shown), comparable to what we previously saw in an *in utero* transplant model using wild type mice [18]. We also performed a competitive repopulation experiment to confirm that the co-injection of donor PCT-4 STC with HSC would target niches in the recipient marrow. Importantly, B6 *mArt*$^{-/-}$ bone marrow that had been previously exposed *in vivo* to PCT-4 STC 24 hours earlier and then injected into irradiated F1 recipients resulted in significantly reduced B6 HSC engraftment compared to B6 *mArt*$^{-/-}$ bone marrow not exposed to PCT-4 STC. The difference suggested that PCT-4 STC can target host HSC and make niches in bone marrow.

When we evaluated varying doses of PCT-4 STC we found that the optimal dose for T, B and granulocyte engraftment was 4×10^5 cells. At the lowest dose (1×10^5 cells) of PCT-4 STC, we found minimal engraftment, possibly due to insufficient generation of niches in recipient bone marrow. Interestingly, at the highest dose (6×10^5 cells) there was significantly reduced T and B cell reconstitution in addition to the lowest survival. The reason for the reduced engraftment and survival at the highest dose used (6×10^5 cells) is due most likely to GvHD as indicated by elevated clinical GvHD scores and a greater extent of liver damage histologically. We believe that GvHD damaged the bone marrow niche early, including the stroma and osteoblasts, resulting in poor engraftment [27].

While the dose of PCT-4 STC that resulted in the highest level of durable multilineage engraftment and T and B cell immune reconstitution was 4×10^5 cells, only 70% of these animals survived (>180 days) with 30% dying at 52.5±4.9 days. In contrast to what was seen in the 6×10^5 non-PCT control animals, we could find no consistent pathologic evidence for acute or chronic GvHD in these animals. In addition to inflammation and duct damage, cholestasis also is common in acute GvHD and was not seen in this treatment group (or any other). Further, the pathology results in the 4×10^5 PCT-4 STC experimental group indicated chronic inflammation in the liver, raising the possibility that chronic infection and/or hepatitis of unclear etiology might have accounted for the 30% mortality. However, we don't know whether Tregs were eliminated by PCT, which may lead to GvHD. It is also possible that the PCT-4 STC were not completely rendered incapable of causing GvHD. Photochemical treatment of donor STC resulted in a significant reduction in lymphocyte proliferation *in vitro* (while maintaining cytotoxic capability and expression of activation markers); however, there was a degree of residual proliferation, although no more than 4% of control. We were concerned that these remaining cells could have resulted in GvHD. However, injection of an equivalent number (2.5×10^4) of STC (without PCT-4) into anti-NK1.1 mAb pre-treated Artemis-deficient recipients resulted in no engraftment, immune reconstitution, or mortality. When we injected 6×10^5 CFSE-labeled PCT-4 STC into anti-NK1.1 mAb pre-treated animals, they were undetectable in the circulation or in various organs after 24 hours post transplant. In contrast, 6×10^5 STC alone (without PCT-4) increased in number over time following transplant and resulted in 100% mortality from GvHD.

Compared with other approaches [28,29], our data demonstrate that major barriers to achieving efficient allogeneic donor engraftment in *mArt*$^{-/-}$ mice include recipient NK cell mediated resistance and occupancy of host bone marrow niches. In this study we

demonstrate that reduction of NK cell function combined with PCT-4 STC results in durable multilineage engraftment of MHC mismatched HSC and restoration of normal T and B cell immunity in Artemis-deficient mice. Not surprisingly, the dose of PCT-4 STC is critical. Too low a dose results in minimal engraftment while too high a dose results in GvHD and poor reconstitution. In this study the optimal dose in terms of producing significant chimerism and full immune reconstitution, was nonetheless associated with some mortality of unclear etiology. This study suggests that the challenge of this treatment strategy will be to find a balance between beneficial and harmful T cell effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH R01grant (5 R01 HL058842-07) and MOD grant (6-FY05-84). We thank Randa Ibeid, Jennifer Sasaki for their valuable technical support and Dr. Lee Ann Baxter-Lowe and Charlyn Dames for helping us with the immunoscope analysis.

