SUPPLEMENTARY RESULTS

Immune defects in ABL are limited to CD1 and CD1-restricted T cells

The composition and function of the immune system in ABL has not been previously studied. To investigate whether immunological alterations are limited to CD1 and CD1-restricted T cells, we broadly analyzed immune cell composition by flow cytometry. Relative and absolute levels of monocytes, B cells, T cells, and NK cells as well as levels of subpopulations of T cells (CD4+, CD8+, double-negative T cells) and NK cells (CD16brightCD56dim, CD16dimCD56bright) were unchanged in ABL (data not shown). In addition, no changes in expression of activation (CD69, CD25), naive (CD62L, CD45RA), memory (CD45RO), and NK markers (CD56, CD161) could be found on conventional T cells or their CD4+, CD8+ and double-negative subpopulations (Fig. 6 and data not shown). B cell function as indirectly assessed by quantitative determination of immunoglobulin levels (IgA, IgM, total IgG, IgG1, IgG2, IgG3, IgG4) was normal in ABL (data not shown). Finally, in accordance with unaltered MHC class I and II-restricted antigen presentation and CD4+ and CD8+ T cell levels, proliferation of conventional T cells in response to phytohemagglutinin and secretion of Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17A) cytokines upon stimulation with PMA and ionomycin were unchanged compared to healthy controls (data not shown). Thus, ABL patients exhibit immune defects specific for CD1 and CD1-restricted T cells.

SUPPLEMENTARY METHODS

Flow cytometry

Cells were pre-incubated with FcR blocking reagent (Miltenyi Biotec) and in the case of tetramer-staining with 1 μg/ml unlabeled streptavidin (Pierce) for 15 min at 4°C before staining with monoclonal antibodies for 30 min at 4°C. Staining with CD1d-αGalactosylceramide (αGalCer) tetramers (NIH tetramer core facility) was performed to detect invariant NKT (iNKT) cells for 1 hour at 4°C. Cells were then washed with staining buffer (PBS, 1% BSA) and analyzed by flow cytometry using a 4 laser LSRII (BD Biosciences) and Flowjo software (Tree Star, Inc.). For intracellular staining, cells were first surface-stained, then permeabilized with Cytofix/Cytoperm (BD Biosciences) and washed with Perm/Wash buffer (BD Biosciences) according to the manufacturer's instructions. Antibodies were added for 30 min at 4°C before washing with Perm/Wash buffer. Because the frequency of NKT cells is low, at least 3×10^6 cells were recorded and analyzed for each staining combination.

For direct detection of αGalCer loading, monocytes were loaded overnight at 37°C with 10 μg/ml αGalCer followed by washing and incubation with 80 μg/mL myc-tagged Fab9b (1, 2) for 1 h at 4°C. After another washing step, cells were incubated with a mouse anti-myc antibody (10 μg/mL, Ebioscience) for 45 min at 4°C followed by washing, incubation with PE-labeled anti-mouse antibody (BD Biosciences) for 30 min at 4°C, additional washing, and analysis.

All antibodies and reagents used for flow cytometry were obtained from BD Biosciences expect for antibodies against MHC class I (W6/32), CD1d (51.1), CD56, FoxP3 (Ebioscience); CD1d (Nor3.2) (AbD Serotec) and CD1d-αGalCer tetramers (NIH tetramer core facility). Via-Probe (BD Biosciences) was added to all stainings for exclusion of dead cells. All flow cytometry stainings were performed with the same lot of antibody under standardized conditions to allow for comparison of mean fluorescence intensities between experiments.

Antigens

The following lipids were used in these studies: αGalactosylceramide (αGalCer, KRN7000; kind gift of Kirin Brewery Co., Ltd. (Tokyo, Japan)), Gal(α1–2)galactosylceramide (3), C20:2 (4), GSL-1 (5) (NIH tetramer core facility), BbGLIIf (6), mannosyl-phosphomycoketide (7), glucose monomycolate (GMM) C32 and C80 (8, 9). For MHC class I and II-restriced antigen presentation, CMV-, EBV-, Flu peptides (NIH AIDS reagent program) and tetanus toxin (Massachusetts Biolab) were used.

Antigen presentation assays

Monocytes, monocyte-derived DCs or B cells were incubated with lipids at the indicated concentrations. After 4 h or overnight incubation, cells were washed three times with RPMI-10 before co-culture of APCs and T cell clones at the indicated cell concentrations in 96-well flat bottom plates. Endogenous antigen presentation was studied by coculture of APCs and T cells in the absence of exogenous antigens. In some cases, blocking antibodies against CD1a (10H3.9, (10)), CD1b (BCD1b3.2, (11)), CD1c (F10/21A, (12))

3

or CD1d (51.1, Ebioscience) were added for 2 h at 37°C before addition of T cells. Pattern recognition receptor-dependent, CD1d-restricted antigen presentation was studied by co-culture of DCs and J3N.5 iNKT cells in the presence of heat-inactivated *Salmonella typhimurium* CS015 (13) or *Staphylococcus aureus* at a multiplicity of infection (MOI) of 20 as described in (14). To study *Mycobacterium tuberculosis*-induced lipid antigen presentation, DCs were infected with *M. tuberculosis* H37Rv at an MOI of 10 for 2 h at 37°C, in a humified atmosphere of 5% CO_2. Extracellular bacteria were removed by low speed centrifugation (1200 rpm) of DCs for 5min. DCs were cultured for the indicated times before washing and co-culture with T cell clones/lines. Supernatants were harvested after 18-20 h and assayed by ELISA (BD Biosciences) according to the manufacturer's instructions. For assays involving antigen presentation to human iNKT cells obtained from peripheral blood, IL-4 and IFN-γ were detected at their peak concentrations on day 1 and 3, respectively.

To investigate presentation of endogenous CD1-restricted antigen presentation, the T cell clones KHO.8 (CD1a-restricted, (15)), EC2.55 (CD1b-restricted, (15)), Ye2.3 (CD1c-restricted, (15)) and J24L.17 (CD1d-restricted, (16)) were used. For investigation of exogenous antigen presentation the following T cell clones/lines were used : LDN5 (CD1b-restricted clone, (8)) for presentation of glucose monomycolates (GMMs), DN6 (CD1c-restricted line, (17)) in response to *M. tuberculosis* infection, CD8-1 (CD1c-restricted line, (7)) in response to mannosyl-phosphomycoketide (MPM) and *M. tuberculosis* infection, and J3N.5 (CD1d-restricted, (16)) in response to CD1d-restricted lipids. In addition, iNKT cells were obtained by α-galactosylceramide-driven expansion

of Vα24-positive peripheral blood mononuclear cells obtained from healthy controls as previously described (18). Since cytokine production of T cell clones varies over time, samples of each ABL and FHBL patient were processed along with samples of three healthy controls to which results were compared.

