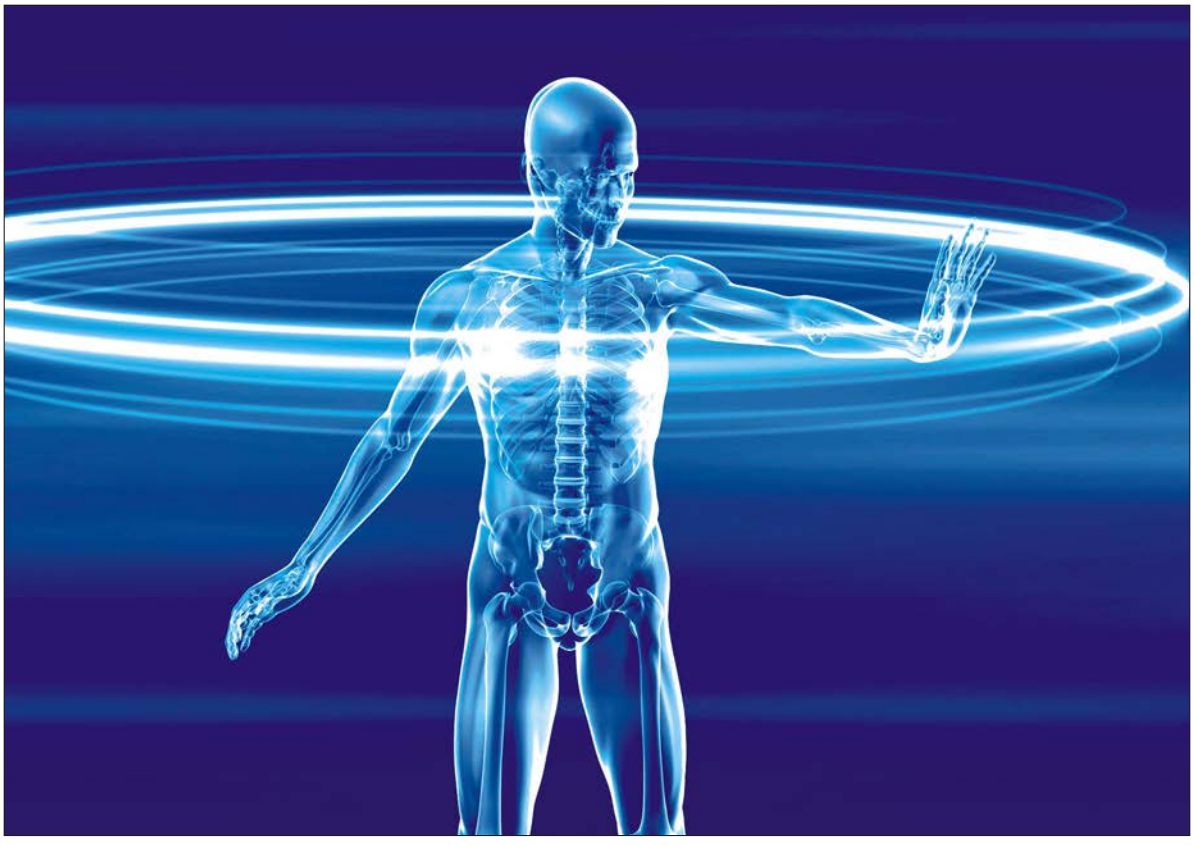

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Homoserine Lactones and Resorcinolic Lipids Inhibit *In vitro* Pro-and Anti-inflammatory Cytokine Production

