SUPPLEMENTARY APPENDIX

The allogeneic HLA-DP-restricted T-cell repertoire provoked by allogeneic dendritic cells contains T cells that show restricted recognition of hematopoietic cells including primary malignant cells

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The allo-HLA-DP restricted T cell repertoire provoked by allogeneic dendritic cells contains T cells that show restricted recognition of hematopoietic cells including primary malignant cells

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Online Supplementary Appendix

Methods

Cell collection and preparation

Peripheral blood mononuclear cells (PB-MNC) were isolated using Ficoll-Isopaque separation. Peripheral blood was obtained from healthy donors and patients after informed consent. Lymphocytes were enriched by using magnetic cell separation (MACS) to deplete $CD14^+$ cells with magnetic CD14-beads and LS columns according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated $CD14^+$ cells were transformed into immature monocyte derived DCs by culturing for 2 days at a concentration of $2x10^6$ cells/ml in Iscove's modified Dulbecco's medium (IMDM; Lonza, Verviers, Belgium) containing 10% heat-inactivated human serum (ABOS) supplemented with 100 ng/ml GM-CSF (Novartis Sandoz Pharmaceuticals, Rotkreutz, Switzerland) and 500 IU/ml IL-4 (Schering-Plough, Innishammon, Cork, Ireland). To generate mature DCs, immature DCs were transformed by culturing for an additional 2-3 days in 10% ABOS IMDM supplemented with 100 ng/ml GM-CSF, 10 ng/ml TNFα (Cellgenix, Freiburg, Germany), 10 ng/ml IL-1β (Sclavo, Siena, Italy), 10 ng/ml IL-6 (Novartis Sandoz Pharmaceuticals), 1 µg/ml PGE-2 (Sigma Aldrich/Merck, Darmstadt, Germany), and IFNγ (500IU/ml, Boehringer Ingelheim, Rijnland-Palts, Germany).

Generation and culturing of stimulator cells for functional analyses

The different stimulator cells used in the functional analyses can be distinguished by the number between brackets, which indicates the individual of which the cells are derived (overview is given in Online Supplementary Table S1). The isolation of $CD14^+$ cells and the

generation of $CD14^+$-derived DCs are described in the section 'Cell collection and preparation'. EBV-transformed lymphoblastoid cell lines (EBV-LCL) were generated using standard procedures and cultured in 10% heat-inactivated FCS supplemented IMDM.[1] PHA (phytohemagglutinin)-blasts were generated by incubating PB-MNC in IMDM supplemented with 10% heat-inactivated ABOS, 100 IU/ml IL-2 and 800 ng/ml PHA-HA16 for seven days after which the PHA-blasts were used in assays. Additionally, third party primary leukemic cell samples (AML, B-ALL, and CLL) expressing the mismatched HLA-DP molecules were selected from the Leiden University Medical Center Biobank for Hematological diseases.

Skin derived human fibroblasts[2] were cultured up to 90% confluency in DMEM (Lonza) supplemented with 10% heat-inactivated FCS and were pre-treated for 4 days with 200 IU/ml IFNγ to upregulate HLA-class II expression. K562 (chronic myelogenous leukemia, cultured in 10% FCS IMDM), HeLa (cervical cancer, cultured in 10% FCS IMDM), WiDR (colon adenocarcinoma, cultured in 10% FCS IMDM), EGI-1 (bile duct carcinoma, cultured in 10% FCS MEM (Lonza) supplemented with 1mM Sodium Pyruvate, 2x MEM Amino Acids and 2x MEM non-essential Amino Acids (Thermo Fisher Scientific, Waltham, MA, USA)) cell lines were retrovirally transduced to induce expression of specific HLA-DP molecules. Different HLA-DP alleles were cloned into a pLZRS retroviral vector (an empty pLZRS retroviral vector was used as a mock vector for our control cell lines) and the constructs were verified by sequencing. To retrovirally transduce the different cell lines, Retronectin (recombinant human fibronectin, r-fibronectin, Takara Bio USA, Mountain View, CA, USA) was used.[3]

Recognition assay

The clonality of expanded T cell clones was confirmed by TCR Vβ repertoire-analysis using the IOTest® Beta Mark kit (Beckman Coulter Life Sciences, Indianapolis, Indiana, USA). To investigate the recognition profile of the expanded T cell clones, 2,000 T cells were exposed in a 384-well culture plate to different autologous and allogeneic HLA-DP expressing hematopoietic cells (DCs, EBV-LCL, $CD14^+$ monocytes, PHA-blasts, K562 transduced with HLA-DP alleles, AML, B-ALL or CLL) in a 1:5 responder to stimulator ratio, and to autologous and allogeneic HLA-DP expressing third party fibroblasts and tissue cell lines transduced with HLA-DP alleles (HeLa's, WiDR, and EGI-1) in a 1:3 responder to stimulator ratio. The supernatants were harvested after overnight incubation of the cells in 10% ABOS IMDM supplemented with 25 IU/ml IL-2, and the amount of IFNγ in the supernatants was

quantified using standard enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Sanquin Reagents, Amsterdam, The Netherlands). The cytotoxic capacity of a proportion of reactive allo-HLA-DP restricted CD4 T cell clones was confirmed using quantitative FACS analysis of cytotoxicity[2] or by using ^{51}Cr-release assay[4].

Flow cytometric analyses

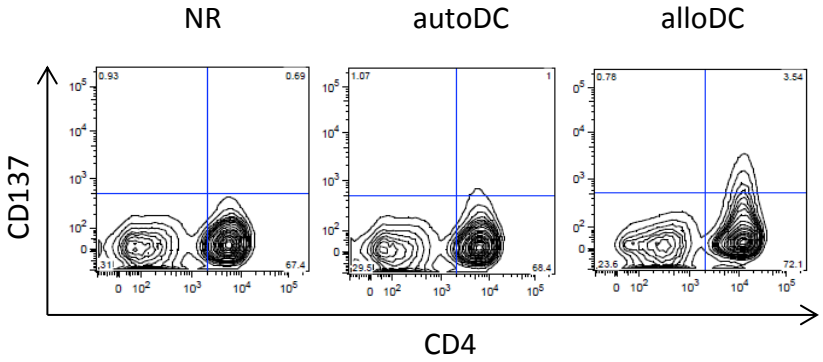
At the initiation and restimulation of the allo-HLA-DP specific immune responses, the percentages of $CD3^+TCR\alpha\beta^+$ T cells were measured in the responder population to determine the correct ratio of stimulation. Flow cytometry was performed using counterstaining with fluorescein isothiocyanate (FITC)-labeled TCRαβ (BD Biosciences), phycoerythrin (PE)-labeled CD4 (BD Pharmingen), peridinin chlorophyll protein (PerCP)-labeled CD3 (BD Biosciences), and APC-labeled CD8 (BD Pharmingen) monoclonal antibodies. The expression of HLA-DP was measured on the stimulator cells using PE-labeled HLA-DP monoclonal antibody (Bioconnect, Huissen, Netherlands). Fluorescent events were analyzed using FACSCalibur (BD Biosciences), Cellquest software (BD Biosciences), and FlowJo (FlowJo LLC, Ashland, Oregon USA).

For the enumeration and isolation of $CD137^+$ CD4 T cells from the *in vitro* HLA-DP immune responses, cells were stained with the monoclonal antibodies PE-labeled CD4 (BD Pharmingen) and APC-labeled CD137 (BD Pharmingen). Alexa Fluor 700 (AF700-) labeled CD8 (Life technologies, Thermo Fisher), FITC-labeled CD14 (BD Pharmingen), CD16 (BD, San Jose, CA, USA), CD19 (BD Pharmingen), and TCRγδ (BD) antibodies were used to set up the dump channel for exclusion. Fluorescent events were analyzed and collected using a FACSAria (BD Biosciences), FACSDiva software (BD Biosciences), and FlowJo (FlowJo LLC, Ashland, Oregon USA).

