Supplementary Material and Methods

Human primary cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from anonymized buffy coats (DRK-Blutspendedienst Hessen GmbH) by applying a density gradient centrifugation (Histopaque® 1077, Sigma-Aldrich). $CD14^+$ monocytes were separated by positive selection using MACS (Miltenyi Biotec). Subsequently, monocytes were cultivated in complete media (CM) consisting of RPMI 1640 (Biowest), 10% fetal calf serum (FCS), 50 µM β-mercaptoethanol (both Sigma-Aldrich), 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 mM HEPES (all from Biochrom AG). For the generation of monocyte-derived dendritic cells (moDCs), media were supplemented with 10 ng/ml interleukin 4 (IL-4; Gibco^{TM}) and 5 ng/ml recombinant human granulocyte macrophage stimulating factor (GM-CSF (Leukine®; Bayer Healthcare Pharmaceutical) for 5-6 days of cultivation, with a medium refresh on day 3 (37°C, 5% CO_2). Autologous peripheral blood lymphocytes (PBL, $CD14^-$ fraction) were collected and frozen in CM containing 30% FCS and 10% DMSO (Sigma-Aldrich).

Cell lines

HLA-A*02:01–positive TAP-deficient 174CEM.T2 (T2) cells (generously gifted by Prof. J.P. Schneck, Johns Hopkins School of Medicine) were cultured in B' medium containing RPMI 1640 (Biowest), 10% FCS (Sigma-Aldrich), 10 mM MEM NEAA, MEM vitamin solution, 100 mM MEM sodium pyruvate (all Gibco^{TM}), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Biochrom AG) at 37°C with 5% CO_2.

DC maturation

Maturation of DCs was assessed after 24 h of adjuvant stimulation by flow cytometry. Surface marker expression (as geometric mean fluorescence intensity on $CD14^-CD1a^+$ DCs) was

examined using the following antibodies and corresponding isotype controls as defined by the manufacturer: CD40 (5C3), CD80 (2D10), CCR7 (G043H7), PD-L1 (29E.2A3, all Biolegend), CD86 (2331, BD Bioscience). The geo. MFI values of the isotype control were subtracted from the geo. MFI values of the specific antibody staining.

Receptor expression

Expression of TLR2, TLR4, TLR7 and MyD88 on Lymphocytes or iDCs was assessed by flow cytometry after staining of cells (CD282/TLR2 clone T2.5, TLR4 clone HTA125, both Biolegend; TLR7 clone 533707; MyD88 clone 316628, both R&D Systems).

Expression of TLR8 and Mincle as well as the housekeeping genes GAPDH and RPL13 was determined by gene-specific PCR. To this end, mRNA was isolated from DCs or PBLs using the RNAeasy kit (Qiagen), followed by reverse transcription into cDNA. Primer sequences for PCR are derived from publications (1, 2) or designed by our lab and are shown in Table S3.

Endocytosis of FITC-dextran

FITC-dextran (50 µg/ml) was given to the culture for an additional hour after 24 h stimulation with the immunomodulators. Replicates at 4°C served as negative control for the phagocytic uptake of FITC-dextran. After thorough washing, cells were stained for CD14 and CD1a to assess the uptake of FITC-dextran (geo. MFI) on $CD14^{-}CD1a^{+}$ DCs. The geo. MFI values obtained at 4°C were subtracted from the geo. MFI values obtained at 37°C. For representative microscopic pictures, DCs were labelled with CellTrace™ Far Red Cell Proliferation Kit (Thermo Fisher Scientific) before stimulation. After harvesting and extensive washing, cells were stained with Hoechst 33342 (Immunochemistry Technologies) and centrifuged on glass slides using a CellSpin II cytocentrifuge (Tharmac). After short air-drying, mounting fluid (IMAGEN™; glycerol solution, pH 10 with an anti-fading agent) was added and cells were analyzed with a Zeiss Axio Observer Z1 fluorescence microscope.

Luminex analysis

DC:PBL co-cultures of a total of 30 donors, (15 men, 15 women), evenly distributed in age (two groups: > 40 years and < 40 years), were set up as described above. Cell supernatants were harvested 24 h after adjuvant stimulation. Briefly, plates were centrifuged and 100µl (75% of well volume) were transferred to another 96 well plate. Plates were tightly sealed and frozen at -80°C immediately until analysis with Luminex *x*MAP technology using a Bio-Plex200 system (Biorad). The 25 analytes (ProCartaplex custom panel 25-plex, Invitrogen) were organized on one multiplex array and a single batch of reagents was used for testing all samples. The least detectable dose (LDD) was derived by averaging the values obtained with sample diluent of 169 blank reads and adding three standard deviations of the mean. LDD and lower limit of quantitation (LLOQ) of the tested analytes are displayed in table S2. For PCA analysis using the Qlucore software, undetected values (OOR <) were replaced by 10% of the least detectable dose, whereas saturated values (OOR >) were replaced by 10x of the upper limit of quantification. The subsequent cytokine analysis is based on the mean of biological duplicates. DC:PBL co-cultures of a second set of 10-12 donors were set up as described above. In addition to 24 h, cell supernatants were harvested after 5 days of adjuvant stimulation. To this end, cells were sedimented, and supernatant was transferred to another 96 well plate. Plates were tightly sealed and frozen at -80°C immediately until analysis employing ELISA (Human IL-4 ELISA MAX Standard Set) or the Legendplex™ *Human Inflammation Panel 1* kit. Legendplex ™ Data Analysis was performed using the Legendplex™ Data Analysis Software Suite (all Biolegend).

Antigen-independent lymphocyte proliferation assay

To assess the proliferation of lymphocytes, moDCs and thawed autologous PBLs were labelled with CFSE (Sigma-Aldrich) at a final concentration of 4µM for 10 min at 37°C prior to co-

culturing and stimulation with the immunomodulators. After 6-7 days, cells were harvested and stained for CD3 (clone: UCHT1), CD4 (RPA-T4), CD8 (SK1), CD56 (HCD56) and CD19 (HIB19, all Biolegend). Proliferating cells were identified by exhibiting a lower intensity of CFSE as measured by flow cytometry. 123count eBeads™ Counting Beads (eBioscience, Thermo Fisher Scientific) were spiked in and acquired during sample analysis, allowing the calculation of the absolute cell count of proliferating lymphocytes within the respective sample. In addition, lymphocyte activation 24 h or 5 days after stimulation with the immunomodulators was determined by staining for CD69 (clone: FN50) or CD25 (BC96; both Biolegend), respectively.

pDC viability

To assess the presence and abundance of viable plasmacytoid dendritic cells within the DC:PBL co-culture 24 h or 6 days after stimulation with the immunomodulators, we determined the cells double positively stained for CD303 (clone: 201A) and CD123 (6H6; both Biolegend) of the viable single $CD14^-/CD3^-/CD56^-/CD19^-/CD45RA^+$ cell fraction (CD14 clone MφP9, BD and CD45RA clone Hl100, Biolegend).

Flow cytometry

To prevent non-specific staining, cells were first incubated in blocking buffer (PBS/ 10 % FCS/ 10 % huAB serum), followed by staining with specific antibodies or isotype controls and a fixable viability dye (Zombie Yellow, Biolegend). Intracellular protein analysis required fixation and permeabilization using BD's Cytofix/Cytoperm™ solution kit according to manufacturer's instruction. Samples were acquired using LSR II SORP or LSR Fortessa flow cytometer (BD Bioscience) and analyzed with FlowJo™ software (v10.16.1, BD).

Antigen-specific T cell experiments

PBMCs from donors with the HLA characteristic DRB1*11:01 were isolated from whole blood as described above. Blood sampling of donors, HLA-typing, and the experimental procedure was approved by the local ethics committee (with ethical allowance #19-226; ethical committee Frankfurt, faculty of Medicine Goethe-University Frankfurt) and informed, written consents were obtained from all donors. HLA-A*02:01 positive donors were selected from anonymized buffy coats obtained from the DRK Blutspendedienst Hessen GmbH using an HLA-A2 antibody (BB7.2, Biolegend). DCs were harvested on day 5-6 and pulsed with 10 µM of the respective peptide (Influenza matrix protein 1, $FluM1_{58-66}$, GILGFVFTL, Innovagen; tetanus toxoid, TT $p2_{829-844}$, MQYIKANSKFIGITEL, MBL) in serum-free media for 2 hours at 37°C. DCs that were not loaded with peptide served as control cells. Peptide-pulsed and control DCs were washed extensively to remove unbound peptide. Autologous $CD14^-$ PBLs were thawed and untouched $CD4^+$ or $CD8^+$ T cells were obtained using respective EasySep™ isolation kits (Stemcell™ technologies). CFSE-labeled (4µM CFSE for 10 min) $CD4^+$ or $CD8^+$ T cells were co-cultured in a 1:5 DC:T cell ratio and stimulated with the adjuvants as written above. Additional 20 IU/ml IL-2 (Norvartis) was given to the DC:$CD8^+$ co-culture. After 6-7 days of culture, T cells were harvested and analyzed for polyfunctionality, the expression of inhibitory markers or peptide specificity. Therefore, T cells were stained using HLA-A*02:01-GILGFVFTL or HLA-DRB1*11:01-MQYIKANSKFIGITEL PE tetramers (MBL) and T cell-identifying antibodies (CD3 (UCHT1), CD4 (RPA-T4) or CD8 (SK1)).