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ABSTRACT

Homoserine Lactones (HSLs) and Resorcinolic Lipids (RLs) originating from microbes and plants influence immune system, however, it is currently unclear whether HSLs and RLs are activators or suppressors of the cytokine network, especially in humans. A study has been carried out to investigate the effects of pure, synthetic HSLs and RLs with different chain lengths on pro-and anti-inflammatory cytokines produced by human blood monocyte cultures. Human monocytes (macrophages and lymphocytes) were collected from leukocyte-rich plasma, separated on a double density gradient, pre-treated with HSLs or Rls and then were inducted with standard either lipopolysaccharide or phytohemagglutinin stimulus. Quantitative assays for pro-and anti-inflammatory cytokines in the monocyte culture supernatants were performed using enzyme immunoassay kits for the quantitative determination of IL-1β, IL-2, IL-4, IL-10, TNF-α and IF-γ. We observed differences in cytokine production according to HSL and RL pre-treatment, increasing in the following order: IL-1β→IL-2≈IL-10≈TNF-α→IL-4→IF-γ. HSLs have been shown more pronounced inhibitory effects than Rls and long-chained homologs were more active than short-chained ones. Our study suggests that some small molecules originating from microbes and plants play an important role in cytokine network regulation and the immunomodulatory effect of HSLs and RLs may obstruct host defenses and affect inflammatory processes.

Key words: Monocytes, cytokines, homoserine lactones, resorcinolic lipids

INTRODUCTION

Small molecules originating from microbes and plants have a wide range of biological activities that suggest their possible importance in inflammation control. For example, bacteria release diffusible, low molecular weight signal molecules that allow individual cells to sense population density, termed «quorum sensing» (Camilli and Bassler, 2006). For many gram-negative bacteria, the most investigated quorum-sensing molecules are the N-acyl homoserine lactones (HSLs) (Fuqua *et al*., 2001) and an increasing body of evidence reveals that HSLs are used for both intra-species and inter-kingdom signaling (Rumbaugh and Kaufmann, 2012), involving host pathways and the immune response. Another group of structurally similar molecules are resorcinolic lipids (RLs), commonly found in plants, mosses, fungi and several bacterial families (Stasiuk and Kozubek, 2010). A number of observations suggest that the biological action of Rls

is not restricted to the source cells, but affects mammalian cell function (Buonanno *et al.*, 2005), including immune system effectors (Komolova *et al.*, 1989). On collecting data HSLs and RLs appear to influence the production of pro-and anti-inflammatory cytokines that are secreted by numerous cells and up-or down-regulate the immune system during infection processes.

Several independent research groups have demonstrated that *Pseudomonas aeruginosa* «quorum sensing» activator C_{12}-oxo-HSL induces interleukin-8 (IL-8) in respiratory epithelial cells and lung fibroblasts (Smith *et al.*, 2001), stimulates IL-2 release by T-cells activated via anti-CD3/anti-CD28 antibodies (Hooi *et al.*, 2004) and also up-regulates IL-1α, IL-6 and gamma-interferon (IF-γ) production in mice (Smith *et al.*, 2002). Similar activity was shown for C_{12}-HSL which increases the expression of tumor necrosis factor-α (TNF-α), IL-1β and IL-8, unlike other short chain lactones (Gomi *et al.*, 2006). However, the direction of immuno-modulation is often controversial, with reports suggesting that C_{12}-oxo-HSL inhibits TNF-α production by murine macrophages (Telford *et al.*, 1998) and is a partial agonist in horse-derived macrophages (Thomas *et al.*, 2006). C_{12}-oxo-HSL also increases IL-12 production in mixed lymphocyte-antigen-presenting cells (Smith *et al.*, 2002), but decreases it in lipopolysaccharide-stimulated bone marrow-derived dendritic cells without altering IL-10 release (Skindersoe *et al.*, 2009).

The influence of ARs on cytokine production has been less intensively investigated and those data that have been reported are conflicting. Thus hexyl-resorcinol and some other phenolic lipids were shown to induce the release of IL-1α from keratinocytes (Newby *et al.*, 2000), whereas other ARs repress TNF-α production (Kawaguchi *et al.*, 2011).

