Mast Cells Act as Phagocytes Against the Periodontopathogen *Aggregatibacter Actinomycetemcomitans*

Heliton G. Lima[*], MSc; Karen H. Pinke[*], BSc; Taiane P. Gardizani[*], BSc; Devandir A. Souza-Júnior[†], PhD; Daniela Carlos[‡], PhD; Mario J. Avila-Campos[§], PhD; Vanessa S. Lara[*], PhD.

[*] Department of Stomatology, Bauru School of Dentistry, São Paulo University, Bauru, SP, Brazil.

[†] Department of Cell and Molecular Biology and Pathogenic Bioagents, School of Medicine of Ribeirão Preto, São Paulo University, Ribeirão Preto, SP, Brazil.

[‡] Department of Biochemistry and Immunology, School of Medicine of Ribeirão Preto, São Paulo University, Ribeirão Preto, SP, Brazil.

[§] Department of Microbiology, Institute of Biomedical Sciences, São Paulo University, São Paulo, SP, Brazil.

Background: Evidence to date shows that mast cells play a critical role in immune defenses against infectious agents, but there have no reports about involvement of these cells in eliminating periodontopathogens. In this study, the phagocytic ability of mast cells against *A. actinomycetemcomitans* in comparison with macrophages was evaluated.

Methods: In vitro phagocytic assays were conducted using murine mast cells and macrophages, incubated with *A. actinomycetemcomitans*, either opsonized or not, with different bacterial load ratios. After one hour, cells were stained with acridine orange and assessed by confocal laser scanning electron microscopy.

Results: Phagocytic ability of murine mast cells against *A. actinomycetemcomitans* was confirmed. In addition, the percentage of mast cells with internalized bacteria in absence of opsonization was higher than those in presence of opsonization. Both cell types showed significant phagocytic activity against *A. actinomycetemcomitans*. However, the percentage of mast cells with non-opsonized bacteria was higher than those of macrophages with opsonized bacteria, in one of ratios (1:10).

Conclusion: This is the first report about the participation of murine mast cells as phagocytes against *A. actinomycetemcomitans*, mainly in the absence of opsonization with human serum. Our results may indicate that mast cells act as professional phagocytes in the pathogenesis of biofilm-associated periodontal disease.

KEY WORDS:

mast cells; macrophages; phagocytosis; gram-negative bacteria.

Periodontal biofilm hosts a wide diversity of potentially harmful bacterial species. *Aggregatibacter actinomycetemcomitans* has been described as member of the indigenous oral microbiota in many healthy individuals but is also largely involved in the pathology of periodontitis, [1, 2] the major cause of tooth loss in the world, [3] and various non-oral infections. [4, 5] *A. actinomycetemcomitans* is an oral Gram-negative bacterium and has shown the ability to invade periodontal tissues, establishing an infection. [6]

Several studies have demonstrated that the evolution of biofilm-associated periodontal diseases is influenced by the host's inflammatory and immune responses against pathogens resulting in the destruction of soft and mineralized periodontal tissues, and involves the participation of different immune cells and their chemical mediators. [7-11] Currently, mast cells (MCs) have been associated with inflammatory periodontal diseases through release of pro-inflammatory and immunoregulatory cytokines, which activate other immune cells, such as neutrophils and lymphocytes, thus contributing to the development and amplification of the host's defense response. [12, 13]

MCs are tissue-dwelling granule-containing immune cells that play a pivotal role in inflammatory and other processes such as the allergic immune response. [14, 15] MCs are commonly found in tissues that interface with the external environment such as the skin and mucosa of the

respiratory and gastrointestinal tracts. [16] Because these sites are also common portals of infection, MCs are likely to be among the first inflammatory cells to interact with invading pathogens. [17]

Studies have shown that MCs have the capacity to modulate the host's innate immune response against bacteria by their ability to phagocytose and kill bacteria, to process and present bacterial antigens to T cells, and to recruit phagocytic help through the release of physiological amounts of pro-inflammatory mediators. [18-21] Moreover, MCs display a phagocytosis-independent antimicrobial activity against extracellular bacteria, producing extracellular traps and antimicrobial compounds. [17, 22, 23]

Although phagocytic and microbicidal activity has been described in MCs against bacteria, the involvement of these cells in the elimination of periodontopathogens, via phagocytosis remains unclear. Since *A. actinomycetemcomitans* is one of the major periodontal pathogens, it is important to obtain more knowledge about the protective role of MCs against this microorganism. In this study, the phagocytic ability of mast cells against *A. actinomycetemcomitans* was determined and compared with the phagocytic capacity of macrophages.

MATERIALS AND METHODS

Isolation and Expansion of Bone Marrow-Derived Mast Cells

Primary mast cell lines were derived from femoral bone marrow of male C57BL/6 mice, 4 to 6-week-old. These animals were bred and maintained in the central animal facility of the Bauru School of Dentistry, University of Sao Paulo, Bauru, SP, Brazil. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use (#028/2010). Mouse femoral bone marrow was removed by washing the medullary cavity with RPMI medium[‖] supplemented with 10% heat-inactivated fetal bovine serum (FBS)[‖], 100 U/mL penicillin[‖], 100 µg/mL streptomycin[‖], 0.05 mM β-mercaptoethanol[¶], and 36 µM sodium bicarbonate[#]. Bone marrow cells were dissociated by aspiration with a Pasteur pipette and the cells were then rinsed twice by centrifugation (400 x*g* for 10 minutes) in RPMI medium. Cells were counted and the viability was assessed by Trypan blue in a hemocytometer. After this, cells were cultured, 0.5 x 10^6 cells/mL, in RPMI supplemented with 100 ng/mL recombinant stem cell factor (SCF)[**] and 20 ng/mL recombinant interleukin-3 (IL-3)[**], in a humidified atmosphere containing 5% CO_2 at 37°C. [24] By the end of 2–3 weeks, a nonadherent population of large granular cells was observed. Over the course of 21 days the bone marrow cells differentiated to mast cells (bone marrow-derived mast cells - BMMC).