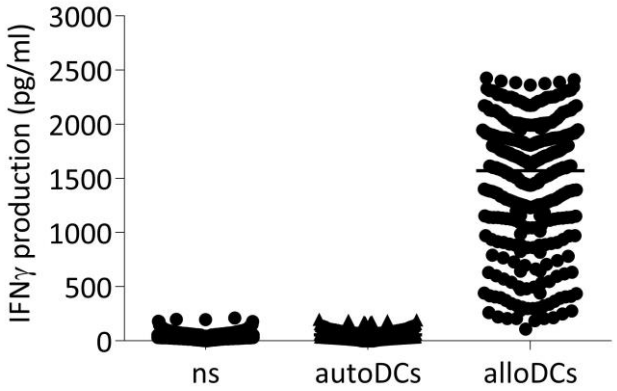
No.	HLA-DP typing	Material
1	DPB1*04:01:01	DCs, EBV-LCL
2	DPB1*03:01:01 & DPB1*04:01:01	DCs, $CD14^+$, EBV-LCL
3	DPB1*03:01 & DPB1*09:01	DCs, PHA-blasts, EBV-LCL
4	DPB1*03:01 & DPB1*04:02	DCs, $CD14^+$, PHA-blasts, EBV-LCL
5	DPB1*02:01:02 & DPB1*04:02:01	DCs, $CD14^+$, PHA-blasts, EBV-LCL
6	DPB1*04:02 & DPB1*05:01	DCs, $CD14^+$, PHA-blasts, EBV-LCL
7	DPB1*02:01:02 & DPB1*03:01:01	DCs, $CD14^+$
8	DPB1*01:01 & DPB1*02:01:02	DCs
9	DPB1*03:01 & DPB1*04:01	DCs, $CD14^+$, EBV-LCL
10	DPB1*03:01 & DPB1*04:01	DCs, $CD14^+$, PHA-blasts, EBV-LCL
11	DPB1*04:01 & DPB1*04:02	DCs, $CD14^+$, EBV-LCL
12	DPB1*02:01:02 & DPB1*04:01:01	$CD14^+$
13	DPB1*02:01:02 & DPB1*04:01	EBV-LCL
14	DPB1*02:01:02 & DPB1*04:01	EBV-LCL
15	DPB1*03:01 & DPB1*04:01	EBV-LCL, Fibro
16	DPB1*02:01 & DPB1*04:02	EBV-LCL
17	DPB1*03:01 & DPB1*04:01	EBV-LCL
18	DPB1*02:01:02	EBV-LCL, Fibro
19	DPB1*02:01:02 & DPB1*04:02	EBV-LCL, Fibro
20	DPB1*02:01 & DPB1*04:01:01	EBV-LCL, Fibro
21	DPB1*02:01:02 & DPB1*04:02	EBV-LCL
22	DPB1*03:01 & DPB1*04:02	EBV-LCL, Fibro
23	DPB1*02:01 & DPB1*04:02	EBV-LCL
24	DPB1*03:01 & DPB1*04:02	EBV-LCL
25	DPB1*02:01:02 & DPB1*04:01:01	EBV-LCL
26	DPB1*03:01 & DPB1*04:02	EBV-LCL, Fibro
27	DPB1*03:01 & DPB1*04:01	EBV-LCL, Fibro
28	DPB1*02:01 & DPB1*03:01	AML
29	DPB1*02:01:02 & DPB1*04:01	AML
30	DPB1*02:01:02 & DPB1*03:01	AML
31	DPB1*02:01 & DPB1*04:01:01	AML
32	DPB1*02:01 & DPB1*03:01	AML
33	DPB1*03:01 & DPB1*04:01:01	AML
34	DPB1*03:01 & DPB1*04:02	AML
35	DPB1*03:01 & DPB1*04:02	AML
36	DPB1*04:01:01	AML
37	DPB1*04:01	AML
38	DPB1*04:01 & DPB1*04:02	AML
39	DPB1*04:02 & DPB1*09:01	AML
40	DPB1*04:02:01 & DPB1*11:01:01	AML
41	DPB1*02:01 & DPB1*04:02	B-ALL
42	DPB1*02:01:02 & DPB1*04:01	B-ALL
43	DPB1*02:01 & DPB1*03:01	B-ALL
44	DPB1*03:01 & DPB1*04:02	B-ALL
45	DPB1*03:01 & DPB1*11:01:01	B-ALL
46	DPB1*03:01:01 & DPB1*11:01:01	B-ALL, Fibro
47	DPB1*04:01 & DPB1*04:02	B-ALL
48	DPB1*04:01:01 & DPB1*04:02	B-ALL
49	DPB1*03:01 & DPB1*04:01:01	B-ALL
50	DPB1*02:01 & DPB1*03:01	CLL
52	DPB1*02:01:02 & DPB1*04:01	CLL
53	DPB1*04:01 & DPB1*04:02	CLL
54	DPB1*04:02 & DPB1*15:01	CLL
56	DPB1*04:01 & DPB1*04:02	CLL
57	DPB1*04:01	DCs, EBV-LCL

Supplementary Table S1. Overview cell materials. No. = number indicates individual of which the cells are derived, DCs = CD14-derived dendritic cells, EBV-LCL = EBV-transformed lymphoblastoid cell lines, $CD14^+$ = monocytes, PHA-blasts = lympho-blasts by phytohemagglutinin treatment, Fibro = skin derived human fibroblasts, AML/B-ALL/CLL = primary malignant cell samples.

