Characterising the mechanisms of non-opsonic uptake of Cryptococci by macrophages

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RUNNING TITLE: Uptake of *Cryptococcus* by phagocytes

KEY WORDS: Syk/Dectin/*Cryptococcus neoformans*/*Cryptococcus gattii*/phagocytosis

SOURCE OF FUNDING

Work in the May Lab is supported by project MitoFun, funded by the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement

No. 614562 and by a Wolfson Research Merit Award from the Royal Society (to RCM). GDB is supported by the Wellcome Trust (102705), Wellcome Trust Strategic Award in Medical Mycology and Fungal Immunology (097377) and the MRC Centre for Medical Mycology at the University of Aberdeen (MR/N006364/1). JL is supported by a start-up fund from the University of Stirling.

ABSTRACT

The pathogenic fungus *Cryptococcus* enters the human host via inhalation into the lung and is able to reside in a niche environment that is serum (opsonin) limiting. Little is known about the mechanism by which non-opsonic phagocytosis occurs via phagocytes in such situations. Using a combination of soluble inhibitors of phagocytic receptors and macrophages derived from knockout mice and human volunteers, we show that uptake of non-opsonised *Cryptococcus neoformans* and *Cryptocccous gattii* via the mannose receptor is dependent on macrophage activation by cytokines. However, while uptake of *C. neoformans* is via both dectin-1 and dectin-2, *C. gattii* uptake occurs largely via dectin-1. Interestingly, dectin inhibitors also blocked phagocytosis of unopsonised Cryptococci in wax moth (*Galleria mellonella*) larvae and partially protected the larvae from infection by both fungi, supporting a key role for host phagocytes in augmenting early disease establishment. Finally, we demonstrated that internalisation of non-opsonised Cryptococci is not accompanied by the nuclear translocation of NFκB or its concomitant production of proinflammatory cytokines such as TNFα. Thus, non-opsonised Cryptococci are recognised by mammalian phagocytes in a manner that minimises proinflammatory cytokine production and potentially facilitates fungal pathogenesis.

INTRODUCTION

Cryptococcus neoformans and *Cryptococcus gattii* are encapsulated human fungal pathogens that cause cryptococcosis in immunocompromised and, more rarely, immunocompetent individuals. Often found as free-living cells in soil and avian excreta, Cryptococci are not intrinsic human pathogens. However, Cryptococci become human pathogens because several defence mechanisms possessed by these fungi also act as virulence factors within a human or animal host (**1**), including the ability firstly, to survive and replicate within free-living soil amoeba and, secondly to evade clearance by the host immune system by hiding and persisting within macrophages (**2, 3**).

As Cryptococci enter hosts via inhalation into the lungs, they are detected and phagocytosed by resident alveolar macrophages (**4**). Phagocytosis is a multi-step process that sequentially involves receptor-mediated particle recognition, actin-driven uptake, phagosome maturation and particle clearance. It is critical during the early innate immune response to ensure the removal of microorganisms and apoptotic cells as well as subsequent priming of the adaptive immune response through the production and release of cytokines, such as Tumour Necrosis Factor-α (TNF-α) (**5**). Phagocytosis of Cryptococci is typically inefficient unless they are opsonised (coated) by antibodies or complement proteins found in serum within the circulatory system. Interestingly, there is a lack of serum opsonins in the alveoli of the lungs, and so the initial uptake of *Cryptococcus* upon colonisation is most likely through a non-opsonised route (**6**).

Non-opsonic phagocytosis requires host cell phagocytic pattern recognition receptors (PRRs) to directly recognise fungal cell wall components (pathogen associated molecular patterns; PAMPs) (**7**) such as β-glucans or mannan polysaccharides, but the nature of this interaction for Cryptococci remains unknown. Here we show that non-opsonised *C. neoformans* and *C. gattii* enter macrophages in a Syk-dependent, mannose receptor-independent manner that involves the receptors Dectin-1 and Dectin-2. This differential uptake of *C. neoformans* and *C. gattii* corresponds to differential exposure of PAMPs found on the fungal cell wall. Phagocytic kinetics of macrophages and insect haemocytes in the

absence or presence of cellular receptor inhibitors were similar in response to fungal targets. Finally, we demonstrate that entry of *Cryptococcus* does not affect NFκB nuclear translocation or subsequent TNF-α release, highlighting the remarkably non-inflammatory capabilities of this organism.

MATERIALS AND METHODS

Reagents

All reagents (e.g. heat-inactivated fetal bovine serum, FBS; Dulbecco's Modified Eagle's Medium (DMEM); L-glutamine; powdered yeast-extract peptone dextrose, YPD and phosphate buffered saline, PBS) were purchased from Sigma unless stated otherwise. Mouse macrophage- and Human granulocyte-macrophage- colony stimulating factors (mM-CSF, 130-094-129 and hGM-CSF, 130-093-862) were purchased from Miltenyi Biotec. Commercially sourced inhibitors tested included the Syk inhibiting plant metabolite, Piceatannol (527948, Calbiochem), the β-1,3-glucan from brown algae *Laminaria digitata*, Laminarin (L9634, Sigma) and mannan from *Saccharomyces cerevisiae* (M7504, Sigma).

The antibodies used in this study were rabbit anti-sheep red blood cells, IgG fraction (#55806, MP Biomedicals), rabbit anti-sheep red blood cells, IgM fraction (CL9000M, VH Bio/Cedarlane), rabbit anti-p65 NFkB monoclonal antibody (clone D14E12, #8242, NEB/Cell Signalling), rat anti-αM (clone 5c6, MCA2289, Bio-Rad/AbD Serotec), rabbit anti-Phospho-Syk (Tyr525/526 in humans, Tyr519/520 in mice, clone C87C1, #2710, NEB/Cell Signalling, a kind gift from Yotis Senis, University of Birmingham). Rhodamine-Phalloidin and Alexa Fluor conjugated secondary antibodies were purchased from Life Technologies and Calcofluor White from Sigma. Glucan-6-phosphate and mouse anti-cryptococcal capsule antibody (clone 18B7) were kind gifts from David Williams (East Tennessee State University) and Arturo Casadevall (Albert Einstein College of Medicine) respectively.

Mice

Mice devoid of specific PRRs (in C57BL/6 background) were published before (**8, 9**) and were housed under pathogen-free conditions in the registered animal facility at the University of Aberdeen. Mice were allocated to experimental groups on the basis of genotype and age-matching. All animal procedures were performed according to the protocols provided by the Animal, Welfare and Ethical

Review Body (AWERB) of the University of Aberdeen and is regulated by the UK Home Office's Animal (Scientific Procedures) Act of 1986 and European Directive 2010/63/EU.

Yeast and bacterial cell growth conditions

C. neoformans strain H99, *C. gattii* strain R265, *Candida albicans* strains SC5314 were incubated in liquid YPD medium for 24hr (unless stated otherwise) at 25^{o}C on a rotator at 20rev/min (or 37^{o}C, 200 rpm for *C. albicans*). *Escherichia coli* strain DH5α was incubated in Luria-Bertani broth for 16hr at 37^{o}C in a shaking incubator at 200rpm. Yeast cells were centrifuged at 3000g for 2.5min (or 6000g for 1min for *E. coli*), washed three times in PBS and counted with a haemocytometer prior to use.

Mammalian cell growth conditions

Cells from the murine macrophage-like cell line J774.A1 (American Type Culture Collection number TIB-67) were cultured in DMEM supplemented with 2mM L-glutamine and 10% heat-inactivated FBS at 37^{o}C, 5% CO_2 (**10**). As required, macrophages were scrapped in PBS, counted and seeded (50 000 per well) onto 13mm acid-washed glass coverslips and incubated for 24hr at 37°C, 5% CO_2 – prior to experimental use.

Macrophages devoid of specific PRRs were derived from mouse bone marrow. Bone marrows were flushed using a 21-gauge needle from the hind leg bones of either receptor knockout or litter-matched wild type mice. Monocytes were differentiated into macrophages with 20ng/ml macrophage-colony stimulating factor (M-CSF, Miltenyi Biotec) for 7 days.

Pooled peripheral blood mononuclear cells (PBMC) were isolated from whole blood from healthy volunteers using density gradient centrifugation with Ficoll-Paque (GE Healthcare). The mononuclear layer was collected and washed with PBS to remove platelets. Monocytes were purified by adherence to plastic in RPMI-1640 media supplemented with 5% heat-inactivated FBS, 2mM glutamine, 100mg/ml streptomycin, 100units/ml penicillin at 37^{o}C, 5% CO_2 for 1hr. Non-adherent cells were

removed with PBS and adherent cells differentiated into macrophages with 20ng/ml recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF, Miltenyi Biotec) for 7 days. This study was covered by the University of Birmingham's Science, Technology, Engineering and Mathematics Ethical Review Committee.

