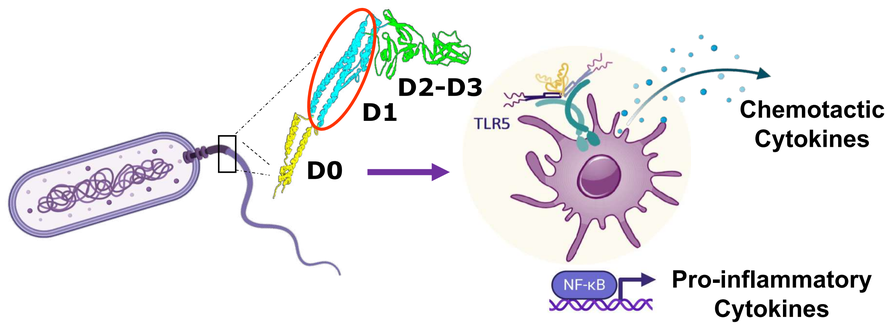
LABORATORIO MEDICINA TRASLACIONAL FALP
Unidad I+I

Recombinant ND1 domain of flagellin from *Vibrio anguillarum* promotes *in vitro* overexpression of proinflammatory cytokines in human immune cells

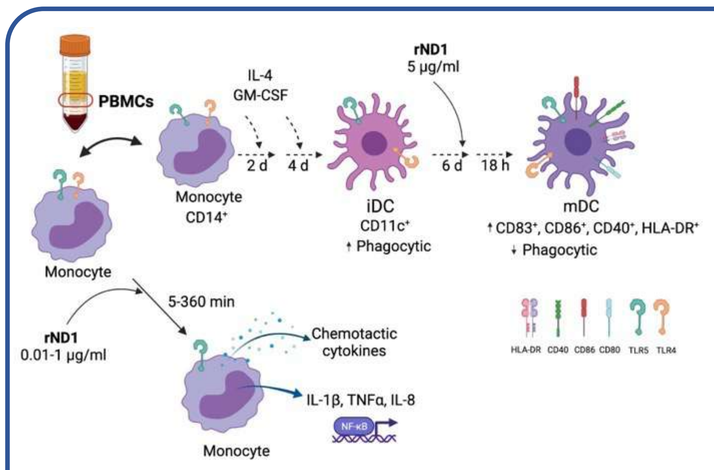
Roxana González-Stegmaier[1], Adam Aguirre[1], Constanza Cárcamo[1], Franz Villarroel-Espíndola[1].
[1]Laboratorio de Medicina Traslacional. Departamento de Investigación del Cáncer. Instituto Oncológico Fundación Arturo López Pérez.
roxana.gonzalez@falp.org

INTRODUCTION



Flagellin is the major component of flagellum in Gram negative and positive bacteria, and is the ligand for Toll-like receptor 5 (TLR5). The activation of TLR5 promotes the expression of proinflammatory cytokines and chemokines. In this work, we evaluated a recombinant peptide from the amino-terminus D1 domain (rND1) of flagellin B from *V. anguillarum*, a fish pathogen, as an immune modulator in mammal cells. This rND1 contains key amino acids needed to bind TLR5, and it has shown IL-8, IL-1β and TNF-α overexpression in lower vertebrates.

EXPERIMENTAL DESIGN



Monocytes and Monocyte-derived Dendritic Cells (MDC) were obtained from human PBMCs using centrifugation in a density gradient, and then used for *in vitro* stimulation.

