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Supplementary Materials for

MHC Class I–Associated Phosphopeptides Are the Targets of Memory-like Immunity in Leukemia

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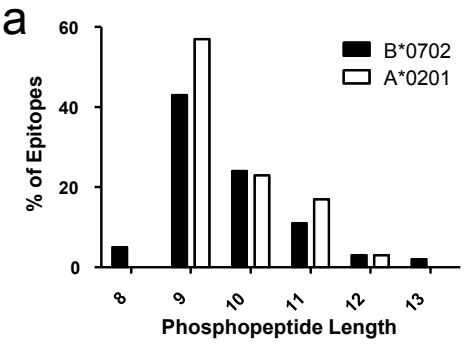
The PDF file includes:

Fig. S1. Detailed HLA-binding motif analysis.
Fig. S2. Immunity against phosphopeptides in healthy donors is phosphate-dependent.
Fig. S3. T cell memory subset mapping of anti-phosphopeptide responses in healthy donors.
Fig. S4. Leukemia-associated phosphopeptide-specific immunity is lacking in CLL patients.
Fig. S5. Deletion (rather than anergy) of anti-phosphopeptide immunity in five patients with CLL.
Fig. S6. Leukemia-associated phosphopeptide-specific immunity is lacking in AML patients but restored after SCT.
Fig. S7. Immunocompetence of patients with AML in complete remission before transplantation.
Table S1. CLL patient characteristics.
Table S2. AML patient characteristics.

a



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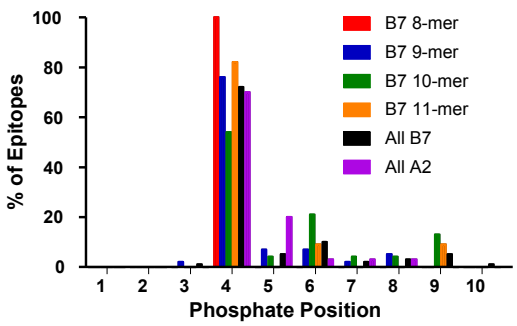


c

9mer HLA-B7 Phosphopeptides

	P1	P2	P3	P4	P5	P6	P7	P8	P9
A	2	2	15	0	2	12	20	12	7
C	0	0	0	0	0	0	0	0	0
D	0	0	0	0	0	5	2	5	0
E	5	0	2	0	0	7	5	7	0
F	0	0	5	0	0	2	0	0	10
G	0	0	2	0	0	20	2	5	0
H	0	0	2	2	0	0	7	0	0
I	0	0	0	0	0	0	2	2	5
K	20	0	12	2	2	2	10	10	0
L	2	0	2	2	5	5	10	0	61
M	0	0	0	0	0	2	0	2	7
N	0	0	0	0	0	0	2	0	0
P	0	85	12	2	63	5	10	5	2
Q	0	0	2	0	2	0	2	0	0
R	61	2	24	7	10	7	10	15	0
S	7	7	2	2	5	15	7	20	2
T	2	0	2	2	0	2	0	2	0
V	0	2	7	0	0	7	7	7	5
W	0	0	2	0	0	0	0	0	0
Y	0	0	2	2	2	0	0	2	0
pSer (X)	0	0	2	71	7	7	2	2	0
pThr (Z)	0	0	0	5	0	0	0	2	0

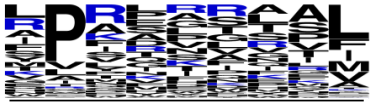
d

9mer Predicted HLA-B7 Phosphopeptides with P4=pSer(X)

	P1	P2	P3	P4	P5	P6	P7	P8	P9
A	11	0	12	0	2	7	8	7	25
C	1	0	0	0	0	1	0	1	0
D	1	0	4	0	1	6	4	5	0
E	9	0	9	0	1	9	9	9	0
F	0	0	1	0	2	1	2	2	3
G	6	0	8	0	0	11	6	5	1
H	2	0	1	0	0	1	0	2	0
I	3	0	2	0	1	4	1	2	8
K	9	0	5	0	0	6	7	2	0
L	4	0	11	0	1	4	5	9	30
M	2	0	1	0	0	1	1	1	2
N	3	0	2	0	0	2	1	1	0
P	13	100	9	0	87	12	22	20	1
Q	4	0	4	0	1	1	3	5	0
R	10	0	8	0	1	11	5	7	0
S	10	0	13	0	1	7	13	10	0
T	7	0	6	0	0	9	7	4	17
V	4	0	5	0	2	5	7	6	13
W	1	0	0	0	0	1	0	0	0
Y	1	0	0	0	1	1	0	2	0
pSer (X)	0	0	0	100	0	0	0	0	0
pThr (Z)	0	0	0	0	0	0	0	0	0