It is currently unclear whether HSLs and RLs are activators or suppressors of the cytokine network, especially in humans. The possible reasons for the conflicting data are: (1) The presence of impurities with immune-modulating activity in natural HSL and RL samples; (2) The dependence of HSL and RL effects on the experimental context, cytokine source and mitogen induction (Ritchie *et al.*, 2003).

The aim of this study was to evaluate the effect of pure, synthetic HSLs and RLs with different chain lengths on a range of pro-(IL-1β, IL-2, TNF-α, IF-γ) and anti-(IL-4, IL-10) inflammatory cytokines produced by human blood monocyte cultures treated with standard lipopolysaccharide and phytohemagglutinin stimuli.

MATERIALS AND METHODS

Chemicals: Two groups of commercially available pure (97% and more) synthetic analogs of small molecules originating from microbes and plants-homoserine lactones (HSLs) and Resorcinolic Lipids (RLs) were used in this study. These compounds have a polar headgroup attached to an alkyl chain of variable length, forming a hydrophobic tail (Fig. 1). The HSLs were α-amino-γ-butirolactone hydrochloride (HSL•HCl) purchased from Sigma-Aldrich (Missouri, USA) and N-hexanoyl-L-homoserine lactone (C_6-HSL) and N-hexadecanoyl-L-homoserine lactone (C_{12}-HSL) from Cayman Chemical Company (Michigan, USA). The RL analogs orcinol (C_1-RL) and 4-hexylresorcinol (C_6-RL) were purchased from Sigma-Aldrich (Missouri, USA) and 5-N-dodecyl resorcinol (C_{12}-AR) was synthesized *de novo* according to standard organic procedures and purified to 99% homogeneity by Enamine Ltd. (Kiev, Ukraine).

While C_1-RL and C_6-RL were dissolved in distilled water, all the HSLs and C_{12}-RL were primarily dissolved in 96% ethanol at a concentration of 0.01 M. These stock solutions were 10-fold serially diluted in Hank's Balanced Salt Solution (HBSS) immediately before experiments.

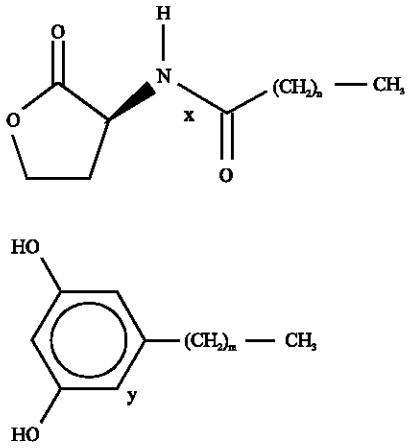

Fig. 1: Structural formulae of HSLs (top) and RLs (bottom) used in this study. Designations: n = 0, 4 or 10 for HSLs; m = 0, 5 or 11 for RLs; x-no bonds in the HSL•HCl molecule; y-hydrocarbon chain in the 4-pair-position in a C_6-RL molecule

Monocyte cell culture: Human monocytes (macrophages and lymphocytes) were isolated from leukocyte-rich plasma originating from heparin-treated peripheral blood <2 h old. The plasma samples were placed on a double ficoll-verografine density gradient (1.077 and 1.092 g mL^{-1}) and were centrifuged at 800 g for 15 min at 24±4°C. The separated cells were gently washed with cold HBSS and resuspended in Medium 199 in an appropriate plasma volume.

Cell treatment for cytokine production: Nine hundred microliters of cell suspension containing 1.0-1.8×10^6 monocytes were seeded in glass vials and 100 μL HSL or RL dilutions (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M final concentration) were added. Controls were HBSS containing an equal amount of distilled water or ethanol. The suspensions were incubated for 1 h at 37°C in a 5% CO_2-humidified atmosphere to allow HSL or RL to act. Lipopolysaccharide (LPS) from *Eschericia coli* O55:B6 (Sigma-Aldrich, USA) at 30 kg mL^{-1} final concentration for TNF-α, IL-1β and IL-10 induction or Phytohemagglutinin (PHA) from *Phaseolus vulgaris* (Sigma-Aldrich, USA) at 50 mkg mL^{-1} final concentration for IL-2, IL-4 and IF-γ induction, was added to treated and control samples. After a stimulation period of 12 h at 37°C in a 5% CO_2 atmosphere, the culture supernatant was collected by centrifugation for 10 min at 500 g and stored at -20°C until cytokine analysis.