***Primary cell culture*:** it was carried out at 37°C with 5% CO_2 in RPMI-1640 supplemented with 10% FBS, and antibiotics.
Time course and doses response: Monocytes in monolayer culture were stimulated with peptide between 5 and 360 min with a unique dose or treated with 0.01 to 1 µg/ml of rND1 by 3 hours. Total RNA and supernatant were recovered for each condition for transcriptional and protein analysis, respectively.
MDC: CD14+ cells were isolated and cultured in presence of 800 IU/ml GM-CSF and 500 IU/ml IL-4. Differentiated dendritic cells were treated by 18 h with rND1 and analyzed by flow cytometry.
Gene expression: Real time PCR was performed in QuantStudio 3 instrument (Applied Biosystem) using SYBR Green Reagents. For each mRNA, gene expression was normalized with the GAPDH and β-actin using the comparative Ct method ($2^{-\Delta\Delta Ct}$).
Protein secretion: 29 targets were detected with the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel kit (HCYTOMAG60, Millipore) using a MAGPIX® Luminex platform.
Flow citometry: APC-anti-huCD11c (#559877), FITC-anti-huCD83 (#556910), PE-anti-huCD86 (#555658), V450-anti-huCD40 (#561219), PerCP-Cy5.5-anti-huHLA-DR (#560652) antibodies (BD) were used for the phenotype characterization in a FACS Canto II flow cytometer. 10,000 events were considered for analysis.