By staining with toluidine blue, the isolated cells were shown to be homogeneous with typical granular appearance. Moreover, to further confirm their differentiation into mast cells, the cells were immunostained with antibodies against specific surface molecules of murine mast cells: anti-mouse CD117 (*c-kit* receptor) conjugated PE-Cy5[††], anti-mouse Fc epsilon receptor (FcεRI) (high-affinity receptor for immunoglobulin E) conjugated PE[††] and anti-AA4 conjugated to FITC (gangliosides derived from GD1b, present only on the surface of rodent mast cells). [24-26] After this, cells were analyzed by flow cytometry[‡‡], according to parameters of size (FSC), granularity (SSC) and fluorescence intensity. Thus, the homogeneity of this cell population also was determined by flow cytometric analysis, revealing a BMMC purity > 98%.

Bacterial Growth

Previous studies conducted by Malaviya et al.[18] demonstrated that different bacterial strains including *Escherichia coli* bind avidly to mouse mast cells. This bacteria/mast cell interaction causes phagocytosis and bacterial killing. Therefore, to ensure that the BMMC were able to cause phagocytosis, the same assay was performed with *E. coli* as control.

The enteroinvasive *E. coli* 0:124 (EIEC) were initially grown on BHI agar[§§], and incubated at 37°C for 24 hours. *A. actinomycetemcomitans* ATCC 29523 was grown on TSA plus 0.6% yeast extract[‖‖] and the plates were incubated in 10% CO_2 at 37°C for 5 days. The bacterial concentration of *A. actinomycetemcomitans* was standardized at $1x10^9$ CFU/mL and *E. coli* at $1x10^7$ CFU/mL by spectrophotometer[¶¶] ($A_{660\ nm}$).

Phagocytosis Assay

This phagocytic assay was performed following the protocols of previous studies,[17, 18, 22, 27] and it was adjusted in accordance with the conditions of our laboratory**.** Initially, bacteria were opsonized via complement with a pool of human serum under shaking at 37°C for 30 minutes, and non-opsonized bacteria were used as control. BMMC were washed twice with RPMI-1640 medium without antibiotics and incubated with *A. actinomycetemcomitans* at 1:5, 1:10 and 1:25 ratios of cell:bacteria or with *E. coli* at 1:10, for 1 hour at 37°C under gentle agitation. Then, cells were centrifuged at 218 xg, at 24°C, for 10 minutes and the pellet resuspended in 200 mL of RPMI-1640 medium. The cell suspensions were transferred to 24-well plates, containing sterile spherical glass coverslip (13 mm in diameter), and pre-treated with tissue adhesive[##] allowing cell adhesion. The wells were washed with PBS to remove the extracellular bacteria. The coverslips with cells attached were fixed with 2% paraformaldehyde (PFA) in PBS for 20 minutes and washed again with PBS. Then they were stained with 0.05 mg/mL acridine orange[***] for 15 minutes in a dark room. After this, coverslips were carefully removed and placed on glass slides with mounting medium[†††]. The slides were qualitatively evaluated by a confocal laser scanning electron microscopy[‡‡‡], which initially allowed entrapped bacteria to be differentiated from bacteria only bound to the mast cell surface molecules. Thereafter quantitative analysis was performed at 100x magnification, by counting the MCs with internalized bacteria, in 20 randomly captured fields[§§§]. Phagocytosis was assessed by considering the number of internalized bacteria per cell (≤ 5 and > 5). Cells with absence of internalized bacteria also were counted. The results were expressed in percentages, and were obtained from the average of three independent experiments, considering the total number of cells counted per coverslip (20 fields) in each experiment.

Simultaneously to BMMC, phagocytosis assays against *A. actinomycetemcomitans* were performed by using mouse peritoneal macrophages (MPM), allowing the phagocytic capacity of cell types, mast cells and macrophages, to be compared. MPM were obtained according to the methodology of Murciano et al. (2008). [28]

Statistical Analysis

The results are expressed as the mean ± SD (standard deviation) from three independent experiments (1 mouse in each experiment for testing mast cells and 4 mice for macrophages). Data were analyzed by using an analysis of variance (two-way ANOVA) and Tukey's test, followed by Student's-*t* test[‖‖‖]. Values of $p<0.05$ were considered statistically significant.

RESULTS

BMMC Act as Phagocytes Against A. Actinomycetemcomitans, Especially in the Absence of Serum Opsonization.

In this study, by using a ratio of 1:25 (BMMC:bacteria), the percentage of BMMC with opsonized *A. actinomycetemcomitans* was higher when compared with BMMC without bacteria ($p=0.011$). Most of the cells displayed more than five internalized bacteria.

Interestingly, in the absence of opsonization, BMMC presenting ingested bacteria were more numerous than cells without bacteria in all BMMC-bacterial ratios used. In addition, a higher

percentage of BMMC with non-opsonized bacteria was detected in comparison with BMMC with bacteria opsonized, in the ratios 1:5 and 1:10 (p=0.02 and p=0.004, respectively).

Considering BMMC with more than five internalized bacteria, the cells incubated with non-opsonized bacteria were more numerous than the BMMC with opsonized bacteria only in the ratio of 1:10 (p=0.0008) (Figures 1 and 2).

In the Absence of Serum Opsonization, BMMC Displayed More Ingested A. Actinomycetemcomitans than MPM.

When comparing the phagocytic activity of BMMC with professional phagocytes (MPM) against opsonized *A. actinomycetemcomitans*, the percentage of MPM with up to five internalized bacteria was higher than BMMC in 1:5 and 1:25 ratios (p=0.03 and p=0.02, respectively). However, the percentage of BMMC that had more than five internalized bacteria was higher than that of MPM, in the ratio of 1:5 (p= 0.04).

In the absence of opsonization, MPM without internalized bacteria were more numerous than BMMC, in all the ratios evaluated. Moreover, there were also higher percentages of BMMC with more than five internalized bacteria than those observed for MPM in all ratios (Figure 3).

Considering only the higher percentages of cells with *A. actinomycetemcomitans*, the values were higher for BMMC with non-opsonized bacteria in comparison with MPM with opsonized bacteria in the ratio 1:10 (p=0.0008) (Figure 4).

Phagocytosis by BMMC Against A. Actinomycetemcomitans and E. Coli was Similar; Serum Opsonization also Appeared to Amplify E. Coli Internalization by MPM but not by BMMC.

When comparing the two cell types with opsonized *E. coli* (1:10), the percentage of MPM with more than five internalized bacteria was higher than BMMC. Moreover, the percentage of MPM without opsonized bacteria was lower than BMMC. In the absence of opsonization, the opposite was observed since the percentage of BMMC with more than five internalized bacteria was higher in comparison with MPM.

