Immunomodulation of human macrophages and myeloid cells by 2-substituted (1-3)-β-D-glucan from *P. parvulus* 2.6

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ABSTRACT

β-glucans produced by eukaryotic cells and by microorganisms are known to modulate immune responses by affecting macrophage activation. Here, we have investigated the effect of purified 2-substituted (1-3)-β-D-glucan, produced by either *Pediococcus parvulus* 2.6 or *Lactococcus lactis* NZ9000[pNGTF], on the effector functions of human PMA-differentiated THP-1 cells and M1 pro-inflammatory monocyte-derived macrophages. The results reveal that this kind of β-D-glucan activates macrophages and has an anti-inflammatory effect.

Key words: 2-substituted (1-3)-β-D-glucan, exopolysaccharides, macrophages modulation, lactic acid bacteria.

1. Introduction

In the intestinal wall there is a crosstalk between the epithelial mucosa and the underlying immune system cells. When this is disturbed, the immune cells may release pro-inflammatory cytokines. Chronic gut inflammation, as in Crohn´s disease or ulcerative colitis, is a debilitating condition with a high prevalence in developed countries (Loftus, 2004). Polysaccharides are of interest as regulators of the inflammatory responses in these pathologies (Cho, 2008). For example, β-glucans synthesized by microorganisms and eukaryotic cells have been shown to exert a potent immunomodulatory activity (Brown & Gordon, 2001; Robinson, Hale & Carlin, 2006). The activation of macrophages, dendritic cells and natural killer cells by β-glucans is mediated by specific receptors (Smiderle, Alquini, Tadra-Sfeir, Iacomini, Wichers & Van Griensven, 2013) including dectin 1 (Brown & Gordon, 2001), Toll-like receptors

Abbreviations: EPS, exopolysaccharide; EPS L, 2-substituted (1-3)-β-D-glucan from *L. lactis* NZ9000[pNGTF]; EPS P, 2-substituted (1-3)-β-D-glucan from *P. parvulus* 2.6; LPS, *Escherichia coli* lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PMA-THP-1, differentiated THP-1; PBMC, human peripheral blood mononuclear cell; SEC, size exclusion chromatography; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanil.

TLR-2 and TLR-6, and complement receptor CR3 (Chan, Chan & Sze, 2009). β-glucan recognition by these receptors triggers a variety of intracellular signaling cascades which ultimately modulate the link between the innate and the adaptive immune systems.

The chemical structure of β-glucans consists of a linear backbone composed of glucose residues linked by (1-3) bonds (in yeast, oat, and bacteria) or (1-6) bonds (in various fungi), with branching located at position 6 (1-6) or 4 (1-4) of the main chain (Wasser, 2002). The different β-glucan linkages significantly affect solubility, mode of action and biological activity. Some linear and branched β-glucans have been characterized as immunomodulators (Chen & Seviour, 2007). The role of 2-substituted (1-3)-β-D-glucan has only been investigated recently. These polymers are bacterial exopolysaccharides (EPS) synthesized by GTF glycosyltransferase (Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008) and we have previously shown that EPS-producing *Pediococcus* and *Lactobacillus* strains modulate monocyte-derived macrophage effector functions (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010). However, these studies did not prove that these modulations were specifically due to their EPS.

Thus, in the present study we have performed a comparative analysis of the effect of purified 2-substituted (1-3)-β-D-glucan derived from strains expressing the pediococcal GTF glycoyltransferase, the parental strain *P. parvulus* 2.6 (Dueñas-Chasco et al., 1997) and the recombinant strain *L. lactis* NZ9000[pNGTF] (Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008) on human macrophages. To further validate the analysis, two kind of macrophages were used: pro-inflammatory M1 macrophages generated from human peripheral blood mononuclear cells using granulocyte-macrophage colony-stimulating factor (de las Casas-Engel et al., 2013) and THP-1 cell line differentiated to macrophages by phorbol 12-myristate 13-acetate

(PMA) treatment (Auwerx, 1991).

