Antibody to the dendritic cell surface activation antigen CD83 prevents acute graft-versus-host disease

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NONSTANDARD ABBREVIATIONS:  allo – allogeneic; ATG - rabbit polyclonal anti-human thymocyte globulin; FMP – influenza matrix protein; GVHD – acute graft versus host disease; GVL – graft versus leukemia; HSCT - hematopoietic stem cell transplantation; hu-SCID - human PBMC transplanted SCID mouse; RA83 – polyclonal affinity purified anti-human CD83 rabbit IgG; RAneg – non-immune negative control rabbit IgG

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CD83 antibody prevents acute GVHD
ABSTRACT

Allogeneic hematopoietic stem cell transplantation is an effective therapy for hematological malignancies but it is limited by acute graft-versus-host disease (GVHD). Dendritic cells (DC) play a major role in the allogeneic T cell stimulation causing GVHD. Current immunosuppressive measures to control GVHD target T cells but compromise post-transplant immunity in the patient, particularly to cytomegalovirus (CMV) and residual malignant cells. We showed that treatment of allogeneic mixed lymphocyte cultures with activated human DC depleting CD83 antibody, suppressed alloproliferation but preserved T cell numbers, including those specific for CMV. We also tested CD83 antibody in the human T cell dependent peripheral blood mononuclear cell transplanted SCID (hu-SCID) mouse model of GVHD. We showed that this model requires human DC and that CD83 antibody treatment prevented GVHD but, unlike conventional immunosuppressants, did not prevent engraftment of human T cells, including cytotoxic T cells (CTL) responsive to viruses and malignant cells. Immunization of CD83 antibody treated hu-SCID mice with irradiated human leukemic cell lines induced allogeneic anti-leukemic CTL effectors in vivo that lysed $^{51}$Cr-labelled leukemic target cells in vitro, without further stimulation. Antibodies that target activated DC are a promising new therapeutic approach to the control of GVHD.

CD83 antibody prevents acute GVHD
Allogeneic hematopoietic stem cell transplantation (alloHSCT) is an effective therapy for many malignant and non-malignant hematological and some non-hematological conditions. Conditioning the recipient with radiation and chemotherapy enables donor hematopoietic and immune systems to engraft and provide immune effectors, which confer protective immunity and, for leukemia patients, the desired therapeutic graft versus leukemia (GVL) effect. Donor T cell mediated acute graft-versus-host disease (GVHD), which targets recipient skin, gut, liver, lung and lymphoid tissue, is an inevitable consequence of alloHSCT and a major cause of morbidity and mortality (1). GVHD arises within the conditioning induced inflammatory milieu as donor CD4+ and CD8+ T cells are stimulated to generate alloreactive anti-host effector cells. Murine alloHSCT models indicate that the donor anti-host T-cell response is stimulated by direct alloantigen presentation, by host APC, particularly dendritic cells (DC) (2, 3). Donor APC also contribute, presumably via the indirect pathway by processing and presenting host antigens to donor T cells (4). That they may be an appropriate therapeutic target in their own right is further supported by recent studies showing that donor APC propagate GVHD initiated by host APC (5), that they can independently induce GVHD (6) and that they play a key role in HSCT rejection (7).

Prophylactic and often additional therapeutic immunosuppression, is used to control GVHD but, being non-specific, this neither spares pre-existing donor memory cells nor discriminates between alloreactive and non-alloreactive donor T cells. Thus, although GVHD can be controlled, it is at the cost of increased incidence of graft failure, leukemia relapse (8) and compromised immunity to post-transplant infection, particularly to CMV (9). GVHD and/or immunosuppression associated complications prevent the application of alloHSCT to older patients and limit its wider use for the treatment of non-

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hematopoietic tumors, common non-malignant conditions (autoimmune disease, thalassemia, immunodeficiencies) and for gene replacement therapy (10).

An alternative strategy that primarily targets DC might prevent GVHD, without the complications associated with T cell immunosuppression. Depletion of APC (including DC) in mice with liposomal clodronate reduced development of liver GVHD (11) and UV radiation, to deplete host skin DC, prevented mouse skin GVHD (12). More practical methods aimed at DC are required for clinical therapy. Antibodies can be used to target specific cells and some are available for therapeutic T cell depletion and immunosuppression. However, there are no pan-DC specific antibodies, therefore it is not currently possible, and possibly not desirable, to specifically deplete all human DC in order to achieve immunosuppression. However, a proportion of human DC spontaneously up-regulate the DC surface activation markers CD83 and CMRF-44, after overnight culture (13). These activated DC are the prime stimulators of allogeneic T cell proliferation \textit{in vitro} and their depletion with antibody specific for CD83(14) or CMRF-44 antigen(15, 16) significantly reduces the allogeneic proliferative response, suggesting that such antibodies may have a role in the control of GVHD. We show here that treatment of mixed lymphocyte cultures (MLC) with anti-human CD83 antibody markedly reduced allogeneic T cell proliferation but preserved pre-existing anti-viral, particularly anti-CMV effector/memory CD8$^+$ T cells. In contrast, the therapeutic immunosuppressive antibody alemtuzumab (Campath-1H) prevented allogeneic T cell proliferation by depleting virtually all cells, including virus specific T cells.

To investigate the anti-human CD83 antibody \textit{in vivo}, we used a chimeric human/mouse model of xenogeneic GVHD (17), in which SCID mice are engrafted with human PBMC. These hu-SCID mice develop a fatal human CD4$^+$ T cell dependent GVHD-like syndrome affecting multiple organs, which has histological features similar
to those seen in allogeneic human and mouse GVHD (18). We show here that human DC were required to induce GVHD in this model. Treatment of the hu-SCID mice with CD83 antibody prevented GVHD, yet allowed human leukocyte engraftment and preserved T cells, including CTL precursors specific for CMV, influenza and the malignancy associated antigen Mart1. Moreover, CD83 antibody treatment of hu-SCID mice did not impair induction, in vivo, of anti-leukemic cytolytic T cell effectors in response to immunization with human leukemic cell lines.
RESULTS

Anti-CD83 antibody in allogeneic MLC reduces T-cell proliferation and IFN-γ expression but maintains cell numbers.

