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β_2-Glycoprotein I (Apolipoprotein H) Modulates Uptake and Endocytosis Associated Chemiluminescence in Rat Kupffer Cells

L.F. GOMES[a,*], L.M. GONÇALVES[a], F.L.A. FONSECA[a], C.M. CELLI[b,e], L.A. VIDELA[c], H. CHAIMOVICH[b] and V.B.C. JUNQUEIRA[d]

[a]*Disciplina de Patologia, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Lineu Prestes n. 580, Bloco 17 superior. CEP: 05508-900, São Paulo, Brazil;* [b]*Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil;* [c]*Programa de Farmacología Molecular y Clínica, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile;* [d]*Discplina de Geriatria, Departamento de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil;* [e]*Department of Pharmacology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, TX 77030, USA*

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β_2-Glycoprotein I (β_2GPI) is known to influence macrophage uptake of particles with phosphatidylserine containing surfaces, as apoptotic thymocytes and unilamellar vesicles *in vitro*. Nevertheless, effects upon macrophage activation induced by this interaction are still unknown. β_2GPI influence upon the reactive species production by Kupffer cells was evaluted in order to investigate whether β_2GPI modulates the macrophage response to negatively charged surfaces. Chemiluminescence of isolated non-parenchymal rat liver cells was measured after phagocytosis of opsonized zymosan or phorbolmyristate acetate (PMA) stimulation, in the presence and absence of large unilamellar vesicles (LUVs) containing 25 mol% phosphatidylserine (PS) or 50 mol% cardiolipin (CL) and complementary molar ratio of phosphatidylcholine (PC). β_2GPI decreased by 50% the chemiluminescence response induced by opsonized zymosan, with a 66% reduction of the initial light emission rate. PMA stimulated Kupffer cell chemiluminescence was insensitive to human or rat β_2GPI. Albumin (500 μg/ml) showed no effect upon chemiluminescence. β_2GPI increased PS/PC LUV uptake and degradation by Kupffer cells in a concentration-dependent manner, without leakage of the internal contents of the LUVs, as shown by fluorescence intensity enhancement. LUVs opsonized with antiphospholipid antibodies (aPL) from syphilitic patients increased light emission by Kupffer cells. Addition of β_2GPI to the assay reduced chemiluminescence due to opsonization with purified IgG antibodies from systemic lupus erythematosus (SLE or syphilis (Sy) patient sera. A marked net increase in chemiluminescence is observed in the presence of Sy aPL antibodies, whereas a decrease was found when SLE aPL were added to the assay, in the presence or absence of β_2GPI. At a concentration of 125 μg/ml, β_2GPI significantly reduced Kupffer cell *Candida albicans* phagocytosis index and killing score by 50 and 10%, respectively. The present data strongly suggest that particle uptake in the presence of β_2GPI is coupled to an inhibition of reactive species production by liver macrophages during the respiratory burst, supporting the role of β_2GPI as a mediator of senescent cell removal.

Keywords: Apolipoprotein H (β_2-Glycoprotein I); Chemiluminescence; Kupffer cell; Macrophage activation; Phospholipid vesicles; Phagocytosis

INTRODUCTION

Macrophage activation is an important biological response to avoid damage caused by infection, neoplasia, and several kinds of chemical and physical cell injury, which triggers the orchestrated set of chemical reactions that compose the respiratory burst.[1,2] The reactive oxygen and nitrogen species produced during the respiratory burst operate the early and unspecific reactions of the inflammatory host defenses from multicellular organisms.[1,2] These reactive species react with biomolecules with low specificity inducing some

*Corresponding author. Tel.: +55-11-38183632. Fax: +55-11-38132197. E-mail: lfgomes@plugnet.com.br

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degree of damage to adjacent tissues.[3] Nevertheless, the physiological process of senescent cell removal by macrophages is not followed by inflammation or adjacent tissue damage.[4] Phagocytes are not activated and an inflammatory reaction is avoided, making the silent ingestion of senescent cells a key complement to normal cell turnover, tissue growth and remodeling.[5] Therefore, phagocyte priming and activation must be regulated in normal homeostasis.[6] Macrophages, neutrophils, and non-professional phagocytes cooperate to enhance the safety of apoptotic cell removal through the use of receptors, preventing the leakage of dying cell contents and secondary necrotic damage. Apoptotic cells are recognized by macrophages through multiple receptors, including vitronectin, lectins, phosphatidylserine (PS) and scavenger receptors.[7,8] However, it remains to be described whether and how these receptors converge to down-regulate inflammatory responses.[4,5]

