**Bacteria expressing a protein of *Wolbachia* induce activation of M1 response, killing *Leishmania* parasite**

***Asaia* symbionts induce activation of M1 response and killing of *Leishmania* parasite, which is potentiated by the expression of a protein form *Wolbachia***

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**ABSTRACT** (max 300 parole)

Leishmaniases are severe vector-borne parasitic diseases affecting humans and dogs, caused by *Leishmania* protozoans. Millions of dogs and over 350 million of people are at risk of infection. Immune polarization is thought to play a major role in determining the outcome of *Leishmania* infection: hosts polarized on the Th1 side are protected, while those displaying a Th2 response acquire a chronic infection, eventually developing into an overt and potentially deadly disease. The identification of M1/Th1-biasing factors, protecting mammalian hosts from leishmaniases, would greatly increase the knowledge on the immunology of the disease and would open the way towards the design of a novel generation of prophylactic and therapeutic vaccines. Accordingly, several lines of evidence indicate that infection by the filarial nematode *Dirofilaria immitis* is one of the factors interfering with dog leishmaniasis. Indeed, filarial nematodes induce a skew of the immune response towards Th1, partially caused by their bacterial endosymbionts, *Wolbachia*. Here we have tested the potential of *Asaia*WSP, a bacterium engineered for the expression of the *Wolbachia* surface protein (WSP) from *D. immitis*, as a M1-skewing factor. We have also tested the efficacy of *Asaia*WSP as an inductor of macrophage activation and of *Leishmania* killing. The experiments have been performed on a macrophage cell line, using *Leishmania* *infantum* as the test parasite. *Asaia*WSP has determined the expression of typical markers of M1 macrophage activation, including Th1-type cytokines, ROS and NO, and an increased phagocytosis process. Furthermore, macrophages pre-stimulated with *Asaia*WSP have shown an increased leishmanicidal activity. In summary, our study provides an additional evidence for the M1 stimulating capability of WSP and highlights the potential of *Asaia*WSP as an immunomodulating agent, suitable to be further investigated for the development of novel tools for the control of leishmaniasis.

**AUTHOR SUMMARY (max 150)**

In this study the authors focus their attention on leishmaniasis, a vector-borne disease widespread in over one hundred countries in tropical, sub-tropical and temperate zones and caused by the protozoan parasite Leishmania infantum. The properties of the symbiont Wolbachia as immunomodulator of the immune system (from insects to mammals) are here exploited: this study investigates the capability of a Asaia-expressing a molecule from Wolbachia to polarize the immune response towards the M1/Th1 phenotype, which is a protective response against visceral leishmaniasis. This chimeric bacterium AsaiaWSP acts as a polarizing agent, stimulating phagocytosis and inducing the release of pro-inflammatory stimuli and microbicidal molecules. Finally, AsaiaWSP determines an anti-leishmanial effect with a reduction of the number of intracellular parasites.

**INTRODUCTION**

Naïve macrophages (M0) can differentiate into two major, functionally distinct, subtypes: the classically activated- and the alternatively activated-macrophages (indicated as M1 and M2, respectively). These myeloid cells play crucial roles non only in the immunity towards microbial and parasitic infections, but also in wound healing, tissue repair and in cancer progression or regression (Zhu et al., 2015; Ruytinx et al., 2018). Classically activated macrophages are the pro-inflammatory subtype with microbicidal properties, and the activation of the M1 response is intrinsically associated with increased phagocyte activity and killing of intracellular pathogens, through the production of reactive oxygen species (ROS) and nitric oxide (NO) (Atri et al., 2018); M1 macrophages are also crucial in anti-cancer immunity. The M2 phenotype is an anti-inflammatory/regulatory subtype, that plays a role in the resolution of inflammation and in tissue repair, as well as in tumor progression, and in variety of diseases associated with excessive antibody production (Weagel et al., 2015; Parisi et al., 2018).

A parasitic infection that is paradigmatic in terms of its clinical outcome in relation with the M1 or M2 polarization is leishmaniasis. The general consensus is that during a Leishmania infection the development of a M1/Th1 response is associated with the production of proinflammatory cytokines such as TNFα, IL-12, and IFN-γ and the release of ROS and NO, with the killing of the parasites, therefore with a protective immunity. On the other hand, a M2/Th2 response is associated with anti-inflammatory cytokine production, such as IL-4/IL-13, IL-10, TGF-β, M-CSF, expression of arginase I (with reduced NO production), inhibition of inflammation, parasite survival, and thus disease progression. In summary, while M1 activation is crucial for a successful elimination of *Leishmania* parasites, in some forms of leishmaniasis the M2 polarization, besides being unprotective, it is associated with disease severity, also in relation with immune-complex pathology (Rossi & Fasel 2018).

Therefore, one of the major aims in leishmaniasis research is the identification of molecules with immunotherapeutic properties, i.e. molecules capable of modulating the immune response, to be used alone, in combination with drugs, or as vaccine adjuvants. Immunotherapy is already applied for the control of several diseases, e.g. cancer, allergies, and viral infections (Papaioannou et al., 2016; Naran et al., 2018; Roatt et al., 2014). In visceral leishmaniasis, patients non-responding to conventional chemotherapy have been treated with success through combination therapies with various immunomodulators, e.g. MDP13, IFNγ, IL-12, and the bacterium Bacille Calmette-Guérin (BCG) (Roatt et al., 2014; El-On et al., 2009). In addition, also in cutaneous leishmaniasis, BCG has been used in combination with a lysate of *Leishmania* parasites, with positive therapeutic effects (Convit et al., 2003; Mayrink et al., 1992).

In this study, we suggest that the bacteria of the genus *Wolbachia* represent a promising source of molecules capable of stimulating and modulating innate immunity, with the potential to be exploited in immunotherapy and prophylaxis. In insects, *Wolbachia* has been shown to be a potent activator of innate immunity, able to determine the upregulation of several immune effectors such as antimicrobial peptides, autophagy-related proteins, and ROS (Zug et al., 2015; Epis et al., 2020). Indeed, the successful use of *Wolbachia* to block the transmission of viruses by mosquitoes has in part been associated with this immune-activating capacity (Rancès et al., 2012). On the other hand, *Wolbachia* from filarial nematodes (or its surface protein, WSP) has been shown to activate macrophages through the stimulation of innate-immunity receptors, determining a M1/Th1-type activation (Brattig et al., 2004; Saint André et al., 2002). In summary, there is strong evidence that *Wolbachia* is an effective inductor of innate immunity, in insects and in mammals, and WSP from the nematode *Wolbachia* represents a promising candidate immunomodulator, with pro-M1 properties.

A strategy to deliver immunomodulators to hosts, for therapeutic or prophylactic purposes, is to engineer non-pathogenic bacteria for their expression; the engineered bacteria are then administered to the host, through different routes (Berlec et al., 2019). For example, Jacouton and colleagues (2019) have modified a *Lactococcus lactis* strain for the expression of the cytokine IL-17A, with tumor prevention in a mouse model, after intranasal delivery of the engineered bacterium. In this context, we recently selected an acetic acid bacterium, *Asaia* sp., as a bacterial vehicle for the expression of the *Wolbachia* surface protein, generating the chimeric symbiont *Asaia*WSP (Epis et al., 2020). Our hypothesis is that *Asaia*WSP should determine innate immune activation with a M1 bias, thus conferring protection against M1-impaired infections. In order to test the potential of *Asaia*WSP as an immunomodulating agent, we assayed its capacity to induce a M1 activation on a macrophage cell line, determining the pattern of immune-activation; we then tested its efficacy in the induction of *Leishmania* killing by macrophages (Fig 1).