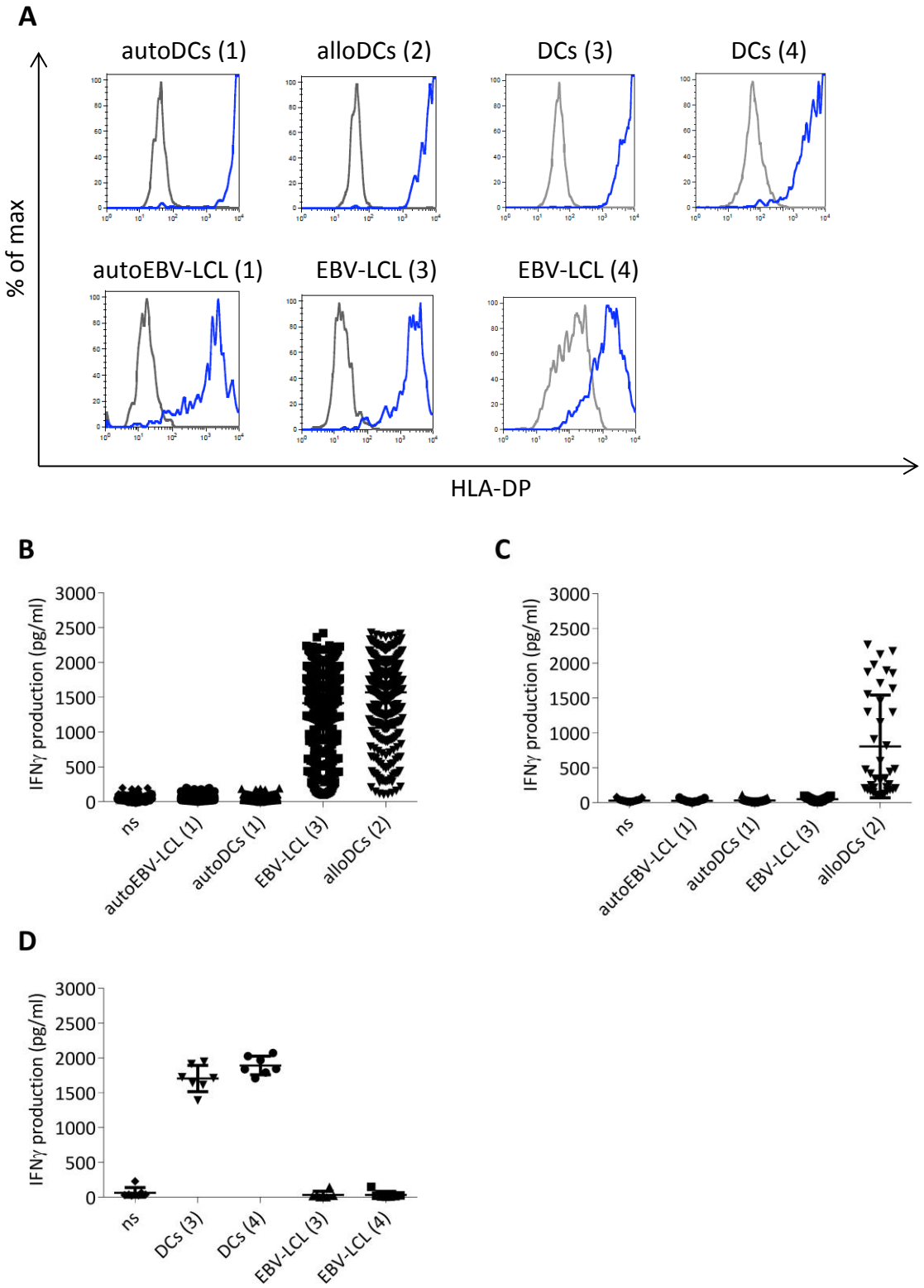
Results



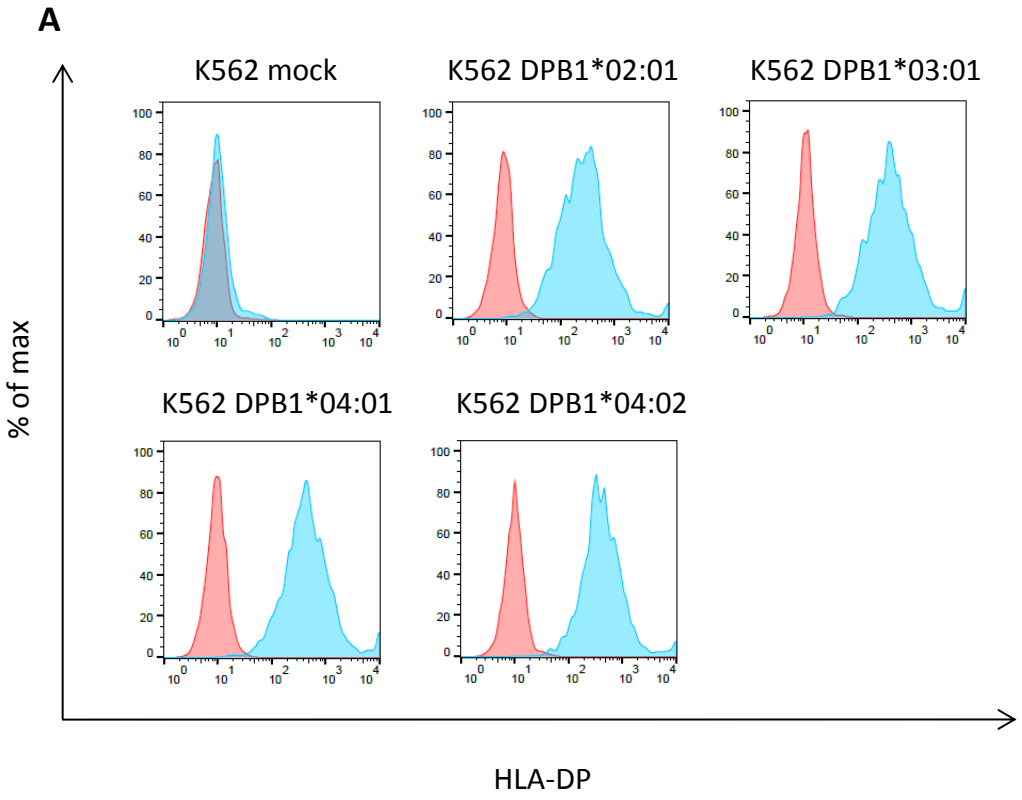
Supplementary Figure S1. Enrichment of allo-reactive T cells. Density plots of CD137 expression on the not restimulated (NR), autologous dendritic cells (autoDC) stimulated and allogeneic dendritic cells (alloDC) restimulated responder cells (representative example, response 4).

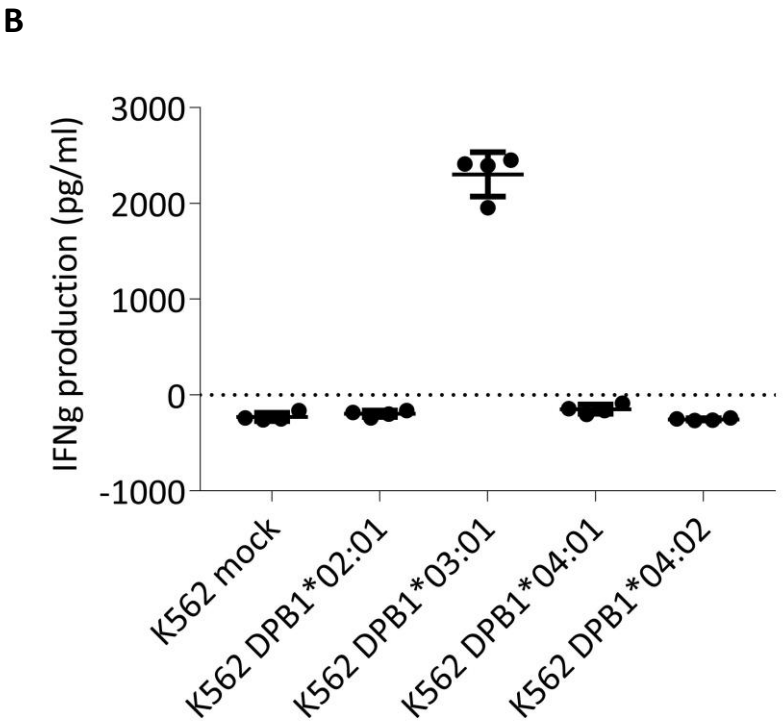


Supplementary Figure S2. IFNγ production by allo-reactive T cells. IFNγ production by allo-reactive T cell clones (response 4 as representative example) after overnight stimulation without stimulator cells (ns = no stimulation), autologous dendritic cells (autoDCs) or allogeneic dendritic cells (alloDCs).

