Leptospira species promote a pro-inflammatory phenotype in human neutrophils

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Summary

Leptospirosis is a global zoonosis caused by pathogenic *Leptospira.* Neutrophils are key cells against bacterial pathogens but can also contribute to tissue damage. Since the information regarding the role of human neutrophils in leptospirosis is scant, we comparatively analyzed the human neutrophil's response to saprophytic *Leptospira biflexa* serovar Patoc (Patoc) and the pathogenic *Leptospira interrogans* serovar Copenhageni (LIC).

Both species triggered neutrophil responses involved in migration, including the up-regulation of CD11b expression, adhesion to collagen and the release of IL-8. In addition, both species increased levels of pro-inflammatory IL-1β and IL-6 associated with the inflammasome and NFκB pathway activation and delayed neutrophil apoptosis. LIC was observed on the neutrophil surface and not phagocytized. In contrast, Patoc generated intracellular ROS associated with its uptake.

Neutrophils express the TYRO3, AXL and MER receptor protein tyrosine kinases (TAM), but only LIC*,* selectively increased the level of AXL.

TLR2 but not TLR4-blocking antibodies, abrogated the IL-8 secretion triggered by both *Leptospira* species.

In summary, we demonstrate that *Leptospira* species trigger a robust neutrophil activation and pro-inflammatory response. These findings may be useful to find new diagnostic markers and therapeutic strategies against leptospirosis.

Keywords: Granulocytes, *Leptospira interrogans*, *Leptospira biflexa*, polymorphonuclear cells, phagocytosis, degranulation.

Introduction

Leptospirosis is a global zoonosis caused by pathogenic spirochetes of the genus *Leptospira* (Bharti *et al.*, 2003). Recent estimates suggest that there are more than 1 million cases and nearly 60,000 deaths due to leptospirosis worldwide per year (Costa *et al.*, 2015). Human leptospirosis ranges from a mild to a fulminant disease. The bacteria is mainly disseminated through the circulatory system, and several organs may be involved (Haake *et al.*, 2014). As a result, the signs and symptoms of leptospirosis are diverse and frequently mistaken for other causes of acute febrile illness (Haake *et al.*, 2014). Although the pathogenesis of leptospirosis is not completely understood, it is accepted that while saprophytic *Leptospira* fail to disseminate, a major characteristic of pathogenic *Leptospira* is the rapid dissemination, implicating a stealthy escape from the host defenses (Ratet *et al.*, 2014).

Neutrophils have a critical role in human acute bacterial infections since they are among the first cells to arrive at the microbial foci and have a primary role in the clearance of extracellular pathogens through three major microbicide mechanisms: phagocytosis that usually requires previous opsonization, degranulation and NETosis (Kobayashi, 2015). Moreover, through the release of multiple cytokines, neutrophils are involved in the activation, regulation and effector functions of innate and adaptive immune cells. Given the broad functions of neutrophils, they have emerged as important players in the pathogenesis of numerous disorders (Mantovani *et al.*, 2011).

In mice models, it was observed that although neutrophil depletion significantly increases the early leptospiral loads in blood (Scharrig *et al.*, 2015), their absence did not modify the overall course of the disease (Werts, 2010, Scharrig *et al.*, 2015, Chen *et al.*, 2017). In addition, also in a murine model, it was recently observed that macrophages but not neutrophils appeared to be the major infiltrating and anti-leptospira phagocytes during leptospirosis (Chen *et al.*, 2017). The effect of *Leptospira* spp. on human neutrophils is much less known and it is worth considering that, with regard to neutrophils, important differences exist between mice and humans. While most of the circulating cells in mice are of the mononuclear lineage, with neutrophils forming only a relatively small percentage, neutrophils represent 60–70% of the

circulating white blood cells in humans (Mestas *et al.*, 2004, Washington *et al.*, 2012). An early *in vitro* study showed that although saprophytic *Leptospira* were phagocytized by human polymorphonuclear leukocytes (PMN), pathogenic *Leptospira* were not (Wang et al., 1984). Together, the findings in human and murine studies suggest that neutrophils do not seem to be an efficient defense factor for pathogenic *Leptospira* (Werts, 2017). On the other hand, it is well known that human leptospirosis presents neutrophilia which correlates with severity (Lindow *et al.*, 2016, Raffray *et al.*, 2016, de Silva *et al.*, 2018) and the pathogenic serovar involved (Craig *et al.*, 2013).

Although *Leptospira* spp. are classified as Gram-negative bacteria, lipopolysaccharide (LPS) from pathogenic *Leptospira*, at least in human macrophages, is not recognized by the canonical toll-like receptor 4 (TLR4), but it is by TLR2, the receptor for lipoproteins (Werts *et al.*, 2001). Interestingly, a recent report showed that although TLR2 was found to be significantly increased in neutrophils from leptospirosis patients, these cells failed to regulate the expression of several of the receptors involved in cell migration, questioning the ability of neutrophils to migrate into inflamed target-tissues during leptospirosis and suggesting an impaired neutrophil response (Raffray *et al.*, 2016). This study suggested that, like macrophages, human neutrophils sense *Leptospira* spp. through TLR2; however, the up-regulation was associated with a partially characterized impaired neutrophil response.

In addition, while little is known about the LPS composition in the saprophytic spp. of *Leptospira*, important differences with the pathogenic species have been reported in the expression of their lipoproteins, which have been reported as virulence factors (Mehrotra *et al.*, 2017, Vk *et al.*, 2018). In this context and considering the lack of studies about the direct effects of *Leptospira* spp. in turning on effector responses in human neutrophils, we compared the effect of saprophytic *Leptospira biflexa* serovar Patoc (Patoc) and the pathogenic *Leptospira interrogans* serovar Copenhageni (LIC) on several relevant human neutrophil effector responses.

Results

Leptospira spp. trigger the up-regulation of neutrophil CD11b expression, adhesion and platelet-neutrophil aggregate formation

Gram-negative bacteria induce several neutrophil activation responses, including an increase in CD11b expression (Weirich *et al.*, 1998, Jamsa *et al.*, 2015). For this reason, we analyzed CD11b levels after *Leptospira* spp. stimulation as an activation marker. Figure 1A shows that neutrophil stimulation with both *Leptospira* spp. triggered an increase in CD11b expression levels after 2 h post-stimulation. Since CD11b is a receptor for several ligands, including proteins of the extracellular matrix (ECM), we evaluated the adhesion of neutrophils to coated plates with collagen type I, followed by phosphatase acid activity quantification. Although non-stimulated neutrophils showed little adhesion, neutrophils incubated 30 and 120 min with both *Leptospira* spp. showed an enhanced binding to collagen (Figure 1B-C). The activation of neutrophils not only allows interactions with endothelial cells but also with other vascular cells, such as platelets, which result in the formation of mixed aggregates. We investigated this possibility by evaluating neutrophil-platelet aggregates by flow cytometry and found that both *Leptospira* spp. induced the formation of these heterotypic aggregates (Figure 1D).

