Supplementary Material

High production of IL-12 by human dendritic cells stimulated with combinations of pattern-recognition receptor agonists

Brian C Gilmour, Alexandre Corthay, and Inger Øynebråten

Table of contents

Supplementary Table 1. Ligands used to stimulate pattern recognition receptors (PRRs), and ligand abbreviations

PRR ligand	Abbreviation	Target receptor	Tested conc. (µg/mL)	Catno.	Provider
5'-triphosphate hairpin RNA¤	3p-hpRNA	RIG-I	10	tlrl-hprna	InvivoGen
Lipopolysaccharide from *E. coli* K12 (Ultrapure)	LPS	TLR4	1	tlrl-peklps	InvivoGen
Lipoteichoic acid from *Staphylococcus aureus*	LTA	TLR2:1, TLR2:6	0.1-100	tlrl-pslta	InvivoGen
N-Glycolyl-muramyl dipeptide	GMDP	NOD2	0.1	tlrl-gmdp	InvivoGen
ODN2395	CpG	TLR9	1	tlrl-2395	InvivoGen
Pam3CSK4	Pam3	TLR2:1	0.1-10	tlrl-pms	InvivoGen
Poly(dA:dT) naked¤	p(dA:dT)	cGAS/ALR	2	tlrl-patn	InvivoGen
Poly(I:C) HMW*	poly(I:C)	TLR3	0.02; 20	tlrl-pic	InvivoGen
Poly(I:C) HMW¤*	poly(I:C)	MDA5/TLR3	20	tlrl-pic	InvivoGen
Resiquimod	R848	TLR7/8	0.5; 1	tlrl-r848	InvivoGen
Zymosan (depleted)	Zym-D	Dectin-1	1-20	tlrl-zyd	InvivoGen

¤ 5'-triphosphate hairpin RNA, poly(dA:dT) naked, and poly(I:C) HMW were mixed with LyoVec™ to transfect the ligands into the cytosol.
* HMW, high molecular weight

Supplementary Table 2. Increased concentrations of the agonists of dectin-1, TLR2:1, or TLR2:6 did not trigger IL-12p70 production

Stimulated PRR	Ligand	Ligand concentration (μg/mL)	Absorbance		IL-12p70 (pg/mL) ¤
Unstimulated	None	0	0.081	0.078	ND
Dectin-1	Zym-D	1	0.083	0.081	ND
		10	0.083	0.081	ND
		20	0.080	0.083	ND
TLR2:1	Pam3CSK4	0.5	0.088	0.082	ND
		1	0.080	0.080	ND
		10	0.083	0.078	ND
TLR2:1; TLR2:6	LTA	0.5	0.083	0.078	ND
		1	0.079	0.079	ND
		10	0.084	0.080	ND
		100	0.082	0.081	ND
MDA5/TLR3	poly(I:C)*	20	1.353	1.455	1470.5

ND, not detectable

¤ Amounts of IL-12p70 (pg/mL) in supernatants from monocyte-derived DCs stimulated with the indicated ligand concentrations for 24 h.

* Poly(I:C) was transfected into the cytosol by LyoVec™, a lipid-based transfection reagent. The ligand was included as positive control.

Supplementary Table 3. Increased concentrations of the ligands of dectin-1, TLR2:1, or TLR2:6 did not trigger IFNβ production

Stimulated PRR	Ligand	Ligand concentration (μg/mL)	IFNβ (pg/mL) ¤	
Unstimulated	None	0	7.1	7.5
Dectin-1	Zym-D	1	7.1	7.4
		10	6.9	7.0
		20	6.9	6.7
TLR2:1	Pam3CSK4	0.5	7.1	7.6
		1	6.9	7.1
		10	7.0	7.2
TLR2:1, TLR2:6	LTA	0.5	7.1	7.0
		1	7.1	7.1
		10	7.1	7.8
		100	7.1	7.2
MDA5/TLR3	poly(I:C) *	20	261.9	289.2

¤ Amounts of IFNβ (pg/mL) in supernatants from monocyte-derived DCs stimulated with the indicated ligand concentrations for 24 h.