NOTA: IN REALTA’ LE DOMANDE A CUI IL LAVORO VOLEVA RISPONDERE SONO LE SEGUENTI:

This study was initiated with goal of answering the following questions: 1) Does WSP, produced by the heterologous system *Asaia*WSP, and not purified, maintain the capability to activate macrophages, inducing M1 polarization? 2) Does the macrophage-stimulating capacity of *Asaia*WSP, if confirmed, increase the killing activity of these cells toward *Leishmania*?

QUESTO E’ UN PEZZO CHE HO SCRITTO PER LA DISCUSSIONE, MA CHE FORSE POTEBBE STARE QUI, AL TERMINE DELL’INTRODUZIONE, SOSTITUENDO LE ATTUALI ULTIME RIGHE.

**RESULTS**

**Uptake and survival of bacteria in macrophage cell line**

In order to determine the bacterial uptake and resistance in a macrophage cell line, J774A.1 cells were exposed to bacteria (*Asaia*WSP or *Asaia*pHM4) at different conditions (see Materials and Methods) of co-incubation at a MOI of 100. After 1h of treatment with streptomycin, the bacteria inside the macrophages were quantified and expressed as CFU/ml. *Ad hoc* experiments showed that the uptakes, after 1h or 2h of incubation, were comparable (Fig 2A, p=0.823); for this reason the second time point (2h) was used in all the successive experiments, also considering published protocols (e.g. Migliore et al., 2018). Survival of bacteria after 24h of infection was quantified and expressed as mean number of phagocyted bacteria in 2h and survived until 24h. As reported in Fig 2A, the number of bacteria phagocyted by macrophages co-incubated with *Asaia*WSP is higher than that of macrophages co-incubated with *Asaia*pHM4 (p=0.0007), and comparable with the uptake of *Asaia*pHM4 by macrophages treated with LPS (positive control). In summary, the mean number of bacteria *Asaia*WSP phagocyted is almost double than that of *Asaia*pHM4 (6.32x105CFU/ml vs 3.52x105 CFU/ml).

As for the survival of bacteria in the macrophage, after 24h of co-infection the vast majority of bacteria phagocyted during 2h of co-incubation is killed by the macrophages with slight differences between the two treated groups and the control, indicating a general bactericidal activity of the macrophages (Fig 2B). In particular, as reported in Fig 2B, a slightly higher number of bacteria *Asaia*WSP were counted in macrophages (1.19x104 CFU/ml), compared both to *Asaia*pHM4 and *Asaia*pHM4 + LPS (6.52x103 CFU/ml and 6.78x103 CFU/ml respectively), but the differences are not statistically significant.

[[[[[ TOGLIERE The phagocytosis activity was also evaluated in pre-stimulated macrophages (with *Asaia* bacteria), subsequently exposed to *Staphylococcus epidermidis.* After 1 hour, the medians related to the number of phagocyted *S. epidermidis* after the pre-treatment with *Asaia* bacteria in general are higher than that of the controls (*Asaia*WSP vs LPS p=0.0012, *Asaia*WSP vs control p=0.0027, *Asaia*pHM4 vs LPS p=0.0065, *Asaia*pHM4 vs control p=0.0161; S1 Fig). After 2 hours of exposure of cells to *S. epidermidis,* only thenumber of phagocyted bacteria in *Asaia*WSP-infected macrophages was significantly different from that of the controls, suggesting a more intense phagocyte activity of these macrophages (*Asaia*WSP vs LPS p=0.0012; *Asaia*WSP vs cells in the medium p=0.0023). In summary, after both 1 hour and 2 hours, there is an evidence for an *Asaia*-induced effect on phagocytosis activity.]]]]]

**TEM analyses of phagocytosis**

The intracellular localization of *Asaia*WSP and *Asaia*pHM4 after 24h of incubation with macrophages was also investigated by transmission electron microscopy. As shown in Fig 3A, in macrophages exposed to *Asaia*WSP, bacteria were observed inside the cell; in some cases, the phagocytic vacuole hosting the bacterium was evident; several empty vacuoles were also observe, which suggest that a strong digestive activity occurred in the infected macrophage. On the other hand, when the macrophages were treated with the *Asaia*pHM4, the bacteria were mostly outside the macrophages and only a few bacteria were observed within the cells (Fig 3B); in general, a reduced number of empty vacuoles were observed in the macrophages exposed to *Asaia*pHM4, compared to those exposed to *Asaia*WSP.

**M1/Th1 cytokines secretion by *Asaia*WSP-infected macrophages**

Macrophages of the J774A.1 cell line were infected with *Asaia* bacteria (MOI 100) or with *Asaia* bacteria plus *Leishmania* (MOI 2) promastigotes; after 24h and 48h of co-infection, the culture supernatants were collected and analyzed by ELISA assay, for the presence of the cytokines IL-1β, IL-12p40, TNFα and IL-6 as markers of M1/Th1 polarization. Fig 4 illustrates the panel of M1/Th1 cytokines tested in this study, after 24h of infection. Twenty-four hours post-infection, the secretion IL-6 cytokine by macrophages treated with *Asaia* bacteria was statistically different compared to controls (means: *Asaia*+= 4934 pg/ml, *Leishmania* = 8.22 pg/ml, untreated = 19.74 pg/ml, LPS = 3543 pg/ml; Fig 4A), indicating a strong effect due to the presence of the bacteria. In particular, Fig 4B showes a higher production of this cytokine by *Asaia*WSP compared to the *Asaia*pHM4 treated cells, both in the presence or in the absence of the *Leishmania* (means: *Asaia*WSP not infected [by leishmania]= 5722 pg/ml; *Asaia*WSP infected= 4805 pg/ml; *Asaia*pHM4 not infected= 4145 pg/ml; *Asaia*pHM4 infected=3495 pg/ml)*.* Moreover*,* the presence of the parasite *Leishmania* determined a general reduction of the IL-6 production that is statistically significant, in particular in *Asaia*pHM4 treated cells (p = 0.0058).

The same trend was obtained for the expression of the cytokine IL-12p40 (Fig 4C-D), 24h post-infection. The macrophages treated by the bacteria *Asaia* produced a greater amount of cytokine IL-12p40 comparable with the production determined by LPS (means: *Asaia*+= 4157 pg/ml, LPS = 5372 pg/ml), and it was statistically different from the expression determined by the macrophages untreated or infected only with *Leishmania* (means: untreated= 1 pg/ml, *Leishmania* = 7.5 pg/ml, Fig 4C). Fig 4D presents the higher production of this cytokine by *Asaia*WSP compared to the *Asaia*pHM4 treated cells, both in the presence or in the absence of the *Leishmania* (means: *Asaia*WSP not infected= 2301 pg/ml; *Asaia*WSP infected= 1919 pg/ml; *Asaia*pHM4 not infected= 986 pg/ml; *Asaia*pHM4 infected= 526 pg/ml).

