ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

Η ΕΠΙΔΡΑΣΗ ΤΟΥ ΠΕΡΙΟΠΑΘΟΓΟΝΟΥ ΒΑΚΤΗΡΙΟΥ *PORPHYROMONAS GINGIVALIS* ΣΤΗΝ ΔΙΕΓΕΡΣΗ ΑΝΘΡΩΠΙΝΩΝ ΜΑΣΤΟΚΥΤΤΑΡΩΝ ΚΑΙ ΤΗΝ ΠΑΡΑΓΩΓΗ ΠΡΟ- ΦΛΕΓΜΟΝΟΔΩΝ ΜΕΣΟΛΑΒΗΤΩΝ

ΚΑΣΣΙΑΝΗ - ΗΡΩ Β. ΠΑΛΑΣΚΑ

ΟΔΟΝΤΙΑΤΡΟΥ-ΠΕΡΙΟΔΟΝΤΟΛΟΓΟΥ

Α’ ΠΑΝΕΠΙΣΤΗΜΙΑΚΗ ΚΛΙΝΙΚΗ ΔΕΡΜΑΤΙΚΩΝ ΚΑΙ ΑΦΡΟΔΙΣΙΩΝ ΝΟΣΩΝ

ΝΟΣΟΚΟΜΕΙΟ ΑΝΔΡΕΑΣ ΣΥΓΓΡΟΣ

ΔΙΕΥΘΥΝΤΗΣ: ΚΑΘΗΓΗΤΗΣ ΔΗΜΗΤΡΙΟΣ ΡΗΓΟΠΟΥΛΟΣ

ΣΕ ΣΥΝΕΡΓΑΣΙΑ ΜΕ ΤΟΝ

ΤΟΜΕΑ ΜΟΡΙΑΚΗΣ ΦΥΣΙΟΛΟΓΙΑΣ ΚΑΙ ΦΑΡΜΑΚΟΛΟΓΙΑΣ, ΕΡΓΑΣΤΗΡΙΟ ΜΟΡΙΑΚΗΣ ΑΝΟΣΟΦΑΡΜΑΚΟΛΟΓΙΑΣ ΚΑΙ ΑΝΑΠΤΥΞΗΣ ΦΑΡΜΑΚΩΝ, ΙΑΤΡΙΚΗΣ ΣΧΟΛΗΣ ΠΑΝΕΠΙΣΤΗΜΙΟΥ TUFTS ΒΟΣΤΩΝΗΣ, ΗΠΑ

ΔΙΕΥΘΥΝΤΗΣ: ΚΑΘΗΓΗΤΗΣ ΘΕΟΧΑΡΗΣ ΘΕΟΧΑΡΙΔΗΣ

ΑΘΗΝΑ 2021

NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS

SCHOOL OF HEALTH SCIENCES

MEDICAL SCHOOL

PHD THESIS

THE EFFECT OF THE PERIODOPATHOGENIC BACTERIA PORHYROMONAS GINGIVALIS ON THE STIMULATION OF MAST CELLS AND THE RELEASE OF PRO-INFLAMMATORY MEDIATORS

KASSIANI IRO V. PALASKA

DDS, MSc (PERIODONTOLOGY)