Lentiviral MTP reconstitution

To obtain lentiviruses expressing MTP, human MTP was amplified using the primers BamHI hMTP_for 5'-GAT<u>GGATCC</u>AATATGGGAATTCTTCTTGCTGTGCTT-3' and XhoI hMTP_rev 5'-GAA<u>CTCGAG</u>TCAAAACCATCCGCTGGAAGT-3' (restriction sites underlined). This fragment was cloned 5' of the IRES-eGFP encoding sequence into the previously described trip/SIN encoding lentiviral transfer plasmid (19). Lentiviral vector particles were generated in human embryonal kidney 293T cells by the transient cotransfection method using the multiple attenuated packaging plasmid pCMVΔR8.9 (Addgene) and the vesicular stomatitis virus glycoprotein (VSV.G) encoding plasmid pMD2.G (Addgene) as described previously (19). Viral titers were determined by infection of 293T cells with serial dilutions of the vector stock and determination of eGFP-positive cells 72 h after infection by flow cytometry. On day 3, monocyte-derived DCs were infected with lentiviruses expressing hMTP or the 'empty' eGFP virus at an MOI of 15 in RPMI-1640 containing 10 μg/ml protamine sulphate (Sigma Aldrich). The immature transduced DCs were cultured at 10^6 cells/ml until day 5.

Quantitative real-time PCR

Total RNA isolation and reverse transcription were performed using the RNeasy mini kit (Qiagen) and High-Capacity cDNA archive kit (Applied Biosystems) according to the manufacturers' instructions. Primer sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/) and sequences are listed in Suppl. Table 2. Real-time PCR was performed using LightCycler 480 SYBR Green I mastermix (Roche) and a Roche LightCycler 480 system. Human GAPDH served as endogenous control and its expression was not affected under experimental conditions. PCR was set up in triplicates and threshold cycle (Ct) values of the target genes were normalized to the endogenous control. Differential expression was calculated according to the $2^{-\Delta\Delta CT}$ method [20].

Immunofluorescence and confocal laser scanning microscopy

Monocyte-derived DCs were allowed to adhere to coverslips pretreated with fibronectin (Sigma-Aldrich) before fixation with paraformaldehyde (4%, 15 min, RT), quenching with 25mM glycine and washing in PBS. In some cases, fluorescently labeled human transferrin or proteasomal inhibitors were added before fixation of DCs. Coverslips were blocked and permeabilized with 10% goat serum, 0.1% saponin (30 min, RT) before addition of fluorescently labeled primary antibodies (30 min, RT), washing and mounting in ProLong Gold antifade reagent with DAPI (Invitrogen). Slides were stored at 4°C before analysis using a Zeiss LSM 510 Meta laser scanning microscope. The following antibodies and reagents were used: anti-CD1a (10H3.9, (10)), anti-CD1b (BCD1b3.2, (11)), anti-CD1c (F10/21A, (12)), anti-CD1d 51.1 (Ebioscience), 488-human transferrin

(Invitrogen), anti-calnexin (Cell Signaling Technology), anti-Lamp 1 (BD Biosciences) and anti-EEA1 (Calbiochem).

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|	Gender	APOB truncation	MTTP mutation nucleotide change	MTTP mutation name	Other mutations	Age	Chol	TG	ABL-related symptoms and medical history	Medication
ABL	F	UD	hom c.2593G>T	hom G865X		26	16	2	diarrhea, otherwise asymptomatic	
	M	UD	hom c.2593G>T	hom G865X		41	59	<1	dysarthria, dysmetria, tremor, diarrhea	
	F	UD	hom c.1820delG	hom 1820del1		46	25	<1	ptosis, basal cell carcinoma, corneal ulcer, ataxia, paresthesia, acanthocytosis,steatorrhea	
	F	UD	comp het; c.2237G>A (p.G746E) and c.2524A>T (p.K842X)	comp het G746E, K842X		48	68	<1	diarrhea, acanthocytosis, dysarthria, ataxia	
	F	UD	comp het c.2593G>T, c.501G>A	comp het G865X, E167E (exon 4 donor splice site mutation)		39	48	15	Marfan's Syndrome, liver cirrhosis, mitral valve prolapse, colorectal cancer	Digoxin, Gabapentin, Fluoxetine, Metoprolol, Spironolactone, Furosemide
	M	UD	hom c.136C>G	hom R46G		57	48	5	steatorrhea, ataxia, retinal degeneration, peripheral neuropathy, neurogenic bladder, dysartric speech, microvesicular steatosis	
FHBL	F	het B-31				48	156	27	asymptomatic	
	M	het B-52				45	118	63	asymptomatic	
	F	het B-52				74	117	58	asymptomatic	
	F	het B-4				56	72	98	asymptomatic	
	F	het B-4				54	117	35	asymptomatic	
	F	UD, comp het (D1086H/Y1476X)				31	97	32	Fat intolerance, diarrhea	
	M	UD, het: I381T, D2272H, E2387D, M4241T	het c.1769G>T	het S590I	SAR1B (het H105R)	67	39	2	Failure to thrive in childhood, diarrhea, fat intolerance	

Supplementary Table 1. Characteristics of patients with ABL and FHBL. UD indicates undetectable serum APOB levels. Hom, homozygous; het, heterozygous; comp het, compound heterozygous; chol, cholesterol; TG, triglycerides.

Primer name	Sequence (5' -> 3')
hBIP_for	CATCACGCCGTCCTATGTCG
hBIP_rev	CGTCAAAGACCGTGTTCTCG
hCANX_for	GGCTGGTTAGATGATGAGCCT
hCANX_rev	GTTGGCAATCTGAGGAGCCT
hCD1A_for	GTGATGGCAATGCAGACGG
hCD1A_rev	CCACAGGAAAACGATGGTGCT
hCD1B_for	CTGCCATTTCAACTGTTAGCTGT
hCD1B_rev	GCCCAGGTACTATTGGTAAAGGA
hCD1C_for	AGACGCATCCCAGGAACAC
hCD1C_rev	GCCATGAGTCTGCAACTCGT
hCD1D_for	TCTCGTCCTTCGCCAATAGC
hCD1D_rev	GGGACCAAGGCTTCAGAGAG
hHLA_A_for	AAAAGGAGGGAGTTACACTCAGG
hHLA_A_rev	GCTGTGAGGGACACATCAGAG
hHFE_for	GAGTCTGAAAGGGTGGGATCA
hHFE_rev	TCTCCAATCCAGTGTGTCAGG
hB2M_for	GGCTATCCAGCGTACTCCAAA
hB2M_rev	CGGCAGGCATACTCATCTTTTT
hGAPDH_for	CTCTGGTAAAGTGGATATTGTTGC
hGAPDH_rev	CATGTAAACCATGTAGTTGAGGTC