Culture supernatants from stimulated macrophages collected at the 24h time point were also checked for the production of the cytokines TNFα (Fig 4E-F) and IL-1β (Fig 4G-H). The secretion TNFα cytokine by macrophages treated with *Asaia* bacteria was statistically significant compared to controls, although lower than in macrophages treated with LPS (means: *Asaia*+= 791 pg/ml, *Leishmania* = 6.46 pg/ml, untreated = 5.81 pg/ml, LPS = 2679 pg/ml; Fig 4E). In Fig 4F, when the macrophages were infected by *Asaia*WSP the production of TNFα was significant compared to the *Asaia*pHM4 when the parasite was present (means: *Asaia*WSP not infected= 1143 pg/ml; *Asaia*WSP infected= 1491 pg/ml; *Asaia*pHM4 not infected= 860 pg/ml; *Asaia*pHM4 infected= 879 pg/ml). Finally, the quantification of IL-1β release was obtained after a pre-stimulation with LPS for 12h before the infection. The production of IL-1β cytokine induced by *Asaia* was higher than the LPS treated macrophages (means: *Asaia*+= 334 pg/ml, *Leishmania* = 49.34 pg/ml, untreated = 16.86 pg/ml, LPS = 121.7 pg/ml; Fig 4G); in details, when the macrophages were infected by *Asaia*WSP the production of IL-1β is significant compared to the *Asaia*pHM4 , but only when the parasite is absent (means: *Asaia*WSP not infected= 358.6 pg/ml; *Asaia*WSP infected= 304 pg/ml; *Asaia*pHM4 not infected= 308.5 pg/ml; *Asaia*pHM4 infected= 292.8 pg/ml; Fig 4H).

The production of IL-6 and TNFα was also evaluated after 48h post infections. As for IL-6 there is a positive effect determined by the bacteria, comparable with the production in LPS positive control (means: *Asaia*+= 2168 pg/ml, *Leishmania* = 10.16 pg/ml, untreated = 5.33 pg/ml, LPS = 3162 pg/ml). In particular, Fig 5B shows a higher production of this cytokine by *Asaia*WSP compared to the *Asaia*pHM4 treated cells, both in the presence or in the absence of the *Leishmania* (means: *Asaia*WSP not infected= 4247 pg/ml; *Asaia*WSP infected= 6073 pg/ml; *Asaia*pHM4 not infected= 1810 pg/ml; *Asaia*pHM4 infected= 3783 pg/ml)*.* Moreover*,* the presence of *Leishmania* determined a general increase of IL-6 production (Fig 5B), which is statistically significant, in particular in *Asaia*WSP treated cells (p = 0.0366). Macrophages treated by *Asaia*for 48h produced a greater amount of TNFα as compared to untreated and *Leishmania* treated controls, although it was comparable with production induced by LPS (means: *Asaia*+= 156.4 pg/ml, *Leishmania* = 28.55 pg/ml, untreated = 29.56 pg/ml, LPS = 1735 pg/ml, Fig 5C). No differences were detected in the production of this cytokine by *Asaia*WSP compared to the *Asaia*pHM4 treated cells (means: *Asaia*WSP not infected= 152 pg/ml; *Asaia*WSP infected= 171 pg/ml; *Asaia*pHM4 not infected= 111 pg/ml; *Asaia*pHM4 infected= 136 pg/ml).

**M2/Th2 cytokines secretion**

Culture supernatants from cells pre-stimulated with LPS and infected with *Asaia* bacteria or *Asaia* bacteria plus *Leishmania* were collected 24h post-infection and tested for the production of IL-10, a typical marker of M2/Th2 polarization. S2A Fig shows that a significant production of IL-10 was obtained when the macrophages were infected with promastigotes (means: *Asaia*+= 576 pg/ml, *Leishmania* = 363 pg/ml, untreated = 2.16 pg/ml, LPS = 491 pg/ml); macrophages infected with *Asaia*pHM4 produced more IL-10 compared to the *Asaia*WSP when the cells were infected only with bacteria (means: *Asaia*WSP not infected= 516 pg/ml; *Asaia*WSP infected= 475 pg/ml; *Asaia*pHM4 not infected= 694 pg/ml; *Asaia*pHM4 infected= 461 pg/ml; S2B Fig).

**NO and ROS production by *Asaia*WSP-infected macrophages**

The capability of macrophages infected with *Asaia*WSP or *Asaia*WSP plus *Leishmania* to produce NO was investigated, after 24h and 48h. After 24h post-infection, there are no significant differences between bacteria and controls, both in presence and in absence of *Leishmania* (S3 Fig). After 48h post-infection, the secretion of NO in the form of nitrites, as measured by the reduction of nitrates in nitrites, by macrophages treated with *Asaia* bacteria is statistically significant compared to controls (means: *Asaia*+= 84 μM, *Leishmania* = 73 μM, untreated = 80 μM, LPS = 119 μM; Fig 6A). As shown in Fig 6B, when the macrophages were infected by *Asaia*WSP the production of NO was significant compared to the *Asaia*pHM4, when the parasite is absent (means: *Asaia*WSP not infected= 119.9 μM; *Asaia*WSP infected= 131.7 μM; *Asaia*pHM4 not infected= 67.7 μM; *Asaia*pHM4 infected= 112.1 μM). In addition to nitrites, the macrophages in response to parasite infection produce the reactive oxygen species as defense mechanism, in the context of the M1 phenotype. The production of ROS after 24h in macrophages was investigated by a fluorometric assay using H2DCF-DA probe. As reported in Fig 7A, cells infected with *Asaia* bacteria showed the same trend of ROS production of the controls, except for the production by macrophages treated with H2O2 (means: *Asaia*+= 8346 FU, *Leishmania* = 6841 FU, untreated [medium] = 3867 FU, H2O2 = 101093 FU).  However, in Fig 7B, when the macrophages were infected by *Asaia*WSP the production of ROS is significantly different compared to the *Asaia*pHM4, when the parasite is absent (means: *Asaia*WSP not infected= 11294 FU; *Asaia*WSP infected= 9660 FU; *Asaia*pHM4 not infected= 5239 FU; *Asaia*pHM4 infected= 9660 FU).

**Analysis of the expression of iNOS and arginase I genes by macrophages**

The cells were collected and analyzed for the expression of iNOS gene, at the first time point (24h) and arginase I gene, at the second time point (48h) by reverse transcription-quantitative PCR. The expression of the two genes *β-Actin* and *cyclophilin* was used to normalize the data. As reported in Fig 8A, iNOS relative expression after exposure to *Asaia* was significantly higher than that of cells infected with *Leishmania* (means: *Asaia*+= 0.772, *Leishmania* = 0.002, untreated = 0.053, LPS = 3.037). The cells exposed to *Asaia*WSP showed an up-regulation in iNOS expression compared to those exposed to *Asaia*pHM4, either in presence or in absence of *Leishmania* (Fig 8B), even though the difference was significant only in the absence of the parasite*.* As for arginase I expression, cells were also analyzed at the second time point (48h). As shown in S4A Fig, no expression was detected when the macrophages were exposed to *Asaia* alone, while S4B Fig shows that when macrophages were exposed to *Asaia*WSP in presence of *Leishmania,* there was a significant downregulation of the gene expression, compared to the *Asaia*pHM4 treatment (means: *Asaia*WSP not infected= 1.025; *Asaia*WSP infected= 0.3694; *Asaia*pHM4 not infected=0.976; *Asaia*pHM4 infected= 0.7554).

**Expression of costimulatory molecules in macrophages exposed to *Asaia*WSP**

To investigate the effect of *Asaia*WSP on the activation of selected cell surface markers (CD80-CD86-CD40), the macrophages exposed to *Asaia* and *Leishmania* were processed for flow cytometry analyses.

As shown in Fig 9, both the treatments with *Asaia*WSP and *Asaia*pHM4 stimulated a higher number of macrophages to present at least one of the three CD molecules, compared to macrophages untreated or stimulated with *Leishmania* alone (*Asaia*WSP = 62%; *Asaia*pHM4 = 53%; LPS = 69%; untreated = 16%; *Leishmania* = 26%. P values < 0.0001 for *Asaia*WSP vs untreated, *Asaia*pHM4 vs untreated, *Asaia*WSP vs *Leishmania, Asaia*pHM4 vs *Leishmania*). Moreover, *Asaia*WSP determined an higher percentage of cell expressing at least one receptor compared to *Asaia*pHM4, similar to that determined by LPS treatment (*Asaia*WSP vs *Asaia*pHM4 p= 0.045, *Asaia*WSPvs LPS p=0.198, *Asaia*pHM4 vs LPS p=0.0002). Notably, *Leishmania* slightly stimulated macrophages to expose the CD markers.