Polyfunctionality

T2 cells were pulsed with 1 µM FluM1 or control peptide (NY-ESO-1; SLLMWITQV, iba) for 1 hour and washed several times. FluM1-expanded $CD8^+$ T cells were harvested and added to the peptide-presenting T2 cells at a 1:1 ratio in the presence of monensin/brefeldin A, anti-CD28/CD49d co-stimulus (all from BD Biosciences) and CD107A (H4A3, Biolegend) antibody. The same setting, but without T2 cells and CD28/CD49d co-stimulus, was used for

the negative control as well as for the positive control, to which the cell activation cocktail PMA/Ionomycin (Biolegend) was added. After 6 hours, cells were washed twice, blocked and stained with viability dye, anti-CD3 (UCHT1), anti-CD8 (SK1) and FluM1-tetramer. After fixation and permeabilization, cells were stained with anti–IL-2 (MQ1-17H12), anti–TNFα (Mab11) and anti–IFNγ (4S.B3, all from Biolegend) for another hour.

Expression of inhibitory markers FluM1-expanded $CD8^+$ T cells were harvested and stained with FluM1-tetramer and for the inhibitory expression markers PD-1 (EH12.2H7), PD-L1 (29E.2A3), LAG3 (11C3C65), CTLA-4 (L3D10), Tim (F38-2E2, all from Biolegend).

Supplementary tables and figures including legends

Table S1: Immunomodulators used to investigate and compare their immunogenic properties

Immunomodulator	Abbreviation	Information	Low concentration	High concentration
Trehalose-6,6-dibehenate VacciGrade™	TDB	Mincle ligand	1 µg/ml	10 µg/ml
Pam3CSK4 VacciGrade™	Pam	TLR1/2 ligand	100 pg/ml	1 µg/ml
Gardiquimod VacciGrade™	GARD	TLR7 ligand	0.35 µM	3 µM
Imiquimod VacciGrade™	IMQ	TLR7 ligand	3.6 µM	36 µM
Resiquimod VacciGrade™	R848	TLR7/8 ligand	0.15 µM	1 µM
Positive control (LPS-EB, ultrapure)	LPS	TLR4 ligand	38.4 pg/ml	0.1 µg/ml
Synthetic monophosphoryl lipid A VacciGrade™	MPL-s	TLR4 ligand	0.01 µg/ml	1 µg/ml
Monophosphoryl lipid A (*S. minnesota R595)* VacciGrade™	MPL-SM	TLR4 ligand	0.01 µg/ml	1 µg/ml
Addavax™	ADX	squalene-based oil-in-water nano-emulsion	15 µg/ml	150 µg/ml
Quil-A® adjuvant	Quil	saponin adjuvant	0.15 µg/ml	1.5 µg/ml
Alhydrogel® 2%	$Al(OH)_3$	aluminum hydroxide wet gel suspension	1 µg/ml	10 µg/ml

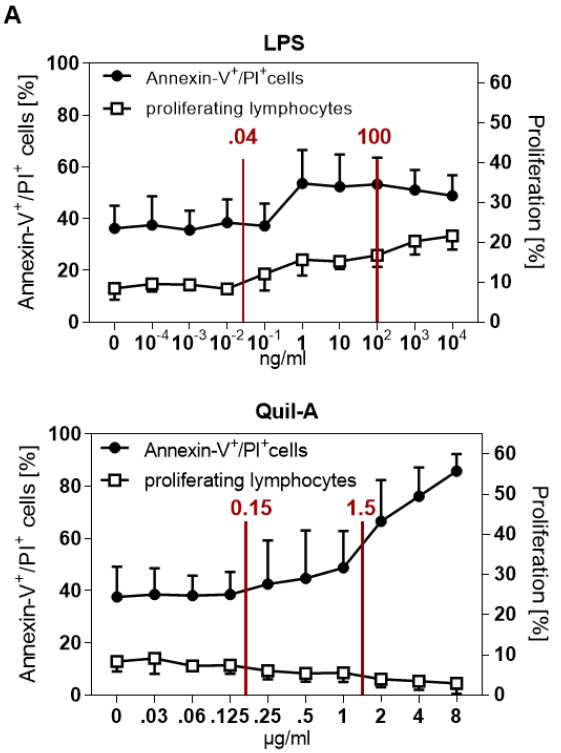
Table S2: Parameters of the Luminex xMAP immunoassay.

Analytes	Abbreviation	Units	mean LDD*	[min;max] - std	mean LLOQ**	[min;max] - std
Granulocyte Colony-Stimulating Factor	G-CSF	pg/mL	159.2	[5.73 ; 648.40] - 194.26	30	[2.56 ; 186.92] - 46.49
Granulocyte-Macrophage Colony-Stimulating Factor	GM-CSF	pg/mL	11.6	[0.01 ; 28.77] - 6.64	8.1	[2.40 ; 14.65] - 5.45
Interferon alpha	IFN-A	pg/mL	1.6	[0.55 ; 3.43] - 0.79	0.4	[0.12 ; 0.59] - 0.18
Interferon gamma	IFN-G	pg/mL	21.5	[7.20 ; 77.40] - 13.01	6.41	[2.62 ; 11.98] - 4.47
Interleukin-1 alpha	IL-1A	pg/mL	6.3	[0.01 ; 13.08] - 3.55	1.7	[0.18 ; 2.98] - 1.10
Interleukin-1 beta	IL-1B	pg/mL	4.1	[1.72 ; 7.55] - 1.26	1.8	[0.49 ; 2.26] - 0.61
Interleukin-10	IL-10	pg/mL	1.3	[0.005 ; 4.41] - 0.93	0.9	[0.48 ; 1.99] - 0.65
Interleukin-12 Subunit p70	IL-12p70	pg/mL	3.4	[0.01 ; 27.16] - 5.44	3.5	[2.68 ; 11.90] - 2.28
Interleukin-17A	IL-17A	pg/mL	9.7	[0.07 ; 29.06] - 8.04	5.3	[0.53 ; 7.75] - 2.68
Interleukin-18	IL-18	pg/mL	6.3	[0.17 ; 30.94] - 5.76	5.6	[3.39 ; 14.45] - 4.20
Interleukin-2	IL-2	pg/mL	16.9	[3.30 ; 39.92] - 7.98	13.2	[5.65 ; 25.83] - 9.46
Interleukin-23	IL-23	pg/mL	56.5	[20.33 ; 133.38] - 26.61	21.9	[0.34 ; 68.55] - 21.39
Interleukin-27	IL-27	pg/mL	239.8	[42.52 ; 2142.96] - 505.76	58.2	[6.88 ; 240.45] - 39.58
Interleukin-3	IL-3	pg/mL	305.3	[62.18 ; 1343.36] - 298.48	58.6	[7.56 ; 261.10] - 60.17
Interleukin-33	IL-33	pg/mL	8.1	[1.11 ; 32.05] - 8.20	3.9	[0.18 ; 12.26] - 4.00
Interleukin-4	IL-4	pg/mL	26.6	[1.88 ; 165.82] - 30.98	10.6	[2.22 ; 39.77] - 9.54
Interleukin-5	IL-5	pg/mL	32.5	[0.68 ; 93.72] - 22.25	19.6	[2.51 ; 41.23] - 15.41
Interleukin-6	IL-6	pg/mL	13.2	[0.11 ; 35.22] - 8.68	3.3	[1.68 ; 7.41] - 2.42
Interleukin-8	IL-8	pg/mL	26.3	[0.05 ; 202.20] - 36.35	5.6	[0.56 ; 9.99] - 4.19
Monocyte Chemotactic Protein 1	MCP-1	pg/mL	8.0	[2.13 ; 24.16] - 5.92	4.3	[0.90 ; 14.94] - 4.07
C-X-C motif chemokine 9	MIG (CXCL9)	pg/mL	27.9	[0.53 ; 74.06] - 20.51	9.6	[1.71 ; 46.11] - 11.67
Macrophage Inflammatory Protein-1 alpha	MIP-1A	pg/mL	8.0	[1.15 ; 25.95] - 4.92	2.1	[0.31 ; 7.10] - 2.11
C-C motif chemokine 5	RANTES (CCL5)	pg/mL	4.3	[0.47 ; 17.81] - 4.34	1.5	[0.27 ; 3.57] - 1.18
Stem Cell Factor	SCF	pg/mL	2.9	[0.01 ; 6.90] - 1.66	2.2	[0.27 ; 6.22] - 2.04
Tumor Necrosis Factor alpha	TNF-A	pg/mL	15.2	[6.31 ; 41.61] - 8.24	5.3	[1.51 ; 9.93] - 3.63

* The least detectable dose (LDD) was determined as the mean + 3 standard deviations of 169 blank readings. Results below the LDD are more variable than results above the LDD.
** The LLOQ (Lower Limit of Quantitation) is the lowest concentration of an analyte in a sample that can be reliably detected.
As the LLOQ and the LDD values are independent from each other, on occasion the LLOQ is lower than the LDD.

Table S3: PCR primers used in this study.