Supplementary Table 2. Sequences of primers used for quantitative PCR analysis.
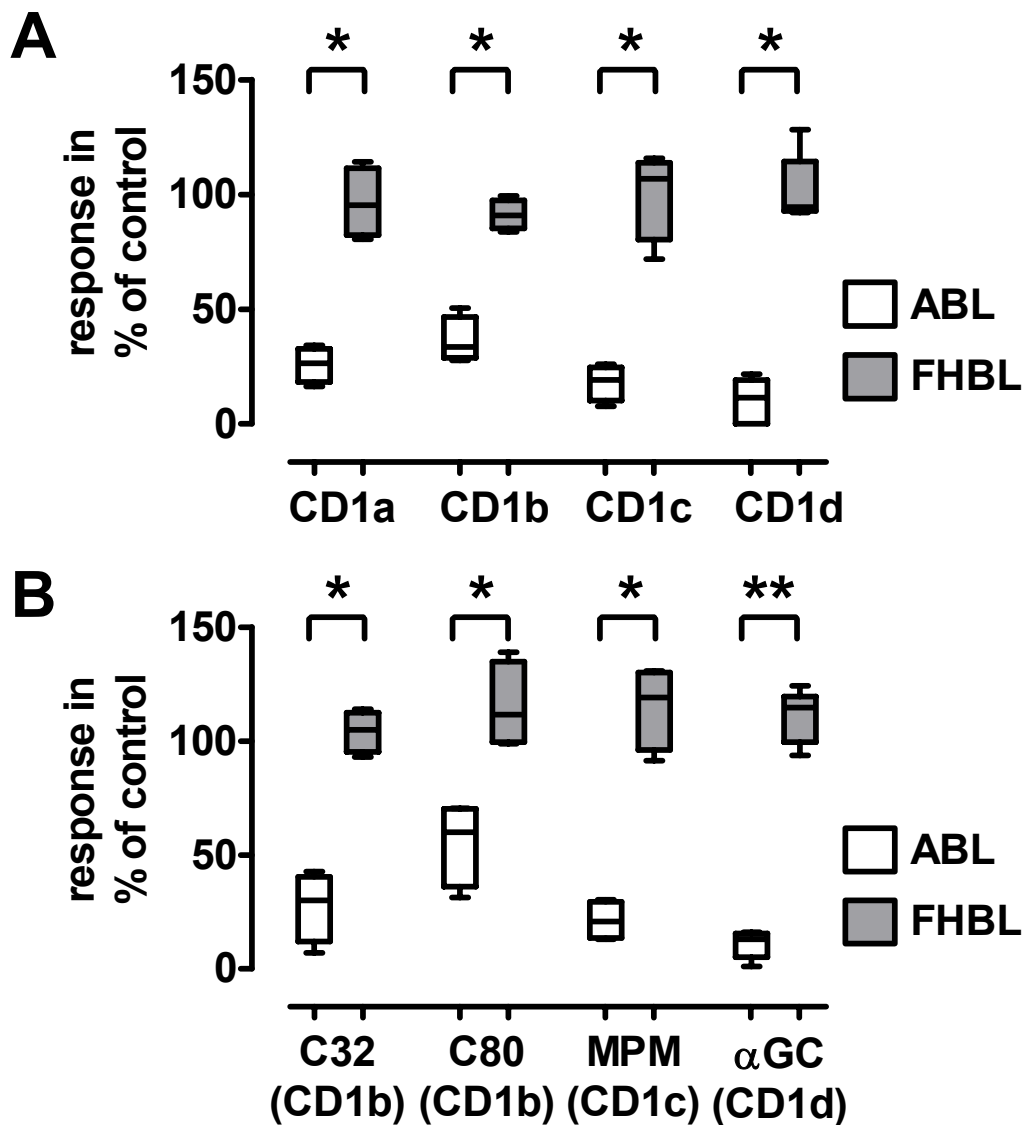
Supplementary Figure 1. Statistical analysis of endogenous (A) and exogenous (B) CD1-restricted antigen presentation in all ABL and FHBL patients studied. DCs were co-cultured with CD1-restricted T or NKT cells in the presence or absence of the indicated antigens. IFN-γ secretion by T cells in response to DCs was determined by ELISA. In each experiment, one ABL or FHBL sample was compared to three controls and relative activation of T cells in response to FHBL and ABL DCs was determined as IFN-γ secretion in percent of all three controls. Box plots show lower and upper quartiles, median and range (whiskers) of the cumulative data of all patients analyzed for each parameter (n=4-5). *p<0.05, **p<0.01. MPM = mannosyl phosphomycoketide, C32 = glucose monomycolate C32, C80 = glucose monomycolate C80, αGC = alpha-galactosylceramide
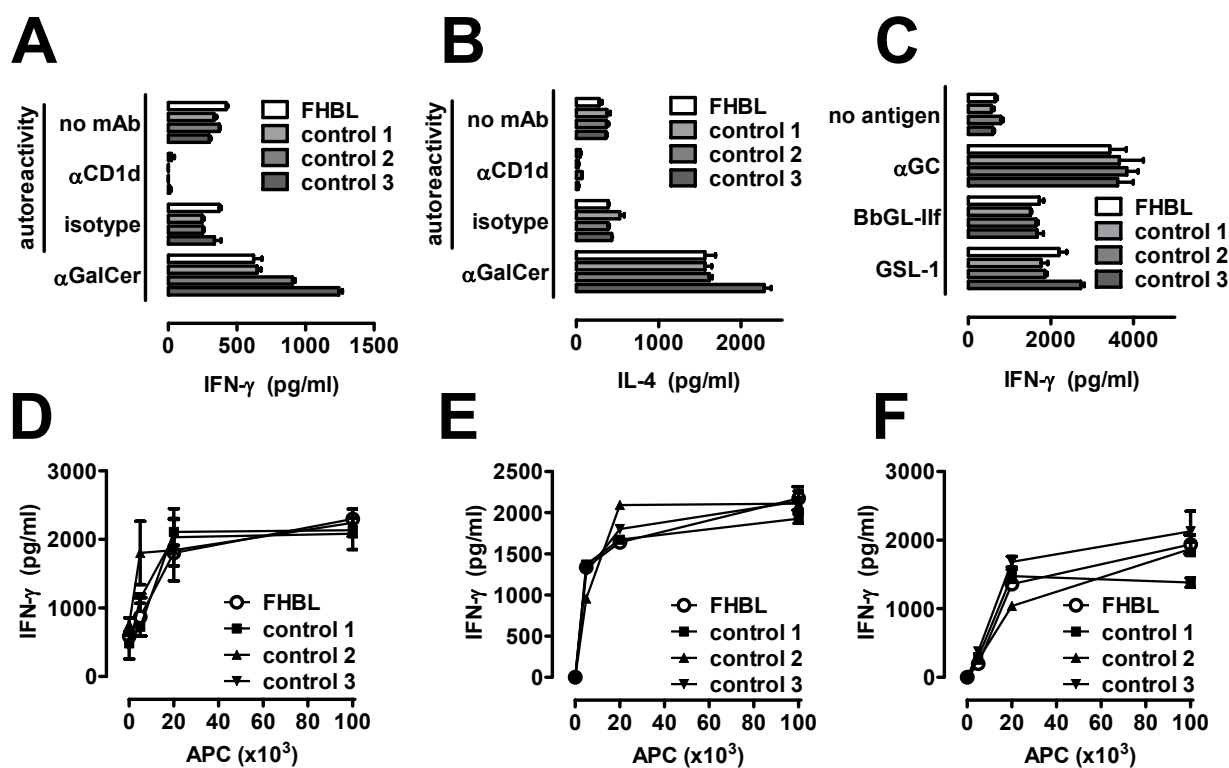
Supplementary Figure 2. Unaltered CD1-restricted antigen presentation in an FHBL subject with compound heterozygous mutations in *APOB* (A-B) and a severely hypolipidemic subject