We then analyzed which class of CD are primarily affected by our treatments; we observed significant differences in the percentage of macrophages presenting CD40 alone or a combination of CD86-CD40 (means CD40+ cells: *Asaia*WSP = 31%; *Asaia*pHM4 = 21%; LPS = 8%; untreated = 0.1%; *Leishmania* = 1%; means CD40+ and CD86+ cells: *Asaia*WSP = 34%; *Asaia*pHM4 = 22%; LPS = 48%; untreated = 0.5%; *Leishmania* = 2%; p values are reported in S1 Table). Notably, macrophages stimulated with LPS have a higher proportion of CD40+ and CD86+ macrophages compared to *Asaia* treated ones, while the opposite was observed for CD40+ cells. When we analyzed the mean fluorescence of the positive cells, we did not detect any differences (apart from a higher levels of CD86 and CD40 in LPS treated cells), probably indicating that positive macrophages expressed the same amount of receptors at the single cell level (S5 Fig).

**Expression of major histocompatibility complex class II (**MHCII**) by *Asaia*WSP**

We also investigated the expression of MHC class II by macrophages exposed to *Asaia* and *Leishmania* after 48h of infection and pre-stimulated with IFNγ. The priming with IFNγ was necessary to allow the expression of MHC class II by this cell line, because the constitutive expression on J774A.1 is very low (Kalupahana et al., 2005). Figure 10 and Table S1 show the results of the MHCII assay in term of percentage of positive macrophages. The higher percentage of MHCII positive cells was observe after the exposure to *Asaia*WSP followed by Leishmania infection (*Asaia*WSP = 47%; *Asaia*pHM4 = 34%; PMA = 30%; untreated = 5%; *Leishmania* = 25%; all p values are significant except for the combination *Asaia*pHM4 vs PMA). As in the case of CD receptors, when we analyzed the geometric means of fluorescence of the positive cells, we did not detect any difference, indicating that positive macrophages exposed the same amount of MHC receptor on their surface (S6 Fig).

**Killing of *Leishmania* by *Asaia*WSP-infected macrophages**

The anti-leishmanial effect of *Asaia*WSP was determined by microscopy observation after 24h and 48h of infection, as reported in Maksouri et al. (2017). J774A.1 cells were pre-infected with *Asaia* for 2h and then incubated with *L. infantum* parasites at a ratio of 1:2. The infection rate, the number of amastigotes per macrophage and the parasitic index were determined. After 24h of infection, considering all the three analyzed parameters (see Material and Methods), there were no significant differences between the three groups (*Asaia*pHM4 plus *Leishmania*; *Asaia*WSP + *Leishmania*; *Leishmania* alone; S7A-C Fig). Figure 11 presents the results observed at 48h. Asfor the infection rate (Fig 11A), the percentage of infected macrophages decreased from 50% (macrophages infected only with *Leishmania*) to 34% when macrophages were treated with *Asaia*WSP or to 37% when treated with *Asaia*pHM4. These differences were however not significant. Interestingly, the number of amastigotes in each macrophage decreased from 0.91 (*Leishmania* alone) to 0.39 (*Asaia*WSP + *Leishmania*), i.e. we observed a reduction of 57% (p=0.042). *Asaia*pHM4 also determined a reduction in amastigote number per macrophage (xx) compared to Leishmania alone, even though the difference was not significant. However, the difference in the effect determined by the two *Asaia* strain was also not significant.

Finally, a similar trend is observed in Fig. 11C, showing the parasitic index (average number of infected macrophages multiplied by the average number of amastigotes per macrophage); shortly, the infection with *Asaia*WSP reduced the parasitic index of 74.3% compared to the infection with *Leishmania* (p=0.043)*.* Moreover, *Asaia*WSP determined a reduction of the parasitic index (37.6%) compared to the macrophages treated with *Asaia*pHM4, though not significant. Fig 11D-F shows the staining of macrophages co-infected with *Asaia*pHM4 or*Asaia*WSP and *Leishmania*, or *Leishmania* alone, after 48h. In panel E, infected macrophages present several vacuoles and the amastigotes show morphological changes e.g. loss of membrane integrity and formation of multiple cytoplasmic vacuoles. These cellular modifications are less noticeable in panel D which report the macrophages pre-infected with *Asaia*pHM4. Panel F (infection only with *Leishmania*) displays numerous intact amastigotes, some of them in replication; part of the amastigotes are out of the macrophages.

**DISCUSSION**

Our first goal was to determine whether WSP, expressed in the heterologous system *Asaia* sp., maintains the expected immune-modulating properties (e.g. Brattig et al 2004; e tutti gli altri …). To this purpose, we set our experiments starting from two engineered strains of *Asaia*, derived from the wild-type strain *Asaia* SF2.1. This native bacterium was previously transformed using the plasmid vector pHM4, either “empty” (i.e. we no insertion of extra-sequences), or containing the WSP gene, thus generating the two engineered strains *Asaia*pHM4 and *Asaia*WSP (Epis et al 2020). Our experiments were then performed using these two strains (Fig. 1), which differ, in their protein repertoire, only for the expression of WSP. *Asaia*pHM4 thus represent an ideal control to determine the immunological properties of WSP, once expressed by *Asaia*. We will first comment the results of the comparison of the effects determined, on a murine macrophage cell line, by these two bacteria.

COME DETTO, DA QUI, PER UNA PAGINA E MEZZA, DISCUTERO’ SOLO IL CONFRONTO TRA *Asaia*WSP  e *Asaia*pHM4, SENZA CONSIDERARE GLI EFFETTI DI LEISHMANIA, DEL MEDIUM, DEGLI LPS. IN PRATICA, AD ESMEMPIO PER LE CITOCHINE, COMMENTANDO SOLO LE FIGURE DI DESTRA NEI NOSTRI PANNELLI ATTUALI DI FIGURE.

The immunomodulatory role of *Asaia*WSP was first investigated analysing the capability to induce phagocytosis (a typical M1 marker) on the macrophage cell line (Fig. 2). These experiments showed that *Asaia*WSP is able to induce a phagocytic activity that is significantly different form that determined by the control bacterium *Asaia*pHM4 (Fig. 2). The increase of the phagocytic activity indicates a good competence of *Asaia*WSP bacteria to invade macrophages and, potentially, to further stimulate them. Indeed, TEM revealed a higher internalization of *Asaia*WSP, compared to *Asaia*pHM4, with a diffuse vacuolization, which is suggestive for an activation of the macrophages (Fig. 3). The analysis of the vitality of bacteria at 24h of infection did not reveal any evident difference between the two strains; this indicates that the bacteria, once internalized, either in a higher (*Asaia*WSP) or in a lower (*Asaia*pHM4) number are anyway almost completely killed at 24h. The recorded survival rates are in accordance with those reported for pathogenic bacteria after similar assays on the same cell line (Fernandez-Cabezulo 2008), with elimination of approximately 90% of phagocyted bacteria. The results of the phagocytic assays on *Asaia* are coherent with those previously obtained on mosquito hemocyte cultures, using the same engineered strains *Asaia*WSP and *Asaia*pHM4 (Epis et al., 2020).

Once verified the phagocytic activity, the production of M1 cytokines was evaluated on the same macrophage cell line used in phagocytosis assays (Fig. 4,5). As detailed in the results section, *Asaia*WSP, compared to the control bacterium *Asaia*pHM4, induced overexpression of most of the tested M1 cytokines at the two time points, with or without *Leishmania* co-infection, with a few exceptions (e.g. TNFα without *Leishmania* and IL1-β at both time points and conditions).