RESULTS

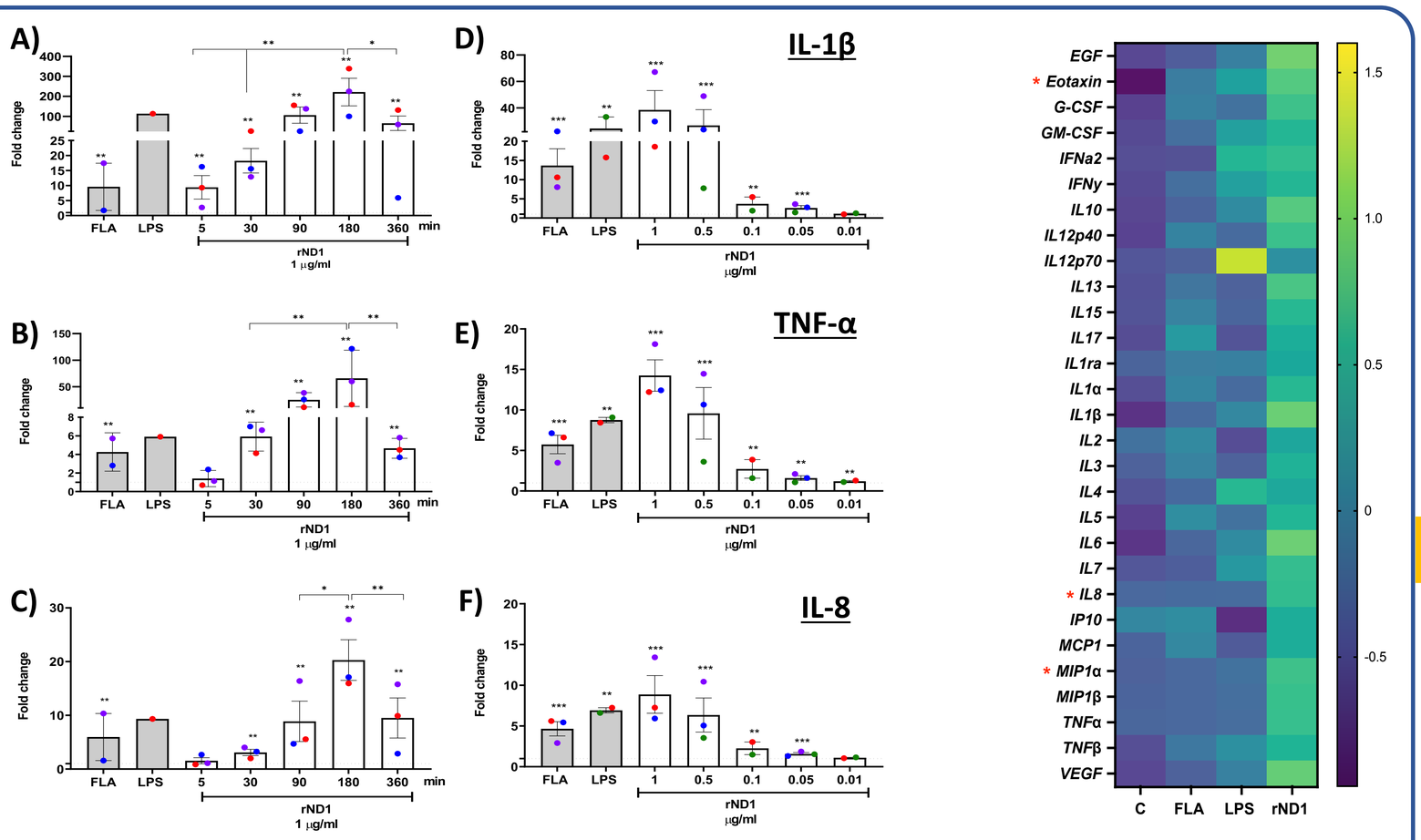


Figure 1: Transcriptional analysis of proinflammatory molecules. Expression levels of IL-1β, TNF-α and IL-8 in human PBMCs under stimulation with 1µg/ml of rND1 during 5 to 360 minutes (A-C) and induction after 3h with different concentrations of rND1 (D-F).
Values of fold change were normalized to the expression of GAPDH and b-actin, and the results are represented as the increase in mRNA for each gene compared to the unstimulated control and presented as the mean ± SEM. Statistical differences using Mann–Whitney test are shown. Color circles represents PBMCs obtained from three independent healthy volunteers in independent experiments. In all cases, the dashed line corresponds to the untreated control.

Figure 2: Induced cytokine profile. Heat map with the differential profile of cytokine and chemokines secreted under stimulation with rND1 (1µg/ml, 3h). Color code represents levels of change folds for each target compared to untreated cells, and normalized using z-score. The results are the mean of four independent experiments. Asterisks indicates significant differences between rND1 and untreated control by an One-way ANOVA and Tukey's multiple comparison test.

For all experiments, a functional control using 50 ng/ml flagellin from *S. typhimurim* (FLA) and 1 µg/ml lipopolysaccharide from *E. coli* K12 strain (LPS) were included to confirm PAMP/TLR activity of human isolated cells.

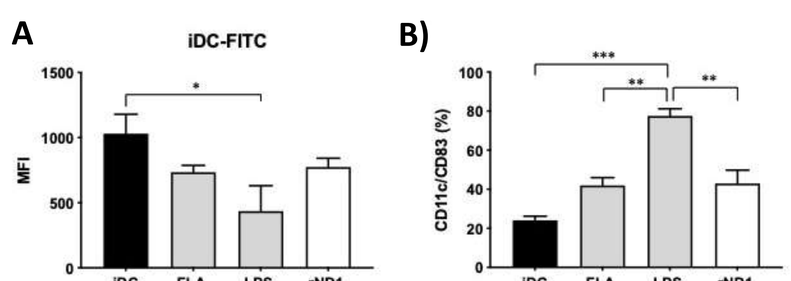


Figure 3: **Effect of rND1 during dendritic cells differentiation.** Measured MFI by FITC-dextran uptake (A) and percentage of expression of CD83 in CD11c positive cells (B) during stimulation with 5 µg/ml of rND1 peptide or 100 ng/ml of full Flagellin from *S. typhimurim*.
The values represent the means of three independent experiments (**p < 0.01, ***p < 0.001). One-way ANOVA and Tukey's multiple comparison test.
As controls, untreated and treated cells with LPS (1 µg/mL) were considered immature (iDC) and mature (mDC) dendritic cells, respectively.

CONCLUSIONS

- The results showed, at transcriptional level, that rND1 induced a time- and concentration-dependent pro-inflammatory response in PBMCs, generating a peak for IL-1β (220-fold), IL-8 (20-fold) and TNF-α (65-fold) at 3h post-stimulation with 1µg/ml.
- The profile of secreted proteins after stimulation of mononuclear cells with rND1 1µg/ml by 3h was concordant with a chemotactic signature and qualitatively different compared to LPS or flagellin.
- The monocyte-derived dendritic cells treated with rND1 showed low levels of co-stimulatory and MHC-II molecules and kept an immature phenotype with a decreased phagocytosis of dextran.

- *Our results suggest that rND1 from a non-human pathogen promotes immune modulation in human cells and it may be considered for further studies in adjuvant therapies based on pathogen-associated molecular patterns.*

Funding: This work was supported by Instituto Oncológico Fundación Arturo López Pérez (FALP-2020-LMT).