e

9mer HLA-B7 non-phosphopeptides



	P1	P2	P3	P4	P5	P6	P7	P8	P9
A	8	5	11	7	8	6	10	19	6
C	1	0	1	1	1	1	1	1	1
D	1	0	2	6	3	3	2	2	0
E	3	1	2	8	3	2	4	3	1
F	7	1	7	4	5	5	4	3	12
G	4	2	3	5	8	8	5	4	1
H	3	0	2	3	4	2	3	1	1
I	7	3	6	4	5	5	4	6	12
K	5	0	7	4	6	3	4	3	2
L	15	7	5	8	8	7	10	4	36
M	5	2	3	3	2	3	3	3	11
N	3	1	3	4	7	4	5	3	0
P	0	60	4	8	7	5	4	10	1
Q	3	2	3	6	2	5	5	3	0
R	12	2	20	6	9	13	7	4	0
S	6	2	6	8	6	10	9	12	1
T	5	3	4	6	6	8	10	8	2
V	4	8	5	4	7	6	6	8	11
W	3	0	2	2	2	2	2	1	1
Y	6	1	4	3	2	2	3	3	1

f

8mer HLA-B7 phosphopeptide ligands

	P1	P2	P3	P4	P5	P6	P7	P8
A	0	0	0	0	20	0	0	0
C	0	0	0	0	0	0	0	0
D	0	0	0	0	0	0	20	0
E	0	0	0	0	0	0	0	0
F	20	0	0	0	0	0	0	20
G	0	0	0	0	0	0	20	0
H	0	0	0	0	0	0	0	0
I	0	0	20	0	0	0	0	0
K	0	0	0	0	0	0	0	0
L	0	0	20	0	20	20	40	60
M	0	0	0	0	0	0	0	0
N	0	0	0	0	0	0	0	0
P	0	60	0	0	60	20	0	20
Q	0	0	0	0	0	0	0	0
R	80	0	40	0	0	0	20	0
S	0	40	20	0	0	0	0	0
T	0	0	0	0	0	20	0	0
V	0	0	0	0	0	40	0	0
W	0	0	0	0	0	0	0	0
Y	0	0	0	0	0	0	0	0
pSer (X)	0	0	0	100	0	0	0	0
pThr (Z)	0	0	0	0	0	0	0	0

g

10mer HLA-B7 phosphopeptide ligands

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
A	0	0	8	0	21	0	4	0	4	8
C	0	0	0	0	0	0	0	0	0	0
D	0	0	0	0	4	0	4	0	4	0
E	0	0	4	0	0	8	0	0	4	0
F	4	0	0	0	0	0	8	4	0	8
G	8	0	4	0	4	4	4	8	0	0
H	0	0	0	0	4	0	4	0	0	0
I	0	0	4	0	0	0	0	8	0	8
K	8	0	0	0	0	0	8	0	4	4
L	0	0	0	4	0	0	13	25	4	63
M	0	0	0	0	0	0	0	0	0	4
N	0	0	4	0	0	0	0	17	0	0
P	0	92	17	13	54	17	25	4	4	4
Q	8	0	8	4	0	4	4	4	0	0
R	50	4	46	13	0	13	8	8	0	0
S	13	0	0	8	4	25	8	8	33	0
T	8	4	0	0	0	8	4	8	0	0
V	0	0	0	0	4	0	0	0	21	0
W	0	0	0	0	0	0	0	0	0	0
Y	0	0	4	0	0	0	0	0	4	0
pSer (X)	0	0	0	42	4	17	4	0	17	0
pThr (Z)	0	0	0	17	0	4	0	4	0	0

