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Role of α-glucan-induced oxygen species in dendritic cells and its impact in immune response against tuberculosis

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ABSTRACT

With 10 million new cases and three million deaths estimated to occur yearly– more than any time in history– tuberculosis (TB) remains the single most widespread and deadly infectious disease. Until recently, it was thought that both latent and active TB was primarily related to host factors. Nonetheless, the participation of bacterial factors is becoming increasingly evident. Minimal variations in genes related to *Mycobacterium tuberculosis (Mtb)* virulence and pathogenesis can lead to marked differences in immunogenicity. Dendritic cells (DC) are professional antigen presenting cells whose maturation can vary depending on the cell wall composition of each particular *Mtb* strain being critical for the onset of the immune response against *Mtb*. Here we evaluated the role played by α-glucan, in the endogenous production of reactive oxygen species, ROS, and the impact on DC maturation and function. Results showed that α-glucans on Mtb induce ROS production leading to DC maturation and lymphocyte proliferation. Even more, α-glucans induced Syk activation but were not essential in non-opsonized phagocytosis. In summary, α-glucans of Mtb participates in ROS production and the subsequent DC maturation and antigen presentation, suggesting a relevant role of α-glucans for the onset of the protective immune response against TB.

1. Introduction

Tuberculosis (TB) remains a major global health problem caused by inhalation of aerosols containing *Mycobacterium tuberculosis (Mtb)*. Ten million new TB cases and 3 million deaths are estimated to occur each year, more than any time in history, thus, approximately one quarter of the world's population are already infected with *Mtb*. The risk of disease is also increased by emergence of acquired immune deficiency syndrome and multidrug-resistant (MDR) *Mtb* strains (WHO, 2018). There is long-standing evidence that some *Mtb* strains are more virulent than others and vary in their ability to cause disease in humans (Reed et al., 2007).

Host-pathogen interactions are determined by cell envelope of pathogenic mycobacteria (Daffé and Draper, 1998). The *Mtb* envelope is composed of a typical plasma membrane, delimited by an outer membrane full of carbohydrates and lipids (Hoffmann et al., 2008) surrounded by an outer layer difficult for the host to degrade. Pathogenic mycobacterial species display a capsule composed of carbohydrates and proteins in the outermost part of the cell envelope, where 80% consist in a high-molecular weight (> 100 000 Da) α-glucan composed of a →4-α-D-Glc-1→ core branched at position 6 every five or six residues by →4-α-D-Glc-1→ oligoglucosides (Lemassu and Daffé, 1994; Ortalo-Magné et al., 1995; Dinadayala et al., 2004). The *Mtb* capsular carbohydrates are absent in some other taxa and mediate specific interactions with the host (Glickman and Jacobs, 2001). It has been demonstrated that α-glucans mediate the non-opsonic binding of *Mtb* to CR3 (CD11b) in (CR3)-transfected Chinese hamster ovary cells (Cywes et al., 1997) and also to CD11b in human neutrophils (Romero et al., 2014) without inducing ROS production. However ROS is induced through Dectin-1 involving the activation of spleen tyrosine

Abbreviation: BCG, Bacillus Calmentte y Guérin; MHC, Major Histocompatibility Complex; APC, antigen presenting cells; CFSE, Carboxyfluorescein Succinimidyl Ester; CPM, counts per minute; LPS, lipopolysaccharide; DC, dendritic cells; DHR, dihydrorhodamine 123; SE, standard error; FITC, fluorescein isothiocyanate; GM-SCF, granulocyte-macrophage colony-stimulating factor; IL, interleuquina; MFI, mean fluorescence intensity; IFN_{γ}, interferon gamma; MAPK, mitogen-activated protein kinases; Mtb, mycobacterium tuberculosis; NADPH, nicotinamide adenine dinucleotide phosphate; OMS, Organización Mundial de la Salud; PBMC, peripheral blood mononuclear cells; PPD, purified protein derivative; Syk, spleen tyrosine kinase; TB, tuberculosis; TLR, toll-like receptors; TNFα, tumor necrosis factor alpha; ROS, reactive oxygen species; PBS, phosphate-buffered saline; FCS, fetal calf serum

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kinase (Syk) (Gagliardi et al., 2007). It is evident that α-glucan accomplishes an important biological function, as it interacts with human dendritic cells (DCs) through DC-specific intercellular-adhesion-molecule-3-grabbing nonintegrin (DC-SIGN) modulating some effector functions of DCs. In this context it has been described that mycobacterial α-glucan blocked CD1 expression and suppressed IL-12 production in monocyte derived DCs (Gagliardi et al., 2007). However the mechanisms are still unknown since DC-SIGN is not expressed in monocytes and α-glucans do not bind TLR (Geurtsen et al., 2009). Finally, α-glucans were described to be the component on *M. bovis* BCG with immunotherapeutic effect against bladder cancer (Zlotta et al., 2000).

DCs are professional antigen-presenting cells (APC) that exhibit the ability to activate naïve lymphocytes against microbial antigens, resulting in the initiation of protective immune response, so that activated CD4+ and CD8+ T-cells migrate back to the lungs to further alveolar macrophages activation (Banchereau and Steinman, 1998). DCs may be differentiated in vitro from CD14+ monocytes (Mo) cultured in the presence of granulocyte macrophage colony-stimulating factor and interleukin-4 (IL-4). These DCs are immature, until activation with lipopolysaccharide (LPS), cytokines (Sallusto et al., 2000) or *Mtb* (Alemán et al., 2007). Once activated, DCs up-regulate the expression of co-stimulatory molecules (CD86, CD80) and maturation markers (CD83, major histocompatibility complex class II) as well as the production of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), IL-12, IL-1β e IL-6, acquiring T-cell stimulatory capacity (Hickman et al., 2002; Fricke et al., 2006). DC maturation is critical for the onset of the protective immune response against TB disease and may vary depending on the nature of the cell wall of *Mtb* strain.

It has been demonstrated that DC maturation could also be affected by oxidative stress (Vulcano et al., 2004). It is now known that relatively low concentrations of ROS serve as second messengers during cellular responses to a variety of physiological stimuli (Reth, 2002). In this regard, in chronic granulomatous disease patients who are incapable of producing ROS, TB progression is much more severe (Lee et al., 2008).

