Supplementary Materials

Supplementary Methods

Participating Transplant Centers

Dana-Farber Cancer Institute, Boston Children's' Hospital and Massachusetts General Hospital (Partners Harvard Cancer Care) (protocol 05030); Children's' Hospital Los Angeles, Los Angeles (05-00079); University of Florida Shands Hospital (117-2009) and MD Anderson Cancer Center (2005-0695).

Phenotyping of DLI before and after alloanergization

Fresh donor PBMC were phenotyped before and after alloanergization by multiparameter flow cytometry. Cells were stained for CD69-FITC, CD25-FITC or -PE, CD4-PE-Cy5, CD8-PE-Cy7, CD3-APC, CD28-FITC, CD278 (ICOS)-PE, CD152 (CTLA4)-PE-Cy5, CD30-FITC, CD70-PE, CD154 (CD40L)-PE-Cy5, CD279 (PD1)-FITC, CD272 (BTLA)-PE, CD127-PE, CD45RA-PE and CD62L-PE-Cy5 and acquired on a BD FACSAria. Viability was assessed by propidium iodide (PI) uptake and PI-positive events were excluded. Compensation controls were single fluorochrome-labelled Compbeads and cells (where appropriate). Isotype controls were used for negative staining controls.

Phenotyping of patient peripheral blood for T-cell subsets

PBMC from patient peripheral blood were phenotyped for T-cell subset markers by flow cytometry. Cells were stained with CD3-ECD, CD4- or CD8-PE-Cy7 and acquired on an FC500 (Beckman Coulter). For naive and memory cell phenotyping cells were additionally stained with CD45RA-PE and CD62L-PE-Cy7. Compensation controls were single fluorochrome-labelled Compbeads (BD) and cells (where appropriate). Isotype controls were used for negative staining controls.

Intracellular cytokine flow cytometry for viral- and WT1- specific T cells

Donor and patient PBMC were stimulated with CMV-, adenovirus- (adeno 6) (both Microbix) or VZV- (ABI) infected human foreskin fibroblast lysates or overlapping WT1-derived peptide pools (JPT technology) for 12 hours in the presence of anti-CD28 (Immunotech, Fullerton, CA) and Brefeldin A (Sigma Aldrich, SA). Positive and

negative controls were Staphylococcal Enterotoxin B (SA) and mock-infected cell lysate/ no peptides respectively. After 12 hours, cells were washed and stained with CD3-ECD, CD4-PE-Cy5, CD8-PE-Cy7 (all Beckman-Coulter), fixed, permeabilized and stained with PE- or FITC-conjugated IFN-γ (BD). The percentage of IFN-γ^+ virus-specific $CD4^+$ and $CD8^+$ T-cells was calculated as (percentage of IFN-γ^+ T-cells after virus lysate stimulation – percentage seen in negative controls) and expressed as the proportion of the $CD4^+$ and $CD8^+$ T-cells. A minimum of 50 IFN-γ^+ events above those seen in negative controls were acquired to give an assay CV of 15% or less.

Table S1 Patients, donors, HLA-mismatches and conditioning

Patient Number	Age (years)	Diagnosis/status pre-HSCT	HSC Donor and age (years)	HLA-mismatch (HvG/GvH)	Conditioning
001	7	AML-IF-CR1	Father, 39	5 Ag/5Ag	TBI
002	41	MDS-IPSS INT2	Sister, 40	4Ag/4Ag	TBI
003	47	Ph+-ALL-CR2	Son, 22	4Ag/5Ag	TBI
004	14	AML-CR2	Sister, 19	4Ag/0Ag	TBI
005	2	AML-CR2	Mother, 43	4Ag/5Ag	MEL
010	25	AML-IF	Father, 50	5Ag/5Ag	MEL
012	22	AML-CR2	Sister, 25	3Ag/2Ag	MEL
013	26	AML-CR1	Brother, 23	2Ag/2Ag	MEL
015	49	MDS-IPSS high	Half-brother, 56	4Ag/5Ag	MEL
016	50	AML-CR2	Daughter, 33	2Ag/2Ag	MEL
018	7	AML-CR1	Mother, 39	2Ag/3Ag	TBI
023	46	AML-CR2	Daughter, 21	2 Ag/4Ag	MEL
025	24	ALL-CR2	Mother, 56	5Ag/5Ag	MEL
026	42	AML-CR2	Brother, 38	5Ag/5Ag	MEL
027	29	ALL-CR2	Sister, 34	5Ag/5Ag	TBI
028	36	MDS/INT1	Brother, 46	5Ag/4Ag	MEL
030	50	AML-IF	Brother, 48	5Ag/5Ag	MEL
033	17	ALL-IF CR1	Father, 40	5Ag/5Ag	TBI
035	37	AML-CR1	Brother, 34	5Ag/5Ag	TBI