h

11mer HLA-B7 phosphopeptide ligands

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
A	0	0	18	9	18	0	9	9	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0
D	0	0	9	0	0	9	0	9	0	9	0
E	0	0	9	0	0	0	9	0	9	9	0
F	0	0	0	0	9	0	9	0	0	0	9
G	0	0	0	0	0	36	27	9	0	27	0
H	0	0	0	0	0	0	0	0	0	0	0
I	0	0	9	0	0	0	0	9	0	0	0
K	9	0	0	9	0	9	0	9	0	9	0
L	0	0	0	0	0	9	0	0	0	0	64
M	0	0	0	0	0	0	0	0	0	0	9
N	0	0	0	0	9	0	0	9	9	0	0
P	0	91	0	0	55	0	9	0	9	0	0
Q	9	9	0	0	0	0	0	9	0	9	0
R	45	0	45	0	9	9	18	18	9	0	0
S	18	0	9	0	0	9	9	0	18	27	0
T	9	0	0	0	0	9	0	0	9	9	0
V	0	0	0	0	0	0	9	9	18	0	18
W	0	0	0	0	0	0	0	0	0	0	0
Y	9	0	0	0	0	0	0	0	9	0	0
pSer (X)	0	0	0	82	0	9	0	9	9	0	0
pThr (Z)	0	0	0	0	0	0	0	0	0	0	0

Fig. S1. Detailed HLA-binding motif analysis. **(a)** proportion of HLA-A2 and -B7 phosphopeptides of given residue length. **(b)** Phosphate position in B7 and A2 phosphopeptides. Residue frequency (%) depicted in combined logoplots and heatmaps for: 41 9-mer HLA-B7 phosphopeptides in this study **(c)**; 164 9-mer phosphopeptides predicted to bind to HLA-B7 where pSer was at P4 (d); 1038 regular non-phosphorylated 9-mer HLA-B7 peptides from the Immune Epitope database (e); five 8-mer (f), twenty-one 10-mer (g) and eleven 11-mer (h) HLA-B7 phosphopeptides identified in this study. Heatmaps denote percentage frequency of residues at each position.

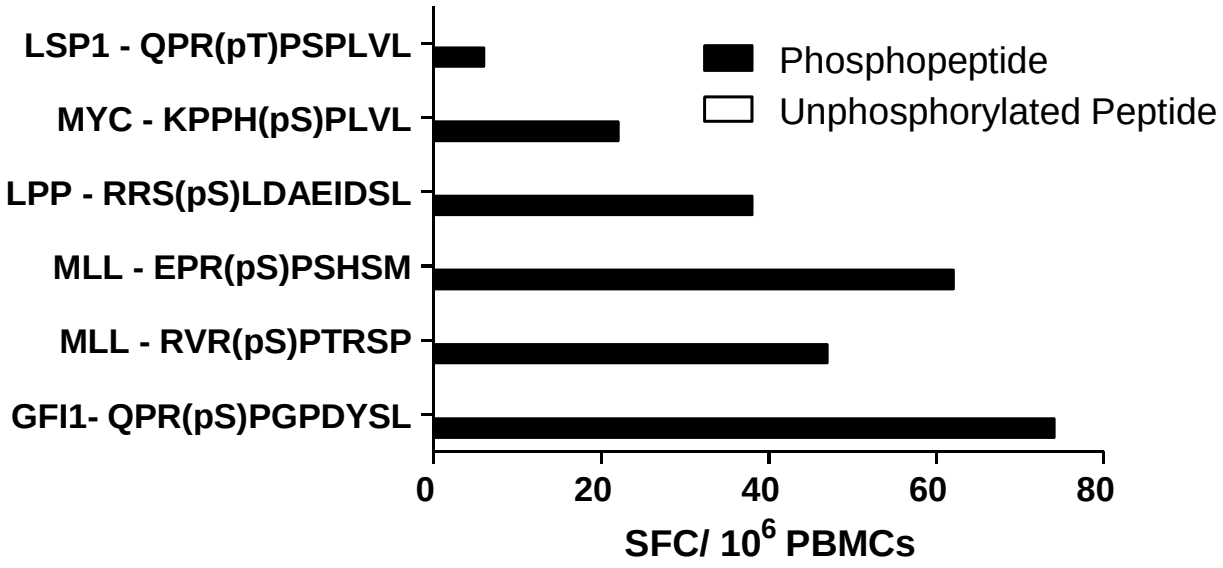


Fig. S2. Immunity against phosphopeptides in healthy donors is phosphate-dependent. Example of responses observed for phosphopeptide antigens (filled) and counterpart non-phosphorylated peptide in a healthy donor.