Other two important factors in M1 macrophage activation are NO and ROS, both involved in *Leishmania* killing (Carneiro et al., 2016). They are produced by macrophages in response to *Leishmania* infection, which, on its side, tend to downregulate their production (Ball et al., 2014). In particular, while at 24h post-infection the production of NO (as revealed by the nitrite assay) was low, after 48h *Asaia*WSP induced a significant increase in the production NO compared with *Asaia*pHM4, but only in absence of Leishmania (Fig. 6). Similar trends were observed for ROS production by macrophages infected with *Asaia*WSP (Fig. 7). The decrease of the levels of ROS in presence of *Leishmania* is a further evidence for the Th2 polarization induced by this parasite, which appear to limit the effect of *Asaia* and WSP.

NO production is governed by the inducible NO synthase (iNOS), and the gene coding for this enzyme is regarded as a typical M1 marker. The presence of *Asaia* bacteria determined an upregulation of the gene and, both in presence and in absence of *Leishmania*, the expression was higher after stimulation by *Asaia*WSP than after stimulation by the control bacterium. We emphasize that the upregulation of iNOS gene is coherent with the significant production of nitrites observed after stimulation of macrophages by *Asaia*, at the 48h time point (see above). iNOS, a typical M1 marker, shares the same substrate with the enzyme arginase, whose upregulation is instead crucial in the process of M2 macrophage polarization. In coherence with all the above evidence for the M1 polarizing properties of *Asaia*WSP, the results of the arginase assays revealed an even lower induction of this M2 enzyme by this bacterium, compared to low level induced by the control bacterium *Asaia*pHM4 (Fig. S4). A similar trend was observed for IL10, another marker of M2 activation. In summary, the two bacteria, while inducing the expression of M1 markers, did not appear to promote M2 activation.

During the immune response, activation of the TCD4 lymphocytes by macrophages requires antigen presentation by MHCII, and the concomitant expression of co-stimulatory molecules (Podojil et al., 2009). Overall, our results show that *Asaia*WSP determined a higher expression of MHCII and of co-stimulatory molecules (Fig. 9,10,S5,S6), compared to *Asaia*pHM4, even though not significant for all counted parameters (for details, see the method section).

In summary, the results of the assays on phagocytosis, on M1 and M2 marker production, and on MHCII and co-stimulatory molecules expression, were all coherent in indicating that *Asaia*WSP possesses a higher capacity to induce classic, M1 activation, compared to *Asaia*pHM4. In other words, the question of whether WSP, expressed in the heterologous system *Asaia* sp., maintains the expected immune-modulating properties, was addressed, with a positive answer.

BENE: FINITO IL CONFRONTO TRA *Asaia*WSP  e *Asaia*pHM4, ORA CONSIDERERO’ ANCHE I RISULTATI DATI DALLA STIMOLAZIONE CON LPS, LEISMANIA, E MEDIUM DI COLTURA.

However, all the results discussed above refers just to the comparison between the two *Asaia* strains (with or without Leishmania), not considering the effects of *Asaia* stimulation, either WSP or pHM4, in comparisons with other stimuli. Indeed, our experiments were conducted also including control M1-promoting and null stimuli (LPS and the culture medium), as well as a M2-promoting stimulus (*Leishmania*). The overall results of the assays are shown in Fig. XX. In general, the comparisons with the control stimuli indicate that *Asaia*, either *Asaia*WSP or *Asaia*pHM4, is a strong inductor of M1 responses, comparable, for several parameters, to LPS. As expected, *Leishmania* did not determine M1 activation and expression, rather it induced expression of the two tested M2 markers; similarly, the expression of macrophage surface markers (MHCII and co-stimulatory molecules) was limited after stimulation with *Leishmania*, compared to the other stimuli. It is interesting to note that both *Asaia* bacteria were able to “revert” the anti-M1 effect of *Leishmania*, e.g. in the cases of IL6, IL1-β, iNOS expression. These results, i.e. the strong M1 stimulating property of *Asaia* bacteria, either expressing or not expressing the Wolbachia protein, was in some way not expected when we planned this study, considering the first immunological studies on wild type Asaia (MANDRIOLI???). However, considering that *Asaia* is a Gram-negative bacterium, with its load of LPS, and likely of other immune-stimulating molecules, a posteriori we can consider the obtained results as not surprising. Anyhow, our experiments revealed that our control bacterium *Asaia*pHM4, possesses an immune-stimulating capacity that is only slightly potentiated when modified for the expression of WSP. Indeed, while the comparison between the two strains, as shown in Fig. xx, and commented in the first paragraphs of Discussion, evidenced a higher capacity *Asaia*WSP to induce M1 responses, compared to *Asaia*pHM4, the inclusion of the data recorded after stimulation with LPS, the medium and Leishmania greatly increased the spectrum of the values, rendering minimal the difference between the two Asaia strains. On the other side, as already emphasized, the effect of both Asaia strains appeared really encouraging in terms of their potential as M1-polarizing agents.

A QUESTO PUNTO HO FATTO OUTING, HO DICHIARATO CHE ANCHE L’ASAIA PHM4 HA EFFETTI FORTI, QUINDI POSSO TRATTARE L’ESPERIMENTO SU LEISHMANIA TUTTO INSIEME, QUNDI I DUE CEPPI, LA LEISHMANIA, GLI LPS ...

In summary, all the tested markers of macrophage activation, i.e. the enhanced phagocytosis and the expression of M1 cytokines, antimicrobial effectors, surface receptors, coherently indicate that both strains of *Asaia* hold the potential to increment the killing capacity of macrophages toward *Leishmania*. We thus decided to investigate in vitro, on the same macrophage cell line, the antileishmanial effect of *Asaia*WSP and *Asaia*pHM4 (Fig. 11). After 24h of co-infection, the two strains of *Asaia* did not determine differences in the parasitic indexes, probability because the majority of the parasites were still in the form of promastigotes, out of the macrophages. At 48h post-infection, a decrease in the number of macrophages infected with parasites was shown in presence of *Asaia* from both strains compared to the control group (macrophages infected with only *Leishmania*). Since the count was performed only on morphological intact macrophages, the result in term of *Leishmania* killing could be likely underestimated; indeed, the parasite killing process is known to increase the fragility of macrophages, part of which is then seen broken on the microscope slide, and thus not included in the counting. We also counted the number of amastigotes per macrophage: pre-stimulation with both *Asaia* strains determined a significant reduction of the intracellular parasite presence compared to the macrophages infected only with *Leishmania*, that was even higher after treatment with *Asaia*WSP, compared to *Asaia*pHM4*.* The same trend was confirmed by the third parameter observed, the parasitic index, which further emphasises the anti-Leishmania effect of *Asaia* stimulation, and the slightly enhanced effect determined by *Asaia*WSP. Comparing the results obtained at 24h and 48h of co-infection, the number of amastigotes per macrophages prestimulated with *Asaia*WSP decreased, passing from 0.80 to 0.39, contrary to infected with only *Leishmania* whose number of amastigotes did not substantially change (from 0.96 to 0.91). Therefore, we would conclude that stimulation of macrophageswith *Asaia* increased the killing activity of these cells toward *Leishmania* at the 48h time point, with a slight, but significant, increase of the effect after the use of *Asaia*WSP. The killing activity on *Leishmania*, after *Asaia* stimulation, is also visible by microscopic observation: the amastigotes showed degeneration of the membrane and the macrophages appeared with several vacuoles, signs of an intense degeneration, while in the controls cells (infected only with Leishmania) the evidence for amastigote degeneration were rare.