* Poly(I:C) was transfected into the cytosol by LyoVec™, a lipid-based transfection reagent. The ligand was included as positive control.

Supplementary Table 4. Table 3 in colors:
Data summary for the production of IL-12p70 and IFNβ
by DCs stimulated with pattern-recognition receptor agonists

Stimulated PRRs [#]	Ligands	IL-12p70 [¤]	IFNβ [¤]
C1: Dectin-1 + TLR7/8	Zymosan-D + R848	+++++	-/+
C2: TLR2:1 + TLR3	Pam3CSK4 + poly(I:C)	+++++	+++
C3: TLR2:1 + MDA5/TLR3[§]	Pam3CSK4 + poly(I:C)[¶]	+++++	+++
C4: TLR3 + TLR7/8	poly(I:C) + R848	+++++	+++
C5: TLR3 + NOD2	poly(I:C) + *N*-GMDP	+++++	+++
C6: TLR7/8 + MDA5/TLR3[§]	R848 + poly(I:C)[¶]	+++++	++
TLR3 + TLR7/8	poly(I:C) [0.02]‡ + R848	++++	+
Dectin-1	Zymosan-D	-/+	-/+
TLR2:1	Pam3CSK4	-/+	-/+
TLR2:1/6	LTA	-/+	-/+
TLR3	poly(I:C) [0.02]‡	+	+
TLR3	poly(I:C)	++++	+++
TLR4	LPS-EK	+++	+++
TLR7/8	R848	+	-/+
TLR9	CpG	-/+	-/+
RIG-I	3p-hpRNA[¶]	-/+	+
MDA5/TLR3[§]	poly(I:C)[¶]	++++	+++
cGAS/ALR[‡]	poly(dA:dT)[¶]	-/+	+++
NOD2	*N*-GMDP	-/+	-/+
Unstimulated	No ligand	-/+	-/+

[#] C1-C6 indicate the identified ligand combinations that induce high production of IL-12p70 by moDCs.

[¤] Graded concentrations of IL-12p70 and IFNβ in the media from stimulated moDCs: -/+ indicates non-detectable to 20 pg/mL cytokine; +, 21-100 pg/mL; ++, 101-350 pg/mL; +++, 351-800 pg/mL; ++++, 801-1500 pg/mL; and +++++, > 1500 pg/mL.

[§] Poly(I:C) was transfected by lipid-based transfection and may engage both MDA5 and TLR3.

[¶] Lipid-based transfection was used to transfer the ligand into the cytosol.

‡ Poly(I:C) concentration: 0.02 µg/mL. When not indicated, poly(I:C) was used in concentration 20 µg/mL.

[‡] Several sensors can recognize dsDNA: cGAS, and ALRs (*e.g*. IFI16 and DAI).