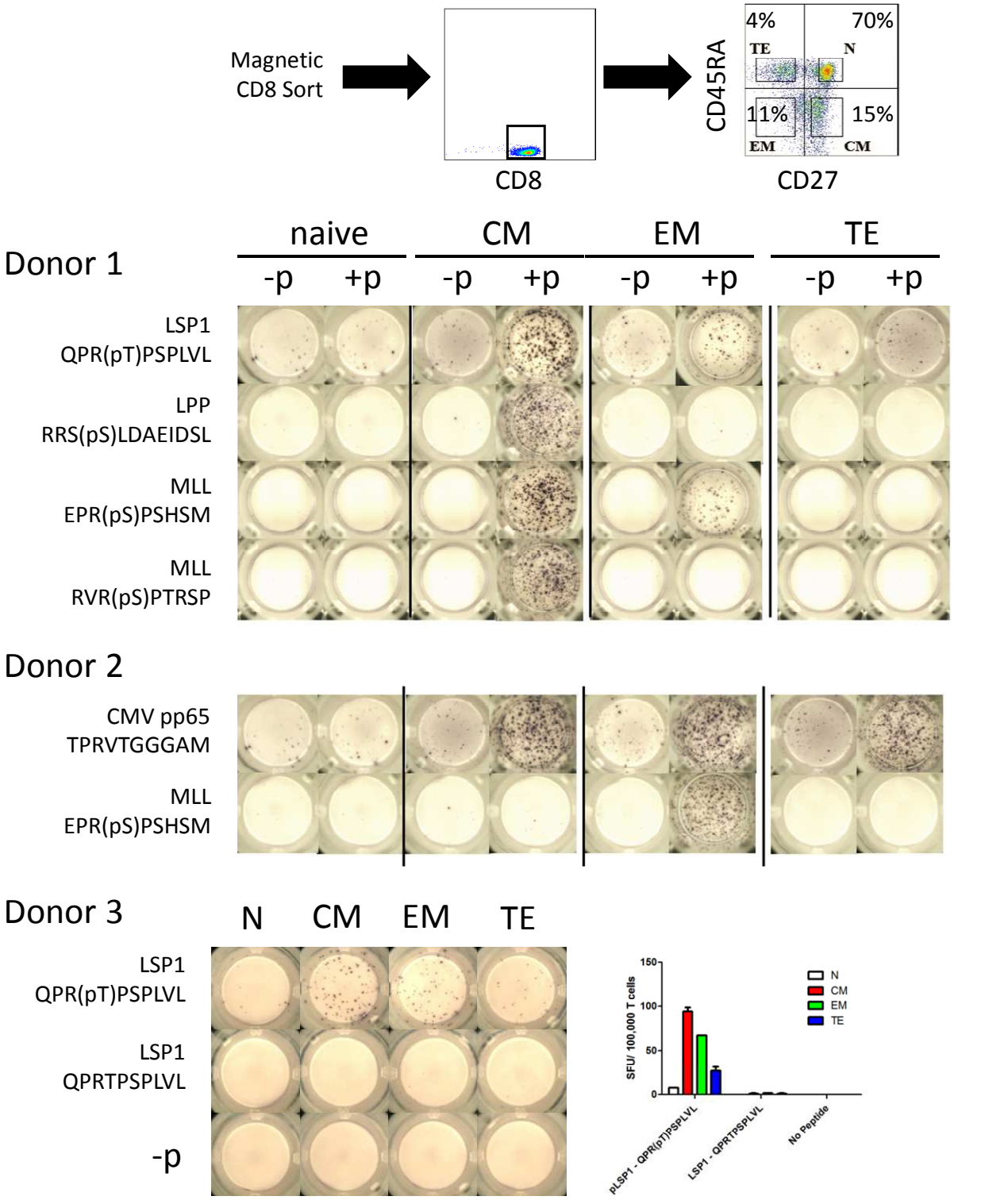


Fig. S3. T cell memory subset mapping of anti-phosphopeptide responses in healthy donors. Representative FACS-sorted T cell subset profile of freshly isolated, magnetically enriched CD8 T cells from peripheral blood of healthy donors (upper panel). 100,000 T cells from each compartment were cultured with phosphopeptides for 7-days. 7-day ELISpot analysis of each T cell memory subset reveals level of immunity against each respective phosphopeptide in 3 donors. For Donor 3 we tested reactivity against the non-phosphopeptide.