with heterozygous mutations in *MTTP*, *SAR1B* and *APOB* (C-F). A-C) DCs were cocultured with iNKT cells in the presence or absence of α-galactosylceramide (100 ng/ml), BbGL-IIf (10 µg/ml, C) or GSL-1 (10 µg/ml, C). CD1d-restriction was confirmed by blocking with a monoclonal antibody directed against CD1d (51.1). D-F) DCs were cultured at the indicated concentrations with 5×10^4 CD1a- (D) CD1b- (E), and CD1c- (F) restricted T cells. Release of IFN-γ (A, C-F) and IL-4 (B) by T cells is shown.
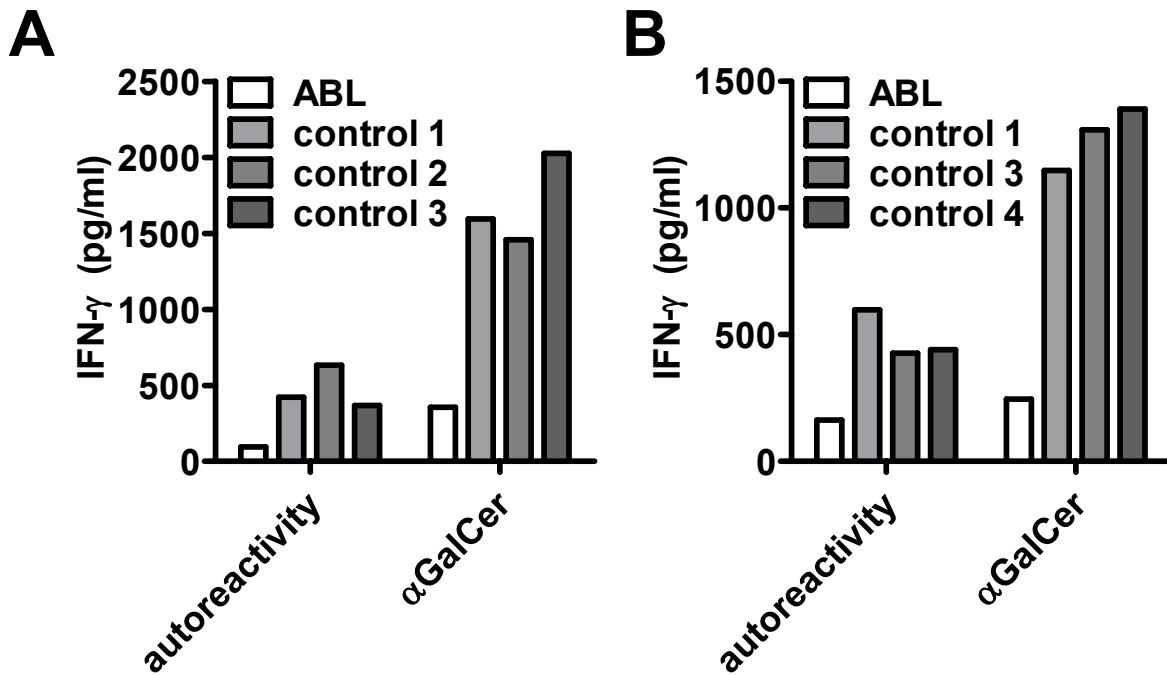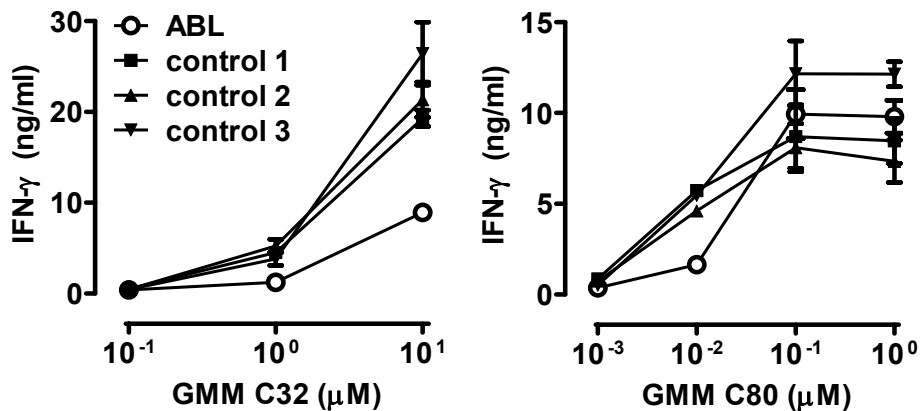
Supplementary Figure 3. Impaired CD1d-restricted antigen presentation in ABL is consistent over time. A and B are analyses of the same ABL patient and partly similar controls as indicated by numbers performed in November 2007 (A) and August 2009 (B). In each experiment, $2x10^4$ DCs were cocultured with $5x10^4$ iNKT cells in the presence or absence of 100 ng/ml α-galactosylceramide. Culture of DCs or T cells alone did not lead to detectable cytokine production. Cytokine release of iNKT cells was determined after 18-20 hours of coculture by ELISA.
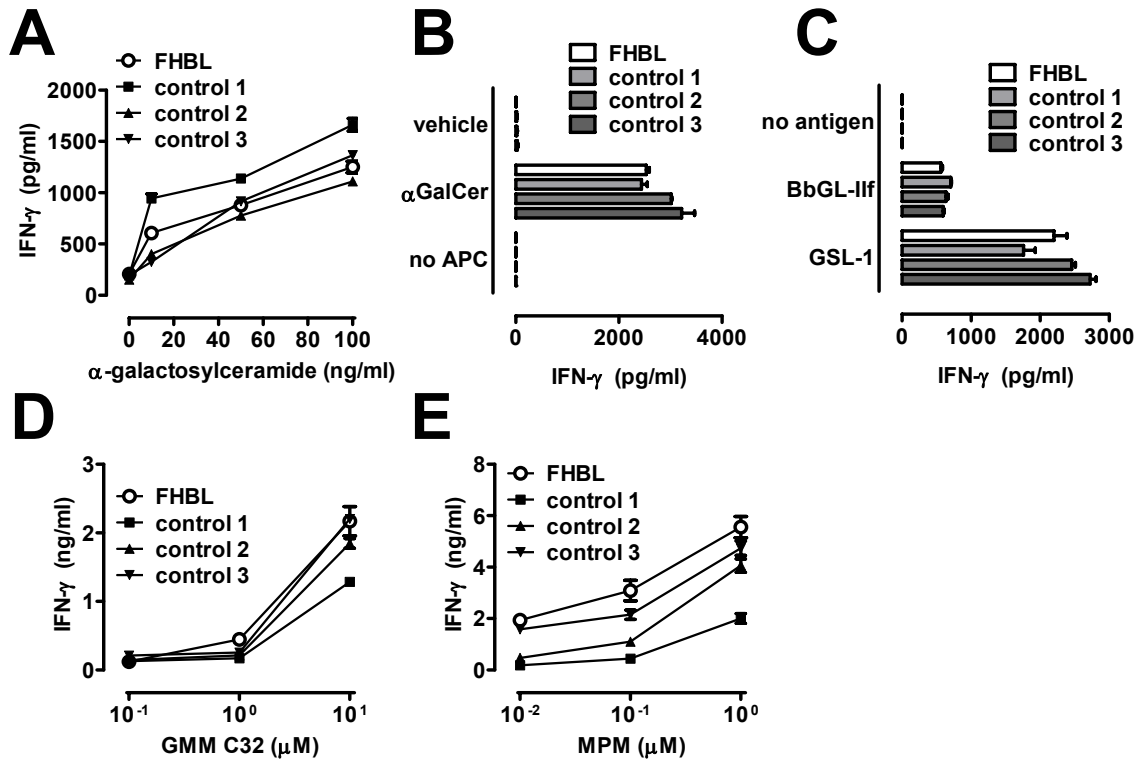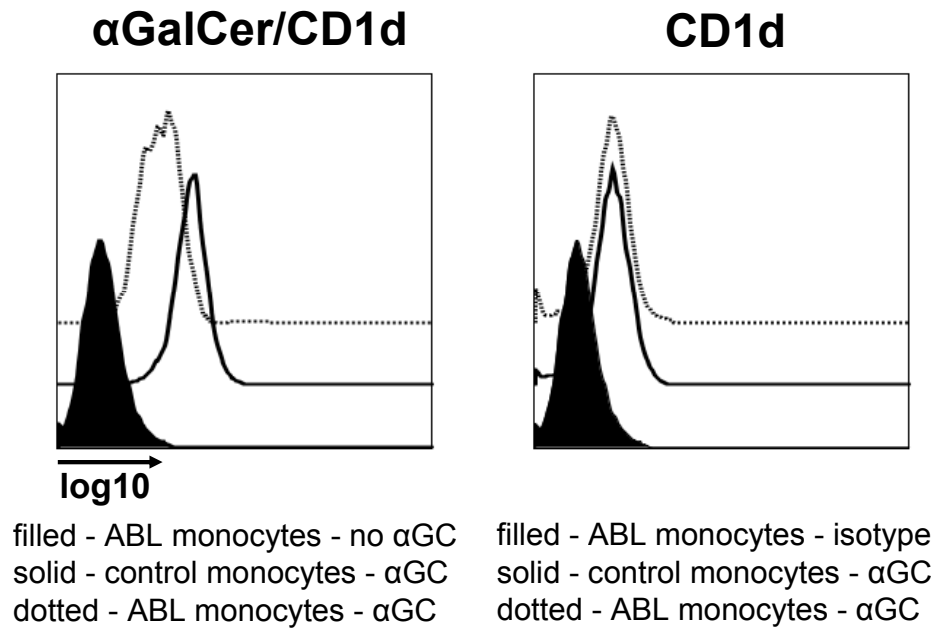
