Boosting immunity to treat parasitic infections: *Asaia* bacteria expressing a protein from *Wolbachia* determine M1 macrophage activation and killing of *Leishmania* protozoans

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ABSTRACT

Leishmaniases are severe vector-borne diseases affecting humans and animals, caused by *Leishmania* protozoans. Over one billion people and millions of dogs live in endemic areas for leishmaniases and are at risk of infection. Immune polarization plays a major role in determining the outcome of *Leishmania* infections: hosts displaying M1-polarized macrophages are protected, while those biased on the M2 side acquire a chronic infection that could develop into a deadly disease. The identification of the factors involved in M1 polarization is essential for the design of therapeutic and prophylactic interventions, including vaccines. Infection by the filarial nematode *Dirofilaria immitis* could be one of the factors that interfere with leishmaniasis in dogs. Indeed, filarial nematodes induce a partial skew of the immune response towards M1, likely caused by their bacterial endosymbionts, *Wolbachia*. Here we have examined the potential of *Asaia*^WSP, a bacterium engineered for the expression of the *Wolbachia* surface protein (WSP), as an inductor of M1 macrophage activation and *Leishmania* killing. Macrophages stimulated with *Asaia*^WSP displayed a strong leishmanicidal activity, comparable to that determined by the choice-drug amphotericin B. Additionally, *Asaia*^WSP determined the expression of markers of classical macrophage activation, including M1 cytokines, ROS and NO, and an increase in phagocytosis activity. *Asaia* not expressing WSP also induced macrophage activation, although at a lower extent compared to *Asaia*^WSP. In summary, the results of the present study confirm the immunostimulating properties of WSP highlighting a potential therapeutic efficacy against *Leishmania* parasites. Furthermore, *Asaia* was designed as a delivery system for WSP, thus developing a novel type of immunomodulating agent, worthy of being investigated for immunoprophylaxis and -therapy of leishmaniases and other diseases that could be subverted by M1 macrophage activation.

1. 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 not only in the immunity towards microbial and parasitic infections, but also in wound healing, tissue repair and in cancer progression or regression [1,2]. "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) [3]. M1 macrophages are also crucial in anti-cancer immunity [4]. 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 a variety of diseases associated with excessive antibody production [5]. Macrophage activations on the M1 or M2 side are associated with corresponding pathways in the polarization of T-helper lymphocyte cells, known as Th1 or Th2 [6].

A parasitic infection that is paradigmatic in terms of its clinical outcome in relation to the M1 or M2 polarization is leishmaniasis [7]. The general consensus is that, during a *Leishmania* infection, the development of an M1/Th1 response is associated with the production of proinflammatory cytokines such as TNFα, IL-12, and IFNγ and the generation of ROS and NO, with the killing of the parasites, therefore with a protective immunity. On the other hand, an M2 response is associated with anti-inflammatory cytokine production, such as IL-4/IL-13, IL-10, TGFβ, 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, the M2 polarization, besides being unprotective, is in general associated with disease severity, being associated with immune-complex pathology [8].

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 [9], or as vaccine adjuvants. Immunotherapy has already been proposed for the control of several diseases, e.g. cancer, allergies, and viral infections, including COVID-19 [10–13]. In visceral leishmaniasis [7], 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) [10,14]. In addition, BCG has also been used in cutaneous leishmaniasis, in combination with a lysate of *Leishmania* parasites, with positive therapeutic effects [15, 16].

In this study, we suggest that 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 [17,18]. Indeed, the successful use of *Wolbachia* to block the transmission of viruses by mosquitoes has in part been attributed to this immune-activating capacity [19–21]. 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 an M1/Th1-type activation [22,23]. In summary, there is strong evidence that *Wolbachia* is an effective inductor of innate immunity, in insects and in mammals, and its surface protein WSP represents a promising candidate immunomodulator, with pro-M1 properties.

The exploitation of *Wolbachia* in macrophage stimulation experiments is however hampered by the characteristics of this bacterium: it is an obligate intracellular symbiont and it is not culturable in cell-free media, and thus not easy to be used in controlled in vitro and in vivo experiments. A strategy to deliver immunomodulators to hosts, for therapeutic or prophylactic purposes, is to engineer culturable nonpathogenic bacteria for their expression; the engineered bacteria are then administered to the host, through different routes [24]. For example, Jacouton and colleagues (2019) [25] 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} [18]. Our hypothesis is that *Asaia*^{WSP} should determine innate immune activation

with an 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 capability to stimulate the killing activity of macrophages towards the parasite *Leishmania infantum*, using a macrophage cell line, and determining the pattern of immune-activation of *Asaia*-stimulated cells.

2. Results

We planned our experiments starting from the engineered strains *Asaia*^{pHM4} and *Asaia*^{WSP}. These had been derived from the wild-type strain *Asaia* SF2.1, using the plasmid vector pHM4, either "empty" (thus generating *Asaia*^{pHM4}) or containing the WSP cassette (thus generating *Asaia*^{WSP}) [18]. The first set of experiments was aimed at assessing whether incubation with *Asaia*^{pHM4} or *Asaia*^{WSP} stimulates the microbicidal activity of macrophages, on bacteria (*Asaia* themselves and *Staphylococcus epidermidis*) and on *Leishmania infantum* (hereafter *Leishmania*). Secondly, we characterized the pattern of macrophage activation determined by *Asaia*, focusing on markers of M1 polarization.

2.1. Uptake and survival of bacteria in macrophages

The microbicidal activity of macrophages on bacteria was determined through the analysis of two processes: i) phagocytosis *sensu stricto*, i.e. the internalization of bacteria by macrophages; ii) the killing of phagocytized bacteria. In order to determine these processes, murine macrophage cells J774A.1 (see Materials and Methods) were exposed to bacteria (*Asaia*^{WSP} or *Asaia*^{pHM4}) at different conditions of co-incubation at a MOI of 100 (100 bacteria:1 macrophage). After 1 h of treatment with streptomycin, the intramacrophagic bacteria were quantified and expressed as CFU/mL. *Ad hoc* experiments showed that the uptakes, after 1 h or 2 h of incubation, were comparable (Supplementary Table S1a; p = 0.823); for this reason the second time point (2 h) was used in all the successive experiments [26]. Survival of bacteria after 24 h of treatment was quantified and expressed as the mean number of phagocytized bacteria in 2 h, surviving until 24 h. As reported in Fig. 1a, the number of bacteria phagocytized by macrophages co-incubated with *Asaia*^{WSP} is higher than that of macrophages co-incubated with *Asaia*^{pHM4}, and comparable with the uptake of *Asaia*^{pHM4} by macrophages treated with LPS (positive control). The mean number of bacteria *Asaia*^{WSP} phagocytized is almost double than that of *Asaia*^{pHM4} (7.89 × 10^5 CFU/mL vs 4.12 × 10^5 CFU/mL). Bootstrap estimation confirmed that the increase in the number of phagocytized *Asaia*^{WSP} was statistically significant (Δ = 3.77 × 10^5 CFU/mL, p = 0.0006). As for the survival of bacteria in the macrophages, after 24 h of co-infection most bacteria phagocytized during 2 h of co-incubation are killed by the macrophages with slight differences between the two treated groups and the control. In particular, as reported in Supplementary Table S1b, a slightly lower number of bacteria *Asaia*^{WSP} were observed within macrophages (1.43 × 10^4 CFU/mL), compared both to *Asaia*^{pHM4} and *Asaia*^{pHM4} + LPS (2.03 × 10^4 CFU/mL and 1.07 × 10^4 CFU/mL, respectively), but the differences are not statistically significant (p = 0.628). The phagocytosis activity was also evaluated in macrophages pre-stimulated with *Asaia*, subsequently exposed to *Staphylococcusepidermidis* for 1 h or 2 h (see Materials and Methods). After both time points, the average number of phagocytized *S. epidermidis* after the pre-treatment with *Asaia*^{WSP} are higher than that of the controls, but with a slight overlap with the 0 intercepts (Supplementary Fig. S1).