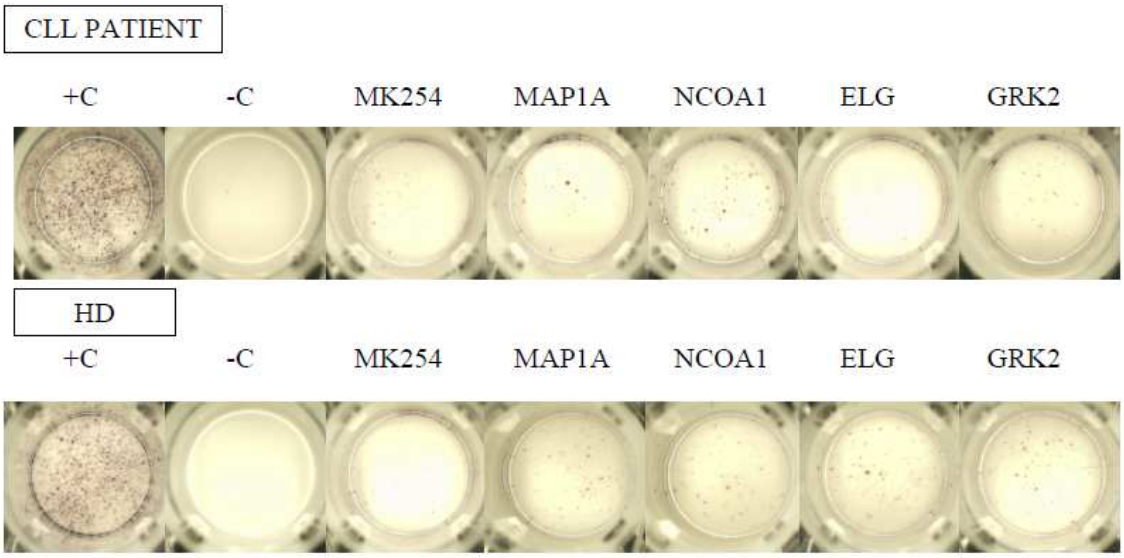


Fig. S4. Leukemia-associated phosphopeptide-specific immunity is lacking in CLL patients. Example of ELISpot of enriched CD8+ T cells from a patient with CLL and a healthy donor against selected CLL-associated antigens.