Many pathogens have evolved mechanisms to avoid being eliminated, and such mechanisms have allowed *Mtb* to become a very successful pathogen (Goldberg et al., 2014). In this way, it was suggested that certain strains of *Mtb* can spread more effectively and cause disease more frequently than others (Fratti et al., 2003), resulting in different profiles in the immune response (Velasco-Velázquez et al., 2003 Portevin et al., 2011). For instance, *Mtb* and BCG differ in their capacity to induce DC maturation in vitro (Mazurek et al., 2012), which might depend on the different nature of cell wall associated molecules produced by the divergent mycobacterial strains. In a previous work, we described an attractive role played by ROS on DC maturation. Actually, the differences in the ability to induce DC maturation and lymphocyte proliferation between different *Mtb* strains, was related to a variance in ROS production, contributing to immune evasion (Romero et al., 2016). In this context, we have demonstrated that in neutrophils, ROS are generated by non-opsonized mycobacteria through α-glucans (Romero et al., 2014). Here we evaluated the role of α-glucans in the endogenous production of ROS and their impact on the maturation of DC and *Mtb*-specific lymphocytes expansion. Here we describe that lack of α-glucans leads to the loss of *Mtb* ability to induce ROS as well as DC maturation and antigen presentation. Thus, because the critical role of DC maturation and function in TB disease, we consider Mtb α-glucans as essential for the onset of the protective immune response.

2. Methods

2.1. Ethics compliance

All procedures were performed in accordance with the World Medical Association Declaration of Helsinki. The relevant institutional committee (Ethics Committee of the National Academy of Medicine in Buenos Aires) approved our research and all healthy volunteers signed a written informed consent.

2.2. Antibodies and reagents

Oxidase inhibitor, diphenyleneiodonium (DPI) was provided by Cayman Chemical (Michigan, USA), the Syk inhibitor Piceatannol were purchased from Calbiochem-Behring (La Jolla, CA, USA). Annexin V-FITC, cytochalasin D, orthovanadate, LPS and α-amyloglucosidase (A3514, EC 3.2.1.3) were purchased from (Sigma Chemical Co., St. Louis, Mo, USA) and dihydrorhodamine 123 (DHR) was purchased from (Invitrogen, Massachusetts, USA). Granulocyte macrophage colony-stimulating factor GM-CSF and rIL-2 were purchased from Peprotech Inc (Rocky Hill, NJ USA) and IL-4 from R&D Systems (Minneapolis, MN, USA). Rabbit antihuman p-(Tyr525/526)-Syk was purchased by Cell Signaling Technology (Cell Signaling Technology, Inc., Danvers, MA), donkey anti-mouse-IgG-FITC, mouse anti-human-dectin-1 (clone 259931), mouse anti-human-DC-SIGN (clone 120507), CD86-PeCy5 (clone IT2.2) and HLA-DR-PE conjugated antibodies were purchased by R&D Systems.

2.3. Amyloglucosidase digestion of the α-glucan on Mtb capsaule

The γ-irradiated *Mtb* H37Rv strain was kindly provided by J. Belisle (Colorado State University, Fort Collins, CO, USA). *Mtb* was treated with the enzyme α-amyloglucosidase to remove α-glucans from the capsule. Briefly, the bacteria were centrifuged at 100 *g* and suspended in 1 ml of α-amyloglucosidase (500 U/ml) prepared in sodium acetate buffer 0.05 M and 0.1 M NaCl, pH 4.5. As a control, the bacteria were suspended in enzyme-free buffer. The bacteria suspensions were incubated at 37 °C for 18 h and then centrifuged at 100 *g*. The supernatants were discarded, and the pellets were washed with PBS. Thereafter, the bacteria (*Mtbe*) were suspended in 1 ml of PBS and stored at −20 °C until their use.

2.4. Dendritic cell differentiation and maturation

Mononuclear cells from peripheral blood samples were isolated by Ficoll-Hypaque gradient and seeded in plates (Corning, NY, USA) for 2 h in RPMI-1640 and 2% fetal calf serum (FCS) (Gibco Lab., NY, USA) for adherence. Non-adherent cells were removed and maintained in culture with suboptimal dose of rIL-2 for 6 days. Adherent monocytes were cultured with of IL-4 (20 ng/ml) and GM-CSF (50 ng/ml) for 6 days in RPMI-1640 supplemented with penicillin–streptomycin and 10% FCS (complete medium, CM) at 37 °C in 5% CO_2. Thereafter, DC were analyzed for the expression of surface markers associated with DC differentiation, being up to 80–90% $CD1a^+$ and 95% $CD14^-$.

DC maturation was achieved by treatment with LPS (100 ng/ml) or H37Rv (2:1 *Mtb*:DC ratio) for 24 h at 37 °C. When indicated, DCs were incubated in the presence of 10 μg/ml blocking antibodies against dectin-1, DC-SIGN or IgG1 isotype control Ab (R&D Systems Inc. Minneapolis, MN, USA). As control, the oxidase inhibitors DPI (10 μM), or inhibitor of Syk piceatannol (15 mM) were added to DC culture 30 min before maturation. H_2O_2 (9 M) were added just before to induce DC maturation. Cytokine expression was evaluated after 24 h of culture. DC apoptosis and necrosis were assessed by Annexin-V (Sigma) and 7-aminoactinomycin (7-AAD) (BD Bioscience Pharmingen TM, USA) staining.

2.5. Flow cytometric analysis of cells

Cell surface expression of CD86 and HLA-DR was evaluated by direct immunofluorescence using saturating concentrations of monoclonal mouse antihuman CD86-PeCy5 (clone IT2.2) and HLA-DR-PE conjugated antibodies (R&D Systems Inc., Minneapolis, MN, USA).

Briefly, 5×10^5 cells were incubated with the antibody for 20 min on ice, and, after 20-min incubation on ice in the dark, cells were washed and fixed in 500 µl of 1% paraformaldehyde. For intracellular staining of the phosphorylated form of Syk, DCs were incubated with *Mtb* for 30 min, in the presence of ortovanadate in order to avoid phosphatases activation. Thereafter, cells were permeated by using a Fix and Perm kit (Caltag, Burlingame, CA, USA) and washed with PBS containing 1% Na azide and 5% fetal calf serum. Afterward, cells were stained with mouse antihuman p-(Tyr525/526)-Syk (Cell Signaling Technology, Inc., Danvers, MA) and revealed with donkey anti-mouse-IgG-FITC antibodies (Cell Signaling Technology, Inc.). For intracellular staining of IFNγ, cells were incubated with 2 mg/ml brefeldin A (Golgi Plug) being added during the last 6 h of culture and thereafter stained with IFNγ-FITC (clone 4S.B3) (BD Pharmigen, San Diego, CA). Thereafter, 10 000 events were collected in linear mode for forward scatter (FSC) and side scatter (SSC), and log amplification for FL-1 and FL-2, using a FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Analysis was performed using the FACS express software (De Novo Software) and isotype matched controls were used to determine auto-fluorescence and nonspecific staining. Results were shown as percentages of positive cells and as mean fluorescence intensity (MFI).

