SUPPLEMENTARY APPENDIX

Human invariant natural killer T cells promote tolerance by preferential apoptosis induction of conventional dendritic cells

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Supplementary Material

Methods

Flow cytometry

PBS57-loaded and unloaded human CD1d tetramers were obtained from the National Institutes of Health Tetramer Core Facility (Atlanta, GA, USA). The following antibodies were purchased from BD Biosciences, Miltenyi Biotech or BioLegend: anti-CD1c (L161), anti-CD1d (51.1), anti-CD3 (HIT3a/OKT3), anti-CD4 (RPA-T4), anti-CD8 (HIT8a), anti-CD25 (BC96), anti-CD69 (FN50), anti-CD11c (MJ4-27G12), anti-HLA-DR (L243) and anti-CD303 (201A). Fluorescence minus one controls were used for proper gating. To stain dead cells, 7-AAD (7-aminoactinomycin, BD Biosciences), eBioscience Fixable Viability Dyes eFluor 506 and 780 (ThermoFisher Scientific) were used. Data were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyses were performed with FlowJo 10.2 (Tree Star, Ashland, OR, USA). For cell sorting, a FACS Aria II cell sorter (BD Biosciences) was used.

iNKT-cell expansion

PBMCs were incubated in iNKT-cell culture medium consisting of RPMI 1640 GlutaMAX Medium (ThermoFisher Scientific), 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany), 100 IU/mL penicillin-streptomycin (Lonza, Basel, Switzerland), 5.5 µM 2-mercaptoethanol (Roth, Karlsruhe, Germany), 0.1 mM non-essential amino acids (NEAA, Gibco, Grand Island, NY, USA), 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Gibco) and 1 mM sodium pyruvate (Gibco) supplemented with 100 ng/ml KRN7000 (Sigma-Aldrich, St. Louis, MO, USA) and 100 IU/mL recombinant human interleukin-2 (rhIL-2, Novartis, Basel, Switzerland). After 7 days, cultured cells were re-stimulated with irradiated (30 Gray, cesium-137 irradiator Gammacell 1000, Atomic Energy

of Canada Limited, Chalk River, Canada) and glycolipid-pulsed autologous PBMCs together with KRN7000 (50 ng/mL) and rhIL-2 (50 IU/mL). After a total of 14 days, cell culture was completed.

Apoptosis assays

To investigate DC apoptosis induction by iNKT cells by conventional flow cytometry, only singlets were analyzed. For proper gating on DCs, iNKT cells were excluded via CD3 and PBS57-loaded CD1d tetramer staining. For image stream analysis, cells were stained with Annexin V-FITC, 7-AAD, HLA-DR and PBS57-loaded CD1d tetramer; 10,000 cells were acquired using the ImageStream mkII (Amnis/Luminex, Austin, TX, USA). To assess cellular interactions, image stream analysis was performed by analyzing HLA-DR+ PBS57-loaded CD1d tetramer+ doublets. Data were analyzed with IDEAS analysis software (Amnis/Luminex).

Pre-treatment for blocking assays

For blocking assays, iNKT cells or DCs were pre-incubated separately for 1 h at 37°C and 5% CO_2 with the respective antibodies or IgG control. The following antibodies/reagents and concentrations were used: anti-CD1d (51.1) 10 µg/mL (BioLegend), anti-FasL (Nok-1) 5 µg/mL (BioLegend), anti-TRAIL (tumor necrosis factor related apoptosis inducing ligand, RIK-2) 10 µg/mL (BioLegend), anti-NKG2D (natural killer group 2 member D, 149810) 10 µg/mL (R&D Systems, Minneapolis, MN, Canada), CMA (concanamycin A) 100 nM (Sigma-Aldrich), zVAD-fmk (N-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethylketone) 100 µM (Adooq Bioscience, Irvine, CA, USA) and monensin and brefeldin A 2 µL/mL (ThermoFisher Scientific). No agonistic effects of these compounds are reported according to our knowledge. Pre-treated cells were used in a MLR and analyzed for

apoptosis. The percentage of apoptosis inhibition was calculated by comparing the amount of living DCs after co-culture with antibody/reagent treatment with respective controls.

Supplemental Figures – Legends

Supplemental Figure 1. T-cell activation and proliferation is impaired by iNKT cells. Representative dot plots showing (A) early activated T cells (CD69+, day 1), (B) late activated T cells (CD25+, day 3) and (C) proliferating T cells (CFSE, day 7). T-cell activation and proliferation was measured after incubation with monocyte-derived DCs together with iNKT cells at indicated ratios. iNKT cells were either added directly to the culture or separated by a transwell insert (TW).

