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Acanthamoeba proteases contribute to macrophage activation through PAR_1, but not PAR_2.

Running title: *Acanthamoeba* macrophage activation by PAR_1.

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Disclosures

None

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Abstract

Aim *Acanthamoeba* infections are characterized by an intense localized innate immune response associated with an influx of macrophages. *Acanthamoeba* protease production is known to affect virulence. Herein, the ability of *Acanthamoeba* trophozoite proteases, of either the laboratory Neff strain, or a recently isolated clinical strain, to stimulate IL-12 and IL-6 and to activate protease-activated receptors, PAR_1 and PAR_2 expressed on murine macrophages, was investigated.

Method and Results Using selected protease inhibitors, leupeptin and E64, we showed that *Acanthamoeba* proteases can stimulate IL-12 and IL-6 by murine macrophages. Subsequently, using specific antagonists to inhibit PAR_1, and bone-marrow derived macrophages from PAR_2 gene deficient mice, we demonstrate that PAR_1, but not PAR_2

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contributes to macrophage IL-12 production in response to *Acanthamoeba*. In contrast, *Acanthamoeba*-induced IL-6 production is PAR_1 and PAR_2 independent.

Conclusion This study shows for the first time the involvement of PARs, expressed on macrophages, in the response to *Acanthamoeba* trophozoites and might provide useful insight into *Acanthamoeba* infections and their future treatments.

Keywords

Acanthamoeba; Macrophages; Cytokines; Proteases; Protease-Activated Receptors; PAR_1; PAR_2.

1. Introduction

Acanthamoeba castellanii is a facultative parasitic free-living amoeba known to be the agent of a serious, painful and potentially blinding keratitis, named *Acanthamoeba* keratitis (AK), as well as usually fatal encephalitis, named granulomatous amoebic encephalitis (GAE). Immunocompromised individuals are susceptible to the deadly brain infection, whereas AK can occur in immune-competent individuals, and especially contact lens (CL) wearers[(1)]. Among the 20 identified *Acanthamoeba* genotypes, *A. castellanii* T4 is the most common genotype in the environment and the most often associated with AK and non-AK infections as it is frequently isolated from the site of infections[(2)]. This has been attributed to its wide distribution in nature as well as in indoor environments, identified virulence factors, and its relative resistance to drugs and disinfectants[(2)].

The release of proteases by *Acanthamoeba* spp. has been associated with virulence[(3,4)]. After binding mammalian cells, *Acanthamoeba* releases proteases that enhance the penetration of the amoeba into the deeper layers of the host tissue[(5)]. As proteases are of major importance

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in the pathogenic process, the pattern of their secretion is commonly used to discriminate between pathogenic and non-pathogenic strains of *Acanthamoeba*[6].

Protease-activated receptors (PARs) are trans-membrane domain G-protein-coupled receptors activated by the proteolytic activity of endogenous and exogenous proteases[7]. PARs are expressed in a ubiquitous manner in various immune, as well as non-immune cells, and have a wide range of physiological functions associated with the maintenance of tissue integrity, as well as with inflammatory and immunological responses[8]. Interestingly, PARs, especially PAR_1 and PAR_2, are expressed on human corneal epithelial (HCE) cells, predominantly in the outer cell layers of the corneal epithelium, and their activation leads to production of pro-inflammatory cytokines, as well as wound healing processes[9]. Consequently, the role of innate immune receptors in *Acanthamoeba* recognition and response at the ocular surface has been an area of recent interest and it has been demonstrated that *Acanthamoeba* plasminogen activator factor stimulates, in HCE cells, the production of IL-8 in a PAR_2 dependent, but PAR_1 independent manner[10]. Once *Acanthamoeba* trophozoites reach the deepest layers of the tissues, macrophages as well as neutrophils are the predominant cell types present at the site of infection[11], where they are found alongside trophozoites and cyst forms. Macrophages are therefore considered the major cells involved in *Acanthamoeba* clearance[6]. Although macrophages are heavily recruited during the development of both GAE and AK, little is known about how *Acanthamoeba* influences macrophage function. Our recent studies have demonstrated the role of TLR4, MyD88 dependent events in the activation of murine macrophages[12]. The potential role of proteases released by *Acanthamoeba*, and their interaction with PARs expressed by macrophages have not until now been reported.