Supplementary Table 5. ELISA kits used for quantification of cytokines

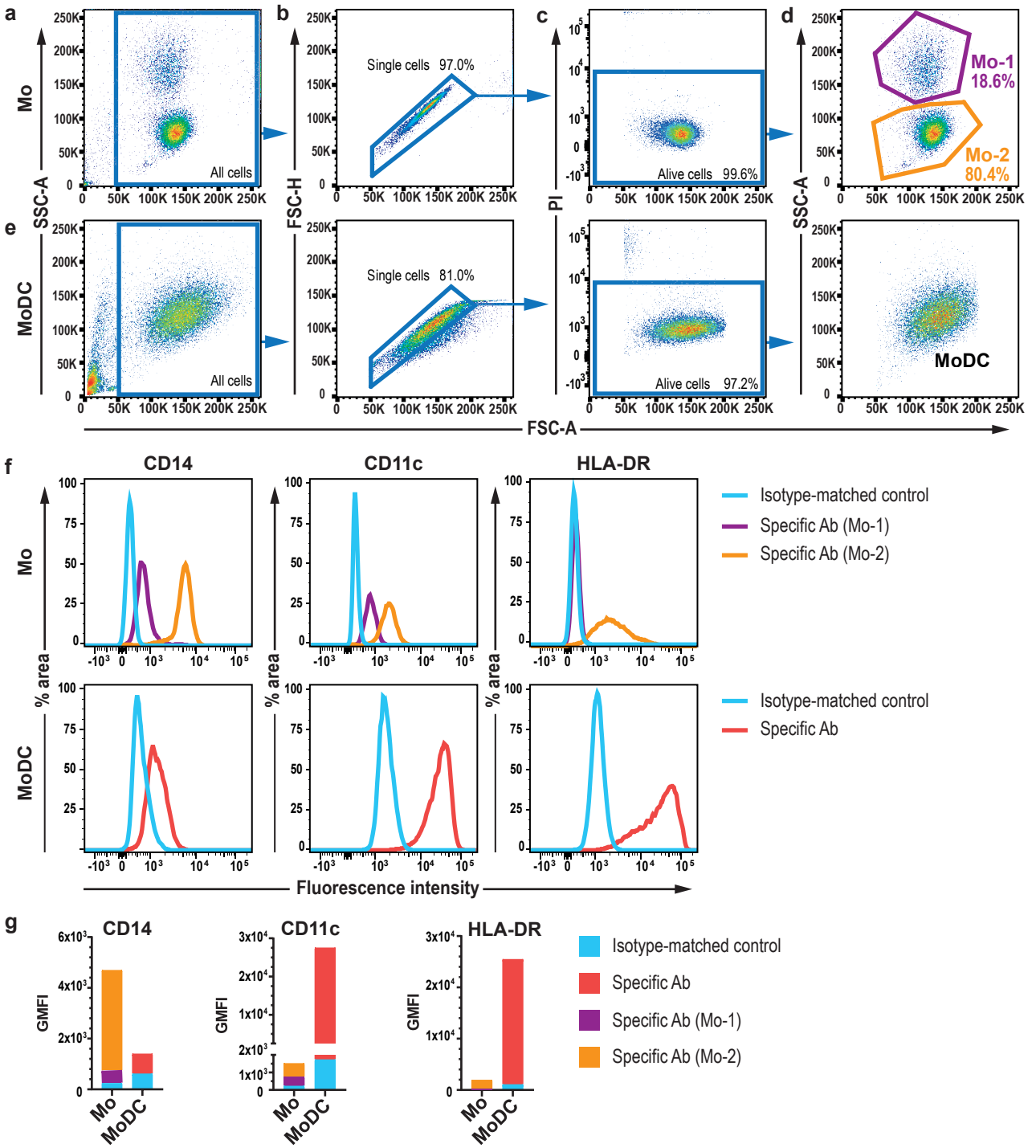
Item	Provider	Catno.
DuoSet® Human IL-12 p70	Bio-Techne	DY1270
DuoSet® Human IFN-β	Bio-Techne	DY814-05
DuoSet® Human IL-10	Bio-Techne	DY217B

Supplementary Table 6. Primary antibodies used for analysis of expression of cell surface molecules on monocytes and monocyte-derived DCs

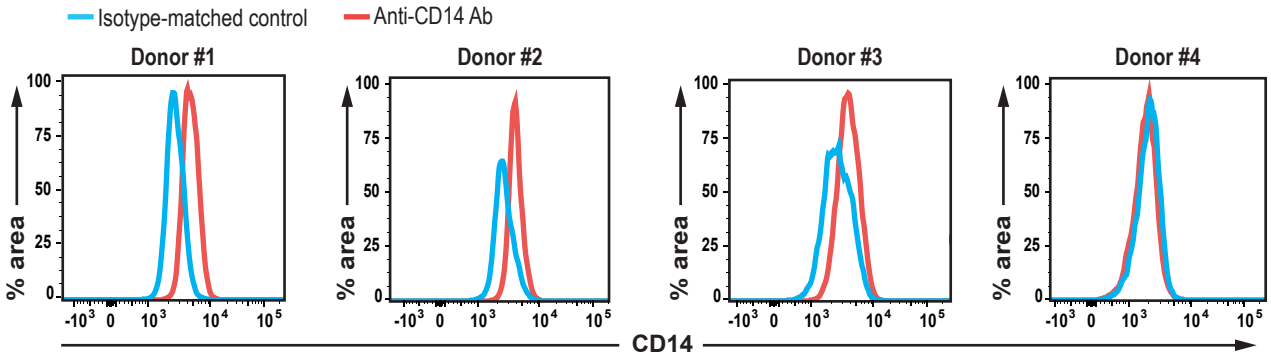
Specificity	Fluorophore	Clone	Isotype	Final conc. µg/mL	Provider	Catno.
CD14	APC/Cy7	HCD14	Mouse IgG1,κ	20	BioLegend	325620
CD11c	Pacific Blue	3.9	Mouse IgG1,κ	20	BioLegend	301626
HLA-DR	BV605	L243	Mouse IgG2a,κ	5	BioLegend	307640
CD80	PE/Cy7	2D10	Mouse IgG1,κ	10	BioLegend	305217
CD86	APC	IT2.2	Mouse IgG2b,κ	10	BioLegend	305411
CCR7	PE/Cy7	G043H7	Mouse IgG2a,κ	10	BioLegend	353225