2. Materials and Methods

2.1 Production and purification of EPS

Two strains were used, both producing the 2-substituted (1-3)-β-D-glucan. *P. parvulus* 2.6 isolated from cider (Dueñas-Chasco et al., 1997), and *L. lactis* NZ9000[pNGTF] recombinant strain, which carries in the pNGTF plasmid, based on the pNZ8040 vector, the *P. parvulus* 2.6 *gft* gene under control of the PnisA promoter (Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008). EPS were produced, purified and analyzed as previously described (Notararigo et al., 2013). Briefly, the polymers were obtained from the supernatants of *P. parvulus* (EPS P), and of *L. lactis* (EPS L) cultures by precipitation with ethanol and then purified by size exclusion chromatography (SEC) and dialysis. Contamination by DNA, RNA, and proteins was measured with the Qubit® 2.0 fluorometer (Invitrogen life-technologies). This technique allows the detection of more than 0.5 μg mL^{-1} of DNA, 20 ng mL^{-1} of RNA, and 1 μg mL^{-1} of proteins.

2.2 Cell cultures

THP-1 cell line was cultured in RPMI 1640 (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), 100 U mL^{-1} penicillin (Gibco) and 100 μg mL^{-1} streptomycin (Gibco), at 37°C under humidified 5% CO_2. Differentiated THP-1 (PMA-THP-1) were generated by treatment of THP-1 with 40 nM phorbol 12-myristate 13-acetate PMA (Sigma-Aldrich) for 72 h in the above RPMI 1640 supplemented medium, cells were seeded in a 24 well plate (Falcon) at a concentration of 5×10^5 cells per well. The treatment led to high morphological similarities to macrophages and cells become

adherent to the culture surface due to the reorganization of the receptors of the outer membrane (Auwerx, 1991). After differentiation, non-attached THP-1 cells were removed by aspiration and the adhered macrophages were washed twice with RPMI 1640 medium.

Monocyte-derived M1 macrophages were obtained as follows. Human Peripheral Blood Mononuclear Cell (PBMC) were isolated from buffy coats from normal donors over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMC by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytes (>95% $CD14^+$ cells) were cultured at 0.5×10^6 cells mL^{-1} for 7 days in RPMI 1640 supplemented with 10% Fetal calf serum medium (Sigma-Aldrich, USA) at 37°C in a humidified atmosphere with 5% CO_2, and containing granulocyte macrophages colony-stimulating factor 1000 U mL^{-1} (ImmunoTools, Friesoythe, Germany) to generate M1 monocyte-derived macrophages. Cytokines were added every 2 days (de las Casas-Engel et al., 2013).

2.3 XTT test

M1 and PMA-THP-1 were analyzed using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanil (XTT) kit (Hoffman-La Roche). Both human cell types were seeded at a concentration of $1x10^5$ per well in a 96 wells plate, and treated for 24 h with either control medium Krebs (Sigma-Aldrich) or Krebs supplemented with different concentrations of EPS L and EPS P. Relative cell viability was determined spectrophotometrically by quantification of the conversion of XTT to formazan, after exposure of the samples to XTT for 3 h (M1) or 4 h (PMA-THP-1).

2.4. Signalling pathway assays

M1 macrophages were exposed for 10 minutes to *Escherichia coli* lipopolysaccharide (LPS, Sigma) (10 ng mL^{-1}), EPS L (100 μg mL^{-1}) or EPS P (100 μg mL^{-1}). Macrophages were then immediately lysed and protein phosphorylation of 46 kinases analyzed using the Proteome profiler® protein array (R&D Systems, Inc, USA), which detects the phosphorylation state of the serine/threonine/tyrosine kinases according to the specifications of the manufacturers. Membranes were subjected to densitometry and the intensity of each spot (in arbitrary pixel units, a.u.) were normalized using the signal yielded by internal positive controls.

2.5. ELISA test

M1 and PMA-THP-1 were treated 24 h with LPS (10 ng mL^{-1}), EPS L (100 μg mL^{-1}) or EPS P (100 μg mL^{-1}) and culture supernatants were tested for the presence of cytokines and growth factors using commercially available ELISA for TNF-α, IL-10, and IL-12p40 (OptEIA IL-12p40 set; BD Pharmingen, San Diego, CA) as indicated by the manufacturers.

2.6. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, Inc, La Jolla, CA USA). Statistical analysis subdued data to D'AgosBno & Pearson normality test, furthermore significance of parametric values was obtained with the Student's t-test; while for no parametric values was performed with Wilcoxon test.