2.6. Oxidative burst assay

Intracellular ROS were measured by Dihydrorhodamine123 assay (DHR). Briefly, DC (5×10^5) matured with LPS for 24 h were incubated with 100 µl DHR (5 µg/ml) for 15 min at 37 °C. Later, DC were re-stimulated with *Mtb* (2:1 *Mtb*:DC) for 2 more hours to induce oxidative stress and washed and collected in a flow cytometer as mentioned above.

2.7. Phagocytosis assay

Phagocytosis of *Mtb* was evaluated by the method described by Busseto (Busetto et al., 2004). Briefly, 3×10^6/ml DC were suspended in media with 10% FCS and incubated in a shaking water bath with opsonized or un-opsonized FITC-labeled *Mtb* for 2 h at 37 °C at a 10:1 ratio *Mtb*:DC. To stop phagocytosis, aliquots of the incubation mixtures were withdrawn within an equal volume of Trypan Blue (250 µg/ml) dissolved in ice-cold citrate buffer (0.1 M, pH 4.0). After 1-min incubation in ice, the samples were analyzed by flow cytometry. Each sample was collected for 30 seconds at the slowest flow rate to minimize the coincidental appearance of free bacteria and DCs in the laser beam. When indicated, bacteria where enzymatically treated to eliminate α-glucans from capsule, as described above. The data were then analyzed by using CellQuest software from Becton Dickinson. The mean number of ingested bacteria per DC was assessed (i.e., both attached and internalized particles).

When indicated, phagocytosis was indirectly determined by measuring ROS production employing DHR-labeled *Mtb* (^{DHR}*Mtb*). Briefly, *Mtb* were incubated with 5 µg/ml DHR for 30 min at 37 °C followed by an extensive washing to remove free DHR and suspended in RPMI 1640 plus 10% FCS. Thereafter, 5×10^5 DCs were incubated with ^{DHR}*Mtb* strains at a 10:1 ratio for 90 min at 37 °C, washed and centrifuged at 100 *g* evaluated immediately in a flow cytometer as previously described.

2.8. Proliferation assays

Mixed leukocyte reaction (MLR) were performed by culturing DCs with 1×10^5 allogeneic lymphocytes (Ly) and specific lymphocyte proliferation (recall) were carried out in cells from PPD + healthy subjects, by culturing DC and lymphocytes at 10Ly:1DC ratio for 5 days. When indicated, 10 mM of H_2O_2 was added 10 min after *Mtb* treatment. Afterward, 0.5 µCi per well of methyl-(^{3}H)thymidine (PerkinElmer, Boston, MA, USA) was added the last 18 h to the culture and radioactivity (cpm) was measured in a liquid scintillation counter. Lymphocyte proliferation was also evaluated by Carboxyfluorescein Succinimidyl ester (CFSE) staining. Briefly, cell suspension was incubated with PBS/2% FCS and 0.5 µM CFSE at 37 °C for 10 min. After several washings, cells were added to *Mtb*-matured DCs (10:1 ratio) and cultured for 5 days. Afterward, cells were stained with mAbs directed to CD3-PE and CD4-PerCP/ Cy5.5 in the cell surface, and later, 10 000 events were collected and CFSE low (proliferating lymphocytes, FL-1) and analyzed as described earlier.

2.9. Statistics

The statistical analysis of the data was performed using one-way ANOVA followed by Tukey's multiple comparison tests to compare more than two groups followed by Wilcoxon test to compare two groups. The significance adopted was $p < 0.05$. The graphical representation of the values is given by mean ± standard error of the mean.

3. Results

3.1. ROS production in DC involves α-glucans on Mtb cell wall

In a previous work we described that the α-glucans present in the bacteria cell wall mediates ROS production in neutrophils and their subsequent apoptosis. Here we proposed to assess the role of α-glucans in ROS production in DCs. To accomplish this aim, we evaluated the conversion of non-fluorescent dihydrorhodamine123 (DHR) to rhodamine123 as a measure of H_2O_2 production. DHR freely enters the cell and it binds to cellular and mitochondrial membranes emitting a bright

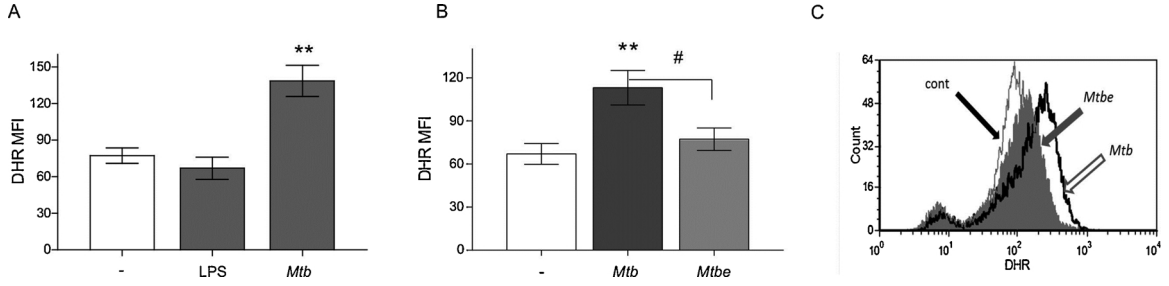

Fig. 1. The α-glucans on *Mtb* cell wall induce ROS production in DCs.
DCs were incubated with LPS for 24 h for priming and thereafter pretreated with DHR and incubated or not with 2:1 *Mtb*:DC ratio for 90 min. Alternatively, bacteria were enzymatically treated to eliminate α-glucans from the capsule (*Mtbe*). Conversion of non-fluorescent dihydrorhodamine123 (DHR) to rhodamine123 as a measure of H_2O_2 production was evaluated. Results are expressed as a mean fluorescence intensity ± SE (n = 20) of oxidized dihydrorhodamine123. The emission of oxidized DHR was evaluated by flow cytometry. A) *Mtb* vs. C and LPS $^{**}p < 0.0005$. B) *Mtb* vs. control $^{**}p < 0.0005$; *Mtb* vs. *Mtbe* $^{\#}p < 0.002$. C) Representative histogram out of 15 experiments is shown.