Supplementary Figure 4. Unaltered presentation of exogenous antigens in FHBL. 2×10^4 DCs (A, D, E), monocytes (C) or B cells (B) were cocultured with 1×10^5 iNKT cells (A-C), CD1b-restricted T cells (D) or CD1c-restricted T cells (E) in the presence of the indicated concentration of α-galactosylceramide (100 ng/ml in B), glucose monomycolate (D), mannosyl-phosphomycoketide (E), BbGL-IIf (10 µg/ml, C) or GSL-1 (10 µg/ml), C). Culture of DCs or T cells alone did not lead to detectable cytokine production. Cytokine release was determined after 18-20 hours of coculture by ELISA. One representative experiment is shown. Two additional experiments with cells from independent FHBL and control subjects showed comparable results.
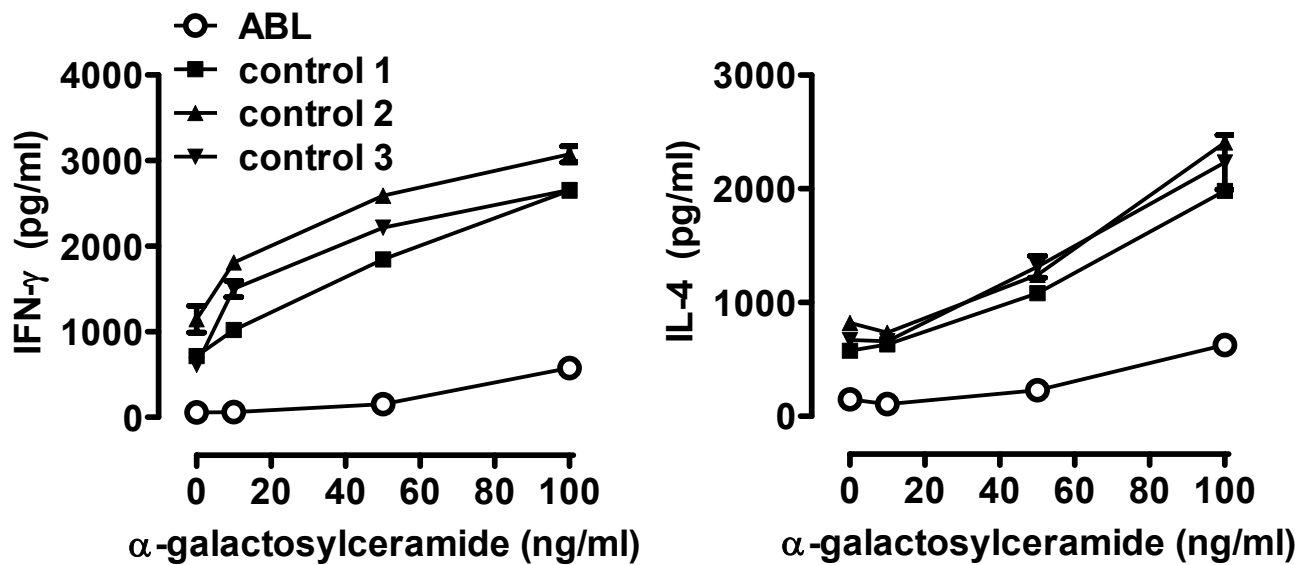
Supplementary Figure 5. Impaired production of Th1 and Th2 cytokines by iNKT cells in response to ABL DCs. $2x10^4$ DCs were cocultured with $5x10^4$ iNKT cells expanded from peripheral blood of healthy donors as described in Supplementary Methods. IL-4 and IFN-γ were determined at their peaks after 1 and 3 days of coculture, respectively. Culture of DCs or iNKT cells