The intracellular localization of *Asaia*^{WSP} and *Asaia*^{pHM4} after 24 h of incubation with macrophages was also investigated by transmission electron microscopy (TEM). As shown in Figs. 1b and c, in macrophages exposed to *Asaia*^{WSP}, bacteria were observed inside the cells. In some cases, the phagocytic vacuole hosting the bacterium was evident; several empty vacuoles were also observed, which suggests that a strong digestive activity occurred in the treated macrophages. On the other
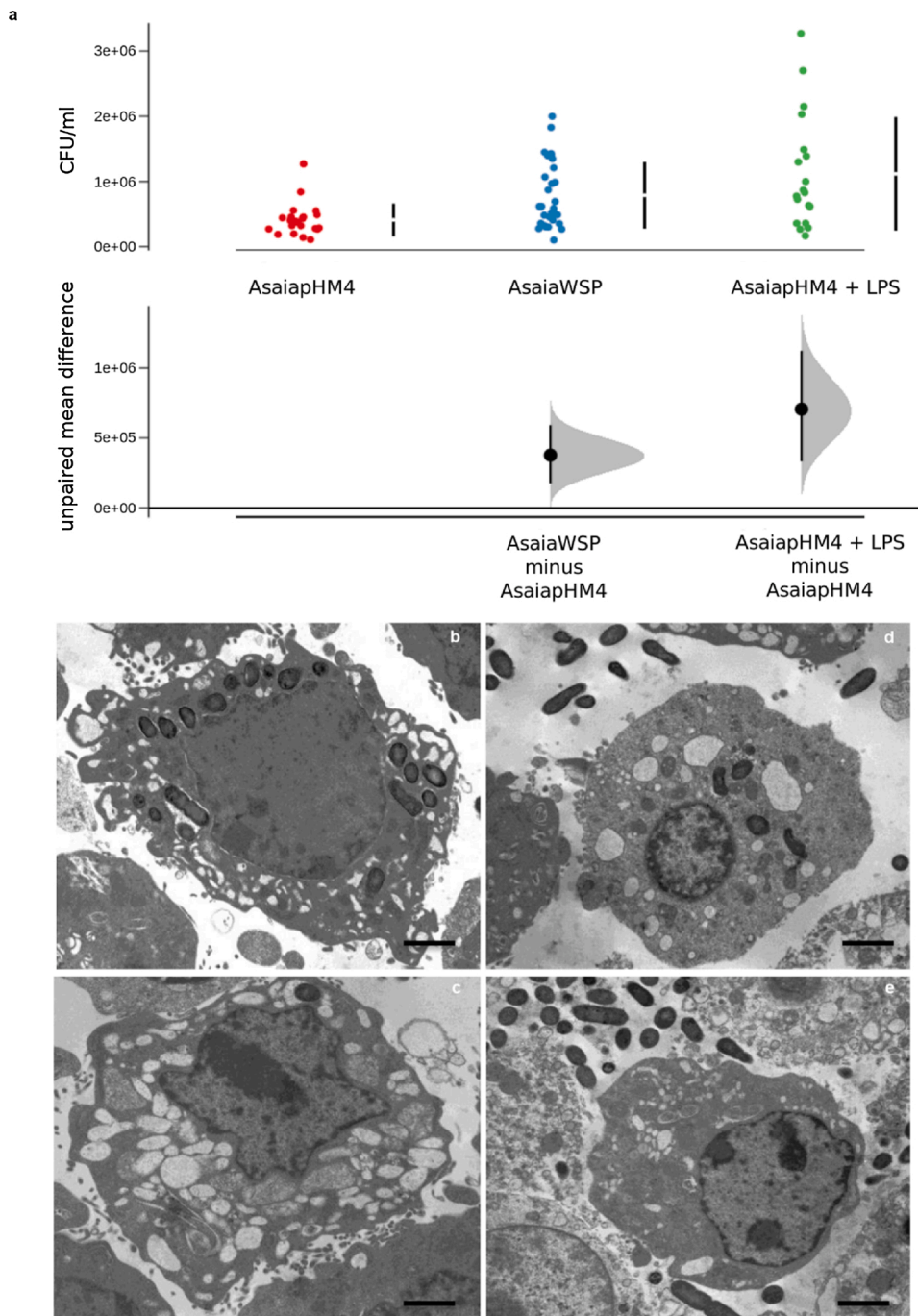
I. Varotto-Boccazzi et al.

Fig. 1. *Bacterial uptake by macrophages and TEM observation*. Murine macrophages were stimulated with *Asaia*[pHM4], *Asaia*[WSP], or *Asaia*[pHM4] plus LPS. In the top of panel a the numbers of bacteria internalized within macrophages are reported as CFU/mL. Vertical error bars: mean and standard deviation. In the bottom part of panel a the unpaired mean differences between treatment groups compared to *Asaia*[pHM4] are reported (see Materials and Methods). The shaded distribution derives from the application of the resampling algorithm. The large black circle represents the average difference between groups and the error bars on the large black circle indicate the 95 % confidence interval for the calculated difference. Estimation Statistics (ES) approach and bootstrapped Welch two-sample *t*-test were applied. The results are representative of triplicate experiments. TEM images of phagocytosis are shown in panels b-d. Murine macrophages were incubated with *Asaia*[WSP] (b and c) or with *Asaia*[pHM4] (d and e) for 24 h. In panels b and c, note the bacteria in the vacuoles and the signs of intense phagocytic activity. In panels d and e, bacteria are mostly outside the macrophage. Bars: 5 μm.

hand, when the macrophages were treated with *Asaia*[pHM4], the bacteria were mostly outside the macrophages and only a few bacteria were observed within the cells (Figs. 1d and e); in general, a reduced number of empty vacuoles were observed in the macrophages exposed to the control *Asaia*[pHM4], compared to those exposed to *Asaia*[WSP].

2.2. Killing of Leishmania by Asaia[WSP]-stimulated macrophages

The above results encouraged us to investigate whether macrophage activation determined by *Asaia*[WSP] could result in the phagocytosis and killing of *Leishmania*. The anti-leishmanial effect of *Asaia*[WSP] was determined by microscopy observation after 24 h and 48 h of incubation, as reported in Maksouri et al. (2017) [27]. J774A.1 cells were pretreated with *Asaia* for 2 h and then incubated with *L. infantum*