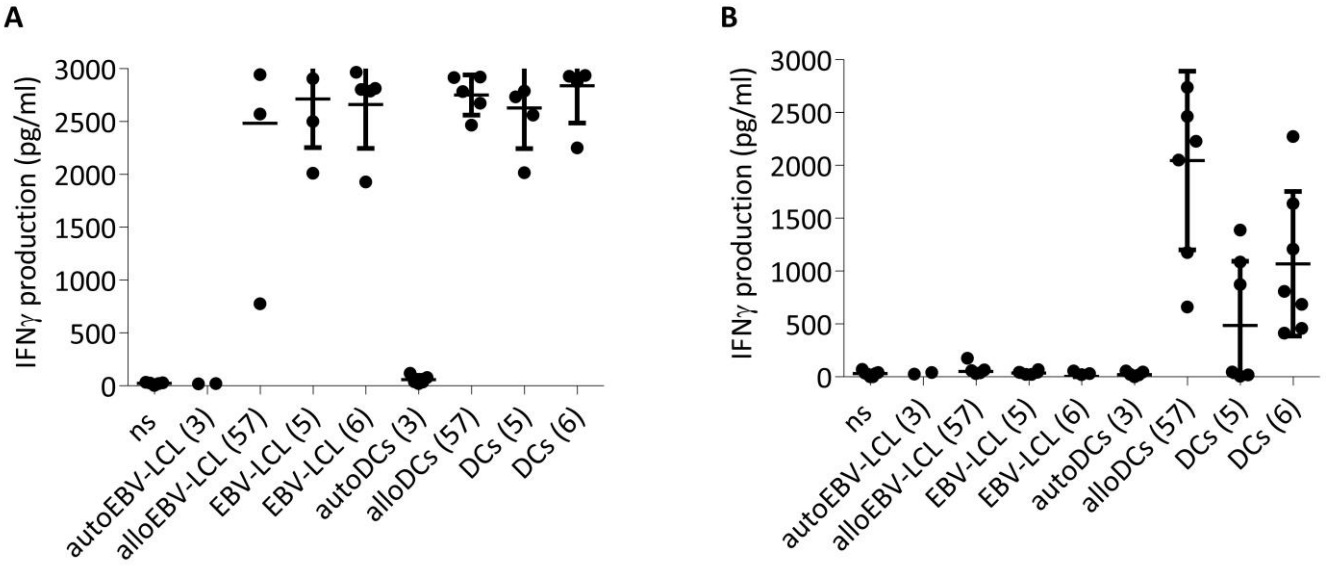


Supplementary Figure S3. IFNγ production by HLA-DP restricted CD4 T cell clones. A. Representative histograms (response 4) show the HLA-DP expression measured by flow cytometry (the blue curves represent stained cells and the grey curves unstained cells) on hematopoietic cells (CD14-derived dendritic cells; DCs, and EBV-transformed lymphoblastoid cell lines; EBV-LCL). **B-D.** IFNγ production by HLA-DPB1*03:01 restricted T cell clones (response 4 as representative example) after overnight incubation with stimulator cells. **B.** Reactivity against allogeneic DCs (alloDCs) and third party EBV-LCL expressing the mismatched HLA-DPB1 allele. **C.** Reactivity against alloDCs, but not against EBV-LCL. **D.** Reactivity by representative alloDC recognizing T cell clones (n=7) against third party DCs. The number between brackets in the figure indicates of which individual the stimulator cells are derived.

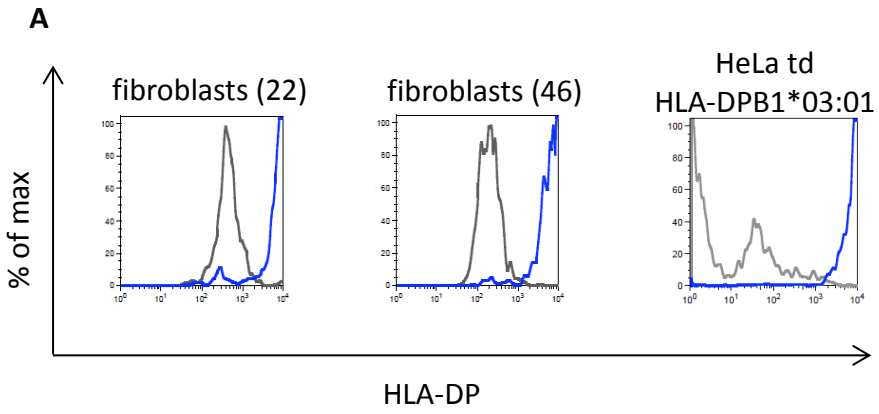




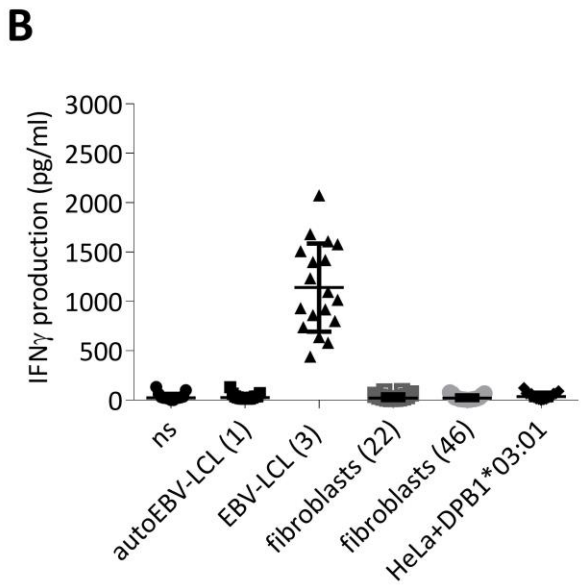
Supplementary Figure S4. IFNγ production by HLA-DPB1 restricted CD4 T cell clones. A. Histograms show the HLA-DP expression measured by flow cytometry (blue represents stained cells and red unstained cells) on the retrovirally transduced K562 cell lines. **B.** IFNγ production of representative HLA-DPB1*03:01 restricted T cell clones (n=4 of response 4) after overnight incubation with different K562 cell lines**.**

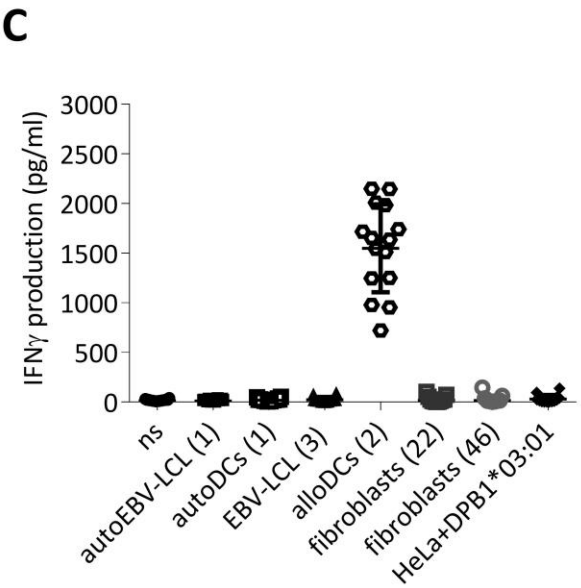


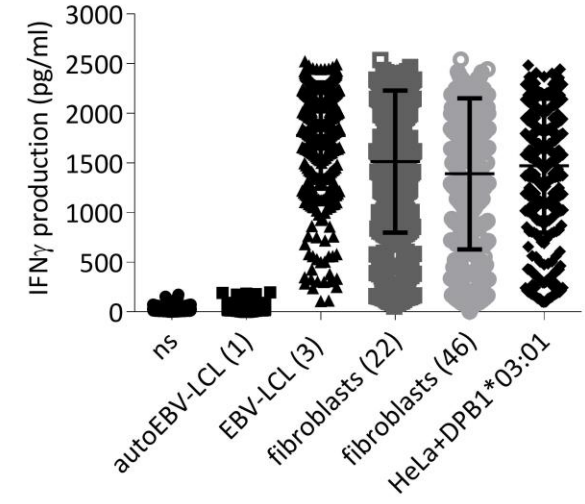
Supplementary Figure S5. IFNγ production by HLA-DQB1 restricted CD4 T cell clones. IFNγ production of representative HLA-DQB1*06:03 restricted T cell clones (response 5) after overnight incubation with stimulator cells. **A.** Reactivity against allogeneic EBV-transformed lymphoblastoid cell lines (alloEBV-LCL), third party EBV-LCL, allogeneic CD14-derived dendritic cells (alloDCs) and third party DCs expressing the mismatched HLA-DQB1 allele. **B.** Reactivity against alloDCs and third party DCs, but not against EBV-LCL. The number between brackets indicates of which individual the stimulator cells are derived.