Phagocytic challenge

Macrophages were serum starved for 2 – 16hr with serum-free medium at 37^{o}C, 5% CO_2. Where needed, inhibitors were added directly and left for a further 30min. Next, media was removed prior to fresh serum-free medium added containing either 1μg/ml 18B7 antibody (against cryptococcal capsule) or unopsonised targets at a multiplicity of infection (MOI) of either 10:1 or 20:1 for 20-180min at 37°C, 5% CO_2. Cells were washed three times with PBS to remove unbound yeast/bacteria cells and fixed in 4% paraformaldehyde for 10min at room temperature.

***Galleria mellonella* maintenance**

Larvae of the greater wax moth, *G. mellonella*, were sourced from Livefoods Direct (UK) and stored in wood shavings in the dark at 13^{o}C. This study was covered by the University of Stirling's Animal, Welfare and Ethical Review Body (AWERB). Healthy larvae weighing between 0.2 and 0.4 g were used in all experiments. Larvae were inoculated with different concentrations of inhibitors via intrahaemocoel injection 1hr prior to infection with 1 million *C. neoformans* H99 per larva as described previously (**11**). Controls consisted of larvae that received a 20μl PBS inoculum. 3-5 larvae were used per treatment, with all treatments being performed on at least three independent occasions.

For phagocytosis, larvae were bled and haemolymph treated as previously described (**12**). Briefly, pooled haemolymph was mixed with 0.5ml PBS and added onto a 13mm coverslip in a 24-well plate. Haemocytes were centrifuged onto the coverslips for 10 min at 500 x g at room temperature (RT),

before washing 3 times with PBS to remove non-internalised yeasts. Cells were then fixed with 4% paraformaldehyde before permeabilisation and immunostained as described below. All determinations were performed on at least three independent occasions.

Immunofluorescence and scoring

Fixed cells on coverslips were permeabilised with 0.1% Triton-X-100 for 5 min (if necessary to identify internalised yeasts), washed with PBS and blocked with 0.5% bovine serum albumin (BSA) in PBS for 30 min. Appropriate primary antibodies (1:200 dilution) were added to cells, left for 30 min at room temperature, washed with PBS, and counter stained with the appropriate fluorophore-conjugated secondary antibody, along with Rhodamine-Phalloidin and Calcofluor White. Coverslips were then washed in PBS and distilled water before mounted in ProLong Gold antifade reagent (Life Technologies) and analysed by microscopy.

For counting of phagocytosed yeast/bacteria, fixed but unpermeabilised cells on coverslips were stained with Calcofluor White to highlight the external yeasts. Coverslips were analysed with a Nikon Eclipse Ti microscope under a 63x oil immersion objective. Between 5 – 10 fields of view of each coverslip were counted for number of macrophages and association of microbial cells. At least 100 macrophages were observed for each cover-slip.

The enrichment in phosphorylated Syk at sites of yeast binding and the translocation of p65 into the nucleus during NFκB activation were studied and scored by the Nikon A1R confocal microscope using 20x – 63x objectives. For the former, a minimum of 25 infected cells per condition were analysed for a discrete local enrichment in marker signal (Syk) at bound particles. For the latter, between 3-5 fields of view for each sample/coverslip were counted for the number of macrophages with p65 marker signal located within the nucleus and expressed as a percentage of the total number of macrophages (%NFκB nuclear translocation).

197

198 ***In vitro* cytokine production**

199 J774.A1 and primary human macrophages were cultured in 96-well microtiter plates (Greiner) at 10000

200 cells/well, in a final volume of 200µl. Cells were stimulated with either control medium or LPS or a

201 range of unopsonised pathogenic yeasts. After 6hr of incubation at 37^{o}C, plates were centrifuged (500g

202 for 10min), and the supernatant was collected and stored at -80^{o}C until cytokine assays were

203 performed. Levels of TNF-α were determined by commercial ELISA kits, used according to the

204 instructions of the manufacturer (R&D Systems).

205

206 **Statistical analyses**

207 Analysis carried out on the results described in this paper was by a generalise linear model (GLM)

208 using a Poisson error distribution in R (R Development Core Team). This was tested for significance

209 using a Posthoc Tukey Honesty Significant Difference (HSD) test.

210

211 **RESULTS**

212 **Uptake of non-opsonised Cryptococci via mannose receptor is activation dependent**

213 As previously observed, the levels of non-opsonic uptake of Cryptococci is very low (e.g. 0.4% of *C.*

214 *neoformans* serotype D was taken up by unstimulated mouse peritoneal macrophages; or 7-21% of *C.*

215 *gattii* R265 was taken up by human dendritic cells; **13, 14**) and our results are in agreement with those

216 findings – 8.89% or 5.83% of primary human macrophages contained one or more *C. neoformans* H99,

217 or *C. gatii* R265, respectively (based on the carrier controls in **Figures 1C** and **2B**) after two hours of

218 incubation. The mannose receptor is broadly expressed on macrophages and important for the non-

219 opsonic uptake of fungal pathogens such as *Candida albicans* and *Pneumocystis carinii* (**15, 16**). The

220 uptake of *C. neoformans* H99, or *C. gatii* R265 by J774.A1 macrophages pre-treated with soluble

mannan (a competitive inhibitor of mannose receptor binding) was unaltered relative to control (untreated) cells (**Figure 1A**). Similarly, M-CSF differentiated bone marrow macrophages from mannose receptor knock-out mice (MR KO) showed no reduction in uptake of either *Cryptococcus neoformans* (Cn) or *Cryptococcus gattii* (Cg) relative to wildtype control cells (**Figure 1B**). Interestingly, however, GM-CSF differentiated primary human macrophages showed a strong inhibition of uptake under the same conditions (**Figure 1C**), suggesting that the mannose receptor may play a greater role in cryptococcal uptake into human cells than those of mice.

Phagocytosis of unopsonised Cryptococci is Syk-dependent

The other major class of non-opsonic phagocytic receptors for fungi are the dectins (**17**). Both dectin-1 and dectin-2 require Syk activity for their function, via immunoreceptor tyrosine-based activation motifs (ITAM) contained within dectin-1 itself or via membrane association with ITAM-containing Fc receptor γ chain in the case of dectin-2 (**18**). Inhibiting Syk activity in J774.A1 cells by using piceatannol (**19**) resulted in a marked reduction in their ability to phagocytose either *C. neoformans* H99 or *C. gattii* R265 (**Figure 2A, $p<0.05$**). The same observation was also seen in GM-CSF differentiated primary human macrophages from pooled monocytes isolated from human volunteers (**Figure 2B**). In line with this, staining with an anti-Phospho-Syk antibody showed intense accumulation of active Syk at phagocytic cups forming around non-opsonised Cryptococci (**Figure 3**). This antibody was raised against the tyrosine phosphorylated residues at positions 525 and 526, located in the activation loop of the Syk kinase domain and essential for Syk function (**20**). Therefore, we propose that the localisation of this antibody to the sites of non-opsonic uptake of Cryptococci and the activity of piceatannol in blocking uptake suggests that Syk activity is required for internalisation.

Phagocytosis of unopsonised Cryptococci is partially dependent on Dectin-1

To test for a role for the dectin family of receptors during phagocytic uptake of non-opsonised *Cryptococci*, we first exposed J774.A1 macrophages (**Figure 4A**) or differentiated primary human macrophages (**Figure 4B**) to the dectin-1 inhibitor glucan-6-phosphate before challenging with either unopsonised *C. gattii* R265 or *C. neoformans* H99. This inhibitor partially blocked the uptake of both species of *Cryptococcus*, suggesting dectin-1 contributes towards Cryptococci uptake but is not the sole recognition receptor involved in this process (**Figures 4A** and **4B**). In line with this, M-CSF-differentiated bone marrow macrophages (BMM) from dectin-1 and dectin-2 knockout mice both showed substantially impaired uptake of *C. neoformans* H99 – surprisingly, this was not the case for *C. gattii* R265 (**Figure 4C**). This suggests either the presence of another Syk-dependent non-opsonic receptor, or that both dectins are redundant with each other for *C. gattii*, but not *C. neoformans* uptake.