Cytokine quantification in culture supernatants: Quantitative assays for pro-and anti-inflammatory cytokines in the monocyte culture supernatants were performed using enzyme immunoassay kits for the quantitative determination of IL-1β, IL-2, IL-4, IL-10, TNF-α and IF-γ in human biological fluids and culture medium (JSC «Vector-Best», Novosibirsk, Russia) according to the manufacturer's instructions. Mouse monoclonal antibodies specific to each cytokine and anti-cytokine antibodies conjugated with biotin and streptavidin-horse radish peroxidase conjugates were used in a three-step sandwich-enzyme-immunoassay. The results were expressed as the mean concentrations (pg mL^{-1}) in duplicate culture supernatants.

Data analysis and statistics: Data are reported as ±standard error of the mean of determinations performed in triplicate on three different samples and were analyzed by Student's

t-test. One-way ANOVA was used for analyses comparing the effect of HSL or RL series. Differences were considered significant if p was less than 0.01. Calculations were performed using Statistica V8 for Windows (StatSoft Inc., USA).

RESULTS

Cytokine profile induced by LPS and PHA: In response to LPS and PHA macrophages and lymphocytes produce and secrete cytokines that are essential for inflammation and the adaptive immune response. Our observations (Fig. 2) revealed that treatment of monocyte cell cultures with LPS at a concentration of 30 mkg mL^{-1} significantly increased the level of TNF-α, IL-1β and IL-10 in supernatants to 6426.97±508.34 (5573.60-7294.20) pg mL^{-1}, 1049.47±59.12 (989.89-1088.13) pg mL^{-1} and 2419.39±191.64 (2136.95-2728.44) pg mL^{-1}, respectively. These levels were 55-, 6- and 12-fold higher than the cytokine levels in non-treated cell culture supernatants. We also found that PHA at a concentration of 50 mkg mL^{-1} induced IL-2, IL-4 and IF-γ to 260.15±17.36 (251.51-279.66) pg mL^{-1}, 10.61±1.03 (9.89-11.28) pg mL^{-1} and 232.60±8.24 (227.64-240.08) pg mL^{-1}, these levels being 19-, 7- and 5-fold higher than in non-treated samples, respectively.

Thus, we suggest that the experimental model was adequate for strong cytokine induction by monocyte cell cultures. Additionally, these data were used as a baseline for subsequent calculation of HSL and RL regulatory effects on cytokine production.

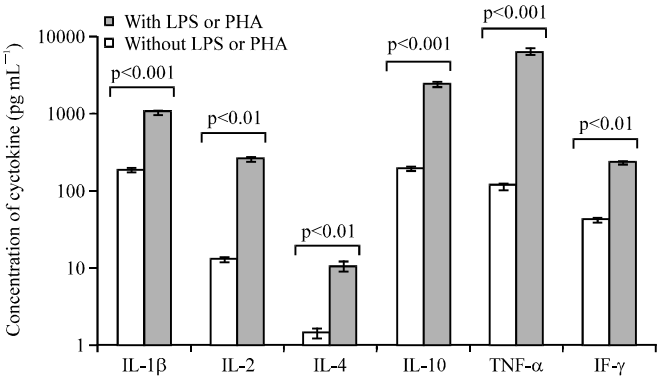

Fig. 2: Level of cytokine production in monocyte cell cultures treated with and without LPS or PHA stimuli

Cytokine production following HSL and RL pre-treatment: Pre-treatment of monocyte cell cultures with HSLs and RLs for 1 h often altered subsequent cytokine production, depending on the type and fine structure of the pre-treatment molecules.