Similar to the phagocytic assays with *A. actinomycetemcomitans*, percentages of BMMC without internalized *E. coli* ranged according to the presence or absence of previous bacterial opsonization. BMMC without opsonized *E. coli* were more numerous than BMMC with absence of non-opsonized *E. coli*. Conversely, MPM without non-opsonized *E. coli* displayed higher percentages compared with MPM without internalization of opsonized bacteria, suggesting that serum opsonization amplified bacterial internalization by MPM but not by BMMC (Figure 5) as observed for *A. actinomycetemcomitans*.

DISCUSSION

The contribution of MCs to the host defense has become increasingly recognized in recent years. It is known that MCs have the ability to recognize and phagocytose infectious agents through specific receptors present on their surfaces. [29-31] From in vitro assays, we are able to demonstrate for the first time, the participation of murine MCs as phagocytes against the periodontopathogen *A. actinomycetemcomitans*, especially in the absence of opsonization with human serum. Considering that the Fc portion of immunoglobulin is variable between species, [32] the bacterial opsonization by human serum supplied only complement system proteins for opsonin-dependent phagocytosis by murine cells.

In the present study, opsonized *A. actinomycetemcomitans* with complement were not phagocytosed by BMMC as efficiently as it was without opsonization. Probably, the recognition of

A. actinomycetemcomitans by murine BMMC, when independent of complement receptor, seems to have remarkable implications, because percentages of BMMC with non-opsonized bacteria were higher than BMMC without these bacteria or than BMMC with opsonized bacteria, after comparisons in the same ratios. In addition, high percentages of BMMC had more than five internalized *A. actinomycetemcomitans,* especially in the absence of opsonization. Similar results were observed with BMMC challenged with *E. coli* by comparing the presence or absence of opsonization.

It is now known that the MCs contain numerous surface receptors, including those that promote recognition and bacterial binding. MCs exhibit two basic mechanisms of microbial recognition: opsonin-dependent (via Fc and C3 receptors) and opsonin-independent (via integrins, CD48 molecule and Toll-like receptors-TLR). [33-36] In agreement with our results, the recognition and ingestion of *A. actinomycetemcomitans* by murine BMMC involve either opsonin-dependent or opsonin-independent receptors, in agreement with Malaviya et al.[35] who demonstrated the recognition of *E. coli* by MCs.

Since *A. actinomycetemcomitans* produces diverse bacterial products such as lipopolysaccharide (LPS) and peptidoglycan (PGN) mediated by TLR2 and TLR4 receptors, [31, 37-39] the TLR pathway may represent another route of recognition and internalization of this periodontopathogen by the MCs studied here, since MCs express TLR1, 2, 3, 4, 6 and 9. [36, 40, 41] However, further studies are needed to unravel which extracellular receptors on these cells are involved more frequently and efficiently in the recognition and ingestion of *A. actinomycetemcomitans.*

In the present study, unlike BMMC, macrophages with opsonized bacteria were more frequent than macrophages with non-opsonized bacteria, suggesting that complement system proteins resulted in more efficient internalization by these cells than BMMC, for both *A. actinomycetemcomitans* and *E. coli.* Furthermore, in a ratio of 1:10, BMMC with non-opsonized *A. actinomycetemcomitans* were more numerous than macrophages with opsonized *A. actinomycetemcomitans*, suggesting that BMMC phagocytosed more efficiently non-opsonized periodontopathogens than macrophages against opsonized bacteria with complement. However, we cannot rule out the possibility that these opsonized bacteria were killed by macrophages after internalization, supporting some findings, [42-44] which observed phagocytosis by macrophages with bacterial death in an opsonin-rich environment. After comparison of phagocytosis by both cell types, our results showed that the BMMC could be considered professional phagocytes against *A. actinomycetemcomitans*, in the same way as macrophages are, irrespective of the bacterial killing. It is important to emphasize that the bacterial phagocytosis by both cell populations occurred in a bacterial load-independent manner.

Based on the methodology used, it was not possible to verify the viability of internalized bacteria. Malaviya et al.[18] demonstrated that murine MCs were able to phagocytose and kill Gram-negative bacteria in a medium containing serum components, by means of non-oxidative and oxidative mechanisms. However, several bacteria can affect this phagocytic ability of MCs to their advantage when in opsonin-poor microenvironments, [45] thus inducing the cell to become an intracellular environment in which bacteria could proliferate and contribute to bacterial spreading. Under these conditions, MCs would then serve as reservoirs of viable bacteria, resulting in harmful effects to the host. [46] In fact, our results revealed high percentages of MCs with more than five internalized *A. actinomycetemcomitans*, mainly in the absence of opsonization through complement. Accordingly, C3-deficient mice presented functional impairment of MCs with delayed elimination of bacteria and reduction in their degranulation and production of TNF-α, suggesting that products of the complement system are essential for the activation and microbicidal activity of MCs after bacterial challenge. [47] Moreover, *E. coli* phagocytosis by MCs through CD48 molecule (GPI-anchored glycosylphosphatidylinositol) promoted bacterial death more efficiently than through CR3. [35]

When MCs interact with opsonized bacteria, and perform their phagocytic and microbicidal functions, they simultaneously release considerable amounts of pro-inflammatory mediators, thereby leading to the migration of more inflammatory cells at the infectious focus. [48-50] When there is excessive release of mediators, it can result in exacerbation of the inflammatory process, causing serious cytotoxic effects on tissues. However, several internalized bacterial pathogens express toxins that can inhibit the release of chemical mediators by MCs. [45] Thus, we are tempted to think that the phagocytic response of MCs against *A. actinomycetemcomitans* can be ambiguous in biofilm-associated periodontal diseases either protecting the individual by phagocytosis and bacterial killing or leading to further destruction and progression of the disease by exacerbated release of mediators. Moreover, MCs could be an intracellular refuge for periodontopathogens. With regard to these considerations, there continue to be conflicting views on whether phagocytosis by mast cells plays a protective or harmful role in relation to clinical progression of periodontal disease. Although there is broad scientific knowledge about the pathogenesis of periodontal diseases, further studies are needed for a better understanding of the biological events involved in this pathology, especially those related to mast cells.