Non-opsonic uptake in the *Galleria* model

The greater wax moth, *Galleria mellonella*, is widely used as a model organism in the study of host-pathogen interactions with a variety of human pathogens (**21**). As with other insects, *G. mellonella* does not possess an adaptive immune system like mammals but possesses a complex innate immune system that includes phagocytic cells, termed haemocytes (**22, 23**). We therefore tested whether non-opsonic uptake of Cryptococci in *G. mellonella* showed similar receptor dependency as in mammalian cells by pre-treating larvae for 1hr with soluble mannan or glucan-6-phosphate or laminarin. The full genome sequence of *Galleria* is currently available though not fully annotated (**24**). However, several β-1,3-glucan binding protein analogues and C-type lectins have been characterised in this species, as well as other Lepidopterans, namely *Manduca sexta* (**25, 26**), *Bombyx mori* (**27**) and *Plodia interpunctella* (**28**). Recognition of fungal PAMPs (e.g. curdlan and mannan) by membrane bound receptors modulate cellular (haemocyte)-directed immunity in insects (encapsulation, nodulation and phagocytosis) (**29**). Whilst soluble mannan did not significantly reduce association of Cryptococci with *Galleria*

haemocytes in data presented here, both glucan-6-phosphate and laminarin led to a marked reduction in uptake (**Figure 5,** $p<0.001$ for both when compared to the PBS controls).

Interestingly, administering glucan-6-phosphate or laminarin for 24 h appeared to partially protect the insect larvae from infection by both unopsonised species of *Cryptococcus* (**Figure 6**) suggesting that disease establishment in this model organism requires the fungus to grow intracellularly, something that has previously been proposed for human hosts (**30**).

Cryptococcal uptake by macrophages does not lead to increased proinflammatory cytokine secretion

Unlike many pathogens, internalisation of opsonised Cryptococci into phagocytes is not accompanied by the production of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1α or IL-1β (**31, 32**). To test whether this is also true of non-opsonic uptake, we measured the secretion of TNFα and nuclear translocation of p65 (a major regulator of cytokine transcription) from J774.A1 macrophages upon challenge with unopsonised or serum-opsonised *C. neoformans* H99 or *C. gattii* R265. Although LPS-stimulated macrophages showed strong nuclear translocation of p65, neither IgG-opsonised nor unopsonised *C. neoformans* H99 or *C. gattii* R265 stimulated NFκB activation (**Figure 7A**). However, NFκB activation could be restored in cryptococcal exposed macrophages by the subsequent addition of LPS (**Figure 7B**).

Furthermore, to test whether internalisation of unopsonised Cryptococci into J774.A1 mouse macrophages or primary human macrophages elicits the production of proinflammatory cytokines such as tumor necrosis factor (TNF), we measured the secretion of TNFα from J774.A1 macrophages or primary human macrophages upon challenge with unopsonised *C. neoformans* H99 or *C. gattii* R265, with *Candida albicans* and LPS as controls. With J774.A1 mouse macrophage and primary human macrophages, *C. albicans*- or LPS- stimulated macrophages showed stronger TNFα production

compared to varying doses of *C. neoformans* H99 or *C. gattii* R265 (**Figure 8**; $p = 0.04$ for Ca vs. media control, $p > 0.05$ for Ca vs. Cn/Cg). Overall this suggests that Cryptococci do not actively block inflammatory signalling in host cells, and do not induce a strong inflammatory stimulus following non-opsonic uptake.

DISCUSSION

In this study, we examined the phagocytic uptake of unopsonised cryptococcal yeast particles by macrophages. This process relies on the use of phagocytic receptors, which can be categorised either as opsonic or non-opsonic. Opsonic phagocytic receptors include the Fc receptor and complement receptor families, which recognises antibody- or complement- opsonised (coated) particles, respectively. Non-opsonic phagocytic receptors are pattern recognition receptors (PRRs), such as the C-type lectin family of receptors recognizes distinct pathogen-associated molecular patterns (PAMPs) on the fungal surface (**33**).

While phagocytosis of *Cryptococcus* within the circulatory system would occur predominantly through an opsonised (coated) uptake route due to the presence of antibodies and/or complement proteins found in serum, this is not always the case. For example, first encounter of the human body with *Cryptococcus* is through the lungs when desiccated yeast cells or spores are breathed in. These cryptococcal particles encounter their initial immunological challenge through resident alveolar macrophages and dendritic cells in a serum-deficient or low-serum environment (**34, 35, 36**). Interestingly, it was reported recently that between 25-40% of mouse lung-resident macrophages are able to phagocytose *C. neoformans* particles through a scavenger receptor pathway (**37**). Therefore, this confirms that initial uptake of *Cryptococcus* by macrophages is most likely through a non-opsonised route and there is a need to understand the mechanisms that underpins this process (**6**). We confirmed that, compared to the bacterium *Escherichia coli* or fungus *Candida albicans*, Cryptococci cells are not readily taken up by mammalian macrophages, most likely due to the presence of the capsule which

renders Cryptococci anti-phagocytic (**38, 39**). By using a combination of a soluble mannose inhibitor and mannose receptor knockout mouse tissue, we demonstrated that mannose receptor was not necessary for the uptake of either species of *Cryptococcus*, in line with recent data from the zebrafish model (**40**), though this is not the case in primary human macrophages. We note that others have shown mannose receptor knockout mice to be more susceptible to *C. neoformans* (**41**) and demonstrated a role for this receptor, along with FcγRII (CD32) in driving cryptococcal uptake into dendritic cells (**42**). Thus, mannose receptor dependency apparently varies across different cell types and tissue contexts.

Next, we pursued a different set of non-opsonic pattern recognition receptors, dectin-1 and dectin-2 which are C-type lectin receptors (CLRs) that are highly expressed in macrophages and are key β-glucan receptors (**43, 44**). Recognition of soluble or surface expressed β-glucans on yeasts is sufficient to initiate and mediate phagocytosis and pro-inflammatory cytokine responses (**45**). Both of these receptors require Syk activity (**46, 47, 18**) and indeed our data clearly demonstrate the activation of Syk at phagocytic cups containing unopsonised Cryptococci, as well as a strong dependency on Syk for particle uptake. Interestingly, pharmacological inhibition of dectins inhibited uptake of both *C. neoformans* and *C. gattii* in J774.A1 mouse and human macrophages, but bone marrow macrophages (BMM) from dectin-1- and dectin-2- knockout mice showed defects only in the uptake of *C. neoformans* and not *C. gattii,* an effect that has been observed before (**48**). The most parsimonious explanation is therefore that the two dectin receptors are redundant for the uptake of *C. gattii*, but not *C. neoformans*, perhaps reflecting differing the surface components between the two species as reported recently (**49**). Such surface variation between species, strains and potentially developmental stages of Cryptococci may explain many of the previous inconsistencies in the literature regarding dectin dependency (or otherwise) (**50, 51**).

Alongside mouse macrophages, we adopted wax worm larvae (*Galleria mellonella*) as an alternative model for understanding cryptococcal virulence and host immune responses (**52 – 54**) in which

cryptococcal phagocytosis has previously been reported (**55**). Our data demonstrate striking similarities in patterns of uptake between this invertebrate host and murine phagocytes. In addition, we showed that inhibiting phagocytosis in this alternative host reduces disease burden, highlighting the importance of host phagocytes as a niche for cryptococcal replication.

We acknowledge that while there are currently no direct dectin receptor homologues identified in *G. mellonella*, many C-type lectins have been characterised in other insect models, for example the tobacco hornworm, *Manduca sexta* (immulectin-2 facilitates phagocytosis of bacteria; **56**), webworm, *Hyphantria cunea* (lectin; **57, 58**) silkworm, *Bombyx mori* (BmLBP and BmMBP; **59, 60**) and the cockroach, *Blaberus discoidalis* (a β-glucan-specific lectin; **61**). These invertebrate C-type lectins show up to 35% similarity with mammalian C-type lectins and can bind to several PAMPs, including LPS, LTA and β-glucan and are inducible when the host is exposed to microbial challenge or ligands and the mechanisms for uptake of pathogenic microbes by *G. mellonella* hemocytes are similar to that of human neutrophils (**62**).

Two key reports have shown that there are at least 3 scavenger receptors involved in the recognition of different serotypes of *Cryptococcus neoformans*, namely the homologous genes from the nematode *Caenorhabditis elegans*, CED-1 and C03F11.3 as well as the mouse MARCO scavenger receptors (**37, 63**). Interestingly, knocking out MARCO gene from mice did not abolish uptake of *Cryptococcus neoformans* by lung-resident mononuclear phagocytes (**37**), suggesting role(s) for the extent and distribution of multiple receptors and ligands on the surface of both host cell and yeast.

Finally, we demonstrate that entry of *Cryptococcus* does not affect NFκB nuclear translocation and its subsequent TNF-α release in the Dectin-1/Syk/NFκB signalling axis – both in J774.A1 mouse macrophages and in primary human macrophages. Although it is known that Dectin-1 coupling to Syk leads to downstream activation of NFκB, which coordinate the transcription of innate response genes

including expression of proinflammatory cytokines such as TNF-α (**64-66**), this appears not to be the case for cryptococcal uptake.

