Supplementary Information

A high-throughput nanoimmunoassay chip applied to large-scale vaccine adjuvant screening

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Supplementary Methods

Automatic Sample Microarraying

We found that 60% humidity gave us the most consistent features in terms of spot diameter (~300 μm). This humidity percentage also prevented the sample channel of the spotting pin to dry and get clogged. For viscous samples, such as serum, we found that using the "Touch Off" feature on the robot reduced blotting –remove excess sample from the pin tip. A 2-step Touch Off with a 500 msec pause after dipping was found to be sufficient. A stringent wash sequence between spotting samples was necessary to prevent sample to sample carry-over. The table below shows the sequence of washing steps we found were adequate to avoid cross-contamination.

Liquid	Wash time (sec)	Dry time (sec)
De-ionized water	5	**5**
De-ionized water	5	**5**
PBS/0.05% Tween 20	3	**3**
De-ionized water	5	**5**
PBS/0.05% Tween 20	3	**3**
De-ionized water	5	**5**

Spotted slides were stored in the dark for at least two hours in an incubator at 40°C before manual alignment of the PDMS device. For high-humidity environments, this step allowed for most of the water to evaporate from the sample and thus facilitate device alignment. The assembled device was incubated overnight in the dark at 40°C.

Supplementary Information

Antibodies and recombinant cytokines

Mouse antibodies and standard proteins used, were purchased from eBioscience (San Diego, USA), and are summarized in the table below.

	Mouse recombinant protein	Capture antibody (Biotin)	Detection antibody (PE)
IL-6	39-8061-60	36-7062-85	12-7061-41
TNF-alpha	39-8321-60	13-7341-81	12-7423-41
IL-23	39-8231-60	16-7232-85	12-7123-41
IL-12 p70	39-8121-60	14-7122-85	12-7123-41

Microfluidic Device Operation

Execution of nanoimmunoassay chip protocol

a. *Reagent loading*. All reagents were aspirated into Tygon tubing (0.020" ID, AAQ02103, Coler-Parmer). 80 µL of PBS buffer with 0.05% Tween-20 was connected to the first inlet of the device. PBS/Tween was used as a washing buffer throughout the experiments. 30 µL of biotinylated BSA (29130, Thermo Fisher Scientific) at a concentration of 2 mg/mL and 15 µL of neutravidin (31000, Thermo Fisher Scientific) at 0.5 mg/mL were connected to the second and third inlet, respectively. 10 µL of 5% milk powder resuspended in PBS was connected to the fourth inlet.

b. Control line priming. Microfluidic control channels were primed with dH_20 at 6 psi. Once the channels were filled the pressure was increased to 20 psi to close all the valves except for the MITOMI 'button' lines.

c. Biotin-neutravidin layer deposition. Reaction chambers were passivated by flowing biotin-BSA for 20 min at 3 psi. At this step, it is possible to use blocking buffers such as BSA, milk, or casein while keeping the buttons closed but this adds another step and consequently increases the assay time. Biotin-BSA was washed by flowing PBS/Tween for 5 min. Neutravidin was then flowed for 20 min through the chambers and washed for 5 min. The pressure in the buttons lines was increased to 20 psi and the rounded valves closed. Closing the buttons mechanically shields a round area of ~2700 μm^2 (60 µm diameter) of the surface and delineates the space where the sandwich immunoassay takes place. Biotin-BSA was flowed again for 20 min followed by a washing step of 5 min. Next 5% of non-fat dry milk in PBS was flushed for 10 min and washed for 5 min.

Supplementary Information

d. Primary antibody immobilization. Each primary antibody is immobilized under its corresponding button. Biotinylated antibodies were diluted in 1% blocker casein in PBS (37528, Thermo Fisher Scientific). Optimal working concentration for all primary antibodies was found to be 2 µg/mL except for anti-IL6 antibody, which was 200 ng/mL. 15 µL of each antibody dilution were loaded into different Tygon tubing and connected to the device. (At this step there is a layer of biotin-BSA-neutravidin under the area protected by the buttons.) One of the buttons was opened while keeping the remaining buttons closed, and the first antibody was flowed for 20 min followed by a 10 min washing step. This process was repeated for the remaining primary antibodies. A final blocking step with milk was performed by opening all the buttons, flowing milk for 10 min, and finally washing for 5 min with PBS/Tween.

e. Sample incubation. Water from the control lines diffuses constantly through the PDMS due to is porosity, thus all spotted samples have fully rehydrated at this step. This also increases considerably the pressure inside the spotting chambers. To incubate the sample, the isolation valves separating each reaction chambers were closed and the chamber valves opened. Rehydrated samples are incubated for at least one hour at room temperature.

f. Sample washing. During incubation the pressure across the three chambers equilibrate (the pressure is higher in the spotting chamber than the reaction chamber before incubation), raising the reaction chamber internal pressure to the point were the rounded valves will not close when actuated. Relief valves are opened to dissipate some of the pressure in the spotting chambers (and therefore in the reaction chamber) and to allow the buttons to fully deflect and protect the antibody-antigen complex. After 1 min the chamber valves are closed, isolation valves open, and unbound material washed away by flowing PBS/Tween for 20 min.

g. Detection step. A cocktail of secondary detection antibodies was diluted in casein/PBS to a concentration of 0.01 µg/mL, 0.05 µg/mL, and 1 µg/mL, for IL6, TNFα, and IL-12/IL-23 p40 antibodies, respectively. The cocktail was flowed through the chip for 10 min, isolating valves closed, and buttons opened. After the secondary antibodies were incubated with the bound complex for 20 min, the buttons were closed to protect the sandwich complex, followed by a final wash of PBS/Tween for 10 min to remove unbound antibodies.

h. Optical Readout. The microfluidic device was scanned using a fluorescent microarray scanner (ArrayWorx e-Biochip Reader, Applied Precision, USA) equipped with a Cy3 filter (540/25 X, 595/50 M). Devices were scanned with an exposure time of 1 sec at the highest resolution of 3.25 µm. Stitched images were exported as a 16-bit TIFF file.

i. Data analysis. Image files were analyzed using a microarray image analysis software (GenePix Pro v6.0, Molecular Devices) and Matlab (Mathworks). An analysis template grid

was manually created containing 1536 circular features that matched the location of the 4 buttons for the 386 reaction chambers on the chip. For each chip, the grid was manually aligned and the diameter of the circular features adjusted for each of the 1536 detection assays. Although this step could be automated, the manual alignment of the PDMS device to the glass slide introduced an inconsistent offset between rows and columns that varied across chips. The positions of each feature and its diameter were saved in a text file, which was fed to a Matlab script. The script automatically computes the mean fluorescent intensity inside each feature and subtracts the local background around the feature. It then generates a file reporting the relative fluorescent unit (RFU) values of the four different biomarkers for each assay. The script also arranges the RFU values of the calibration curves for each biomarker.

I. Statistics Analysis. A statistical software (Prism v5.0, GraphPad) was used to perform a non-linear regression analysis on the standard curves. Data from the calibration curves were fit using a dose-response model (variable slope, four-parameters), weighting data points by the observed standard deviation. Unknown RFU values were interpolated from the standard curves.