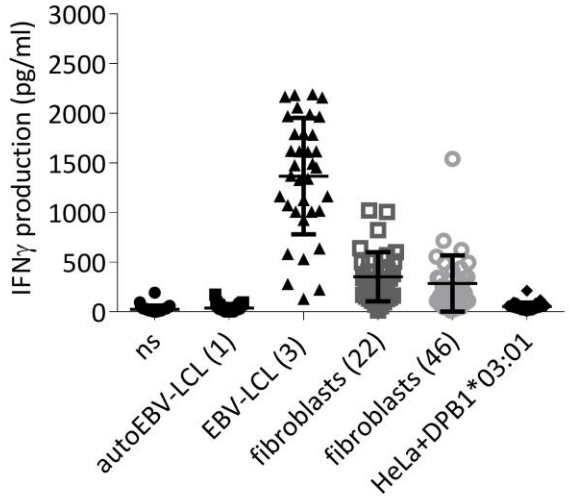


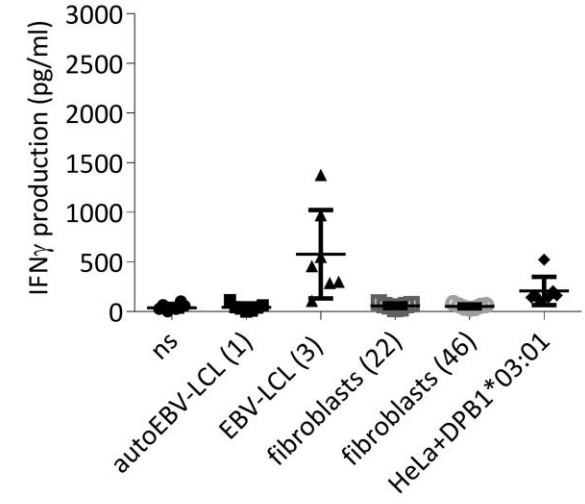
Supplementary Figure S6. IFNγ production by HLA-DP restricted CD4 T cell clones with different recognition patterns (figure is continued on next page). A. Representative histograms (DPB1*03:01 positive stimulator cells used for analysis of T cell clone reactivity in response 4) show the HLA-DP expression (the blue curves represent stained cells and the grey curves unstained cells) measured by flow cytometry on non-hematopoietic cell lines: third party fibroblasts and HeLa's transduced with the mismatched HLA-DP allele. **B-F.** IFNγ production by HLA-DPB1*03:01 restricted T cell clones (response 4 as representative example) after overnight incubation with stimulator cells. **B.** Reactivity against third party EBV-transformed lymphoblastoid cell lines (EBV-LCL). **C.** Restricted reactivity against only allogeneic CD14-derived dendritic cells (alloDCs). **D.** Reactivity against third party EBV-LCL, third party fibroblasts and HeLa's transduced with the mismatched HLA-DPB1 allele. **E.** Restricted reactivity against third party EBV-LCL and third party fibroblasts. **F.** Restricted reactivity against allogeneic EBV-LCL (alloEBV-LCL) and HeLa's transduced with the mismatched HLA-DPB1 allele. The number between brackets in the figure indicates of which individual the stimulator cells are derived.

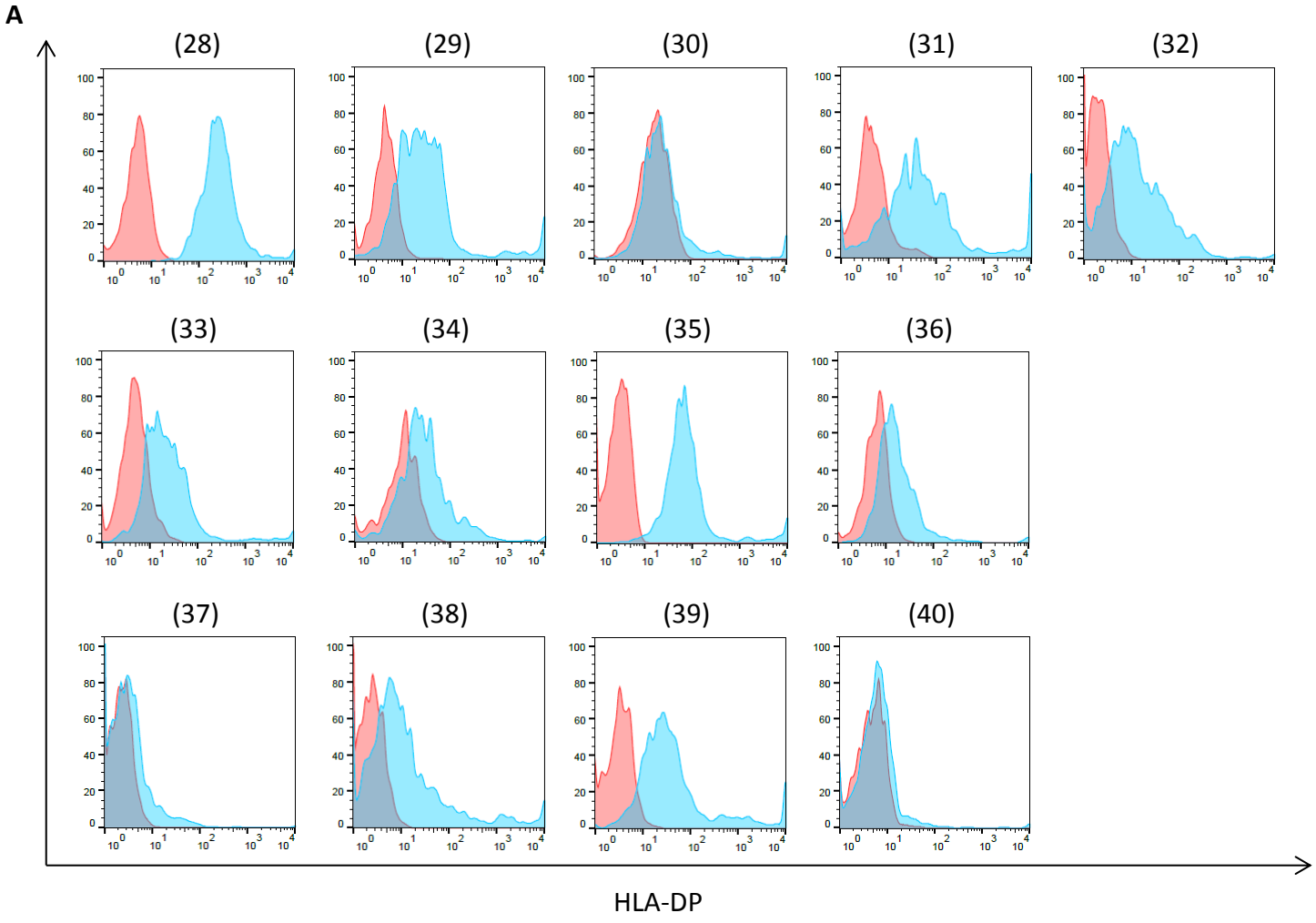

B
IFNγ production (pg/ml)
3000
2500
2000
1500
1000
500
0
ns
autoEBV-LCL (1)
EBV-LCL (3)
fibroblasts (22)
fibroblasts (46)
HeLa+DPB1*03:01