Polyclonal rabbit anti-human CD83 (RA83) induces antibody dependent cellular cytotoxic (ADCC) lysis of activated DC, thereby reducing DC stimulated allogeneic T cell proliferation in MLC (Fig.1a)(14). The therapeutic antibody alemtuzumab, which depletes most human PBMC by ADCC and complement dependent cytotoxicity (CDC), also reduced T cell proliferation (Fig.1a) but, unlike RA83, it substantially reduced the total number of viable leukocytes recovered at day 7 from the alloMLC (Fig.1b). Of the five cytokines assayed at day 7, RA83 treatment reduced only IFN-γ secretion into the culture medium (Fig.1c). Alemtuzumab reduced IFN-γ, IL-5 and IL-10. However, TNF and IL-4 were not affected by either antibody treatment, despite the large reduction in cell numbers induced by alemtuzumab.

Anti-CD83 treatment of the MLC preserves specific T cell immunity.

The RA83 mediated reduction in T cell alloproliferation (Fig.1a), without T cell loss (Fig.1b), suggested the hypothesis that this approach to immunosuppression would not compromise T cell memory. To investigate this, antibody treated 7 day alloMLCs using CMV⁺ HLA-A*0201⁺ responder PBMC were tested for the presence of CMV and influenza specific CD8⁺ T cells. For each of three donors, approximately similar absolute numbers of CMVpp65 specific CD8⁺ T cells were recovered from untreated MLCs and those treated with RA83 or negative control antibody (RAneg). Much lower numbers were recovered from alemtuzumab treated MLCs (0%, 1.3%, 7.7% of respective RA83 values for the 3 donors, Fig.2a). The same number of total live cells from each MLC were stimulated with CMVpp65 peptide or influenza matrix protein (FMP) peptide and...
irradiated autologous PBMC, then tested for specific cytotoxic activity. Cells expanded from RA83, RAneg and untreated MLCs all lysed CMV peptide or FMP peptide loaded, \(^{51}\text{Cr}\) labeled T2 target cells. In contrast, alemtuzumab treated MLC derived cells lysed significantly fewer target cells, indicating that most CMV and FMP specific precursors in the MLC had been eliminated by this antibody (Fig.2b). These data support our hypothesis that antibody that targets activated DC could control GVHD yet maintain protective T cell memory.

**Human DC are required for GVHD in the chimeric human PBMC transplanted SCID mouse model (hu-SCID).**

To test this hypothesis *in vivo*, we used the well established chimeric hu-SCID mouse model, in which human donor CD4\(^+\) T cells mediate GVHD in SCID mice (17, 18). In our hands, conditioned SCID mice injected *i.p.* with 50 x 10\(^6\) human PBMC reliably developed a fatal GVHD-like syndrome within 8-13 days. Histological examination showed peri-portal lymphocytic infiltration (Supplementary Fig.1) typical of GVHD in this model (19) and in patients (20). Mice were sacrificed when an overall GVHD score of 5 was attained, reflecting severe GVHD, at which time human donor cells were detected in spleen, bone marrow and peritoneal cavity (Supplementary Fig.2).

Mouse APC are weak stimulators of human T cells *in vitro* (21, 22), therefore we considered it likely that APC in the human donor PBMC graft provided the primary stimulation of the human CD4\(^+\) T cell GVHD effectors in the hu-SCID model. Supporting this view, purified human T cell (97% CD3\(^+\)) grafts alone induced GVHD in only 30% of SCID mice (Fig.3a). A high incidence of GVHD was fully restored by co-administration of 2.5% human autologous monocyte derived DC with the purified T cells (Fig.3a). Thus human APC are required to induce full GVHD in the hu-SCID model. To
explore the role of other human APC besides DC, and of other human leukocytes, we depleted PBMC, prior to transplant, of CD14+ cells (monocytes), CD19+ cells (B cells), CD16+/CD56+ cells (NK cells) and CD8+ (T) cells. None of these depletions significantly affected induction of GVHD (≥80% of mice achieved a GVHD score of 5 within 13 days, Fig.3b). This confirmed that human DC are required to stimulate the human anti-mouse CD4+ T cell effectors to induce GVHD and validated the hu-SCID model for evaluating human DC targeted therapy.

**In vivo anti-CD83 antibody treatment prevents GVHD and alters circulating human cytokine concentrations in the hu-SCID model.**

We administered RA83 to hu-SCID mice, after determining by ELISA that the antibody had a circulating half life of approximately 10 days (data not shown). RA83 administration attenuated GVHD in the hu-SCID model, as assessed by blinded GVHD scoring, in a dose dependent manner (Fig.3c). Ninety four percent of mice injected i.p. with 125µg RA83 per mouse at the time of transplant survived for 30 days, in contrast to RAneg treated control mice, all of which developed severe GVHD within 11 days. In the same experiment, a lower dose of 25µg RA83 per mouse protected only 47% of the mice for the full 30 day experimental period.

Alemtuzumab also prevented GVHD in this model (Fig.3c). From day 3 post-transplant, body weights increased and GVHD scores decreased for mice treated with RA83 (125µg/mouse), alemtuzumab (Supplementary Fig.3) or for control untransplanted mice. Higher circulating human IFN-γ (p=0.01) and IL-5 (p<0.05) concentrations were observed in RA83 treated mice at day 30 compared with alemtuzumab treated mice, indicating greater T cell engraftment of the RA83 treated mice at this time.
Administration of RA83 i.p. was as effective as the i.v route (data not shown), so i.p. injections were used in subsequent experiments.