promastigotes. The infection rate of macrophages and the average numbers of *Leishmania* amastigotes in infected macrophages are reported, for each treatment group, in Supplementary Table S2a and b. The most informative time to evaluate an in vitro killing effect was 48 h after *Leishmania* infection. Interestingly, based on the binomial negative count model (Fig. 2a and Supplementary Table S2c), the mean number of amastigotes in each macrophage significantly decreased from 1.61 (control group, macrophages exposed only to *Leishmania*) to 0.67 (*Asaia*[WSP] + *Leishmania*), i.e. we observed a reduction of 58.4 % (p < 0.001). *Asaia*[pHM4] also caused a slight reduction in amastigote number per macrophage (1.44) compared to *Leishmania* alone, but the difference was not significant (p = 0.265). Moreover, a significant decrease of the mean number of *Leishmania* was recorded after the treatment with amphotericin B (0.45, p < 0.001) (Supplementary Table S2c), an
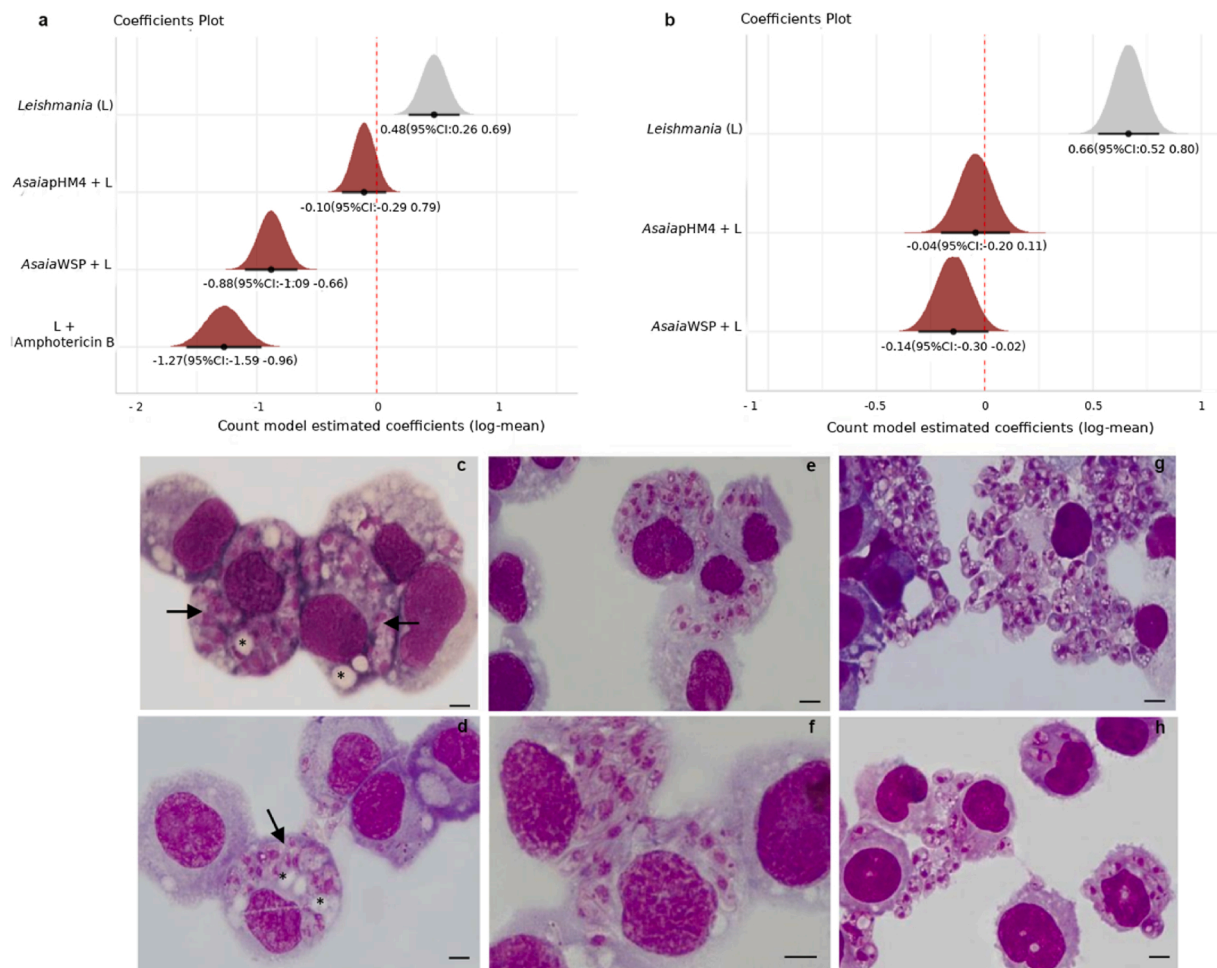
Fig. 2. *Killing of* Leishmania *by* Asaia^WSP*-stimulated macrophages after 48 h of incubation.* Macrophages were pre-stimulated with *Asaia*^WSP or *Asaia*^pHM4 or untreated, and then infected with *Leishmania* promastigotes (*Asaia*^WSP + L, *Asaia*^pHM4 + L, *Leishmania* (L), respectively). Amphotericin B was used as control for the killing, for the observations at the 48 h. Coefficients plots refers to the 48 h (a) or 24 h (b) after *Leishmania* infections. Horizontal bars indicate the 95 % confidence interval; vertical red lines correspond to the difference between the control group and the other groups. In panel a, the exponential of the estimated coefficient in group L (0.48) corresponds to the average number of amastigotes in this group. The other estimated coefficients (-0.10, -0.88, -1.27) correspond to the difference between the estimate of the reference group (L) and that of the treatment group. Similarly, in panel b, the exponential of the estimated coefficient in group L (0.66) corresponds to the average number of amastigotes in this group. The other estimated coefficients (-0.04, -0.14) correspond to the difference between the estimate of the reference group (L) and that of the treatment group. c-h) Giemsa staining of macrophages infected with *Leishmania* and *Asaia*^WSP (c and d), *Leishmania* and *Asaia*^pHM4 (e and f) and *Leishmania* alone (g and h). Arrows indicate groups of killed amastigotes and asterisks indicate the presence of vacuoles in macrophages treated with *Asaia*^WSP, signs of a high leishmanicidal activity. Bars: 5 μm. The experiments were performed in triplicate.

anti-*Leishmania* drug.

The Zero-inflated analysis in the ZINB model (see Materials and Methods) shows that the factor "type of treatment" was not significant as a predictor of the zero *Leishmania* counts in the macrophages (no significant differences were detected in the number of non infected macrophages in the different treatment groups compared to the control group, Supplementary Table S2c).

Fig. 2 (panels c-h) shows the staining of macrophages co-infected with bacteria and *Leishmania*, after 48 h. In panels c and d, 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 panels e and f which show the macrophages pre-treated with *Asaia*^pHM4. Panels g and h (infection only with *Leishmania*) display numerous intact amastigotes, some of them in replication; part of the amastigotes is out of the macrophages.

Finally, the other time-point we analyzed was 24 h. After 24 h of infection, considering the analyses obtained applying the zero-inflated model and the count model, there were no significant differences between the groups. However, as for the results of the count model, we can

observe that *Asaia*^WSP led to a reduction in the amastigote number per macrophage, with very limited overlap with the 0 intercept (Fig. 2b and Supplementary Table S2c).

2.3. NO and ROS production by Asaia-stimulated macrophages

Two major effectors in macrophage microbial killing are NO and ROS [28], both involved in the killing of *Leishmania* parasites [29]. These are produced by macrophages in resistant hosts, in response to *Leishmania* infection; which, on its side, tends to downregulate their production, especially in susceptible hosts [30]. Considering the reduced survival of *Leishmania* within macrophages stimulated with *Asaia*^WSP, we investigated whether this effect was associated with NO and ROS production. The capability of macrophages infected with *Asaia*^WSP or *Asaia*^WSP + *Leishmania* to produce NO was investigated, after 24 h and 48 h. After 24 h post-infection, there are no significant differences between bacteria and controls, both in presence and in absence of *Leishmania* (Supplementary Fig. S2). However, as shown in Fig. 3a, after 48 h post-infection, the generation of NO in the form of nitrites by macrophages treated with *Asaia*^WSP bacteria is significantly higher compared to the untreated
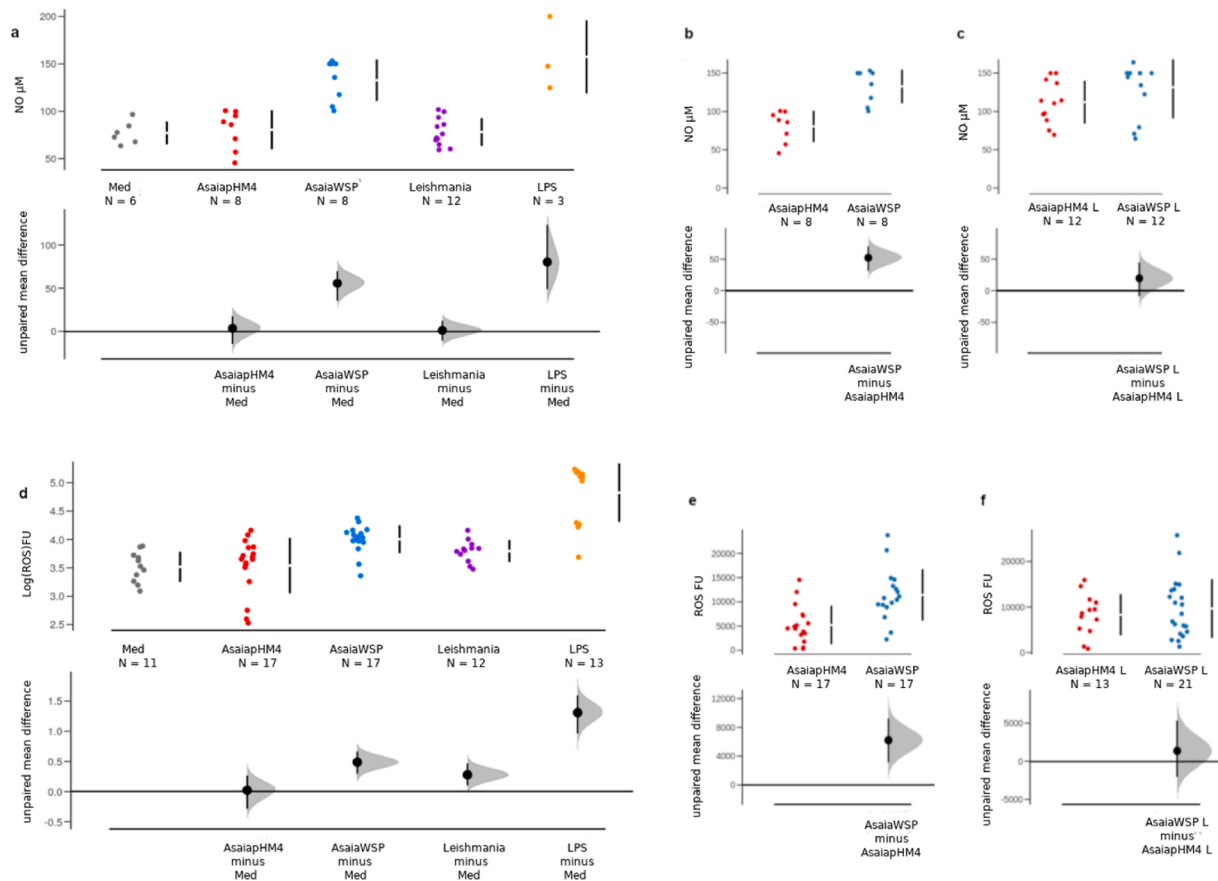
Fig. 3. *Nitrites and ROS production by macrophages stimulated with* Asaia^WSP. Nitrites and ROS levels were measured after 48 h and 24 h of incubation, respectively. In the top parts of panels a and d, the estimation plot show the observed data for nitrites (μM) (a) and ROS (Log fluorescence unit, FU) (d) production by macrophages incubated with *Asaia*^pHM4, *Asaia*^WSP, *Leishmania*, positive control or untreated (Med) (see Materials and Methods). Vertical error bars: mean and standard deviation. In the bottom parts of the panels, the unpaired mean differences between treatment groups compared to Med are reported. The shaded distribution derives from the application of the resampling algorithm. The large black circle represents the average difference between groups and the error bars on the large black circle indicate the 95 % confidence interval for the calculated difference. In the top parts of the panels b and c, the estimation plot show the production of nitrites by macrophages incubated with *Asaia*^WSP or *Asaia*^pHM4 in absence of the parasite *Leishmania* (b) or in presence of the parasite (c). The bottom parts of the panels show the comparisons between *Asaia*^WSP and the control bacterium *Asaia*^pHM4. FU released by macrophages incubated with *Asaia*^WSP or *Asaia*^pHM4 w/o *Leishmania* (e) or with *Leishmania* (f) at the top and the unpaired mean comparisons between the two bacteria at the bottom; Estimation Statistics (ES) approach and bootstrapped Welch two-sample *t*-test were applied. The results are representative of triplicate experiments.