Leptospira spp. induce IL-8 secretion and chemotaxis of neutrophils

In the following experiments, we studied whether *Leptospira* spp. were capable of inducing interleukin (IL)-8 secretion, since this is a cytokine known to facilitate neutrophil extravasation (Harada *et al.*, 1994). Figure 2A-B shows that the exposure of neutrophils to both strains of bacteria markedly increased the levels of IL-8 in the supernatant compared to non-stimulated samples (Mock) in a time and concentration-dependent manner. To find a functional correlation for this increase in IL-8, the chemoattractant capacity of the *Leptospira*-stimulated and non-stimulated neutrophil-derived supernatants was compared using a Boyden chamber. The chemoattractant activity of supernatants derived from neutrophils stimulated with both the saprophytic and pathogenic species was significantly higher than mock samples. In addition, supernatants from neutrophils treated with LIC showed a significantly higher migration capacity compared to Patoc (Figure 2C).

Leptospira spp. increase neutrophil survival and IL-1β and IL-6 secretion

Since several components of the bacterial cell wall are known to prolong neutrophil half-life through the inhibition of apoptosis (Colotta *et al.*, 1992), we explored whether *Leptospira* spp. had a similar effect. Analysis of nuclear morphology showed that at 18 h post-stimulation, the percentage of apoptotic cells was reduced from almost 60% to 20–40% in the *Leptospira*-stimulated neutrophils (Figure 3A)**.** In addition, flow cytometry studies also showed that the number of Annexin V^+ cells was significantly lower in the *Leptospira*-stimulated cells compared to mock-stimulated neutrophils (Figure 3B). To determine whether the increase in cell survival was associated with the production of cytokines, we evaluated the levels of several cytokines known to modulate the fate of neutrophils and found that both *Leptospira* spp. induced a significant increase in the levels of IL-1β and IL-6, in a time and concentration-dependent manner (Figures 3C-F). The IL-1β secretion induced by LIC at 1 and 10 MOI was significantly higher than that triggered by Patoc.

Activation of neutrophils by *Leptospira* spp. involve NFκB and inflammasome pathways

Activation of the transcription factor NFκB is a major pathway involved in cytokine release upon neutrophil activation. Accordingly, we observed that the release of IL-8 mediated by *Leptospira* stimulation was significantly inhibited by pre-incubation of the neutrophils with BAY 11-7082 or Ro 106-9920, selective inhibitors of IkBα phosphorylation and ubiquitination, respectively, indicating that the NFκB pathway was activated upon *Leptospira* stimulation (Figure 4A). Although activation of the inflammasome pathway and pro-IL-1β processing has been studied more in macrophages and dendritic cells, several reports indicate that neutrophils are also a major source of IL-1β in infectious and inflammatory diseases (Mankan *et al.*, 2012, Karmakar *et al.*, 2015, Perez-Figueroa *et al.*, 2016). To evaluate whether inflammasome-driven caspase-1 activation by neutrophils is involved in the cleavage of pro-IL-1β upon *Leptospira* challenge, we performed RT-PCR studies of the different components of the inflammasome. Although non-stimulated (Mock) neutrophils express basal RNA levels of caspase-1, NLRP3 and pro-IL-1β, the RNA of these molecules was markedly increased in

Leptospira-treated cells (Figure 4B). Furthermore, the treatment of neutrophils with a selective and irreversible inhibitor of cysteine protease caspase-1 (Ac-YVAD-cmk) or with a blocker of caspase-1 and pro-IL-1β maturation (Glibenclamide), inhibited the release of IL-1β (Figure 4C).

Leptospira spp. fail to induce neutrophil MPO release and only Patoc induces ROS activity

Neutrophil MPO and ROS activity were evaluated as bactericidal mechanisms for human neutrophils after *Leptospira* stimulation at MOI of 10 over 2 h. It should be noted that *Leptospira*, especially the saprophytic spp., have peroxidase activity (Austin *et al.*, 1981). The results showed that, after discounting the production by *Leptospira* (data not shown), the MPO activity of neutrophils was not detected (Figure 5A). With regard to ROS generation, Patoc was the only one capable of inducing significant levels of intracellular ROS (Figure 5B).

Selective modulation of a TAM receptor by pathogenic *Leptospira*

Several studies on mononuclear immune cells have shown that the TYRO3, AXL and MER (TAM) receptor Tyrosine Kinases family are key negative regulators of the immune response (Lemke, 2013). However, their expression in neutrophils has barely been explored. We analyzed the expression of the three receptors by flow cytometry in neutrophils after exposure to *Leptospira* spp., and observed that while the three receptors are expressed in human neutrophils (Figures 6A-C), only AXL showed an increased percentage after challenge with LIC (Figure 6C).

Human neutrophils phagocytize saprophytic but not pathogenic *Leptospira*

To clarify some contradictions from early studies as whether human neutrophils phagocytize the saprophytic but not the pathogenic *Leptospira* spp. (Wang *et al.*, 1984) or fail to phagocytize both in the absence of specific immune serum (McGrath *et al.*, 1984), neutrophils were incubated with both *Leptospira* spp. for 2 h and then processed for electron microscopy analysis. At the ultrastructural level, while non-stimulated neutrophils (Mock) have no phagocytic vacuoles (Figure 7A), multiple phagocytic vacuoles containing Patoc were in the cytoplasm of neutrophils and few bacteria remained in the extracellular space (Figure 7B). In

contrast, most LIC were observed either by transmission or scanning electron microscopy to be forming extracellular aggregates in close proximity to the surface of neutrophils, with very few in phagocytic vacuoles (Figures 7C and 7D).

Blocking TLR2 abrogated responses triggered by *Leptospira* spp.

In order to explore the role of human neutrophil TLRs in *Leptospira* sensing, we evaluated the secretion of IL-8 triggered by *Leptospira* spp. in neutrophils that have been previously incubated with blocking antibodies against human TLR2, TLR4, or both. As expected, the increased expression of IL-8 induced by Patoc or LIC was suppressed in neutrophils that were previously treated with an anti-TLR2, but not with an anti-TLR4 (Figure 8). LPS and PAM3CSK4, used as positive controls, are shown in Supplementary Figure 1.

Discussion

In this study, we demonstrate that both *Leptospira* spp. trigger activation effector responses and prolong the life span of human neutrophils. The stimulation of neutrophils with *Leptospira* spp. resulted in up-regulation of the integrin CD11b together with increased cell adhesion to an ECM protein such as collagen. Even though the up-regulated expression of CD11b might be involved in the increased adhesion to collagen, we do not discard the possibility of a direct interaction between bacteria and collagen since *Leptospira* spp. have outer membrane proteins capable of binding collagen (Ito *et al.*, 1987, Barbosa *et al.*, 2006, Atzingen *et al.*, 2008). Taken together, these findings indicate that *Leptospira* spp. trigger neutrophil activation responses that facilitate the transmigration of neutrophils into tissues. We also found that supernatants of neutrophils that had been exposed to *Leptospira* exerted robust chemotactic activity and also contained high amounts of IL-8, one of the major molecules responsible for the attraction of phagocytic cells to the infection site (Harada *et al.*, 1994), suggesting that *Leptospira* spp. can induce the recruitment of neutrophils to sites of infection, thus boosting the host's acute inflammatory responses against infection.