AML, acute myeloid leukemia; IF, induction failure; CR, complete remission; MDS, myelodysplasia; IPSS, international prognostic scoring system; INT1, intermediate -1, INT2, intermediate-2; Ph+, Philadelphia chromosome; ALL, acute lymphoblastic leukemia; ag, antigen; MEL, melphalan/, thiotepa/fludarabine/anti-thymocyte globulin/methylprednisolone ; TBI, total body irradiation/ thiotepa/fludarabine/anti-thymocyte globulin/methylprednisolone

Table S2 Cell doses in stem cell transplant, engraftment and chimerism

Patient Number	CD34+ cell dose (x 10^6/kg)	CD3+ cell dose (x 10^6/kg)	Neutrophil engraftment	Chimerism %/day first measured
001	17.42	2.67	19	100/D+25
002	6.02	2	10	100/D+40
003	10.4	2	10	100/D+27
004	11.04	0	17	100/D+27
005	7.57	2	17	99/D+32
010	8.82	2	12	100/D+29
012	6.81	0.40	11	100/D+30
013	17.54	1	9	99/D+30
015	7.30	3	11	>98/D+28
016	9.4	1	9	100/D+28
018	14.4	2.6	12	100/D+17
023	7.8	2.6	9	>98/D+28
025	8.36	1	10	100/D+30
026	9.2	NA	14	100/D+31
027	15.15	1.15	12	>98/D+25
028	5.17	2.79	N/A*	Not done
030	16.5	2.34	8	>98/D+ 18
033	10.8	2.98	16	100/D+26
035	11.31	1.06	14	100/D+26

*autologous reconstitution

Table S3 ADLI dose and timing and allostimulator source

Patient Number	aDLI dose $CD3^+$ cells/kg	Stimulator Donor	Time of aDLI infusion
001	10^3	Mother	D+35
002	10^3	Father	D+35
003	10^3	Son	D+35
004	10^3	Patient	D+42
005	10^4	Father	D+42
010	10^4	Patient	D+34
012	10^4	Mother	D+35
013	None given†	-	-
015	10^5	Patient	D+36
016	10^5	Son	D+35
018	10^5	Father	D+35
023	10^5	Patient	D+35
025	None given§	-	-
026	10^4	Patient	D+35
027	10^4	Patient	D+42
028	None Given‖	-	-
030	10^4	Cousin	D+42
033	10^4	Mother	D+35
035	10^4	Mother	D+35

† died D+32 of bacterial sepsis prior to infusion of aDLI. § Donor not able to donate.
‖ non-engraftment followed by autologous reconstitution.

Table S4 Patient outcomes

Patient Number	aDLI dose $CD3^+$ cells/kg	Relapse	Overall survival	Cause of Death
001	10^3	No	Died D+78	Pulmonary VOD
002	10^3	No	Died D+738	Bleeding post liver biopsy
003	10^3	No	Died D+59	Respiratory Failure
004	10^3	No	Alive 5.8 years*	
005	10^4	No	Alive 9.3 years	
010	10^4	D+180	Died D+473	Disease
012	10^4	D+150	Died D+361	Disease
013	None given	No	Died D+32	Sepsis
015	10^5	No	Alive 9 years	
016	10^5	No	Died D+264	Respiratory failure
018	10^5	No	Alive 8.9 years	
023	10^5	No	Died D+118	Adenovirus
025	None given	No	N/E	
026	10^4	No	Died D+78	Acute GvHD
027	10^4	No	Died D+313	Respiratory Failure
028	None Given	No	N/E	
030	10^4	No	Died D+152	Respiratory Failure
033	10^4	No	Died D+321	Sepsis
035	10^4	No	Died D=99	Respiratory Failure

* lost to follow up at this point

Figure S1 Schema for clinical study

Schematic design of clinical study of delayed infusion of alloanergized donor lymphocyte infusion after CD34-selected haploidentical haematopoietic stem cell transplantation. Pre-transplant conditioning was either TBI-based (TBI 200cGy bid D-11to D-9, Fludarabine 40mg/m^2 D-7 to D-3, Thiotepa 5 mg/kg D-8 to D-7) or chemotherapy-based (Melphalan 140mg/m2 D-8 only, Fludarabine 40mg/m^2 D-6 to D-3, Thiotepa 10 mg/kg D-7 only). All patients received rabbit ATG (Sangstat) 1.5 mg/kg/day from D–6 to D–3. GCSF; Granulocyte colony stimulating factor; PBMC, peripheral blood mononuclear cells; TBI, total body irradiation; ATG, anti-thymocyte globulin; GvHD, graft-versus-host disease.