Supplementary Information

Supplementary Data

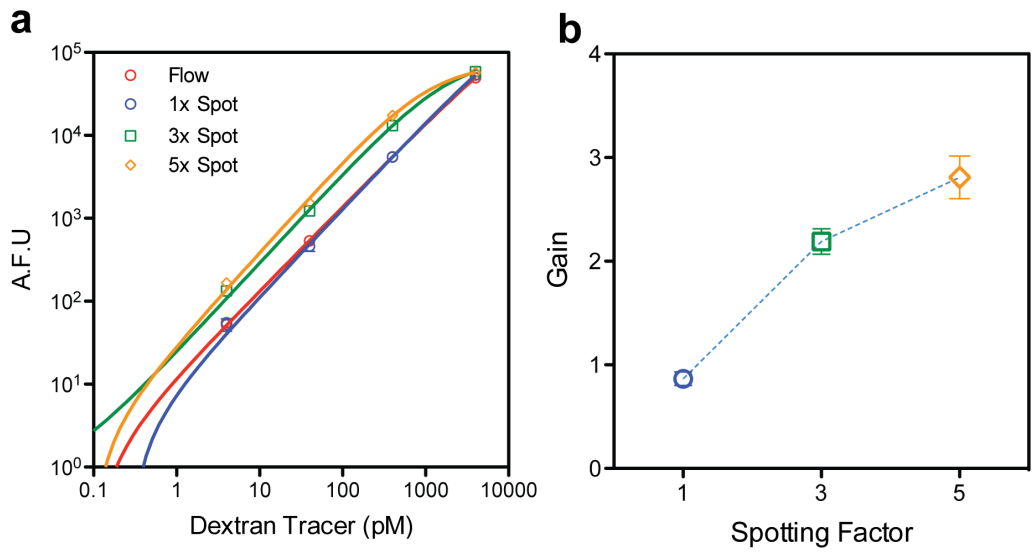
	Nanoimmunoassay chip	ELISA
Effective assay volume	5 nL	100 uL
Sample volume	10 nL	100 µL
Capture antibody amount	20-160 pg	50-400 ng
Detection antibody amount	20-160 pg	50-400 ng
Standard protein volume	10 nL	100 uL
Enzymatic amplification step	No	Yes
Multiplexing	4	1
LOD (TNFα, IL6)	100 fM	100 fM
Hands-on time	10 min	100 min
Automation	Microfluidics	Robot
Pipetting steps	1	30
Type of samples	Various (culture media, serum, BAL)	
Total reagent consumption volume	0.5 µL	7700 uL
Total cost of reagents	~$0.005-0.020	~$5-20

Supplementary Table 1. Comparison of nanoimmunoassay chip and a 96-well plate ELISA per single assay unit

Agonist	TLR	Concentration (h – m – l)	Reference
Pam3CSK4	TLR1/2	100 – 10 – 1 ng/ml	[1,2]
Pam2CSK4	TLR2/6	10 – 0.1 – 0.01 ng/ml	[3,4]
polyI:C	TLR3	20 – 5 – 1 µg/ml	[4-7]
Lipopolysaccharide (LPS)	TLR4	10 – 1 – 0.1 ng/ml	[8]
Monophosphoryl lipid A (MPLA)	TLR4	1– 0.1 – 0.01 µg/ml	[9-11]
Extra domain A of fibronectin (EDA)	TLR4	50 – 10 – 1 nM	[12,13]
Gardiquimod	TLR7	5 – 0.5 – 0.1 µg/ml	[10,14,15]
CpG A	TLR9	2 – 0.5 – 0.1 µM	[16,17]
CpG B	TLR9	50 – 10 – 5 nM	[18-21]
CpG C	TLR9	100 – 50 – 10 nM	[22,23]

Supplementary Table 2. TLR ligands and concentration used in the screening study.

Supplementary Information



Supplementary Figure 1. Immunoassay performance characterization using a fluorescent tracer. Different concentrations of a fluorescent tracer (Alexa647-labeled Dextran 10 KDa) were diluted in serum and spotted onto the chip. The same solutions were flowed onto the chip and fluorescent intensity values were compared to the spotted values. **(a)** A 100% reconstitution of the tracer into the reaction chambers was observed. As up to 93% of blood serum consist of water, we reasoned that spotting multiples times with intermittent pauses to allow evaporation, could lead to sample concentration and thus increase the amount of protein spotted. **(b)** Multi-spotting allows for up to three fold sample concentration by multi-spotting five times onto the same position. Notably, higher multi-spotting numbers are limited by the size of the microfluidic assay units, nevertheless this technique demonstrates to be a simple alternative to other microfluidic pre-concentration methods.

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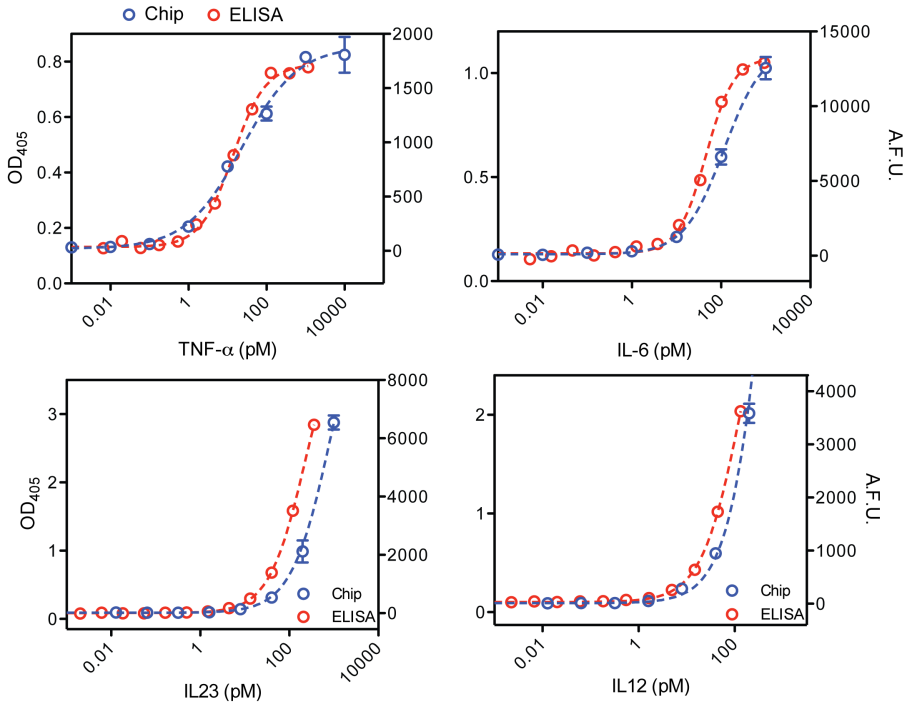
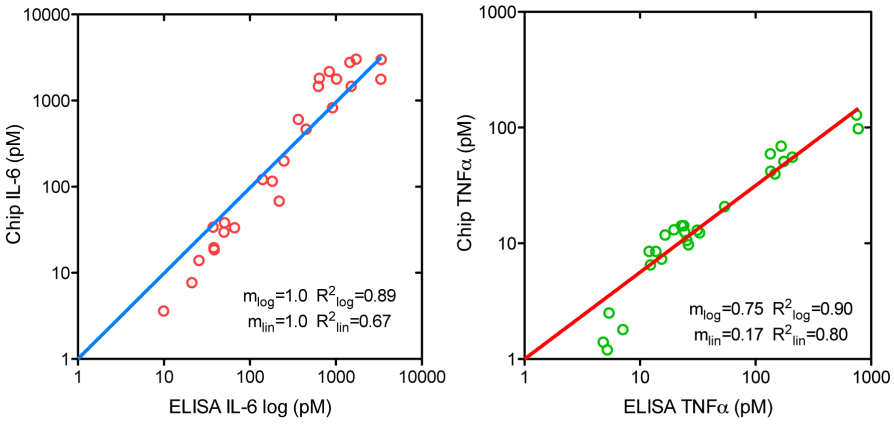


Supplementary Figure 2. Comparison of calibration curves for TNFα, IL-6, IL-12, and IL-23 obtained with the nanoimmunoassay chip and a typical ELISA. Calibration curves for cytokines IL-6, TNFα, IL-12p70, IL-23 in cell culture media were spotted on to the chip and found to be comparable to ones obtained with a standard ELISA.



Supplementary Figure 3. Comparison of blind tests run with the nanoimmunoassay chip and an ELISA. We determined the concentration of IL-6 and TNFα in stimulated cell culture samples known to express both cytokines at a wide concentration range. Samples were spotted in triplicate on-chip and also analyzed with standard 96-well plate ELISA's. A log correlation of 0.89 and 0.90 between ELISA and on-chip results was found for IL-6 and TNFα, respectively.