Besides the interaction with endothelial cells, the activation of neutrophils also allows interactions with platelets. Interestingly, we found that *Leptospira* spp. promoted the formation of mixed platelet-neutrophil aggregates. Thrombocytopenia is a common feature of leptospirosis, but its origin is still not clear (De Silva *et al.*, 2014, Goeijenbier *et al.*, 2015, Raffray *et al.*, 2016, Adiga *et al.*, 2017, Tunjungputri *et al.*, 2017). In this regard, the formation of circulating mixed aggregates could represent a possible mechanism that accounts for the clearance of platelets and the consequent thrombocytopenia. Furthermore, the association of platelets with neutrophils could contribute to the amplification of the inflammatory response as a direct consequence of the reciprocal cell activation and the release of several pro-inflammatory molecules (Wang *et al.*, 2014).

We also found that *Leptospira* spp. increased the viability of neutrophils by diminishing spontaneous apoptosis. These findings are in agreement with other studies which

demonstrated that *E. coli* and other bacteria or their products exert similar responses (Colotta *et al.*, 1992, Hiroi *et al.*, 1998), which are usually related to the synthesis and release of cytokines known to promote cell survival. In this context, we found that both strains induced the release of IL-1β, IL-6 and IL-8, cytokines that promote not only cell survival but also the increased expression of CD11b and the adhesion to collagen triggered by *Leptospira* spp*.* IL-6 is up-regulated not only in response to a variety of signals, including bacterial components, but also by IL-1β and other cytokines, suggesting a complex activation network (Tuyt *et al.*, 1999, Moon *et al.*, 2000). Moreover, acting synergistically, they could also be responsible for the neutrophilia (Chiba *et al.*, 2017) observed during acute leptospirosis in humans (De Silva *et al.*, 2014, Lindow *et al.*, 2016, Raffray *et al.*, 2016).

The main molecular signaling pathway involved in the release of pro-inflammatory cytokines and neutrophil survival under inflammatory conditions is the NFκB pathway (Walmsley *et al.*, 2005). Accordingly, we observed that the inhibition of either IkBα phosphorylation or ubiquitination resulted in a significant inhibition of the *Leptospira*-mediated release of IL-8. Furthermore, we present evidence that *Leptospira* spp. triggered inflammasome activation, since the up-regulation of pro-IL-1β, NLRP3 and caspase-1 transcription was observed in neutrophils exposed to *Leptospira* spp. and blocking caspase-1 maturation or its activity suppressed the release of IL-1β. In the same line, previous studies from Werts's group showed that *L. interrogans* induces NLRP3 inflammasome-dependent secretion of IL-1β through the alteration of potassium transport in murine bone marrow-derived macrophages (Lacroix-Lamande *et al.*, 2012).

The correlation of an increased pro-IL-1β and IL-1β with less apoptosis was quite unexpected since in macrophage, where the secretion of IL-1β has been most studied, the NLRC4 inflammasome drives caspase-1-dependent IL-1β and pyroptotic cell death (Chen *et al.*, 2013). However, neutrophils may behave differently. Unlike macrophages, neutrophils did not undergo pyroptosis upon NLRC4 inflammasome activation triggered by *Salmonella* infection (Chen *et al.*, 2014). Interestingly, it has been recently shown, that an unconventional autophagy-mediated secretory pathway mediates IL-1β secretion in human neutrophils (Iula

et al., 2018). Since autophagy usually blocks apoptosis (Marino *et al.*, 2014), it could be that this or a similar mechanism may be involved in the enhanced IL-1β and reduced apoptosis triggered by *Leptospira* in human neutrophils, a fact that future studies might clarify. Together, these findings suggest that these signaling pathways are involved in neutrophil activation mediated by *Leptospira* and represent potential targets for decreasing inflammation during leptospirosis.

The release of MPO and the generation of ROS together with NETosis are potent antimicrobial strategies used by neutrophils to fight against bacteria (Dale *et al.*, 2008). We found that *Leptospira* spp. did not induce MPO activity, and that intracellular ROS was induced only by Patoc. In the case of LIC, the inability to detect MPO activity at the extracellular level could be associated with the recent report that the LipL21 protein from LIC is a potent inhibitor of neutrophil MPO activity (Vieira *et al.*, 2017), and its binding to peptidoglycans enables *Leptospira interrogans* to escape NOD1 and NOD2 recognition in mice (Ratet *et al.*, 2017). Moreover, it was shown that the killing of *Leptospira* spp. by hydrogen peroxide may be independent of the presence of neutrophil MPO (Murgia *et al.*, 2002). Furthermore, *Leptospira,* particularly pathogenic spp., may degrade peroxide more easily than saprophytic spp., (Kebouchi *et al.*, 2018) making these spp. less susceptible to neutrophil killing.

The lack of ROS generation by LIC correlated with the failure of neutrophils to phagocytize the bacteria. In agreement with (Wang *et al.*, 1984), our studies by both transmission and scanning electron microscopy showed that while Patoc were phagocytized, LIC were rarely observed inside neutrophils but were abundant in the extracellular space, especially close to the surface, where binding to CR3 was reported to be involved (Cinco *et al.*, 2002). How pathogenic *Leptospira* spp. avoid being phagocytized by neutrophils is not clear. In this regard, it has been reported that neutrophils sense microbe size and selectively release neutrophil extracellular traps (NETs) in response to large pathogens, such as *Candida albicans* hyphae and extracellular aggregates of *Mycobacterium bovis*, but not in response to small yeast or single bacteria. Moreover, phagocytosis acts as a sensor of microbe size and prevents the release of NETs (Branzk *et al.*, 2014). We have previously demonstrated that both *Leptospira*

spp. induce the formation of NETs, affecting bacterial viability in a concentration-dependent manner, and that LIC induces more than Patoc*.* Also, levels of circulating nucleosomes were increased after infection but decreased in neutrophil-depleted mice (Scharrig *et al.*, 2015). In addition, the presence of NETs was observed in lung tissue in one human case (Scharrig *et al.*, 2017). These findings, together with our ultrastructural observations showing that LIC was rarely phagocytized and that the main microbicide substances, such as MPO and ROS, are not produced by LIC suggest that neutrophils sense pathogenic *Leptospira* spp. in a way that favors NETosis over phagocytosis.

The TAM tyrosine kinase receptors in mononuclear immune cells are well characterized as negative regulators of the immune response. However, their expression, function and regulation depend on the setting, since AXL and MER were related to function in inflammatory and tolerogenic settings, respectively (Zagorska *et al.*, 2014). The expression of TAM receptors in human neutrophils is still not known. Here, we demonstrate that TAM receptors are expressed in resting human neutrophils. Interestingly, the levels of AXL were significantly increased after incubation with LIC*,* but not with Patoc, suggesting a specific response to the pathogenic bacteria that, considering the findings in mice (Fujimori *et al.*, 2013), may limit the innate immune response favoring *Leptospira* dissemination. Considering the lack of early markers (Vk *et al.*, 2018), these data suggest that neutrophil AXL may be a potential useful marker of leptospirosis, although further studies are needed to link this observation with its pathophysiological relevance.