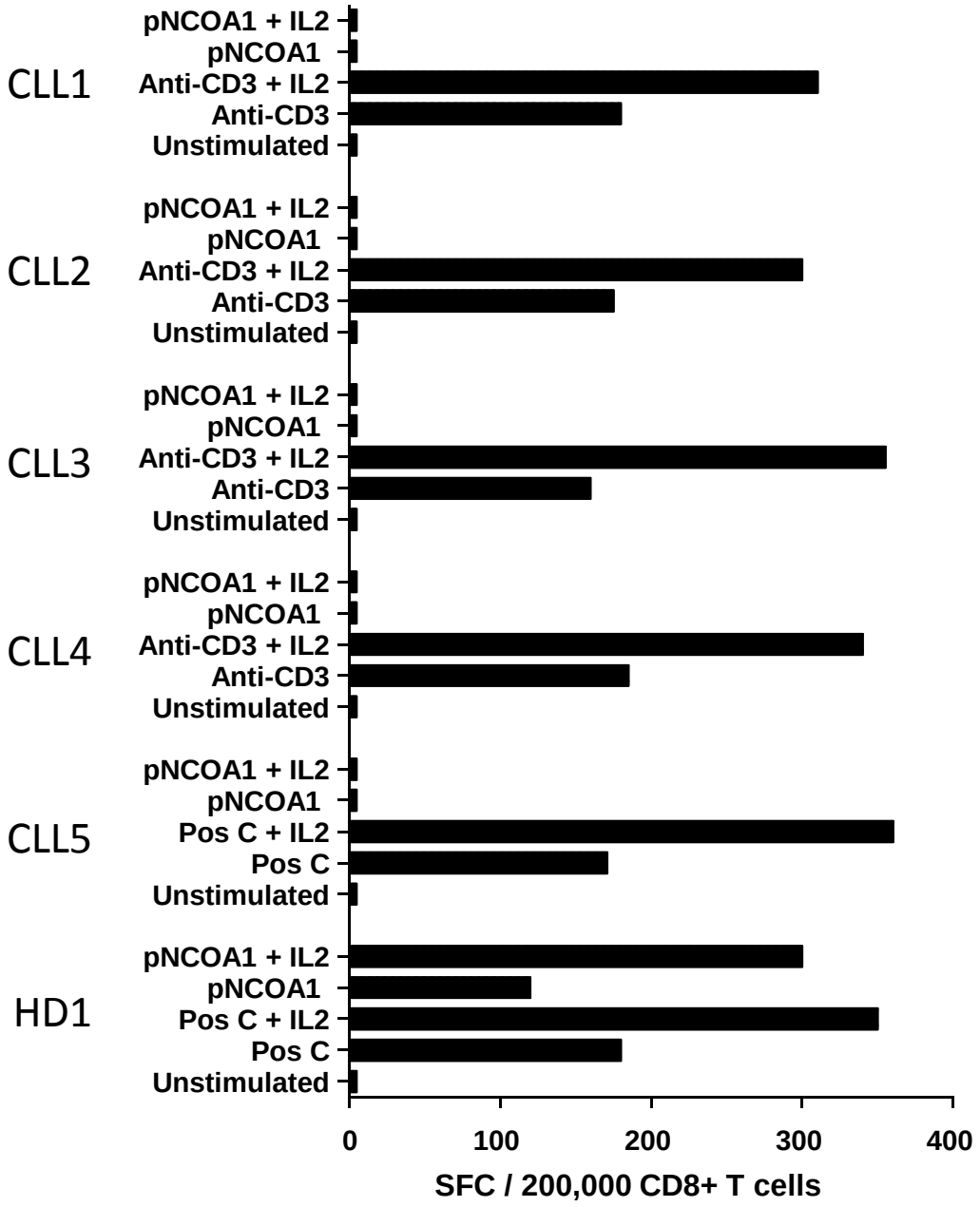


Fig. S5. Deletion (rather than anergy) of anti-phosphopeptide immunity in five patients with CLL. Representative 24-hour ELISpot from enriched CD8 T cells from a healthy donor (HD) and five patients with CLL who lacked anti-phosphopeptide immunity stimulated in presence or absence of IL-2. Responses to anti CD3 (positive control) and the HLA-B7 restricted phosphopeptide (pNCOA1) was assessed.