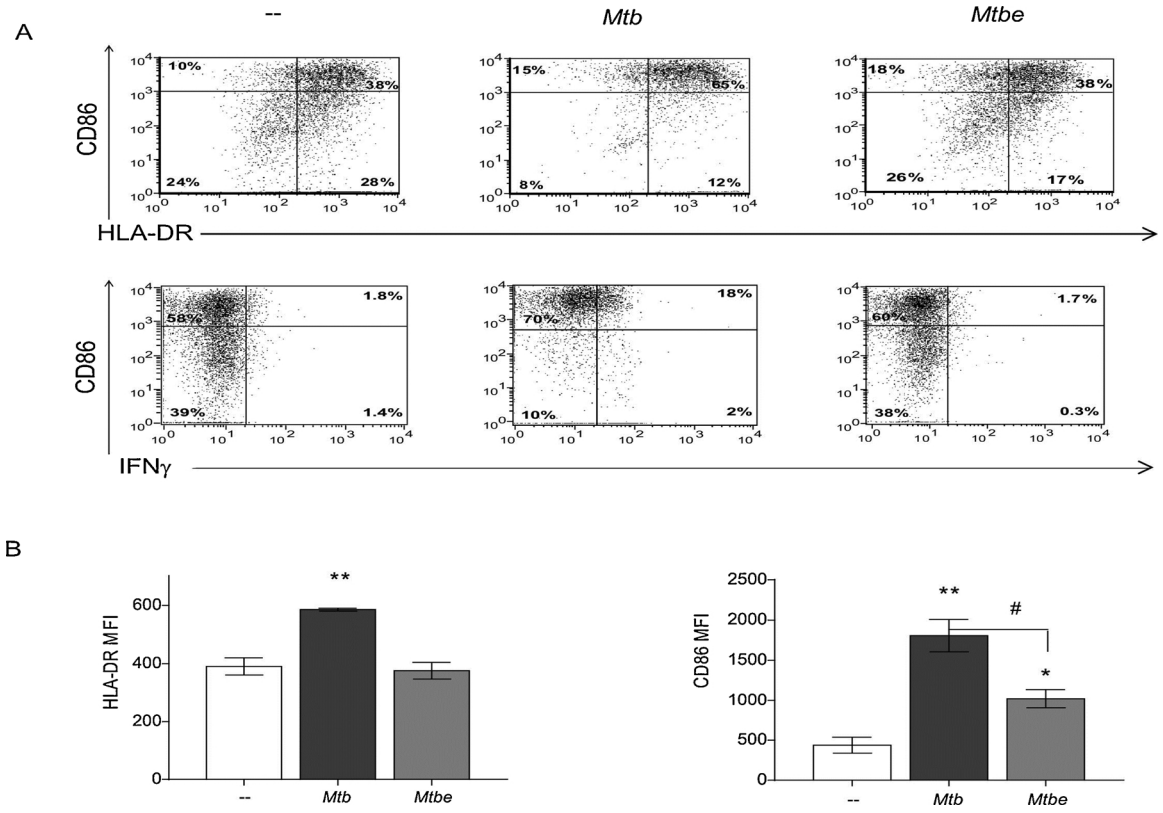

Fig. 2. The α-glucans on *Mtb* cell wall induce DC maturation.
DC population was gated on the basis of CD86 and DC-SIGN expression. A) Surface expression of HLA-DR and intracytoplasmic expression of IFNγ were evaluated in this gate by flow cytometry, and dot plots show a representative experiment of 15 carried out.
B) Immature DCs, control (white bars), were stimulated with *Mtb* H37Rv (dark grey bars) or *Mtbe* (gray bars) and maturation markers were evaluated by flow cytometry. The results are expressed as mean ± standard error (n = 25): HLA-DR expression: *Mtb* versus control, **p < 0.001; CD86 expression: *Mtb* versus control, **p < 0.001; *Mtbe* versus control *p < 0.02; *Mtb* versus *Mtbe* #p < 0.02.

fluorescent signal mainly localized inside the cell (Emmendörffer et al., 1990). As it is shown in Fig. 1A, whereas LPS alone did not induce ROS, *Mtb* was able to induce ROS production in LPS primed DC (matured) as previously described (Romero et al., 2016). However, when α-glucans were removed by enzymatic digestion with 1,4-α-glycosidase (*Mtbe*), a reduction in ROS production to basal levels was observed (Fig. 1B-C), suggesting that intact structure of bacteria is necessary to induce ROS and that α-glucans participate in the oxidative process.

3.2. DC maturation involves α-glucan-induced ROS production

It has been demonstrated an essential role played by ROS in DC maturation and function (Vulcano et al., 2004; Matsue et al., 2003). In this way, we have demonstrated that *Mtb*-induced DC maturation is mediated by ROS involving TLR2/dectin-1 interaction (Romero et al., 2016). Considering that α-glucans mediate ROS production in DCs and that maturation of DCs may be related to ROS production we wondered whether *Mtbe,* which fails at inducing ROS, would exhibit any deficiency to induce DC maturation. As can be seen in Fig. 2A, the removal of α-glucans from the *Mtb* capsule abrogated the *Mtb*-induced HLA-DR and IFNγ positive DCs, suggesting that α-glucans mediates DC maturation very likely mediated by ROS. Even though, *Mtb*-induced CD86 expression was significantly but partially induced with *Mtbe*, suggesting that CD86 up-regulation may be mediated by other mechanisms different than ROS production (Fig. 2B). In order to assess whether α-glucans induced DC maturation through ROS production or by other mechanism, immature DCs were incubated with or without the addition of H_2O_2 (9 M) ten minutes before *Mtbe* treatment. As can be seen in Fig. 3, the failure at inducing MHC-II and CD86 expression by *Mtbe* (measured as MFI and % of positive cells) was totally restored with H_2O_2, confirming the idea that the α-glucan-induced DC maturation involves ROS production.

