In vitro differentiation of murine hematopoietic progenitor cells toward the myeloid lineage occurs in response to *Staphylococcus aureus* and yeast species.

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Running head: Microbes induce *in vitro* differentiation of HPCs.

ABSTRACT

We have studied the effect of inactivated microbial stimuli (*Candida albicans, Candida glabrata, Saccharomyces boulardii,* and *Staphylococcus aureus*) on the *in vitro* differentiation of lineage negative (Lin^-) hematopoietic progenitor mouse cells. Purified Lin^- progenitors were co-cultured for 7 days with the stimuli, and cell differentiation was determined by flow cytometry analysis. All the stimuli assayed caused differentiation toward the myeloid lineage. *S. boulardii* and particularly *C. glabrata* were the stimuli that induced in a minor extent differentiation of Lin^- cells, as the major population of differentiated cells corresponded to monocytes, whereas *C. albicans* and *S. aureus* induced differentiation beyond monocytes: to monocyte-derived dendritic cells and macrophages, respectively. Interestingly, signalling through TLR2 by its pure ligand Pam3CSK4 directed differentiation of Lin^- cells almost exclusively to macrophages. These data support the notion that hematopoiesis can be modulated in response to microbial stimuli in a pathogen-dependent manner, being determined by the pathogen-associated molecular patterns and the pattern-recognition receptors involved, in order to generate the populations of mature cells required to deal with the pathogen.

Key words: Mouse hematopoietic progenitors; pattern-recognition receptors; *Candida albicans*; *Candida glabrata*; *Saccharomyces boulardii*; *Staphylococcus aureus*; *in vitro* differentiation.

Highlights:

- Inactivated microbes induce *in vitro* differentiation of mouse HPCs.
- Yeasts (*C. albicans, C. glabrata, S. boulardii)* and *S. aureus* were tested.
- Differentiation of HPCs is modulated in a pathogen-dependent manner.
- This modulation would generate the mature cells required to deal with the pathogen.

1. INTRODUCTION

Cells of the immune system are prepared for recognizing a variety of pathogen organisms through pattern-recognition receptors (PRRs), which recognize molecular signatures of microbial agents and function as sensors for infection. Toll-like receptors (TLRs) are a family of PRRs that induce the activation of innate immune responses and modulate the subsequent development of adaptive immune responses [1]. Recent studies have indicated that TLRs are not only expressed in mature immune cells, but also in hematopoietic stem and progenitor cells (HSPCs) and their progeny, suggesting a role for them in hematopoiesis during infection [2].

Our group has focused on the involvement of TLRs in the recognition of *Candida albicans*, the most frequent cause of opportunistic human fungal infections, and we have described that inactivated yeasts of *C. albicans* interact *in vivo* with murine HSPCs causing their differentiation toward the myeloid lineage in a TLR2-dependent manner [3-5], and that *in vitro* this differentiation produced monocyte-derived dendritic cells (moDCs), via TLR2- and Dectin-1-dependent pathways [6].

Apart from this, little is known about the effect of other species of yeast and bacterial microbes on the *in vitro* differentiation patterns of hematopoietic progenitor cells (HPCs). For that reason, the objective of the present work has been to study the effect of other microorganisms on *in vitro* differentiation of murine HPCs. In addition to *C. albicans*, we have tested other two yeast and one bacteria species: (a) *Candida glabrata*, the second fungus causing candidiasis worldwide [7]; (b) *Saccharomyces boulardii*, a non-pathogenic yeast used as a probiotic agent against acute gastroenteritis and diarrhea [8] which can produce fungaemia in immunodepressed patients [9, 10], and (c) the Gram-positive bacterial species *Staphylococcus aureus*, a relevant pathogen causing a plethora of infections in humans, which is recognized by TLR2 [11].

2. MATERIALS AND METHODS

2.1. Mice

Wild-type C57BL/6 mice (Harlan Ibérica, Barcelona, Spain) were used as lineage negative (Lin^-) progenitor cells donors. Females between 8 and 12 weeks old were used, and all assays involving mice were approved by the Institutional Animal Care and Use Committee (A1264596506468, Universitat de València).

2.2. Microbial strains and culture conditions

Three yeast species (*C. albicans* ATCC 26555, *C. glabrata* CECT 1448 and *S. boulardii* CECT 1043) and one Gram-positive bacterial species (*S. aureus* CECT 4630) were used in this study. Yeast strains were routinely cultured in YPD plates at 28 ºC for 24-48 h; liquid cultures were grown in YPD at 28 ºC up to exponential growth phase. *S. aureus* was routinely cultured on TSA plates and exponentially growing cells were obtained following incubation in LB medium at 37 ºC.