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2. Materials and Methods

2.1 *Acanthamoeba* strains

In this study we examined the effects of co-cultivation of trophozoites of either a laboratory strain of *Acanthamoeba* (Neff), isolated from soil over 60 years ago, and kindly donated by the late Prof. Keith Vickerman (University of Glasgow, United Kingdom), or a clinical isolate (clinical) of *Acanthamoeba* with murine bone marrow-derived macrophages (MBDM). Both *Acanthamoeba* strains are of the T4 genotype[13]. Trophozoites of either strain were maintained in culture and prepared for the co-incubation experiments as previously reported[12].

2.2 Ethical background

All animal care and experimental procedures were conducted in accordance with relevant guidelines and regulations with the approval of the University of Strathclyde Animal Welfare and Ethical Review Body (AWERB), under UK Home Office regulations (Animals (Scientific Procedures) Act 1986. Animals were housed according to or above the standard of the Home Office Code of Practice for the housing and care of animals bred, supplied or used for scientific purposes. They had nesting material, huts and chew sticks placed in the cages. The GA mice were bred under PPL 70/8369 and have no clinical phenotype. The Appendix D Schedule 1 procedure, 'dislocation of the neck'. was used to sacrifice the animals to obtain tissue. This was followed by a confirmation method.

(https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/662364/Guidance_on_the_Operation_of_ASPA.pdf). The procedure was undertaken by a trained person. The name of the staff member performing the performing the Sch1 is held locally on a register of competent people.

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2.3 Inhibition of secreted amoebic proteases

In order to investigate the role of *Acanthamoeba* secreted proteases in influencing trophozoite-induced macrophage cytokine production, MBDM were obtained from the femurs of 7-weeks-old male BALB/c mice as previously reported[14], and subsequently treated, prior to infection, with either leupeptin (Sigma Chemical Co, Poole, UK), a serine and cysteine proteases inhibitor, or E64 (Sigma Chemical Co, Poole, UK) that selectively inhibits cysteine proteases. The choice of these two protease inhibitors relied on the specific composition of the *Acanthamoeba* secretome that mainly comprises serine and cysteine proteases[15]. Macrophages in complete RPMI (cRPMI) were considered the negative inhibitor control. Immediately after addition of protease inhibitors, macrophage cultures were incubated with either Neff or the clinical isolate trophozoites (ratio 1:1). IL-12 and IL-6 production was evaluated after 24 h, by Enzyme Linked Immunosorbent Assay (ELISA).

2.4 Infection of C57BL/6 macrophages with *A. castellanii* trophozoites

The study was performed using C57BL/6 wild-type mice and C57BL/6 mice deficient for the PAR_2 gene. MBDM were obtained from both mouse strains and co-incubated with either Neff or the clinical isolate trophozoites at a ratio of 1:1. In addition, macrophages were stimulated with LPS as a control for their ability to produce IL-12 and IL-6.

2.5 Inhibition of PAR_1 using RWJ 56110 synthetic antagonist

$PAR_1^{-/-}$ mice were not available to perform this study, and therefore RWJ 56110 (Tocris Bioscience, Bristol, UK), a synthetic selective antagonist, for PAR_1, was used to block the receptor activity. This synthetic compound can directly bind to PAR_1 blocking its activation and internalization without interfering with the cleavage of the N-terminus of the receptor[16,17]. MBDM, obtained from the femur of 7 weeks old BALB/c mice, were either pre-treated with RWJ 56110 or left untreated as controls. Subsequently, macrophages were

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stimulated with LPS or co-cultured with *Acanthamoeba* Neff strain or clinical isolate trophozoites at a ratio of 1:1. In addition, in order to evaluate the intrinsic activity of the antagonist, unstimulated macrophages pre-treated with RWJ 56110 were included in the experimental design. IL-12 and IL-6 production was evaluated at 24 h, by ELISA.

3. Results

3.1 *A. castellanii*-secreted proteases stimulate the production of pro-inflammatory cytokines by MBDM.