Figure S2 Alloanergization efficiency of DLI by centre and by dose level

(A) Efficiency of alloanergization of DLI is shown according to clinical centre. There was no significant differences between centres. (B) Efficiency of alloanergization of DLI is shown according to dose level. There was no significant differences between dose levels.

Figure S3 Residual alloreactivity in aDLI and occurrence of acute GvHD after T-cell depleted haploidentical HSCT and aDLI

Median residual alloreactivity in aDLI was higher in patients who went on to develop acute GvHD although this did not reach statistical significance (median 35% versus 20%, p=0.15). Horizontal lines are medians.

.

Figure S4 Phenotype of $CD4^+$ regulatory T-cell (Treg) in patient peripheral blood after T-cell depleted haploidentical HSCT and aDLI

$CD4^+$ Treg were identified phenotypically as $CD25^+CD127^{lo}$ cells (upper left panel). 90% of these cells were $FOXP3^+$ (upper middle panel) whereas only 1% of

$CD4^+CD25^-$ cells were $FOXP3^+$ (upper right panel). Similarly, 80% of $CD4^+FOXP3^+$ cells (lower left panel) were $CD25^+Cd127^{lo}$ (lower middle panel) whereas only 1% of $CD4^+FOXP3^-$ cells were $CD25^+CD127^{lo}$ (lower right panel). Representative dot plots form the peripheral blood of P02016 at D +90 after haploidentical HSCT at aDLI (at D+35) are shown.