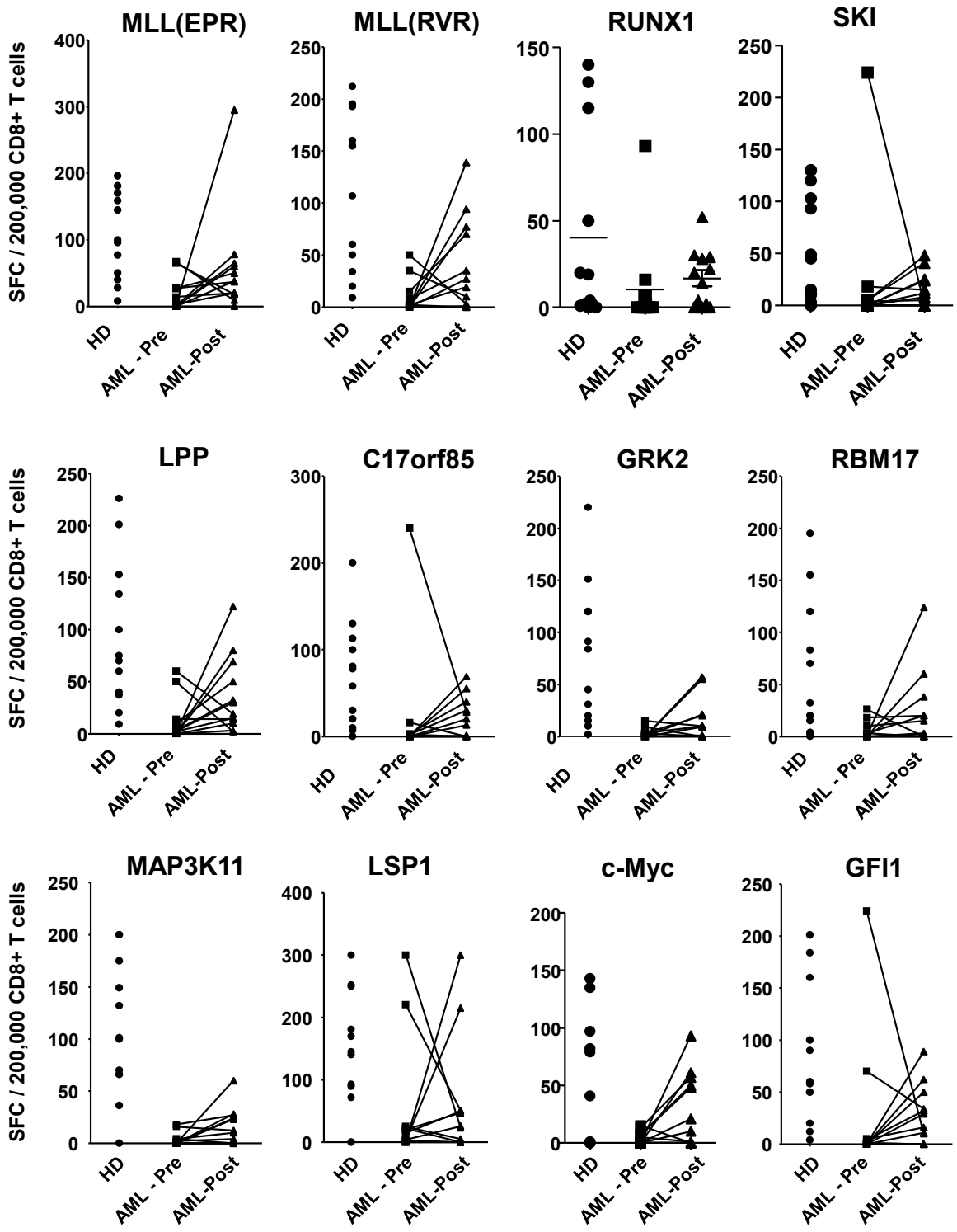


Fig. S6. Leukemia-associated phosphopeptide-specific immunity is lacking in AML patients but restored after SCT. ELISpot data for each phosphopeptide between AML patients pre- and post-transplant compared with healthy donors.

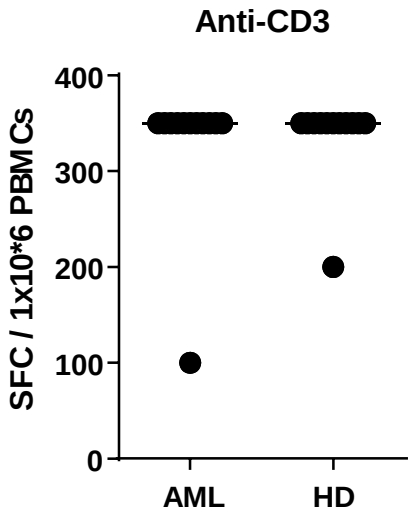


Fig. S7. Immunocompetence of patients with AML in complete remission before transplantation.

Anti-CD3 responses measured by ELISpot among healthy donors (HD) and patients with AML whilst in complete remission pre-transplant

Patient ID	Time Since Sample	Age	Sex	Stage at presentation (Binet stage)	CD38	Zap-70	Genetic aberration	IgVH	Time to first treatment (days)	Progression free survival after first treatment	Timing of sample(s)	Treatment, status
CLL1	890	79		A	POS	POS	Normal	UM	2387	Progressed through chlorambucil	Previously treated, slow progressive disease	Died, Chl, FC, methylpred, died of disease +4695
CLL2	420	71		A	NEG	NEG	NA	NA	Untreated	Untreated	Untreated	Alive day +506
CLL3	1048	64		A	NEG	NA	NA	NA	685	Progressed 301 days post first tx	Untreated	Alive, FCR, transformed to Hodgkins ABVD, day +1142
CLL4	945	75		A	NEG	NEG	Normal	M	Untreated	Untreated	Untreated	Alive day +5341
CLL5	688	63		A	NEG	NA	NA	NA	Untreated	Untreated	Untreated	Alive day +1749
CLL6	586	86		A	NEG	NEG	Normal	M	3334	Died on tx	Prior to first treatment for progressive disease	Died, Chl, died of disease day +3467
CLL7	359	70		A	NEG	NEG	NA	NA	Untreated	Untreated	untreated	Alive, day +1201
CLL8	359	67		A	NEG	NA	13q	NA	1141	Not progressed	Prior to first treatment for progressive disease	Alive, FCR, day +1460
CLL9	897	78		A	NEG	NEG	Normal	M	Untreated	Untreated	Untreated	Alive day +6133
CLL10	1091	89		C	NA	NA	NA	NA	0	Partial response	All taken on treatment (chlorambucil)	Died, Chl, ?died of disease day +362
CLL11	540	65		B	NEG	POS	NA	NA	30	Progressed 631 days post first tx	Previously treated, progressive disease	Alive, Chl, FCO, day +1399
CLL12	1064	84		C	NEG	NEG	NA	NA	68	Not progressed	18/2/09 – Prior to first treatment for progressive disease 6/1/10 – 4 months post treatment	Alive, Chl/R, day +1129
CLL13	523	63		A	NEG	NEG	NA	NA	Untreated	Untreated	6/1/10 – 4 months post treatment	Alive, day +993
CLL14	579	63		A	POS	NEG	NA	M	Untreated	Untreated	Untreated	Alive day +2708