To make direct temporal comparisons between treatments, each time a mouse attained a GVHD score of $\geq 5$, it was sacrificed together with the highest scoring mouse from each other treatment group in the experiment. This occurred 8-13 days post-transplant, depending on the donor. At the time that control hu-SCID mice were suffering severe GVHD, untransplanted mice and transplanted mice treated with either RA83 or alemtuzumab had significantly reduced GVHD scores (Fig.4a) and body weight loss (Fig.4b). Liver and lung from the RA83 treated hu-SCID mice had reduced GVHD associated lymphocytic infiltration and cell damage (Fig.4c, d). RA83 and alemtuzumab treatments each substantially reduced the circulating concentrations of the human cytokines IFN-$\gamma$, IL-8 and IL-10 ($p<0.05$), but maintained IL-4 (Fig.4e). TNF and IL-5 concentrations were also reduced by alemtuzumab treatment ($p<0.05$) but any reductions due to RA83 did not reach statistical significance (Fig.4e).

**In vivo anti-CD83 antibody treatment prevents GVHD without ablative immunosuppression and loss of T cell immunity.**

To assess the effects of RA83 treatment on the recovery of CMV specific human CD$^8^+$ T cells, we repeated the above temporal comparison using a CMV$^+$ HLA-A*$^{*0201}$ PBMC donor. For this donor, 25$\mu$g of RA83 per mouse prevented GVHD as effectively as 125$\mu$g. Median GVHD scores were 0.5 (range 0.5-1.5) for 25$\mu$g RA83, 1.0 (0.5-2.25) for 125$\mu$g RA83, and 0.5 (0-0.5) for 5$\mu$g alemtuzumab ($p<0.001$ for each treatment when compared with 6.0 (5.0-6.5) for 25$\mu$g RAneg and 5.5 (5.0-6.0) for 125$\mu$g RAneg treated controls; $n = 4-5$ mice per treatment, 8-11 days post-transplant). We also tested the therapeutic immunosuppressant ATG, a rabbit polyclonal anti-human thymocyte globulin.
which, like alemtuzumab, has broad specificity for human leukocytes. ATG was as effective as RA83: the mean GVHD score for hu-SCID mice treated with 125µg ATG was 1.0 (0-2.25).

The numbers of live cells in spleen, femoral bone marrow and peritoneal washings from each sacrificed mouse for all treatments were counted at the time that the control hu-SCID mice developed severe GVHD. The three samples from each mouse were then pooled for flow cytometric analysis, enabling calculation of absolute numbers of human CD45+ leukocytes, total human CD8+ T cells and CMVpp65 pentamer+ CD8+ T cells recovered from each mouse. Very low numbers of human CD45+ leukocytes were recovered from hu-SCID mice treated with ATG and alemtuzumab, compared with controls (Fig.5a). The median number of human CD45+ leukocytes recovered from RA83 (125µg) treated mice was 147 times greater than for ATG treated mice (p<0.001, Fig.5a), even though both antibodies protected mice from GVHD equally well. Recoveries of total CD8+ T cells and CMVpp65 pentamer+ CD8+ T cells followed similar trends (Fig.5b,c, Supplementary Fig.5). Notably, the median number of CMVpp65 pentamer+ CD8+ T cells recovered from each RA83 (125µg) treated mouse was 255, compared with zero for alemtuzumab treated mice (p<0.05).

To further assess the effect of RA83 treatment on retention of immunity we harvested cells, as above, from RA83 and RAneg treated hu-SCID mice transplanted with PBMC from a different HLA-A*0201+ donor. Human cells were isolated (see Materials and Methods) and stimulated with irradiated autologous PBMC loaded with HLA-A*0201+ peptides from FMP and from the model tumor antigen Mart1. This induced expansion of FMP and Mart1 pentamer+ CTL (not shown), which specifically lysed FMP and Mart1 peptide loaded T2 cells, respectively, regardless of RA83 or RAneg treatment of the hu-SCID mice from which the precursor cells were recovered (Fig.6a,b).
We assessed the potential for GVL by stimulating, \textit{in vitro}, cells recovered from RA83 and RAneg treated hu-SCID mice with irradiated autologous PBMC and irradiated allogeneic human leukemic cell lines. For three of the four leukemic cell lines tested, cells from RA83 treated mice produced T cell mediated lytic responses equal to or greater than the cells from RAneg treated hu-SCID mice (Fig.6c-f).

The effect of RA83 treatment on the induction of GVL \textit{in vivo} was investigated in two separate experiments using different PBMC donors. RA83 and RAneg treated hu-SCID mice were immunized, on days 0 and 7 post-transplant, with irradiated U937 or Raji cells ($10^7$ cells, \textit{i.p.}). All hu-SCID mice were sacrificed on day 9 or 10 (donor dependent), when the GVHD clinical score for RAneg treated mice was significantly greater than for RA83 treated mice ($p \leq 0.001$). Human cells recovered from the mice (see Materials & Methods) were used as effectors, without further stimulation, in a chromium release assay with $^{51}$Cr labelled U937 or Raji cells as targets. Human cells recovered from non-immunized hu-SCID control mice were poor lytic effectors against U937 or Raji targets, whereas cells from immunized hu-SCID mice lysed the appropriate targets, regardless of the previous RA83 or RAneg antibody treatment of the mice (Fig.7).

\textit{CD83 antibody prevents acute GVHD}
DISCUSSION

We show here that antibody specific for the DC activation marker CD83 is a potential new therapeutic option for the control of GVHD in alloHSCT. Our *in vitro* and *in vivo* evidence shows that CD83 antibody, not only limits the uncontrolled T cell proliferative response that characterizes GVHD but preserves the donor T cell repertoire, in particular, potentially life saving anti-viral memory T cells and anti-leukemic effectors. Current GVHD prophylaxis, be it *ex vivo* T cell depletion of the graft prior to transplant or non-specific immunosuppression, does not spare these vital components of the donor graft (23).