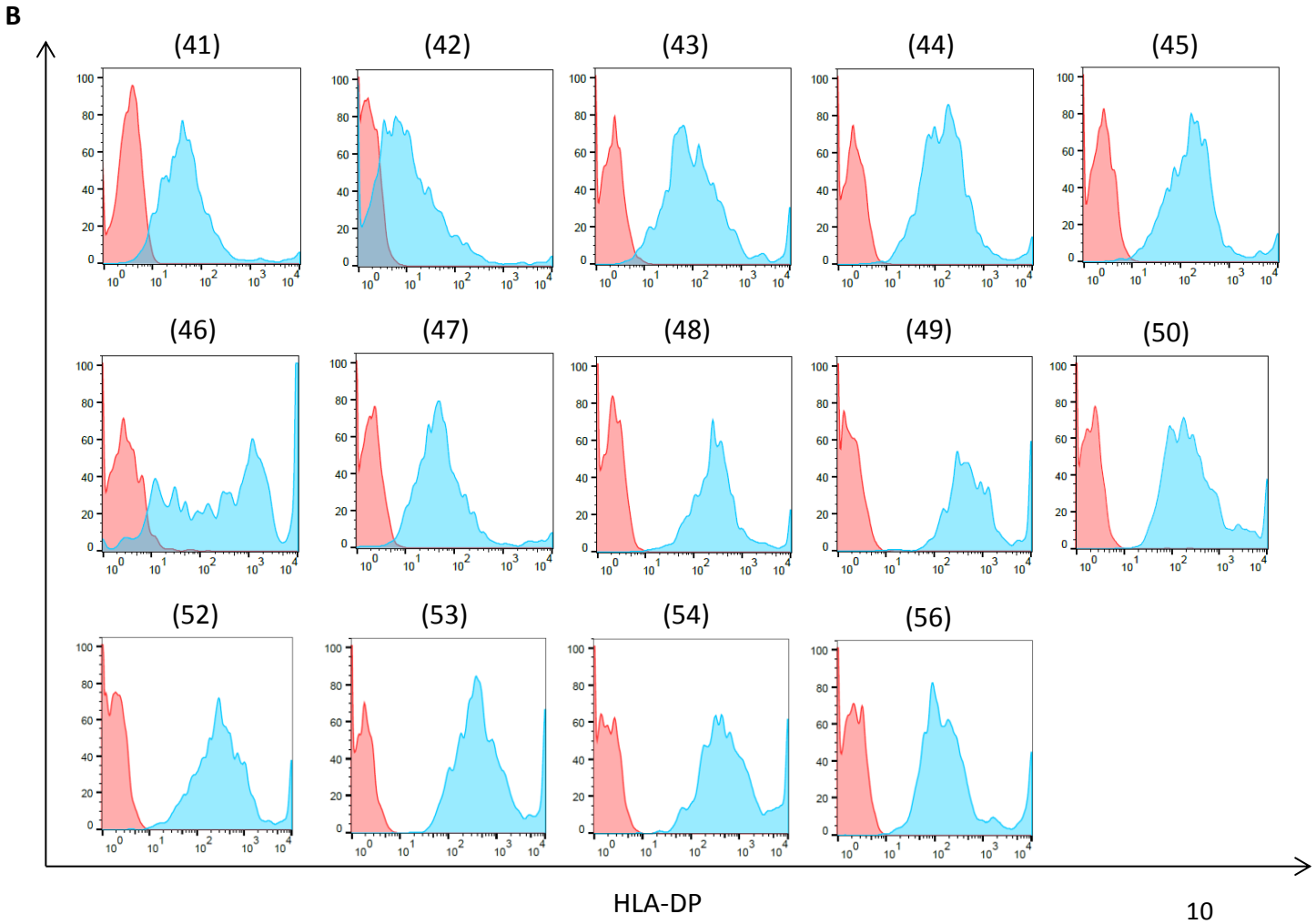

C
IFNγ production (pg/ml)
3000
2500
2000
1500
1000
500
0
ns
autoEBV-LCL (1)
autoDCs (1)
EBV-LCL (3)
alloDCs (2)
fibroblasts (22)
fibroblasts (46)
HeLa+DPB1*03:01

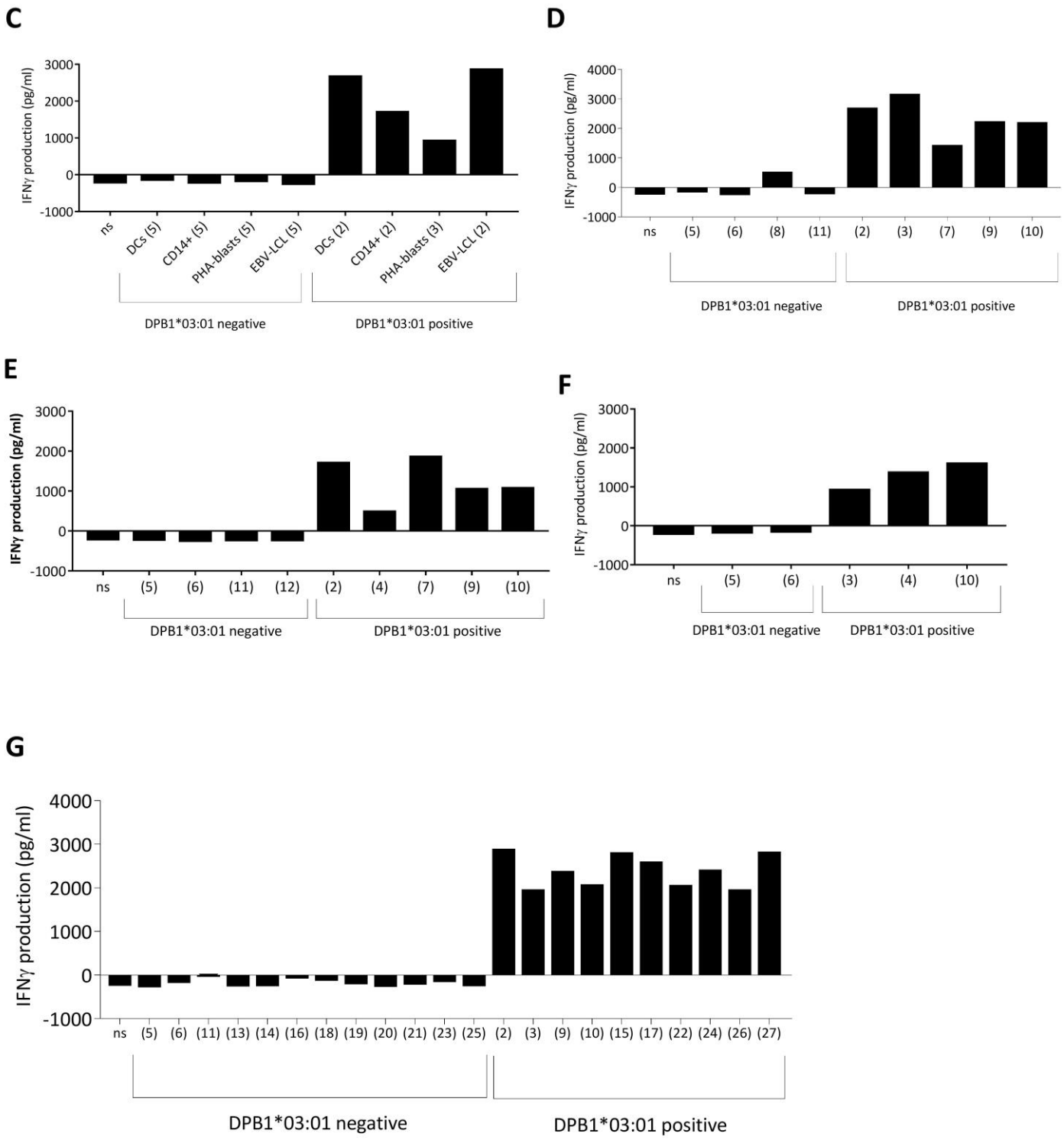

D
IFNγ production (pg/ml)
3000
2500
2000
1500
1000
500
0
ns
autoEBV-LCL (1)
EBV-LCL (3)
fibroblasts (22)
fibroblasts (46)
HeLa+DPB1*03:01