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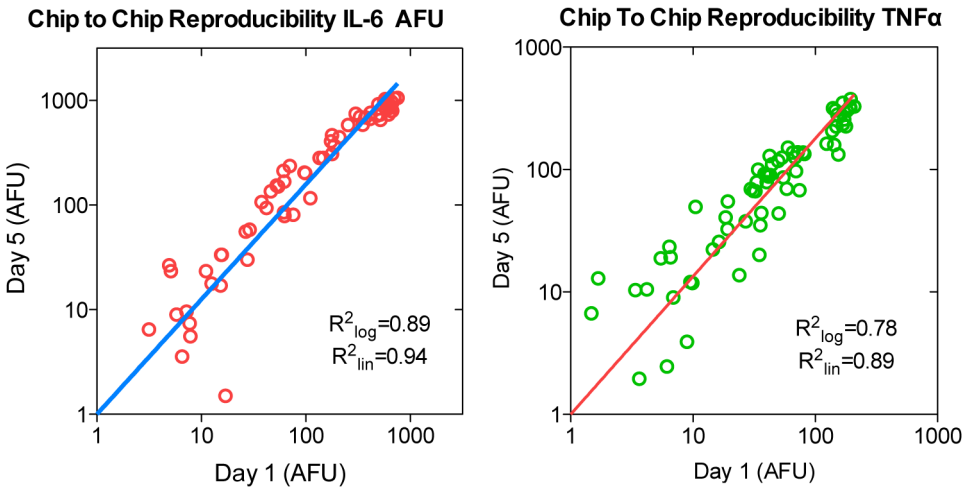

Supplementary Figure 4. Chip to chip reproducibility over time. Two slides were spotted on the same day and PDMS chips bonded to the slides. The first chip was run the following day after fabrication and the second chip five days later to study the reproducibility of our chips and stability of the samples. Log correlations of 0.89 and 0.78 for IL-6 and TNFα, respectively, were observed.

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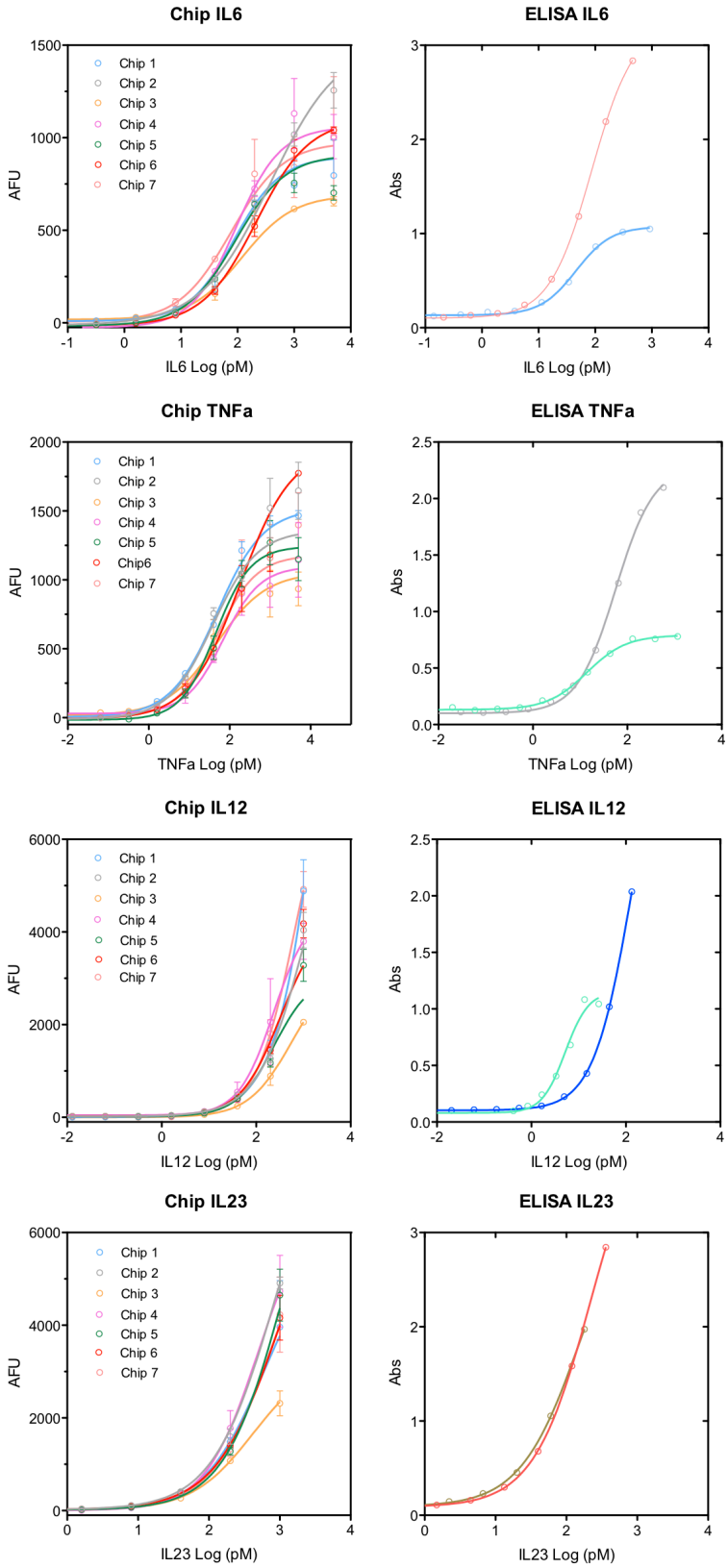
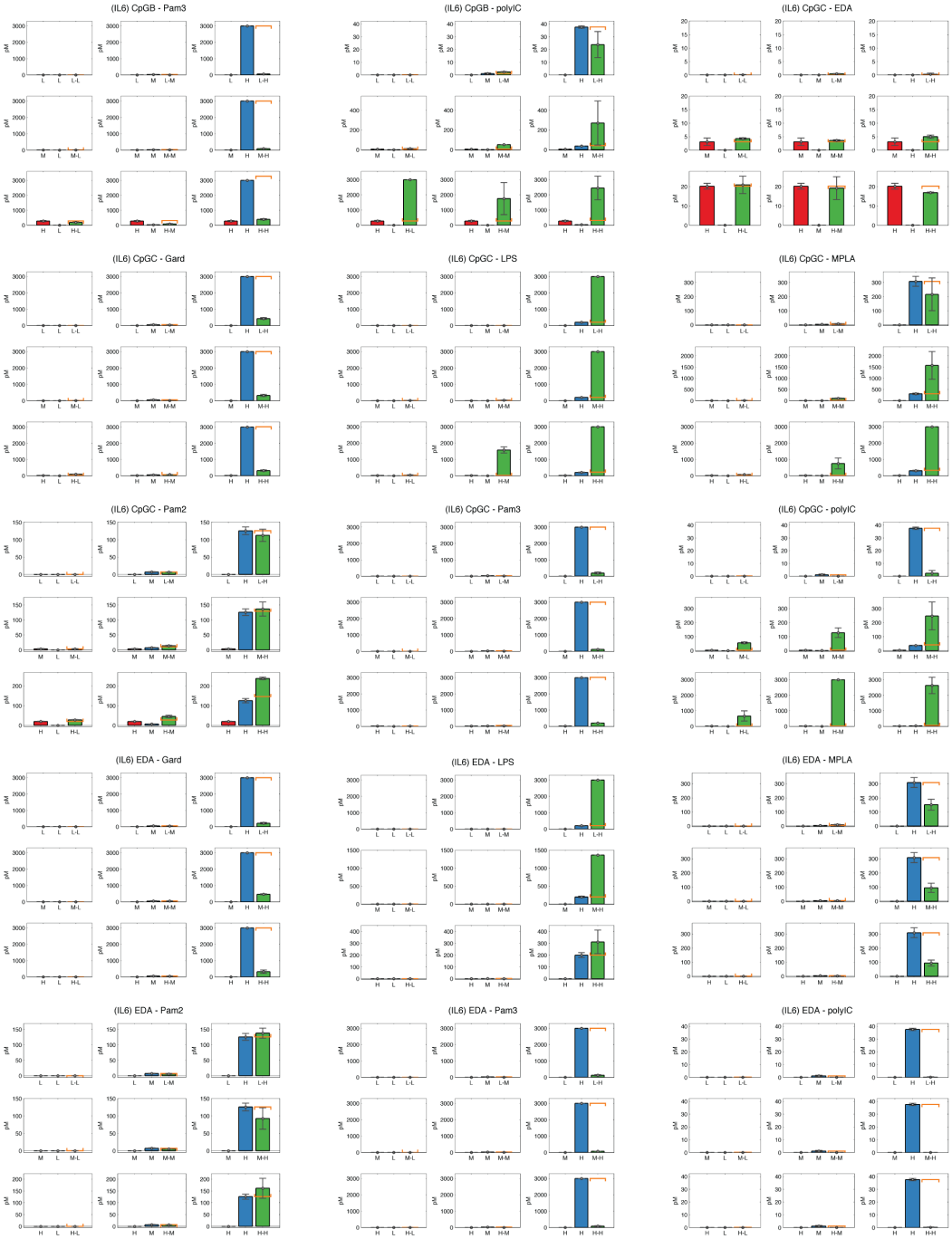