Our *in vitro* comparison of the polyclonal anti-CD83 antibody (RA83) with the CD52 mAb, alemtuzumab, provided insight into their mechanisms of immunosuppression. Both antibodies reduced allo-immune T cell proliferation and expression of the TH1 cytokine IFN-γ, but alemtuzumab also reduced the TH2 cytokine IL-5 and the immunosuppressive cytokine IL-10, presumably as a result of its pan leukocyte depleting capacity. RA83 had little direct effect on T cells, as it did not significantly reduce the number of viable mononuclear cells recovered from the alloMLC (Fig.1b), nor did it destroy pre-existing CMV specific nor flu specific CD8+ T cells (Fig.2).

Alloproliferating CD4+ T cell blasts, generated during MLC and found in highest numbers after 96 hours, express low levels of CD83 and are also susceptible targets of RA83 mediated ADCC lysis (14) but to a lesser extent than activated CD83+ DC (4.4-fold less lysis at 10:1 E/T ratio, calculated from data in (14)). Delayed addition of RA83 to the MLC reduced its inhibitory effect on alloproliferation, completely negating its effect, when administered after 96 hrs to target the alloreponding T cells (14). Thus the
functional cellular target of RA83 is present early, rather than late during the course of MLC, consistent with the principal target being the DC.

To obtain in vivo evidence for immunosuppressive efficacy of CD83 antibody we chose a chimeric human/murine model of GVHD because antibodies specific for human CD83 and CD52 antigens, such as RA83 and alemtuzumab, can be tested. The SCID mouse was used in preference to other immunodeficient strains, despite the requirement for higher donor cell doses (24), because the hu-SCID model of GVHD has been used more extensively than others (eg, (25)), it is complement replete (26) and functional human NK cells are present (27), allowing for antibody mediated CDC and ADCC lysis of human target cells in vivo. The hu-SCID model of GVHD requires human CD4⁺ T cells (17) but the stimulatory cells had not previously been identified and murine antigen presenting cells are known to be weak stimulators of human T cells (21, 22, 28, 29). We therefore considered it likely that human DC in the graft would play a major role and this was supported by finding that purified human T cells required supplementation with human DC to be fully effective at inducing GVHD in conditioned SCID mice (Fig.3a).

Using the hu-SCID model, we found that RA83 prevented GVHD (Fig.3c). RA83 treated mice had significantly lower GVHD scores and less weight loss than RAneg treated or untreated controls when the latter had severe GVHD (Fig.4a,b). Circulating levels of human IFN-γ and IL-8 were substantially reduced in RA83 treated mice, but not IL-4 (Fig.4e), consistent with a GVHD-ameliorative TH2 cytokine milieu predicted by the in vitro studies (Fig.1c). RA83 treatment is expected to leave potentially tolerogenic non-activated (CD83⁻) DC intact and these may induce regulatory T cells (30) with potential allo-suppressive benefits. RA83 may also selectively retain TH2 inducing plasmacytoid DC (31), as they express comparatively low levels of CD83 when activated (13).
Alemtuzumab treatment also prevented GVHD in the hu-SCID model, but at the expense of T cell engraftment, particularly CMV specific CD8+ T cells (Fig.5c). In marked contrast, RA83 treatment prevented GVHD without the loss of specific donor T cell immune memory. Treating transplant patients with CD83 antibody at the time of conditioning should limit the generation of a large pool of allo-reactive GVHD inducing effector/memory T cells immediately after transplantation, which, at least in an allogeneic mouse model, can induce GVHD at any time subsequently in the absence of host DC (32-34). Antibody that targets activated DC, such as that studied here, should also preserve donor T cell immunity to common infections such as CMV, which cause major post transplant mortality and morbidity. Current immunosuppressants that target T cells, exemplified here by alemtuzumab and ATG, compromise post-transplant immunity, particularly to CMV (35) and other infectious agents.

T cell depletion also compromises the GVL effect and predisposes to recurrence of leukemia (8, 36). Theoretically, specific depletion of activated DC to control GVHD in clinical alloHSCT should preserve the anti-leukemia T cell repertoire. Supporting this, we obtained, from cells recovered from RA83 treated hu-SCID mice, human effector T cell responses to the naïve tumor associated antigen Mart1 (Fig.6b) and to allogeneic human leukemic cell lines, particularly to U937 (AML, Fig.6c) and Raji (B cell lymphoma, Fig.6d) cells.

A potential disadvantage of targeting CD83+ DC for the prevention of GVHD in alloHSCT patients is that these DC may be required for the induction of GVL effectors from anti-leukemic precursors, be they T or NK cells. Reddy et al showed in a mouse “acute leukemia” (sic) model that host and, to a lesser extent, donor DC are required for effective GVL post-alloHSCT (37), although the role of DC activation was not explored. Encouragingly, we found that RA83 did not prevent in vitro induction of allogeneic
cytotoxic anti-leukemic cell line activity by co-cultured PBMC (Supplementary Fig.6). Furthermore, RA83 treatment of hu-SCID mice immunized with the human leukemic cell lines U937 and Raji did not impair in vivo induction of anti-leukemic cytotoxic T cell effectors (Fig.7). Nevertheless, if GVL proves to be compromised by peri-transplant DC targeted treatment, it could be managed by subsequent vaccination with leukemia antigen loaded donor DC or by donor leukocyte infusions, perhaps boosted by donor vaccination prior to transplantation. Alternatively, to retain peri-transplant anti-leukemia priming by host CD83+ DC, antibody treatment might await the appearance, post-transplant, of activated DC in the circulation, an event which precedes clinical GVHD (38).

Any significant improvement in the control of GVHD as a result of targeting DC may allow wider utilization of alloHSCT for malignant conditions and for non-malignant conditions, which do not require GVL. Our data provides compelling evidence that depletion of activated human DC is a promising alternative GVHD prevention strategy that warrants further investigation. A DC targeted therapy, which prevents alloreactive GVHD inducing T cell generation, even allowing immature DC mediated tolerance induction, which nonetheless, still preserves protective and therapeutic T cells would also have wider applications in allotransplantation.
MATERIALS AND METHODS

Antibodies. Rabbit polyclonal IgG anti-human CD83 (RA83) was prepared as described previously (14, 39), but with an added CD83 antigen affinity purification step (see Online Supplementary Method for preparation and validation). Clinical grade ATG (Fresenius) and alemtuzumab (Schering) were obtained from the Mater Health Services Pharmacy.