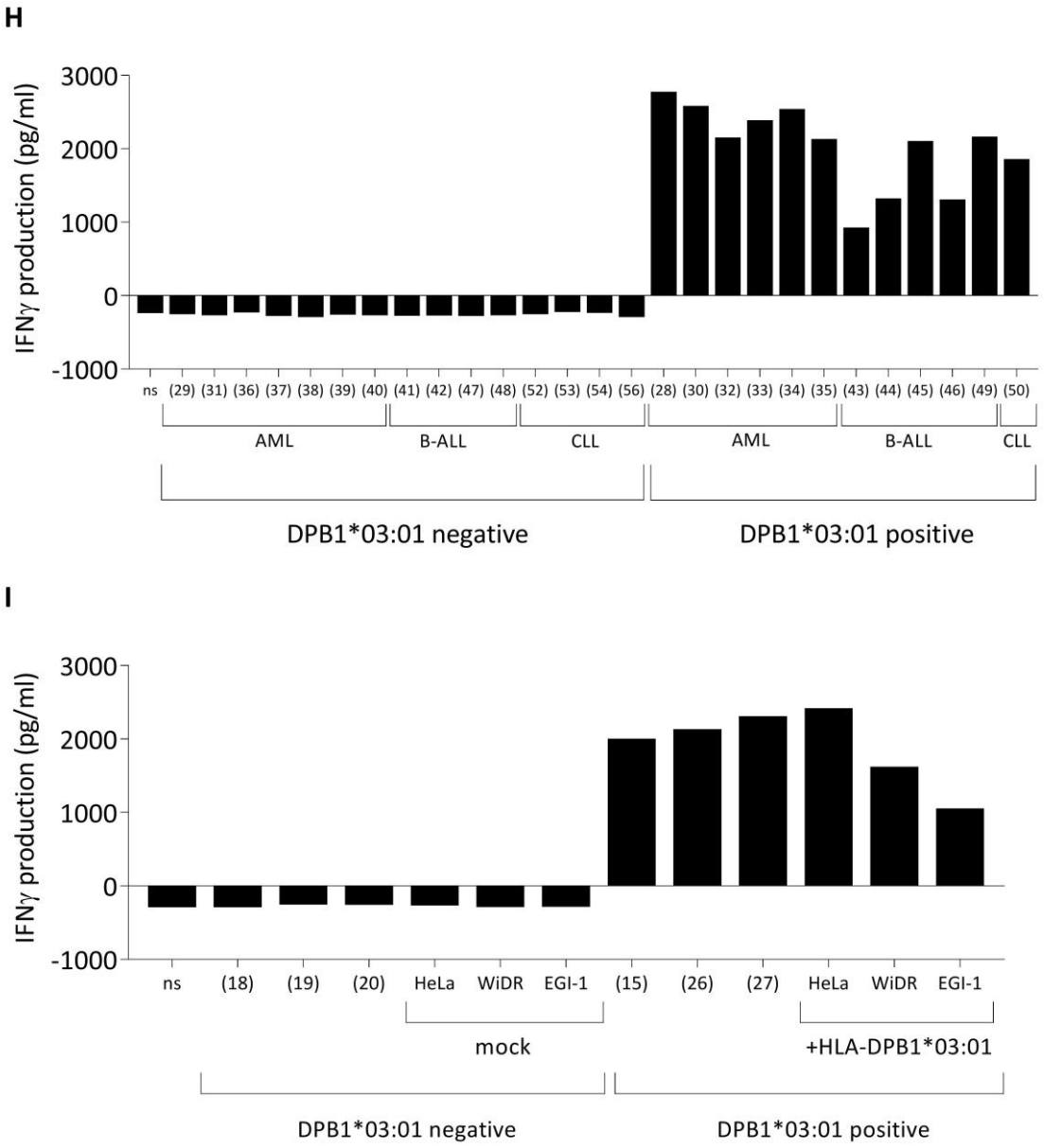

E
IFNγ production (pg/ml)
3000
2500
2000
1500
1000
500
0
ns
autoEBV-LCL (1)
EBV-LCL (3)
fibroblasts (22)
fibroblasts (46)
HeLa+DPB1*03:01


F
IFNγ production (pg/ml)
3000
2500
2000
1500
1000
500
0
ns
autoEBV-LCL (1)
EBV-LCL (3)
fibroblasts (22)
fibroblasts (46)
HeLa+DPB1*03:01

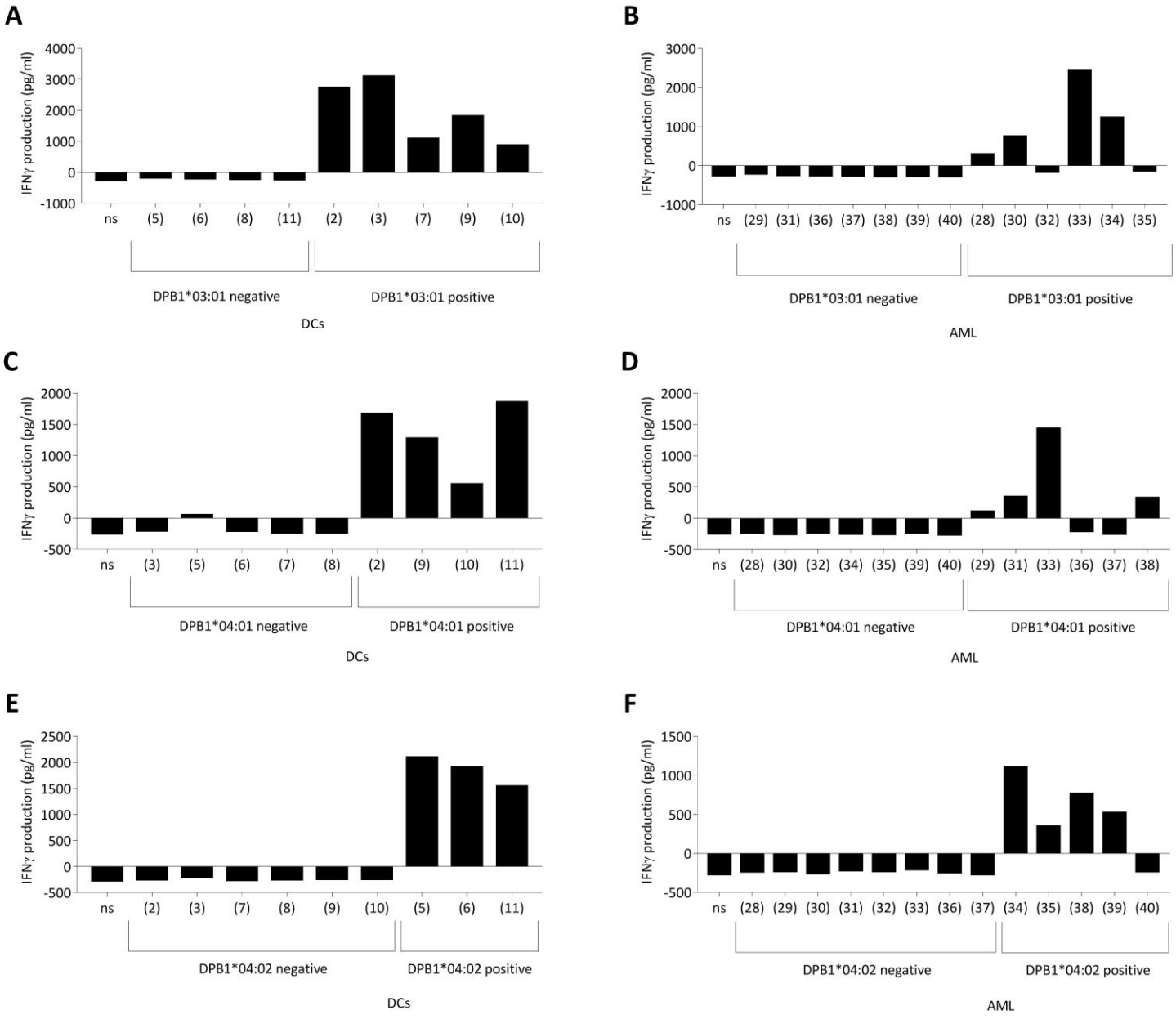

A
(28)
(29)
(30)
(31)
(32)
(33)
(34)
(35)
(36)
(37)
(38)
(39)
(40)
% of max
HLA-DP