2.3. Preparation of inactivated microbial stimuli

For inactivation, exponentially yeast and bacteria cells were collected by centrifugation, resuspended in 4% paraformaldehyde ($20x10^6$ cells mL^{-1}) and incubated for 1 hour at room temperature. After extensive washing with PBS, cells were resuspended in PBS at the desired concentration. Inactivation was checked by absence of growth following plating on YPD/TSA medium. All procedures were performed under conditions designed to minimize endotoxin contamination as previously described [12].

2.4. *In vitro* assays

Lin^- cells were purified as previously described [4], and immediately cultured in flat-bottomed 96-well plates (40×10^3 cells in 200 μL per well) in complete Lin^- cell culture medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 5% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin stock solution; Gibco, Barcelona, Spain) containing three cytokines: stem cell factor (SCF, 20 ng mL^{-1}), Flt-3 ligand (FL, 100 ng mL^{-1}) (Peprotech, Rocky Hill, NJ) and IL-7 (10 ng mL^{-1}) (MBL, Woburn, MA). Cells were challenged for 7 days with Pam3CSK4 (1 μg mL^{-1}), and inactivated *C. albicans, C. glabrata, S. boulardii* (7.5/1 ratio, yeasts/Lin^- cells) and *S. aureus* cells (18/1 ratio, bacteria/Lin^- cells). The ratio microbial cells/Lin^- cells was determined according to preliminary dose-response assays, and the selected ratio was the lower dose inducing the higher differentiation.
Control cultures were performed without stimuli. At day seven, cells were collected, labelled with various combinations of antibodies, and analyzed by flow cytometry.

2.5. Antibodies and flow cytometry analyses

The following antibodies used in flow cytometry analyses were purchased from Miltenyi Biotec (Madrid, Spain): cocktail of biotinylated anti-lineage antigens, APC-labeled anti-CD11c (clone N418) and APC-labeled anti-mPDCA-1 (clone JF05-

1C2.4.1). The following antibodies were from eBioscience (San Diego, CA): PE-labeled anti-CD11b (clone M1/70) and PE-Cy7-labeled anti-F4/80 (clone BM8), or from BD Pharmingen (San Jose, CA): PerCP-Cy5.5-labeled anti-Ly6C (clone AL-21). Flow cytometry analyses were performed on a FACSCanto cytometer (BD Biosciences) and the data were analyzed with FlowJo 7.6.5. software.

2.6. Statistical analysis

Data are expressed as mean ± SD. Statistical differences were determined using the Mann-Whitney *U* test for multiple comparisons. Significance was accepted at $*P < 0.05$ and $**P < 0.01$ level.

3. RESULTS & DISCUSSION

Lin^- progenitor cells, purified from C57BL/6 mice bone marrow, were cultured for 7 days in complete Lin^- cell culture medium, without stimuli or in the presence of Pam3CSK4 (pure TLR2 ligand), inactivated yeasts (*C. albicans, C. glabrata, S. boulardii*) or inactivated bacteria (*S. aureus*). In the described conditions, unstimulated progenitors differentiated to a mixed population of cells (Fig.1A, medium; Fig.1B, empty bars). These differentiated cells did not express the CD19 lymphoid marker (data not shown), indicating that in these conditions B cells were not produced. Analysis of the expression of lineage markers allowed the identification of five different cell populations: (a) plasmacytoid dendritic cells (pDCs, orange gate in Fig.1A), that represented approximately 24% of cells, and were $CD11b^-$ $CD11c^+$ and also expressed mPDCA-1 (data not shown); (b) pre-DCs or classic DCs (cDCs, green gate and subgate), that represented 15% of cells, and were $CD11b^+$ $CD11c^+$ $Ly6C^-$ $F4/80^-$; (c) moDCs (moDCs, blue gate), that represented 2% of cells, and were $CD11b^{+high}$ $CD11c^+$ as well as $Ly6C^+$ $F4/80^+$ (data not shown); (d) $Ly6C^{high}$ monocytes (Mcs, red gate and subgate), that corresponded to 3% of cells, and were $CD11b^+$ $CD11c^-$ $Ly6C^+$ $F4/80^-$, and (e) macrophages (Mphs, red gate and subgate), 1% of cells that were $CD11b^+$ $CD11c^-$ $F4/80^+$. In conclusion, results show that, in the absence of stimuli, Lin^- cells differentiate preferentially into pDCs and cDCs. As expected, treatment with Pam3CSK4 in the same conditions changed completely the differentiation pattern (Fig. 1A, Pam3CSK4; Fig. 1B, black bars), with a dramatic decrease of pDCs and cDCs