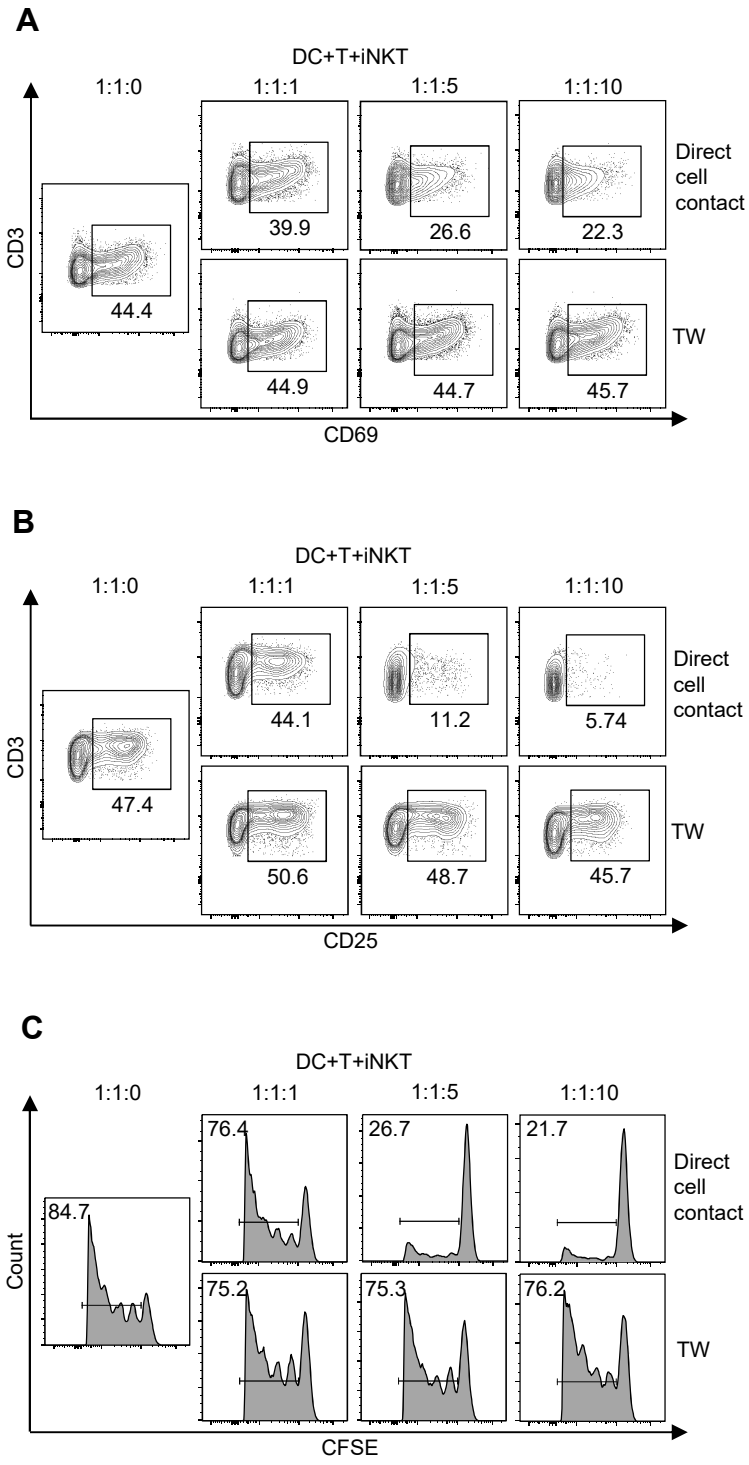
Supplemental Figure 2. Direct impact of human culture-expanded iNKT cells on T-cell activation and proliferation. Representative dot plots and histograms of (A) early activated T cells (CD69+, day 1), (B) late activated T cells (CD25+, day 3) and proliferating T cells (CFSE, day 7) after stimulation with anti-CD3/CD28-coated beads in absence and presence of iNKT cells at indicated ratios. Histograms show the mean of three independent experiments (n=3). Error bars show SEM. ns not significant, ** $p<0.01$.

Supplemental Figure 3. Culture-expanded iNKT cells require cell contact to induce apoptosis through degranulated effector molecules. (A) Representative dot plots showing the percentage of DC apoptosis inhibition after blocking of the receptors CD1d, FasL, TRAIL, NKG2D and specific inhibition by zVAD-fmk, CMA and monensin/brefeldin A.