B
(41)
(42)
(43)
(44)
(45)
(46)
(47)
(48)
(49)
(50)
(52)
(53)
(54)
(56)
% of max
HLA-DP

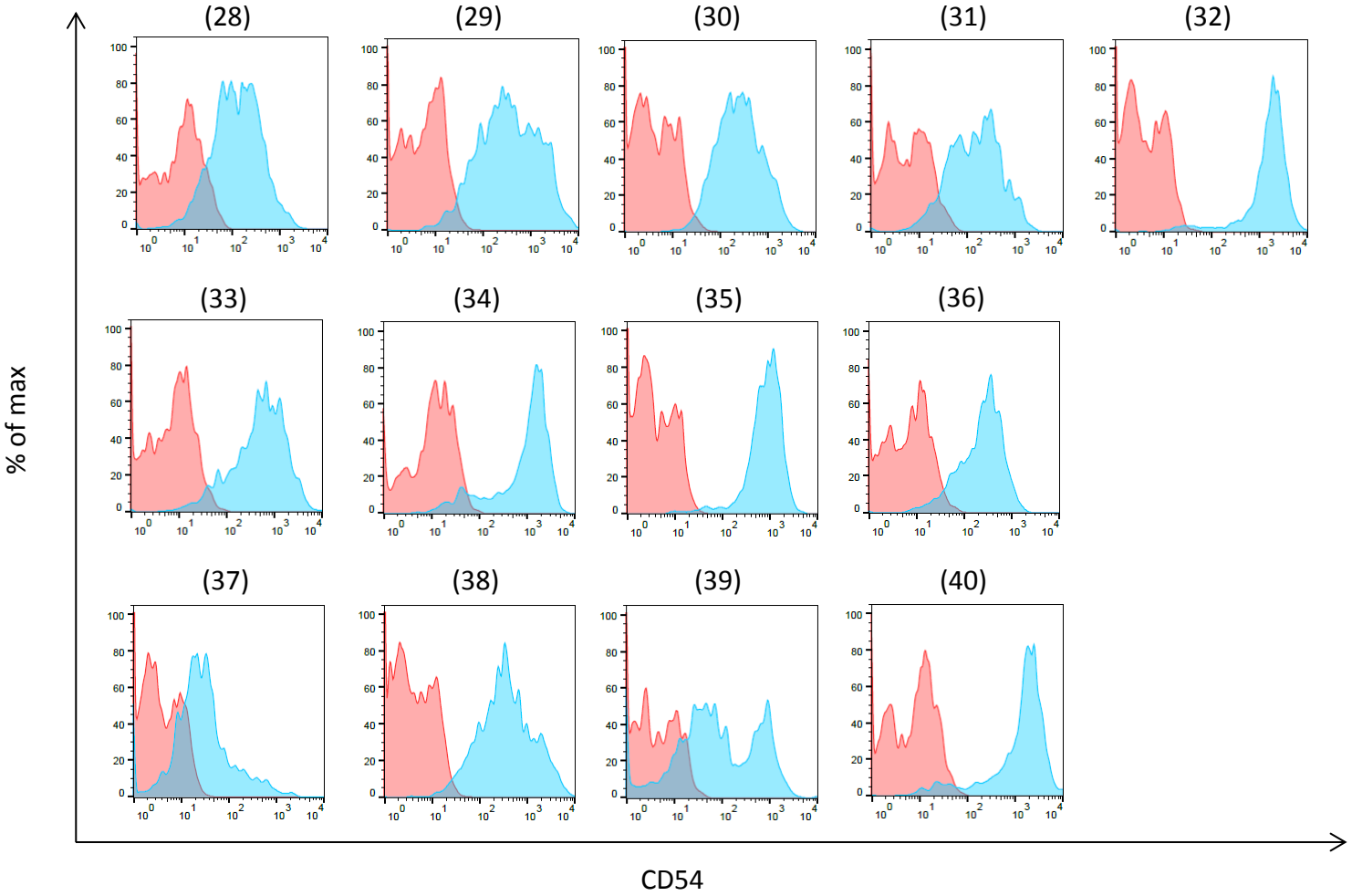

C
IFNγ production (pg/ml)
3000
2000
1000
0
-1000
ns
DCs (5)
CD14+ (5)
PHA-blasts (5)
EBV-LCL (5)
DCs (2)
CD14+ (2)
PHA-blasts (3)
EBV-LCL (2)
DPB1*03:01 negative
DPB1*03:01 positive
D
IFNγ production (pg/ml)
4000
3000
2000
1000
0
-1000
ns
(5)
(6)
(8)
(11)
(2)
(3)
(7)
(9)
(10)
DPB1*03:01 negative
DPB1*03:01 positive
E
IFNγ production (pg/ml)
3000
2000
1000
0
-1000
ns
(5)
(6)
(11)
(12)
(2)
(4)
(7)
(9)
(10)
DPB1*03:01 negative
DPB1*03:01 positive
F
IFNγ production (pg/ml)
3000
2000
1000
0
-1000
ns
(5)
(6)
(3)
(4)
(10)
DPB1*03:01 negative
DPB1*03:01 positive
G
IFNγ production (pg/ml)
4000
3000
2000
1000
0
-1000
ns (5) (6) (11) (13) (14) (16) (18) (19) (20) (21) (23) (25) (2) (3) (9) (10) (15) (17) (22) (24) (26) (27)
DPB1*03:01 negative
DPB1*03:01 positive



Supplementary Figure S7. Recognition of primary malignant and non-malignant hematopoietic and non-hematopoietic cells by allo-HLA-DP restricted T cells. A-B. Histograms show the HLA-DP expression (the blue represents stained cells and the red unstained cells) measured by flow cytometry on leukemia cell samples; **A.** AML samples, **B.** B-ALL (41-49) and CLL (50-56) samples. **C-I.** Representative HLA-DPB1*03:01 restricted T cell clone (derived from response 4) with broad tissue recognition, showing reactivity against **C.** hematopoietic cells, **D.** panel of DCs, **E.** monocytes, **F.** PHA-blasts and **G.** EBV-LCL. The T cell clone also shows reactivity against **H.** primary malignant cells and **I.** non-hematopoietic cells expressing the mismatched HLA-DP allele. This recognition pattern was seen for the tested T cell clones of response 2 (n=3), response 3 (n=3), response 4 (n=3) and response 5a (n=3). The number between brackets in the figure indicates of which individual the stimulator cells are derived.



Supplementary Figure S8. Differential recognition of primary AML blasts. IFNγ production by different representative HLA-DPB1 restricted T cell clones. **A.** Reactivity of a representative HLA-DPB1*03:01 restricted T cell clone against HLA-DPB1*03:01 positive CD14-derived dendritic cells (DCs) and **B.** against HLA-DPB1*03:01 positive primary AML blasts. **C.** Reactivity of a representative HLA-DPB1*04:01 restricted T cell clone against HLA-DPB1*04:01 positive DCs and **D.** against HLA-DPB1*04:01 positive primary AML blasts. **E.** Reactivity of a representative HLA-DPB1*04:02 restricted T cell clone against HLA-DPB1*04:02 positive DCs and **F.** against HLA-DPB1*04:02 positive primary AML blasts. The number between brackets indicates of which individual the stimulator cells are derived.



Supplementary Figure S9. CD54 expression on primary AML blasts. Histograms show CD54 expression (the blue represents stained cells and the red unstained cells) measured by flow cytometry on primary AML blasts. The number between brackets indicates of which individual the stimulator cells are derived.

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