3. Results and discussion

For testing the functional effects of 2-substituted (1-3)-β-D-glucan synthesized by the pediococcal GTF glycosyltransferase, EPS were purified from culture supernatants of *P.*

parvulus 2.6 and *L. lactis* NZ9000[pNGTF]. Analysis of EPS preparations by SEC, infrared spectroscopy and methylation, as well as monosaccharide composition, revealed that both purified EPS were 2-substituted (1-3)-β-D-glucan with an average molecular mass of 9.6 x 10^6 Da and 6.6 x 10^6 Da for EPS P and EPS L, respectively (results not shown). Moreover, analysis of EPS solutions at 1 mg mL^{-1} for purity with the fluorescent Qubit® assays did not reveal (<0.1%) the presence of DNA, RNA or proteins.

The EPS were tested by using human pro-inflammatory M1 macrophages derived from buffy coat from normal donors and the PMA-THP-1 cell line. Differentiated THP-1 macrophage-like cells were also used for testing the functional effects of EPS because they exhibit macrophage characteristics *in vitro* (Kohro et al., 2004; Schwende, Fitzke, Ambs & Dieter, 1996; Tsuchiya et al., 1982). Neither of the two EPS had a deleterious effect on cell viability for either type of macrophage, when their influence was analyzed by the XTT assay (Figure 1). In this regard, it is important to emphasize that M1 macrophages and PMA-THP-1 cells lack the ability to proliferate (Kohro et al., 2004). Interestingly, the cell respiration rate of M1 macrophages directly correlated with the concentration of tested EPS, since high concentration of EPS P (50-100 μg mL^{-1}) resulted in a significant increase of the metabolic rate of cells ($P < 0.0005$). This effect was also observed with EPS L at 100 μg mL^{-1} ($P < 0.05$). These results showed a beneficial influence of both EPS in M1 macrophage metabolism though the reason why EPS L is less efficient remains unknown; it could be related to its lower molecular mass. However, no influence of EPS P or EPS L on PMA-THP-1 cells was detected.

To verify the hypothesis that M1 macrophages had been activated by the 2-substituted (1-3)-β-D-glucans, the activation/phosphorylation of several kinases involved in intracellular signaling was explored. The results, indicated that both EPS promote

numerous changes in the phosphorylation state of proteins involved in signaling (Figure 2). In particular, the phosphorylation levels of Fyn, FAK, AKT(T308) and p70S6K were much higher than in untreated cells. The phosphorylation levels of ERK1/2, MSK1/2, AKT (S473) JNKpan and p38 were found to be higher in LPS- than in EPS-treated cells, implying that LPS and EPS trigger different intracellular signals, and confirming that the EPS preparations were devoid of LPS contamination. In fact, no influence of either EPS L or EPS P on the p38 phosphorylation was detected. Therefore, EPS is a weak inducer of JNK and p38 phosphorylation. Since the activation of these two kinase pathways correlates with the acquisition of M1-associated effector functions (Han et al., 2013), our results further support the idea that EPS does not promote the production of pro-inflammatory activities.

To understand these differences between LPS and EPS stimuli, the influence of the 2-substituted (1→3)-β-D-glucan on macrophage cytokine production (TNF-α, IL-10 and IL-12p40) was investigated. LPS was capable of inducing the production of the three cytokines by either M1 macrophages or PMA-THP-1 cells (Armstrong, Jordan & Millar, 1996; Saraiva & O'Garra, 2010) (Figure 3). Regarding EPS, both EPS L and EPS P also significantly triggered the release of the three cytokines by M1 macrophages and at higher levels than the LPS (Figure 3a). However, comparison of the ratio TNF-α/IL-10 between LPS (107) and the EPS: EPS L (17) and EPS P (10) revealed a more pronounced induction of the TNF-α pro-inflammatory cytokine by LPS. Moreover, both EPS exclusively augmented the production of IL-10 by PMA-THP-1 cells, without significantly affecting the production of either TNF-α or IL-12p40 (Figure 3b). These results are in agreement with the results of the cell respiration test (Figure 1), and suggest that EPS preferentially promotes the production of anti-inflammatory cytokines such as IL-10. Previous analysis of the immunomodulatory effect of *P. parvulus* EPS-