The LPS-induced pro-inflammatory cytokine IL-1β was relatively stable (Fig. 3a). Significant ($p<0.01$) changes only occurred after monocyte pre-treatment with C_{12}-RL, whereby high concentrations (10^{-5} and 10^{-4} M) decreased IL-1β production to 355.77±16.01 and 350.59±21.04 pg mL^{-1}, respectively.

Conversely, LPS-induced pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 were more sensitive to HSL and RL pre-treatment, with more pronounced effects as alkyl chain length increased. Our observations revealed that 1 h incubation of monocytes with C_{12}-HSL ($p<0.001$) and C_6-HSL ($p<0.01$) but not HSL•HCl led to a reduction of both cytokines in culture supernatants (Fig. 3b, c). At the highest concentrations, C_{12}-HSL decreased the TNF-α and IL-10

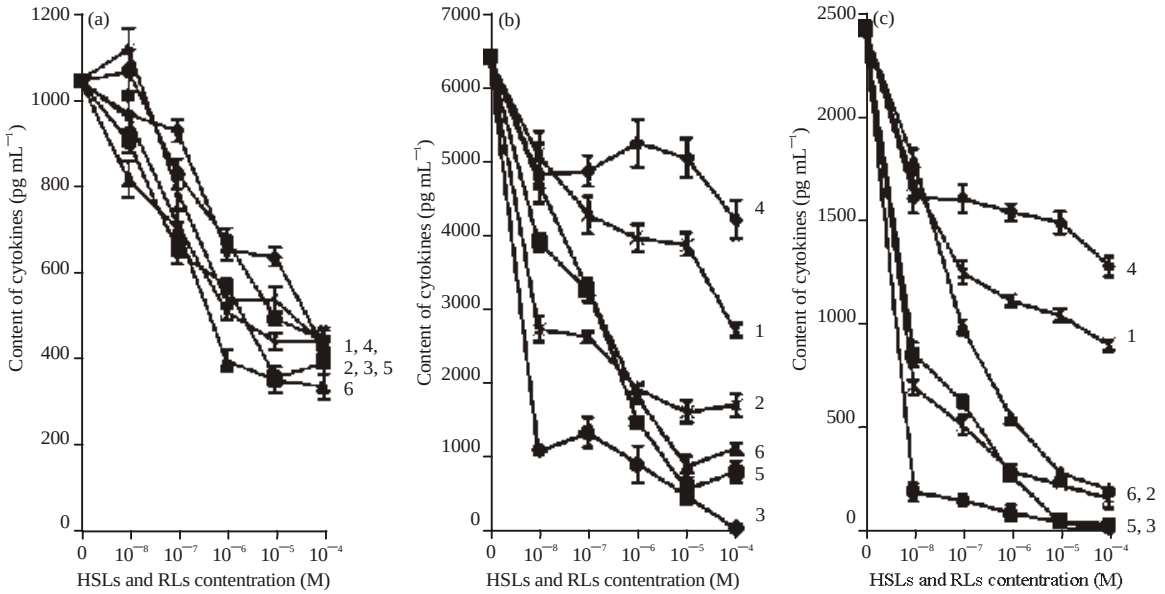

Fig. 3(a-c): (a)IL-1β, (b) TNF-α and (c) IL-10 production by LPS-stimulated monocyte cell cultures pre-treated with various concentrations of (1) HSL•HCl, (2) C_6-HSL, (3) C_{12}-HSL, (4) C_1-RL, (5) C_6-RL and (6) C_{12}-RL

levels to 0-1073.60±73.00 pg mL^{-1} and 146.91±5.73-579.17±31.28 pg mL^{-1}, respectively. There was no difference in the supernatant levels of TNF-α and IL-10 between control and C_1-RL treated samples, whereas C_6-AR and C_{12}-AR caused a dose-dependent inhibition of cytokine production. This effect was less pronounced in comparison with HSLs (see below) and only the 10^{-4}-10^{-6} M concentrations were sufficient to affect TNF-α, resulting in 781.00±50.77-1786.80±112.27 pg mL^{-1} and IL-10, resulting in 360.66±12.26-729.58±43.05 pg mL^{-1} ($p<0.01$).