Supplementary Figure 5. Calibration curves obtained with chips and ELISA. Calibration curves for IL-6, TNFα, IL-12, and IL-23 (left column) obtained with chips used in this study. For comparison, examples of two ELISA's run on different days are shown in the right column.

Supplementary Information

Supplementary Information

IL6 page2



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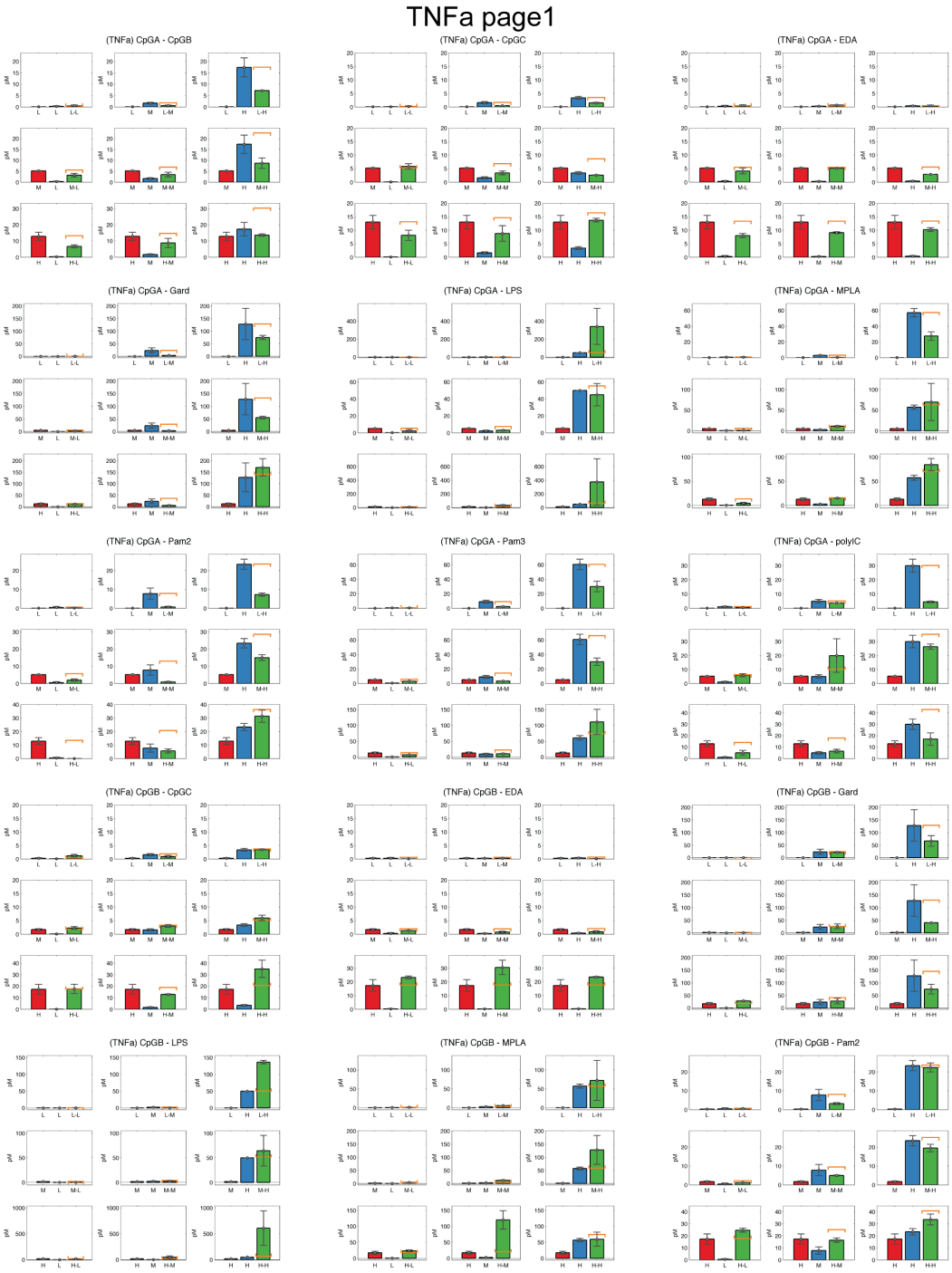

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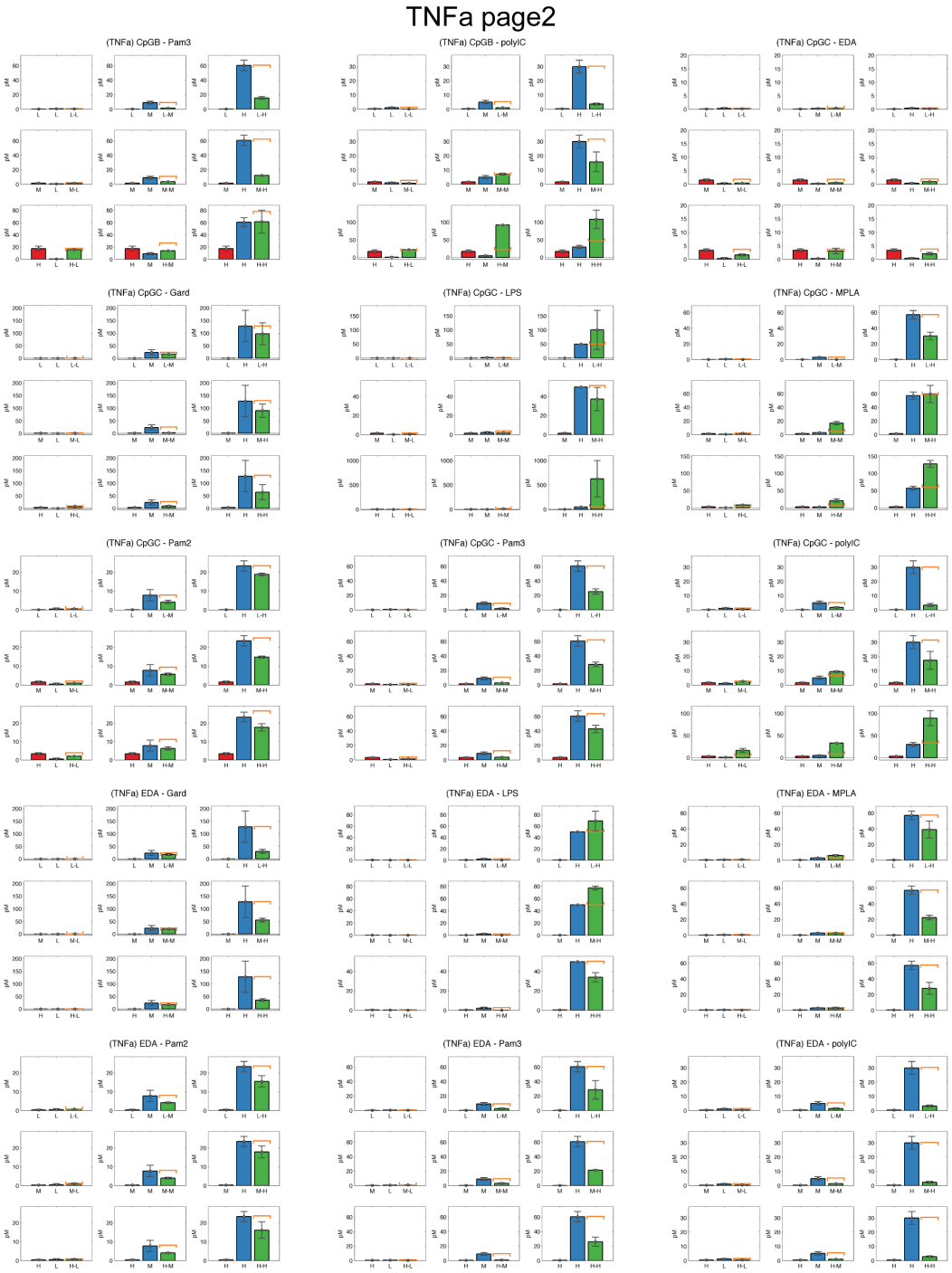