alone did not lead to detectable cytokine production. One representative experiment is shown. Three additional experiments with cells from independent ABL and control subjects showed comparable results.
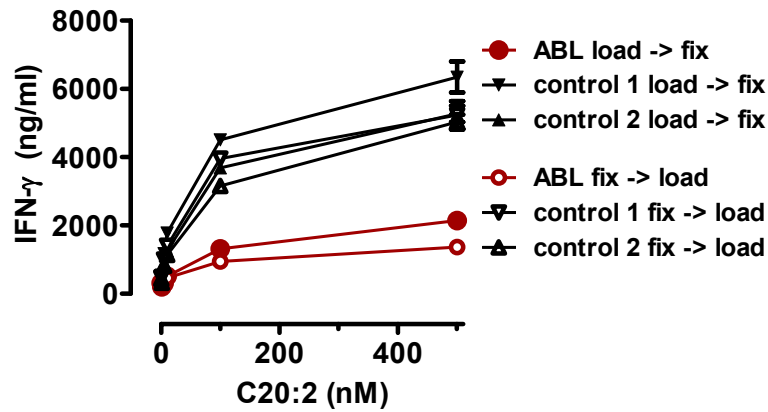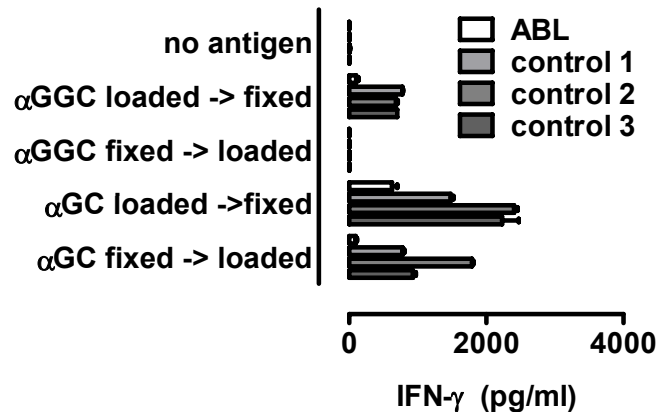
Supplementary Figure 6. Impaired CD1-restricted antigen presentation is not dependent on endolysosomal trafficking. A, B. Monocytes were loaded with the indicated antigens (αGGC, 100 ng/ml Gal(α1–2)galactosylceramide ; αGC, 100 ng/ml α-galactosylceramide) for 4 h (αGC, C20:2) or 16 h (αGGC). To delineate the effects of endolysosomal trafficking on antigen loading, cells were fixed with glutaraldehyde before or after antigen loading. iNKT cells were used as responders. C. Impaired CD1b-restricted presentation of the cell surface-loaded lipid GMM C32 but largely unaltered presentation of the endolysosomally loaded antigen GMM C80 in ABL. DCs were cocultured with CD1b-restricted T cells in the presence of the indicated amounts of antigen. One representative experiment is shown. One additional experiment with cells from independent ABL and control subjects showed comparable results.