Production of PHA-induced cytokines was highly sensitive to HSL and RL pre-treatment. The character of pro-inflammatory cytokine IL-2 regulation was similar to that of TNF-α and IL-10, such that short-chained HSL•HCl and C_1-RL were inactive, but C_{12}- more than C_6- and HSLs had a greater inhibitory effect than RLs, in a concentration-dependent manner (Fig. 4a). Thus monocytes induced with PHA did not produce detectable levels of IL-2 when stimulation occurred after pre-treatment with C_6-HSL at 10^{-5} M or C_{12}-HSL at 10^{-6} M and other higher concentrations.

The production of both anti-inflammatory cytokine IL-4 and pro-inflammatory cytokine IF-γ dramatically decreased after HSL and RL pre-treatment (Fig. 4b, c). HSLs at 10^{-6} M were sufficient for absolute IL-4 repression whereas the 10^{-7}-10^{-8} M concentrations led to cytokine levels of 0.07±0.003-2.56±0.09 pg mL^{-1} ($p<0.001$). No IF-γ was produced when monocyte cell cultures were treated with HSL•HCl at 10^{-4} M, C_6-HSL at 10^{-5} M or C_{12}-HSL at 10^{-6} M, whereas, low HSL concentrations decreased IF-γ in culture supernatants to 0.37-16.09 pg mL^{-1} ($p<0.001$). RLs inhibited IL-4 and IF-γ production less effectively than HSLs and the effects decreased from long-chained C_{12}-AR to short-chained C_1-RL. So if C_{12}-AR showed activity comparable with HSLs whereas C_1-RL effects became statistically significant ($p<0.01$) for IF-γ at concentration 10^{-8} M and higher and on IL-4 at 10^{-6} M and higher.