Supplementary Table 7. Isotype- and fluorophore-matched, irrelevantly targeted antibodies used as negative controls

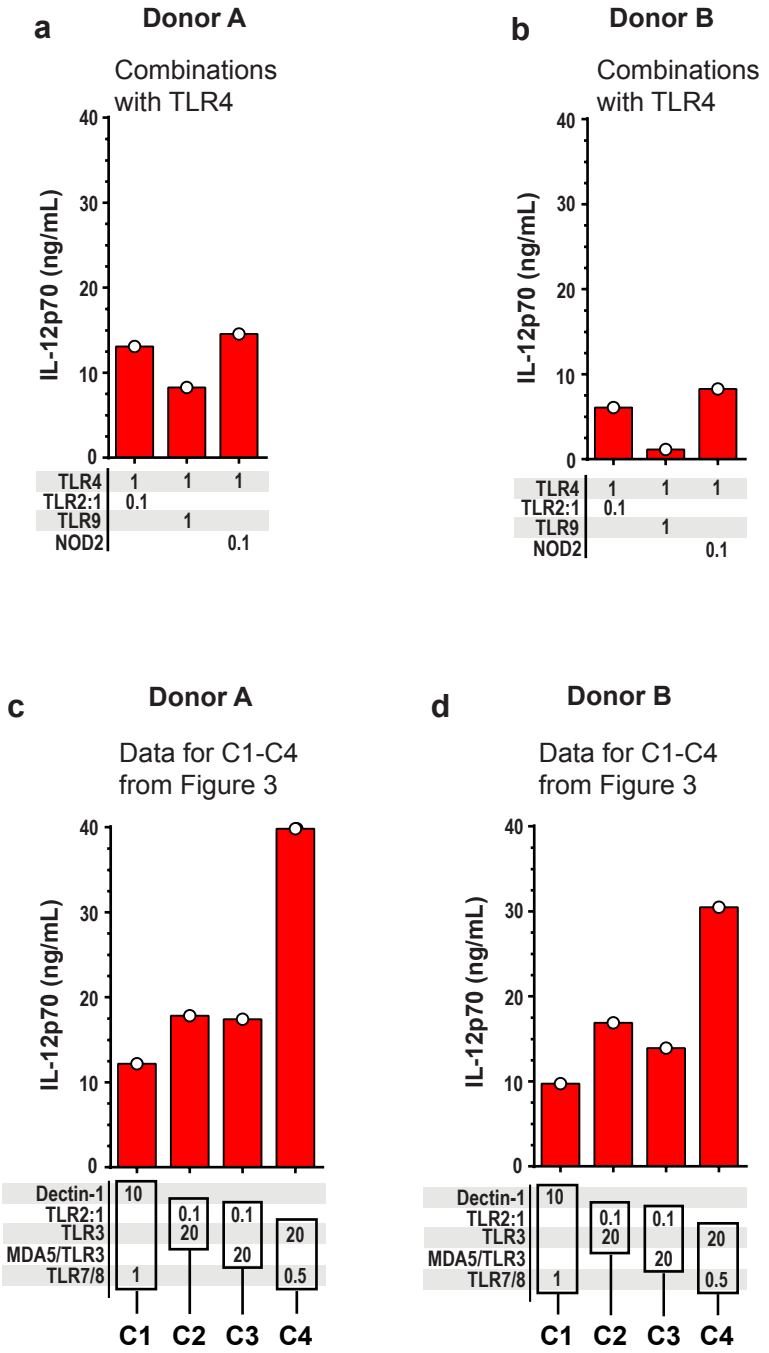
Fluorophore	Isotype	Clone	Sepcificity	Provider	Catno.
APC/Cy7	Mouse IgG1,κ	MOPC-21	Unknown	BioLegend	400128
Pacific Blue	Mouse IgG1,κ	MOPC-21	Unknown	BioLegend	400151
BV605	Mouse IgG2a,κ	MOPC-173	Unknown	BioLegend	400270
PE/Cy7	Mouse IgG1,κ	MOPC-21	Unknown	BioLegend	400126
APC	Mouse IgG2b,κ	MG2b-57	Trinitrophenol + KLH	BioLegend	401210
PE/Cy7	Mouse IgG2a,κ	MOPC-173	Unknown	BioLegend	400231