Finally, this early in vitro investigation proved the phagocytic capacity of MCs against *A. actinomycetemcomitans*. Although this is an in vitro study and only confocal scanning electron microscopy was used as method to analyze phagocytosis, the results obtained here lead to the hypothesis of a possible role of mast cells as professional phagocytes in a manner similar to that of macrophages. In a further study, we intend to investigate the microbicidal activity of MCs against internalized *A. actinomycetemcomitans*, whether opsonized or not, thus contributing to a better understanding of the real importance of bacterial phagocytosis by MCs in inflammatory PD. In the context of infectious diseases, especially PD, it is likely that this will not only expand the scope of our knowledge of the role of MCs in innate immunity with regard to these diseases, but may provide new therapeutic targets to control the inflammatory response in gingival and periodontal tissues against the periodontopathogens.

ACKNOWLEDGEMENTS

The authors thank Dr. Fernando de Queiroz Cunha (Department of Pharmacology) and Dr. Maria Célia Jamur (Department of Cell and Molecular Biology, School of Medicine of Ribeirão Preto, São Paulo University, Ribeirão Preto, SP, Brazil) for providing antibodies. They also thank Dr. Maura Rosane Valério Ikoma, Marcimara Penitenti (Laboratory of Hematology, Amaral Carvalho Hospital, Jau, São Paulo, Brazil), Dr. Camila Peres Buzalaf, Msc. Márcia Sirlene Zardin Graeff and Marcelo Milanda Ribeiro Lopes (Integrated Research Center, Bauru Dental School, University of São Paulo, Bauru, Brazil) for their technical support, and professors José Roberto Pereira Lauris and Heitor Marques Honório (Department of Pediatric Dentistry, Orthodontics and Public Health, Bauru Dental School USP) for the statistical analysis.

SOURCE OF FUNDING STATEMENT AND CONFLICT OF INTEREST.

This study was supported by grants from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo grant 2009/14152-1). The authors have no financial conflict of interests.

REFERENCES

1. Aberg CH, Sjodin B, Lakio L, Pussinen PJ, Johansson A, Claesson R. Presence of Aggregatibacter actinomycetemcomitans in young individuals: a 16-year clinical and microbiological follow-up study. *J Clin Periodontol* 2009;36:815-822.
2. Faveri M, Figueiredo LC, Duarte PM, Mestnik MJ, Mayer MP, Feres M. Microbiological profile of untreated subjects with localized aggressive periodontitis. *J Clin Periodontol* 2009;36:739-749.
3. Berezow AB, Darveau RP. Microbial shift and periodontitis. *Periodontol 2000* 2011;55:36-47.
4. van Winkelhoff AJ, Slots J. Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in nonoral infections. *Periodontol 2000* 1999;20:122-135.

5. Henderson B, Nair SP, Ward JM, Wilson M. Molecular pathogenicity of the oral opportunistic pathogen Actinobacillus actinomycetemcomitans. *Annu Rev Microbiol* 2003;57:29-55.
6. Henderson B, Ward JM, Ready D. Aggregatibacter (Actinobacillus) actinomycetemcomitans: a triple A* periodontopathogen? *Periodontol 2000* 2010;54:78-105.
7. Kinane DF, Attstrom R. Advances in the pathogenesis of periodontitis. Group B consensus report of the fifth European Workshop in Periodontology. *J Clin Periodontol* 2005;32 Suppl 6:130-131.
8. Graves D. Cytokines that promote periodontal tissue destruction. *J Periodontol* 2008;79:1585-1591.
9. Liu YC, Lerner UH, Teng YT. Cytokine responses against periodontal infection: protective and destructive roles. *Periodontol 2000* 2010;52:163-206.
10. Garlet GP, Cardoso CR, Campanelli AP, et al. The dual role of p55 tumour necrosis factor-alpha receptor in Actinobacillus actinomycetemcomitans-induced experimental periodontitis: host protection and tissue destruction. *Clin Exp Immunol* 2007;147:128-138.
11. Trombone AP, Ferreira SB, Jr., Raimundo FM, et al. Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection. *J Periodontal Res* 2009;44:443-451.
12. Steinsvoll S, Helgeland K, Schenck K. Mast cells--a role in periodontal diseases? *J Clin Periodontol* 2004;31:413-419.
13. Batista AC, Rodini CO, Lara VS. Quantification of mast cells in different stages of human periodontal disease. *Oral Dis* 2005;11:249-254.
14. Taylor ML, Metcalfe DD. Mast cells in allergy and host defense. *Allergy Asthma Proc* 2001;22:115-119.
15. Arzi B, Murphy B, Cox DP, Vapniarsky N, Kass PH, Verstraete FJ. Presence and quantification of mast cells in the gingiva of cats with tooth resorption, periodontitis and chronic stomatitis. *Arch Oral Biol* 2010;55:148-154.
16. Mekori YA, Metcalfe DD. Mast cells in innate immunity. *Immunol Rev* 2000;173:131-140.
17. von Kockritz-Blickwede M, Goldmann O, Thulin P, et al. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* 2008;111:3070-3080.
18. Malaviya R, Ross EA, MacGregor JI, et al. Mast cell phagocytosis of FimH-expressing enterobacteria. *J Immunol* 1994;152:1907-1914.
19. Malaviya R, Twesten NJ, Ross EA, Abraham SN, Pfeifer JD. Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. *J Immunol* 1996;156:1490-1496.
20. Shin JS, Gao Z, Abraham SN. Involvement of cellular caveolae in bacterial entry into mast cells. *Science* 2000;289:785-788.
21. Malaviya R, Abraham SN. Mast cell modulation of immune responses to bacteria. *Immunol Rev* 2001;179:16-24.
22. Ronnberg E, Guss B, Pejler G. Infection of mast cells with live streptococci causes a toll-like receptor 2- and cell-cell contact-dependent cytokine and chemokine response. *Infect Immun* 2010;78:854-864.
23. Abel J, Goldmann O, Ziegler C, et al. Staphylococcus aureus evades the extracellular antimicrobial activity of mast cells by promoting its own uptake. *J Innate Immun* 2011;3:495-507.
24. Jamur MC, Grodzki AC, Berenstein EH, Hamawy MM, Siraganian RP, Oliver C. Identification and characterization of undifferentiated mast cells in mouse bone marrow. *Blood* 2005;105:4282-4289.
25. Basciano LK, Berenstein EH, Kmak L, Siraganian RP. Monoclonal antibodies that inhibit IgE binding. *J Biol Chem* 1986;261:11823-11831.
26. Guo NH, Her GR, Reinhold VN, Brennan MJ, Siraganian RP, Ginsburg V. Monoclonal antibody AA4, which inhibits binding of IgE to high affinity receptors on rat basophilic leukemia cells, binds to novel alpha-galactosyl derivatives of ganglioside GD1b. *J Biol Chem* 1989;264:13267-13272.
27. Gasparoto TH, Vieira NA, Porto VC, Campanelli AP, Lara VS. Ageing exacerbates damage of systemic and salivary neutrophils from patients presenting Candida-related denture stomatitis. *Immun Ageing* 2009;6:3.
28. Murciano C, Yanez A, O'Connor JE, Gozalbo D, Gil ML. Influence of aging on murine neutrophil and macrophage function against Candida albicans. *FEMS Immunol Med Microbiol* 2008;53:214-221.
29. Marshall JS. Mast-cell responses to pathogens. *Nat Rev Immunol* 2004;4:787-799.