macrophages (*Asaia*^WSP = 132.71 μM, untreated macrophages = 77.11 μM; p = 0.0038). *Asaia*^pHM4 and *Leishmania* did not induce a significant increase in NO generation compared to the unstimulated macrophages (*Asaia*^pHM4 = 80.46 μM, *Leishmania* = 78.10 μM; p = 0.7092 and p = 0.8687, respectively). Moreover, as shown in Figs. 3b and c, when the macrophages were incubated with *Asaia*^WSP the production of NO was significantly higher compared to *Asaia*^pHM4, when the parasite is absent (*Asaia*^WSP w/o *Leishmania* = 132.71 μM; *Asaia*^pHM4 w/o *Leishmania* = 80.46 μM; p = 0.0002).

NO status was also assessed by measuring inducible NO synthase (iNOS). The cells were collected and analysed for the expression of the iNOS gene, at the first time point (24 h) by reverse transcription-quantitative PCR. As reported in Supplementary Table S3a, iNOS relative expression after exposure to *Asaia*^WSP was higher than that of cells infected with *Leishmania* or *Asaia*^pHM4, supporting what we observed analysing NO production (*Asaia*^WSP = 1.35, *Asaia*^pHM4 = 0.2, *Leishmania* = 0.002, untreated = 0.05, LPS = 3.04).

In addition to nitrites, macrophages in response to microbial infection produce reactive oxygen species (ROS) as a killing mechanism, in the context of the M1 phenotype. The production of ROS after 24 h in macrophages was investigated by a fluorometric assay. In Fig. 3d bootstrap estimation analyses show the significant production of ROS determined by the treatment of macrophages with *Asaia*^WSP (4 Log FU)

or with LPS (4.82 Log FU), compared with untreated macrophages, as control (3.51 Log FU) (p = 0.0002 and p = 0.0002, respectively). Interestingly, *Leishmania* alone (3.79 Log FU) determined an appreciable increase of ROS compared to untreated macrophages (p = 0.008). As reported in Figs. 3e and f, when macrophages were stimulated by *Asaia*^WSP the production of ROS is significantly different compared to the *Asaia*^pHM4, when the parasite is absent (*Asaia*^WSP w/o *Leishmania* = 11454 FU; *Asaia*^pHM4 w/o *Leishmania* = 5237 FU; p = 0.0006).