The exposure of negatively charged phospholipids, mainly PS, in the outer leaflet of cell membranes is associated with several physiologic and pathologic phenomena.[9] PS has been suggested to trigger macrophage recognition and promotion of phagocytosis,[9–11] as it is actively expressed on the outer leaflet of cell membranes during platelet activation or programmed cell death.[9,10] Evidence that a rapid *in vivo* clearance of PS-containing liposomes is associated with β_2-glycoprotein I (β_2GPI), an autologous plasma protein, led to the proposal that the clearance of PS-expressing cells by macrophages could be mediated by the β_2GPI–PS complex.[12] Although the precise physiologic role of β_2GPI is unclear, its binding to negatively charged phospholipid on activated platelet membranes inhibits phospholipid-dependent prothrombinase activity and ADP-induced platelet aggregation.[13,14] β_2GPI has also been proposed to modulate the metabolism of triglyceride-rich lipoproteins.[15] β_2GPI itself or bound to negatively-charged phospholipid may represent the target epitope for antiphospholipid antibodies (aPL) found in autoimmune disorders such as systemic lupus erythematosus (SLE) and primary or secondary antiphospholipid syndrome, suggesting its participation in the pathophysiological mechanism of thrombosis associated with these aPL[16,17] (and references therein). β_2GPI was also reported to mediate the binding of autoantibodies to apoptotic thymocytes *in vivo*.[18]

Although β_2GPI is known to influence the binding and macrophage uptake of PS-expressing membranes such as PS-containing liposomes and apoptotic thymocytes *in vitro*,[11,19] whether macrophage activation is induced by this interaction is still unknown. Since conformational changes of β_2GPI structure, during its binding to negatively charged phospholipid containing surfaces, have been described,[20] we hypothesized that β_2GPI interaction with cell membranes results in a topographic modification of cell surface, recognizable by phagocytes, which may modify macrophage activation. For this purpose, the influence of β_2GPI upon the recognition of negatively charged surfaces by macrophages was assessed by *in vitro* assays using PS-containing large unilamellar vesicles (LUVs) as a model of apoptotic cells membrane. LUVs uptake by rat Kupffer cells was measured in the presence of β_2GPI, concomitantly with the chemiluminescence associated with the process. β_2GPI effects upon chemiluminescence of Kupffer cell activated by phagocytosis of opsonized zymosan or by phorbol-myristate acetate (PMA) stimulation were also evaluated, in addition to its effect on the capacity of Kupffer cells to kill *Candida albicans*.

MATERIALS AND METHODS

Materials

PS and cardiolipin (CL) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Egg phosphatidylcholine (PC) and 5-carboxyfluorescein (CF) (Eastman, New York, NY) were purified as described by Rodes *et al.*[21] and by Evelyn *et al.*,[22] respectively. Rat and human β_2GPI were purified from pools of sera from healthy rats or human subjects according to Polz *et al.*[23] β_2GPI purity was estimated by SDS-PAGE electrophoresis and potential binding to negatively charged phospholipids by ELISA.[24] Antiphospholipid antibodies of the IgG isotype were purified from human sera (a gift from Dr A.E. Gharavi, Morehouse School of Medicine, Atlanta, GA) as described previously.[24,25] Binding of aPL to negatively charged phospholipids was confirmed by ELISA.[24] Bovine serum albumin (BSA) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

LUVs Preparation

LUVs were prepared in a LiposoFast-100 Jacketed, Avestin, Inc., apparatus by extrusion through polycarbonate membranes with 0.1 μm (i.d.) pores (Millipore). Lipids dissolved in chloroform were dried under 10^{-2} Torr vacuum for 1 h to obtain lipid films containing 25% PS or 50% CL and complementary molar ratio of PC. The films were suspended in 50 mM CF/10 mM Tris (pH 7.4) or phosphate buffered saline-glucose (PBS-G, pH 7.4) by vigorous vortexing to obtain multilamellar vesicles (MLV). MLV suspension was then extruded 5 times through 3 superimposed membranes. CF-containing LUVs were passed through a Sephadex G-25 medium

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column (Pharmacia) to remove non-entrapped CF from the vesicles. The final concentration of LUVs was measured by phosphorus analysis.[26]

Kupffer Cell Preparation

Non-parenchymal liver cell preparation was performed by liver perfusion with collagenase in pentobarbital (50 mg/kg ip) anaesthetized male Sprague–Dawley rats, 12–14 weeks old, weighting 250–300 g, according to Doolitle and Richter.[27] Whole non-parenchymal liver cell suspensions were used without further fractionation. Viability was measured immediately before using by Trypan blue exclusion, being always higher than 95%. Viable cells were counted with an hemocytometer immediately before preparing the reaction mixtures.