producer (2.6R) and non-producer (2.6NR) isogenic strains indicated that the presence of the biopolymer counteracts the pro-inflammatory activation of M1 macrophages in response to the bacterium. (Fernández de Palencia et al., 2009) Thus, the results obtained in this work, showing that in the absence of the producer strain the 2-substituted (1→3)-β-D-glucan provokes immunomodulation, provides a new role for the EPS. Finally, comparison of the results obtained with M1 macrophages and PMA-THP-1 cells (Figs. 1 and 3) indicates that to detect the influence of the biopolymer in the latter it is necessary to expose the cells simultaneously to an inflammatory agent such as LPS. Further experiments are required to validate this hypothesis.

4. Conclusions

We have demonstrated that the 2-substituted (1-3)-β-D-glucan isolated from the original and the recombinant strains do not have any cytotoxic effect on human macrophages, rather they have an activation effect on the rate of metabolism, which results in the triggering/mobilization of the signal transduction pathway of Fyn, FAK, AKT(T308) and p70S6K. This differed from the results obtained with LPS. Similarly in the induction of the cytokines, TNF-α, IL-10 and IL-12p40, the effect of the β-glucan, differed from that of the positive control (LPS). The possibility that the EPS activates macrophages by way of TLR-4 has to be discarded as EPS did not stimulate the production of p38.

According to the results obtained in this work with the purified EPS, 2-substituted (1-3)-β-D-glucan continues to be an interesting candidate as a modulator of the immune system and its recombinant origin does not interfere with its immunomodulating properties. Consequently, and in line with previous reports on the probiotic ability of DC-SIGN-binding sugars (Konieczna et al., 2012; Konstantinov et al., 2008; Smits et

al., 2005), further studies should be performed to evaluate *ex vivo* and *in vivo* the potential of this biopolymer as co-adjuvant therapy for chronic inflammatory diseases such as Crohn's and inflammatory bowel diseases.

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Legend to the Figures

Figure 1. Cytotoxic effect of EPS L and EPS P on M1 and PMA-THP-1 macrophages. Cells were treated either with EPS L or EPS P at the concentrations indicated in the figure. Data are expressed as mean with standard deviation, median interquartile range (IRQ) and whisker plots (minimum and maximum). Results are presented as a percentage of untreated control samples. *P < 0.05 EPS L vs untreated cells; *P < 0.0005 EPS P vs untreated cells.

Figure 2. Screening of intracellular signaling triggered by LPS or EPS in M1 macrophages. M1 macrophages were untreated (CNT) or exposed to LPS, EPS L or EPS P. After lysis, a human phospho-kinase array kit was used to detect the relative levels of kinase phosphorylation. Membrane hybridization (upper panel) and folds of induction of sample vs CNT (lower panel) are depicted.

Figure 3. Comparative analysis of cytokines produced by M1 and PMA-THP-1 macrophages. Cells were treated either with LPS, EPS L or EPS P**.** Data are represented as box (median and IQR) and whisker plots. The statistical analysis comprehended the comparison of treated vs untreated cells (*). **a)** In M1 macrophages, variations of TNF-α, IL-10 and IL-12p40 were statistically significant for LPS (*P: < 0.0001, = 0.0009 and < 0.05) as well as for EPS L and EPS P (*P: <0.0009, < 0.0001 and < 0.05) **b)** In PMA-THP-1 there were statistically significant variations of TNF-α, IL-10 and IL-12p40 for LPS (*P: < 0.0001, = 0.0078 and < 0.0003) and of TNF-α for EPS L and EPS P (*P < 0.0001).