In conclusion, we propose that unopsonised Cryptococci are recognised and engulfed via mannose receptor- or dectin-based recognition *in vitro* depending on the activation state of the host cells. The absence of an associated pro-inflammatory cascade allows the yeast to exploit this intracellular niche for rapid disease establishment.

ACKNOWLEDGEMENTS

We thank Neil Hotchin, Yotis Senis, David Williams and Arturo Casadevall for sharing reagents and members of the Krachler/Voelz/Hall/May lab for helpful scientific discussions as well as Charles Eliot Timothy Paine for his assistance with the statistical analysis. JL would like to thank his family for their help and support during the writing of this manuscript.

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559 **65.** Goodridge, H. S., R. M. Simmons, and D. M. Underhill. 2007. Dectin-1 stimulation by Candida
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566
567 **FIGURE LEGENDS**
568 **FIGURE 1.** Mannose receptor is important but dispensable during uptake of *Cryptococcus* particles.
569 Mouse macrophage cell line J774.A1 (**A**) or differentiated bone marrow macrophages (**B,** wildtype,
570 WT or mannose receptor knock out, MR KO) or differentiated primary human macrophages (**C**) were
571 challenged with either *Cryptococcus neoformans* H99 (Cn, black bars) or *Cryptococcus gattii* R265
572 (Cg, white bars) for 60min, processed for immunofluorescence and scored for phagocytosis as
573 described in Materials and Methods. Where indicated, J774.A1 and primary human macrophages were
574 pretreated with 100μg/ml mannan (MAN) for 30min before the addition of *Cryptococcus* particles.
575 Phagocytosis indices were related to the values obtained from the negative controls. Number in bars
576 indicate the total number of phagocytes counted. Results are expressed as the mean± SD of at least
577 three independent experiments.
578
579 **FIGURE 2**: Uptake of *Cryptococcus* particles is Syk dependent. Mouse macrophage cell line J774.A1
580 (**A**) or differentiated primary human macrophages (**B**) were challenged with unopsonised *Cryptococcus*
581 *neoformans* H99 (Cn, black bars) or *Cryptococcus gattii* R265 (Cg, white bars) for 60 min, processed
582 for immunofluorescence and scored for phagocytosis as described in Materials and Methods.
583 Phagocytosis indices were related to the values obtained from the negative controls. Number in bars

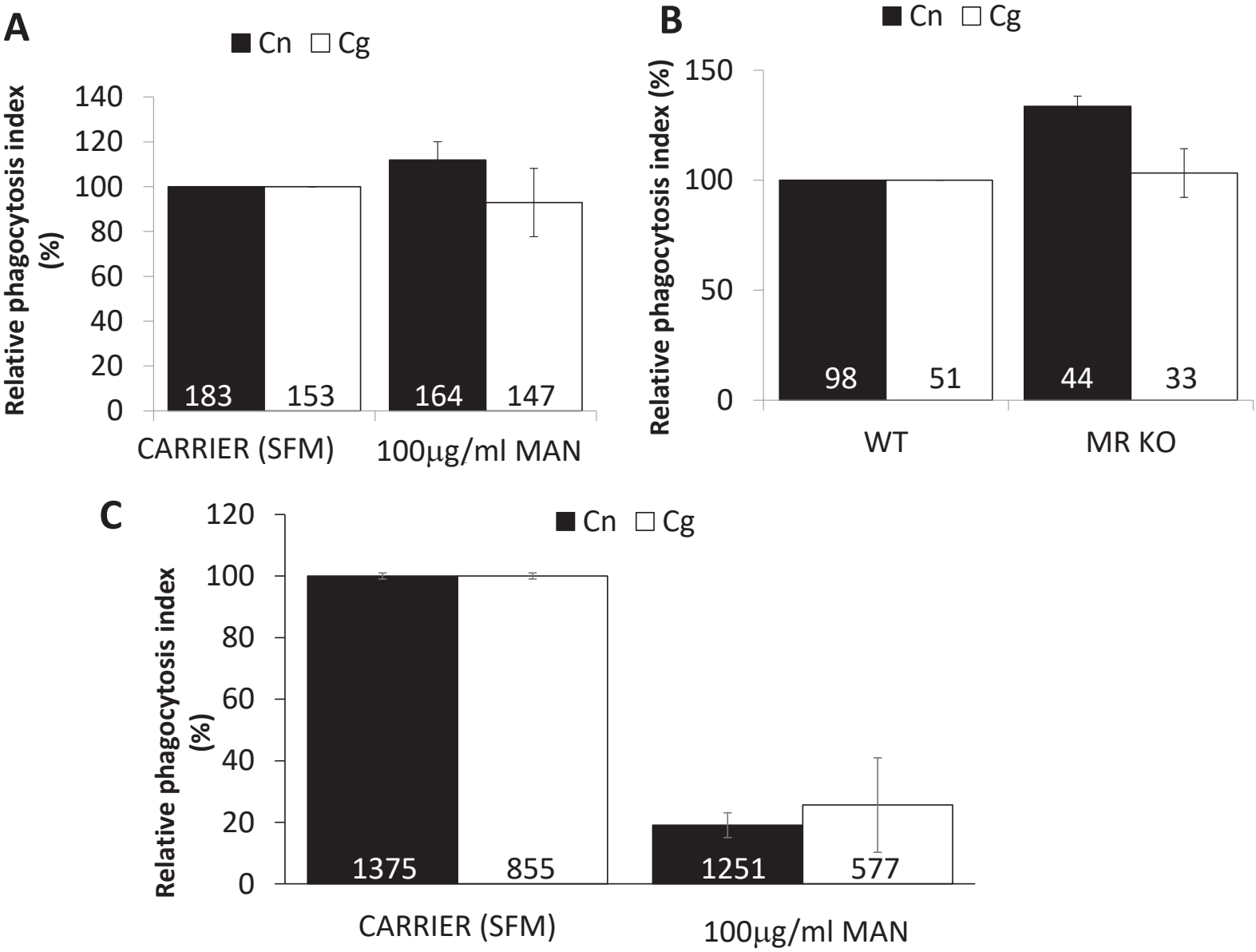
584 indicate the total number of phagocytes counted. Results are expressed as the mean± SD of at least
585 three independent experiments.
586
587 **FIGURE 3.** Activated Syk is essential for the uptake of *Cryptococcus* particles. Mouse macrophage
588 cell line J774.A1 was challenged with either (IgG-opsonised or unopsonised, U/O) *Cryptococcus*
589 *neoformans* H99 or *Cryptococcus gattii* R265 for 15min (**B**), processed for immunofluorescence and
590 analysed by confocal microscopy of localised phospho-Syk (**B, C**) as described in Materials and
591 Methods. (**A**) Schematic diagram J774.A1 macrophage with intracellular actin cytoskeleton (red) and
592 yeast particles (blue). To confirm phospho-Syk localisation, the bottom of the cells was observed first
593 (**A**, green dash line and **B**, bottom panels), before moving to the middle of the cells (**A**, purple dash
594 line, **B**, top panels). Pixel intensities for 20 cells per sample were determined (**C**, right) and normalised
595 to the intensity at the centre of the cell (**C**, left). Results are expressed as the mean± SD of at least three
596 independent experiments.
597
598 **FIGURE 4.** Dectins are required for uptake of *Cryptococcus* particles. Mouse macrophage cell line
599 J774.A1 (**A**), differentiated primary human macrophages (**B**) or differentiated bone marrow
600 macrophages (**C**, wildtype, WT or Dectin-1 or Dectin-2 knockout, KO) were challenged with either
601 *Cryptococcus neoformans* H99 (Cn, black bars) or *Cryptococcus gattii* R265 (Cg, white bars) for 60
602 min, processed for immunofluorescence and scored for phagocytosis as described in Materials and
603 Methods. Where indicated, J774.A1 were pretreated with 100μg/ml glucan-6-phosphate (G6P) for
604 30min before the addition of *Cryptococcus* particles. Phagocytosis indices were related to the values
605 obtained from the negative controls. Number in bars indicate the total number of phagocytes counted.
606 Results are expressed as the mean± SD of at least three independent experiments. ns, not significant, $p \geq 0.05$; *, $p < 0.05$
607
608