IgVH, Immunoglobulin Variable Region Mutation status:- M indicates mutated; UM, unmutated; NA, not assessed;
NA, Not available; FC, fludarabine+cyclophosphamide; FCR, fludarabine+cyclophopshamide+rituximab; FCO, fludarabine+cyclophopshamide+ofatumumab; Chl, chlorambucil; Chl/R, chlorambucil+rituximab; Methylpred, methylprednisolone

Table S1. CLL patient characteristics.

AML Patient ID	Age	Gender	Diagnosis and Treatment	Cytogenetics	Transplant History	Samples Analyzed	GvHD	ALC	Clinical Condition at the end of study (12/2012)
AML1	65	M	AML, ADE x2	monosomy 7	MUD	21 months post SCT	No	1.2	23 months post transplant: Death due to Relapse †
AML2	67	F	AML, DAx3, CR1	normal	MUD	11 months post SCT	No	1.3	26 months post transplant: In Remission and well
AML3	64	F	AML DA x2, MIDAC, CR1	FLT3pos	2 Cord Bloods	9 months post SCT	No	1.3	26 months post transplant: In Remission and well
AML4	67	M	AML MIDAC x3 CR1	normal	Sibling	10 months post SCT	Skin	2.3	10 months post transplant: Relapsed. Patient died 13 months post trasnplant
AML5	62	F	AML, DAx2, MACE	normal	MUD	21 months post SCT	No	1.3	39 months post transplant: In Remission and well
AML6	65	M	AML DA/Myelotarg, DA CR1	normal	MUD	3 months post SCT	No	1.5	3 months post transplant: Death †
AML7	54	F	AML, AML 17 (ADE X2) CR1	monosomy 7	MUD	7 months post SCT	Gut	0.9	25 months post transplant: In Remission and well
AML8	56	M	MDS transformed to AML, [DAx2, MACE, MIDAC] [ARA-C], CR2	normal	MUD	6 months post SCT	Skin now resolved	1	25 months post transplant: In Remission and well
AML9	51	F	MDS no treatment	trisomy 6	Sibling	113 months post SCT	Eyes, mouth, skin and liver	2.2	131 months post transplant: In Remission and well
AML10	66	F	AML, [AML 16] [FLAG x2] CR2	normal	MUD	34 months post SCT	Skin and gut	2.7	46 months post transplant: In Remission and well
AML11	67	F	AML, [DA x2, MIDAC] [FLAG/Myelotarg, FLAG] [FLAG x 2] CR3	normal	2 Cord Bloods	9 months post SCT	Gut	5.4	27 months post transplant: In Remission and well
AML12	41	M	AML, DA x 2, MIDAC, CR1	normal	Sibling	15 months post SCT	Skin	1.8	31 months post transplant: In Remission and well

MUD, matched unrelated donor; ADE, Ara-C, daunorubicin, etoposide; FLAG, fludarabine, Ara-C, idarubicin; DA, daunorubicin, Ara-c; MIDAC; amsacrine, Ara-C, etoposide, mitozantrone; CR = complete remission. (CR1,2,3 = 1st 2nd or 3rd CR). ALC = absolute lymphocyte count ($x10^9$/L)

Table S2. AML patient characteristics.