2.4. M1 cytokines secretion by Asaia-stimulated macrophages

Macrophages were stimulated with *Asaia* (MOI 100) or with *Asaia* bacteria plus *Leishmania* (2 *Leishmania*:1 macrophage, MOI 2) promastigotes; after 24 h and 48 h of co-infection, the culture supernatants were collected and analyzed by ELISA assay, for the secretion of the cytokines TNFα, IL-12p40, IL-1β and IL-6, as markers of M1 polarization (Fig. 4; for a complete description of the choice of cytokines see Supplementary Text S1). Twenty-four hours post-infection, the production of TNFα cytokine by macrophages treated with both *Asaia* bacteria was statistically different compared to the untreated macrophages, indicating a strong effect due to the presence of the bacteria (*Asaia*^WSP = 2.95 Log pg/mL, p < 0.001; *Asaia*^pHM4 = 2.88 Log pg/mL, p < 0.001; untreated macrophages = 0.76 Log pg/mL; Fig. 4a). Comparing the effect of the
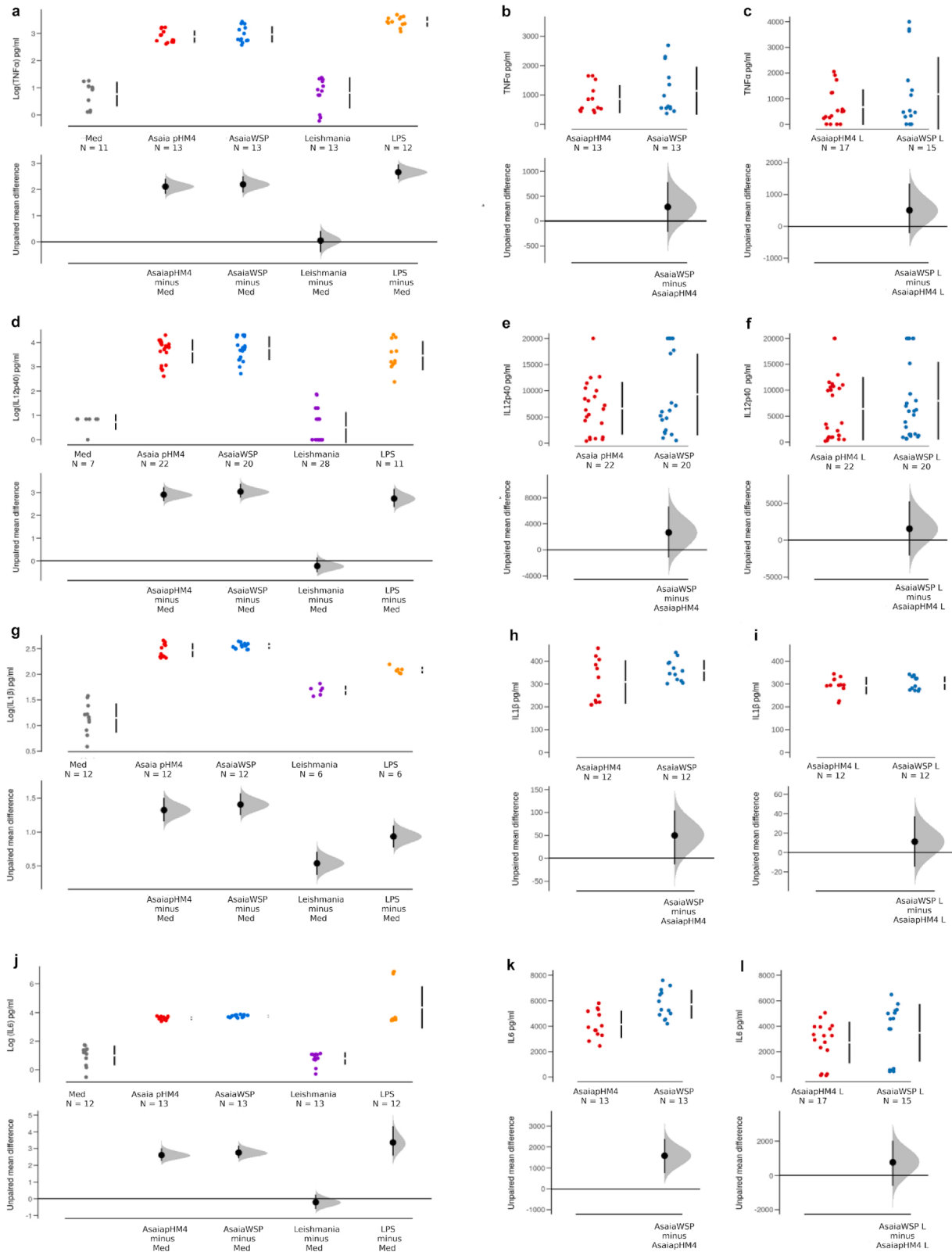
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Fig. 4. *TNFα, IL-12p40, IL-1β and IL-6 cytokines produced by macrophages stimulated with* Asaia *bacteria*. Levels of TNFα (a), IL-12p40 (d), IL-1β (g) and IL-6 (j) cytokines expressed as Log pg/mL and produced by macrophages treated with *Asaia*^WSP, *Asaia*^pHM4, *Leishmania*, LPS or untreated (Med) after 24 h of co-incubation are represented in the top panels (see Materials and Methods). Vertical error bars: mean and standard deviation. In bottom parts of panels a, d, g, j the unpaired mean comparisons of TNFα (a), IL-12p40 (d), IL-1β (g) and IL-6 (j) between treatment groups and untreated macrophages, as control group, are shown. The shaded distribution derives from the application of the resampling algorithm. The large black circle represents the average difference between groups and the error bars on the large black circle indicate the 95 % confidence interval for the calculated difference. The top parts of the panels b and c report observed data for TNFα production by macrophages incubated with *Asaia*^WSP or *Asaia*^pHM4 in absence of the parasite *Leishmania* (b) or in presence of the parasite (c). The bottom parts of the panels show the comparisons between *Asaia*^WSP and the control bacterium *Asaia*^pHM4. IL-12p40 produced by macrophages incubated with *Asaia*^WSP or *Asaia*^pHM4 w/o *Leishmania* (e) or with *Leishmania* (f) at the top and the unpaired mean differences between the two bacteria at the bottom. The top parts of panels h and i report the data observed for IL-1β production by macrophages incubated with *Asaia*^WSP or *Asaia*^pHM4 in absence of the parasite *Leishmania* (h) or in presence of the parasite (i). The bottom parts of the panels show the comparisons between *Asaia*^WSP and the control bacterium *Asaia*^pHM4. IL-6 produced by macrophages incubated with *Asaia*^WSP or *Asaia*^pHM4 w/o *Leishmania* (k) or with *Leishmania* (l) at the top and the unpaired mean differences between the two bacteria at the bottom. Estimation Statistics (ES) approach and bootstrapped Welch two-sample *t*-test were applied. Observed data are representative of three independent experiments.

two bacteria, we can observe (Figs. 4b and c) 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*, although there was no significant difference between the groups. The same trend of differences was observed for the secretion TNFα cytokine after 48 h post-infection (Supplementary Fig. S3). The same tendency was obtained for the expression of the cytokine IL-12p40, 24 h post-infection (Figs. 4d-f). The macrophages treated by *Asaia*^WSP and *Asaia*^pHM4 produced a greater amount of cytokine IL-12p40 comparable with the production determined by LPS treatment (*Asaia*^WSP = 3.74 Log pg/mL, *Asaia*^pHM4 = 3.63 Log pg/mL, LPS = 3.46 Log pg/mL) and statistically higher than the control (0.72 Log pg/mL) (*Asaia*^WSP: p < 0.001; *Asaia*^pHM4: p < 0.001; LPS: p < 0.001). *Leishmania* did not statistically affect the production of this cytokine (0.50 Log pg/mL, Fig. 4d). As observed for TNFα, we obtained a higher production of IL-12p40 by *Asaia*^WSP compared to the *Asaia*^pHM4 treated cells (i.e. with differences between the sample means), though there was no significant difference between the groups (Figs. 4e and f). Culture supernatants from stimulated macrophages were also checked for the production of the cytokines IL-1β (Figs. 4g-i) and IL-6 (Figs. 4j-l). The secretion of IL-1β cytokine by macrophages treated with both *Asaia* bacteria was statistically significant compared to untreated macrophages (*Asaia*^WSP: 2.55 Log pg/mL, p < 0.001; *Asaia*^pHM4: 2.47 Log pg/mL, p < 0.001). Noteworthy, the unpaired mean differences of macrophages treated with both bacteria are higher than that of macrophages treated by LPS (2.08 Log pg/mL) (Fig. 4g). However, the unpaired mean comparison of IL-1β of *Asaia*^WSP vs *Asaia*^pHM4 was not significant (Figs. 4h and i). Finally, the quantification of IL-6 induced by *Asaia*^WSP and *Asaia*^pHM4 after 24 h was comparable with those of LPS treated macrophages and higher than that of the control (*Asaia*^WSP = 3.74 Log pg/mL, *Asaia*^pHM4 = 3.60 Log pg/mL, LPS = 4.35 Log pg/mL; untreated macrophages = 0.99 Log pg/mL; p < 0.001 for all comparisons. Fig. 4j). As reported in Figs. 4k and l, when the macrophages were infected by *Asaia*^WSP the production of IL-6 is significantly higher compared to the *Asaia*^pHM4; bootstrap analysis confirmed the significance when the parasite was absent (p = 0.0012).

2.5. M2 cytokine secretion and expression of arginase I

Culture supernatants from cells pre-stimulated with LPS and infected with *Asaia* bacteria or *Asaia* bacteria + *Leishmania* were tested for the production of IL-10, a typical marker of M2 polarization. In Supplementary Table S3bbB we reported the means, the standard deviation and the corresponding p-value. We observed that macrophages incubated with *Asaia*^pHM4 produced more IL-10 compared to the *Asaia*^WSP when the cells were infected only with bacteria (*Asaia*^WSP w/o *Leishmania* = 565.81 pg/mL; *Asaia*^pHM4 w/o *Leishmania* = 692.05 pg/mL). As for *arginase I* expression (see Materials and Methods), cells were analyzed at 48 h. As shown in Supplementary Table S3c when macrophages were exposed to *Asaia*^WSP in presence of *Leishmania*, there was a downregulation of the gene expression, compared to the *Asaia*^pHM4 treatment (*Asaia*^WSP + *Leishmania* = 0.37; *Asaia*^pHM4 + *Leishmania* = 0.76).

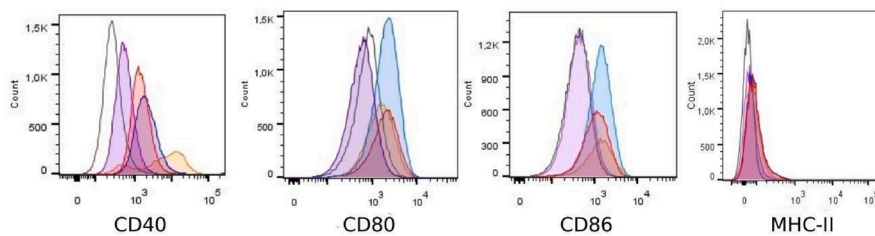## 2.6. Costimulatory molecules and MHC-II expression by Asaia-stimulated macrophages

To investigate the effect of *Asaia* on the expression of selected surface markers (CD80-CD86-CD40) involved during *Leishmania* infection, after 24 h of infection with bacteria and *Leishmania*, macrophages were processed for flow cytometry analyses.

