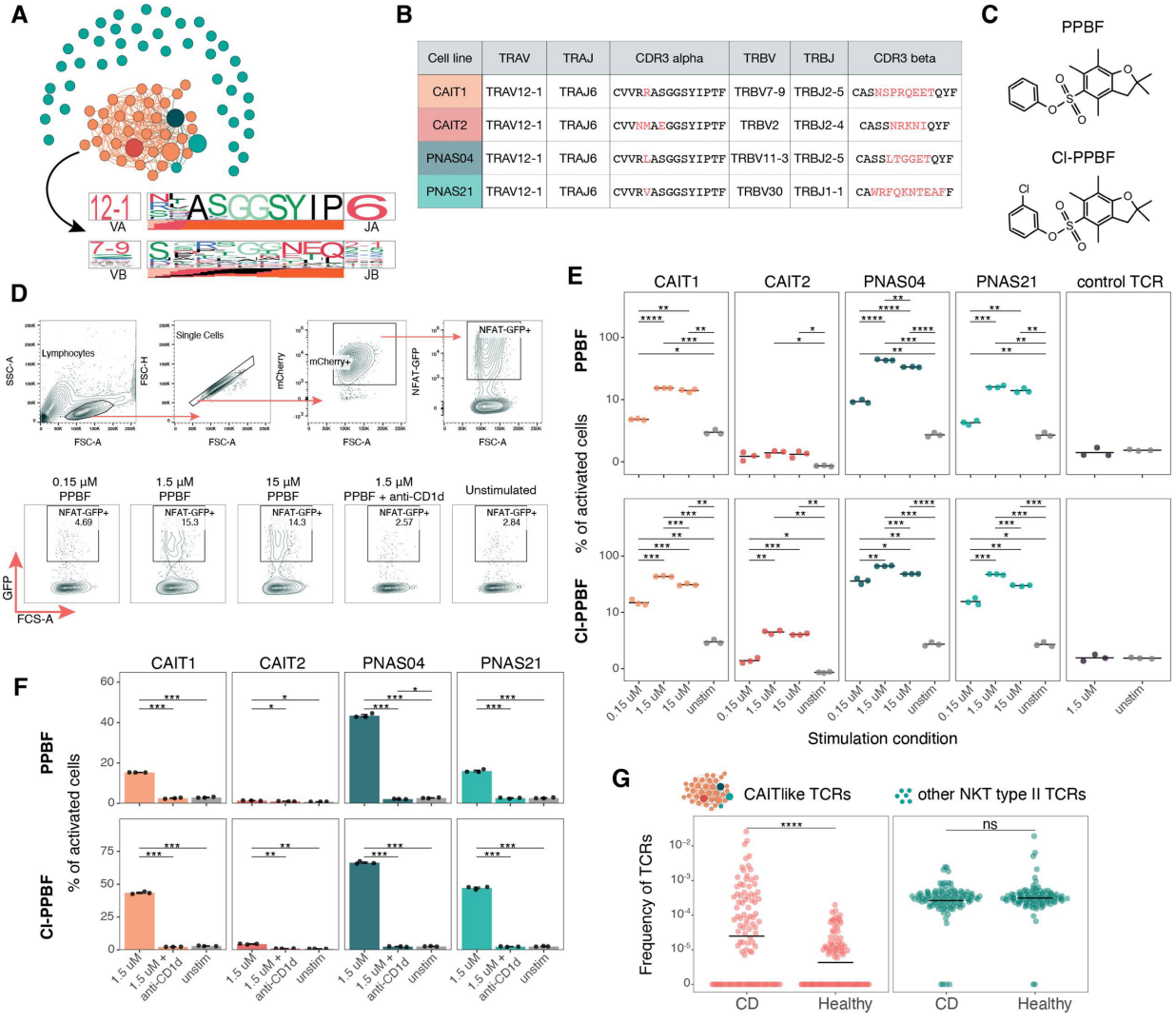
Letters

Crohn's-associated invariant T cells (CAITs) recognise small sulfonate molecules on CD1d

MAIN

In the recent study by Rosati *et al*, we described a novel unconventional T cell population enriched in the peripheral blood of patients with Crohn's disease (CD) and characterised by a semi-invariant T cell receptor (TCR) repertoire.[1] However, the specificity of these Crohn's-associated invariant T (CAIT) cells was not defined. Identifying the specificity of CAIT cells is essential to understand the origin of the antigen triggering their enrichment in CD.