αGalCer/CD1d   **CD1d**

log10

filled - ABL monocytes - no αGC   filled - ABL monocytes - isotype
solid - control monocytes - αGC   solid - control monocytes - αGC
dotted - ABL monocytes - αGC   dotted - ABL monocytes - αGC

Supplementary Figure 7. Altered CD1d loading with α-galactosylceramide in ABL. Monocytes from ABL or control were loaded with saturating concentrations of α-galactosylceramide (10 μg/ml) for 16 h at 37°C followed by staining with antibodies specific for the α-galactosylceramide/CD1d complex (left panel). To control for CD1d expression, cells were stained with 51.1 anti-CD1d antibody (right panel). Expression was determined by flow cytometry. Shown is the mean fluorescence intensity on a log10 scale. One representative experiment is shown. One additional experiment with cells from independent ABL and control subjects showed comparable results.
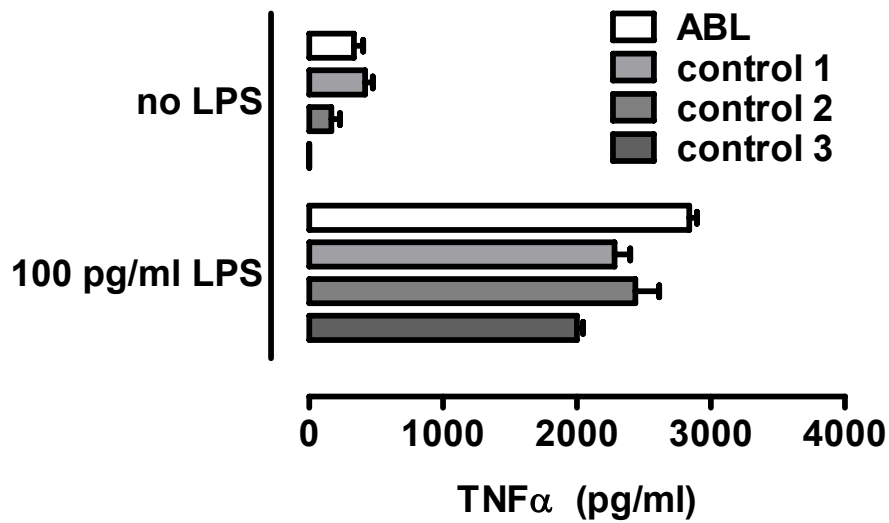
Supplementary Figure 8. Unaltered response of ABL DCs to lipopolysaccharide (LPS). 1×10^5 DCs were cultured in the presence or absence of LPS for 16 h and TNFα release was measured by ELISA. One representative experiment is shown. One additional experiment with cells from independent ABL and control subjects showed comparable results.
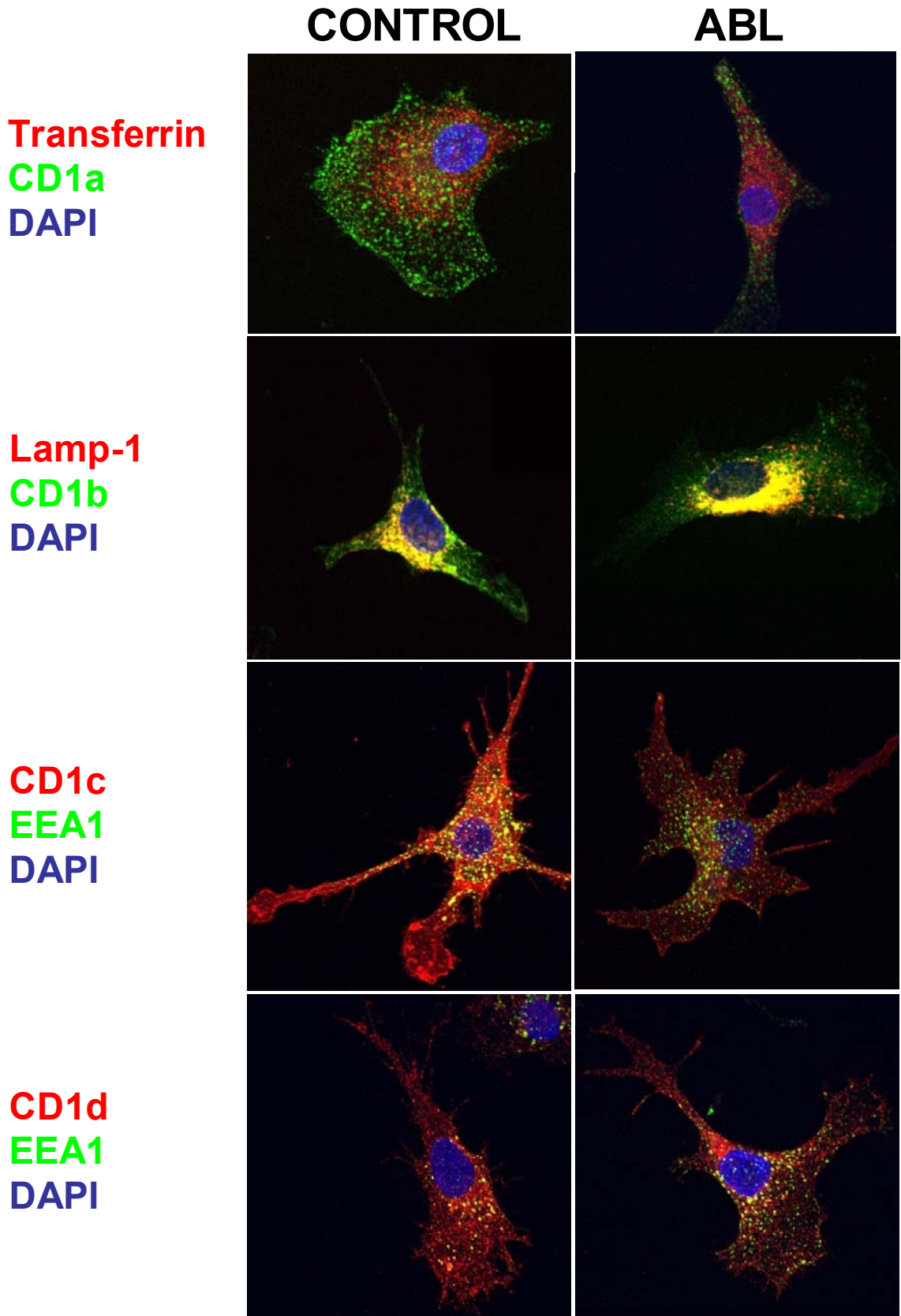
Supplementary Figure 9. Unaltered intracellular distribution of CD1 molecules in ABL DCs. DCs were stained on coverslips with the indicated antibodies. Merged pictures as obtained by confocal microscopy are shown in 600x magnification. All images were taken with the same instrument settings. One representative experiment is shown. One additional experiment with independent ABL and control subjects showed comparable results.
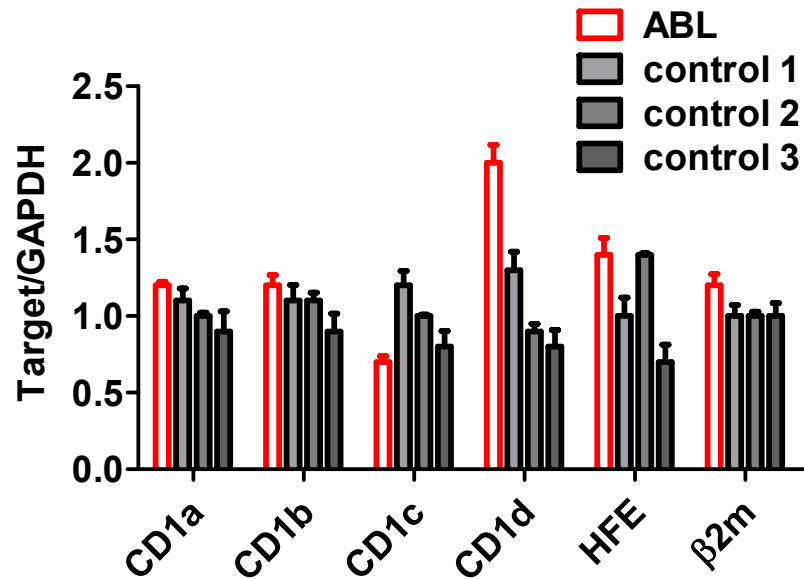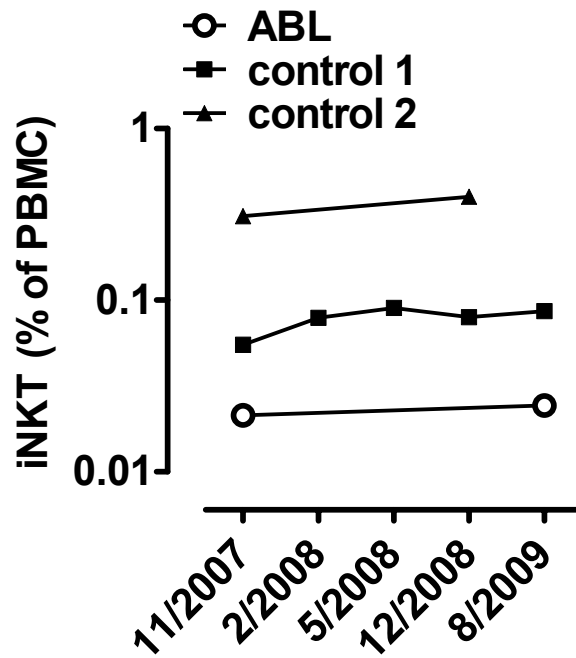