Human PBMC and cell preparations. PBMC were obtained with informed consent from normal healthy donors either as whole blood donations or by leukapheresis (approved by the Mater Human Research Ethics Committee). PBMC were purified by Ficoll-Hypaque centrifugation, cryopreserved and stored at -180°C until required. Specific leukocyte populations were depleted from PBMC using Miltenyi AUTOMACS and either directly conjugated CD14 or CD16+CD56 microbeads (Miltenyi), or indirectly, with CD8 or CD19 antibody followed by anti-murine IgG microbeads. T cells (97.0% CD3+) were prepared by staining PBMC with a mixture of unconjugated antibodies (CD11c clone MCA 2087 Serotec; CD14 CMRF-31 in-house; HLA-DR L243 ATCC; CD16 3G8, CD19 J4.119, CD20 B9.E9, CD56 n901 Beckman Coulter; CD34 HPCA-1 BD Biosciences), followed by anti-mouse Ig microbeads. Monocyte derived DC (MoDC) were generated as described (13), from monocytes purified from PBMC by CD14 immunomagnetic selection. CMV positive donors were identified by serological testing from a panel of HLA-A*0201+ normal donors, and confirmed by CMVpp65 pentamer staining (see below).

MLC. Irradiated human PBMC stimulators and non-irradiated human PBMC responders from another donor were cultured together, each at 10^6/ml for 7 days. Cultures were
performed in 96 well microplates for proliferation assays (14), or in T25 flasks (10ml volume) for cytokine and anti-viral immune memory assays.

Cytokine analysis. Human cytokines in MLC supernatants and in hu-SCID mouse sera were analyzed using BD Biosciences Human Cytokine Flex Sets and a LSR II flow cytometer (data analysis by BD FCAP software). Cross-reactivity with murine cytokines was minimal, as determined by analyzing sera from murine alloHSCT experiments and untransplanted controls.

CTL induction. Cells recovered from MLCs or from hu-SCID mice, at 10^6/ml in media containing IL-7 (10ng/ml), were stimulated with peptide antigen (HCMV pp65 495-504 NLVPVMVATV, Influenza A MP 58-66 GILGFVFTL, Mart1/MelanA 27-35 ELAGIGILTV, Malaria CS 334-342 YLNKIQNSL or HIV Gag 77-85 SLYNTVATL) or with irradiated human leukemic cell lines (ALL-19 (40), U937, Raji, Nalm6) and irradiated (30Gy) autologous PBMC. IL-2 (25 IU/ml) was added every 2-3 days. At 7 day intervals, cells were restimulated with irradiated autologous PBMC and peptide or irradiated leukemic cell line. T cell mediated lysis of ^51Cr labelled leukemic cell lines or peptide loaded T2 cells (10^3/well) was assayed (41). Excepting the experiment shown in Supplementary Fig.6, NK cell mediated lysis was blocked with unlabelled K562 cells (≥5x10^4/well). Lysis of malaria or HIV peptide negative controls was minimal. CD3-PE, CD8-PerCPCy5.5, HLA-A*0201/NLVPVMVATV pentamer-APC (Proimmune Ltd) cells were enumerated by flow cytometric staining.

Hu-SCID mouse model of GVHD. Animal procedures were approved by the University of Queensland Animal Ethics Committee. Female SCID mice (C.B-17-Igh-1^b-Prkdc^scid)
were purchased from the Animal Resource Centre (Perth, WA, Australia), housed in sterile micro-isolator cages and given autoclaved food and water. On day -1, 5-7 week old mice were injected i.p. with 20µl asialo-GM-1 (WAKO) and irradiated (137Cs, 325cGy). 50x10^6 washed human PBMC in 200µl were injected i.p. on day 0 (17). Mice were assessed daily using a GVHD scoring system that assesses weight loss, posture, activity, fur texture, and skin integrity (42) modified by addition of diarrhea. The overall score for each mouse was the sum of the 6 individual scores (0-2 for each). Mice with severe GVHD (overall score ≥5) were sacrificed and tissues, blood and peritoneal washings (in RPMI1640) taken for analysis. Some hu-SCID mice were immunized on day 0 and day +7 with the U937 or Raji human leukemic cell lines (i.p., 10^7 cells, irradiated at 3000cGy).

Antibodies (RAneg, RA83, alemtuzumab, ATG) were administered by i.p. injection 3 hours prior to human PBMC injection on day 0. The RA83 circulating half-life was estimated by ELISA of blood samples drawn from SCID mice up to 14 days after a single i.p. injection.

**Cell and tissue analysis.** Flow cytometric analyses were performed using BD FACS Calibur and LSR II flow cytometers. Cells from peritoneal cavity, femoral bone marrow and spleen were treated with red cell ACK lysis buffer and live cells counted by Trypan blue exclusion. PBMC and cells from mice were stained with fluorophore conjugated antibodies (human CD3 clone SK7, CD8 SK1, CD45 2D1, mouse CD45 30-F11 BD). Flow cytometry data were analyzed using FCS Express software.

In some experiments, cells harvested from hu-SCID mice as above were combined and human leukocytes recovered by density gradient centrifugation (Ficoll
Hypaque) and depletion with mouse CD45 immunomagnetic beads (Miltenyi), for subsequent *in vitro* CTL experiments.

Mouse tissues were fixed in 10% formalin, paraffin embedded, sectioned and stained with haematoxylin and eosin. The degree of lymphocytic infiltration was assessed by examination of 1-2 entire full face sections. Liver was scored between 0 (nil lymphocytes) and 2.5 (moderate infiltration), to which 1.0 was added for any focal apoptosis and hepatitis. Lung was scored between 0 and 3.5 for degree of perivascular lymphocytic infiltration, and 1.0 added for any peribronchiolar infiltration.