In our previous study, we observed that CAIT cells have TCRs similar to those reported for some natural killer T (NKT) type II cells.[2 3] Here, we performed a sequence similarity analysis[4] and identified a large cluster composed of CAIT clonotypes and three reported NKT type II clonotypes (figure 1A). While the NKT type II and CAIT clonotypes all had highly similar TCR alpha chains carrying TRAV12-1/TRAJ6 genomic segments, their beta chains were highly diverse (figure 1A, bottom). Dash *et al* have shown that TCRs with similar sequences frequently have the same specificity.[5 6] In the original publications describing these clonotypes, the authors reported that the NKT type II cells recognise small molecules of the pentamethylbenzofuransulfonates (PBFs)



Cell line	TRAV	TRAJ	CDR3 alpha	TRBV	TRBJ	CDR3 beta
CAIT1	TRAV12-1	TRAJ6	CVVRRASGGSYIPTF	TRBV7-9	TRBJ2-5	CASNSPRQEETQYF
CAIT2	TRAV12-1	TRAJ6	CVVNMAEGGSYIPTF	TRBV2	TRBJ2-4	CASSNRKNIQYF
PNAS04	TRAV12-1	TRAJ6	CVVRLASGGSYIPTF	TRBV11-3	TRBJ2-5	CASSLTGGETQYF
PNAS21	TRAV12-1	TRAJ6	CVVRVASGGSYIPTF	TRBV30	TRBJ1-1	CAWRFQKNTEAFF

Figure 1 Comparison of natural killer T (NKT) type II and Crohn's-associated invariant T (CAIT) cells. (A) Sequence similarity analysis of NKT type II[2 3] and CAIT T cell receptors (TCRs). Each node corresponds to the unique alpha/beta TCR sequence and edges connect highly similar TCRs (tcrdist metric<150). Larger nodes indicate TCRs used for cloning. TCRdist sequence logos for TCRalpha and TCRbeta chains of TCRs from the cluster are shown at the bottom. (B). Genomic segments and amino acid CDR3 sequences of TCRs picked for experimental validation. Red font indicates differences in CDR3 regions. (C). Chemical structures of phenyl-pentamethyldihydrobenzofuransulfonate (PPBF) and chlorophenyil-pentamethyld ihydrobenzofuransulfonate (ClPPBF). (D.) Gating strategy and representative flow plots for pentamethylbenzofuransulfonates (PBFs) stimulation experiment. (E). Frequency of activated cells reactive to PPBF (top) and ClPPBF (bottom). Only significant p values from a T-test with Holm method for multiple testing correction are shown (*<0.05, **<0.01, ***<0.001, ****<0.0001). (F). Anti-CD1d antibody prevents activation of all four cell lines with PPBF (top) and ClPPBF (bottom). Only significant p values from a t-test with Holm method for multiple testing correction are shown (*<0.05, **<0.01, ***<0.001, ****<0.0001). (G.) Frequency of CAITlike NKT type II TCRs from Almeida *et al* (attached to a large cluster on figure 1A) and non-CAIT like NKT type II TCRs in Crohn's disease and healthy cohorts from Rosati *et al* .[1] Only significant p value (****<0.0001) from a Mann-Whitney U-test is shown.

family presented by the invariant HLA-like CD1d protein.[2 3] Thus, we investigated whether CAIT TCRs shared the specificity of the NKT type II cells.

We transduced TCR-null NFAT-GFP reporter Jurkat cells with constructs encoding two representative CAIT TCRs and two coclustering NKT type II TCRs (figure 1B). We synthetised two of the PBF compounds, phenyl-pentamethyldihydro benzofuransulfonate (PPBF), the original compound identified as a CD1d-dependent activator of NKT type II cells,[2] and a more potent PPBF analogue chlorophenyil-pentamethyldihydrobenzofuransulfonate (ClPPBF)[3] (figure 1C). Importantly, Jurkat cells naturally express CD1d and thus can act as antigen-presenting cells for CD1d-dependent antigens. To evaluate TCR activation, we cultured Jurkat cell lines with incremental concentrations of PPBF and ClPPBF (figure 1D).