	Sequence	Reference
hTLR8 for	CAGATTAGCAGGCGTAACACATCA	Caron et al. (2005) J Immunol
hTLR8 rev	AATGTCACAGGTGCATTCAAAGGG	Caron et al. (2005) J Immunol
hMincle for	TCGAGGGTCAGTGGCAATG	Ostrop et al. (2015) J Immunol
hMincle rev	GTTGTGCCTTCTGTTCTTAAAGAGATT	Ostrop et al. (2015) J Immunol
RPL 13 for	TCAAAGCCTTCGCTAGTCTCC	
RPL 13 rev	GGCTCTTTTTGCCCGTATGC	
GAPDH for	GAG TCA ACG GAT TTG GTC GT	
GAPDH rev	TTG ATT TTG GAG GGA TCT CG	



B

Adjuvant	Conc.	Endo-toxin [EU/ml]	Spike recovery [%]
TDB	10 µg/ml	< 0.02	N/A
Pam	1 µg/ml	< 0.02	111
LPS	38.4 pg/ml	0.04	109
LPS	100 ng/ml	> 10	N/A
MPL-s	0.01 µg/ml	2.31	109
MPL-s	1 µg/ml	> 10	83
MPL-SM	0.01 µg/ml	> 10	N/A
MPL-SM	1 µg/ml	> 10	N/A
GARD	3 µM	< 0.02	114
IMQ	36 µM	< 0.02	122
R848	1 µM	< 0.02	144
ADX	150 µg/ml	< 0.02	135
Quil	1.5 µg/ml	< 0.02	146
$Al(OH)_3$	10 µg/ml	< 0.02	145
Complete Media (CM)	-	< 0.02	133
CM+1% DMSO	-	< 0.02	137

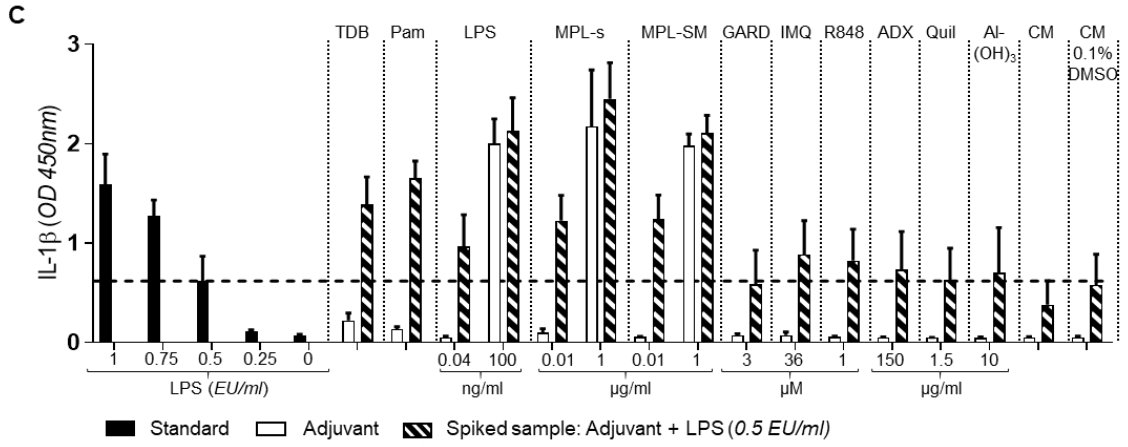


Fig. S1: Definition of suitable working concentrations.

A) Adjuvants' concentrations were defined by assessing cytotoxicity with an annexin-V/ PI staining (filled black dots) and the induction of lymphocyte proliferation (white squares). Red vertical lines indicate the chosen low and high concentration for the examplarily depicted LPS and Quil-A. B) Adjuvants were tested for endotoxin contamination using Limulus amoebocyte lysate (LAL; turbidimetric method) and an endotoxin spike-in of 0.5 EU/ml LPS. C) Pyrogenicity of the adjuvants was analyzed by the monocyte activation test (MAT) on cryopreserved human blood pooled from several donors. After 24 hour incubation with the controls, adjuvants or standards, IL-1β concentrations in cell supernatants were analyzed by ELISA. Data are representative of at least three independent experiments.

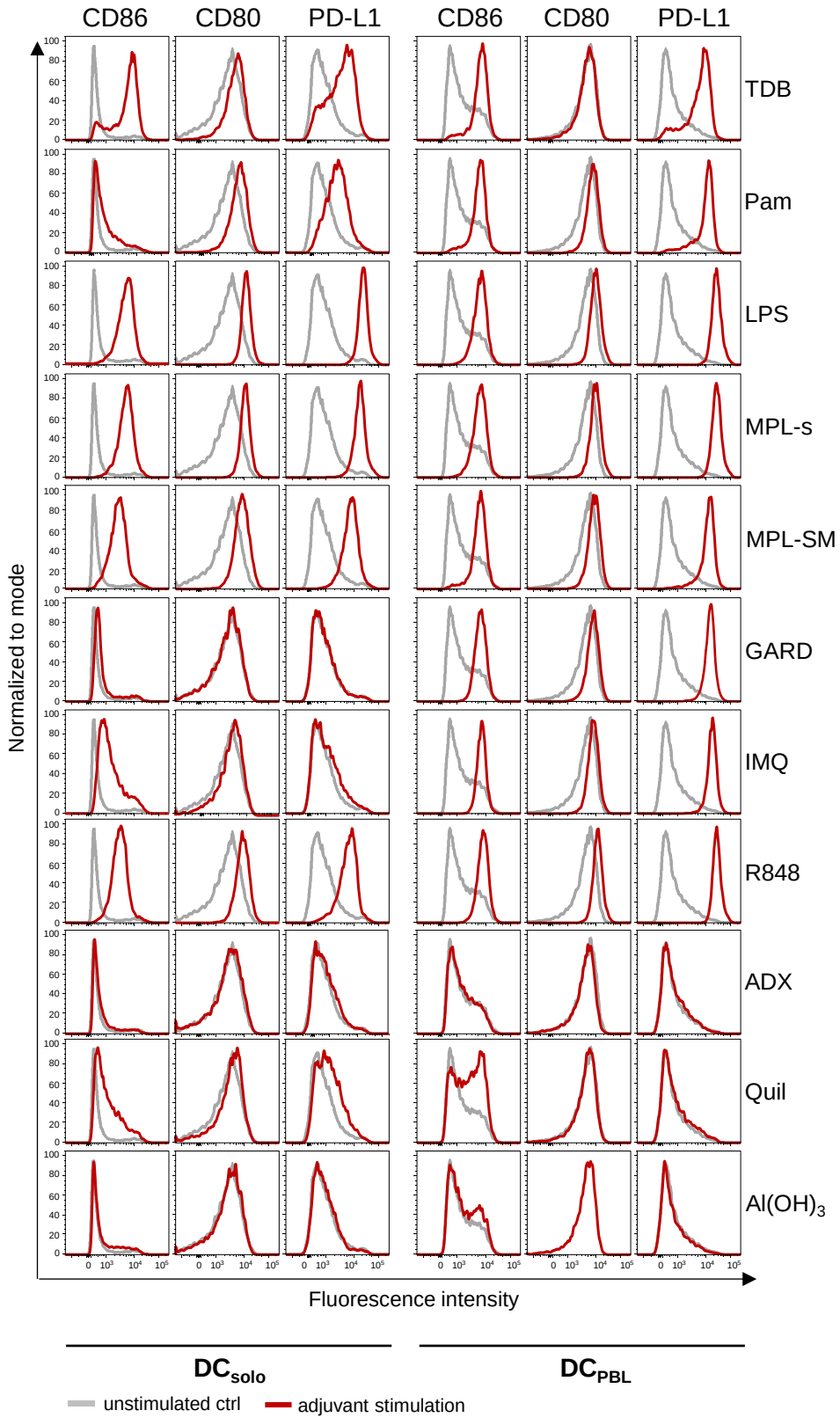

CD86
CD80
PD-L1
CD86
CD80
PD-L1
TDB
Pam
LPS
MPL-s
MPL-SM
GARD
IMQ
R848
ADX
Quil
Al(OH)3
Normalized to mode
Fluorescence intensity
DCsolo
DCPBL
unstimulated ctrl
adjuvant stimulation

Fig. S2: The TLR7 ligands GARD and IMQ increase the expression of CD80, CD86 and PD-L1 on DC_{PBL}, but not on DC_{solo}.

Representative histogram overlays of the maturation markers CD80, CD86 and PD-L1 showing the differences in fluorescence intensity between DC_{solo} and DC_{PBL} when stimulated with the adjuvants GARD or IMQ. The solid grey line represents the fluorescence intensity of the unstimulated control of the respective culture, whereas the solid red line represents the fluorescence intensity of the respective adjuvant stimulation.