30. Dawicki W, Marshall JS. New and emerging roles for mast cells in host defence. *Curr Opin Immunol* 2007;19:31-38.

31. Chen K, Xiang Y, Yao X, et al. The active contribution of Toll-like receptors to allergic airway inflammation. *Int Immunopharmacol* 2011;11:1391-1398.

32. Abbas AK, Lichtman, A. H., Pillai, S. . Antibodies and Antigens. In: *Cellular and Molecular Immunology*. Philadelphia: Saunders/Elsevier, 2010:75-96.

33. Sher A, McIntyre SL. Receptors for C3 on rat peritoneal mast cells. *J Immunol* 1977;119:722-725.

34. Abraham SN, Malaviya R. Mast cells in infection and immunity. *Infect Immun* 1997;65:3501-3508.

35. Malaviya R, Gao Z, Thankavel K, van der Merwe PA, Abraham SN. The mast cell tumor necrosis factor alpha response to FimH-expressing Escherichia coli is mediated by the glycosylphosphatidylinositol-anchored molecule CD48. *Proc Natl Acad Sci U S A* 1999;96:8110-8115.

36. Marshall JS, Jawdat DM. Mast cells in innate immunity. *J Allergy Clin Immunol* 2004;114:21-27.

37. Stassen M, Muller C, Arnold M, et al. IL-9 and IL-13 production by activated mast cells is strongly enhanced in the presence of lipopolysaccharide: NF-kappa B is decisively involved in the expression of IL-9. *J Immunol* 2001;166:4391-4398.

38. Supajatura V, Ushio H, Nakao A, Okumura K, Ra C, Ogawa H. Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4. *J Immunol* 2001;167:2250-2256.

39. Gelani V, Fernandes AP, Gasparoto TH, et al. The role of toll-like receptor 2 in the recognition of Aggregatibacter actinomycetemcomitans. *J Periodontol* 2009;80:2010-2019.

40. Applequist SE, Wallin RP, Ljunggren HG. Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int Immunol* 2002;14:1065-1074.

41. Carlos D, Frantz FG, Souza-Junior DA, et al. TLR2-dependent mast cell activation contributes to the control of Mycobacterium tuberculosis infection. *Microbes Infect* 2009;11:770-778.

42. Drevets DA, Leenen PJ, Campbell PA. Complement receptor type 3 (CD11b/CD18) involvement is essential for killing of Listeria monocytogenes by mouse macrophages. *J Immunol* 1993;151:5431-5439.

43. Warren J, Mastroeni P, Dougan G, et al. Increased susceptibility of C1q-deficient mice to Salmonella enterica serovar Typhimurium infection. *Infect Immun* 2002;70:551-557.

44. Geier H, Celli J. Phagocytic receptors dictate phagosomal escape and intracellular proliferation of Francisella tularensis. *Infect Immun* 2011;79:2204-2214.

45. Feger F, Varadaradjalou S, Gao Z, Abraham SN, Arock M. The role of mast cells in host defense and their subversion by bacterial pathogens. *Trends Immunol* 2002;23:151-158.

46. Shin JS, Abraham SN. Co-option of endocytic functions of cellular caveolae by pathogens. *Immunology* 2001;102:2-7.

47. Prodeus AP, Zhou X, Maurer M, Galli SJ, Carroll MC. Impaired mast cell-dependent natural immunity in complement C3-deficient mice. *Nature* 1997;390:172-175.

48. Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature* 1996;381:77-80.

49. Arock M, Ross E, Lai-Kuen R, Averlant G, Gao Z, Abraham SN. Phagocytic and tumor necrosis factor alpha response of human mast cells following exposure to gram-negative and gram-positive bacteria. *Infect Immun* 1998;66:6030-6034.

50. Malaviya R, Abraham SN. Role of mast cell leukotrienes in neutrophil recruitment and bacterial clearance in infectious peritonitis. *J Leukoc Biol* 2000;67:841-846.

Corresponding author: Vanessa Soares Lara, e-mail: vanessa@fob.usp.br, Bauru School of Dentistry, University of São Paulo, Al. Dr. Otávio Pinheiro Brisolla 9-75. CEP: 17.012-901. Bauru, SP, Brazil. Tel/fax: +55 (14) 3235-8251.

Submitted February 3, 2012; accepted for publication March 30, 2012.

Figure 1 –

*BMMC act as phagocytes against A. actinomycetemcomitans, especially in the absence of serum opsonization. Bars show the percentage of BMMC with A. actinomycetemcomitans internalized or not. Results are expressed as the mean ± SD from three independent experiments. Data were analyzed by Tukey's test and Student's-t test. Identical symbols represent statistical differences, * and † p<0.05; ‡ p<0.01; §, || and ¶ p<0.001.*

Figure 2 –

*Phagocytosis of BMMC against A. actinomycetemcomitans and E. coli. **A through C)** BMMC stained green, with A. actinomycetemcomitans inside, either red or green. **D)** BMMC with E. coli, either green or red, internalized or only adhered to their surfaces. The cell:bacteria ratios are indicated in the top right side of the photomicrographs. Confocal laser scanning electron microscopy (original magnification x630).*

Figure 3 –

*In the absence of serum opsonization, BMMC presented more ingested A. actinomycetemcomitans than MPM. Bars show the percentage of BMMC and MPM with A. actinomycetemcomitans internalized or not. Both cell types were classified as follows: absence of bacteria **(a)**, with up to five **(b)** or more internalized bacteria **(c)**. Results are expressed as the mean ± SD from three independent experiments. Data were analyzed by Tukey's test and Student's-t test. Identical symbols represent statistical differences, *, † and ‡ p<0.05; §, || and ¶ p<0.01; # p<0.001.*