All four transgenic cell lines (CAIT1, CAIT2, PNAS04, PNAS21) reacted to both compounds in a dose-dependent manner (figure 1E). Consistent with the original study, all tested TCRs reacted more strongly to ClPPBF. The CAIT2 cell line, with the lowest level of activation, (figure 1E) carried the most dissimilar TCRalpha sequence compared with the other cell lines. CAIT2 has mismatches at CDR3 positions 4 and 7 (figure 1B), suggesting the importance of these positions for TCR avidity. A control cell line with known TCR specificity was not activated by the compounds, indicating that compound recognition is TCR-dependent (figure 1E). The reactivity of all cell lines dropped to unstimulated levels in the presence of the CD1d-blocking antibody, demonstrating that the TCR interaction is CD1d-restricted (figure 1F).

To further investigate the possible role of NKT type II cells in CD, we searched all NKT type II sequences reported in Almeida *et al* in our previously published TCR data from patients with CD and healthy controls.[1 3] Only TCRs from NKT type II cells with the characteristic CAIT TCRalpha chain motif showed significant enrichment in patients with CD, while other TCRs were found in comparable amounts in patients and controls (figure 1G). Thus, only a subgroup of NKT type II cells with specific TCR features is enriched in CD.

While we show the specificity of CAIT cells for PBF small molecules in the context of CD1d, many questions remain. Many other small molecules similar to PBFs exist, including drug derivatives and microbial metabolites.[3 7 8] It is thus reasonable to hypothesise that different small molecules may be triggering CAIT cells in vivo. Importantly, NKT cells can differentiate into opposing phenotypes, from proinflammatory to regulatory,[8–10] necessitating further characterisation on the functional profile of the NKT type II CAIT cell subset and its behaviour in patients with CD.

Detailed methodologies are described in online supplemental file 1.

Anastasia A Minervina,[1] Mikhail V Pogorelyy,[1] Steffen Paysen,[2] Ulrich Luening,[2] Frauke Degenhardt,[3] Andre Franke,[3] Paul G Thomas,[1] Elisa Rosati [3,4]

[1]Department of Immunology, St Jude Children's Research Hospital, Memphis, Tennessee, USA
[2]Otto-Diels-Institute for Organic Chemistry, Christian-Albrecht University of Kiel, Kiel, Germany
[3]Institute of Clinical Molecular Biology, University Hospital Schleswig Holstein, Kiel, Germany
[4]Institute of Immunology, Christian-Albrecht University of Kiel, Kiel, Germany

Correspondence to Dr Andre Franke, Institute of Clinical Molecular Biology, University Hospital Schleswig Holstein, Kiel, Schleswig-Holstein, Germany; a.franke@ikmb.uni-kiel.deDr Paul G Thomas; Paul.thomas@STJUDE.org

Twitter Elisa Rosati @elisarosix

Contributors Conceptualisation: ER, MVP, AAM; analysis: ER, MVP, AAM; Visualisation: AAM; Resources: PGT, AF, FD, SP, UL; Supervision and coordination: AF, PGT, ER. Writing original draft: ER, MVP, AAM. Funding acquisition: PGT, ER, AF. All authors provided discussion, participated in revising the manuscript, and agreed to the final version.

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AAM and MVP contributed equally.

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AF, PGT and ER are joint senior authors.

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ORCID iD
Elisa Rosati http://orcid.org/0000-0002-2635-6422

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SUPPLEMENTARY MATERIALS AND METHODS

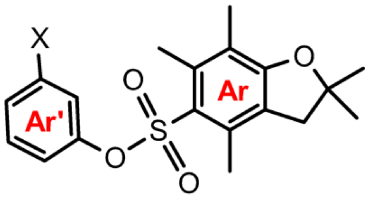
Compound synthesis

General information

Chromatography was carried out with Isolera™ spectra system with ACI™ and assistant Fa. Biotage with automated fraction collector. The used columns were Biotage® SNAP Ultra cartridge filled with Biotage® HP-Sphere™ spherical silica. Reactions were monitored with a UV detector and the R_f-values were determined by TLC-plates (Alugram® Xtra Sil G/UV254 Fa. Machery-Nagel). All solvents were destilled before use.