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Abbreviations used in this article

HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation
***mArt*$^{-/-}$**	Artemis-deficient
PCT	photochemically –treated
STC	sensitized T cells
GvHD	Graft-versus-host disease
B6	C57Bl/6
WT	wild type
NP24-KLH	4-Hydroxy-3-nitrophenylacetyl hapten 24-keyhole limpet hemocyanin

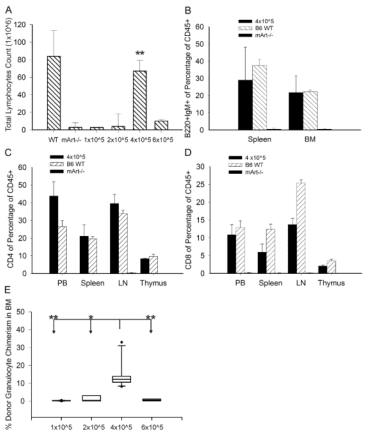

Figure 1. Effects of anti-NK mAb 1.1 and various doses of PCT-4STC on lymphoid and myeloid long-term reconstitution after allogeneic HSCT

(A) Total number of lymphocytes in the spleen of recipient mice 6 months after HSCT compared with unmanipulated WT B6 (positive control) and B6 *mArt*$^{-/-}$ (negative control) mice. Mean values ± SEM are showed (n=3-7); **p<0.01. **(B)** Donor mature B cells ($B220^{+}IgM^{+}$) in spleen and bone marrow (BM) and **(C)** $CD4^{+}$, **(D)** $CD8^{+}$ mature T cell phenotyping in peripheral blood (PB), spleen, lymph node (LN), and thymus from WT B6 mouse (n=3), B6 *mArt*$^{-/-}$ untransplanted mouse (n=6), and B6 *mArt*$^{-/-}$ mice following allogeneic HSCT with anti-NK 1.1 mAb and PCT-4STC (4×10^{5}) (n=7) ≥6 months post transplant. Mean values ± SEM are showed. **(E)** Donor granulocyte chimerism in BM from B6 *mArt*$^{-/-}$mice following allogeneic HSCT with anti-NK 1.1 mAb and various PCT-4 STC ($1\text{-}6\times10^{5}$). Mean values ± SEM are showed (n=3-7); **p<0.01, *p<0.05.