LUV Uptake Assay

Kupffer cell suspensions (1×10^6 cells/ml in PBS-G) were incubated with CF-containing LUVs (1 mM total lipid) in tight sealed polypropylene vials at 37°C with agitation. LUVs were pre-incubated with 52 μg/ml β_2GPI (unless otherwise indicated) for 5 min at room temperature to allow β_2GPI binding to LUVs surface.[24] At fixed incubation times, 200 μl aliquots were removed and centrifuged at 4000 rpm for 5 min. Cell pellets were washed twice by resuspension in PBS-G and centrifuged at 4000 rpm for 5 min. Cell pellets were resuspended into 2 ml PBS-G buffer and CF fluorescence intensity (F_0) was monitored in a Hitachi F-2000 fluorescence spectrophotometer, at 490 nm (excitation) and 520 nm (emission) wavelengths.[22,24] F_0 measurements estimate intracellular degradation of endocytosed LUVs. Maximum fluorescence intensity (F_{TX}) was obtained after rupture of the cells with Triton X-100 (0.5% v/v). Uptake of non-degraded LUVs was calculated from the difference in emission fluorescence intensity before and after the cell lysis ($F_{TX} - F_0$). Control tubes were prepared with LUVs or with β_2GPI, incubated in the absence of cells in the same conditions and dilutions and collected at the same time intervals. F_0 and ($F_{TX} - F_0$) of unwashed controls were obtained in order to rule out CF leakage during incubation.

Chemiluminescence Assay

Kupffer cells were stimulated with zymosan (2×10^8 particles) or PMA (0.5 mg/ml) and the respiratory burst was evaluated by a luminol-dependent chemiluminescence assay,[28] using a Bio-Orbit luminometer model 1251, with temperature control (37°C) and permanent shaking. Control tubes received PBS-G instead of zymosan or PMA. Reaction mixtures (0.8 ml) containing 10^6 cells and 25 μM luminol in PBS-G were assayed with 5–105 μg/ml β_2GPI. Purified IgG-aPL (100 μg/ml) and BSA (500 μg/ml) were used as indicated. Light emission was followed for 90 min. The results were expressed in mV/min for the initial rates of chemiluminescence increase (slope), in mV × min for total chemiluminescence (integral), and in mV for the emission peak values.

Killing Ability Assay

Kupffer cells (10^6 cells) were incubated with a suspension of *C. albicans* (50 yeast per cell), as described by Zeligs.[29] Slides were prepared after 10 min incubation, dried on air, fixed on methanol, and stained with hematological stain (May–Grunwald–Giemsa, Merck). Phagocytosis (%) was quanti fied as the fraction of cells with ingested yeast, either alive or dead. The killing score was determined by counting the alive and dead ingested yeast per cell, and graded as 0 (cells with only alive yeast inside), 1 (cells with 1 or 2 dead yeast inside), 2 (cells with 3 or 4 dead yeast inside), and 3 (cells with more than 4 dead yeast inside). The scores were calculated over at least 400 cells, as previously described.[30]

RESULTS

Chemiluminescence of Kupffer cells induced by zymosan was reduced in the presence of human (Fig. 1) or rat (data not shown) serum β_2GPI, as evidenced by the significant decrease in total light emission (Fig. 1A) or in the initial luminescence rates (Fig. 1B), at 53 μg/ml β_2GPI. These parameters were not significantly modified when Kupffer cells were stimulated with PMA or subjected to BSA addition (Fig. 1).