Although there are no previous studies reporting how saprophytic *Leptospira* spp. are sensed by neutrophils, our observation that Patoc was mainly recognized by TLR2 was not surprising. Among the few previous studies of neutrophils in human leptospirosis, a significant increase in circulating PMNs has been reported during the acute phase of the disease (Lindow *et al.*, 2016, Raffray *et al.*, 2016). Interestingly, this neutrophilia correlated with the increased expression of CD15 (Raffray *et al.*, 2016), a fact that supports our observation of enhanced chemotactic mechanisms, considering the role of this molecule in neutrophil chemotaxis (Kerr *et al.*, 1992). However, the same study reported no differences in CD11b surface levels on

PMNs or in circulating levels of IL-8 between controls and leptospirosis cases (Raffray *et al.*, 2016). The discrepancies between our *in vitro* studies may be explained by several factors, including: a) we did not use CD16 to counterstain CD11b; b) the relatively low number of studied cases (15), an important issue considering the tendency toward higher values of neutrophil activation markers shown in the previous study; and c) the number of circulating bacteria was below 1 MOI; in most of our data, the *Leptospira*-mediated neutrophil effector responses were observed at higher MOI values.

In conclusion, our study demonstrates that *Leptospira* spp. interact with and activates human neutrophils to a pro-inflammatory state through activation of the NFkB and inflammasome pathways, endowing these innate immune cells with a potentially relevant role in human leptospirosis. A better understanding of the role of neutrophils will reveal important information about disease outcome and potential treatment.

Experimental procedures

Bacteria

The virulent strain Fiocruz L1–130 of *Leptospira interrogans* serovar Copenhageni (LIC) (Ko *et al.*, 1999) and the saprophyte *Leptospira biflexa* serovar Patoc strain Patoc 1 (Patoc) (Scharrig *et al.*, 2015) were cultured at 30ºC under aerobic conditions in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, USA), supplemented with 10% rabbit serum (vol/vol), 0.015% L-asparagine (wt/vol), 0.001% sodium pyruvate (wt/vol), 0.001% calcium chloride (wt/vol), 0.001% magnesium chloride (wt/vol), 0.03% peptone (wt/vol) and 0.02% meat extract (wt/vol). Prior to their use, bacteria were pelleted, washed twice wirth PBS, resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA) and counted using dark field microscopy, as described previously (Scharrig *et al.*, 2015). For each neutrophil response evaluated, cells were stimulated with the same bacterial culture collected in the logarithmic phase. The bacterial culture was prepared every week from a stock frozen vial.

Isolation of neutrophils

This study was performed according to institutional guidelines (National Academy of Medicine, Buenos Aires, Argentina) and approved by the Institutional Ethics Committee. All subjects provided written informed consent for the sample collection and subsequent analysis.

Neutrophils were isolated from peripheral blood drawn from healthy donors, as previously described (Lapponi *et al.*, 2013). Briefly, blood samples were centrifuged on Ficoll-Hypaque gradient (1078 g/ml density, GE Healthcare, Buckinghamshire, UK) at 400 x *g* for 30 min at room temperature (RT). Then neutrophils were obtained by dextran sedimentation (Sigma, St. Louis, MO, USA), followed by the hypotonic lysis of erythrocytes. All manipulations were performed under endotoxin-free conditions. Cells, suspended in RPMI-1640 supplemented with 2% FBS, contained >99.5% neutrophils, as determined by May-Grunwald-Giemsa-stained cytopreps. All neutrophil preparations were FACs analyzed after purification to guarantee that their FSC/SSC parameters were compatible with those of non-activated cells. Levels of contamination were always <0.25%, as evaluated by CD14 staining and flow cytometry. To minimize neutrophil spontaneous activation, cells were used immediately after isolation.

Preparation of washed human platelet suspensions

Blood samples were obtained from healthy donors who had not taken non-steroidal anti-inflammatory drugs in the 10 days before sampling. Platelet rich plasma (PRP) was obtained from anti-coagulated blood (sodium citrate 3.8%) by centrifugation at 180×g for 10 min. Platelets were washed as described previously (Etulain *et al.*, 2011), PRP was centrifuged in the presence of PGI_2 (75 nM) and platelets were then rinsed in wash buffer (140 mM NaCl, 10 mM $NaHCO_3$, 2.5 mM KCl, 0.5 mM Na_2HPO_4, 1mM $MgCl_2$, 22 mM sodium citrate, 0.55 mM glucose, 0.35% BSA, pH 6.5). Finally, washed platelets (WPs) were resuspended in RPMI + 2% SFB.

Expression of CD11b and TAM receptors

To evaluate CD11b or TAM receptor expression, neutrophils (3 x 10^5/ml) were stimulated over 2 h with *Leptospira* spp. at different MOIs and then cells were fixed and stained with PerCP-CD11b or FITC-TYRO3, PE-AXL, APC-MERTK (TAM receptors) or irrelevant IgG1

(Biolegend, San Diego, CA, USA and R&D System Minneapolis, MN, USA) and analyzed via fluorescence-activated cell sorting (FACS).

Neutrophil adhesion assay

A 96-well plate was coated with collagen Type I (10 µg/ml) (BD Biosciences, San José, CA, USA) and left 10 min at 37ºC. After discarding the excess of collagen**,** aliquots of neutrophils (1 x 10^5) were added to each well and stimulated or not with *Leptospira* spp at different MOIs at 37°C for 30 or 120 min. After washing, a solution of 5 mM p-nitrophenyl phosphate in 0.1 M citrate buffer containing 0.1% Triton X-100, pH 5.4 was added and incubated for 1 h at 37ºC. The reaction was stopped by adding 2N NaOH, and absorbance at 405 nm was measured with a microplate reader (Dynatech MR 5000; Dynatech Laboratories).

Platelet-leukocyte mixed aggregate assays

Platelet-leukocyte aggregates were detected as described (Pacienza *et al.*, 2008). Briefly, equal volumes of washed platelet suspensions (2 x 10^8/ml) and neutrophils (5 x 10^6/ml) were incubated, followed by stimulation with *Leptospira* spp. (MOI = 10) for 45 min. After stimulation, the samples were fixed with 1% PFA, stained with FITC-CD45 and PE-CD61 Abs or equivalent amounts of FITC and PE-isotype matched controls and then analyzed by flow cytometry using a FACScalibur flow cytometer (BD Biosciences, San José, CA, USA). The results were expressed as the percentage of $CD45^+/CD61^+$ events within the total $CD45^+$ population.

Production of pro-inflammatory cytokines

After neutrophil (3 x 10^5/ml) incubation with *Leptospira* spp. at different MOIs for 3 or 18 h, cells were centrifuged at 600 x *g* for 10 min, and the production of IL-6, IL-1β and IL-8 were determined in the supernatants with commercial ELISA kits (eBioscience, CA, USA) according to the manufacturer's instructions. In selected experiments, neutrophils were incubated for 45 min with BAY 11-7082 (Biomol, USA) and Ro 106-9920 (Tocris, USA) as specific inhibitors of IκBα phosphorylation and IκBα ubiquitination, with glybenclamide (InvivoGen, CA, USA) and Ac-YVAD-cmk (InvivoGen) as selective blockers of caspase-1 maturation and cysteine protease caspase-1, respectively, or with neutralizing monoclonal antibodies against TLR2 or TLR4 and the corresponding isotypes (InvivoGen) before incubation with *Leptospira* spp. LPS

(100 ng/ml) from *E. coli* O111:B4 or PAM3CSK4 (1 ng/ml) were used as TLR4 or TLR2 agonists, respectively.