The percentage of cells pretreated with *Asaia*^WSP or *Asaia*^pHM4 and expressing CD40 marker, was comparable (i.e. no statistical differences were observed) to that of macrophages treated with LPS, the positive control (LPS: 79 %; *Asaia*^WSP: 70 %; *Asaia*^pHM4: 58 %) (Fig. 5). The expression of CD40 on untreated macrophages or on macrophages infected only with *Leishmania* was statistically lower than the LPS positive control (untreated macrophages: 8%, *Leishmania*: 18 %, p < 0.001 for both comparisons). The same trend was obtained from CD80 and CD86 analyses. For both markers, pre-treatments with *Asaia*^WSP or *Asaia*^pHM4 stimulated a percentage of macrophages to present these molecules comparable with the positive control (CD80^+ cells: LPS = 33 %, *Asaia*^WSP = 22 %, *Asaia*^pHM4 = 17 %; CD86^+ cells: LPS = 59 %, *Asaia*^WSP = 40 %, *Asaia*^pHM4 = 34 %). On the contrary, the proportion of positive cells in untreated macrophages or in macrophages infected only with *Leishmania* was statistically lower than LPS (CD80^+ cells: untreated macrophages = 9% p = 0.008, *Leishmania*: 8%, p = 0.003; CD86^+ cells: untreated macrophages = 4% p < 0.001, *Leishmania* = 15 % p = 0.001; Supplementary Table S4 and Fig. 5). Fig. 5 also shows the expression of MHC-II by macrophages infected with bacteria and *Leishmania* after 48 h of infection and pre-stimulated with IFNγ. The priming with IFNγ was necessary to stimulate the expression of MHC-II by this cell line, because the constitutive expression on the cell line J774A.1 is very low [31]. The proportion of MHC-II positive cells treated with *Asaia*^WSP was statistically higher than the positive control treatment with PMA (Phorbol 12-Myristate 13-Acetate) (*Asaia*^WSP: 43 %, PMA: 30 %, p = 0.027; S4 Table). The group *Asaia*^pHM4 showed a higher proportion of positive cells compared with the positive control, but not statistically significant (p = 0.545). On the contrary, the proportion of positive cells in the untreated group was low (4.3 %) and statistically significant compared with the positive control (p < 0.001; S4 Table).

3. Discussion and conclusion

Our main goal was to determine whether *Asaia*^WSP is able to induce a macrophage activation, strong enough to result in microbicidal effects, particularly on *Leishmania*. A first set of assays showed that *Asaia*^WSP indeed activates an antibacterial response in macrophages, on both *Asaia* itself and on *S. epidermidis*. The study was then focused on *Leishmania*, an intracellular parasite that targets monocytes and macrophages [8,32]. The experiments were realized using a macrophage cell line derived from a *Leishmania*-susceptible mice strain. In susceptible hosts, macrophages are typically anergic towards *Leishmania*, which replicates in these cells [33]. As expected, our experiment showed that macrophages infected with *Leishmania* alone, with no pre-stimuli, reached a high load of parasites. However, after *Asaia*^WSP pre-stimulation,

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I. Varotto-Boccazzi et al.   Pharmacological Research xxx (xxxx) xxx

Predictors	cellsCD40+ Estimate/ Percentage of positivity 95%CI	cellsCD80+ Estimate/ Percentage of positivity 95%CI	cellsCD86+ Estimate/ Percentage of positivity 95%CI	cellsMHC-II+ Estimate/ Percentage of positivity 95%CI
Positive Control (Reference)	-0.24/78.7% -0.83 – 0.35	-1.10/33% -2.19 – -0.01	-0.52/59% -1.22 – 0.19	-1.23/30% -1.68 – -0.78
Medium	**-2.25/8% ***** -2.89 – -1.60	**-1.27/9% **** -2.22 – -0.33	**-2.60/4% ***** -3.46 – -1.74	**-1.92/4.3% ***** -2.29 – -1.55
Leishmania	**-1.46/18% ***** -2.07 – -0.85	**-1.33/8% **** -2.22 – -0.44	**-1.36/15% ***** -2.16 – -0.55	-0.18/24% -0.52 – 0.17
Asaia pHM4 +L	-0.31/58% -0.91 – 0.30	-0.64/17% -1.52 – 0.25	-0.54/34% -1.35 – 0.26	0.11/32% -0.24 – 0.45
Asaia WSP +L	-0.12/70% -0.73 – 0.48	-0.40/22% -1.29 – 0.49	-0.39/40% -1.20 – 0.42	**0.39/43% *** 0.04 – 0.73

Fig. 5. *Costimulatory molecules and MHC-II expression by macrophages stimulated with* Asaia *bacteria.* a) Differences in the proportions of macrophages positive for CD40, CD80 and CD86 costimulatory molecules and MHC-II marker between different treatment groups are reported. $e^{(intercept+estimated\ coefficient)}$ can be interpreted as the proportion of cells in the different treatment groups. b) Flow cytometry histograms of CD40, CD80 and CD86 and MHC-II expression, representative of three independent experiments are shown (orange curve: positive control; grey curve: negative control; violet curve: *Leishmania* (L); red curve: *Asaia*[pHM4] + L; blue curve: *Asaia*[WSP] + L).

Leishmania survival was dramatically reduced, with an effect that approximated that of amphotericin B, a choice drug to achieve the killing of *Leishmania* parasites. On the other hand, a higher amastigote survival was observed after pre-stimulation with the control bacterium *Asaia*[pHM4], which indicates that the stronger effect determined by *Asaia*[WSP] is likely associated with the expression of WSP. In summary, *Asaia* expressing WSP is capable of reverting the anergy of macrophages from a *Leishmania* susceptible host, restoring their capability to mount an effective microbicidal response.

The strong reduction in the number of *Leishmania* amastigotes determined by *Asaia*[WSP] likely derives from the activation of the killing activity of macrophage cells. Indeed, at 48 h, after *Asaia*[WSP] stimulation, the number of amastigotes per macrophage was significantly lower compared to the number observed at 24 h. In particular, comparing the results obtained at 24 h and 48 h after *Asaia*[WSP] stimulation, the average number of amastigotes per macrophage decreased passing from 1.68 to 0.67 (60 % of reduction), contrary to macrophages infected with *Leishmania* alone, in which the number of amastigotes changed only slightly (from 1.93 to 1.61, 17 % of reduction). Microscopic observations are coherent with the occurrence of leishmanicidal activity in *Asaia*[WSP]-stimulated macrophages: amastigotes showed degeneration of the membranes and macrophages displayed several vacuoles, signs of an intense digestive activity. Indeed, in our experiments *Asaia*[WSP] determined a significant increase in the production of NO and ROS, two major effectors in macrophage anti-bacterial and anti-*Leishmania* responses [28,29]. The increased killing activity after *Asaia*[WSP] stimulation can thus be attributed to a classical M1 macrophage activation, with production of well-established microbicidal effectors. NO production is governed by the inducible NO synthase (iNOS), whose expression was actually upregulated after macrophage stimulation with *Asaia*[WSP]. iNOS, a typical marker of M1 macrophage activation, shares the same substrate with the enzyme arginase, whose upregulation is instead crucial in the process of the alternative, M2 macrophage polarization [34]. The arginase assay revealed that *Asaia*[WSP] reduces the expression of this

M2-associated enzyme, in the presence of *Leishmania*. Furthermore, assays on cytokine production revealed that both *Asaia*[WSP] and *Asaia*[pHM4] induced overexpression of most of the tested M1 cytokines, with a slight, but coherent, overexpression of these cytokines after stimulation with *Asaia*[WSP] (for a discussion about the choice of the cytokines to be quantified and their role in leishmaniases, see Supplementary Text S1). As for type-2 cytokines, IL-10 was significantly downregulated by *Asaia*[WSP] in the presence of *Leishmania*. In summary, the activation of macrophage phagocytosis and killing activity, the induction of NO and ROS production, and the opposed effects on the expression of M1 and M2 markers (iNOS, arginase and cytokines) coherently point to the capability of *Asaia*[WSP] to activate macrophages, with a bias towards the classical, M1 phenotype [35]. *Asaia*[WSP] proved to be very effective also in the induction of IL-6, a pro-inflammatory cytokine that however does not perfectly fit into the Th1/Th2 paradigm [36].