LUVs containing entrapped CF were used to evaluate β_2GPI influence upon LUVs endocytosis by macrophages. In agreement with previous observations,[31] the ingestion process reached maximum values at incubation times earlier than 20 min (Fig. 2). CF fluorescence is quenched at concentrations higher than 50 mM, thus increases in F_0 are associated with CF release within the cells after endocytosis and degradation of LUVs. Total uptake (including non-degraded LUVs) is associated with CF fluorescence intensity after lysis of the cells and remaining intracellular LUVs with Triton X-100. To exclude any possible background from CF, cells were carefully washed to eliminate extracellular remaining LUVs. LUVs constituted by PS/PC (1:4), but not by pure PC, were taken up by live Kupffer cells, however, endocytosis of PS/PC LUVs by dead cells did not occur (data not shown), suggesting that liposome uptake by Kupffer cells requires more

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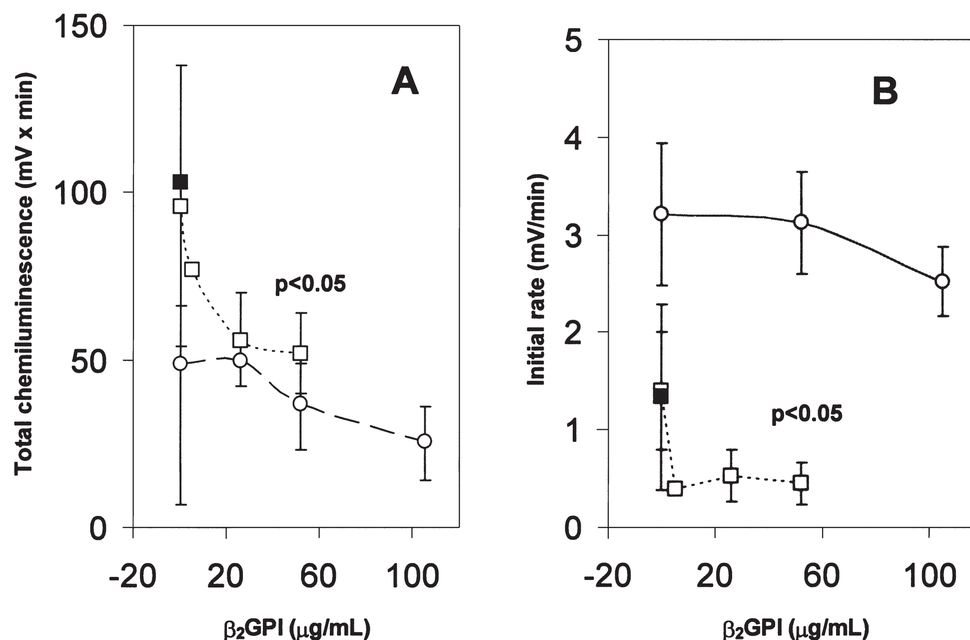

FIGURE 1 Effects of human β2-glycoprotein I (β2GP I) upon Kupffer cell chemiluminescence after stimulation with opsonized zymosan or phorbolmyristate acetate (PMA). Total light emission (A) or initial chemiluminescence rates (B) of reaction mixtures containing 10^6 cells, 25 μM luminol, and 2×10^8 zymosan particles (□) or 500 μg/ml PMA (○), in the presence of 0, 5, 26, 52, and 105 μg/ml β2GP I. The effect of 500 μg/ml BSA (ν) is also shown. Results represent means ± SEM for four separate experiments and significance between mean values was assessed by one-way ANOVA followed by Bonferroni test.

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than the LUVs membrane binding and fusion with the cell membrane. β2GPI increased PS/PC LUVs uptake and degradation by liver macrophages in a concentration-dependent manner, as shown by

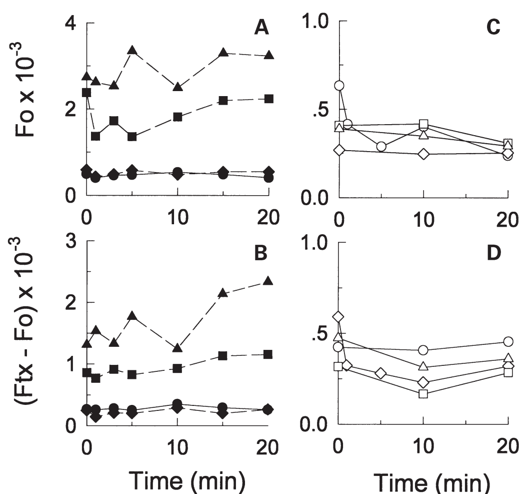

FIGURE 2 Effects of human β2-glycoprotein I (β2GP I) upon the uptake of phosphatidylserine/phosphatidylcholine (PS/PC, 1:3 mol:mol) large unilamellar vesicles (LUVs) containing entrapped 5-carboxyfluorescein (CF) by Kupffer cells. The amount of LUVs uptake and degradation by Kupffer cells are associated with CF fluorescence (F_0). Total LUVs uptake was obtained after cell lysis with 0.5% Triton X-100 (F_{TX}). Total non-degraded LUVs uptake is calculated by the difference ($F_{TX} - F_0$). A representative experiment is shown. The reaction mixture contains (A and B) 10^6 cells, LUVs (1 mM total lipid) (•), in the presence of either 500 μg/ml bovine serum albumin (BSA) (◆) or human β2GP I at concentrations of 53 (■) and 105 μg/ml (▲). Data shown in C and D were obtained in the absence of Kupffer cells (open symbols).