NMR-Spectroscopy

In addition to the ^{1}H and ^{13}C NMR Spectra, two-dimensional measurement methods for further structural elucidation were measured (COSY, HSQC, HMBC). The following designations were used for the assignment of the signals:



Chemicals

Chemical name	Purity	Company
chlorosulfuric acid	99 %	Fluka
3-chlorophenol	98 %	TCI
cyclohexane	99 %	Walter-CMP
dichloromethane	99 %	Walter-CMP
ethyl acetate	99 %	Walter-CMP
isobutyraldehyde	98 %	TCI
potassium carbonate	99 %	Grüssing
phenol	99.5 %	TCI
toluene	99 %	Walter-CMP
2,3,5-trimethylphenol	98 %	TCI
sulfuric acid	96 %	Grüssing

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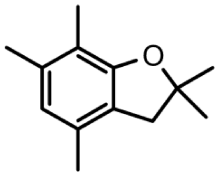
Synthesis of 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran (1)

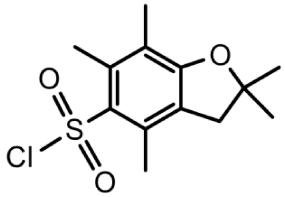


Isobutylaldehyde (78 mmol) and 300 µL of sulfuric acid were added to a stirred solution of 8.88 g of 2,3,5-trimethylphenol (65.3 mmol) in 50 mL of toluene. The reaction mixture was refluxed for 20 h. After cooling to room temperature, 200 mL of ethyl acetate and 50 mL of demineralized water were added. The organic layer was washed with saturated potassium carbonate solution (2 x 100 mL) and demineralized water (2 x 100 mL). The solvent was removed under reduced pressure. The residue was purified by chromatography (cyclohexane (R_f = 0.24) → ethyl acetate/cyclohexane 1:9 (R_f = 0.47) to afford **1** as a white solid.

yield: 6.90 g (36.3 mmol, 56 %) Ref.: 76 % [1]

^{1}H-NMR (200 MHz, $CDCl_3$): δ = 6.48 (s, 1H, 5-Ar-*H*), 2.90 (s, 2H, C*H*$_2$), 2.19 (s, 3H, 4-Ar-C*H*$_3$), 2.14 (s, 3H, 6-Ar-C*H*$_3$), 2.07 (s, 3H, 7-Ar-C*H*$_3$), 1.46 (s, 6H, 2-Ar-C*H*$_3$) ppm.

5-(2,2,4,6,7-Pentamethyl-2,3-dihydrobenzofuran)-sulfonyl chloride (2)



Under nitrogen, a solution of **1** (502 mg, 2.64 mmol) in 25 mL of dry dichloromethane was cooled to 0 °C. 0.35 mL of chlorosulfuric acid (5.26 mmol) was added dropwise and the reaction mixture was stirred for 3 h at room temperature. The crude product solution was used for the synthesis of **3** and **4** without further purification or analysis.

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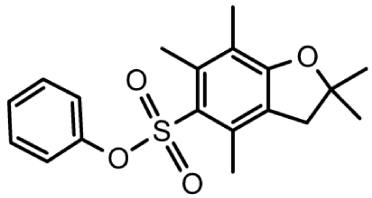
Phenyl-5-(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran)-sulfonate (3)



Under nitrogen, 735 mg of phenol (7.92 mmol) was stirred in 25 mL of dry dichloromethane at 0 °C. During 1 h, freshly prepared sulfonyl chloride **2** was added dropwise to the reaction. The reaction mixture was stirred for 20 h and quenched with 50 mL of demineralized water. Dichloromethane (200 mL) were added to the organic layer, and it was washed with a saturated potassium carbonate solution (1 x 100 mL) and demineralized water (2 x 100 mL). The solvent was removed under reduced pressure. The residue was purified by chromatography (cyclohexane (R_f = 0.11) □ ethyl acetate/cyclohexane 1:9 (R_f = 0.26) to afford **1** as a yellow oil.