FIGURE 5. Administration of polysaccharides blocks uptake of *Cryptococcus* particles to hemocytes in the *Galleria mellonella* larvae model. Larvae were inoculated with 60μg of blocking sugars **1hr** prior to infection for **2hr** with 10^6 *Cryptococcus neoformans* H99 (black bars) or *Cryptococcus gattii* R265 (white bars). Uptake of yeast of hemocytes was determined under light microscopy. Results are expressed as the mean± SD of at least three independent experiments. ns, not significant, $p \geq 0.05$; *, $p < 0.05$ (related to PBS control)

FIGURE 6. Glucan administration protects *Galleria mellonella* larvae from infection by *Cryptococcus neoformans* or *Cryptococcus gattii*. Larvae were inoculated with 60μg of blocking sugars **24hr** prior to infection for further **24hr** with 10^6 *Cryptococcus neoformans* H99 (black bars) or *Cryptococcus gattii* R265 (white bars). Fungal load was determined by serially diluting homogenized larvae and plating aliquots onto erythromycin containing agar plates. Yeast cell density were related to the values obtained from the negative (PBS) controls and expressed as cfu $*10^5$/larva. Results are expressed as the mean ± SD of at least three independent experiments. ns, not significant, $p \geq 0.05$; *, $p < 0.05$ (related to PBS control)

FIGURE 7. Uptake of *Cryptococcus* did not affect NFκB nuclear translocation. J774.A1 macrophages were challenged with a variety of opsonised or unopsonised pathogenic fungi or sheep red blood cells (SRBC) or soluble agonists (LPS or PMA), processed for immunofluorescences and analysed by microscopy (**A**) and scored for p65 nuclear translocation (**B**), as described in Materials and Methods. (**A**) Representative images of PMA- (top) or LPS- (bottom) stimulated J774.A1 macrophages and stained to highlight either actin or p65. Bar, 20μm.

FIGURE 8. Uptake of *Cryptococcus* did not affect proinflammatory cytokine response. J774.A1 macrophages (**black bars**) or differentiated primary human macrophages (**white bars**) were challenged with a variety of unopsonised pathogenic fungi (*Cryptococcus neoformans*, Cn; *Cryptococcus gattii*. Cg; *Candida albicans*, Ca) or LPS and subsequent supernatants were analysed by ELISA, as described in Materials and Methods. Results are expressed as the mean ± SD of at least three independent experiments. *, $p < 0.05$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Cn
Cg
Relative phagocytosis index (%)
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120
100
80
60
40
20
0
183
153
164
147
CARRIER (SFM)
100μg/ml MAN
B
Cn
Cg
Relative phagocytosis index (%)
150
100
50
0
98
51
44
33
WT
MR KO
C
Cn
Cg
Relative phagocytosis index (%)
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100
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60
40
20
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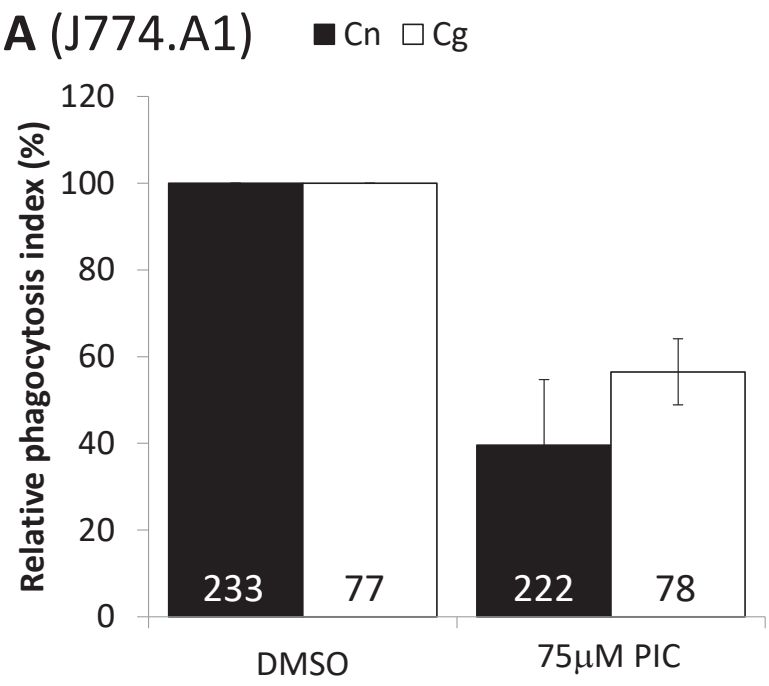
855
1251
577
CARRIER (SFM)
100μg/ml MAN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Cn
Cg
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100
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60
40
20
0
233
77
222
78
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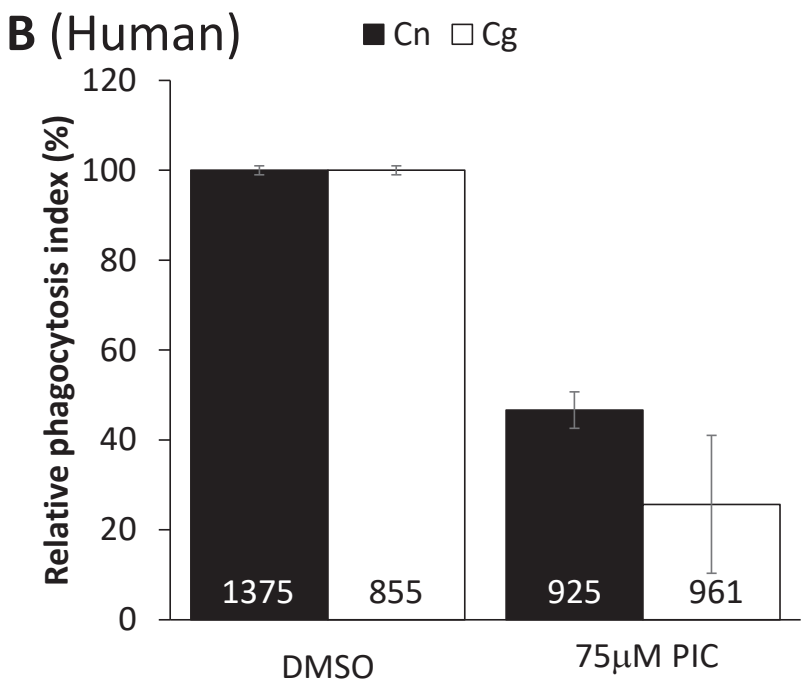
75μM PIC


B (Human)
Cn
Cg
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20
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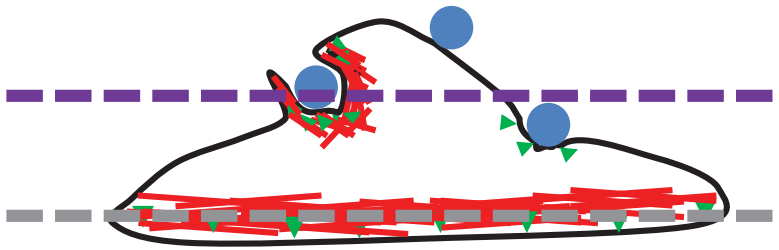
855
925
961
DMSO
75μM PIC

A



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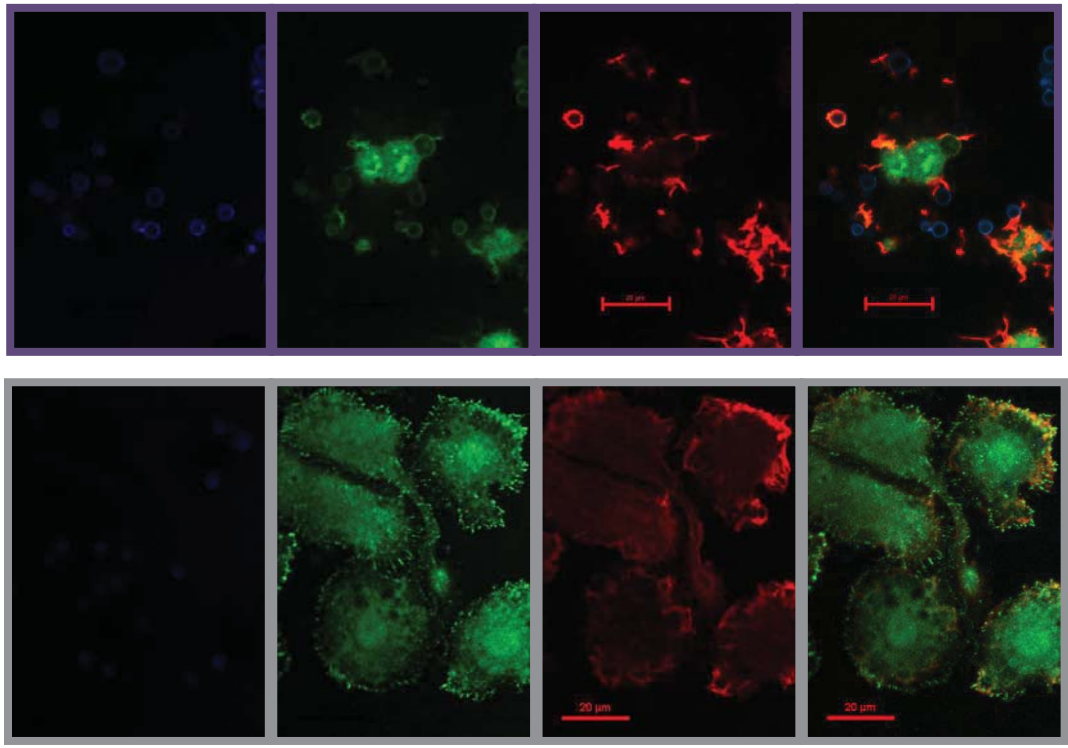