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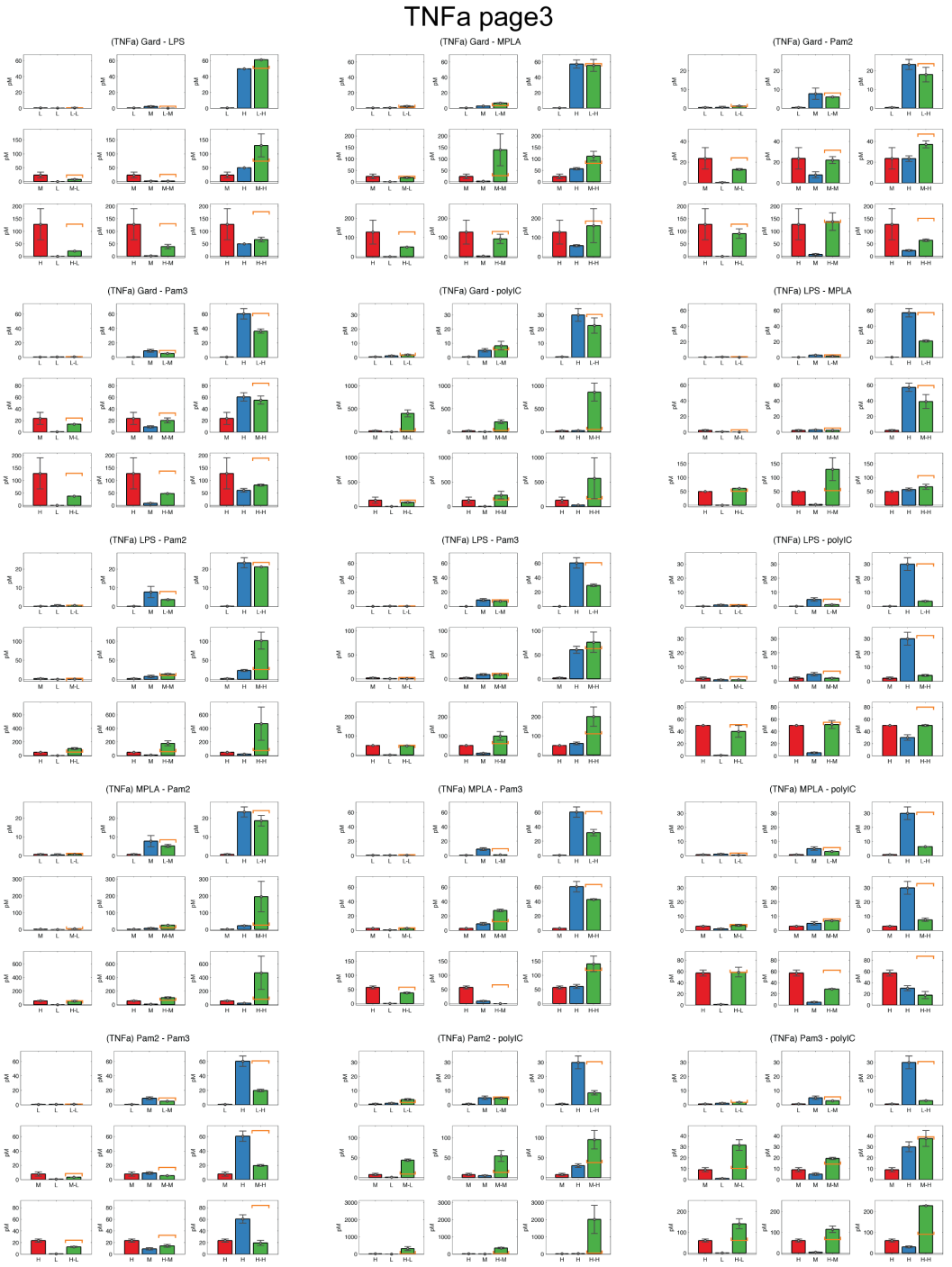

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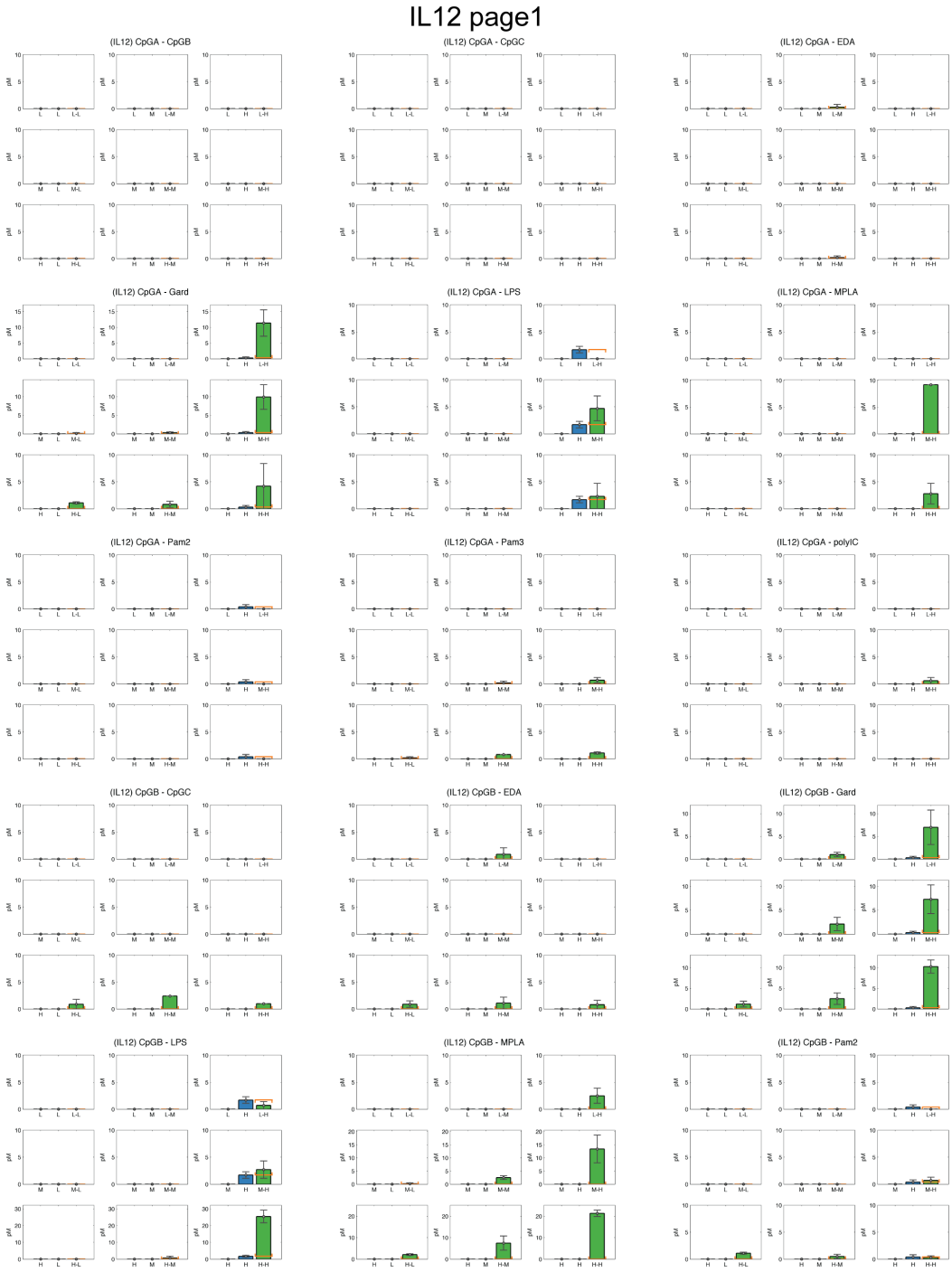