During the immune response, activation of T CD4+ lymphocytes by macrophages requires antigen presentation through MHC-II, and the concomitant expression of co-stimulatory molecules, which also play a role in immune-modulation [37] (for a discussion about the choice of co-stimulatory molecules and their role in leishmaniases, see Supplementary Text S1). The analysis of the expression of co-stimulatory molecules showed that pre-treatment with *Asaia*[WSP] or *Asaia*[pHM4] stimulated a higher number of macrophages to present the tested CD receptors, compared to the control (Fig. 5). *Asaia* bacteria also stimulated the expression of MHC-II, an effect that was higher in cells pretreated with *Asaia*[WSP], compared to the PMA control treatment (Fig. 5). The evidence that *Asaia* stimulates the expression of both MHC-II and co-stimulatory molecules highlights the potential of these bacteria to determine a complete macrophage activation, with expression of the molecules required for antigen presentation and for the co-stimulation of T CD4+ cells.

Our study was not aimed at the investigation of the immune response to *Leishmania* parasites, a deeply explored field (e.g. [38]). However, we emphasize that the results of our experiments on *Leishmania* alone

confirmed the ability of this parasite to determine low level of M1 activation, limited expression of MHC-II and co-stimulatory molecules, and increased expression of M2 markers, in agreement with its well-documented adaptation to intra-macrophage survival and multi-plication (e.g. [39]).

In this study, the stronger capability of *Asaia*^WSP to induce an effec-tive macrophage activation in comparison with *Asaia*^pHM4, as revealed by the *Leishmania* killing assay, provides sound evidence for the capacity of WSP to determine macrophage activation, with clear signs for M1 polarization. Therefore, WSP from the *Wolbachia* endosymbionts of filarial nematodes is confirmed as a candidate immune-modulating factor in filarial diseases, in agreement with the suggestions of Brattig and colleagues [23]. Interestingly, preinfection of hamsters with *Brugia malayi* (a *Wolbachia*-harbouring filaria) determined significant protec-tion against a successive challenge with *Leishmania donovani* [40]. In addition, epidemiological studies have so far revealed a limited number of cases of co-infection in dogs, with *Dirofilaria* and *Leishmania* [41,42]. On the other hand, in dogs co-infected by *D. immitis* and *L. infantum* the presence of circulating *Wolbachia* DNA negatively correlates with clin-ical signs of leishmaniasis [43]. We could thus speculate that in dogs infected by the blood-dwelling filaria *D. immitis* the continuous release of WSP-loaded *Wolbachia* into the blood stream could modulate the immunity of the host, protecting it from *Leishmania* infection (even though chronic filarial infections in humans is also associated with macrophage tolerization; e.g. see [44]).

A first issue addressed by this study was whether WSP, the *Wolbachia* surface protein, possesses the capability to activate macrophages, determining an increased microbicidal activity. The second main issue was whether the engineered bacterium *Asaia*^WSP could be used to induce macrophage activation and *Leishmania* killing. Our results, while providing a positive answer to both the above questions, revealed that also the tested control bacterium *Asaia*^pHM4 displays macrophage acti-vating properties. The fact that both *Asaia* strains are able to activate macrophages is not surprising, considering their bacterial Gram-negative nature. It is interesting that *Acetobacter pasteurianus*, a mem-ber of the *Acetobacteraceae*, phylogenetically related with the genus *Asaia*, has recently been shown to induce macrophage activation in the same cell line used in this study, through the release of micro-vesicles [45].

With few exceptions, *Leishmania* parasites are paradigmatic for their capability to skew macrophages into an anergic, M2 state [46]. Our study reveals that both *Asaia* bacteria, but particularly *Asaia*^WSP, possess the ability to revert this anergic status, towards a classical M1 activation. The M1-polarizing properties of *Asaia* could be exploited without further manipulations, or after its engineering for the expression of specific antigens, from *Leishmania* or other pathogens. Based on our results, *Asaia*^WSP appears as the most encouraging, considering its ability to induce the killing of *Leishmania*. The effective internalization of *Asaia*^WSP in macrophages, followed by proper M1 activation, including expression of MHC-II and costimulatory molecules, suggest that this bacterium, once further modified for antigen expression, holds the po-tential to stimulate specific T CD4$^+$ lymphocytes as Th1-polarized cells. Therefore, *Asaia*^WSP appears very promising in terms of its potential applications in the prevention and treatment of leishmaniasis and other M1-impaired diseases, as an antigen vehicle capable of determining a correct activation of the immune response. The sharp reduction in the infection rate of *Leishmania* in macrophages, which we observed after stimulation with *Asaia*^WSP, suggests that this bacterium deserves to be investigated in in vivo models of leishmaniasis, to evaluate its potential utility as a therapeutic agent. We emphasize that the therapies currently used in the treatment of leishmaniasis in dogs and humans can cause side effects and are not always resolutive, e.g. in immunocompromised subjects [47,48]. A therapy based on immunomodulation could display limited side effects and could represent an alternative, or could be used in co-administration with leishmanicidal drugs. The eventual use of *Asaia* bacteria as engineered vehicles for immunomodulation or

vaccination will obviously require that safety issues be adequately addressed [49].

As a general conclusion we highlight that *Asaia* bacteria prove to be suitable for genetic manipulation, safe and easy to handle [50], and capable of inducing activation of macrophages, with a killing of *Leish-mania* parasites that is significantly potentiated by the expression of the *Wolbachia* surface protein.

4. Materials and methods

4.1. Cell and parasite cultures

The J774A.1 ATCC® TIB-67 macrophage cell line, derived from *Leishmania*-susceptible BALB/c 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% CO_2 at 37 °C in incubator. All reagents for the cell cultures were purchased from ATCC (Manassas, VA, USA). The *L.infantum* promastigotes derived from 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) supple-mented with 10 % FBS and gentamycin (5 μg/mL).

4.2. Bacterial strains and growth conditions

The bacterium *Asaia*^WSP derived from the engineering of the bacte-rium *Asaia* SF2.1 strain, originally isolated from the *Anopheles stephensi* mosquito [51], with the plasmid pHM4-WSP [18]. Briefly, the WSP cassette, inserted in the plasmid pHM4, is composed by the *Wolbachia* surface protein gene sequence (from the *Wolbachia* of the nematode *D. immitis*), the neomycin phosphotranferase promoter PnptII, the E-TAG epitope and the transcription terminator Trrn. *Asaia*^pHM4 was also ob-tained from strain *Asaia* SF2.1, but transformed with the empty plasmid (without the WSP-coding gene) and was used as control bacterium [18]. 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.

4.3. Phagocytosis assays on bacteria and transmission electron microscopy

Macrophages were seeded in 24-well plates (2×10^5/mL) and allowed to adhere overnight at 37 °C in a humidified 5% CO_2 atmo-sphere. Phagocytosis assay was performed applying the gentamicin protection assay as reported in [52], with minor modifications.

Asaia bacteria were washed with sterile PBS and resuspended in complete DMEM medium. The macrophages were treated at a multi-plicity of infection (MOI) of 100 bacteria per macrophage. Macrophages were co-incubated 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 1000 rpm, macrophages were incubated 1 h or 2 h at 37 °C to allow internalization of the bacteria [26]. Then, the macrophages were washed 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 was lysed using deionized water containing 1% (vol/vol) Triton X-100 (Sigma Aldrich, USA) for 15 min at 37 °C, to release phagocytized bacteria. The bacterial titre was determined by plating ten-fold serial dilutions of the cell lysates on GLY plates and CFU/mL were counted after growth for 48 h at 30 °C. In addition, to determine the bacterial survival inside the cells, the remaining part of the macrophages, after the treatment with strepto-mycin 100 μg/mL, were incubated with streptomycin 20 μg/mL until 24 h of infection, followed by the final step of the protocol, as described above.