3.3. The α-glucan-induced ROS production affects lymphocyte proliferation

We have previously demonstrated that *Mtb*-induced ROS production affected lymphocyte proliferation as it improves DC maturation and antigen processing/presentation. Considering that α-glucans induced ROS production in DC, we evaluated whether α-glucan-induced DC maturation, could be reflected in DC functionality. In order to answer this question, we evaluated DC-induced MLR (Mixed Lymphocyte Reaction) by two methods: methyl-(^{3}H)thymidine incorporation (Fig. 4A) and CFSE staining (Fig. 4B). As can be observed, *Mtbe*-induced lymphocyte proliferation was significantly lower than that induced by intact *Mtb*, suggesting that α-glucans are necessary for DC maturation and function. The addition of H_2O_2 ten minutes before the addition of *Mtbe* improves the effect, reinforcing the notion that α-glucan-induced DC maturation and function, may occur through ROS production.

3.4. The α-glucans on Mtb cell wall are not essential in non-opsonized phagocytosis

In a previous report, we demonstrated that α-glucans participate in non-opsonized phagocytosis through CD11b in neutrophils. In this context, it has been reported that *Mtb* interacts with CD11b, through capsular polysaccharides, which may be dependent on the strain; however, the α-glucan-induced ROS production did not involve CD11b interaction in neutrophils (Romero et al., 2014).

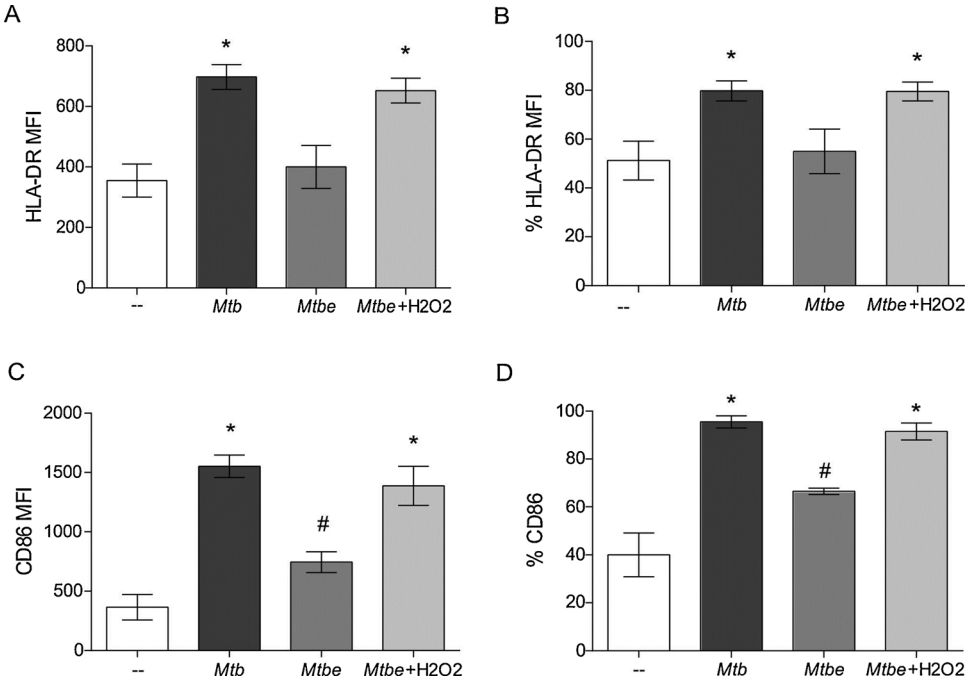

Fig. 3. The α-glucan-induced DC maturation involves ROS production.
DC population was gated on the basis of CD86 and DC-SIGN expression. Immature DC, control (white bars), were stimulated with *Mtb* (dark grey bars), *Mtbe* (gray bars) or treated with H_2O_2 plus *Mtbe* (light gray bars) and maturation markers were evaluated by flow cytometry. The results are expressed as mean ± standard error (n = 25) (A and C) and as % of positive cells within the gate (B and D). A) HLA-DR MFI: *Mtb* versus control, $^{**}p < 0.001$; *Mtbe* + H_2O_2 versus control, $^{**}p < 0.001$. B) Percentage of HLA-DR + cells: *Mtb* versus control, $^{**}p < 0.001$; Mtbe + H_2O_2 versus control, $^{**}p < 0.001$. C) CD86 MFI: *Mtb* versus control, $^{**}p < 0.001$; *Mtbe* versus *Mtb* $^{*}p < 0.02$; *Mtb* versus *Mtbe* $^{\#}p < 0.02$; *Mtbe* + H_2O_2 versus control, $^{**}p < 0.001$. D) Percentage of CD86 + cells: *Mtb* versus control, $^{**}p < 0.001$; *Mtbe* versus *Mtb* $^{*}p < 0.02$; *Mtb* versus *Mtbe* $\#p < 0.02$; *Mtbe* + H_2O_2 versus control, $^{**}p < 0.001$.

In order to evaluate whether α-glucan of the *Mtb* capsule participates in the entrance to DCs, phagocytosis of enzymatically treated bacteria was compared to intact bacteria. To evaluate if low ROS production in DCs was associated with a low entrance, we quantified ROS with DHR-labeled *Mtb* (^{DHR}*Mtb*), so that oxidized DHR represents the level of ingested bacteria, as a measure of indirect phagocytosis. Figs. 5A and 5B show that intact *Mtb* was able to induce ROS whereas *Mtbe* did not, tempting us to consider that α-glucans are necessary to enter DC. However, when phagocytosis was directly measured by FITC-*Mtb* uptake (Fig. 5C-D), we observed that the lack of α-glucans improve bacterial entrance in DCs suggesting that they are not essential in non-opsonized phagocytosis. In particular, the fact that *Mtbe* was not able to induce ROS in this condition might not be related to the low phagocytic rate but a poor ability to induce ROS.