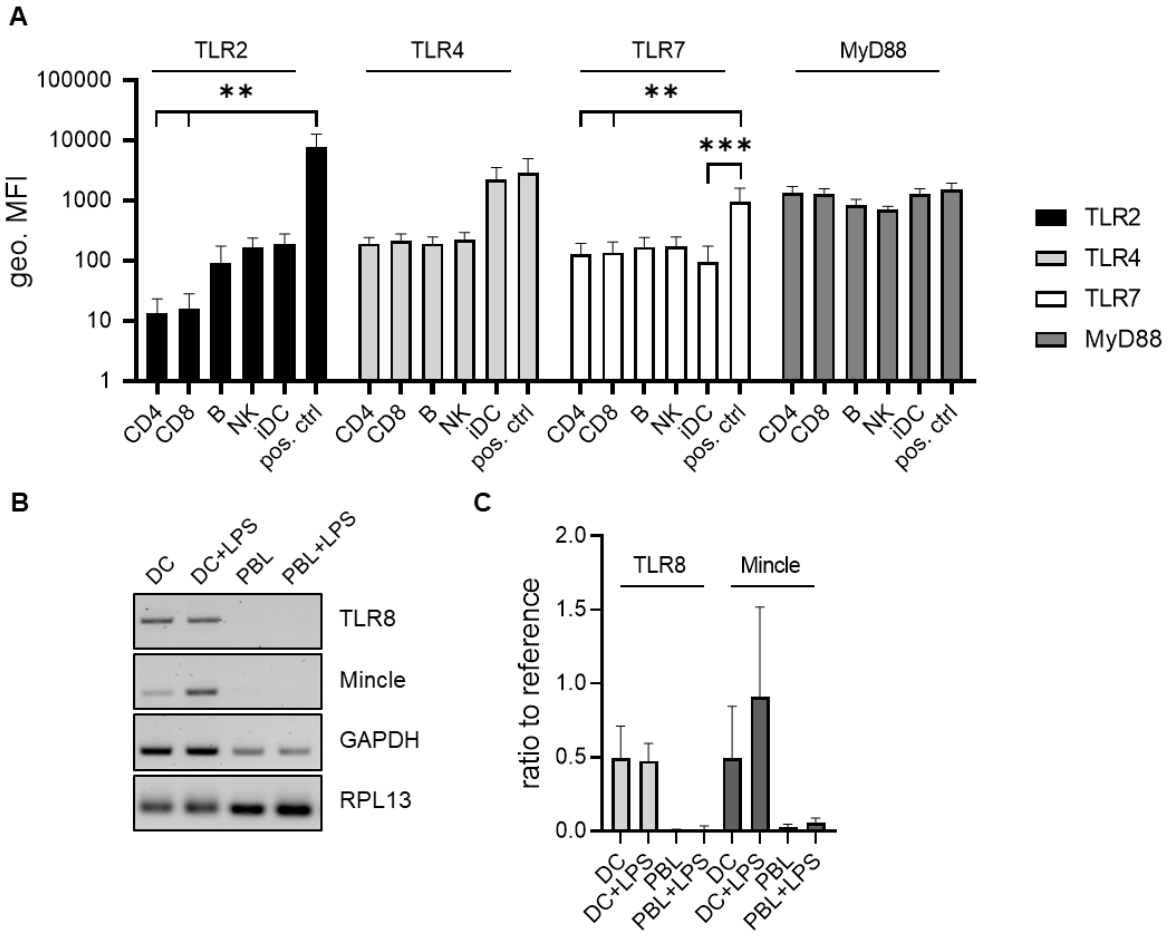


Fig. S3: Expression of TLRs, MyD88 and Mincle on moDCs or PBLs

A) Immature DCs or PBLs were stained for their specific phenotype as well as the receptor or adapter proteins as indicated, and were subsequently analyzed by flow cytometry (n=4-11). B, C) Isolated mRNA from untreated or LPS-treated (10ng/ml) DCs or PBLs was reversely transcribed into cDNA, and gene-specific primers were used to amplify TLR8 or Mincle as well as GAPDH or RPL13 transcripts. B) PCR products visualized from an agarose gel showing representative gene expression. C) Densitometric analysis of PCR products from agarose gels. DC samples were normalized to the GAPDH reference whereas PBL samples were normalized to the more suitable RPL13 reference (n=6).

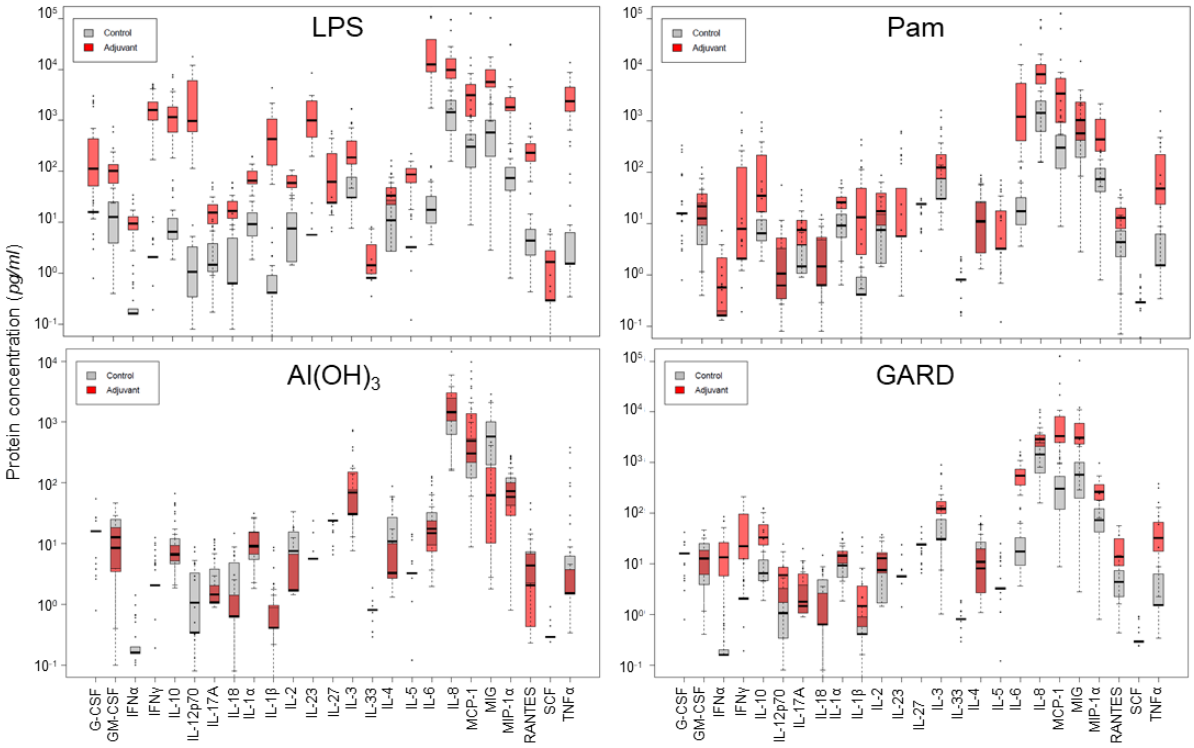


Fig. S4: Induction of cytokine and chemokine expression varies between adjuvants.

The DC:PBL co-culture was stimulated with the different adjuvants or was left untreated (null response). After 24 hours, supernatant was harvested and analyzed for cytokine and chemokine secretion (25 proteins in total) using the Luminex xMAP technology. Box-whisker plots (Tukey) represent the induced cytokine and chemokine response of four exemplified stimuli (high concentration). Induced responses of alphabetically listed analytes are colored in red, the null response is overlaid in grey. Data points beyond the whiskers are outliers (n=30 donors).