Figure 4 –

*Highest percentages of both cell types with internalized A. actinomycetemcomitans. This Figure shows the mean of the highest percentages of mast cells and macrophages with internalized A. actinomycetemcomitans from three independent experiments. These higher means were obtained from mast cells in the absence of opsonization, and from macrophages in the presence of opsonization. Data were analyzed by Tukey's test, * p<0.05.*

Figure 5 –

*Analysis of the phagocytes against E. coli. Bars show the percentage of BMMC and MPM with A. actinomycetemcomitans internalized or not. Macrophages and mast cells were classified as follows: absence of bacteria **(a)**, with up to five **(b)** or more **(c)**, internalized bacteria, only the proportion of 1 cell to 10 bacteria (1:10). Results are expressed as the mean ± SD from three independent experiments. Data were analyzed by Tukey's test and Student's-t test. Identical symbols represent statistical differences, * p<0.05; †, ‡ and §; p<0.01; || p<0.001.*

|| GIBCO, Invitrogen, Grand Island, NY, USA.

¶ Sigma, St Louis, MO, USA.

Merck, Rio de Janeiro, RJ, BR.

** Peprotech, Rocky Hill, NJ, USA.

†† eBioscience, San Diego, CA, USA.

‡‡ FACScalibur/CellQuest, BD Biosciences, Sparks, MD, USA.

§§ Acumedia, Neogen Corporation, Lansing, MI, USA.

|||| DIFCO™, Becton Dickinson Company, San Jose, CA, USA.

¶¶ Biotek, Winooski, VT, USA.

Biobond, Electron Microscopy Sciences, Hatfield, PA, USA.

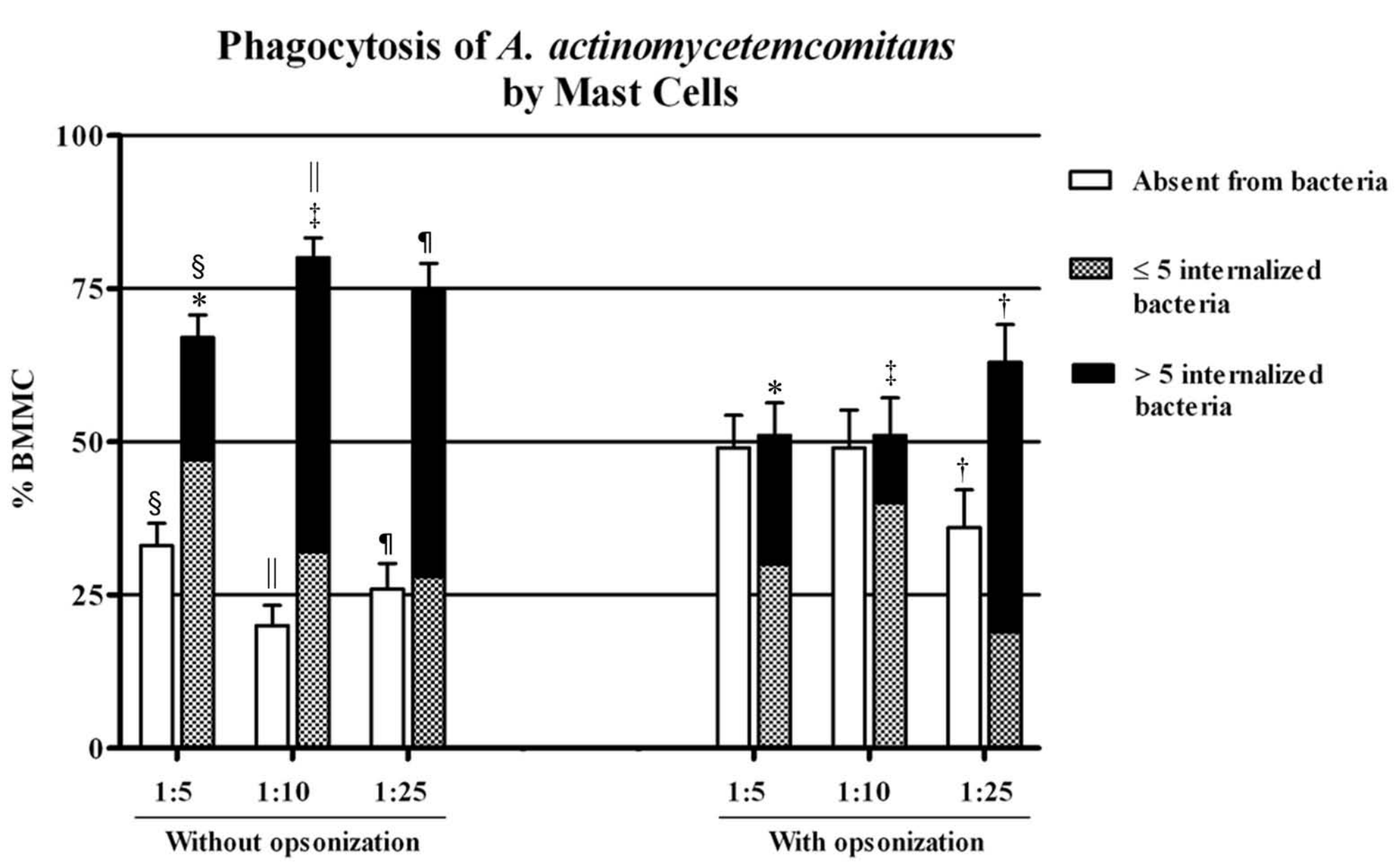
*** Merck, Darmstadt, HE, Germany.