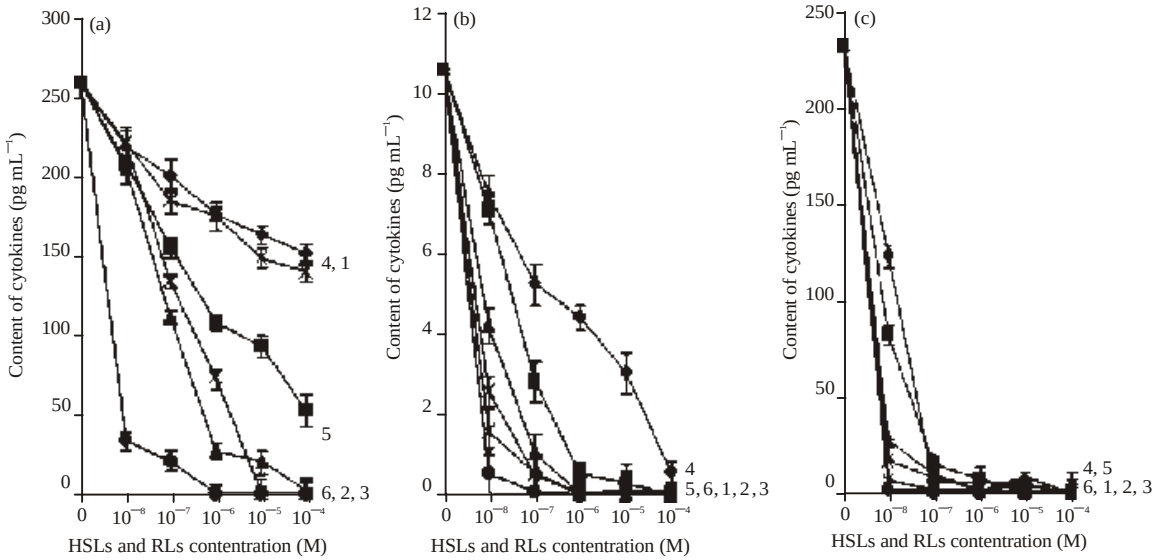

Fig. 4(a-c): (a) IL-2, (b) IL-4 and (c) IF-γ production by PHA-stimulated monocyte cell cultures pre-treated with various concentrations of (1) HSL•HCl, (2) C_6-HSL, (3) C_{12}-HSL, (4) C_1-RL, (5) C_6-RL and (6) C_{12}-RL

Table 1: ANOVA F-test values characterizing significance of cytokine inhibition after monocyte cell culture pre-treatment with HSL and RL homologs

		HSLs			RLs		
Cytokine	Stimulus	HSL•HCl	C_6-HSL	C_{12}-HSL	C_1-RL	C_6-RL	C_{12}-RL
IL-1β	LPS	3.83	3.94	4.01	3.06	3.90	6.93*
IL-10		1.68	10.95*	15.5**	1.45	10.99*	7.12*
TNF-α		2.55	10.75*	43.12**	1.01	9.85*	8.93*
IL-2	PHA	1.79	48.1**	54.11**	1.73	8.41*	45.42*
IL-4		53.01**	55.15**	59.35**	12.36*	27.14**	49.33**
IF-γ		75.05**	76.91**	79.45**	53.04**	58.00**	75.80**

*p<0.01; **p<0.001

ANOVA of HSL and RL effects: Thus, both groups of pure, synthetic HSLs and RLs clearly inhibited the production of both pro-and anti-inflammatory cytokines by human blood monocyte cultures. Moreover, we also revealed the variable sensitivity of various cytokines to HSL and RL pre-treatment and the different effects of long-chained and short-chained homologs. To quantify these differences we used ANOVA.

We reached three conclusions based on the ANOVA F-test values (Table 1). First, cytokines demonstrated various responses to HSL and RL pre-treatment increasing as follows: IL-1β→IL-2 ≈IL-10≈TNF-α→IL-4→IF-γ which was shown in growth of F-test values from 3.06-6.93 ($p<0.01$) to 53.04-79.45 ($p<0.001$). Secondly, the inhibition of PHA-induced cytokines was more pronounced than that of LPS-induced ones (F-values 1.73-79.45 against 1.01-43.12, respectively). Thirdly, there was no distinct differences between pro-and anti-inflammatory cytokine suppression. In a context of small molecules comparison the ANOVA also showed that: (1) HSLs had more pronounced inhibitory effects than RLs; (2) expressed activity of long-chained HSLs and RLs against short-chained homologs which often had no statistically significant effect.

DISCUSSION

The production and secretion of a wide range of cytokines are central in inflammation and the adaptive immune response, involving cell proliferation, maturation, migration, adhesion, differentiation and activation (Brocker *et al.*, 2010). These molecules are under constant pressure to evolve due to continual competition between the host's immune system and infecting organisms or food sources containing small diffusible signal molecules. Several independent research groups have demonstrated that bacteria and plants are capable of affecting cytokine production via low molecular weight molecules such as homoserine lactones (Smith *et al.*, 2001; Hooi *et al.*, 2004; Gomi *et al.*, 2006; Telford *et al.*, 1998; Thomas *et al.*, 2006; Skindersoe *et al.*, 2009) and resorcinolic lipids (Newby *et al.*, 2000; Kawaguchi *et al.*, 2011). The present study contributes to our knowledge of this immunomodulation by means of pure, synthetic HSLs and RLs.