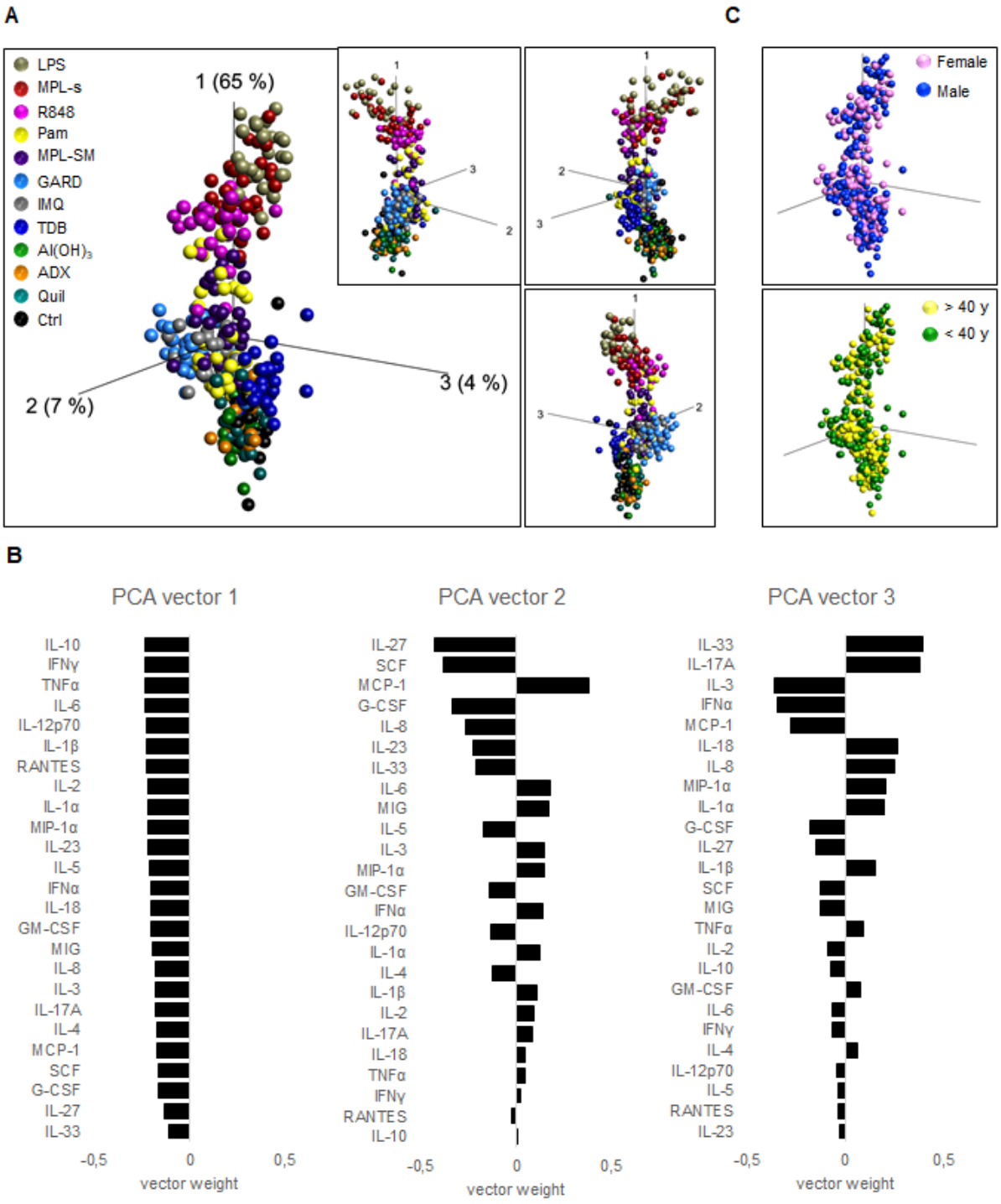


Fig. S5: Adjuvants are characterized by their differential cytokine and chemokine expression profiles.

The DC:PBL co-culture was stimulated with the different adjuvants or left untreated (control) for 24 hours. Subsequently, the supernatant was analyzed for cytokine and chemokine secretion (25 proteins in total) using the Luminex xMAP technology. A) Principal component analysis with each dot representing one donor (n=30) and each color one of the 12 different conditions (Ctrl or high adjuvant concentrations). The PCA plot captures 76 %

of the total variance within the selected data set (PCA-1: 65%, PCA-2: 4%, PCA-3: 7%). Data was set to a mean of zero, scaled to unit variance and logarithmized before performing the PCA. Q-value was not adjusted here (q-value=1). B) Bar graphs representing the contribution of each protein analyte to the 3 principal component axes. The positioning of the bars is arbitrary and is not considered positive or negative, except in relation to the other analytes. C) PCA plots showing the distribution of induced cytokine and chemokine expression within donors of different age or gender.

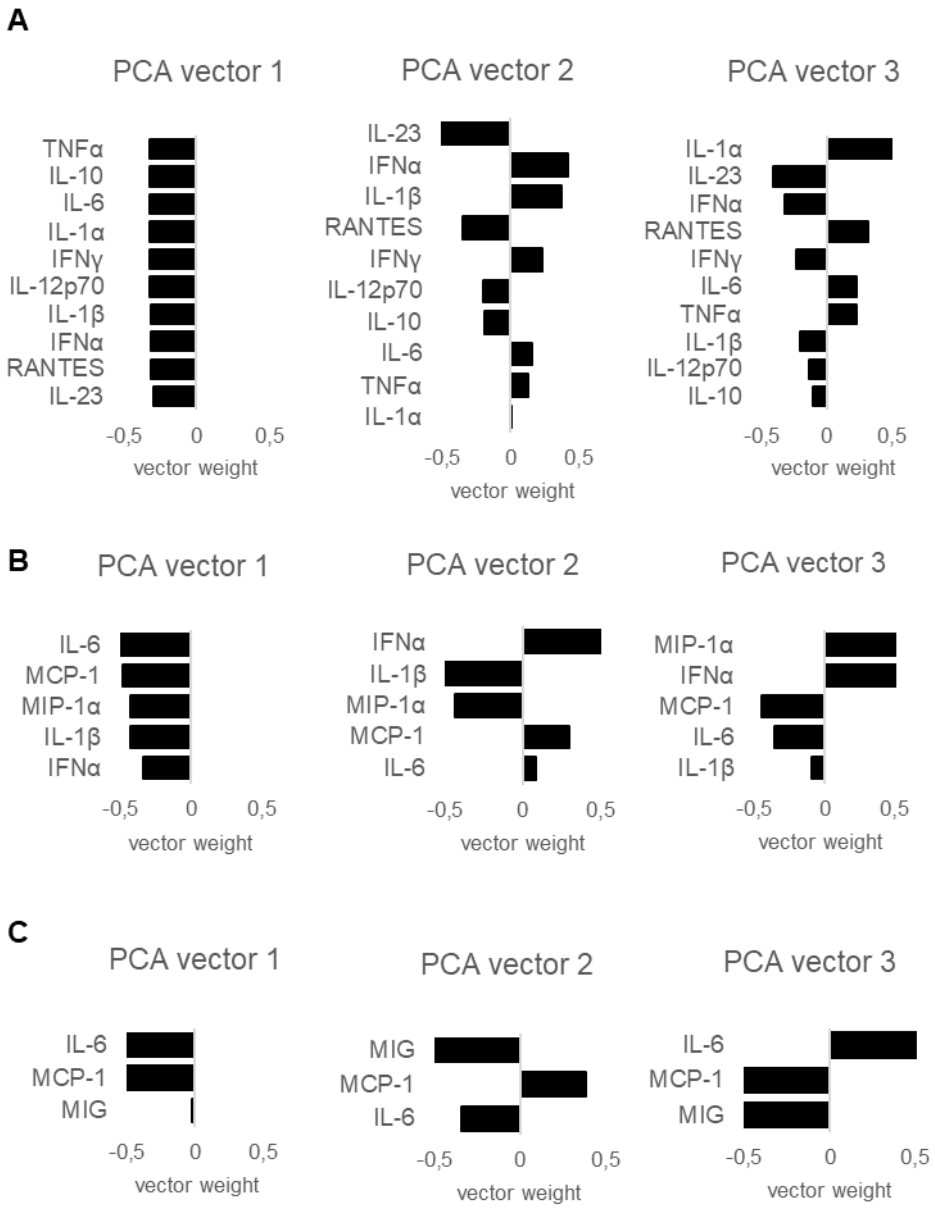
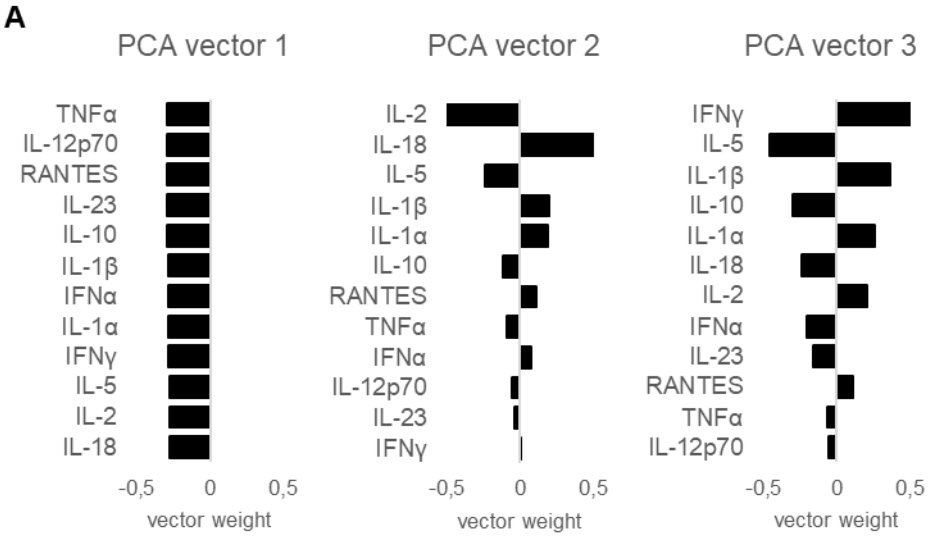


Fig. S6: Contribution of protein analytes to PC axes in the analysis of strong, intermediate or weak immunomodulators.

Bar graphs show the contribution of each protein analyte to the respective axis of the PCA plot for A) strong immunomodulators, B) intermediate immunomodulators, or C) weak immunomodulators in Figure 3B-E). The positioning of the bars is arbitrary and is not considered positive or negative, except in relation to the other analytes.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PCA vector 1
PCA vector 2
PCA vector 3
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RANTES
IL-23
IL-10
IL-1β
IFNα
IL-1α
IFNγ
IL-5
IL-2
IL-18
IL-2
IL-18
IL-5
IL-1β
IL-1α
IL-10
RANTES
TNFα
IFNα
IL-12p70
IL-23
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TNFα
IL-12p70
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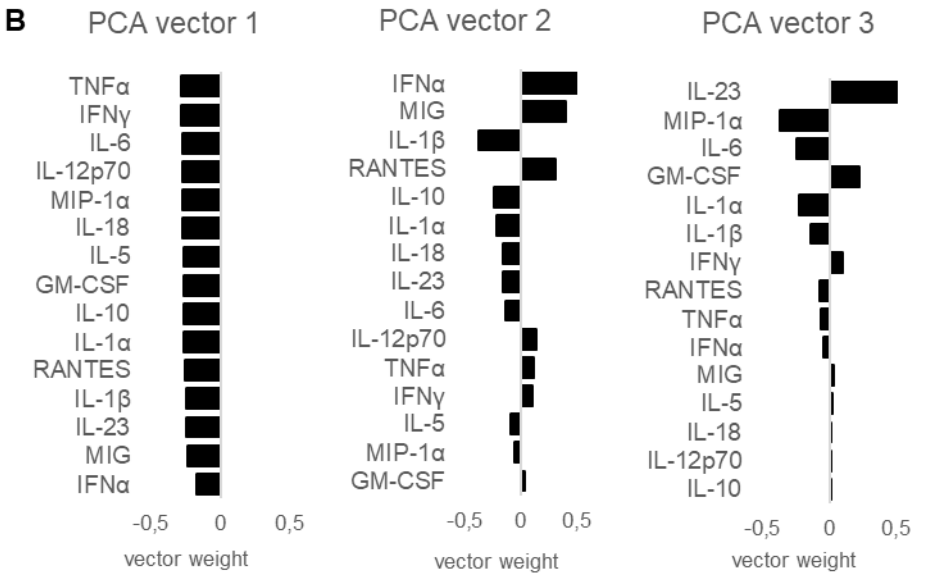
0
0,5
vector weight