As for phagocytosis data analysis after the assays on *Asaia* bacteria,

all data were stored in a spreadsheet and then analysed according to a one-way factor design with "Treatment" as the main experimental factor of interest. For experiments testing the effect of the "Treatment" ($Asaia^{WSP}$, $Asaia^{pHM4}$, *Leishmania*, LPS vs Med) the Estimation Statistics (ES) approach was applied [53]. ES is a simple framework 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 calculating effect size (mean differences) with his 95 % confidence interval, using the bias-corrected and accelerated (BCa) bootstrap confidence interval of Efron and Tibshirani [54,55] and suggested by [56]. The ES approach produces a plot which presents the raw data (top panel) and the bootstrap confidence interval of the effect size (the difference in means, bottom panel) aligned as a single integrated plot (e.g. see Fig. 1).

However, in order to quantify the strength of evidence against null hypothesis (mean difference = 0), Fisher's significant test was applied (bootstrapped Welch two-sample *t*-test) and the exact p-values are reported. Data analysis were performed in R language using dabestr package for ES (bias-corrected and accelerated (BCa) bootstrap confidence interval and Cumming plot) and Mkinfer package for bootstrapped Welch two-sample *t*-test, boot.t.test function (see Supplementary References S2).

Efficiency of the phagocytosis was evaluated also against the bacterium *S.epidermidis* (ATCC 155) following the protocol described above. *S. epidermidis* was grown in LB medium broth buffered to 7.0–7.4 pH with NaOH under constant rotation at 37 °C, overnight. Briefly, macrophages were first incubated with *Asaia* for 2 h (100 bacteria:1 macrophage, MOI 100) and then were incubated with *S. epidermidis* (10 bacteria:1 macrophage, MOI 10) for 1 h or 2 h at 37 °C. The protocol described above was then applied. The analyses of the data were performed applying the ES approach, as deeply described above. The *Asaia* uptake by macrophages was also evaluated by TEM. After 24 h 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% O_sO_4 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 uranyl acetate and lead citrate and examined under an EM900 TEM (Zeiss).

4.4. Leishmania infection assay and killing determination

Macrophages were seeded in 6-well plates (2×10^5/mL) and allowed to adhere overnight at 37 °C in humidified 5% CO_2 atmosphere. The macrophages were stimulated with $Asaia^{pHM4}$ or $Asaia^{WSP}$ at a MOI of 100 bacteria per macrophage for 2 h and then treated with streptomycin for 1 h. Subsequently, 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 24 h post infection by washing with PBS and fresh DMEM was replaced. Cells were then maintained at 37 °C for further 24 h (for a total of 48 h from the infection). At designated time points (24 h and 48 h) the culture supernatants were collected and stored until determination of cytokines and nitrites (see below). For the assessment of leishmanicidal activity, macrophages after 48 h of treatment were washed with PBS, collected by using a cell scraper, centrifugated at 1200 rpm for 6 min and washed with PBS. Finally, they were suspended in 200 μl PBS at the final concentration of 10^6cells/mL and cytocentrifuged (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 number of parasites in infected macrophages and the percentage of infected macrophages (infection rate) were determined with a microscope at 100X; ten areas of two slides per treatment were used to determine these indices. The experiments were performed in triplicate.

The association between the treatment factor ($Asaia^{WSP}$, $Asaia^{pHM4}$, amphotericin B, *Leishmania* alone) and the count outcome, i.e. the number of amastigotes in the macrophages, was determined using a zero-inflated negative binomial (ZINB) model [57] (see Supplementary References S2), since the recorded data were overdispersed and with an excess of zeros (we indeed recorded an overall 63 % macrophages with zero amastigotes in the four groups). The amastigote number in the macrophages infected with *Leishmania* alone was used as the reference. A ZINB model assumes that a zero outcome can derive from two different processes. In our model the two processes are: macrophages had not been infected by *Leishmania* (not infected); macrophages had been infected by *Leishmania*, but have the potential to clear the infection (infected). For the not infected, the only possible outcome is zero. For the infected ones, it is a count process with integer values $> = 0$. The two parts of the zero-inflated model form a binary model: a logit model, to determine to which of the two processes the zero outcome is associated with; a count-negative binomial model, to analyze the count outcome. The expected counts derive from the combination of the two processes (see Supplementary References S2).

4.5. Determination of M1 and M2 cytokines and NO production

All the cytokines were determined by ELISA kits: IL-12p40, IL-10 (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 bacterial stimulation and/or *Leishmania* infection. Simultaneous determination of nitrate and nitrite concentrations induced by the bacteria was done 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 [58]. In brief, saturated solutions of Vanadium (III) chloride (VCl_3) were prepared in 1 M HCl. Culture supernatants, collected at 24 h and 48 h of co-infection, were mixed with the same volume of VCl_3 and reacted with an equal volume of the Griess reagents. The absorbance at 540 nm was measured using a plate reader following the incubation. The analyses of the data were performed applying the ES approach, as deeply described in the previous section "Phagocytosis assays on bacteria and transmission electrone microscopy".

4.6. Arginase and iNOS expression by real time quantitative PCR

To evaluate arginase expression, cells were pre-treated with IL-4 (200 U/mL) (R&D Systems, Minneapolis, MN) for 12 h at 37 °C and then stimulated and infected as above, while for iNOS (inducible nitric oxide synthase) expression no priming was performed. After 24 h of infection for iNOS and 48 h for arginase, the cells were collected. Total RNA was extracted using the ReliaPrep™ RNA Tissue (Promega, Madison, WI, USA) following manufacturer's instructions. cDNAs were synthesized from 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 (S5 Table), according to standard PCR conditions and software analysis.

4.7. ROS determination

Intracellular reactive oxygen species (ROS) were measured by a fluorometric assay using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) as probe. In brief, macrophages were seeded (35,000/well) in a final volume of 200 μl/well in 96-well microplates and allowed to adhere 24 h at 37 °C. After an overnight incubation, the supernatants were discarded, and the cells were washed with PBS. H_2DCF-DA was added to macrophages, and incubated 1 h at 37 °C. Subsequently, the

cells were washed and stimulated with the two strains of bacteria and then infected with *Leishmania*, as reported above. The cells were incubated, protected from light, for approximately 14 h. Half an hour before ending of incubation, a group of cells was treated with 1 mM H2O2 and the fluorescence was measured at 485 nm (Ex) / 535 nm (Em). The analyses of the data were performed applying the ES approach, as described in the previous section "Phagocytosis assays on bacteria and transmission electron microscopy".

4.8. Cell surface markers analysis by flow cytometry

Expression of CD40, CD80, CD86 and MHC-II was evaluated by Cytofluorimetric Analysis using FACSCanto II cytometer (Becton Dickinson, Franklin Lakes, NJ). For the evaluation of co-stimulatory molecules, the cells were harvested 24 h 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 (Biolegend San Diego, CA) for 15 min at 37 °C. The cells were washed, resuspended in the FACS washing buffer and finally analyzed. LPS treatment (0.3 µg/mL) was used as control. For the evaluation of MHC-II molecules, the cells were pre-stimulated with INF-γ (1 ng/mL) 12 h before the infection. After 48 h of co-infection the cells were harvested and processed as above using an anti-MHC-II FITC-conjugated antibody (Biolegend San Diego, CA). Treatment with PMA (50 ng/mL) was used as positive control. FACS data were analyzed with FlowJo software (TreeStar, Ashland, Ore). The differences in the proportions of macrophages presenting CD40, CD80, CD86 or MHC-II between the different treatment groups were analysed by a generalized linear mixed model (GLMM) with two random effects: the biological and the technical replicates, and the treatment with LPS or PMA as the reference effect, using poisson family as log link function. $e^{(\text{intercept}+\text{estimated coeff})}$ can be interpreted as the proportion of cells in the different treatment groups (see Supplementary References S2).

Author contributions

C.B. and S.E. conceived and coordinated the study. I.V.B, I.A. and S. E. designed and performed the majority of the experiments. Y.C., N.B., M.G., L.G. contributed to cell culture and *Leishmania* maintenance. L.S. performed TEM analysis. R.N., M.P. contributed to phagocytosis assays and flow cytometry analyses. L.G. contributed to the design of the assay on Leishmania killing. P.G., V.T. analyzed all the data. I.V.B, C.B. and S. E. wrote the paper with input from all of the authors.

Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2020.105288.

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