(from 24% and 15% to 1% and 2%, respectively) and a huge increase of Mphs (from 1% to 32%), whereas Lin$^-$ cells in presence of *C. albicans* (Fig. 1A, *C. albicans*; Fig. 1B, light blue bars) experienced an important reduction of pDCs, but an increase of Mcs and in a major extent of moDCs (from 2% to 27%), as previously described by our group [13, 14]. MoDCs are involved in the synthesis of inflammatory molecules and oxidant compounds which help the organism to fight the infection. The moDCs differentiated from Lin$^-$ progenitors are phenotypically similar to a subset of DCs named TipDCs ("TNF and inducible nitric oxide synthase (iNOS)-producing Dendritic Cell") that display a remarkable microbicidal activity [15].

In the presence of *C. glabrata* (Fig. 1A, *C. glabrata*; Fig. 1B, deep blue bars) a different differentiation pattern was produced, with a minor level of differentiation (pDCs decreased to 50%, as compared to unstimulated control) and an increased population of Mcs. Co-culture with *S. boulardii* (Fig. 1A, *S. boulardii*; Fig. 1B, light purple bars) resulted in a marked reduction of pDCs and cDCs, accompanied with a predominant population of Mcs. The pattern observed with *S. aureus* (Fig. 1A, *S. aureus*; Fig. 1B, deep purple bars) showed a reduction in pDCs and cDCs, with progenitors differentiating toward Mcs and their derivatives Mphs.

Overall, these results suggest that *S. boulardii* and particularly *C. glabrata* are the stimuli that induce differentiation of Lin- cells in a minor extent, as the major population of differentiated cells correspond to Mcs, whereas *C. albicans* and *S. aureus* induced differentiation beyond Mcs, to moDCs and Mphs, respectively. Interestingly, signalling through TLR2 by its pure ligand directs differentiation of Lin$^-$ cells almost exclusively to Mphs. Differences in the *in vitro* response of Lin$^-$ cells between a soluble pure TLR2 ligand (Pam3CSK4) and microbial cells indicate the participation of the TLR2 signalling pathway together with other PRRs, which would be activated by diverse ligands present on the microbial cell surface. In the case of *C. albicans*, differentiation of Lin$^-$ cells toward moDCs is partially dependent on the interaction of the glucan from the yeast cell wall (ligand which is absent in the prokaryotic cell walls) with its Dectin-1 receptor [4]. Unlike *C. albicans*, none of the other yeasts strains induced a massive differentiation to moDCs, suggesting a minor role of Dectin-1 (and therefore of glucan) in the response to *C. glabrata* and S. *boulardii*. The fact that pDCs decrease when Lin$^-$ cells were incubated in presence of all microbial stimuli tested supports that these cells are not much relevant in the defence against yeast and bacterial

infections, which agrees with evidence indicating that pDCs are producers of interferon α and β, and so they are more specialized in the defence against viral infections.

4. CONCLUSION

These data support the notion that hematopoiesis can be modulated in response to infection, in a pathogen-dependent manner, being determined by the patoghen-associated molecular patterns and the PRRs involved, in order to generate the populations of mature cells required to deal with the pathogen, and that this response is mediated by a direct interaction between microbial ligands and HSPCs. In this sense, it should be noted that in our study the highest degree of Lin^- cell differentiation was reached in response to *C. albicans*, the most pathogen yeast species, and to *S. aureus*, and that this response is pathogen-dependent. This response may be mediated by a direct interaction between microbial ligands and HSPCs, which requires colocalization, since HSPCs can be found as resident or migratory populations in both uninfected and infected tissues where microbes could induce them to differentiate by extramedullary hematopoiesis. Although HSPCs in infected tissues are most likely to directly detect microbial components, HSPCs in the vascularised bone marrow may also be exposed to circulating microbial ligands, or even to intact microbial cells following invasion of bone marrow during systemic infection [16].