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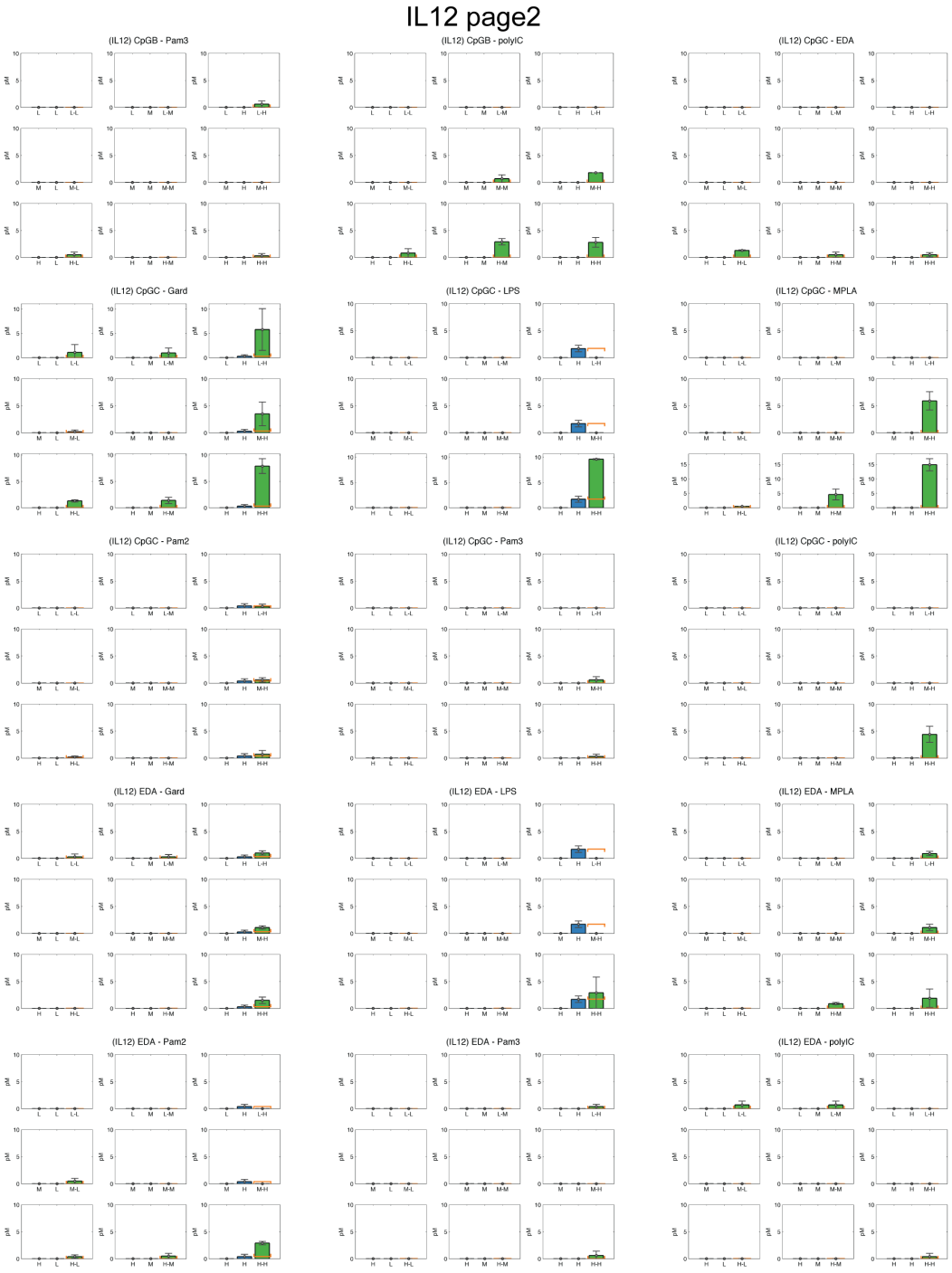

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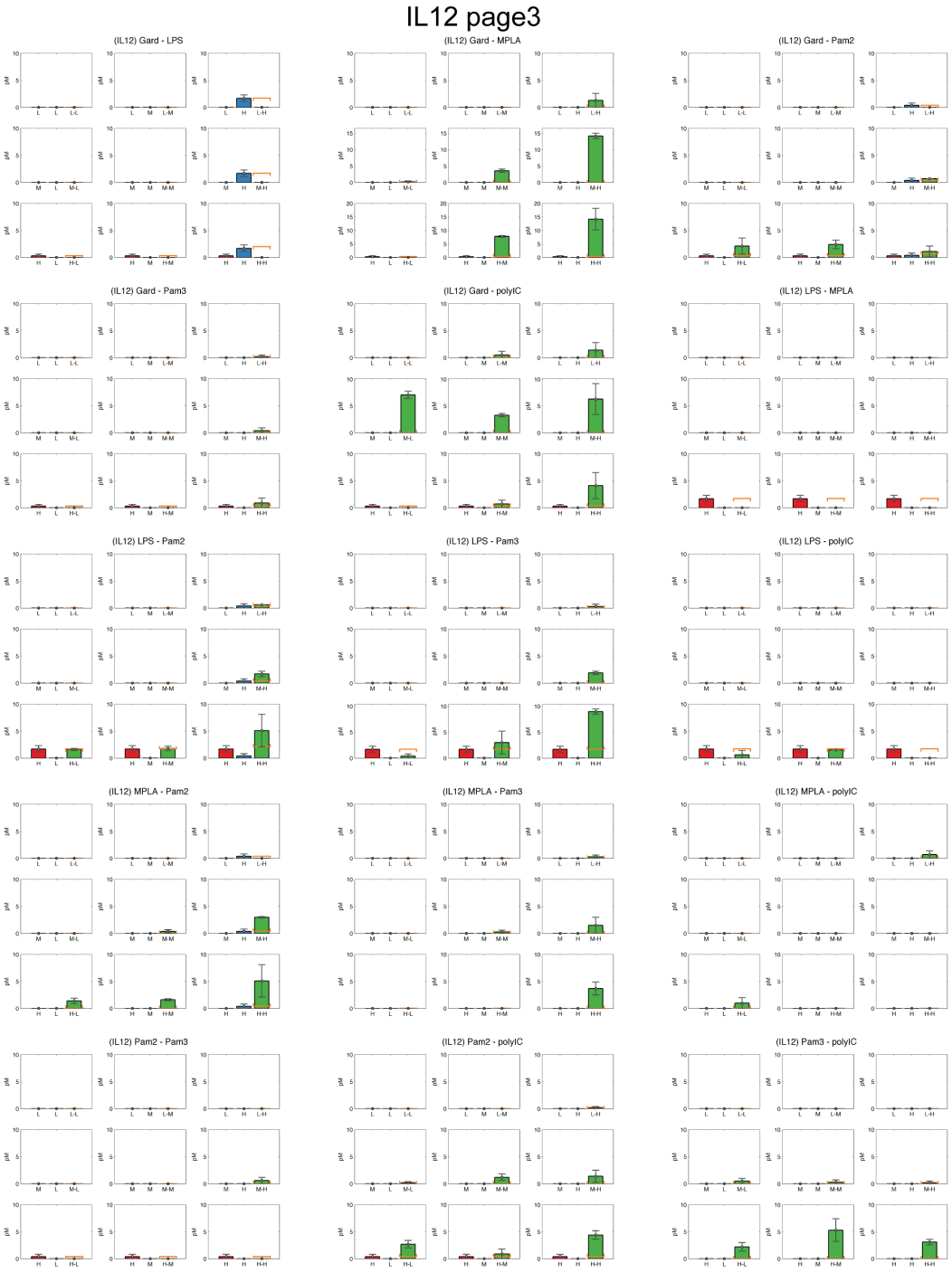