**Statistical Analysis.** We used GraphPad Prism 4.0 (GraphPad Software, Inc) and SYSTAT 10.2 (SYSTAT Software, Inc). Survival data were analyzed using the Kaplan-Meier log-rank test, with Bonferroni corrected post-test multiple comparisons. All other data (log transformed where clearly non-Gaussian, indicated in figure legends) were analyzed by ANOVA (repeated measures where indicated, in legends) and, if statistically significant (p<0.05), Bonferroni corrected multiple comparisons post-tests were done.
ONLINE SUPPLEMENTAL MATERIAL

Supplementary Figure 1 shows histology of liver from a hu-SCID mouse with GVHD and Supplementary Figure 2 shows human leukocyte engraftment of hu-SCID mouse spleen, bone marrow and peritoneal cavity, by flow cytometric staining. Supplementary Figure 3 shows weight change and GVHD scores for antibody treated and control hu-SCID mice, while Supplementary Figure 4 shows human leukocyte engraftment 30 days post-transplant in hu-SCID mice treated with RA83. Supplementary Figure 5 shows human CMV specific CD8+ T cells from a RA83 treated hu-SCID mouse. Supplementary Figure 6 shows the effect of RA83 on in vitro induction of anti-leukemic cell line cytotoxicity. Supplementary Method, with Supplementary Figures 7-10 embedded, describes the preparation and validation of the RA83 antibody. The online supplemental material is available at …………

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efficiency compared to monocyte-derived dendritic cells despite higher levels of MHC class I expression. *J Immunother* 29:596-605.

LEGENDS TO FIGURES

Figure 1. RA83 reduces T cell proliferation and expression of IFN-γ in alloMLC without non-specific ablation of leukocytes. (a) Cell proliferation ($^3$H-thymidine incorporation, CPM) was significantly reduced in MLCs treated with 5µg/ml of RA83 or with 5µg/ml of alemtuzumab (Alem), compared to 5ug/ml of RAneg (non-immune rabbit IgG negative control antibody). Median and interquartile range shown for n = 9 stimulator/responder combinations. (b) The number of viable leukocytes recovered from 7 day MLCs was not affected by RA83 but was substantially reduced by alemtuzumab. Median viable cell count and interquartile range shown for n = 11 stimulator/responder combinations. (c) RA83 reduced 7-day MLC concentrations of IFN-γ (note log scale) by a median of 64%, but TNF, IL-4, IL-5 and IL-10 were not significantly affected. Alemtuzumab similarly reduced 7-day MLC concentrations of IFN-γ (median 75% reduction), IL-5 and IL-10. Graphs show, for each antibody treatment, individual cytokine concentrations for n = 6 stimulator/responder combinations, each linked by lines. Raw cytokine data contained zero values, therefore 1.0 pg/ml was added to all cytokine data to enable log transformation for statistical analysis. (NS – not significant, p>0.05).

NS and p-values are for repeated measures ANOVA followed by Bonferroni corrected multiple comparisons post-tests for RA83 and alemtuzumab each compared to RAneg treatment. Data is also shown for untreated (Nil) MLCs, which were not statistically significantly different from RAneg treated cultures.

Figure 2. RA83 treatment preserves virus specific T cell immunity in allogeneic MLC. (a) Number of CMVpp65 pentamer positive CD8$^+$ T cells surviving after 7 days in antibody treated 10ml MLCs. Day 0 column shows the starting number of cells. (Data
shown for 3 different HLA-A*0201\(^+\) CMV\(^+\) donors; lines link data from the same donor).

(b) A substantial viral antigen specific functional CTL response was generated from 7 day RA83 (▲), RA\(^-\) (▼) and Nil (■) treated MLCs, but not from alemtuzumab (♦) treated MLCs. Graphs show mean percentage lysis of CMV and FMP peptide loaded, \(^{51}\)Cr labelled target cells by CTL effectors generated from the treated MLCs. \(p<0.0001\) for CMV (n=3 donors), \(p<0.02\) for FMP (n=4 donors) for repeated measures ANOVA. Subsequent Bonferroni corrected multiple comparisons testing showed that alemtuzumab treatment was significantly different from each of the other treatments (\(p<0.001\) for CMV and \(p<0.01\) for FMP).

**Figure 3. Hu-SCID model of GVHD.** (a) Human DC enable full GVHD induction: Administration of purified T cells (97\% CD3\(^+\)) alone induced severe GVHD in only 3 out of 10 mice (□, not significantly different from untransplanted controls, ∆) but co-administration of 2.5\% autologous monocyte derived DC restored the incidence of severe GVHD (6 out of 7 mice; ○) to PBMC levels (10 out of 10 mice; ◊, \(p>0.05\) for MoDC + T cells \(vs\) PBMC). \(p = 0.025\) for T cells only \(vs\) MoDC + T cells. (b) Monocytes and B cells are not required for GVHD induction: *In vitro* depletion of monocytes (×), B cells (+), CD8\(^+\) T cells (○) and NK cells (✳) from human PBMC, prior to administration to mice, did not prevent or delay development of GVHD (n = 5 mice for each depletion, \(p>0.05\) for each depletion \(vs\) undepleted PBMC transplanted mice, ◊). Administration of irradiated (3000cGy) PBMC (∇) or of vehicle alone (untransplanted, ∆) did not induce GVHD as assessed by GVHD score. (c) *In vivo* treatment with anti-CD83 antibody prevents GVHD: *i.p.* injection of conditioned SCID mice with RA83 (125µg - ∆, 25µg - ▲) or alemtuzumab (5µg – ♦) 3 hr prior to PBMC administration prevented GVHD (combined data for 3 PBMC donors, 8 - 18 mice for each treatment; ✤ = no transplant, ■)
Figure 4. *In vivo* treatment of hu-SCID mice with anti-CD83 antibody significantly reduced GVHD score (a), weight loss (b), lymphocyte infiltration in liver (c) and in lung (d) and circulating human IFN-γ, IL-8 and IL-10 (e). RA83 (125µg/mouse) was always compared with RAneg (125µg); alemtuzumab (5µg) and untransplanted (“No tx”) mice were always compared with Nil antibody treated, transplanted mice. p-values are shown only when <0.05. (The RA83 outlier in (d) and the alemtuzumab outliers in (e) for IL-5 & IL-10 were omitted for statistical analysis). n = 5 - 7 mice per treatment, each sacrificed 8 - 11 days post-transplant, when a Nil or RAneg treated control mouse developed severe GVHD (score ≥5). Horizontal lines are median values.