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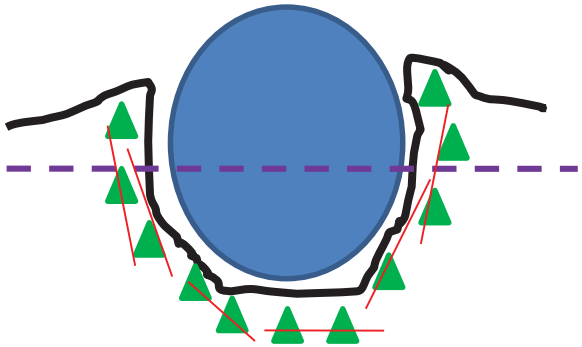

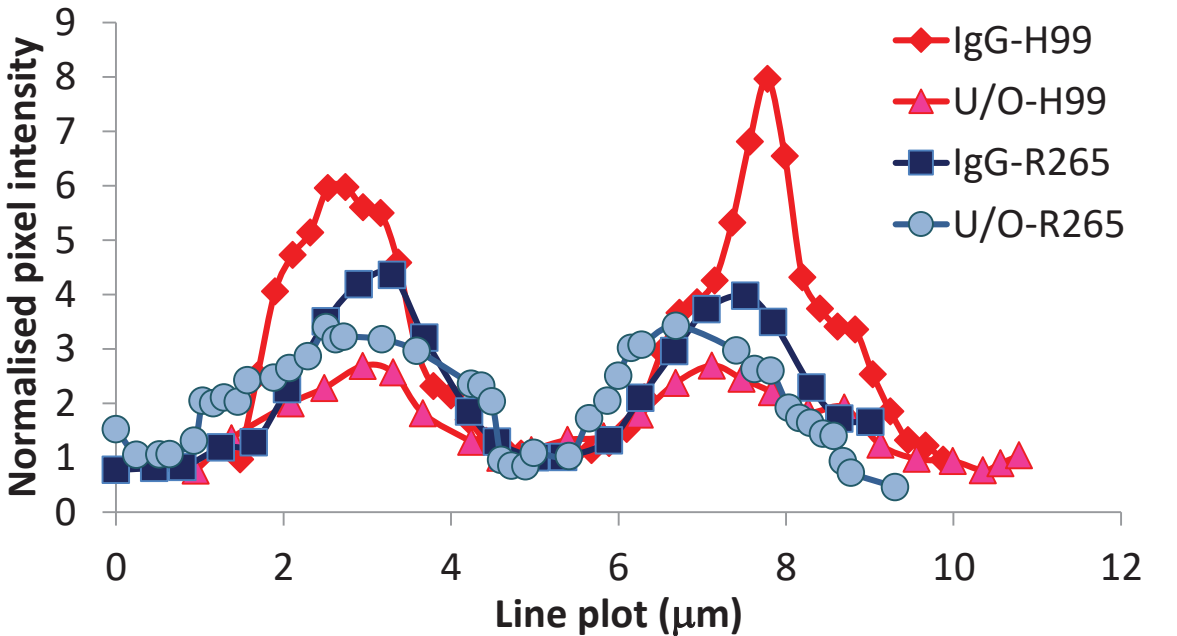
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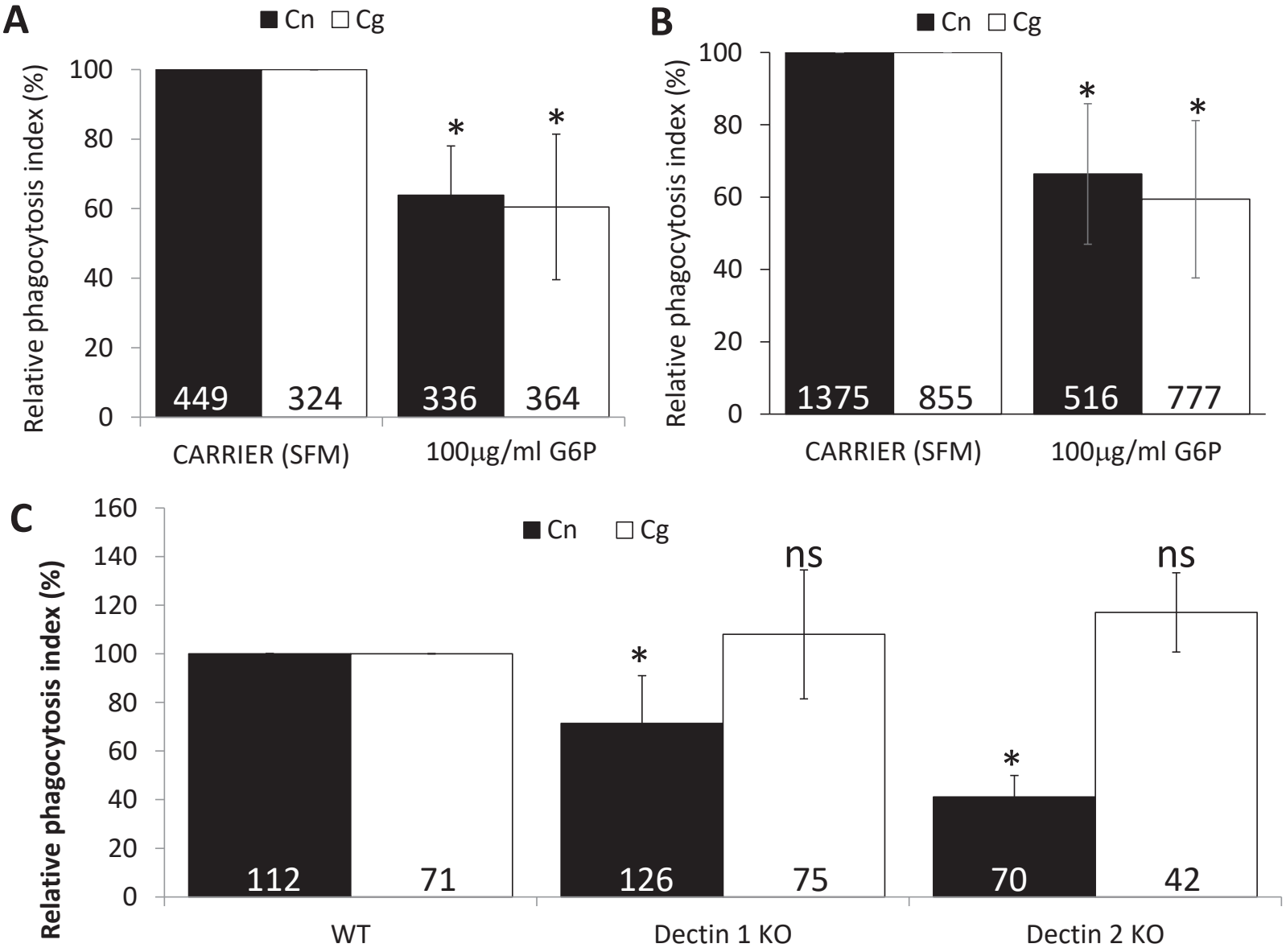
IgG-H99
U/O-H99
IgG-R265
U/O-R265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Cn
Cg
Relative phagocytosis index (%)
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60
40
20
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449
324
336
364
*
*
CARRIER (SFM)
100µg/ml G6P
B
Cn
Cg
Relative phagocytosis index (%)
100
80