We have previously demonstrated that *Mtb* induces DC maturation through TLR2/dectin-1 by generating ROS and, through DC-SIGN in a ROS independently manner (Romero et al., 2016). Given that the main phagocytic receptor in DC, DC-SIGN, has been proposed to bind α-glucans inducing host immune modulation (Geurtsen et al., 2009) we wondered whether this receptor could affect ROS and/or α-glucan-mediated entrance to DCs. As shown in Fig. 5B, the generation of ROS induced by *Mtb* involves both DC-SIGN and dectin-1, as observed by using blocking antibodies for DC-SIGN (Tailleux et al., 2003) and dectin-1 (Brown et al., 2003). However, phagocytosis per se does not involve dectin-1 (Fig. 4D). In fact, when α-glucans were removed from the *Mtb* capsule, the increase in phagocytosis induced by *Mtbe* was also reduced by blocking DC-SIGN indicating that, although it is the main receptor, other ligands that are not glucans would be participating in DC-SIGN-mediated entrance to DC.

3.5. The α-glucan-induced lymphocyte proliferation is not mediated by DC-SIGN

As mentioned above, the α-glucans have an impact on the processing/presentation of antigens through the production of ROS (Fig. 3). However, they would not play an important role in the DC entrance (Fig. 5B), but would rather hinder the binding of ligands more related to DC-SIGN (Fig. 5D). In this way, we wondered whether all these factors would affect the processing/presentation at the end. To that, we evaluated antigen presentation in terms of specific proliferation employing lymphocytes from PPD + donors (Fig. 6A) and MLR (Fig. 6B) evaluated by methyl-(^{3}H)thymidine incorporation, and CFSE staining (data not shown). As expected, *Mtb*-induced MLR and specific lymphocyte proliferation was significantly reduced by the blockade of DC-

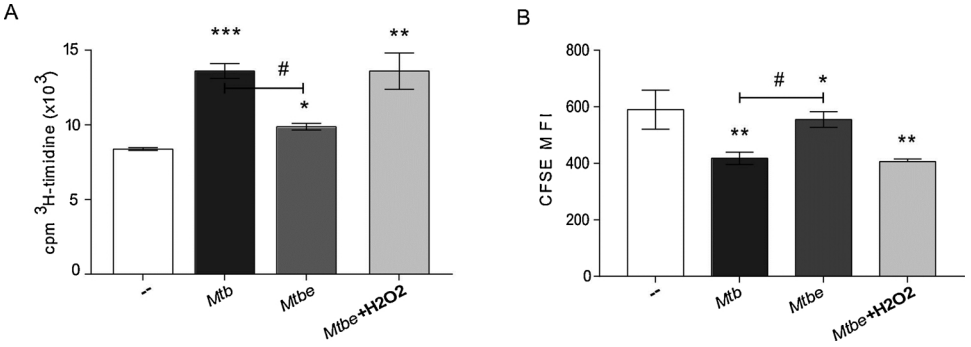

Fig. 4. The α-glucans on *Mtb* cell wall promote lymphocyte proliferation.
Lymphocyte proliferation (MLR) in response to *Mtb* and *Mtbe* was evaluated. Immature DC, control (white bars), were stimulated with *Mtb* (dark grey bars), *Mtbe* (gray bars) or H_2O_2 plus *Mtbe* (light gray bars) were measured by A) ^{3}H-thymidine incorporation or B) CFSE staining on lymphocyte gated on CD3 + cells cultured for 5 days at 1:10 of DC to T-cell ratio. Results are expressed as mean ± standard error (n = 12) of the counts of ^{3}H-thymidine incorporation per minute (CPM), or mean ± standard error (n = 15) of the mean fluorescence intensity. *Mtb* versus control, $^{**}p < 0.001$; *Mtbe* versus control $^{*}p < 0.02$; *Mtb* versus *Mtbe* $^{\#}p < 0.02$; *Mtbe* + H_2O_2 versus control, $^{**}p < 0.001$.

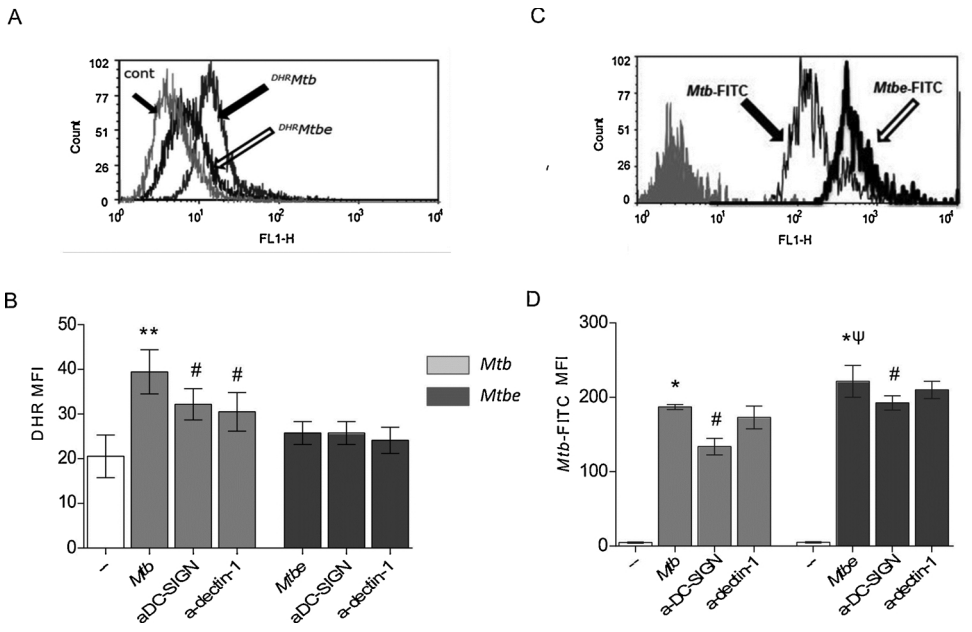

Fig. 5. The α-glucans on *Mtb* are not essential in non-opsonized phagocytosis.
(A,B) DHR-labeled *Mtb* (^{DHR}*Mtb*) or ^{DHR}*Mtbe* or control *Mtb* (without DHR) were incubated with 5×10^5 DC at a 10:1 ratio for 90 min. Thereafter, the emission of oxidized DHR was evaluated by flow cytometry. When indicated, DCs were incubated with blocking antibodies against DC-SIGN or dectin-1. A) Representative histogram out of 15 experiments is shown. B) Results are expressed as a mean ± SE (n = 20) of MFI of oxidized DHR (phagocytosed *Mtb*). ^{DHR}*Mtb* versus control and ^{DHR}*Mtbe* $^{**}p < 0.001$; ^{DHR}*Mtb* + anti DC-SIGN or + anti-dectin-1 $^{\#}p < 0.02$. (C,D) FITC labeled-*Mtb* were incubated with 5×10^5 DCs at a 10:1 ratio for 90 min. C) Representative histogram out of 6 experiments is shown D) Results are expressed as a mean ± SE (n = 6) of MFI of FITC (phagocytosed *Mtb*); *Mtb* versus control $^{*}p < 0.005$; *Mtb* + anti DC-SIGN $^{\#}p < 0.02$; *Mtbe* versus control $^{*}p < 0.005$, *Mtbe* versus *Mtb* $^{\Psi}p < 0.02$, *Mtbe* + anti DC-SIGN $^{\#}p < 0.02$.