IL-12 production by macrophages was induced by co-culture with both Neff and clinical isolate trophozoites in the absence of protease inhibitors. In contrast, IL-12 production was significantly reduced when leupeptin was present in the co-culture with both Neff ($p<0.005$) and the clinical isolate ($p<0.005$) trophozoites. Similarly, macrophage IL-12 production was significantly lower after challenge with either Neff ($p<0.005$) or the clinical isolate ($p<0.0005$) trophozoites when macrophages were treated with E64, in comparison with the control cultures lacking the inhibitor (Fig 1-A). Both Neff and the clinical isolate trophozoites induced IL-6 production by murine macrophages in the absence of proteases inhibitors (0.583 ng/ml and 0.216 ng/ml, respectively). IL-6 levels were significantly reduced in leupeptin-treated macrophages challenged with Neff or the clinical isolate trophozoites ($p<0.0005$). Treatment of cultures with E64 also induced a significant reduction in the levels of IL-6 produced by macrophages after challenge with either Neff (0.388 ng/ml, $p<0.0005$) or the clinical isolate (0.149 ng/ml $p<0.005$) trophozoites. Furthermore, a difference in IL-6 production was found between the protease inhibitors in macrophage cultures incubated with Neff trophozoites; leupeptin (0.303 ng/ml) was more efficient at inhibiting IL-6 than E64 ($p<0.005$). This difference in macrophage IL-6 production was not demonstrated following incubation with the clinical isolate trophozoites (Fig 1-B). Neither of the inhibitors

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significantly influenced LPS induced macrophage IL-12 or IL-6 production indicating that their activities were targeting trophozoite protease activity specifically (Fig 1-A and B).

3.2 *Acanthamoeba*-induced IL-12 and IL-6 production by murine macrophages is PAR_2-independent

After demonstrating that amoebic serine and cysteine proteases could stimulate IL-12 and IL-6 production by murine macrophages, we investigated if PARs were involved in this event. PAR_2 was considered a potential target for our investigation, since it is an extracellular receptor widely expressed on immune cells and involved in inflammatory and immunological processes[18]. Both IL-6 and IL-12 production by $PAR_2^{-/-}$ macrophages co-incubated with trophozoites of either Neff strain or the clinical were similar to that produced by WT macrophages (Fig 2).

3.3 *A. castellanii* clinical strain induces IL-12 production by murine macrophages in a PAR_1-dependent manner.

In the absence of evidence of PAR_2-dependent activation of macrophages by *Acanthamoeba*, further investigations were undertaken to determine whether PAR_1 was involved. Similar to PAR_2, PAR_1 is an extracellular receptor widely expressed on immune cells and involved in inflammatory and immunological processes[18]. As expected pre-treatment with RWJ 56110 did not induce either IL-12 or IL-6 production by murine macrophages (Fig 3-A, B). Stimulation of macrophages with LPS induced IL-12 and IL-6 production and this was significantly diminished by pre-treatment with RWJ 56110 ($p<0.05$) (Fig 3-A, B). Pre-treatment of macrophages with RWJ 56110 significantly reduced IL-12 production in response to the clinical isolate trophozoites compared with control macrophages ($p<0.05$); however, it did not significantly reduce Neff trophozoite-induced IL-12 production (Fig 3-A).

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Pre-treatment of macrophages with RWJ 56110 did not modify the *Acanthamoeba*-induced IL-6 production (Fig 3-B).

4. Discussion

During parasitic infections proteases are known to be important virulence factors and to be involved in cell differentiation, tissue penetration, nutrient acquisition and immune evasion mechanisms[19]. The importance of *Acanthamoeba* proteases in pathogenicity[4,20,21], immune evasion[22] and differentiation mechanisms[23] has been widely discussed. However, more recently, studies have focused their attention on the role of these molecules in triggering the immune response. Indeed, it has been demonstrated that the amoebic serine protease MIP-133, is not only involved in the invasion of the trophozoites within the host tissue, but also in stimulating pro-inflammatory cytokine production by HCE cell line, via the cytosolic phospholipase $A_{2\alpha}$[24]. Our study demonstrates, for the first time, that both serine and cysteine amoebic proteases were capable of inducing IL-12 and IL-6 production by murine macrophages. Trophozoite-induced macrophage IL-12 production was inhibited equally when either leupeptin (serine and cysteine proteases inhibitor) or E64 (selective cysteine proteases inhibitor) were present, suggesting that IL-12 was equally stimulated by serine and cysteine proteases. Macrophage IL-6 production induced by either Neff or clinical trophozoite decreased in the presence of either inhibitor. However, during Neff strain infection of macrophages, leupeptin was more effective than E64 in inhibiting IL-6 production, indicating that serine proteases might be the major protease responsible for inducing this cytokine. By using PAR_2 deficient macrophages, we found that *Acanthamoeba*-induced IL-12 and IL-6 production was PAR_2-independent. On the other hand, *Acanthamoeba*-induced IL-12 production by macrophages was shown to be induced through PAR_1-dependent mechanisms. Interestingly, a very recent study has reported that