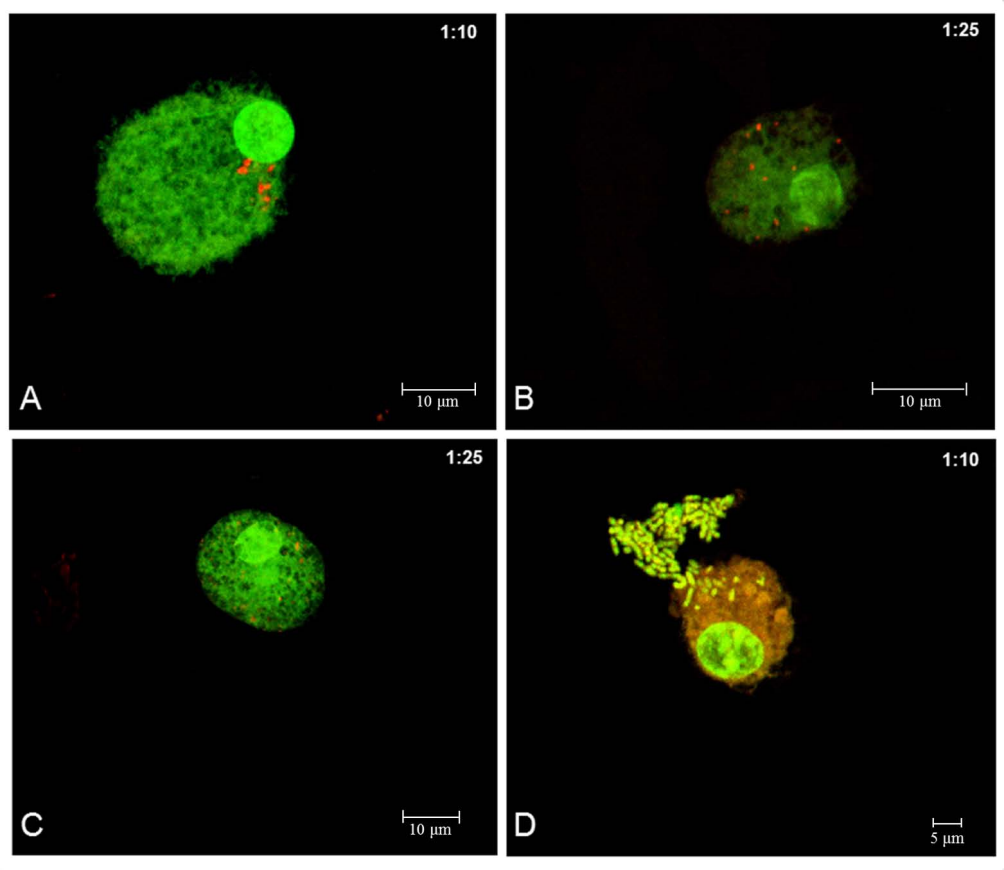
††† Permount, Fisher Scientific, Morris Plains, NJ, USA.

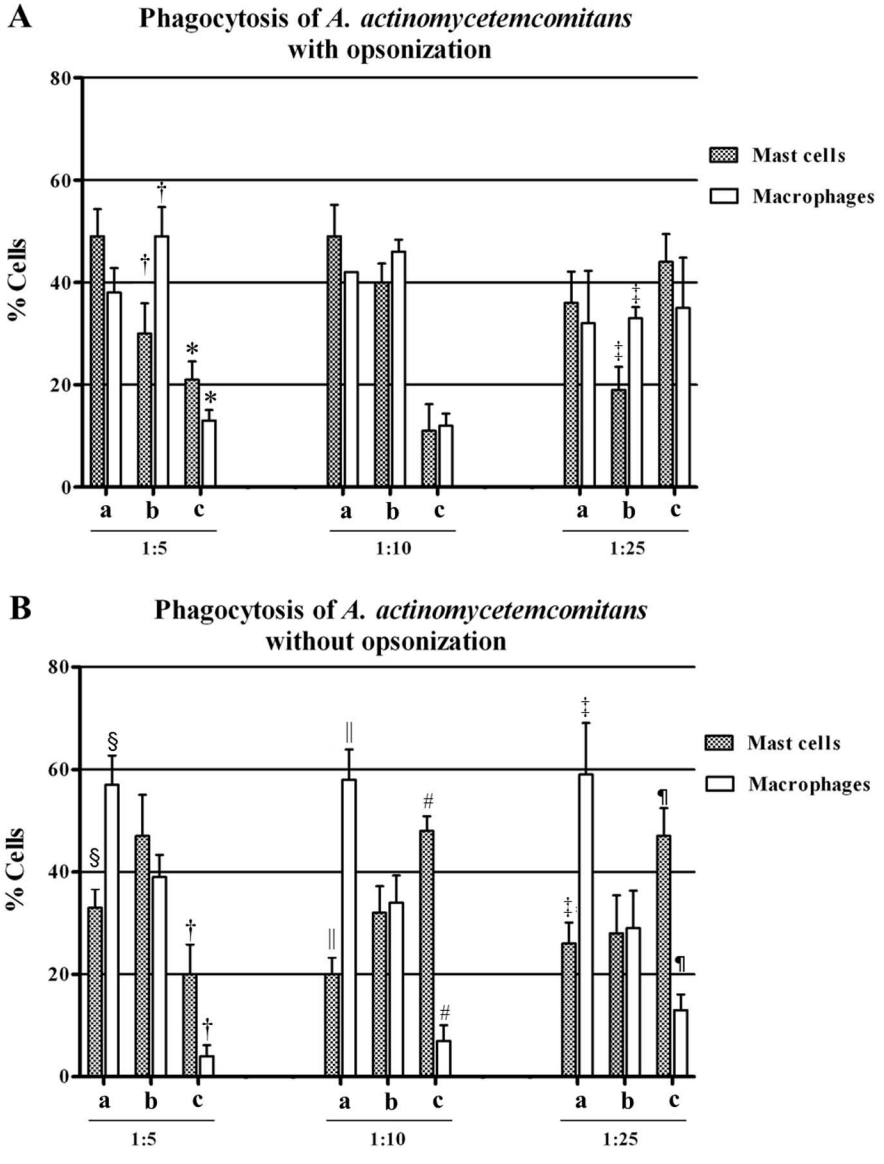
‡‡‡ Leica TCS-SPE, Wetzlar, HE, Germany.