Figure S1

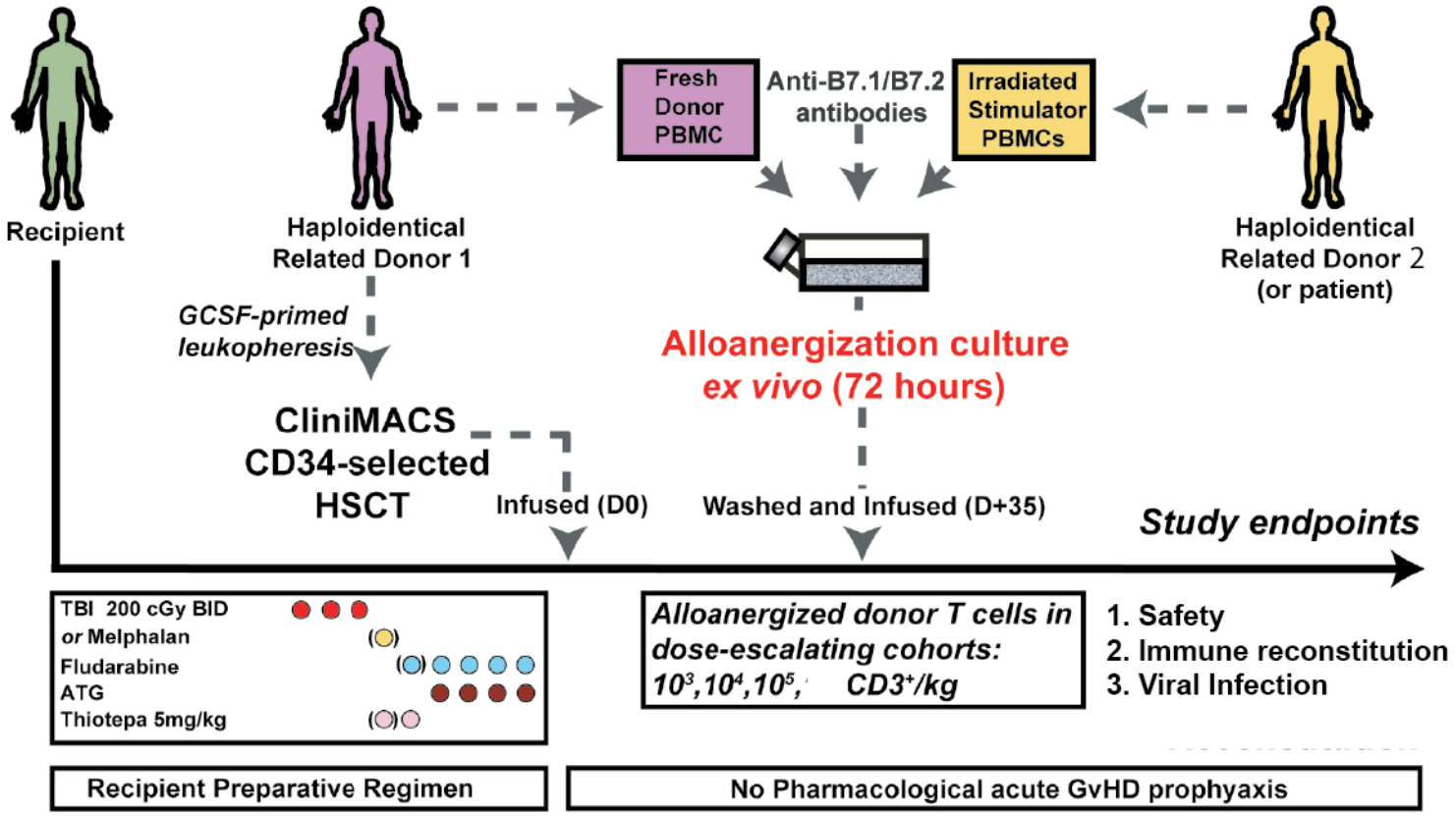

Recipient
Haploidentical Related Donor 1
Fresh Donor PBMC
Anti-B7.1/B7.2 antibodies
Irradiated Stimulator PBMCs
Haploidentical Related Donor 2 (or patient)
GCSF-primed leukopheresis
Alloanergization culture ex vivo (72 hours)
CliniMACS CD34-selected HSCT
Infused (D0)
Washed and Infused (D+35)
Study endpoints
TBI 200 cGy BID
or Melphalan
Fludarabine
ATG
Thiotepa 5mg/kg
Alloanergized donor T cells in dose-escalating cohorts:
10³,10⁴,10⁵, CD3⁺/kg
1. Safety
2. Immune reconstitution
3. Viral Infection
Recipient Preparative Regimen
No Pharmacological acute GvHD prophyaxis