60
40
20
0
1375
855
516
777
*
*
CARRIER (SFM)
100µg/ml G6P
C
Cn
Cg
Relative phagocytosis index (%)
160
140
120
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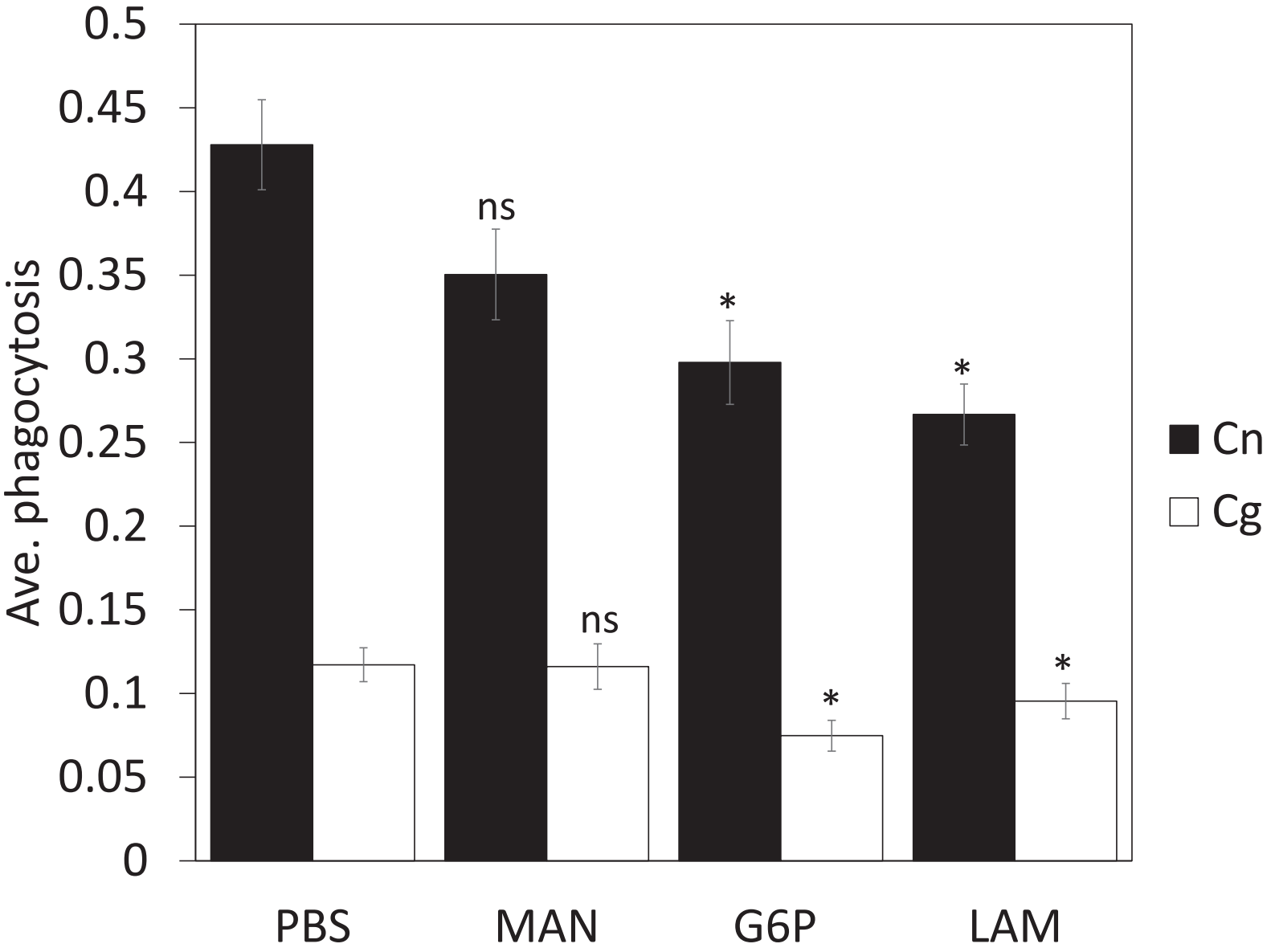
Dectin 1 KO
Dectin 2 KO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0.25
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0.15
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ns
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*
*
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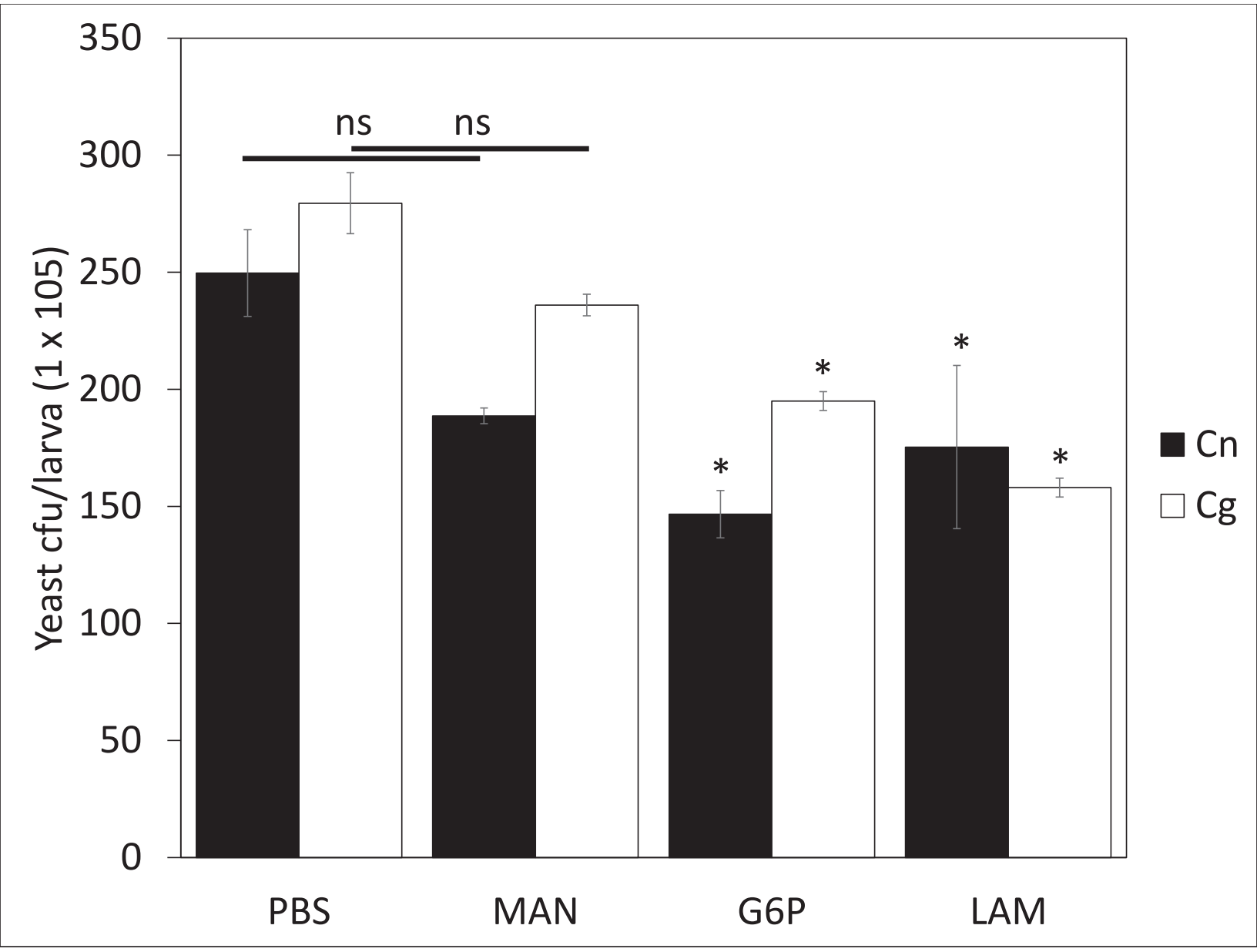
LAM
Cn
Cg