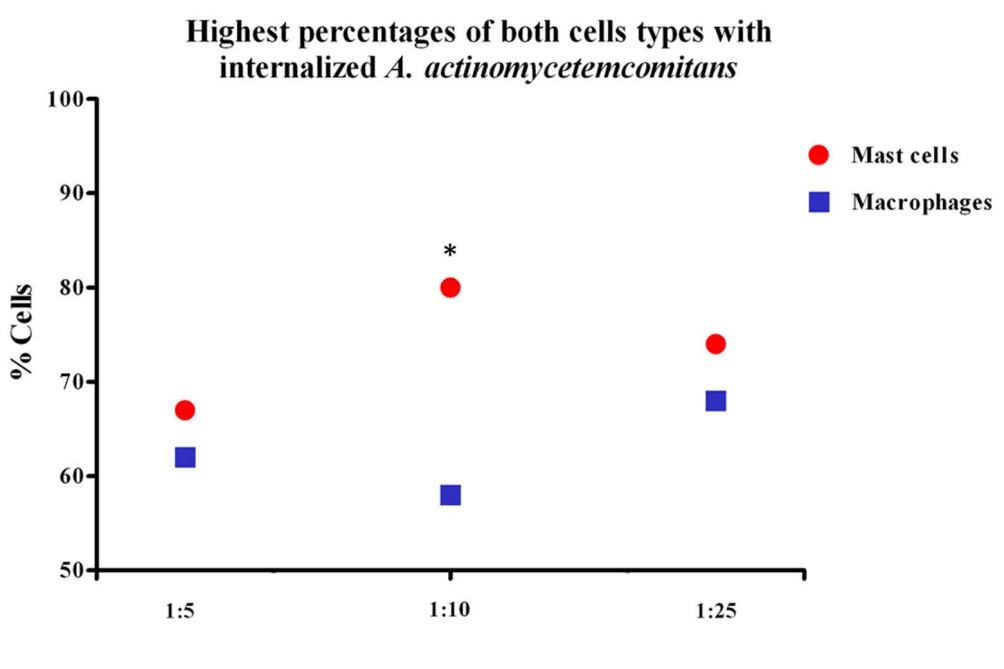
§§§ Carl Zeiss/AxioVision Rel 4.6, Goettingen, NI, Germany.

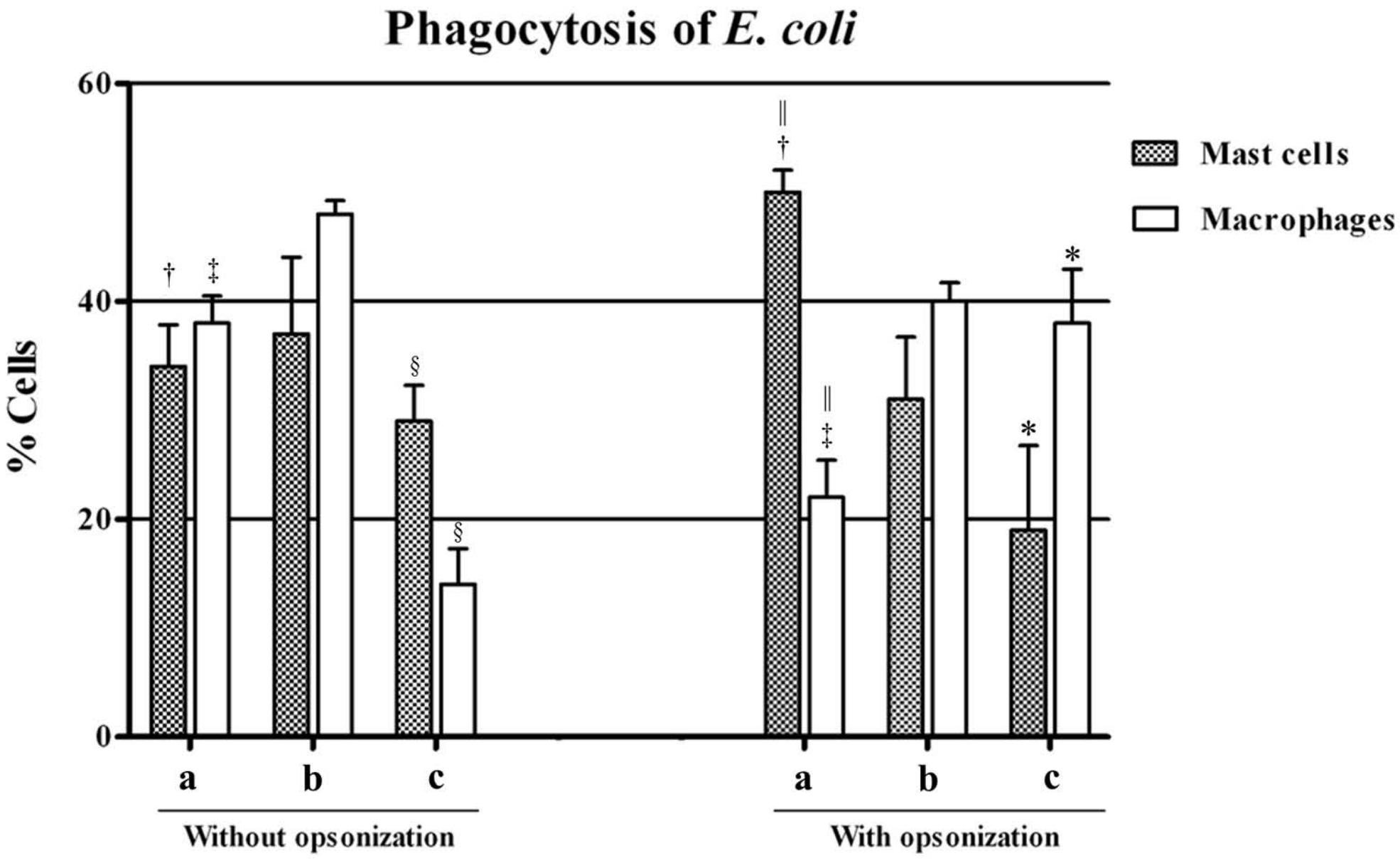
|||||| PRISM Software; GraphPad, San Diego, CA, USA.


Phagocytosis of A. actinomycetemcomitans by Mast Cells
100
75
50
25
0
% BMMC
Absent from bacteria
≤ 5 internalized bacteria
> 5 internalized bacteria
1:5
1:10
1:25
Without opsonization
1:5
1:10
1:25
With opsonization


1:10
A
10 µm
1:25
B
10 µm
1:25
C
10 µm
1:10
D
5 µm


A
Phagocytosis of A. actinomycetemcomitans
with opsonization
% Cells
80
60
40
20
0
Mast cells
Macrophages
a
b
c
1:5
1:10
1:25
B
Phagocytosis of A. actinomycetemcomitans
without opsonization
% Cells
80
60
40
20
0
Mast cells
Macrophages
a
b
c
1:5
1:10
1:25


Highest percentages of both cells types with internalized A. actinomycetemcomitans
100
90
80
70
60
50
% Cells
*
Mast cells
Macrophages
1:5
1:10
1:25


Phagocytosis of E. coli
60
40
20
0
% Cells
Mast cells
Macrophages
a
b
c
a
b
c
Without opsonization
With opsonization