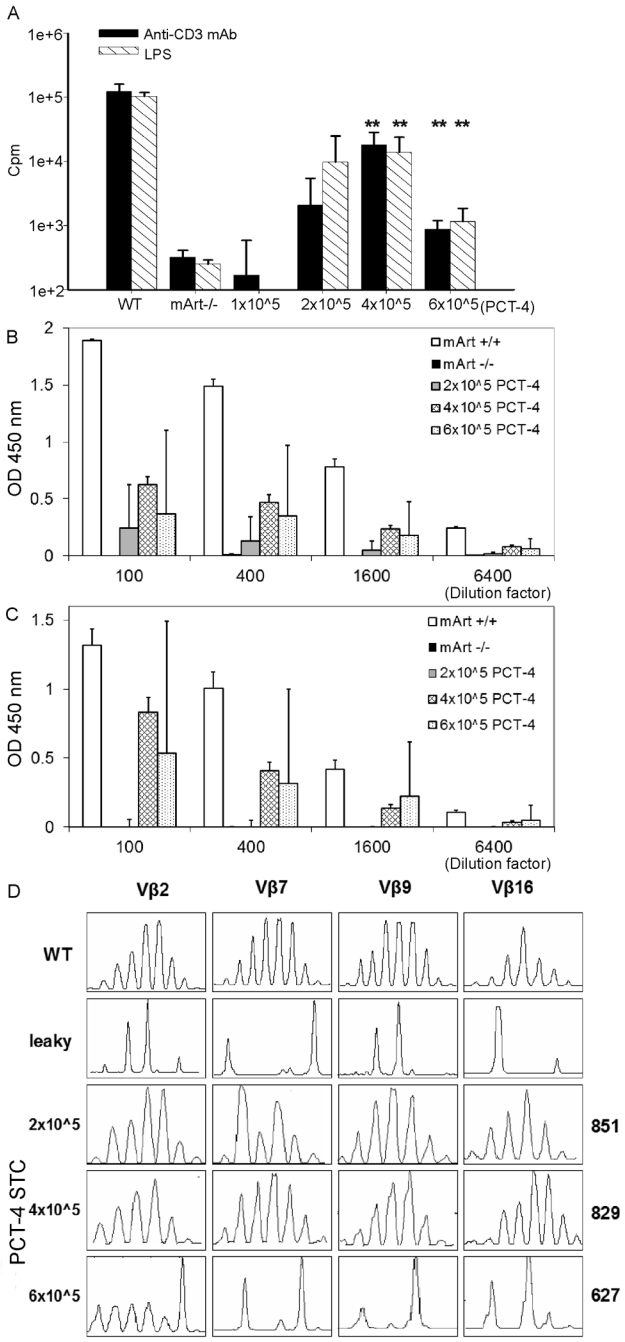

Figure 2. T and B cell proliferative responses, specific IgM and IgG responses post immunization, and T cell receptor diversity post HSCT

(A) Proliferative responses of lymphocytes to anti-CD3 and LPS. WT B6 mice were compared to B6 *mArt*$^{-/-}$untransplanted mice (negative controls), recipients of allogeneic HSCT with anti-NK1.1 mAb and various doses of PCT-4 STC ≥6 months post transplant. Results (stimulated minus resting) are expressed as counts per minute (cpm) on a log scale. **p<0.01 (n>3). **(B), (C)** Serum IgM (B) and IgG-specific (C) antibody response to NP24-KLH after immunization (minus pre-immune response). Average response from recipients (n>3) of allogeneic HSCT with anti-NK 1.1 mAb and various dose of PCT-4 STC are compared to B6 *mArt*$^{+/+}$ (n=3) and B6 *mArt*$^{-/-}$ (n=3) controls with serial dilutions. **(D)** TCR

Vβ repertoire analysis. Four representative Vβ regions are shown. T cells were enriched from either thymus or spleen and prepared as in Materials and Methods. WT is wild type mouse and "leaky" is from representative 129/SvJ *mArt*$^{-/-}$ mouse [17]. Examples of the repertoire from *mArt*$^{-/-}$ recipients of allogeneic HSCT (851, 829, 627 are serial numbers) with 2, 4, 6×10^5 PCT-4 STC also are shown.