yield.: 245 mg (707 µmol, 13 %) Ref.: 55 % [1]

^{1}H-NMR (600 MHz, $CDCl_3$): δ = 7.29 (t, ^3J = 7.4 Hz, 2H, 3-Ar'-*H*, 5-Ar'-*H*), 7.22 (t, ^3J = 7.4 Hz, 1H, 4-Ar'-*H*), 7.03 (d, ^3J = 7.4 Hz, 2H, 2-Ar'-*H*, 6-Ar'-*H*), 2.95 (s, 2H, CH_2), 2.56 (s, 3H, 4-CH_3), 2.33 (s, 3H, 6-CH_3), 2.13 (s, 3H, 7-CH_3), 1.46 (s, 6H, 2-CH_3) ppm.

^{13}C-NMR (150 MHz, $CDCl_3$): δ = 161.0 (s, 5-Ar-*C*), 149.6 (s, 1-Ar'-*C*), 141.2 (s, 7a-Ar-*C*), 135.1 (s, 7-Ar-*C*), 129.5 (d, 3-Ar'-*C*, 5-Ar'-*C*), 126.8 (d, 4-Ar'-*C*), 125.2 (s, 4-Ar-*C*), 125.1 (s, 6-Ar-*C*), 122.4 (d, 2-Ar'-*C*, 6-Ar'-*C*), 118.2 (s, 3a-Ar-*C*), 87.3 (s, 2-*C*), 42.8 (t, 3-*C*H_2), 28.5 (q, 2-*C*H_3), 19.2 (q, 6-Ar-*C*H_3), 17.8 (q, 4-Ar-*C*H_3), 12.5 (q, 7-Ar-*C*H_3) ppm.

^{1}H-NMR-purity ≥ 95 %

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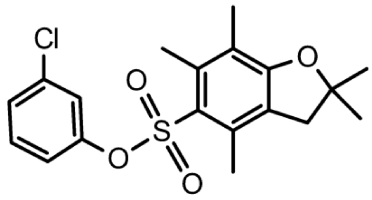
3-Chlorophenyl-5-(2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran)-sulfonate (4)



Under nitrogen, 1.47 g of 3-chlorophenol (11.5 mmol) was stirred in 25 mL of dry dichloromethane at 0 °C. During 1 h, freshly prepared sulfonyl chloride **2** was added dropwise to the reaction. The reaction mixture was stirred for 20 h and quenched with 50 mL of demineralized water. Dichloromethane (200 mL) were added to the organic layer, and it was washed with a saturated potassium carbonate solution (2 x 100 mL) and demineralized water (2 x 100 mL). The solvent was removed under reduced pressure. The residue was purified by chromatography (ethyl acetate/cyclohexane 9:1 (R_f = 0.11) □ 1:1 (R_f = 0.27) to afford **1** as a yellow oil.

yield.: 175 mg (460 µmol, 9 %) Ref.: 44 % [1]

^{1}H-NMR (500 MHz, $CDCl_3$): δ = 7.23 (m_c, 1H, 4-Ar'-*H*), 7.22 (d, ^4J = 1.2 Hz, 1H, 2-Ar'-*H*), 7.05 (m_c, 1H, 5-Ar'-*H*), 6.96 (m_c, 1H, 6-Ar'-*H*), 2.95 (s, 2H, 2-CH_2), 2.56 (s, 3H, 4-CH_3), 2.33 (s, 3H, 6-CH_3), 2.13 (s, 3H, 7-CH_3), 1.46 (s, 6H, 2-CH_3) ppm.

^{13}C-NMR (125 MHz, $CDCl_3$): δ = 161.0 (s, 1C, 5-Ar-*C*), 150.0 (s, 1C, 1-Ar'-*C*), 141.0 (s, 1C, 7a-Ar-*C*), 135.1 (s, 1C, 7-Ar-*C*), 134.6 (s, 1C, 3-Ar'-*C*), 130.2 (d, 1C, 4-Ar'-*C*), 127.4 (d, 1C, 2-Ar'-*C*), 125.4 (d, 1C, 4-Ar-*C*), 124.5 (s, 1C, 6-Ar-*C*), 122.9 (d, 1C, 5-Ar'-*C*), 120.7 (d, 1C, 6-Ar'-*C*), 118.5 (s, 1C, 3a-Ar-*C*), 87.5 (s, 1C, 2-*C*), 42.9 (t, 1C, 3-*C*H_2), 28.5 (q, 2C, 2-*C*H_3), 19.1 (q, 1C, 6-Ar-*C*H_3), 17.9 (q, 1C, 4-Ar-*C*H_3), 12.5 (q, 1C, 7-Ar-*C*H_3) ppm.