QUI SOTTO RIPRENDIAMO LE DUE DOMANDE A CUI SI VOLEVA RISPONDERE, RIPRENDIAMO IL NOSTRO OUTING SUL FATTO CHE ANCHE ASAIA PHM4 FA COSE IMPORTANTI, QUINDI UNA PICCOLA SINTESI DEI RISULTATI.

This study was initiated with goal of answering the following questions: 1) Does WSP, produced by the heterologous system *Asaia*WSP, and not purified, maintain the capability to activate macrophages, inducing M1 polarization? 2) Does the macrophage-stimulating capacity of *Asaia*WSP, if confirmed, increase the killing activity of these cells toward *Leishmania*? Our experiments provided a positive answer to both questions, but also revealed that the tested control bacterium, *Asaia*pHM4, also possesses M1 polarizing properties, promoting the killing of the parasite by stimulated macrophages. In summary, stimulation of macrophages with both *Asaia* strains induced M1 polarization of the response, with production of pro-inflammatory cytokines and effector molecules, NO and ROS. On the contrary, typical M2 markers such IL10 and arginase expression were downregulated when the cells were stimulated with *Asaia*. In addition, *Asaia* induced an increase in the proportion of cells positive for the co-stimulatory molecules, and an increase of the geometric mean of the MHCII positive cells was also observed. This type of immunological activation by both *Asaia*WSP and *Asaia*pHM4 was suitable to inhibit *L. infantum* development inducing a reduction of the number of amastigotes in challenged macrophages, with evidence for an increase in amastigote killing. In conclusion, the tested *Asaia* stains appear as promising immunomodulator, worth of further investigations toward the development of novel therapeutic or prophylactic tools, for the control of leishmaniases and other Th1- impaired diseases.

E SI CHIUDE CON CONSIDERAZIONI FINALI (FORSE UN PO’ PERICOLOSE DOVE DICHIARIAMO CHE ASAIA WSP DI WSP NE PRODUCE POCA, E DOVE DICHIARIAMO CHE ALLA FINE UN EFFETTO DI ASAIA COME TALE POTEVAMO ASPETTARCELO …)

We admit that, while expecting a strong immune-stimulating capacity of *Asaia*WSP, we also expected a substantially lower stimulating capacity by *Asaia*pHM4. As already discussed, the strong macrophage stimulation determined by this strain is perhaps not surprising if we consider its Gram-negative nature (and LPS load). As for *Asaia*WSP, the overall expression of WSP, and its exposure at the bacterial surface, are perhaps not very efficient, as revealed by the immunogold staining and Western blotting, performed in a previous paper (Epis et al 2020). For future developments of *Asaia* as bacterial vaccine vehicle, and/or as an immunomodulating agent, both the issue of the immunopolarizing property of native strains, and the generation of strains capable of a higher production of WSP should be addresses. As a general conclusion we highlight that *Asaia* prove to be suitable for genetic manipulation, safe and easy to handle, capable of inducing activation of macrophages and *Leishmania* killing in in vitro experiments. Of course, the eventual use of *Asaia* as an engineered vehicle for immunomodulation or antigen vehicle will require genetic manipulations that do not require the insertion of resistance genes, and genetic streamlining to remove undesired genes (resistance factors; gene that could facilitate the recombination, that acquisition of undesired properties; etc).

**MATERIALS AND METHODS**

**Cell and parasite cultures**

J774A.1 ATCC® TIB-67 macrophage cell line, derived from *Leishmania*-susceptible BALB/Cn mice, was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained under an atmosphere of 5% CO2 at 37°C in incubator. All reagents for the cell cultures were purchased from ATCC (Manassas, VA, USA).

The *Leishmania* *infantum* promastigotes derived form a strain maintained at the Istituto Superiore di Sanità, Rome, Italy (strain MHOM/TN/80/IPT1). The parasites were grown at 23°C in Schneider's *Drosophila* medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS and gentamycin (5 μg/ml) within 3 to 4 days.

**Bacterial strains and growth conditions**

The bacterium *Asaia*WSP derived from the engineering of the bacterium *Asaia* SF2.1 strain, originally isolated from the *Anopheles stephensi* mosquito (Favia et al., 2007), with the plasmid pHM4-WSP (Epis et al. 2020). Briefly, the WSP cassette, inserted in the plasmid pHM4, is composed by the *Wolbachia* surface protein gene sequence (from the *Wolbachia* of the nematode *Dirofilaria immitis*), the neomycin phosphotranferase promoter PnptII, the E-TAG epitope and the transcription terminator Trrn. *Asaia*pHM4 was also obtained from strain *Asaia* SF2.1, but transformed with the empty plasmid (without the WSP-coding gene) and was used as control bacterium (Epis et al. 2020). Both bacteria were grown overnight in GLY medium broth (glycerol 25 g/l and yeast extract 10 g/l, pH 5) supplemented with kanamycin 100 μg/ml, under constant agitation at 30°C overnight.

**Phagocytosis Assay**

Macrophages were seeded in 24-well plates (2x105/ml) and allowed to adhere overnight at 37°C in humidified 5% CO2 atmosphere. Phagocytosis assay was performed applying the gentamicin protection assay as reported in Glasser, 2001, with minor modifications.

*Asaia* bacteria, grown overnight, were washed with sterile PBS and resuspended in complete DMEM medium. The macrophages were infected at a multiplicity of infection (MOI) of 100 bacteria per macrophage. Macrophages were infected with *Asaia*pHM4 or *Asaia*WSP; as a positive control, macrophages were infected with *Asaia*pHM4 in presence of *Escherichia coli* lipopolysaccharide (LPS) (0.3 μg/ml) (R&D Systems, Minneapolis, MN). After a 10 min centrifugation at 1,000 rpm, macrophages were incubated 2h at 37°C to allow internalization of the bacteria (Migliore et al., 2018). The choice of time of incubation was defined after preliminary tests. Then, the macrophages were washed with PBS once and treated with complete DMEM containing 100 µg/ml streptomycin for 1 h at 37°C to kill extracellular bacteria. After two washes with PBS, part of the macrophages were lysed using deionized water containing 1% (vol/vol) Triton X-100 (Sigma Aldrich, USA) for 15 min at 37°C, to release phagocyted bacteria. The bacterial titre was determined by plating ten-fold serial dilutions of the cell lysates on GLY plates (glycerol 25 g/l and yeast extract 10 g/l, agar 20 g/l with kanamycin 100 μg/ml acidified to pH 5) and CFU/ml were counted after growth for 48h at 30°C. In addition, to determine the bacterial survival inside the cells, the remaining part of the macrophages, after the treatment with streptomycin 100 μg/ml and the two washes, were incubated with streptomycin 20 μg/ml until 24h of infection, followed by the final step of the protocol, as described above.

[[[[[[ TOGLIERE Efficiency of the phagocytosis and killing by macrophages activated with *Asaia*pHM4 or *Asaia*WSP was evaluated also against the bacterium *Staphylococcus epidermidis* following the protocol described above. *S. epidermidis* was grown in LB medium broth (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l) buffered to 7.0-7.4 pH with NaOH under constant rotation at 37°C over night. Briefly, macrophages were first incubated with *Asaia* for 2h (MOI of 1:100) and washed with PBS; then were incubated with *S. epidermidis* (MOI of 1:10) and incubated for 1h or 2h at 37°C. Cell monolayers were washed once in PBS to remove extracellular bacteria and treated with DMEM containing 300 μg/ml gentamycin for 1 h to kill non-internalized bacteria. Then, the macrophages were treated as above for the determination of viable colonies growth on LB agar plates after 24h of incubation at 37°C.