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Acanthamoeba plasminogen activator factor stimulates IL-8 production by human corneal epithelial cells in a PAR_2-dependent manner[10]. Furthermore, it has been demonstrated, by using an *in vivo* murine model, that *Acanthamoeba* trophozoites and their excreted/secreted molecules stimulate allergic responses and induce T_H2 responses through the activation of dendritic cells in a PAR_2-dependent manner[25]. Our study has demonstrated a different activation pattern of PARs expressed on murine macrophages, by *Acanthamoeba*, in comparison with what has been observed in murine dendritic cells and human corneal epithelial cells[10]. Importantly, the studies reported herein demonstrate that pre-treatment of macrophages with specific PAR_1 antagonist significantly inhibits, but does not eliminate IL-12 production: this would be consistent with our recently published data also demonstrating a role for TLR4, MyD88-dependent mechanisms in IL-12 production. Collectively these studies might suggest a potential cross-talk between receptors either directly (receptor association) or indirectly (at signalling level). Examples of TLRs-PARs cross-talk, especially involving PAR_2, have been reported and known to be associated with bacterial, viral and fungal infections, but not as yet during protozoan infections [26]. Consequently, the present study suggests previously unobserved interactions between PARs and TLRs during *Acanthamoeba* infections that influence cytokine production by macrophages, although more investigations are needed to elucidate these interactions.

In conclusion, the current study demonstrates for the first time a contribution of *Acanthamoeba* serine and cysteine proteases and host PAR_1 in macrophage activation and inflammatory responses. Such knowledge might ultimately provide insight into the pathogenesis of *Acanthamoeba* induced-diseases and their treatments.

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Figure Legends

Fig 1. Release of macrophage IL-12 (A) and IL-6 (B) at 24 h after treatment with protease inhibitors prior to co-incubation with *A. castellanii* trophozoites. $1x10^6$ murine macrophages, obtained from BALB/c mice, were infected with $1x10^6$ trophozoites of either *A. castellanii* Neff (Neff) or clinical (Clinical) in three different experimental conditions: in cRPMI (- Inh), in cRMPI supplemented with leupeptin 50 μM (+ Leu) and in cRPMI supplemented with E64 10 μM (+ E64). LPS, at a concentration of 200 ng/ml (LPS) was included as positive control, whereas uninfected macrophages (Control) consisted of the negative control. The experiment was repeated twice. Results represent the mean ± standard error of n=3. One-way ANOVA was applied and Tukey's multiple comparison test was performed to evaluate differences within the three different conditions means. In the graphs,

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significant differences within each condition are indicated as follows: for values of $p<0.005$ **; $p<0.0005$ ***. Values below the detectable levels are indicated in the graphs as ND (not detected). Note that *Acanthamoeba*-induced macrophage production of IL-6 and IL-12 is significantly inhibited in the presence of serine and cysteine proteases. This inhibition is observed in both the infection with either Neff or clinical trophozoites.

Fig 2. Release of macrophage IL-12 (A and B), and IL-6 (C and D): comparison between C57BL/6 WT (A and C) and C57BL/6 $PAR_2^{-/-}$ (B and D) macrophages at 24 h after co-incubation with *Acanthamoeba* trophozoites. $1x10^6$ murine macrophages, obtained from C57BL/6 WT and $PAR_2^{-/-}$mice, were challenged with $1x10^6$ trophozoites of either *A. castellanii* Neff (Neff) or clinical isolate (Clinical). LPS at 200 ng/ml (LPS) was also included in the experimental design as positive control. Uninfected macrophages (Control) were considered the negative control. The experiment was performed twice. Results represent the mean ± standard error of n=3. Mann-Whitney *U* test was applied to evaluate differences between C57BL76 WT and C57BL/6 $PAR_2^{-/-}$ conditions. Macrophages derived from C57BL/6 $PAR_2^{-/-}$did not show reduced IL-12 or IL-6 production, relative to macrophages derived from wild-type mice, when co-incubated with trophozoites. Thus, the production of these pro-inflammatory cytokines by macrophages, in response to *Acanthamoeba*, is not PAR_2-dependent.