Supplementary Figure 10. Altered CD1 expression in ABL DCs is not caused by impaired transcription. Expression of the indicated transcripts was detected by quantitative PCR. Relative expression as normalized to the endogenous control GAPDH is shown. One representative experiment is shown. One additional experiment with independent ABL and control subjects showed comparable results.
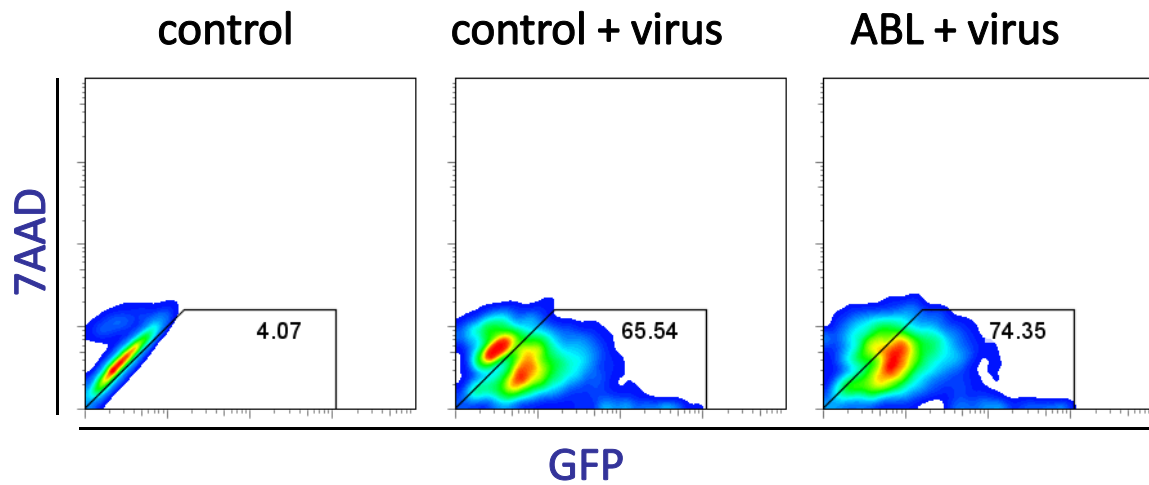
Supplementary Figure 11. Transduction of ABL and control DCs by lentiviruses expressing MTP. Transduction and viability is shown by flow cytometric analysis of DCs two days after lentiviral transduction at a multiplicity of infection of 15. Lentiviruses express GFP via an IRES sequence which can be used for conformation of transduction. Viability is indicated by lack of 7AAD uptake.
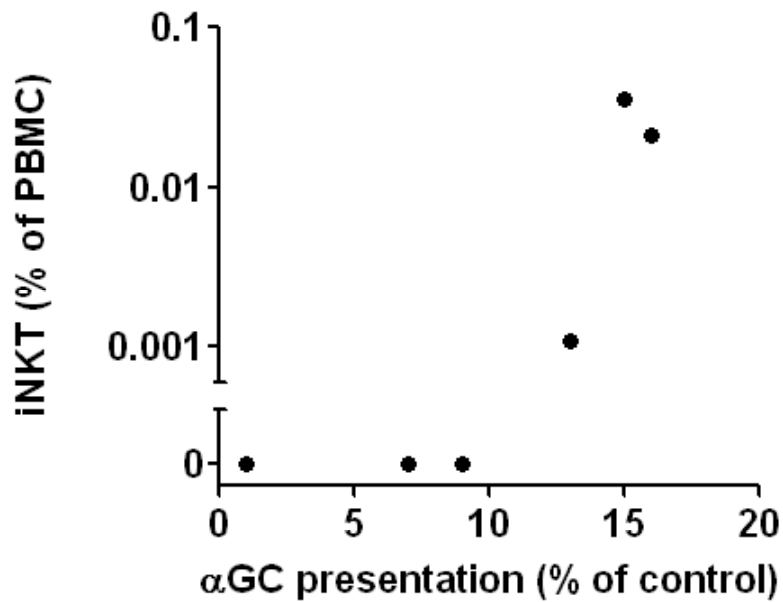
Supplementary Figure 12. Impaired CD1d-restricted antigen presentation in ABL correlates with reduced peripheral iNKT cell numbers. Shown is a comparison of periperal iNKT cell numbers and residual CD1d-restricted antigen presentation in ABL. CD1d-restricted antigen presentation was determined as IFN-γ release by the J3N.5 iNKT cell clone upon presentation of 100 ng/ml α-galactosylceramide by ABL and control monocytes. Antigen presentation of each ABL patient was compared to three healthy controls and iNKT IFN-γ release is given as percentage of the average of all three healthy controls.

Supplementary Figure 13. Individual peripheral iNKT cell levels are stable over time. iNKT cell levels in peripheral blood were determined by staining with αGalCer-loaded CD1d tetramers. iNKT cell levels are expressed as percentage of PBMC. Dates (month/year) are indicated on the x axis.