Phagocytosis data and the *Staphylococcus* survival data were Log transformed to reach normality, as determined using a Shapiro-Wilk test, and analysed using a two-way ANOVA with repeated measures followed by Sidak’s post-hoc test to detect differences between treatments and also taking in account the inoculation period (1 and 2h).]]]]]]

**Electron microscopy**

The *Asaia* uptake by macrophages was also evaluated by transmission electron microscopy (TEM). After 24h of infection, cells were pelleted, washed with PBS and immediately fixed in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 2 h at 4°C and postfixed in 1% OsO4 in 0.1 M cacodylate buffer (pH 7.2) for 1.5 h at 4°C. Subsequently, the samples were subject to dehydration in ethanol and then were embedded in Epon 812. Finally, thin sections were stained with uranil acetate and lead citrate and examined under an EM900 transmission electron microscope (Zeiss).

***Leishmania* infection assay**

Macrophages were seeded in 6-well plates (2x105/ml) and allowed to adhere overnight at 37°C in humidified 5% CO2 atmosphere. The macrophages were infected with the two strains of *Asaia* at a MOI of 100 bacteria per macrophage, as described above.

After the streptomycin treatment for 1h, cell monolayers were washed once in PBS and then infected with *L. infantum* stationary phase promastigotes at a ratio of 2:1 (2 parasites per 1 macrophage). Non-internalized promastigotes were removed at 24h post infection by washing with PBS and fresh DMEM was replaced. Cells were then maintained at 37°C for further 24h (for a total of 48h from the infection). At designated time points (24h and 48h) the culture supernatants were collected, centrifugated (14,000 rpm, 15 min at 4°C) and stored at -20°C until cytokines and nitrite determination (see below).

For the assessment of leishmanicidal activity, macrophages after 24h and 48h of infection were washed with PBS, collected by using a cell scraper, centrifugated at 1,200 rpm for 6 min and washed with PBS. Finally, they were suspended in 200 μl PBS at the final concentration of 106cells/ml and cytocentrifugated (Cytospin Hettich) for 5 min at 500 rpm on a slide and stained with Giemsa solution following the standard protocol (Sigma-Aldrich, USA). As control of the leishmanicidal activity, we treated the macrophages with the anti-*Leishmania* drug Amphotericin B (0.3 μg/ml). The infection rate (percentage of infected macrophages/100 macrophages), the number of parasites in each macrophage and the parasitic index (average number of infected macrophages multiplied by the average number of amastigotes per macrophage) were determined with a microscope at 100X. Ten areas of the cover slip was used to determine these indices in duplicate.

The experiments were performed in triplicate. ~~Considering that we scored a large proportion of macrophages not containing amastigotes, the William’s mean (Mw) was used to calculate a geometric mean. The Mw data were then analyzed using Friedman test for repeated measures followed by a Dunn’s post-hoc test. The proportions of macrophages containing at least one amastigote was analyzed using a Cochran-Mantel-Haenszel (CMH) test for contingency tables followed by Bonferroni correction to avoid type II errors. The Parasite index, calculated as the product of the first two indexes, was analyzed as Mw data. The Friedman test was performed using GraphPad Prism 8, while the CMH test was performed in RStudio from the “dplyr” package.~~

**Determination of M1 and M2 cytokines and NO production**

All the cytokines were determined by ELISA kits: IL12p40, IL10 (Biolegend, USA), IL-1β, IL-6, TNFα (Thermo Fisher, USA), according to manufacturer’s instructions. Only for IL-10 and IL-1β quantification, the cells were pre-stimulated with LPS 1 µg/ml for 12 h, before the infection.

Simultaneous evaluation of nitrate and nitrite concentrations induced by the bacteria was measured by Vanadium assay with the reduction of nitrate to nitrites by Vanadium (III) combined with detection by the acidic Griess reaction (Sigma-Aldrich, USA), as reported in Miranda, 2001. In brief, saturated solutions of Vanadium (III) chloride (VCl3) were prepared in 1M HCl. One hundred μl of culture supernatants, collected at 24h and 48h of co-infection, were mixed with the same volume of VCl3 and reacted with an equal volume of the Griess reagents (1% sulfanilamide and 0.1% naphthylethylenediamine-HCl in 2.5% phosphoric acid). The absorbance at 540 nm was measured using a plate reader following the incubation (usually 30-45 min at 37°C).

The data (pg/ml) were first checked for normality using a Shapiro-Wilk test and Log transformed if needed and analyzed in two groups: a first test was performed comparing the samples containing *Asaia*, LPS, the Medium alone or *Leishmania* only. This set was meant to detect if the bacteria have an effect comparable to LPS and different from other controls. A second set was meant to detect the effect of *Asaia* strain during *Leishmania* infection. ~~For the first set, data were analysed using a ONE-WAY Brown-Forsythe ANOVA followed by a Dunnet’s T3 post-hoc test if normal, otherwise using a Kruskal-Wallis test followed by a Dunn’s test. For the second set, data were analysed using a two-WAY Mixed-effects model with repeated measures matching followed by Sidak’s post-hoc test. The tests were performed using GraphPad Prism 8.~~

**Arginase and iNOS expression by real time quantitative PCR**

To evaluate arginase expression, the cells were pre-treated with IL4 (200 U/ml) (R&D Systems, Minneapolis, MN) for 12h at 37°C and then infected as above, while for iNOS (inducible nitric oxide synthase) expression no priming was performed. After 24h of infection for iNOS and 48h for arginase, the cells were collected and stored in RNA later (Qiagen, Germany) at -80°C till further use. Total RNA of infected cells was extracted using the ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI, USA) following manufacturer’s instructions. RNA purity was checked by determining the 260/280 nm absorbance ratio. cDNAs were synthesized from 200 ng of total RNA using the LunaScript™ RT SuperMix Kit from New England BioLabs (NEB, USA) according to the manufacturer’s instructions. Quantitative real-time PCR (qPCR) was performed using a BioRad CFX Real-Time PCR Detection System (Bio-Rad, USA) using *β-Actin* and *cyclophilin* genes for normalization (S2 Table) at the following conditions in a final volume of 20 μl: 10 μl of Supermix SsoAdvanced SYBR Green, 10 μM of primers, 2.4 μl of cDNA. After initial denaturation for 95°C for 30 sec, the amplification was performed at 95°C (15 s), 58°C (30 s) for 45 cycles then 65°C (5 s).

~~The data (relative expression) were first checked for normality using a Shapiro-Wilk test and Log transformed if needed and analyzed in two groups as described before. For the first set, data were analysed using a Kruskal-Wallis test followed by a Dunn’s test. For the second set, data were analysed using a two-WAY ANOVA followed by Sidak’s post-hoc test.~~

**ROS determination**

Intracellular reactive oxygen species (ROS) were measured by a fluorometric assay using 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) as probe. In brief, macrophages were seeded (35,000/well) in a final volume of 200 μl/well in 96well microplates and allowed to adhere 24h at 37°C in humidified 5% CO2 atmosphere. After an overnight incubation, the supernatants were discarded, and the cells were washed with PBS. Two hundred µl/well of H2DCF-DA (5 µM) were added to the macrophages, and incubated 1h at 37°C. Subsequently, the cells were washed and infected with the two strains of bacteria first and then with *Leishmania,* as reported above. The cells were incubated in humidified 5% CO2 atmosphere at 37°C, protected from light, for approximately 14h. Half an hour before ending of incubation, a group of cells were treated with 1mM H2O2 and the fluorescence at 485 nm (Ex) / 535 nm (Em) was measured. Data were analysed as described for the secreted cytokines.