Fig 3. IL-12 (A), and IL-6 (B) production by murine macrophages, pre-treated with the PAR_1 antagonist RWJ 56110, after 24 h co-incubation with *Acanthamoeba*. $1x10^6$ murine macrophages, obtained from BALB/c mice, were pre-treated with 20 μM RWJ 56110 solution (+ RWJ56110) or not pre-treated (- RWJ56110). Macrophages were incubated for 10

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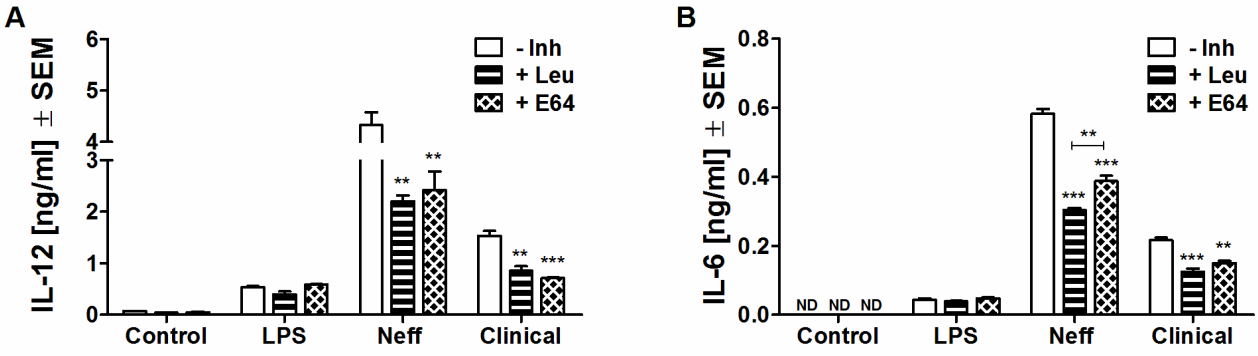
min at 37° 5% CO_2. Subsequently, macrophages were stimulated with LPS at a concentration of 200 ng/ml (LPS). Unstimulated macrophages, treated or not treated with RWJ 56110, were included in the experimental design (Control). The experiment was performed twice. Results represent the mean ± standard error of n=3. Student's *t*-test was applied to evaluate differences between – RWJ56110 and + RWJ56110 conditions. In the graphs, significances between – RWJ56110 and + RWJ56110 conditions are indicated as follow: for values of $p<0.05$ *. Values below the detectable levels are indicated in the graphs as ND (not detected). Data shown in the graphs indicate that IL-12 production by murine macrophages induced by clinical isolate trophozoites is at least in part PAR_1-dependent, although it does not appear to be the main receptor involved in this event.