Figure 5. RA83 treatment did not prevent engraftment of human leukocytes (a), total CD8⁺ T cells (b), or CMV specific CD8⁺ T cells (c) in the hu-SCID mouse model of GVHD. Dots show, for each treated hu-SCID mouse, the total number of human cells recovered from bone marrow, spleen and peritoneal cavity, combined, 8 - 11 days post- transplant. Heavy horizontal lines show median values. Raw CMV data contained zero values, therefore 1.0 was added to all CMV data to enable log transformation for statistical analysis (p-values shown for selected post-tests, NS – not significant).

Figure 6. RA83 treatment of hu-SCID mice did not impair subsequent *in vitro* induction of flu (a) and Mart1 (b) specific cytotoxic T cell effectors and allogeneic anti-leukemic cytotoxic T cell effectors (c)-(f) from cells recovered from hu-SCID

CD83 antibody prevents acute GVHD
mice. Ten-day post-transplant hu-SCID mice treated with 125ug RA83 (n=19; GVHD score = 0.5 on d9) or RAneg (n=5; GVHD score = 3.25 on d9) were sacrificed, cells from spleen, bone marrow and peritoneal washings were combined and human leukocytes recovered (see Materials and Methods). These cells and, as a control, an equal number of freshly thawed PBMC from the same donor, were stimulated in vitro with irradiated autologous PBMC plus either peptide antigen or irradiated leukemic cell lines. After two rounds of stimulation, T cell mediated lysis of FMP peptide loaded T2 cells (a), U937 (c), Raji (d), Nalm6 (e), and ALL-19 (a human primary ALL passaged in NOD-SCID mice (40)) (f) leukemic cell lines was measured by $^{51}$Cr release assay. Specific killing of T2 cells loaded with peptide from the naïve melanoma associated antigen Mart1 was assayed after three rounds of stimulation (b). (▲- RA83, ▼- RAneg, ■ – freshly thawed donor PBMC). Dotted lines in (a) & (b) show minimal lysis of T2 cells loaded with irrelevant HIV peptide (RA83: p<0.01 for FMP, 0.001 for Mart1 c.f. HIV).

Figure 7. RA83 treatment did not impair in vivo induction of human allogeneic anti-leukemic cytotoxic T cell effectors as a result of immunization of hu-SCID mice with irradiated leukemic cell lines. RA83 or RAneg treated hu-SCID mice were immunized by i.p. injection on day 0 and day 7 with $10^7$ irradiated (3000cGy) U937 cells (a), Raji cells (b) or vehicle alone. All mice were sacrificed on day 10-11 and cells from peritoneal cavity, spleen and bone marrow were combined within each cohort. After removal of dead cells, erythrocytes and murine CD45$^+$ cells the remaining cells (>95% human CD45$^+$) were tested for cytotoxic activity, without further stimulation, in a $^{51}$Cr release assay using U937 (a) or Raji (b) cells as Targets. Effectors from ▲- immunized RA83 treated hu-SCID mice, ▼- immunized RAneg treated hu-SCID mice, ■ – freshly thawed donor PBMC, □ - non-immunized RAneg treated hu-SCID mice.
Figure 1.
Figure 2.

a) CMV specific CD8$^+$ T cells

b) Specific lysis (%) of CMV and Flu at different Effector : Target ratios.

Day 0, Day 7 RA$	ext{neg}$, Day 7 RA$	ext{pos}$, Day 7 Alen, Day 7 Nil
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.

a (U937 targets)

b (Raji targets)
Supplementary Fig.1. Periportal liver apoptotic bodies (short arrow) and lymphocytic infiltration (long arrow) in hu-SCID mouse with GVHD (x400). H & E stained formalin fixed section of liver.
**Supplementary Fig.2.** Human leukocyte engraftment in hu-SCID mice. Flow cytometry dot-plots show human CD45+ leukocytes in peritoneal cavity, spleen and bone marrow 9 days after *ip* injection of 50 x 10^6 PBMC into a conditioned SCID mouse. GVHD score at time of sacrifice = 5.25 (severe GVHD). Human leukocyte engraftment levels for this animal were 98.2% for peritoneal cavity (mouse CD45+ = 1.7%); 76.2% for spleen (mouse CD45+ = 3.8%); 47.1% for bone marrow (mouse CD45+ = 34.1%).
Supplementary Fig.3. Mean (& 1SEM) percent weight changes and GVHD scores for RA83, alemtuzumab (Campath 1H) and RAneg negative control antibody treated hu-SCID mice for up to 25 days post-transplant. Mice were sacrificed when GVHD score attained 5.
Supplementary Fig. 4. Human leukocytes were found in spleen 30 days post-transplant in hu-SCID mice treated with RA83 (25µg/mouse). (0.33% of live gated cells in the example above, mouse CD45⁺ cells = 56.9%). Circulating human IFN-γ and IL-5 concentrations at day 30 were significantly higher in RA83 treated mice compared to alemtuzumab (Campath) treated mice (Mann-Whitney U-test, p=0.001, p<0.05 respectively), consistent with higher engraftment in RA83 treated hu-SCID mice. “No Tx” mice did not receive a human PBMC graft.
Supplementary Fig.5. Detection of human CMV specific CD8+ T cells in hu-SCID mice. A conditioned SCID mouse was injected with 50 x 10^6 human PBMC from a CMV+ HLA-A*0201+ donor and treated with RA83 (125ug/mouse). After 10 days, cells from spleen, peritoneal cavity and bone marrow were pooled and analysed for CMVpp65 pentamer positive human CD8+ T cells by flow cytometric staining. Human CD45+ mouse CD45- leukocytes were gated and CMVpp65 pentamer positive CD8+ T cells within enumerated and expressed as a percentage of human CD45+ leukocytes.
Supplementary Fig.6. RA83 does not substantially affect *in vitro* induction of anti-leukemic cell line activity by allogeneic PBMC. Irradiated (30Gy) U937 (AML) or K562 (CML) cells (0.5 x 10^6/ml) were cultured with PBMC (10^6/ml) and 5ug/ml of RA83, RAneg or Nil antibody. On day 6, 50% of the medium was replaced with fresh medium and IL-2 (20 U/ml). On day 14, fresh medium, antibodies, irradiated leukemic cells and irradiated (30Gy) autologous PBMC were added, and fresh medium & IL-2 as above on day 19. On day 22 the cells were harvested and tested in a 4 hour 51Cr release assay using 51Cr labelled U937 and K562 cells as targets.
Online Supplementary Method