350
300
250
200
150
100
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ns
*
*
*
*
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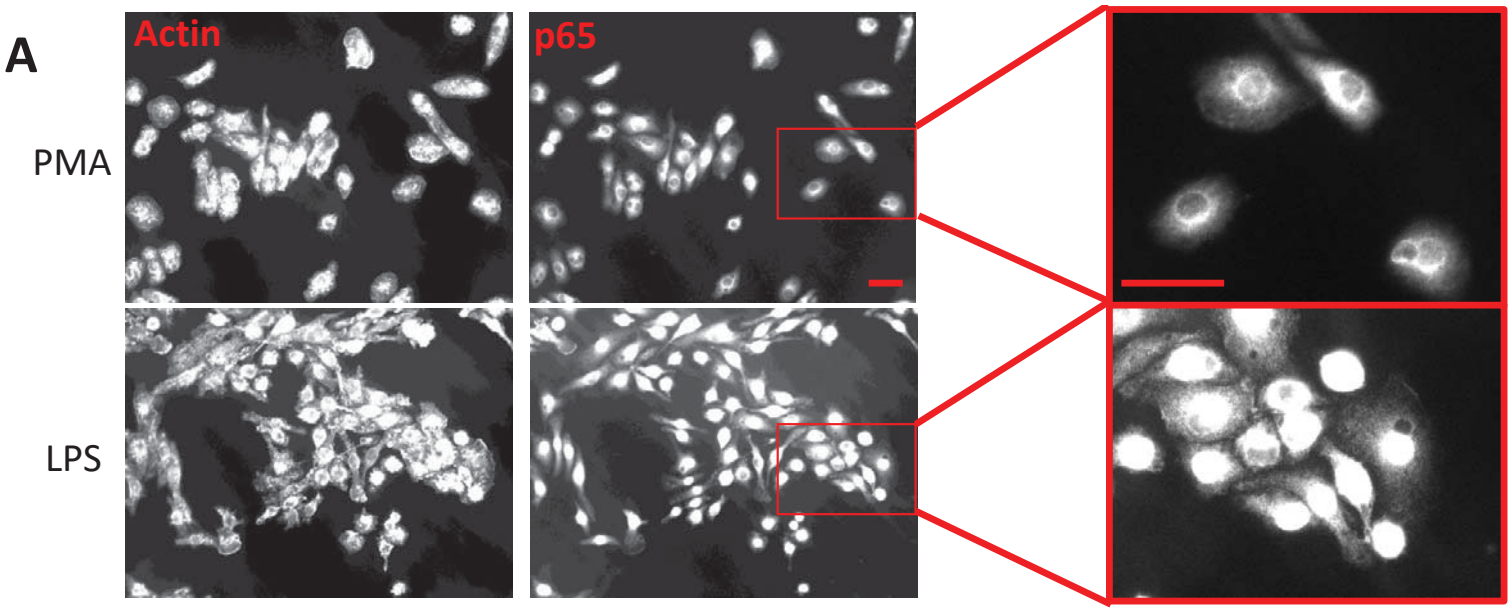
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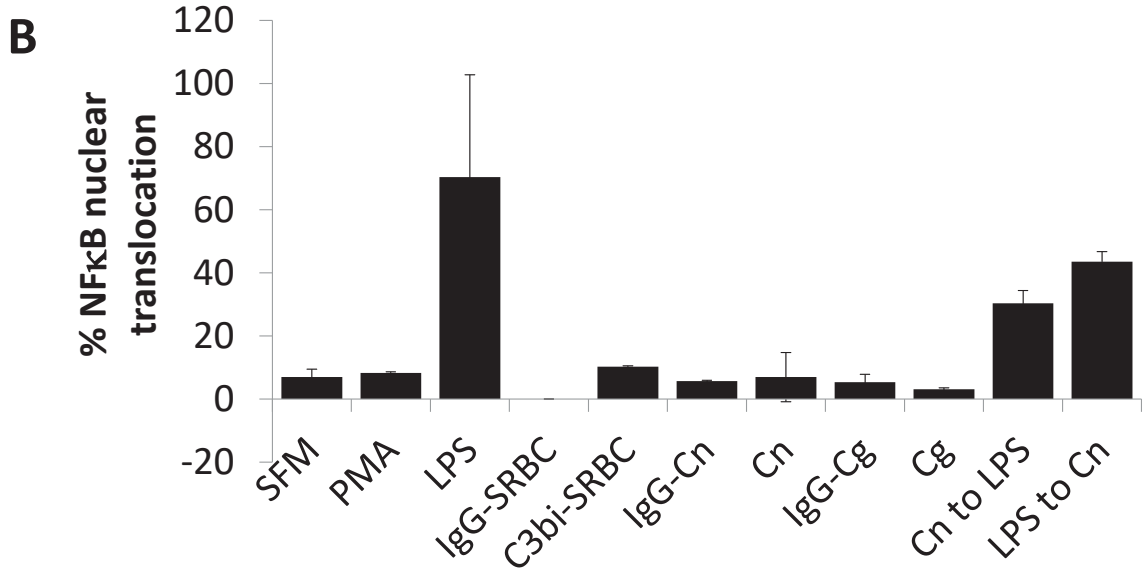
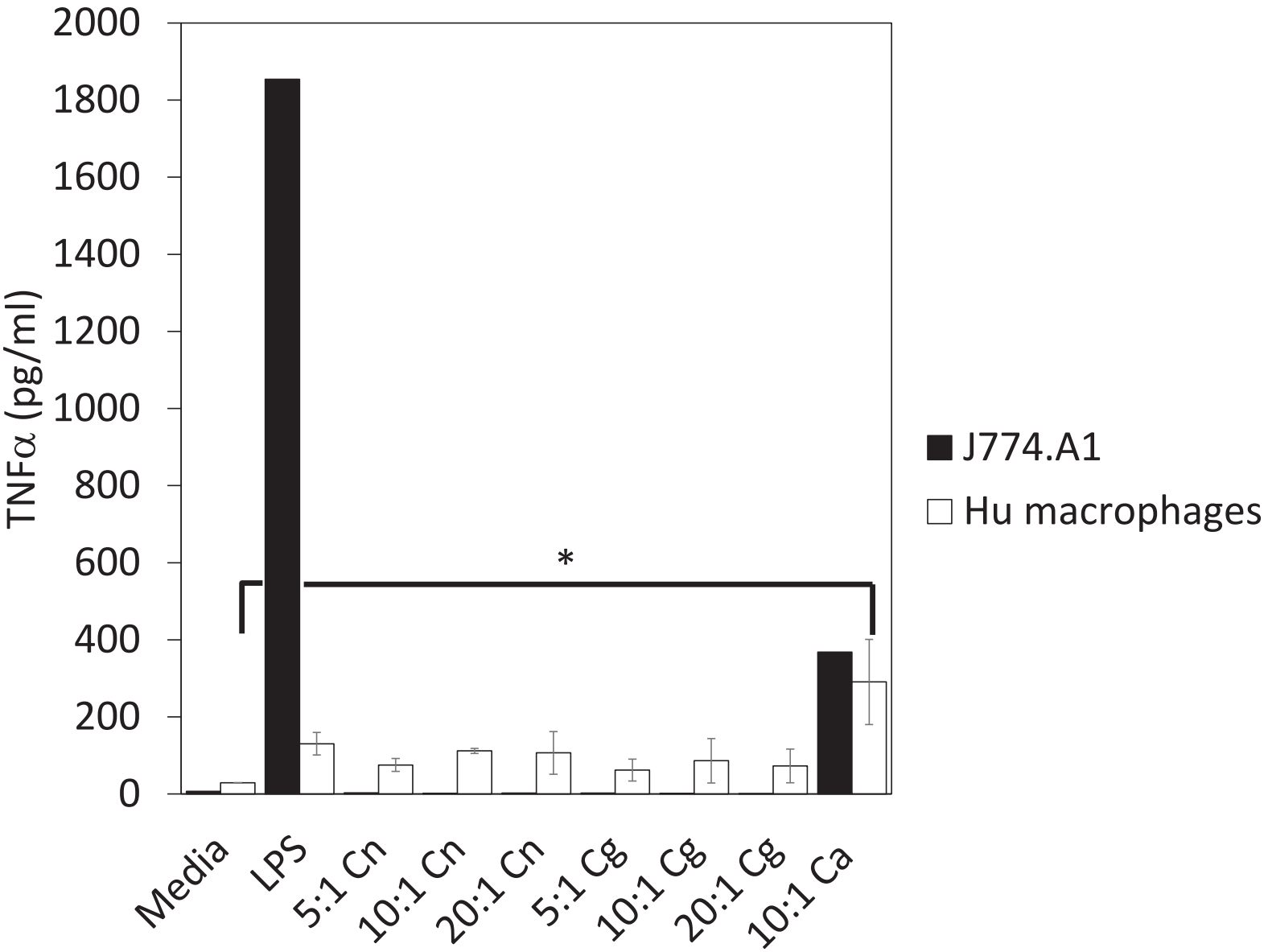
p65
PMA
LPS


B
% NFκB nuclear translocation
120
100
80
60
40
20
0
-20
SFM
PMA
LPS
IgG-SRBC
C3bi-SRBC
IgG-Cn
Cn
IgG-Cg
Cg
Cn to LPS
LPS to Cn


2000
1800
1600
1400
1200
1000
800
600
400
200
0
TNFα (pg/ml)
*
Media
LPS
5:1 Cn
10:1 Cn
20:1 Cn
5:1 Cg
10:1 Cg
20:1 Cg
10:1 Ca
J774.A1
Hu macrophages