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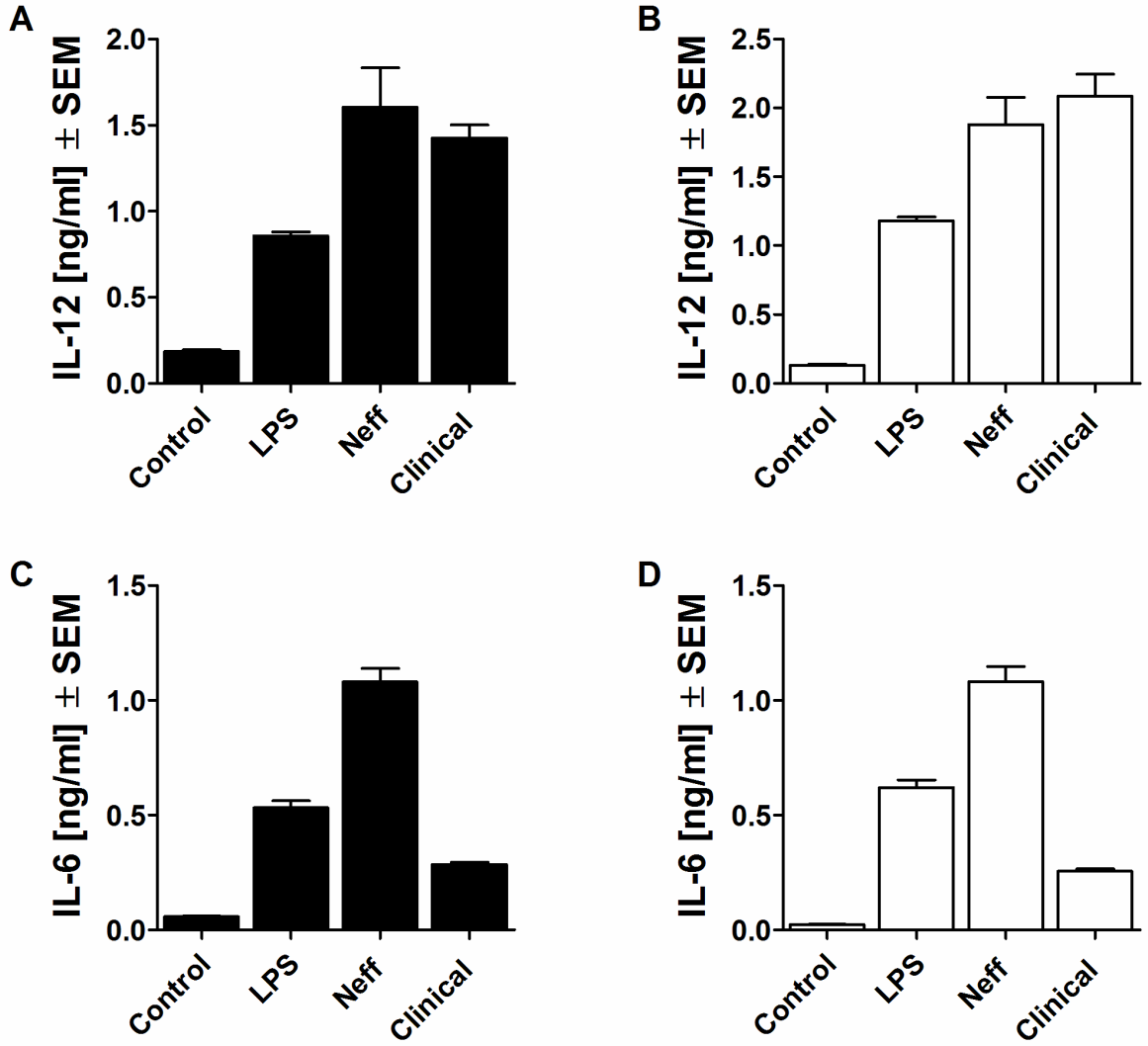

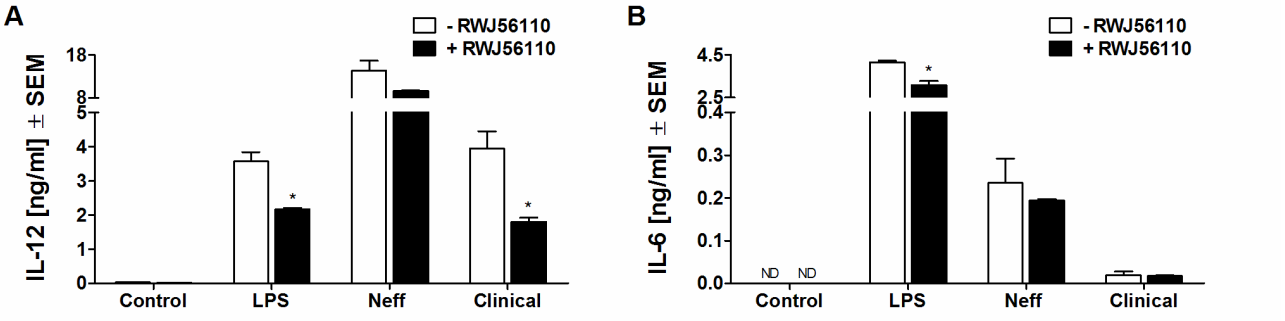

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