SIGN as it is the main phagocytic receptor in DCs (Alemán et al., 2007). However, the low *Mtbe*-induced lymphocyte proliferation was also reduced by blocking DC-SIGN, suggesting that this reduction while small was significant because other ligands are mediating DC-SIGN entrance to DCs. The fact that the lack of α-glucans improves bacterial entrance in DC (Fig. 5B) but do not improve processing/presentation, confirms the importance of ROS in DC functionality because *Mtbe* fails at induce ROS without losing its ability to enter DCs. Moreover, MLR which highly depends on DC maturation returns to the basal value in the absence of glucans, which confirms the role of ROS in the activation and functionality of the DC (Fig. 6B).

3.6. The α-glucan on Mtb capsule participates in Syk activation in DC

We have previously demonstrated that in neutrophils, the activation of the spleen tyrosine kinase (Syk) which is necessary to induce ROS production, might involve α-glucans on *Mtb* (Romero et al., 2014). In addition, in DCs, Syk activation is significantly induced by *Mtb* and moreover, *Mtb*-induced ROS production is abrogated by using the Syk inhibitor piceatannol (Alemán et al., 2007). Thus, we asked whether α-glucan of *Mtb* capsule induces Syk activation on DC. Fig. 7 shows, that *Mtb* significantly induced the activated form of Syk (pSyk), whereas removing α-glucans by enzymatic digestion reduced its activation over baseline, suggesting a role for α-glucans in Syk activation and ROS production in DCs.

4. Discussion

The intracellular replication of *Mtb* and its spread from the lungs to other sites occur before the development of adaptive immune responses. Dendritic cells (DCs) are professional antigen presenting cells whose maturation is critical for the onset of the protective immune response against TB disease and may vary depending on the nature of the cell wall of *Mtb* strain. In this context, *Mtb* has evolved successful strategies to invade and persist within host cells and these interactions appear to involve surface polysaccharides and glycolipids present in the *Mtb* surface (Reed et al., 2004; Torrelles and Schlesinger, 2010).

Unlike other pathogens, *Mtb* lacks typical virulence factors such as toxins; therefore, epidemiologic fitness of a strain can be influenced by a range of factors, for instance, the genetic background of host and pathogen, host–pathogen interactions, and the environment (O'Sullivan et al., 2010). The ability of a pathogen to avoid inducing inflammatory cytokine release, as well as to limit the expression of those molecules implicated in antigen presentation, may allow certain strains to remain undetected by DCs evading an effective immune response (Urban et al., 1999). In this context, certain intracellular pathogens have evolved mechanisms to escape from the host immune response, and in this manner, some *Mtb* strains are able to manipulate DCs as a means of

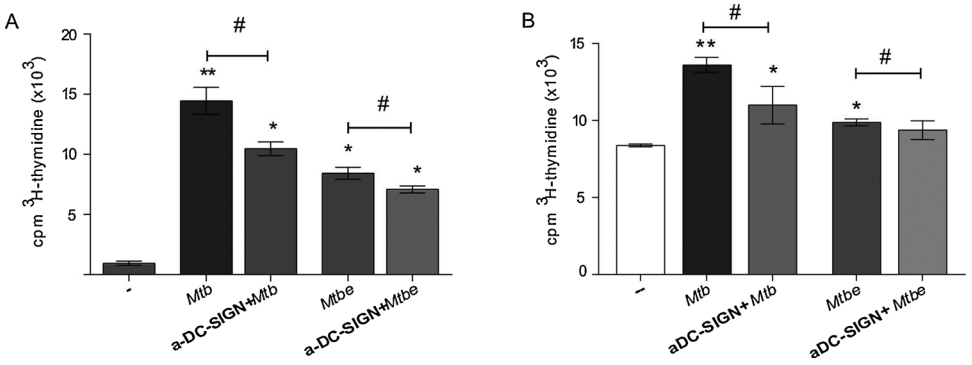

Fig. 6. The α-glucan-induced lymphocyte proliferation is not mediated by DC-SIGN.
Immature (control) and *Mtb*-matured or *Mtbe*-matured DCs were conducted in proliferation assays either by using (A) autologous lymphocytes from healthy donors with positive response to the antigen-purified protein derivative (PPD+) as a specific response, or (B) human lymphocytes from non-related donor (MLR) for 5 days at 1:10 ratios of DCs to T-cells. The results show the lymphocyte proliferative response and are expressed as mean ± SE (n = 9) of the counts of ^{3}H-thymidine incorporation per minute (cpm). Specific response: *Mtb* versus control (-) $^{**}p < 0.001$; *Mtb* + anti-DC-SIGN, *Mtbe* and *Mtbe* + anti-DC-SIGN versus control $^{*}p < 0.02$; *Mtb* + anti-DC-SIGN versus *Mtb* $^{\#}p < 0.02$, *Mtbe* + anti-DC-SIGN versus *Mtbe* $^{\#}p < 0.02$. MLR: *Mtb* versus control (-) $^{**}p < 0.001$; *Mtb* + anti-DC-SIGN and *Mtbe* versus control $^{*}p < 0.02$; *Mtb* + anti-DC-SIGN versus *Mtb* $^{\#}p < 0.02$, *Mtbe* + anti-DC-SIGN versus *Mtbe* $^{\#}p < 0.02$.