Quantitation of LPS

LPS levels were determined by using the *limulus* test (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific). The concentration of LPS in the supernatants from non-stimulated, *Leptospira*-stimulated neutrophils, and in the RPMI plus serum (the media used for all experiments) was below the detection level of the kit (0.01 ng/ml).

MPO activity and ROS levels

MPO activity was measured in supernatants from neutrophils stimulated or not with *Leptospira* spp. at MOI = 10 for 2 h at 37°C in RPMI 1640 medium supplemented with 2% FBS. MPO was evaluated by adding the high sensitivity substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Thermo Fisher Scientific, MA, USA) and absorbance was read at 450 nm after stopping the reaction with 2N sulfuric acid. The calibration curve was constructed using a purified MPO of a known concentration.

ROS production by neutrophils was determined as previously described (Gorgojo *et al.*, 2017), with minor modifications. Briefly, tubes containing 2 x 10^7 bacteria were transferred to a Luminometer (Luminoskan TL Plus, Thermo LabSystems, Finland), in which the chemoluminescence response of 2 x 10^5 neutrophils was measured every 10 min for 3 h at 37°C after injection of 120 µM luminol (Sigma). Neutrophils incubated with phorbol myristate acetate (PMA) were used as positive controls.

Neutrophil chemotaxis assay

Neutrophils (1 x 10^5) were suspended in RPMI with 2% FBS, and chemotaxis was quantified using a modification of the Boyden chamber technique, as described (Kviatcovsky *et al.*, 2017). Briefly, the cell suspension was placed in the upper wells of an 18-well micro chemotaxis chamber (Neuro Probe, Bethesda, MD, USA) separated by a polyvinylpyrrolidone (PVP)-free polycarbonate filter (3-µm pore size; Poretics Products, Livermore, CA, USA). The lower wells contained the different conditioned media. The chamber was incubated for 30 min at 37ºC in a 5% CO_2 humidified atmosphere. Following incubation, the chamber was

disassembled and the membrane removed, fixed and stained with Staining 15 (Biopur Diagnostics, Argentina). The number of neutrophils migrating through the filter was counted in five random high-power fields (400X) per condition (each condition by triplicated) employing an optical microscope. Results are expressed as number of neutrophils migrated/field.

Determination of neutrophil apoptosis and viability

Neutrophils (4 x 10^6/ml) were cultured with RPMI and 2% FBS in the presence or absence of *Leptospira* spp. at different MOIs. After 18 hpi, cells were analyzed for changes in nuclear morphology and viability by labelling with a mixture of acridine orange and ethidium bromide (100 µg/ml) to determine the percentage of cells that had undergone apoptosis. The cover-glass preparations were counted under a fluorescence microscope with a minimum of 200 cells scored from each sample. The scorers were blinded to treatment and scoring was: A) live cells with normal nuclei staining which present green chromatin with organized structures, B) apoptotic cells with condensed or fragmented chromatin (green or orange) and C) necrotic cells with normal nuclei staining as live cells, except that chromatin is orange instead of green (Negrotto *et al.*, 2006)(Supplementary Figure 2).

Phosphatidylserine expression was detected by Annexin-V-FITC binding using a commercial kit (BD Pharmingen). In brief, 18 h after different treatments, 1 x 10^6 neutrophils were washed and incubated for 25 min with Viability dye Alexa fluor 780 (Ebioscience) at 4°C. Then, neutrophils were washed and incubated for 15 min with Annexin-V-FITC at RT. Finally, cells were washed, fixed with 1% PFA and then analyzed by flow cytometry.

RT-PCR

Total RNA was isolated from cell pellets using TriReagent (Genbiotech, BsAs, Argentina). RNA was quantified with a spectrophotometer (Nanodrop 1000). Before cDNA synthesis, DNase treatment was performed with an RNase-free DNase kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1.5 µg of total RNA using 15 mM of random hexamers (Byodinamics, BsAs, Argentina) and Moloney Murine Leukemia Virus reverse transcriptase (Promega, BsAs, Argentina). The primers sequences (5'-3') were: Pro-IL1β F ACCAAACCTCTTCGAGGCAC, R ATCGTGCACATAAGCCTCGT; NLRP3 F

GGAGAGACCTTTATGAGAAAGCAA, R GCTGTCTTCCTGGCATATCACA; Caspase-1 F CCGAAGGTGATCATCATCCA, R ATAGCATCATCCTCAAACTCTTCTG; Eef1A F TCGGGCAAGTCCACCACTAC, R CCAAGACCCAGGCATACTTGA.

Ultrastructural studies

Neutrophils and *Leptospira* spp. were incubated for 2 h and fixed in 4% formaldehyde, 2% glutaraldehyde and 5% sucrose in phosphate-buffered saline (PBS) pH 7.4, then post-fixed in osmium tetroxide and embedded in Vestopal. Slides were obtained using glass knives, and grids were stained with uranyl acetate and lead citrate. Studies were performed using a transmission electron microscope (JEOL 1011, Pleasanton, CA, USA) at 60 kV and scanning electron microscope (JEOL T100). Images were recorded with a cooled charge coupled device digital camera (MegaView III; Olympus, Center Valley, PA, USA).

Statistical analysis

Results are expressed as means ± SEM. Parametric and non-parametric analysis were performed using Graph Pad Prism Version 6.00 (GraphPad Software Inc, San Diego, CA, USA). Normality and homoscedasticity of residuals from parametric models were tested by using SPSS software (SPSS, Chicago, IL, USA). Data or transformed-data from samples that fitted the assumptions of normality and homoscedasticity of residuals were analyzed with one- or two-way ANOVA, followed by Bonferroni multiple comparison tests. When the assumptions for the ANOVA were not met, data were analyzed using the Friedman test, followed by Dunn's multiple comparisons. A P value lower than 0.05 was considered statistically significant.

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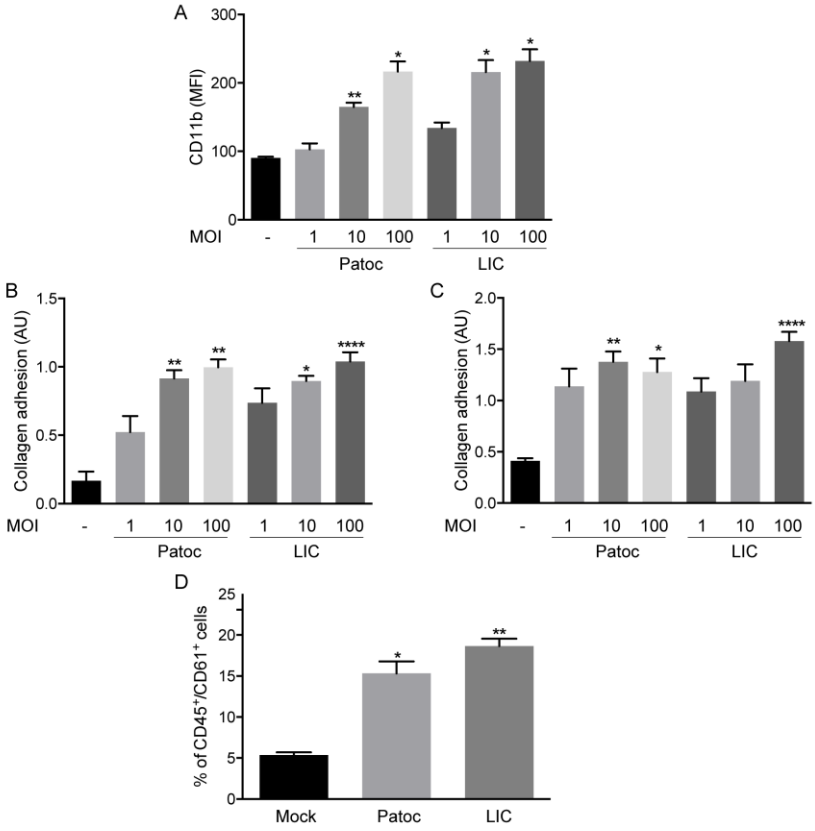