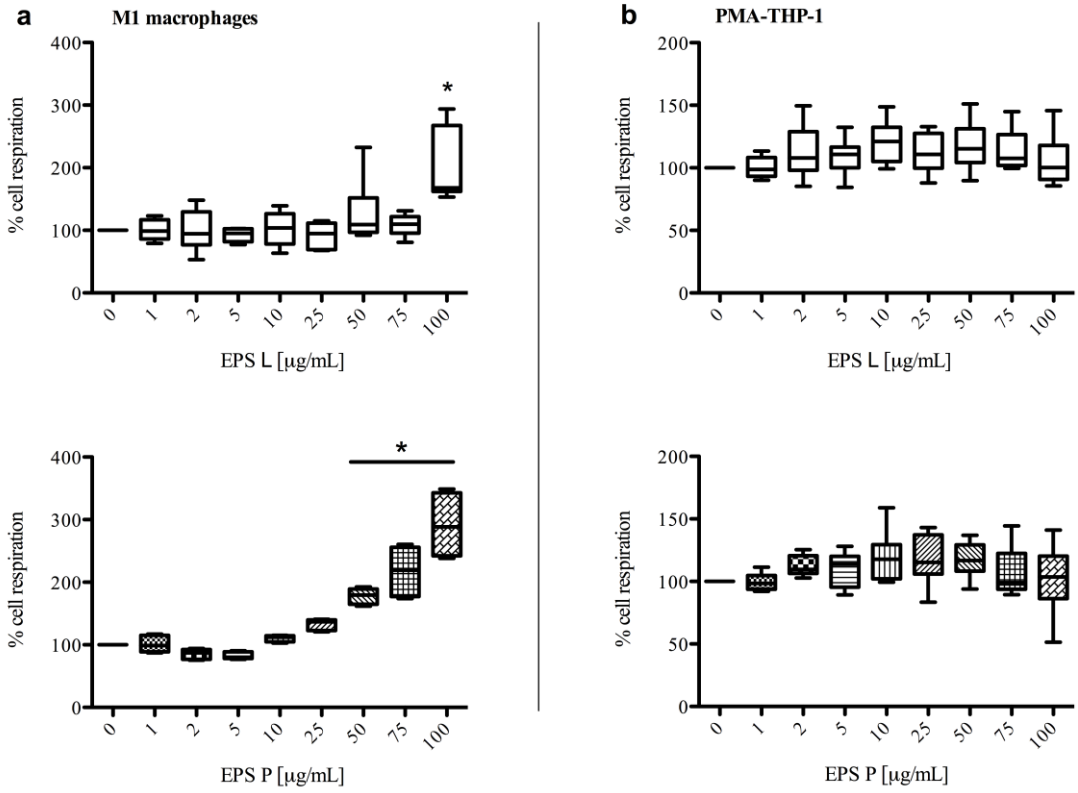
a
M1 macrophages
b
PMA-THP-1
% cell respiration
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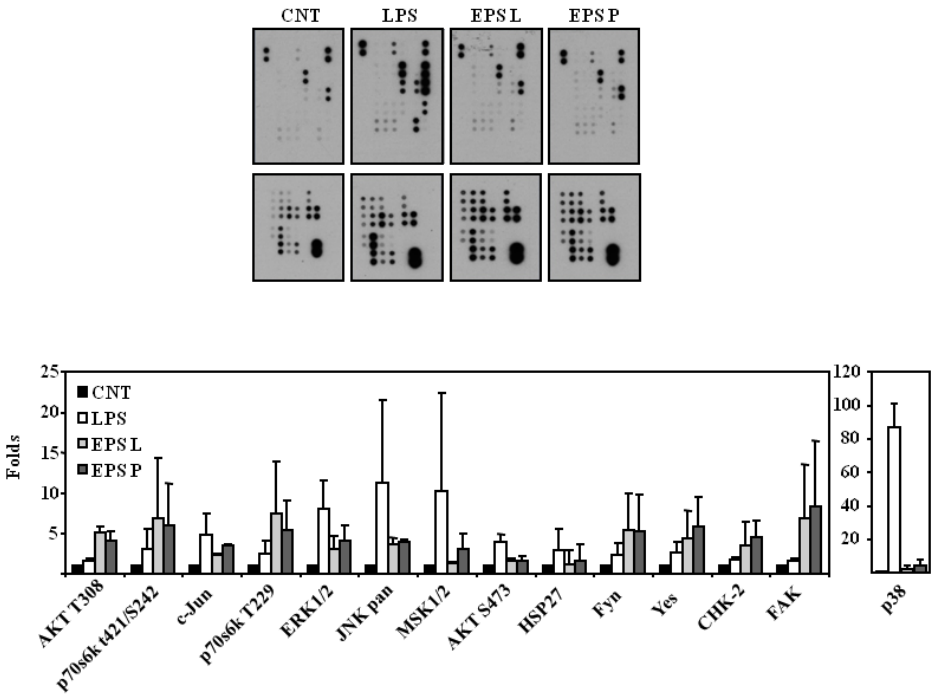
Figure 1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Fyn