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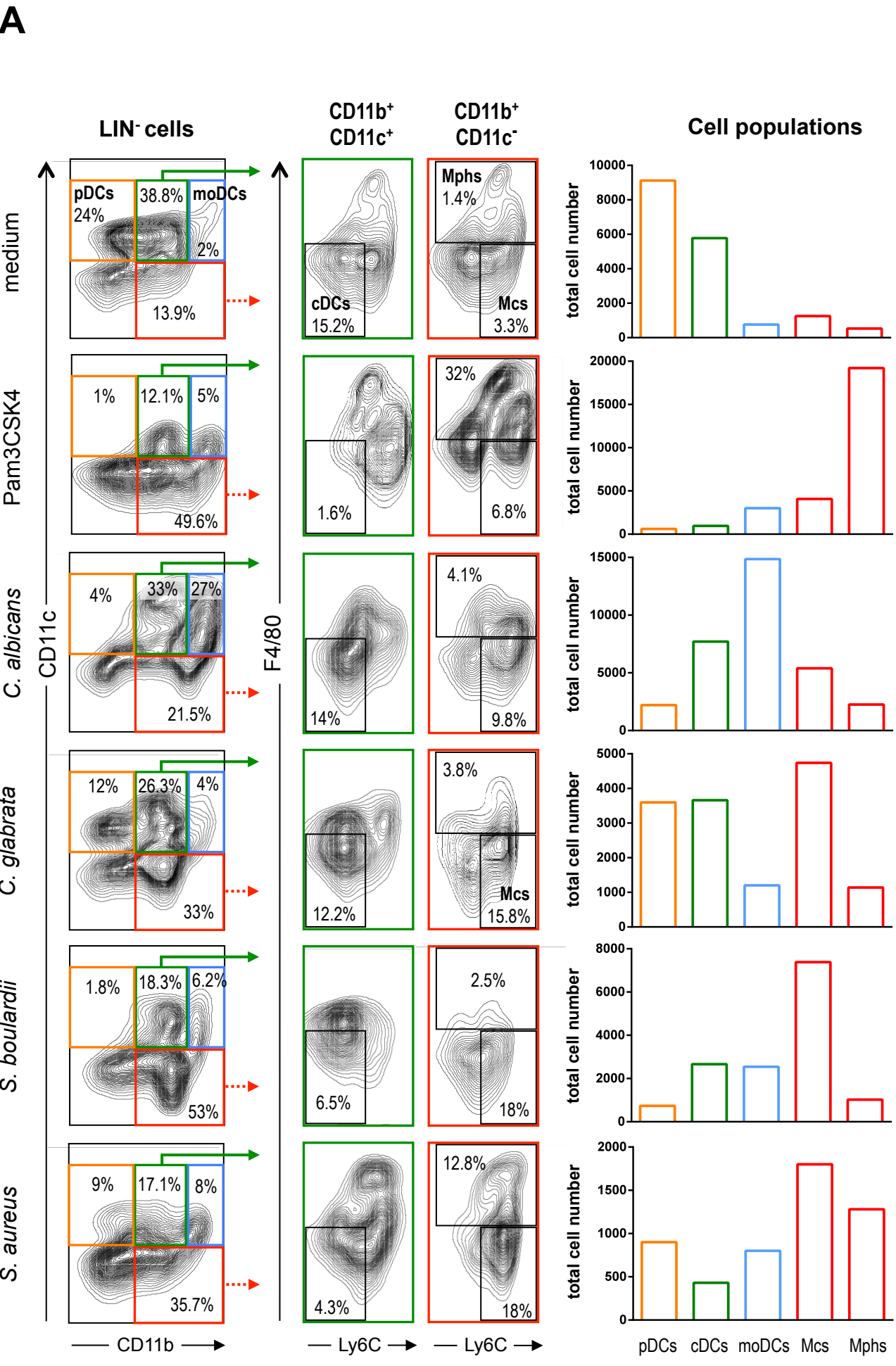
FIGURE LEGEND

Figure 1. *In vitro* differentiation of Lin$^-$ cells in response to stimuli. Figure 1A: Lin$^-$ progenitor cells from C57BL/6 mice were cultured with medium alone (medium), Pam3CSK4 (1 μg mL^{-1}) and *C. albicans, C. glabrata, S. boulardii* (7.5/1 ratio, yeasts/Lin$^-$ cells) or *S. aureus* cells (18/1 ratio, bacteria/Lin$^-$ cells) for 7 days, labelled with antibodies and analyzed by flow cytometry. Cells were gated as pDCs (plasmacytoid cells: CD11b$^-$ CD11c$^+$ mPDCA$^+$), cDCs (pre-classical or classical DCs:

$CD11b^{+}$ $CD11c^{+low}$ $Ly6C^{-}$ $F4/80^{-}$), moDCs ($CD11b^{+high}$ $CD11c^{+}$ $Ly6C^{+}$ $F4/80^{+}$), Mcs (monocytes: $CD11b^{+}$ $CD11c^{-}$ $Ly6C^{+}$ $F4/80^{-}$) and Mphs (macrophages: $CD11b^{+}$ $CD11c^{-}$ $F4/80^{+}$). Ly6C vs. F4/80 plots are subgated from CD11b vs. CD11c plot, as indicated by the arrows. Gates were drawn according to the values obtained by labelling with isotype control antibodies. Percentages represented in the contour plots refer to total analyzed Lin^{-} population. The bar graphs (right) depict the total cell number of each gate. Contour plots from one representative experiment of four are shown. Figure 1B shows the changes in percentages of pDCs, cDCs, moDCs, Mcs and Mphs in the presence or absence of stimuli, analyzed following the same schedule in figure 1A. Data represent means ± SD, from four experiments. *$P < 0.05$ and ** $P < 0.01$ level (Mann-Whitney U test) for each individual variable with respect to cells incubated with medium alone (medium).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Mcs
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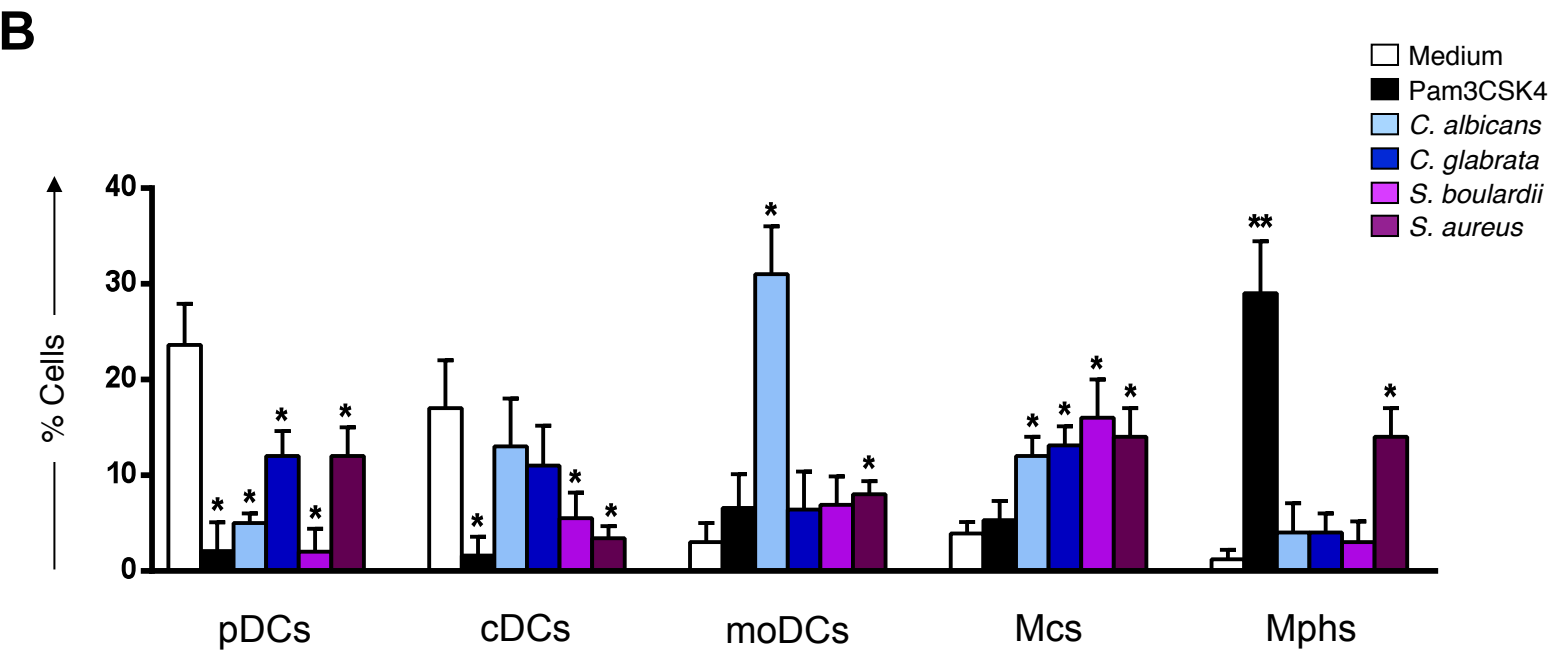
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