Figure 1. *Leptospira* spp. trigger neutrophil adhesion responses and mixed aggregates. All responses were determined in non-stimulated (mock) and *Leptospira*-stimulated neutrophils. A) CD11b expression was determined by flow cytometry, and the results were expressed as the mean fluorescence intensity (MFI) (One-way ANOVA followed by the Bonferroni multiple comparisons test; **P* < 0.05 and ***P* < 0.01 vs. mock). Neutrophils were seeded on collagen type I coated-wells for 30 (B) or 120 min (C) and then adhesion was evaluated by phosphatase acid activity quantification. AU: arbitrary units. (Friedman followed by Dunn's multiple comparisons test; **P* < 0.05, ***P* < 0.01 and *****P* < 0.0001 vs. mock). D) Generation of mixed platelet-leukocyte aggregates was determined at 45 min post-incubation with *Leptospira* spp. Equal volumes of washed platelet suspensions (2 x 10^8/ml) and neutrophils (5 x 10^6/ml) were incubated together in RPMI plus 1% FBS, followed by stimulation with *Leptospira* spp. (MOI=10, bacteria/neutrophil) for 45 min. The results are expressed as the percentage of $CD45^+/CD61^+$ events within the total $CD45^+$ population (One-way ANOVA followed by the Bonferroni multiple comparisons test; **P* < 0.05 and ***P* < 0.01 vs. mock). The results are the mean ± SEM of 4 to 5 independent experiments.

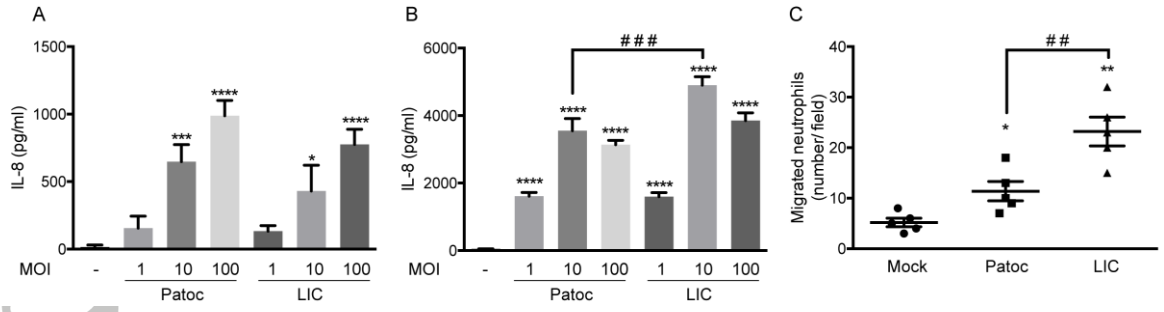

Figure 2. *Leptospira* spp. induce IL-8 secretion and enhances chemotaxis in neutrophils. IL-8 levels were determined in the supernatants at 3 h (A) and 18 (B) h post-incubation (Two-way ANOVA followed by the Bonferroni multiple comparisons test; **P* < 0.05, ****P* < 0.001 and **** P < 0.0001 vs. mock; ### P < 0.001 between Patoc and LIC). C) Neutrophil chemotaxis was quantified using the Boyden chamber technique. The cells were placed in the upper wells, and the supernatant from non-stimulated or *Leptospira*-stimulated neutrophils for 18 h was added to the lower wells. The number of neutrophils that migrated through the filter was counted in the five random higher-power fields (400X) per condition (One-way ANOVA followed by the Bonferroni multiple comparisons test; **P* < 0.05 and ***P* < 0.01 vs. mock; ## P < 0.01 between Patoc and LIC). The results are the mean ± SEM of 4 to 7 independent experiments.