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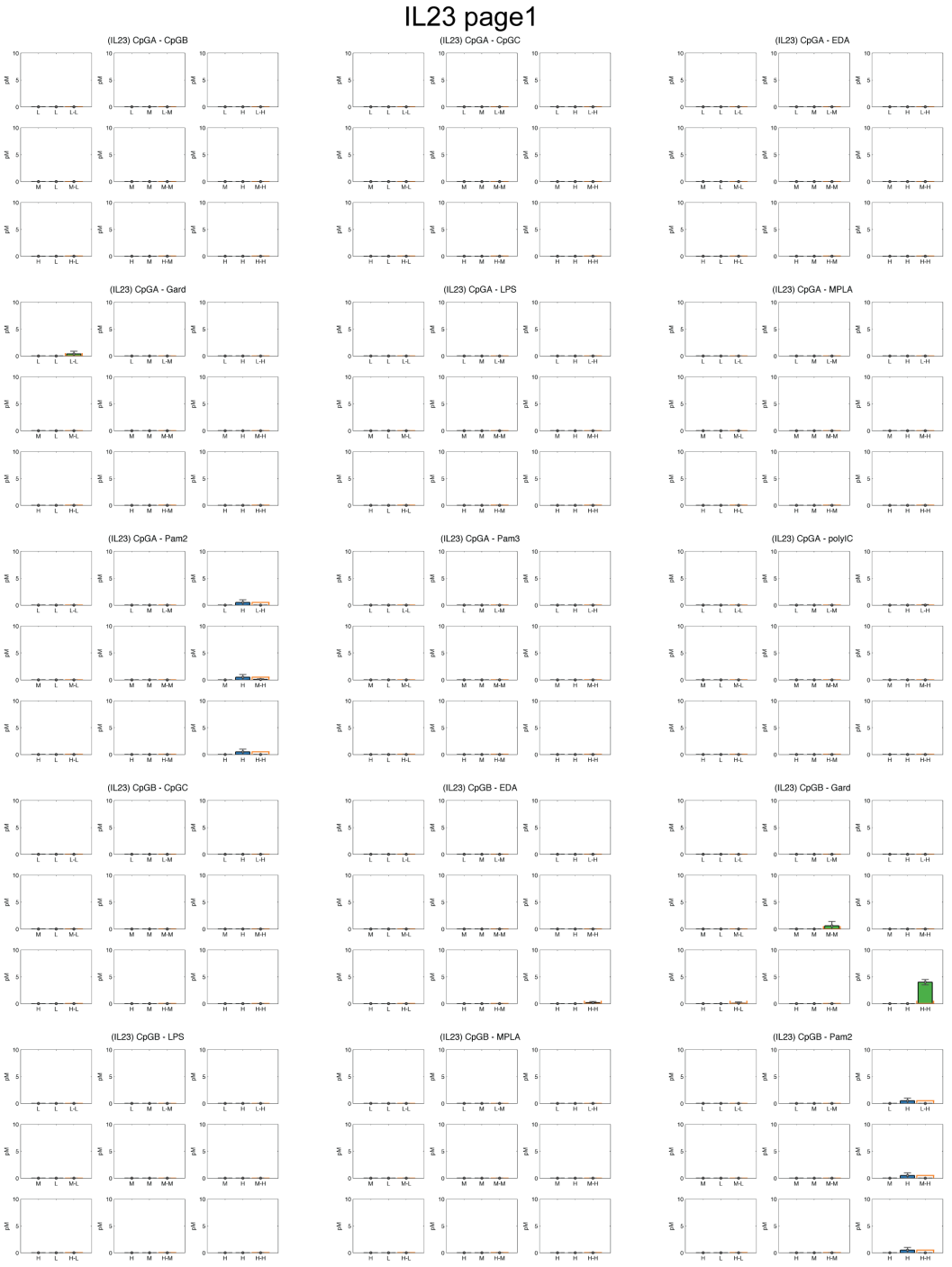

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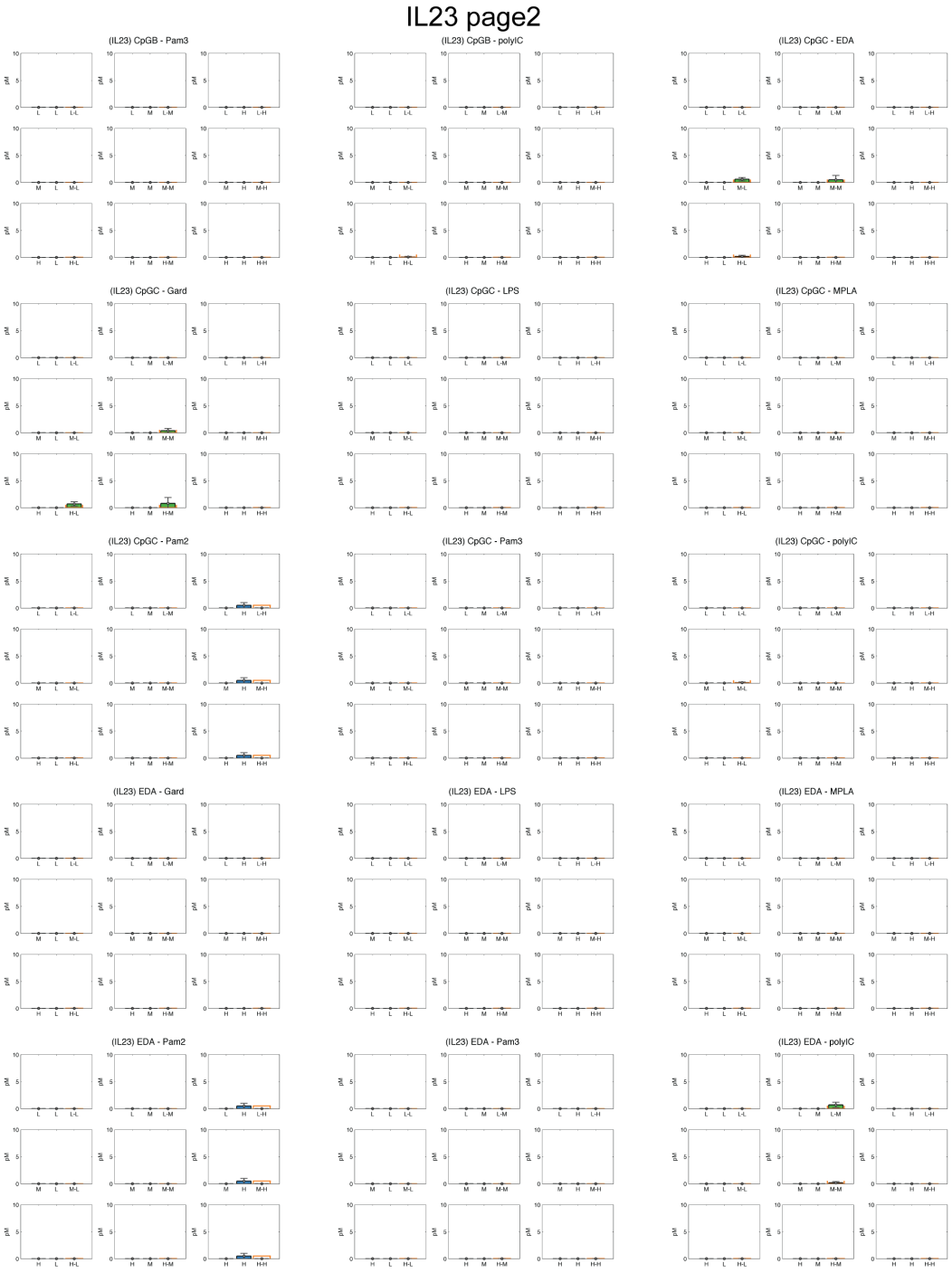