^{1}H-NMR-purity ≥ 90 %

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Jurkat Cell Line generation

Gene fragments encoding TCRalpha (modified with mouse TRAC), TCRbeta (modified with mouse TRBC), and mCherry construct connected with the T2A(TRA-TRB junction) and P2A linkers (TRB mCherry junction) were ordered from Genscript and cloned into pLVX-EF1a-IRES-Puro lentiviral expression vector (Clontech). 293T cells (ATCC CRL-3216) were transfected with the parental plasmid together with psPAX2 packaging plasmid (Addgene plasmid #12260), and pMD2.G envelope plasmid (Addgene plasmid #12259) using Lipofectamine 3000 kit (Invitrogen). Lentivirus-containing media was collected 24 and 48 hours post-transfection. Lenti-X Concentrator (Clontech) was used for lentivirus precipitation according to the manufacturer's protocol. TCR-null Jurkat cell line expressing endogenous NFAT-GFP reporter (gift from Wouter Scheper) was transduced with a lentivirus and antibiotic selected for 1 week using 1 mg/mL puromycin in RPMI (Gibco) containing 10% FBS and 1% penicillin/streptomycin. Transduction of Jurkat cell line was confirmed by expression of mCherry.

Transgenic cell lines stimulation and flow analysis

Jurkat cells were seeded into round-bottom 96-well plates at 100 000 cells per well. Cells were stimulated with serial dilution of PPBF (15uM, 1.5 uM, 0.15uM in DMSO) and ClPPBF compounds (15uM, 1.5 uM, 0.15uM in DMSO) in 100 ul of stimulation media, containing 1 mg/mL each of anti-human CD28 and CD49d (BD Biosciences). An unstimulated (CD28, CD49d) and positive control (CD28, CD49d, 1X Cell Stimulation Cocktail, PMA/ionomycin; eBioscience) were included in each assay. Each experimental condition was performed in triplicates. For the CD1d blocking experiment 5ul of anti-CD1d (clone 51.1, Biolegend) was added to each well. Transgenic Jurkat cell line expressing the TCR known to be specific for SARS-CoV-2 epitope was used as a negative control. TCR activation was assessed using NFAT-GFP expression after 18 h incubation (37 C, 5% CO2) by flow cytometry on a BD Fortessa using FACSDiva software. Flow data were analyzed in FlowJo software.

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TCR sequence analysis

To identify a cluster of highly similar TCR sequences we used a TCRdist network approach as described in ref Cell reports Medicine. The pairwise TCRdist metric and motif logo for the largest CAIT-like cluster was generated with *conga*[1]. The TCR similarity network (with tcrdist<150 threshold for edges) was built with the igraph package and visualized using *gephi*.

All TCR alpha chains from published NKT type II cells reactive to the small sulfonate compounds[2 3] were searched in previously published bulk TCR repertoire dataset[4] including 109 Crohn's disease (CD) patients and 99 healthy controls. TCRs were looked for by the combination of their amino acid CDR3 sequence, their V gene and J gene. To identify whether NKT type II clonotypes were enriched in CD patients, we extracted from the reference dataset all TCR alpha sequences carrying the same VJ combination and differing by maximum one amino acid from any of the query TCRs. The so identified clonotypes were then grouped in two categories (i) CAIT-like clonotypes, carrying the TRAV12-1, TRAJ6 genes and motif CVV**A*GGSYIPTF as previously described[4] and (ii) other NKT type II clonotypes. The relative abundance of each clonotype in the respective category was summed thus obtaining the cumulative abundance of (i) CAIT-like and (ii) other NKT type II clonotypes in each individual.

Reference

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