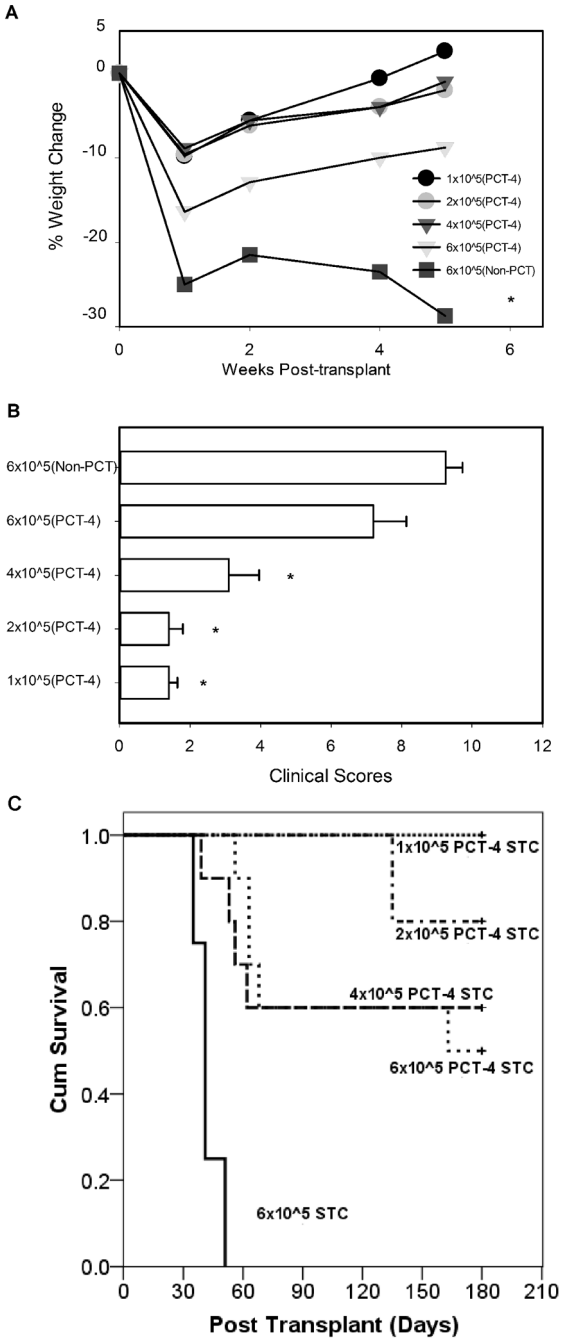

Figure 3. PCT-4 of sensitized donor T cells limits GvHD and prolongs survival

(A) Percentage of weight change in mice after allogeneic HSCT. Transplanted mice were weighed individually on day +1 and then weekly up to the day of analysis. At 5 weeks after allogeneic HSCT, the percentage of weight loss was significant only in the group treated with 6×10^5 non PCT-4 STC. Values are expressed as the mean ± SEM (n=4 to 10 per group, *p<0.05). (B) Clinical GvHD scores of transplanted mice 5 weeks after allogeneic HSCT. Transplanted mice were evaluated for evidence of clinical GvHD as described in the Materials and Methods. Scores are expressed as the mean ± SEM (n=4 to 10 per group, *p<0.05). (C) Kaplan-Meier survival 180 days after HSCT with 1×10^5 HSC plus varying doses of PCT-4 STC. Control mice received 1×10^5 HSC plus 6×10^5 STC. Analysis by

independent sample t-test of the mean survival (days) were significant only for recipients of 1 vs 4 $\times 10^5$ PCT-4 STC (p=0.04) and 1 vs 6×10^5 PCT-4 STC (p=0.03). There was no significant difference in the overall comparison of survival curves between recipients of 1, 2, 4, and 6×10^5 PCT-4 STC (p=0.5) using Log Rank or Generalized Wilcoxin analysis.

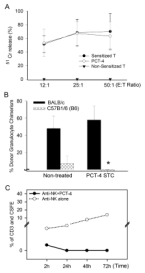

Figure 4. PCT STC makes niches in recipient bone marrow and PCT-4 decreases survival of STC

(A) Cytotoxicity of sensitized CD8a^{+}T cells (BALB/c) with or without PCT-4. Killing of B6 lin^{-}c-kit^{+} HSC was assessed in an overnight chromium-release assay. The control was non-sensitized T cells. Each dot represents the mean value ± SEM for killing by T cells (n=3) using a ratio of 12:1, 25:1, 50:1, respectively. **(B)** Competitive repopulation experiments with equal amount untreated BALB/c and B6 lin^{-}c-kit^{+} HSC with or without pre-conditioning with donor PCT-4 STC. This panel displays a bar graph regarding engrafted granulocyte percentages for BALB/c and C57B1/6 (B6) in B6 X BALB/c F1 recipients 2 months post transplant, n=3, *p<0.05. (For details, see Materials and Methods). **(C)** Survival kinetics of STC in vivo. Purified STC (donor, BALB/c) with or without PCT-4 were labeled with CFSE and administered intravenously to B6 *mArt*$^{-/-}$mice pre-conditioned with anti-NK1.1 mAb. Bone marrow was harvested and evaluated for CFSE-positive cells at various time points. One representative result from three independent experiments. (For details, see Materials and Methods).