Supplementary Figure 1. Gating and flow cytometry analysis of monocytes and monocyte-derived DCs. Positively selected, freshly isolated CD14+ monocytes and monocytes differentiated for 5 days by GM-CSF and IL-4 into monocyte-derived dendritic cells (moDC) were gated and analysed by flow cytometry as indicated: **a** The monocytes (Mo) were first gated based on size and complexity in a forward scatter (FSC-A) and side scatter (SSC-A) plot; **b** Next, a FSC-A and FSC-H plot was used to gate single cells and exclude doublets; **c** Dead cells were removed by their uptake of propidium iodide (PI); **d** Gated live, single cells distributed in two populations, Mo-1 and Mo-2, which constituted 18.6 and 80.4 % respectively, of the gated monocytes. **e** The same gating strategy was used for moDCs, and gated live, single moDCs distributed in one population in the FSC-A and SSC-A plot. **f** Mo-1 showed low CD14 and CD11c expression, and was negative for HLA-DR. Mo-2 on the other hand, was positive for CD14, CD11c, and HLA-DR. The moDCs showed reduced expression of CD14 compared to Mo-2, and increased expression of CD11c and HLA-DR compared to both Mo-1 and Mo-2. **g** The graphs show geometric mean fluorescence intensity (GMFI) for CD14, CD11c, and HLA-DR calculated from the experiment shown in f. The specific antibodies that were used: anti-CD14, clone HCD14; anti-CD11c, clone 3.9; and anti-HLA-DR, clone L243. The antibodies were used alongside isotype- and concentration-matched controls.



Supplementary Figure 2. The blood donors gave rise to monocyte-derived DCs with low CD14 expression. Monocyte-derived dendritic cells (moDCs) were differentiated with GM-CSF (100 ng/mL) and IL-4 (20 ng/mL) for 5 days before flow cytometry analysis of CD14. The moDCs were gated as indicated in Supplementary Figure 1 before evaluation of CD14 expression. The control antibody (blue line, clone MOPC-21) matched the isotype and concentration of the anti-CD14 antibody (red line, clone HCD14). MoDCs generated from different donors (Donor 1-4), showed low to no expression of CD14, suggesting that the monocytes were successfully differentiated into moDCs. The proportion of CD14+ moDCs varied from 13.8-31.8 % with an average value of 22 % across the donors.



Supplementary Figure 3. Levels of IL-12p70 induced by combinations containing the TLR4 ligand LPS-EK. MoDC from two donors were stimulated for 24 h with different combinations of ligands: **a**, **b** LPS-EK (TLR4) in combination with an agonist of TLR2:1, TLR9 or NOD2; and **c**, **d** the combinations C1, C2, C3, and C4. IL-12p70 was quantified in the cell culture supernatants. The concentrations of the PRR ligands are indicated in µg/mL below the bars. Each bar present one experiment from one donor. The data presented in c and d for the combinations C1-C4 are part of Figure 3.