B
PCA vector 1
PCA vector 2
PCA vector 3
TNFα
IFNγ
IL-6
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MIP-1α
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IL-10
IL-1α
RANTES
IL-1β
IL-23
MIG
IFNα
IFNα
MIG
IL-1β
RANTES
IL-10
IL-1α
IL-18
IL-23
IL-6
IL-12p70
TNFα
IFNγ
IL-5
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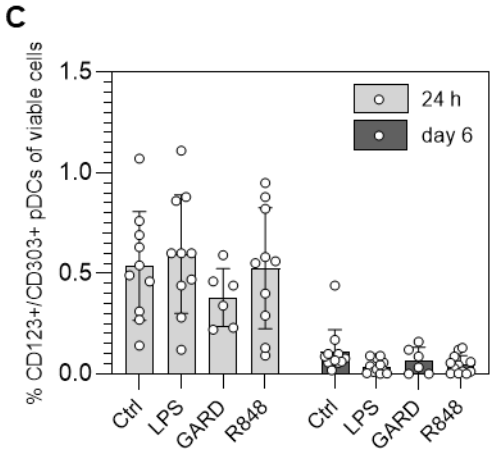
C
% CD123+/CD303+ pDCs of viable cells
1.5
1.0
0.5
0.0
24 h
day 6
Ctrl
LPS
GARD
R848
Ctrl
LPS
GARD
R848

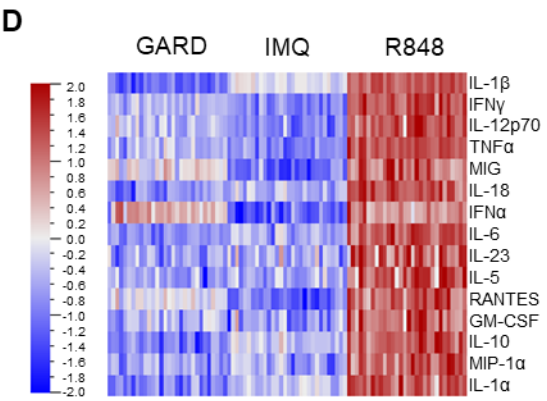
D
GARD
IMQ
R848
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
IL-1β
IFNγ
IL-12p70
TNFα
MIG
IL-18
IFNα
IL-6
IL-23
IL-5
RANTES
GM-CSF
IL-10
MIP-1α
IL-1α

Fig. S7: Expression profiles and contribution of analytes to PCA for TLR4 or TLR7 and TLR7/8 agonists

Contribution of protein analytes from Luminex analysis to each PC vector of the PCA shown for A) TLR4 ligands or B) TLR7 and TLR7/8 ligands corresponding to Figure 4 A and B). C) Proportion of pDCs within the DC:PBL co-culture 24h or 6 days after high concentration adjuvant treatment, shown as mean ± SD. Cells were stained for the specific surface markers and were analyzed by flow cytometry (n=6-12). D) Expression levels (Luminex analysis) of the 15 most differentially expressed proteins (q-value < $1x10^{-15}$) from cells stimulated with the TLR7 ligands GARD, IMQ or the TLR 7/8 ligand R848, displayed as heat map.

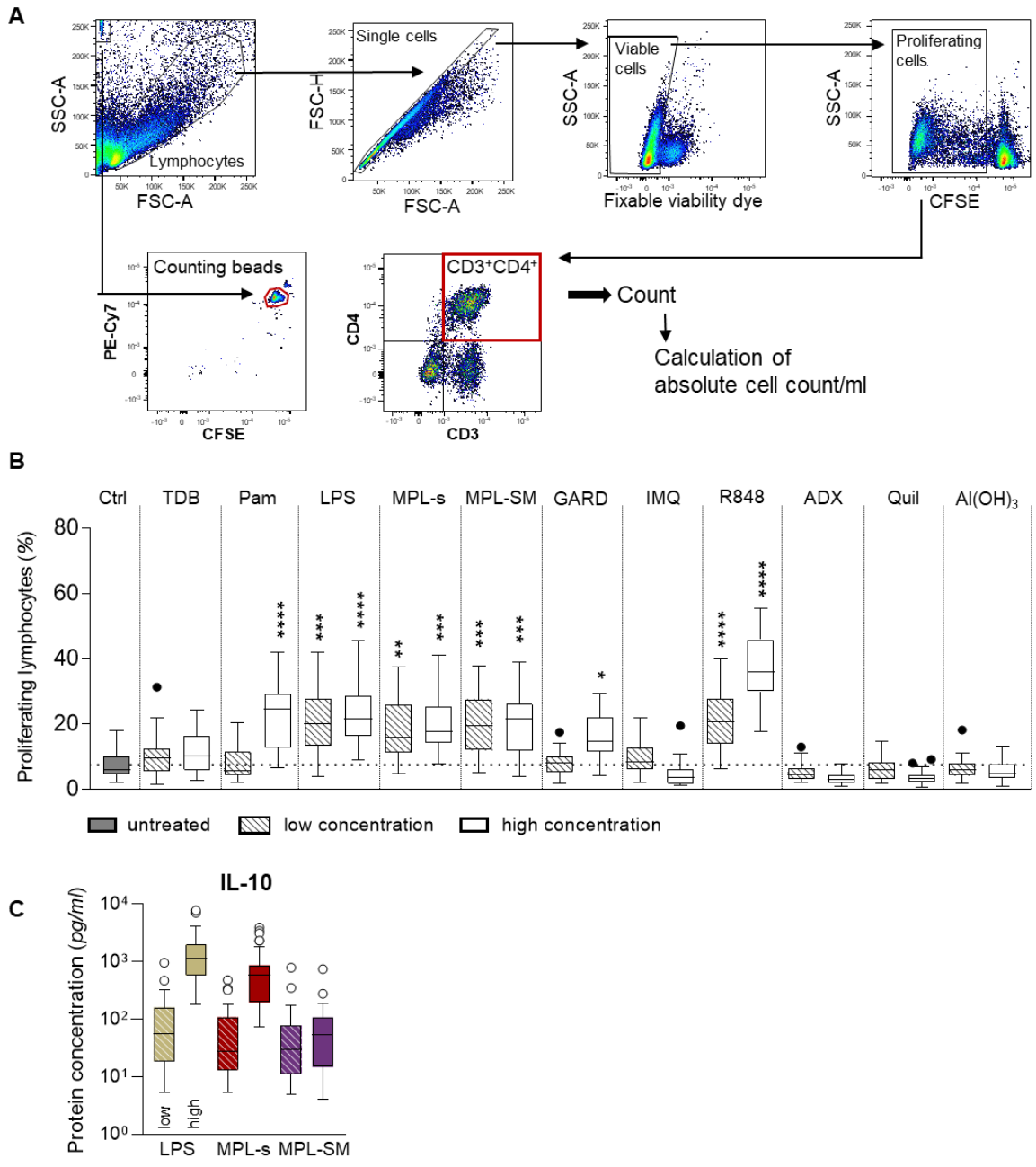

Fig. S8: Adjuvants stimulate the proliferation of lymphocytes to different degrees.

CFSE-labeled DC:PBL co-cultures were stimulated with the different adjuvants for 6 days, followed by antibody staining and flow cytometry analysis. A) Gating strategy to assess the proliferation of different lymphocyte populations (as shown in figure 5). Doublets and apoptotic cells were excluded. Proliferating cells were identified by exposing a lower intensity of the CFSE dye. Within the proliferating lymphocytes, various sub-populations were identified by specific antibody staining. Here, an example for $CD3^+CD4^+$ T cells is shown. Counting beads were acquired during sample analysis allowing the calculation of the absolute cell count of proliferating $CD3^+CD4^+$ T cells within the respective sample. B) Box Whisker plots represent adjuvant-induced total

lymphocyte proliferation. Statistical comparisons of the unstimulated control and the adjuvant was performed using the Kruskal-Wallis test with Dunn's correction (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$) (n=26 donors). If not otherwise specified, comparison revealed no significance. Data is representative for at least three independent experiments. C) IL-10 protein concentrations measured by Luminex analysis after stimulation with TLR4 ligands LPS, MPL-s or MPL-SM. Data are neither transformed nor batch corrected.