Preparation and validation of rabbit polyclonal anti-human CD83 (RA83)

RA83 was prepared from serum of rabbits immunized with CD83-Ig, a recombinant fusion protein consisting of the extra cellular domain of human CD83 fused to human IgG1-Fc (reference 39, Hock et al., 2001. Int Immunol 13;959). Total IgG was prepared from rabbit serum by Protein A affinity chromatography, which was then depleted of anti-human IgG-Fc immunoreactivity by passage through immobilized human IgG (Intragam P, a human IgG preparation for iv injection, CSL Ltd, Parkville, Australia; immobilized on a 1ml NHS-activated HiTrap column, Amersham), as described (reference 14, Munster et al., 2004. Int Immunol 16;33). To ensure specificity for CD83 of the RA83 used in the present study, an additional purification step was added in which specific anti-human CD83 immunoreactive IgG was affinity isolated on CD83-Ig immobilized on a 1ml NHS-activated HiTrap column. This final antigen affinity isolation step increased the antibody potency by >25-fold (see ELISA results below, Supplementary Fig.7).

Negative control rabbit IgG (RAneg) was prepared from serum from non-immunized rabbits by Protein A affinity chromatography followed by passage through a column of immobilized human IgG, as above.

Both RA83 and RAneg were pure IgG by SDS-PAGE. RA83, but not RAneg, was strongly immunoreactive by ELISA with CD83-Ig but not with human IgG (Supplementary Fig.7). RA83, but not RAneg, stained stable human full length CD83 transfected FDCP1 cells but not untransfected FDCP1 cells (Supplementary Fig.8) nor mouse CD83 transfected cells (not shown). RA83, but not RAneg, blocked staining of the human CD83 transfectant above with the Hb15a mAb for human CD83 (Beckman Coulter, Supplementary Fig.8). There were no obvious differences
between RA83 and RAneg in staining fresh human PBMC. Both antibodies stained monocytes, due to non-specific Fc receptor binding, but not B cells nor T cells. Overnight cultured PBMC had a small population of RA83⁺ CD14⁻ CD19/20⁻ cells (activated DC) and a small subset of RA83⁺ B cells (not shown), as previously observed with Hb15a (Hock et al., 1999. Tissue Antigens 53;320).

RA83, but not RAneg, maximally inhibited allogeneic MLC proliferation at 1 ug/ml (Supplementary Fig.9). We have previously reported (reference 14, Munster et al., 2004. Int Immunol 16;33), but not shown the data, that the inhibitory effect of RA83 is specific for CD83 by blocking it with CD83-Ig but not with human IgG. This data is shown in Supplementary Fig.10.
Supplementary Fig.7. Sandwich ELISA of anti-CD83 preparations, showing non-reactivity of RAneg, >25-fold greater potency of the antigen affinity purified RA83 antibody used in the work described here compared to the two step purified preparation, and minimal residual cross-reactivity of RA83 with human IgG (300-fold excess Intragam was added to the RA83 containing test sample). NUNC Maxisorb ELISA plate wells were coated with CD83-Ig (2ug/ml) for capture. After blocking with fish gelatin, diluted antibody containing samples were added, incubated for 90min, washed, bound rabbit Ig was detected with HRP-conjugated sheep anti-rabbit Ig (Silenus, <0.2% cross-reactivity with human Ig) and OPD substrate colour development.
Supplementary Fig.8. RA83 recognises human CD83 expressed on the cell surface. FDCP1 cells expressing full length human CD83 were stained with RA83 or RAneg. Bound rabbit Ig was detected with FITC-conjugated goat anti-rabbit Ig (upper panel). Prior incubation of human CD83 transfected FDCP1 cells with RA83 blocked subsequent binding of FITC-conjugated Hb15a mAb for human CD83 (lower panel).
Supplementary Fig.9. RA83 inhibits proliferation in allogeneic two-way MLC. PBMC from two donors were cultured together, each at $10^5$ cells/well in a 96-well plate, with different concentrations of RA83 or RA neg. On day 5, each well received 1µCi of $^3$H-thymidine. The cells were harvested 18 hours later and incorporated $^3$H quantitated by scintillation counting (CPM).
Supplementary Fig.10. The inhibitory effect of RA83 is mediated specifically via CD83. CD83-Ig (10ug/ml, diagonal lines), but not human IgG (10ug/ml, vertical lines), abrogates the inhibitory effect of RA83 (5ug/ml), but has no effect by itself (Nil) or with RAneg, on alloproliferation in MLC (³H-thymidine incorporation. Histogram shows mean CPM ±2SEM). Horizontal lines show proliferation in the presence of RA83, RAneg or Nil antibody in the absence of CD83-Ig and human IgG.