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FAK
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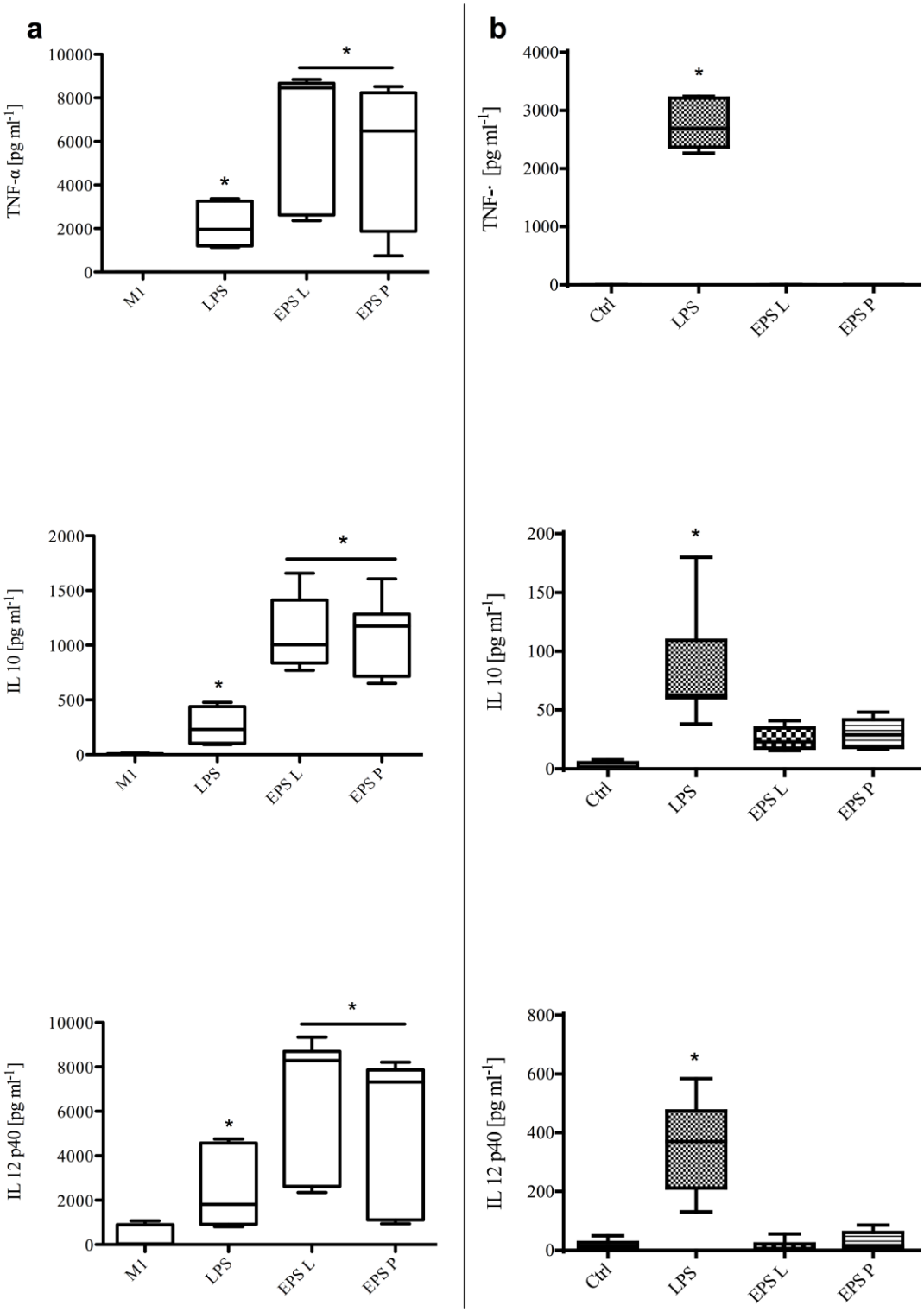
Figure 2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IL 10 [pg ml-1]
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Figure 3