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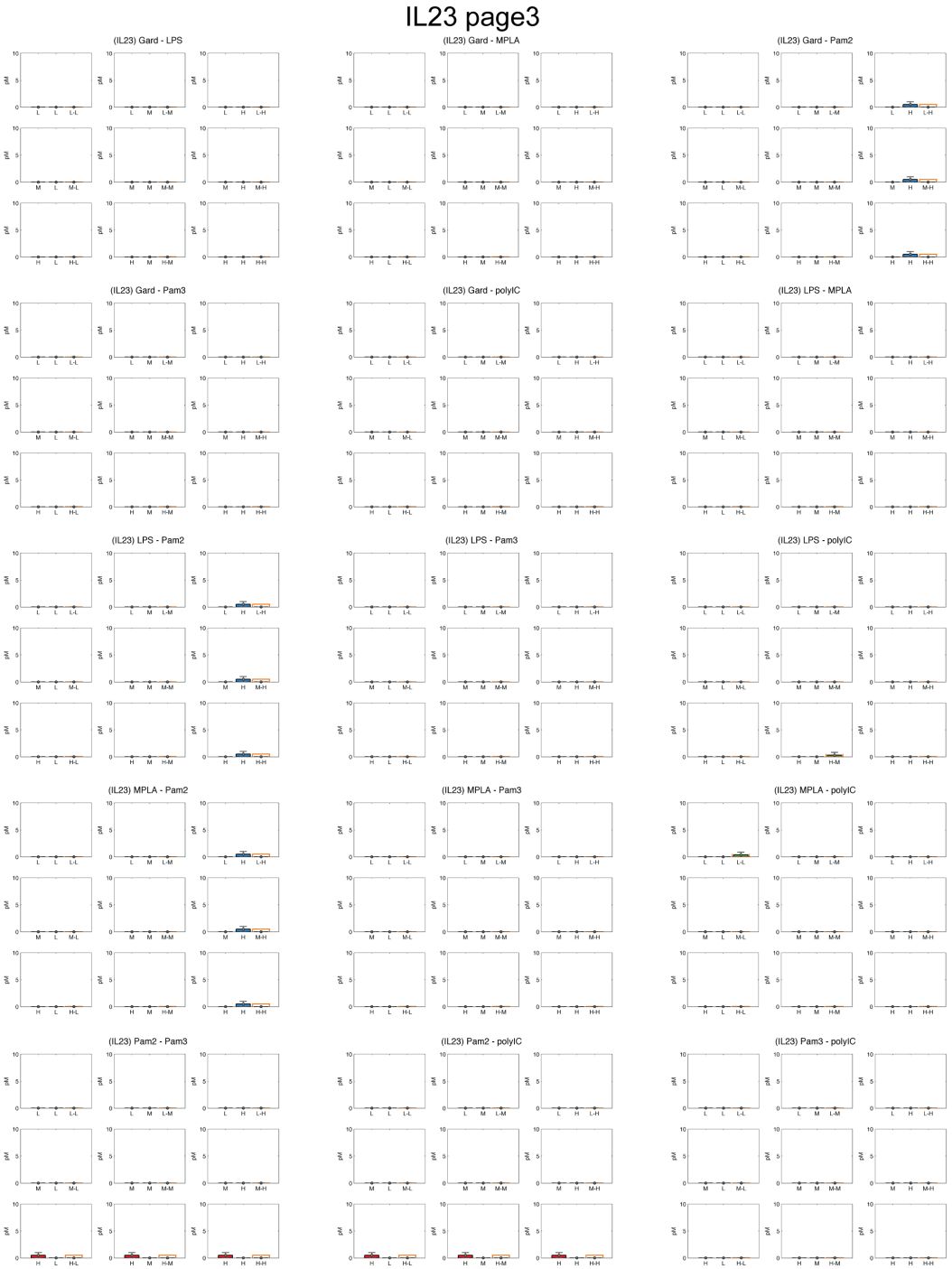

Supplementary Figure 6. Cytokine secretion of the different combinations used in this study.

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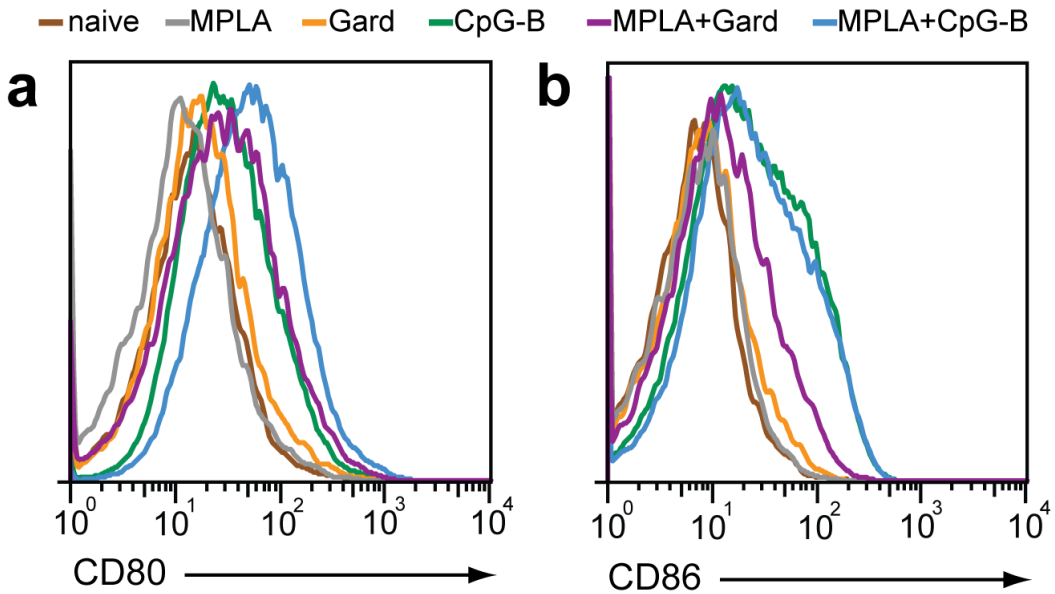

Suppl. Fig. 7. Upregulation of activation markers on lung DCs by agonist combinations. MPLA, Gardiquimod, CpG-B and combinations were administered to mice through the pulmonary route. After 24h, lung leukocytes were isolated and stained for DC surface markers, naïve mice were used as controls. Histograms are gated on autofluorescence^{low}, CD11c^{high}, MHC-II^{high} lung DCs. The expression of CD80 (a) and CD86 (b) of one representative animal per group is shown. Experiment was performed once with n=5 mice in every group except naïve (n=2).

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Supplementary Information

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