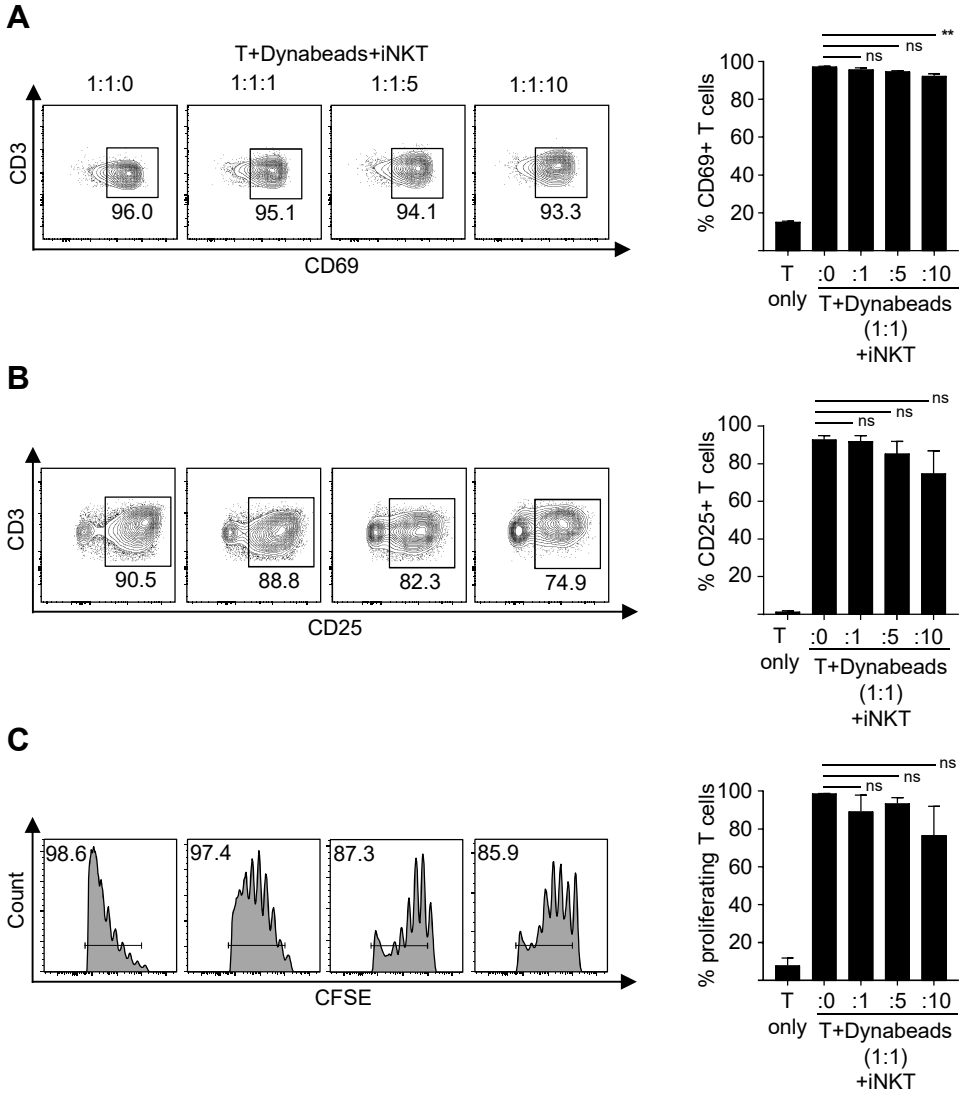
Supplemental Figure 4. Preferential apoptosis induction of blood conventional DCs by iNKT cells. (A) Representative histogram and pooled data showing the CD1d expression

(mean fluorescence intensity, MFI) of pDCs and cDCs from peripheral blood of healthy volunteers. (B) Representative dot plots and histograms showing early activated T cells (CD69+, day 1), late activated T cells (CD25+, day 3) and proliferating T cells (CFSE, day 7) after incubation with sorted pDCs or cDCs. (C) Representative histogram and pooled data showing the CD1d expression (MFI) of pDCs and cDCs from peripheral blood of GvHD patients. Histograms show the mean of three independent experiments (n=3). Error bars indicate standard error of the mean (SEM). ns not significant, * $p<0.05$, *** $p<0.001$.

Supplemental Figure 1

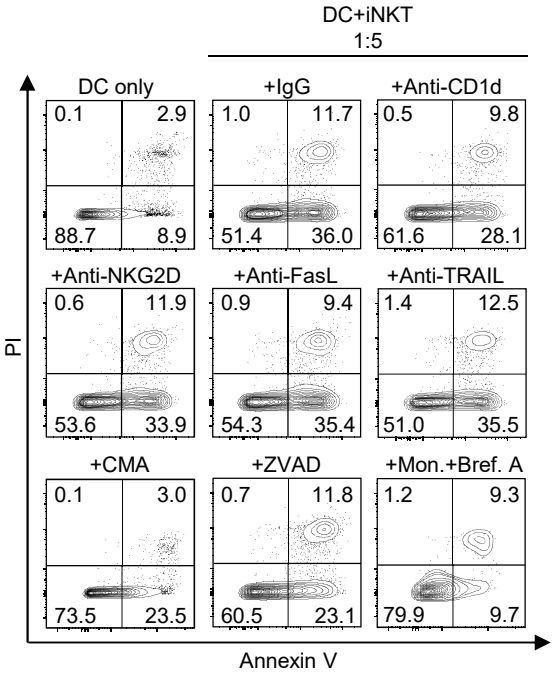


Supplemental Figure 2



Supplemental Figure 3

A



Supplemental Figure 4