**Cell surface markers analysis by flow cytometry**

Expression of CD40, CD80, CD86 and MHC class II was evaluated with a FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ). For the evaluation of co-stimulatory molecules, the cells were harvested 24h post infection with bacteria and *Leishmania* as indicated previously, washed with PBS and stained with appropriate dilutions of the following fluorochrome-conjugated antibodies: CD40-PE, CD80-Alexa Fluor488 and CD86-PE/Cy7 (all purchased from Biolegend San Diego, CA) for 15 min at 37°C. The cells were washed, resuspended in the Facs washing buffer (PBS/FCS 1%) and finally analyzed.

For the evaluation of MHC class II molecules, the cells were pre-stimulated with INFγ (1ng/ml) 12h before the infection. After 48h of co-infection the cells were harvested and processed as above using MHC class II-FITC (Biolegend San Diego, CA) as antibody. Fluorescence-activated cell sorting (FACS) data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

~~The proportions of macrophages presenting the studied markers were analyzed using a Cochran-Mantel-Haenszel (CMH) test for contingency tables followed by Holm correction to avoid type II errors, performed in RStudio from the “dplyr” package. The geometric means of fluorescence of the fluorescent cells in each set was compared using one-way ANOVA followed by Sidak’s post-hoc test for multiple comparisons performed in GraphPad Prism 8.~~

**DATA ANALYSIS**

All data were stored in a spreedsheet and then analysed according to a one-way factor design with Treatment factor as main experimental factor of interest.

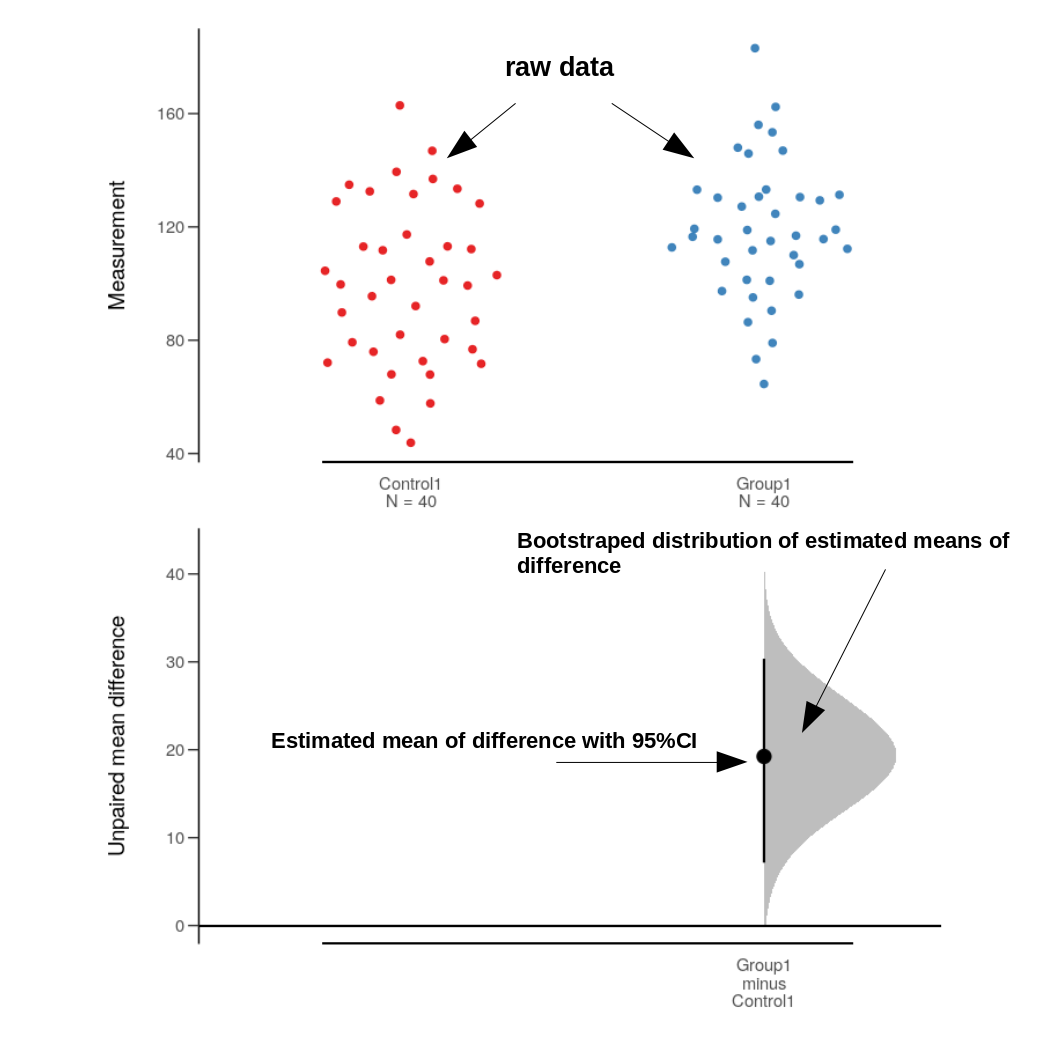
For experiments testing the effect of Treatment (AsaiaWSP vs AsaiapHM4, Leishmania, LPS and Med) on cytokine expression and phagocitos we used Estimation Statistics (ES) approach ( Cumming, Geoff (2012). *Understanding The New Statistics: Effect Sizes, Confidence Intervals, and Meta-Analysis*. New York: Routledge.). Estimation statistics is a simple [framework](https://thenewstatistics.com/itns/) that—while avoiding the pitfalls of significance testing () —uses familiar statistical concepts: means, mean differences, and error bars. More importantly, it focuses on the effect size of one's experiment/intervention, as opposed to significance testing by calculatin effect size ( mean differences) with his 95% confidence interval , using bootstrap resampling methods as described by Efron 1981 (BRADLEY EFRON, Nonparametric estimates of standard error: The jackknife, the bootstrap and other methods, Biometrika, Volume 68, Issue 3, December 1981, Pages 589-599, <https://doi.org/10.1093/biomet/68.3.589>).

Bootstrap resampling gives us two important benefits:

1. Non-parametric statistical analysis. There is no need to assume that our observations, or the underlying populations, are normally distributed. Thanks to the Central Limit Theorem, the resampling distribution of the effect size will approach a normality.

2. Easy construction of the 95% CI from the resampling distribution. For 1000 bootstrap resamples of the mean difference, one can use the 25th value and the 975th value of the ranked differences as boundaries of the 95% confidence interval. (This captures the central 95% of the distribution.) Such an interval construction is known as a percentile interval.

The ES approach produce a plot which presents the rawdata (top panel) and the bootstrap confidence interval of the effect size (the difference in means) (bottom panel) aligned as a single integrated plot, called Cumming plot (fig.x)



Anyway in order to have a measure of strong evidence against Null Hypothesis ( mean difference = 0) we applied for each contrast a Fisher’s significant test ( bootstraped Welch two-sample t-test ) and exact p-values are reported.

For experiment testing the effect of Treatment (AsaiaWSP vs AsaiapHM4, Leishmania, Anfotericina) in reduction of number of parasites (Leishmania) in Macrophages median with 95% credibility intervals (95%CI) of number of Leishmania in Macrophages were estimated fitting a Bayesian Negative Binomial Regression to take account of the large zero observation ( >60%) in data that induce overdispersion in the variance.

Data analysis and graphics associated were performed in R language using dabestr (ref.) package for Estimation Statistics, and Mkinfer package

for bootstraped Welch two-sample t-test using boot.t.test() function.

we use and rstarnm package (ref.) with tidybayes package (ref.) for Bayesian Negative Binomial Regression

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**Supporting information captions**

**S1 Table. Statistical analyses**

**S2 Table. List of primers used in this study.**