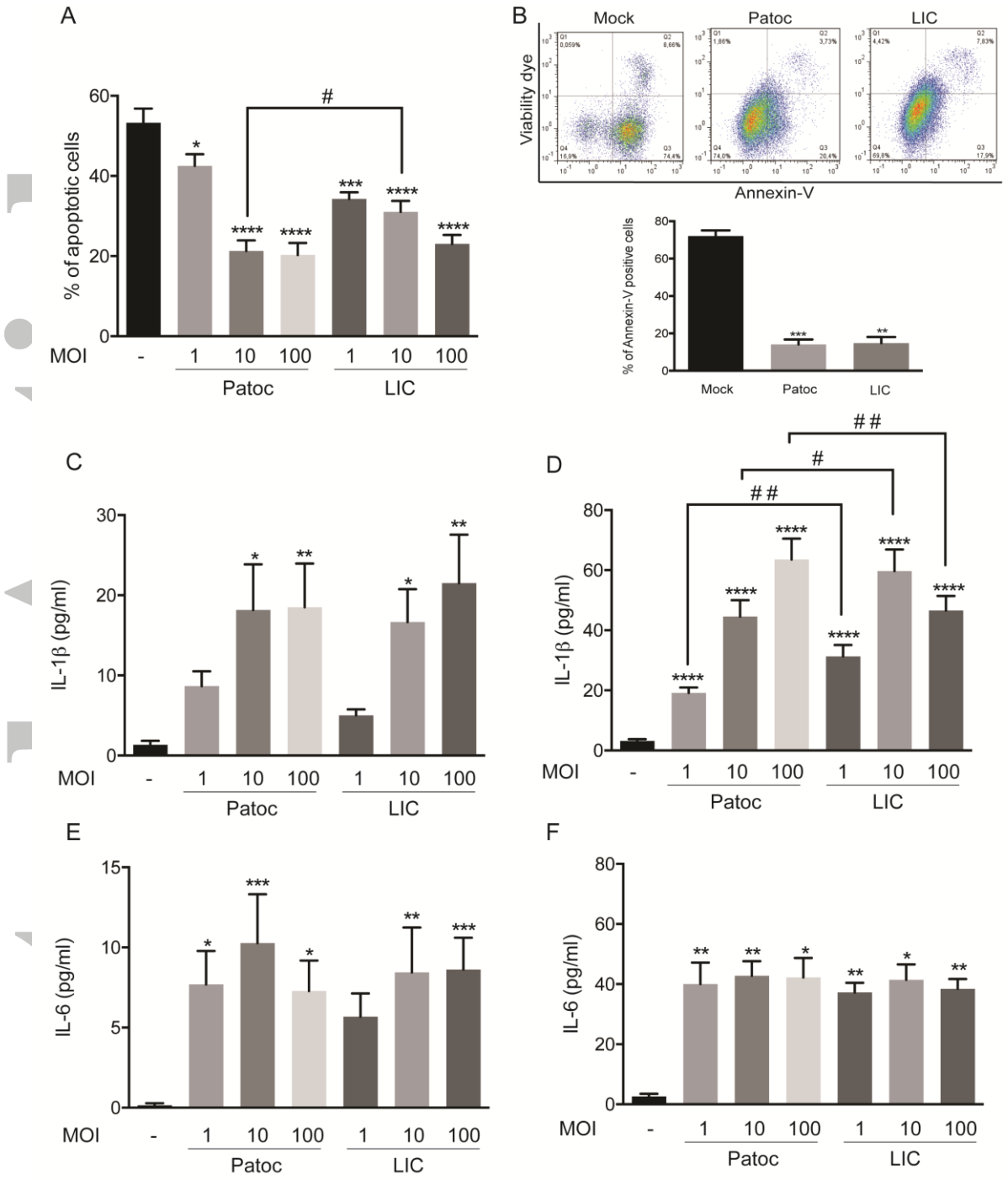

Figure 3. *Leptospira* spp. inhibit apoptosis and induces cytokine release in neutrophils. All responses were determined in non-stimulated (mock) and *Leptospira*-stimulated neutrophils. Percentage of cell apoptosis was evaluated by (A) monitoring nuclear morphology changes and viability by labelling cells with a mixture of acridine orange and ethidium bromide (100 µg/ml) (Two-way ANOVA followed by the Bonferroni multiple comparisons test (*$P < 0.05$, ***$P < 0.001$ and **** $P < 0.0001$ vs. mock; # $P < 0.05$ between Patoc and LIC), and (B)

Annexin-V-FITC/ Viability dye Alexa 780 staining. (One-way ANOVA followed by the Bonferroni multiple comparisons test; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. mock). The release of IL-1β and IL-6 was determined in the supernatants at 3 h (C-E) and 18 h (D-F) post-incubation by ELISA (Two-way ANOVA followed by the Bonferroni multiple comparisons test; ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ vs. mock; # $P < 0.05$ and ## $P < 0.01$ between Patoc and LIC). The results are the mean ± SEM of 3 to 7 independent experiments.

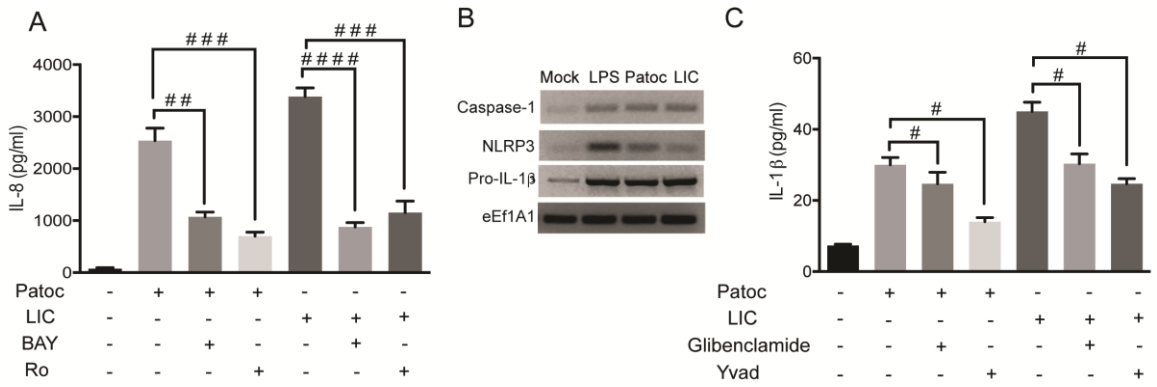

Figure 4. *Leptospira* spp. activate NFκB and inflammasome pathways in neutrophils. All responses were determined in non-incubated (mock) and *Leptospira*-stimulated neutrophils 18 h post-stimulation. A) Activation of NFκB was evaluated by pre-incubating the neutrophils for 45 min with BAY 11-7082 (10 µM) and Ro 106-9920 (5 µM) as specific inhibitors of IκBα phosphorylation and IκBα ubiquitination, respectively, and then evaluating IL-8 levels in the supernatants. Values represent the mean ± SEM of 3 to 5 independent experiments (One-way ANOVA followed by the Bonferroni multiple comparisons test; ## $P < 0.01$, ### $P < 0.001$ and #### $P < 0.0001$ between the marked groups). B) Activation of the NLRP3 inflammasome was evaluated in mock samples and *Leptospira*-stimulated neutrophils by comparison of NLRP3, pro-IL-1β and caspase-1 mRNA levels in mock and *Leptospira*-stimulated cells; the housekeeping gene used was Eef1A and one representative experiment of three is shown. LPS (100 ng/ml) from *E. coli* O111:B4 was used as a positive control. C) inhibition of IL-1β secretion by pre-incubation of neutrophils with glibenclamide (200 µM) and Ac-YVAD-cmk (30 µM) as selective blockers of caspase-1 maturation and cysteine protease caspase-1, respectively (One-way ANOVA followed by the Bonferroni multiple comparisons test; # $P < 0.05$ between the marked groups). Values represent the mean ± SEM of 3 to 5 independent experiments.

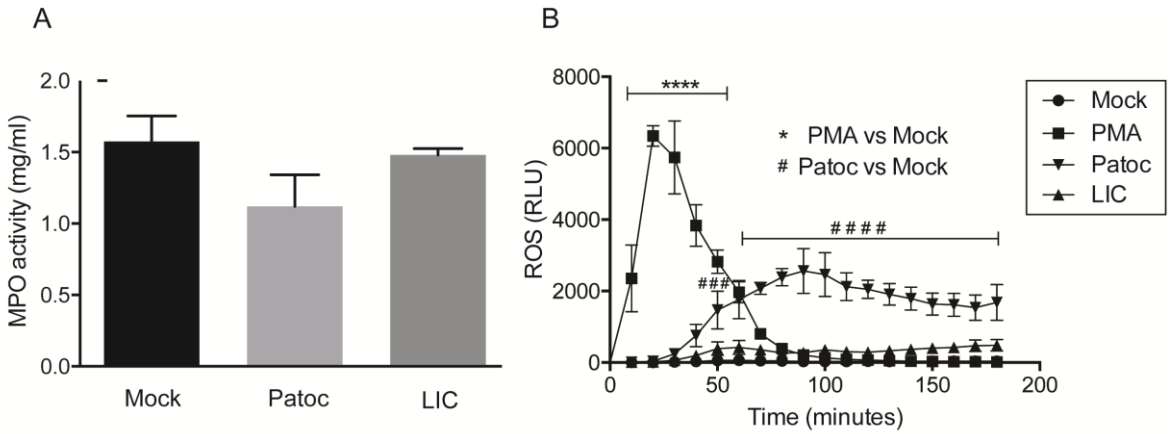

Figure 5. LIC fails to induce neutrophil bactericidal responses. Some effector responses were determined in non-stimulated (mock) and *Leptospira*-stimulated neutrophils. A) Myeloperoxidase (MPO) activity was measured in supernatants after 2 h of incubation (One-way ANOVA followed by the Bonferroni multiple comparisons test). B) Reactive oxygen species (ROS) generation was evaluated by chemoluminescence after the addition of luminol. PMA was used as a positive control. The results are the mean ± SEM of 4 to 5 independent experiments (Two-way ANOVA followed by the Tukey's multiple comparisons test).