fluorescence intensity increase (Fig. 2A and B). Absence of LUVs' leakage due to β2GPI binding was observed during the assay time course (Fig. 2C and D). Uptake measurements longer than 30-min incubation were avoided due to liposome instability at 37°C (fluorescent probe spontaneous leakage increased F_0).[24]

Binding of aPL antibodies to LUVs provides opsonized LUVs prompting endocytosis via Fc receptor,[24] a process that induces phagocyte activation and chemiluminescence response.[28] LUVs opsonized with either unspecific IgG antibodies (neg) or aPL from an autoimmune disorder such as SLE did not significantly alter chemiluminescence emission in LUVs containing PS or CL, whereas aPL from syphilitic patients induced a higher luminescent response ($p < 0.05$) than LUVs alone (Fig. 3A). Addition of β2GPI to LUVs alone or to neg aPL opsonized LUVs has no effect on Kupffer cell chemiluminescence (Fig. 3A). This parameter is significantly ($p < 0.05$) reduced in the presence of either SLE aPL-opsonized LUVs containing PS (45%) or in those opsonized with aPL from syphilitic patients containing PS (37%) or CL (32%) (Fig. 3A and B). The net effect of different aPL antibodies on Kupffer cell chemiluminescence elicited by opsonized PS and CL LUVs is shown in Fig. 3B. Addition of unspecific IgG antibodies (neg) elicited a marginal enhancement in light emission, whereas a marked increase or a reduction is elicited by Sy aPL or SLE aPL, respectively, either in the presence or absence of β2GPI (Fig. 3B).

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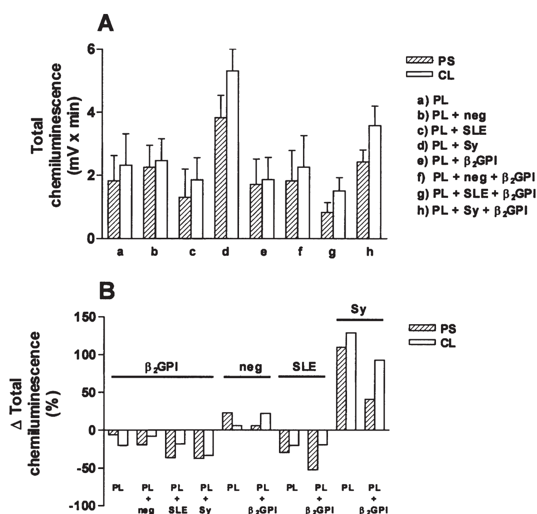

FIGURE 3 Effects of human β_2-glycoprotein I (β_2GP I) upon Kupffer cell chemiluminescence after stimulation with antiphospholipid antibodies (IgG-aPL) opsonized large unilamellar vesicles (LUVs). (A) Total chemiluminescence emission of reaction mixtures containing 10^6 cells, 25 μM luminol, and either phosphatidylserine/phosphatidylcholine (PS/PC, 1:3) (dashed bars) or cardiolipin/phosphatidylcholine (CL/PC, 1:1) (empty bars) LUVs (1 mM total lipid) in PBS-G buffer (PL); PL plus 100 μg/ml unspecific IgG antibodies (neg); PL plus 100 μg/ml aPL from patients with systemic lupus erythematosus (SLE); PL plus 100 μg/ml aPL from patients with syphilis (Sy). When indicated, 105 μg/ml β_2GP I was added. (B) Net effect of the indicated proteins was calculated from total chemiluminescence (TC) values in A, according to the equation: $\Delta TC = [(TC_{protein\ presence}) - (TC_{protein\ absence})/(TC_{protein\ absence})] \times 100$. Results represent means ± SEM for 4–9 separate experiments and significance was calculated by one-way ANOVA followed by Bonferroni test: a versus b, c, and e (N.S.); a versus d ($p < 0.05$); b versus f (N.S.); c versus g and d versus h ($p < 0.05$). N.S., not significant.