Figure S2

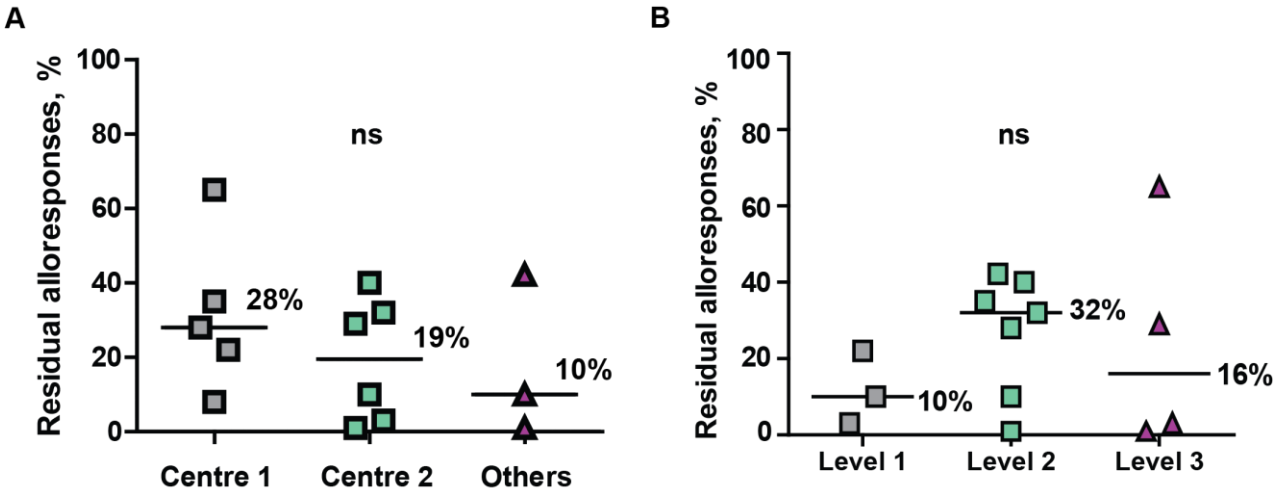


Figure S3

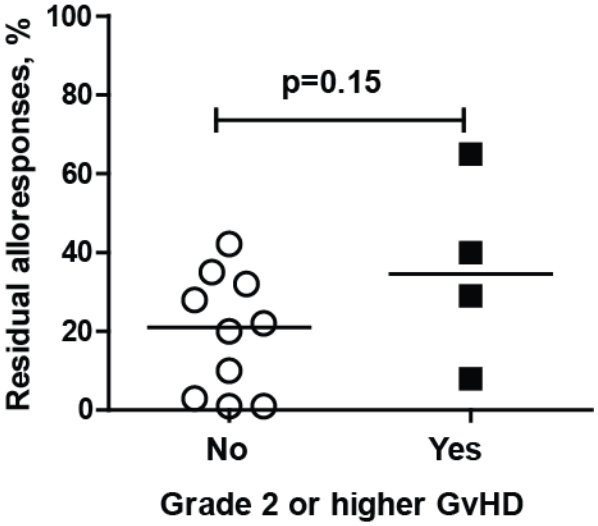
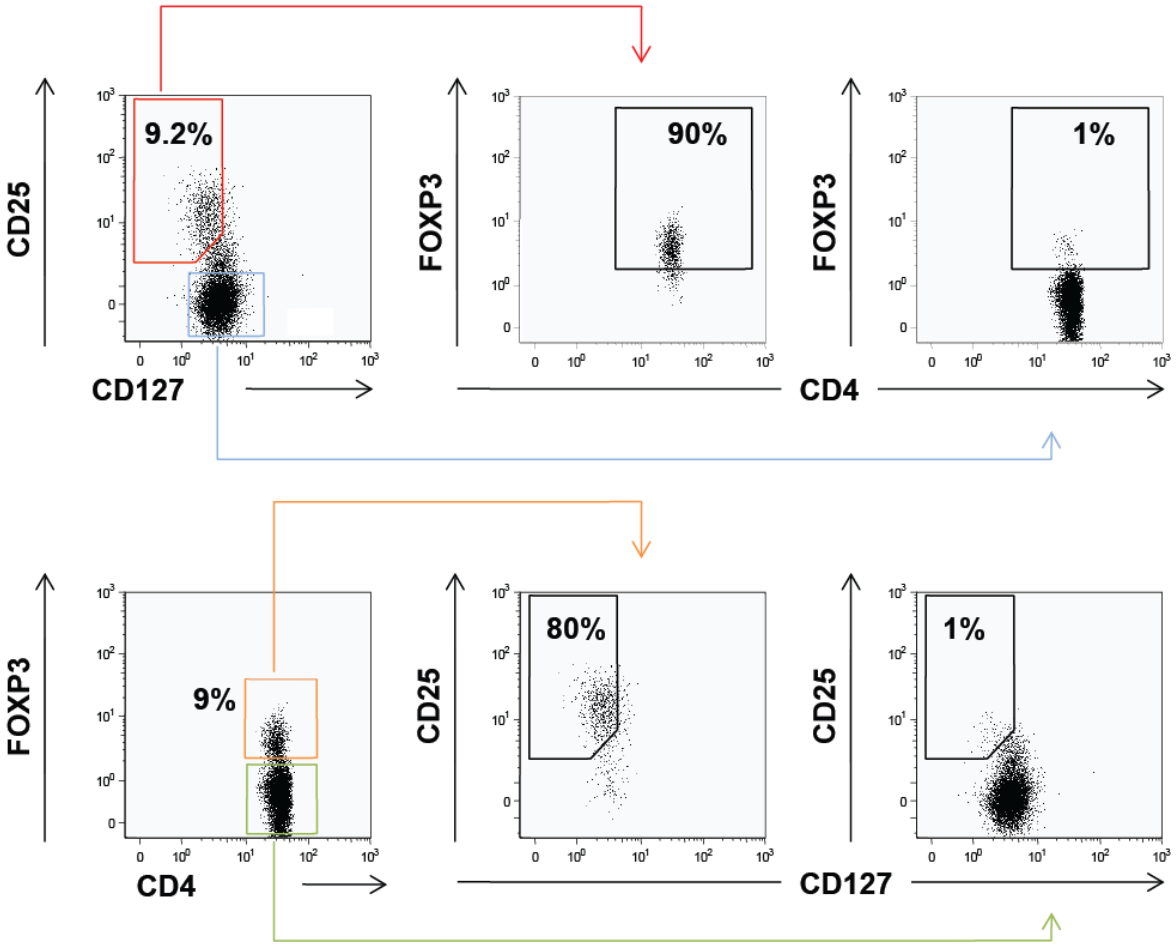


Figure S4


CD25
9.2%
CD127
FOXP3
90%
CD4
FOXP3
1%
CD4
FOXP3
9%
CD25
80%
CD127
CD25
1%