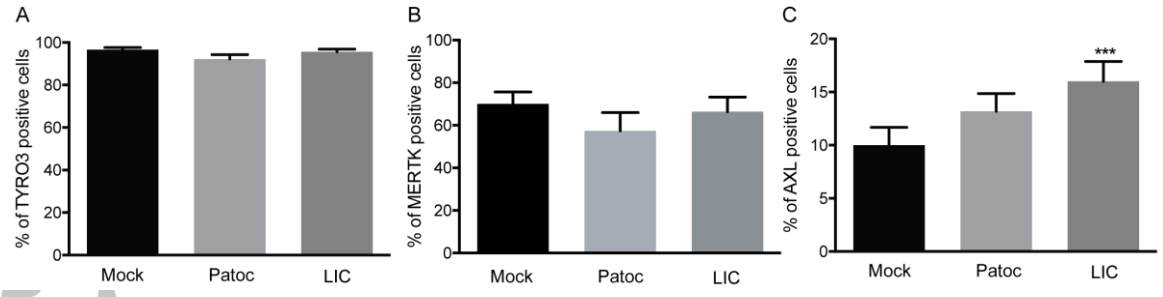

Figure 6. LIC selectively enhances AXL expression in neutrophils. TAM receptor expression was determined by FACS in mock cells and *Leptospira*-stimulated cells at 120 min post-stimulation (One-way ANOVA followed by the Bonferroni multiple comparisons test; ****P* < 0.001 vs. mock). The results are the mean ± SEM of 5 independent experiments**.**

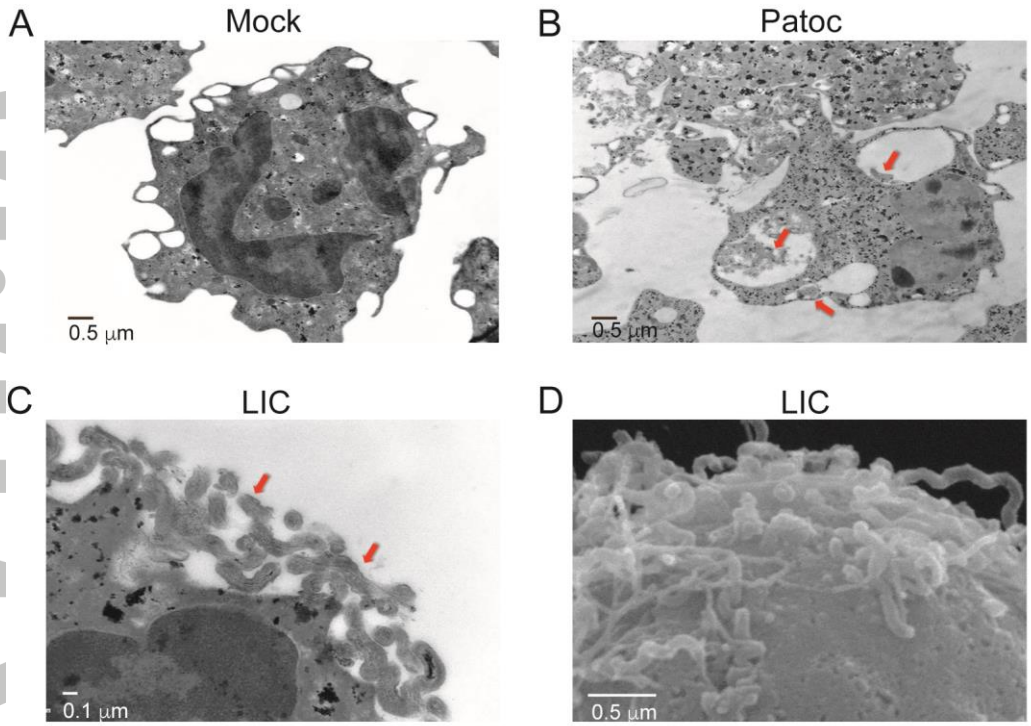

Figure 7. Human neutrophils phagocytize Patoc but not LIC. All responses were determined in non-stimulated (mock) and *Leptospira*-stimulated neutrophils. Human neutrophils (2 x 10^5/ml) were incubated with *Leptospira* spp. (MOI = 10) for 2 h and then processed for transmission (A–C) and scanning (D) electron microscope analysis. A) Non-stimulated neutrophils (mock); B) Incubated neutrophils with Patoc. The original magnification was 12,000X. C) Incubated neutrophils with LIC. The original magnification was 50,000X. D) Incubated neutrophils with LIC. The original magnification is 33,000X. Images are representative of three independent experiments.

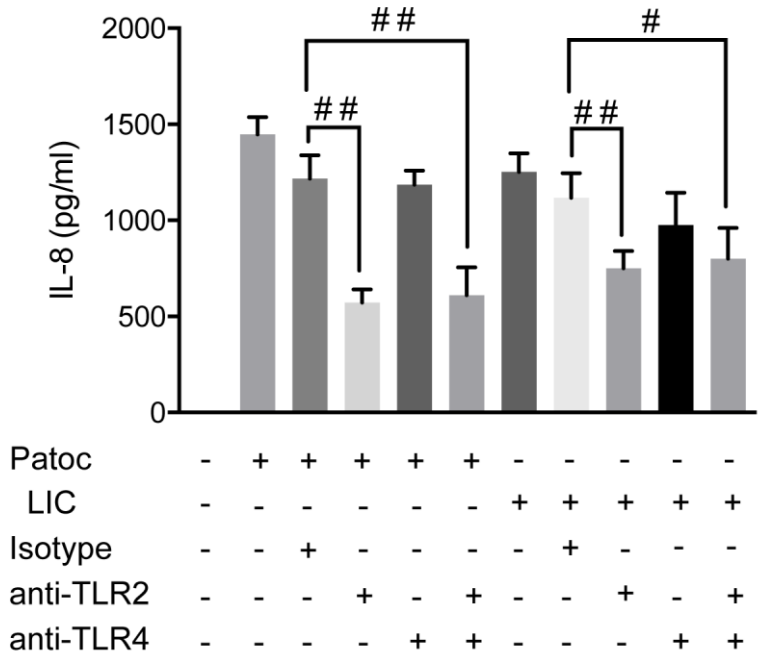

Figure 8. Neutrophils sense *Leptospira* spp. through TLR2. Neutrophils were pre-incubated for 45 min with blocking antibodies to TLR2 (5 µg/ml) or TLR4 (5 µg/ml) or both blocking Abs, then were incubated with *Leptospira* spp. and the release of IL-8 was determined in the supernatants 3 h post-incubation by ELISA. The results are the mean ± SEM of 6 independent experiments (One-way ANOVA followed by the Bonferroni multiple comparisons test; #$P < 0.05$ and ##$P < 0.01$ between the marked groups).