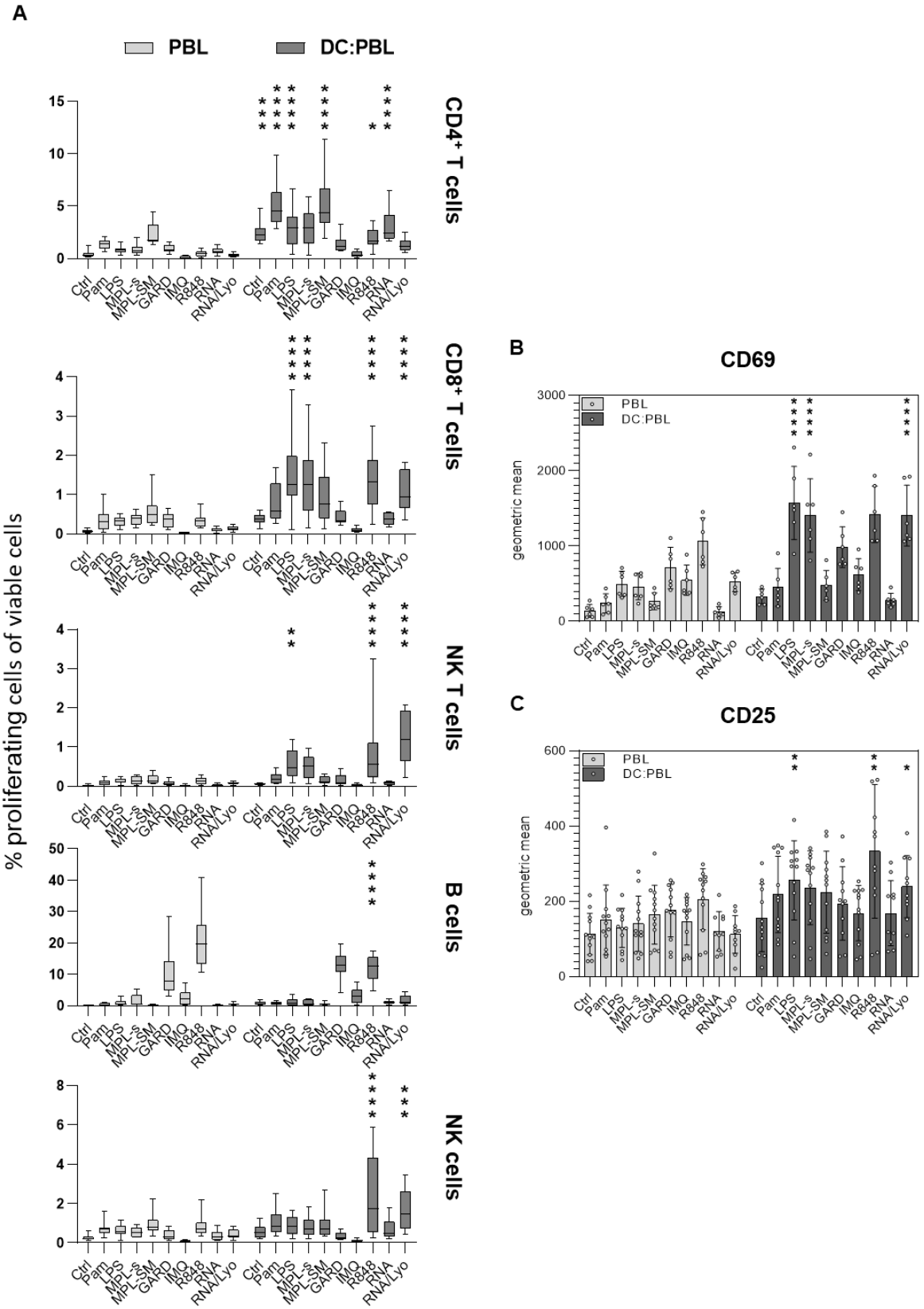
A
PBL
DC:PBL
% proliferating cells of viable cells
CD4+ T cells
CD8+ T cells
NK T cells
B cells
NK cells
Ctrl
Pam
LPS
MPL-s
MPL-SM
GARD
IMQ
R848
RNA
RNA/Lyo
B
CD69
geometric mean
C
CD25
geometric mean

Fig. S9: Influence of moDCs on proliferation and activation of lymphocytes

PBLs in absence or presence of moDCs were treated with the high concentrations of the different adjuvants/stimuli for 5 days, and A) proliferation of lymphocyte subpopulations or expression of B) CD69 or C) CD25 was determined by flow cytometry (n=10-12; ≥4 independent experiments; shown as mean ± SD). B, C) Unspecific antibody binding was tested by isotype control. Statistical significance was calculated employing two-way ANOVA with Sidak's multiple comparison test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$), displaying the significant differences in proliferation between the DC:PBL co-culture and the respective condition of PBL only.

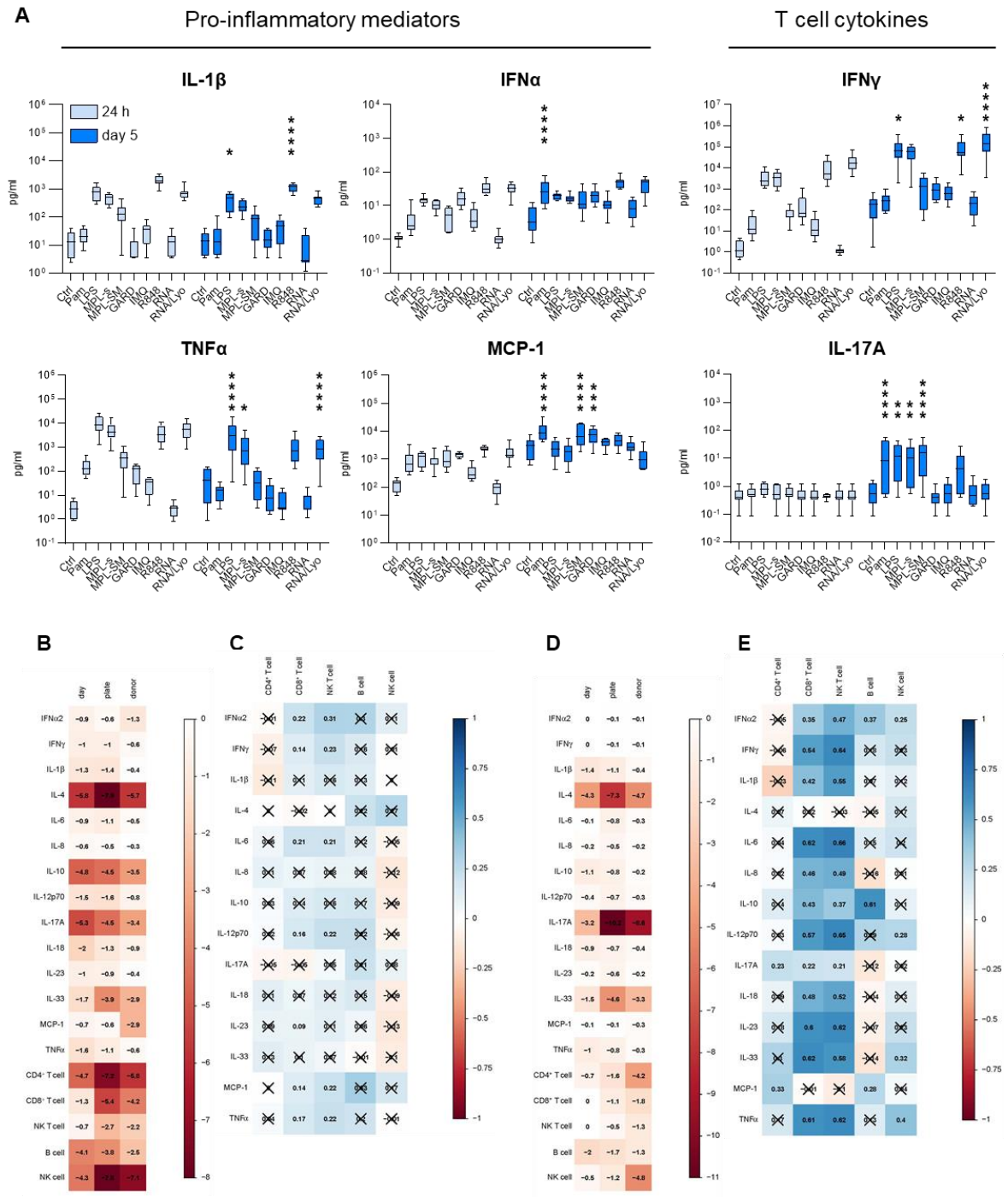

Fig: S10: Expression of cytokines or chemokines 24 h or 5 days upon adjuvant treatment positively correlates with lymphocyte proliferation.

A) DC:PBL co-cultures were treated with the high concentrations of the different adjuvants/stimuli for 24 h or 5 days. Cytokine and chemokine concentrations, displayed as box-whisker (min to max) plots, were measured by flow cytometry from ≥ 3 independent experiments (n=10) employing the Legendplex™ 'Human Inflammation panel 1' (n=10). Statistical analysis, determining the significant differences in analyte concentration between day

5 and 24 h of the respective condition, was performed using two-way ANOVA with Sidak's multiple comparison test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Data sets (n=10) of adjuvant-induced cytokine and chemokine secretion after B, C) 24 h or D, E) 5 days were linked to adjuvant-induced lymphocyte proliferation assessed after 5 days using Spearman's rank correlation. B, D) The influence of several factors on the levels of cytokine and chemokine concentration as well as of absolute lymphocyte count has been assessed individually by Kruskal-Wallis testing. The presented values are the common logarithms of the p-values analyzed by the Kruskal-Wallis test. White color represents a high p-value, whereas red color indicates a very low p-value. All analyses are exploratory without corrections for multiple testing. C, E) The correlation matrix is based on normalized values which were obtained by setting the unstimulated control for each donor to 1. Spearman correlation coefficients associated with a p-value above 0.01 are crossed out. A value of +1 (blue) indicates a positive, whereas a value of -1 (red) represents negative correlation.