Our results show that pre-treatment of human blood monocyte cultures with these molecules decreases, in a concentration-dependent fashion, both pro-and anti-inflammatory cytokine production. The mechanisms involved remain unclear, but recent data suggest that changes in the cytokine profile may be caused by cytotoxic effects (Tateda *et al.*, 2003; Gasiorowski *et al.*, 2001), as well as by biochemical pathways that have no effect on cell viability (Kravchenko *et al.*, 2006; Deryabin *et al.*, 2013).

Surprisingly, the results of the present *in vitro* study differed from *in vivo* stimulation of the same cytokines by HSLs (Smith *et al.*, 2002) and RLs (Newby *et al.*, 2000) which may be attributed to the multi-target interaction of these molecules in poly-component live systems, in which secondary cytokine induction by the released products is possible. The results also support opinions about the multilevel control of cytokine production and the dependence of HSL and RL effects on experimental context, including the underlying immune status of the host (Ritchie *et al.*, 2003). It should be considered while comparing *in vitro* and *in vivo* data and may be subject to further studies in the near future.

We also found that different cytokines were inhibited to different extents by identical pre-treatments. This may be due to differences in the sensitivity of various monocyte subpopulations (Katial *et al.*, 1998). For example, the inhibition of PHA-induced cytokines was more pronounced than that of LPS-induced cytokines which means that lymphocytes have a higher sensitivity to HSL and RL pre-treatment than macrophages. It may also be due to differences in the cytokine induction pathways (Kravchenko *et al.*, 2006).

Recently, small molecules originating from microbes and plants such as C_{12}-oxo-HSL (Hooi *et al.*, 2004; Skindersoe *et al.*, 2009) and polyphenols have been shown to inhibit cytokine production (Kawaguchi *et al.*, 2011). Our data demonstrate for the first time the similar immune-modulating activity of a wide range of synthetic HSL and RL analogs. Simultaneously, our findings suggest that the carbon chain moiety of these molecules is important for recognition by monocytes (Hooi *et al.*, 2004; Gomi *et al.*, 2006), probably determining the targeting receptors or stability to degrading enzymes (Stoltz *et al.*, 2007). On the other hand, the whole fine structure determines biological activity, with HSLs having a more pronounced effect than RLs.

To ensure that HSLs and RLs were responsible for the changes in cytokine production we tested the final concentrations, from micro- to nano-mole, in monocyte cell cultures and revealed the concentration-dependent down-regulation of the cytokines. If the high (micromolar) concentrations have been used as a model for cytokines regulation, then nanomolar concentrations corresponded to experimentally detected presence of HSLs and RLs in biological liquids and tissues of human (Middleton *et al.*, 2002; Ross *et al.*, 2010). Thus our data suggest that these two molecular groups

play an essential role in real systems and that the immunosuppressive effect of HSLs and RLs may affect inflammation in humans and animals (Kravchenko *et al.*, 2011; Fito *et al.*, 2008).

In summary, the *in vitro* results presented here provide a new insight into the effects of homoserine lactones and resorcinolic lipids on the cytokine network and will be essential for the understanding of their role in host defense. Although the bioactivity of these molecules in the immune system seems to be multilevel and further *in vivo* work is needed to characterize the immune-modulating effects of HSLs and RLs, these results are consistent with the hypothesis that host defense and inflammation may be obstructed by small molecules originating from microbes and plants. On the other hand, the immune-suppressive effects of HSLs and RLs could have therapeutic potential (Thomas *et al.*, 2006) and could be used to protect the host in cases of immune system hyperactivation such as bacterial septic shock and autoimmune diseases. This will require further investigation.

CONCLUSION

The chemical analogues of microbial and plant homoserine lactones and resorcinolic lipids treated human monocytes cell culture showed the inhibitory effect on both pro-and anti-inflammatory cytokines production. The variable sensitivity of various cytokines to HSL and RL pre-treatment has been revealed, also as structure-activity relationships for these small molecules. Thus, the present study confirms the HSLs and RLs possible role in cytokine network regulation that may obstruct host defenses and repress immune response.

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