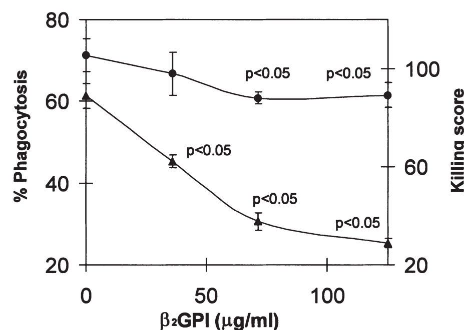

FIGURE 4 Effects of β_2-glycoprotein I (β_2GP I) upon Kupffer cell phagocytosis index (Δ) and killing score (○) of *C. albicans*. Reaction mixtures containing 10^6 cells and 50 *C. albicans* particles per cell, were incubated for 10 min in the presence of 0, 35, 70, and 125 μg/ml β_2GP I. Results represent means ± SEM for 4–5 separate experiments and significance was assessed by one-way ANOVA followed by Bonferroni test ($p < 0.05$ compared with control values at zero β_2GP I concentration).

Phagocytosis of yeast (*C. albicans*) by Kupffer cells exhibits a concentration-dependent diminution by β_2GPI, with a 50% reduction at 125 μg/ml ($p < 0.05$), whereas the killing score is decreased by only 10% ($p < 0.05$) at 125 μg/ml β_2GPI (Fig. 4).

DISCUSSION

β_2GPI, a glycosylated serum protein, has been suggested to mediate the uptake of PS-containing vesicles by human macrophages and to be an apoptotic cell signal for macrophages.[11,18,19] β_2GPI belongs to the short consensus repeat superfamily of proteins which are composed by homologous repeating units such as regulatory proteins of the complement system, and several non-complement proteins like interleukin-2 receptor and leukocyte adhesion molecules from the selectin family[32] (and references therein). β_2GPI is mainly found in the chylomicron and VLDL fractions of lipoprotein *in vivo*.[23] Data presented indicate that uptake of PS/PC LUVs by rat Kupffer cells is completed within 15–20 min. Close similar time-spans were described for apoptotic lymphocytes uptake by human Kupffer cells.[33] Upon stimulation with particulate material, chemiluminescence is induced as a result of reactive species production by Kupffer cells.[28] This response is inhibited by β_2GPI when liver macrophages are stimulated by zymosan, but not by PMA, suggesting that β_2GPI affects membrane receptor-related Kupffer cell activation. This effect of β_2GPI seems to be specific, as BSA not significantly changes chemiluminescence.

The binding of β_2GPI to negatively charged membranes has been shown to result in major protein conformation and lipid architecture changes that might trigger an autoimmune epitope generation.[20,24] Although the ultimate epitopes of aPL are still unknown, β_2GPI enhances the phospholipid binding of aPL from autoimmune disorders (such as SLE) and inhibits that of infection associated aPL (such as from syphilis (Sy))[24,34] (and references therein). β_2GPI is proposed to be part of the antigen of this group of antibodies, considering that the fifth domain of β_2GPI holds both the phospholipid binding site and the antiphospholipid antibody binding site,[35] though other protein domains are also implicated in antibody binding.[36] Either unspecific IgG-antibodies or IgG-aPL purified from patients with syphilis increased chemiluminescence of Kupffer cells as expected for inflammatory opsonins (Fig. 3). In contrast, IgG-aPL from SLE patients seemed to help β_2GPI inhibitory effect upon chemiluminescence of Kupffer cells (Fig. 3). These data support that autoimmune IgG-aPL could provide a tighter binding of β_2GPI to LUVs surface, dependent on β_2GPI density on the surface, as recently suggested by Sheng *et al.*[37] Therefore, the chemiluminescence decrease induced by autoimmune IgG-aPL may also result from keeping β_2GPI bound to the LUVs surface in a conformation recognizable by the Kupffer cell.

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The decreasing effect of β_2GPI upon phagocytosis and killing of *C. albicans* performed by Kupffer cells are quite different in magnitude. Despite a high diminution of the phagocytosis index, a small decrease in killing of yeast is observed at the maximum β_2GPI concentration used (Fig. 4). In this condition, less Kupffer cells carried out uptake, maintaining the killing efficiency, despite less reactive species being produced, as measured by respiratory burst in chemiluminescence assay (Fig. 1). Taken together these results suggested that binding of β_2GPI to Kupffer cells may couple a more efficient antigen uptake and degradation with a lower reactive species output, supporting a physiological role for β_2GPI during senescent cell removal *in vivo*.

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