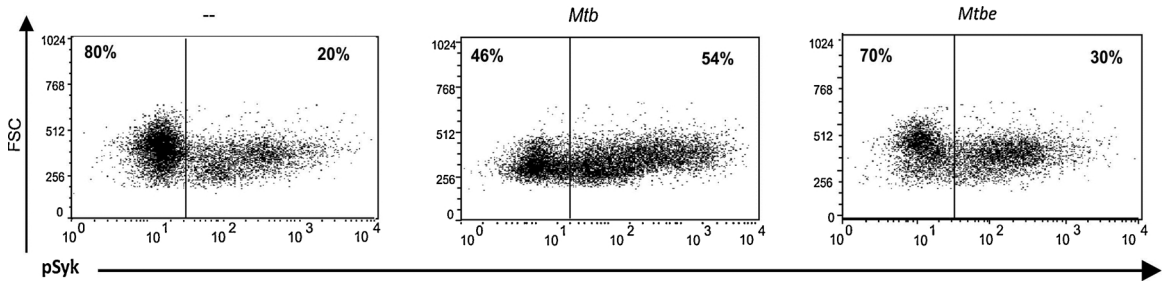

Fig. 7. The α-glucan on *Mtb* participates in Syk activation in DCs.
DCs were incubated with *Mtb* or *Mtbe* for 30 min, in the presence of ortovanadate in order to avoid phosphatases activation. Thereafter, cells were permeated and stained with mouse antihuman p-(Tyr525/526)-Syk. Results were shown as percentages of positive cells that express the activated form of Syk in control DCs or DCs stimulated with *Mtb* or *Mtbe*. A representative experiment of seven carried out is depicted.

immune evasion (Buchan et al., 2009). For instance, it was suggested that certain strains of *Mtb* can spread more effectively and cause disease more frequently than others, resulting in different profiles in the immune response (Velasco-Velázquez et al., 2003; Portevin et al., 2011). In this way, we have previously shown that *Mtb*-induced DC maturation comprised the up-regulation of CD86, HLA-DR and IL-12 and IFNγ production and secretion suggesting autocrine DC activation upon bacterial encounter (Romero et al., 2016). The role of ROS in the maturation of DC has been strongly documented (Vulcano et al., 2004; Matsue et al., 2003). In this way, we have demonstrated that ROS affect DC maturation and expansion of *Mtb*-specific lymphocytes. Moreover, the different capacity to induce DC maturation exerted by *Mtb* strains was related to a variation in ROS production (Romero et al., 2016). In this context, we showed that a clinical strain fails in their ability to induce ROS and apoptosis in neutrophils, a mechanism putatively governed by carbohydrate composition of mycobacteria cell wall as ROS production was significantly reduced with laminarin, a soluble β-glucan (Romero et al., 2014). Therefore, herein we studied the role of α-glucans in the endogenous production of ROS in DC by evaluating the ability of *Mtbe* (that lack α-glucans) to induce ROS. We found that in this condition, the bacteria were not able to induce ROS in LPS primed DCs (matured) compared to intact *Mtb,* revealing a relevant role of α-glucans in the oxidative process in DCs. Accordingly, since maturation of DCs is associated with ROS production, the lack of α-glucans will directly impair DC maturation. In this context, *Mtb*-induced DC maturation is a prerequisite for an efficient T-cell activation as it involves the up-regulation of co-stimulatory molecules (Hertz et al., 2001; Tsuji et al., 2000; Giacomini et al., 2001; Tailleux et al., 2005; Mellman and Steinman, 2001). The addition of H_2O_2 ten minutes before recovers *Mtbe*-induced T-cell proliferation permitting us to conclude that α-glucan-induced DC functionality may occur through ROS production.

Mtb interacts with immune receptors on cell surface including TLR, CD11b, mannose receptor, scavenger receptors, and DC-SIGN, leading to the induction of an inflammatory response (Dheda et al., 2010). Considering that the ingestion of *Mtb* is essential for antigen presentation to T cells (Korbel et al., 2008), we aimed to evaluate whether α-glucans participate in *Mtb* entrance to DC. We observed a significant increase in phagocytosis when α-glucans of *Mtb* capsule were removed, thus they seem not to be essential in non-opsonized phagocytosis. Moreover, the increase of phagocytosis in the absence of glucans could be due to the exposure of ligands of higher affinity in the bacterial cell wall. Therefore, the fact that *Mtbe* was not able to induce ROS in this condition might not be related to the low phagocytic rate but a poor ability to induce ROS. Taking together, we can consider as a strategy of certain strains that have limited glucans, to enter DC without inducing ROS, converting DC into a reservoir.

It has been proposed that DC-SIGN -the main *Mtb* phagocytic receptor in DCs- could bind α-glucans inducing host immune modulation (Geurtsen et al., 2009). However, in absence of α-glucans, *Mtbe* phagocytosis was significantly reduced by the blockage of DC-SIGN, suggesting again that other ligands mediate phagocytosis through this receptor. These results differed in part what was observed in neutrophils where the enzymatic digestion of α-glucans reduced phagocytosis of non-opsonized bacteria (Romero et al., 2014). This could be ascribed to the fact that neutrophils do not express DC-SIGN so their main phagocytic receptor would be CD11b that interact with *Mtb* capsular polysaccharides through the lectin site, and may be dependent on the strain (Cywes et al., 1997). We have previously described a dual role of ROS, one directly on antigen presentation and other by inducing DC maturation, all of this leading to *Mtb*-specific lymphocyte proliferation (Romero et al., 2016). Besides, it has been reported that ROS generated by DCs during antigen presentation, enhances the antigen-specific T-cell proliferation (Matsue et al., 2003). In this way, we observed that the lack of α-glucans improved bacterial entrance in DC but do not improve processing/presentation, confirming the importance of ROS in DC functionality.

We have previously demonstrated that *Mtb*-induced ROS production in neutrophils and DCs was remarkably reduced by the specific inhibitor of Syk activation, piceatannol. In line with this, certain strains that fail at induce ROS, lack the ability to drive Syk activation being reflected on a poor neutrophil apoptosis and DC maturation (Romero et al., 2014, 2016). Here we showed that, as in neutrophils, α-glucans in *Mtb* capsule triggered Syk signaling and ROS production leading to maturation of DC. These results, together with the notion that mycobacteria shed high amounts of α-glucan in TB patients (Yu et al., 2012) suggest that α-glucans may fulfill an important role in immune response against tuberculosis and should be taken into account when designing an anti-tuberculous vaccine.

Author contribution statement

María Mercedes Romero, conceived the study, carried out experimental procedures, helped write the manuscript

Alejandra Duarte, participated in performing assays

Mercedes Pastorini, carried out experimental procedures

Mercedes Alemán, conceived the study, revised experimental procedures and drafted the manuscript

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