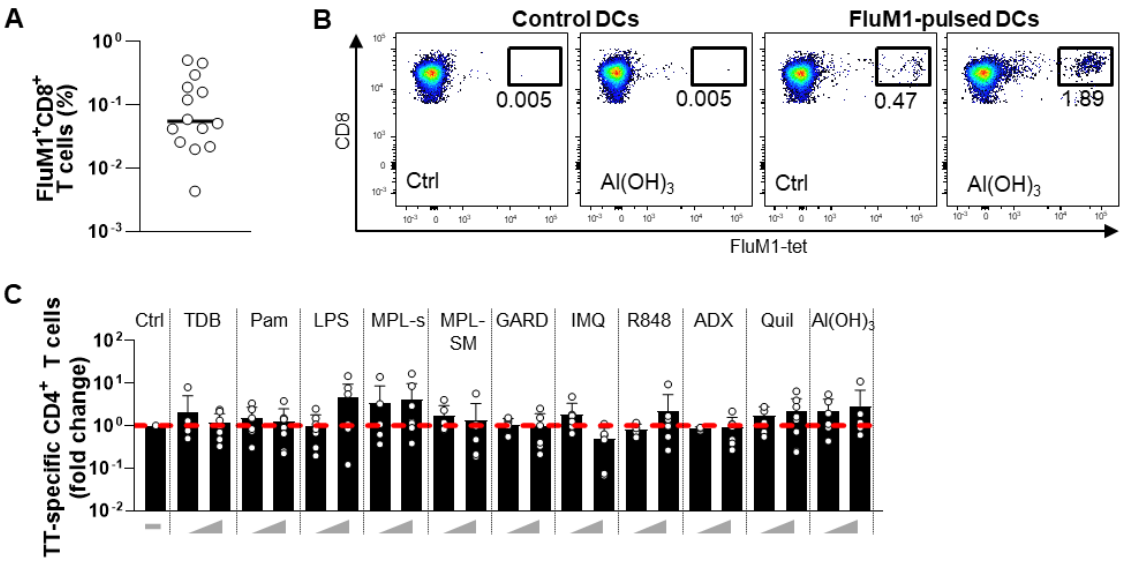

Fig. S11: Effects of adjuvants on the antigen-specific CD8$^+$ or CD4$^+$ T cell population.

DCs loaded with the FluM1$_{58\text{-}66}$ peptide (GILGFVFTL) were co-cultured with autologous CD8$^+$ T cells and stimulated with the different adjuvants. A) Donor individual baseline levels of FluM1-specific CD8$^+$ T cells as measured using the corresponding MHC class I tetramer (GILGFVFTL HLA-A*0201-PE) after CD8$^+$ T cell isolation (n=14). B) Representative dot plots of the FluM1-specific CD8$^+$ T cell population on day 7 of the DC^{FluM1}:CD8 co-culture, which was stimulated either with Al(OH)$_3$ or left untreated (ctrl). DCs not loaded with the FluM1 peptide served as control (control DCs). C) DCs loaded with the TT p2$_{829\text{-}844}$ peptide (MQYIKANSKFIGITEL) were co-cultured with autologous CD4$^+$ T cells and stimulated with the different adjuvants. The negative control was left untreated. On day 6 or 7, CD4$^+$ T cells were stained with a peptide-corresponding MHC class II tetramer (MQYIKANSKFIGITEL HLA-DRB1*1101-PE) and anti-CD3 and -CD4 antibodies for analysis by flow cytometry. Single, viable cells were used for pairwise comparison of the adjuvant-stimulated condition with the unstimulated control employing the Wilcoxon test (no significance revealed; n=6-8). Data is representative for at least three independent experiments.

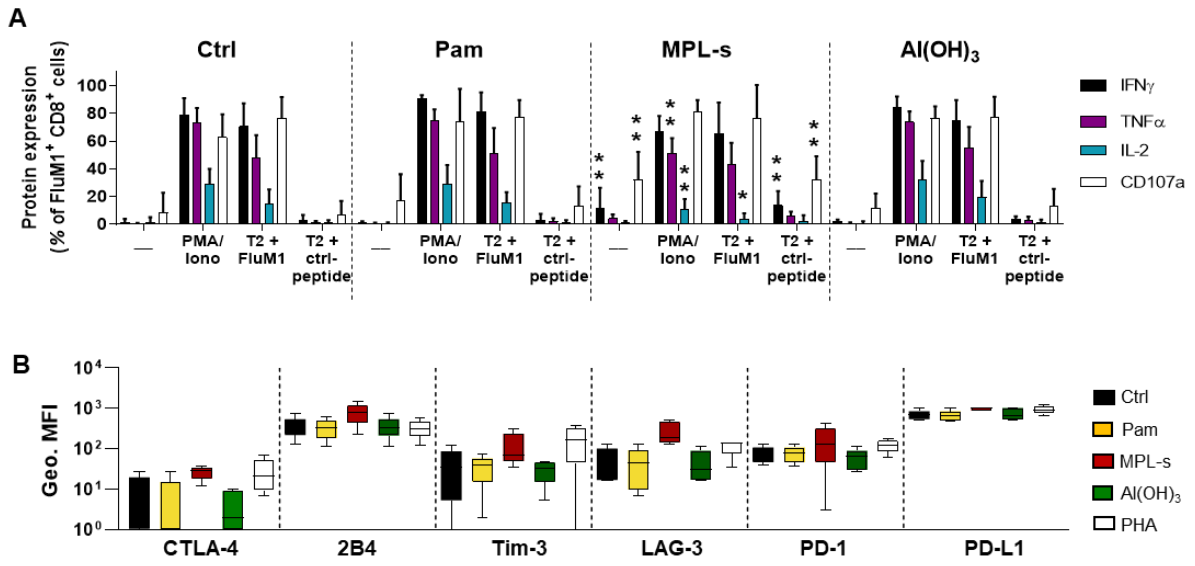

Fig. S12: Polyfunctionality of Pam-, MPL-s- and Al(OH)$_3$-treated CD8$^+$ T cells upon FluM1-restimulation.

A) FluM1-specific CD8$^+$ T cells were expanded without adjuvant or with Pam, MPL-s or Al(OH)$_3$. Expression of IFNγ, TNFα, IL-2 and CD107a were measured in FluM1-specific CD8$^+$ T cells after being stimulated with PMA/Iono (positive control), peptides being presented on T2 cells (FluM1 or control; n=5-10). B) Analysis of inhibitory ligand expression on CD8$^+$ T cells after being stimulated for 6-7 days with Pam, MPL-s, Al(OH)$_3$, PHA or left untreated (Ctrl; n=5). Statistical differences between the adjuvant stimulations and the unstimulated control were compared by (A) Kruskal-Wallis or (B) Friedman test with Dunn's correction (n=5). A p-value of * $p < 0.05$, ** $p < 0.01$ was considered as statistically significant. Data are representative of at least three independent experiments.

Abbreviations

ADX	Addavax
Al(OH)3	Aluminium hydroxide
APC	Antigen presenting cell
AS01/04	Adjuvant system 01/04
BCR	B cell receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
ELISA	Enzyme-linked immunosorbent assay
EU/ml	Euopean Units/ milliliter
FluM1	specific peptide of the Influenza A virus matrix protein
GARD	Gardiquimod
Geo. MFI	Geometric mean fluorescence intensity
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
iDC	immature DC
IFN	Interferon
IgG/ IgM	Immunoglobulin G/ M
IL-	Interleukin
IMQ	Imiquimod (R837)
LAG-3	Lymphocyte-activation gene 3, CD223
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MAT	Monocyte activation test
MCP-1	Monocyte chemotactic protein-1; CCL2
MF59	Oil-in-water emulsion adjuvant
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma interferon; CXCL9
MoA	Mode of action
moDC	monocyte-derived DC

MPL	Monophosphoryl lipid A
MPL-s	synthetic monophosphoryl lipid A
MPL-SM	Monophosphoryl lipid A derived from Salmonella minnesota R595
MyD88	Myeloid differentiation primary response 88
n	Number of donors
NK cell	Natural killer cell
NKT cell	Natural killer T cell
Pam	Pam3CSK4
PAMP	Pattern associated molecular pattern
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cells
PCA	Principal component analysisi
pDC	plasmacytoid DC
PD-L1	Programmed death ligand 1
PHA	Phytohemagglutinin
PI	Propidium iodid
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
Quil	Quil-A
R848	Resiquimod
ss polyU	Single-stranded RNA polymer; polyuridylic acid
SCF	Stem cell factor
SD	Standard deviation
TDB	Trehalose-6,6-dibehenate
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TT	Tetanus toxoid
WHO	World Health Organization

References

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