ANDREAS SIGGROS HOSPITAL OF CUTANEOUS AND VENEREAL DISEASES

DIRECTOR: PROFESSOR RIGOPOULOS DIMITRIOS

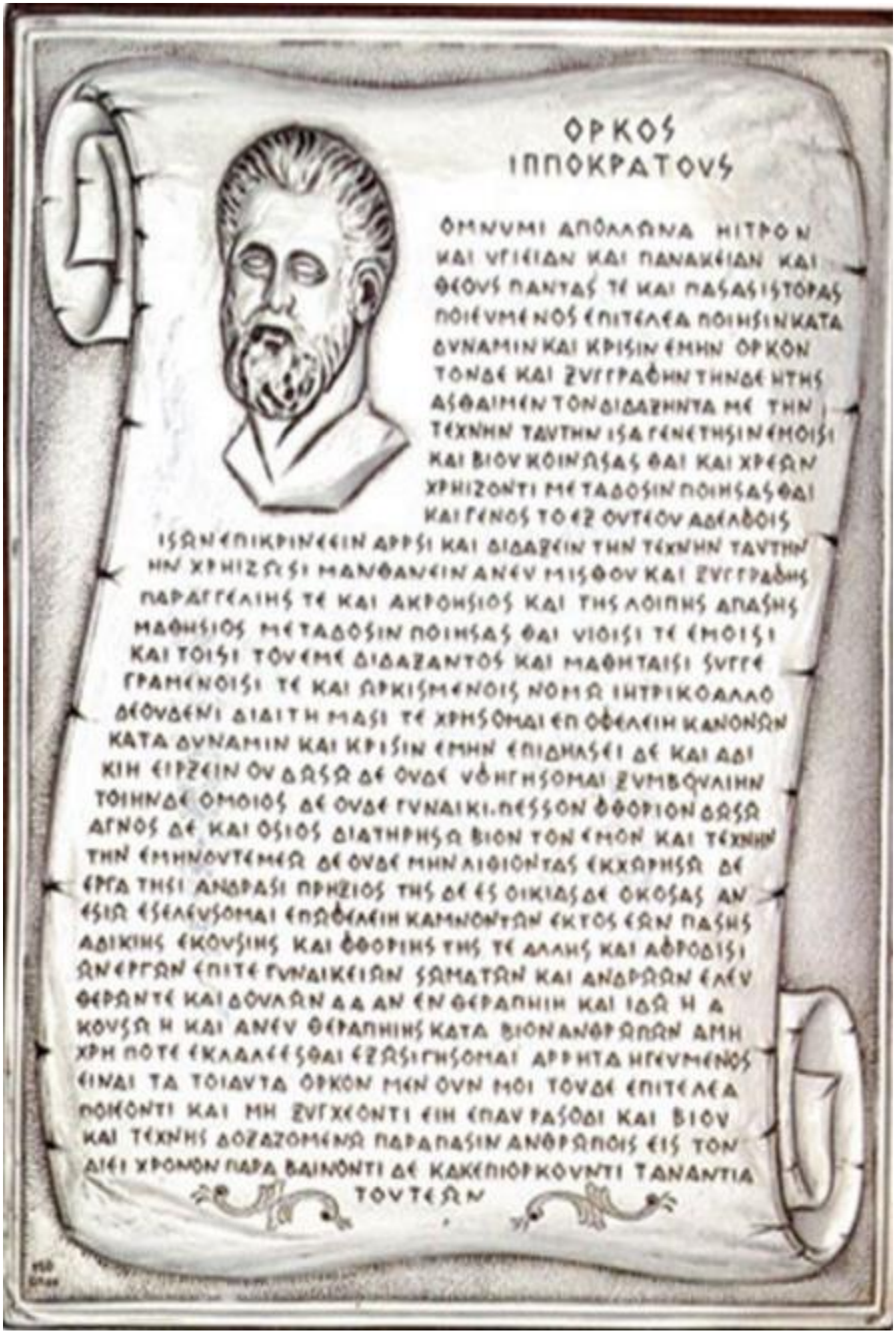
IN COLLABORATION WITH

THE DEPARTMENT OF MOLECULAR IMMUNOPHARMACOLOGY AND DRUG DISCOVERY

LABORATORY, GRADUATE SCHOOL OF BIOMEDICAL SCIENCES, TUFTS, BOSTON, MA

DIRECTOR: PROFESSOR THEOHARIDES THEOHARIS

ATHENS 2021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ΑΙΕΙ ΧΡΟΝΟΝ ΠΑΡΑΒΑΙΝΟΝΤΙ ΔΕ ΚΑΚΕΠΙΟΡΚΟΥΝΤΙ ΤΑΝΑΝΤΙΑ
ΤΟΥΤΕΩΝ

ΠΡΟΟΔΟΣ ΕΚΠΟΝΗΣΗΣ ΤΗΣ ΔΙΔΑΚΤΟΡΙΚΗΣ ΔΙΑΤΡΙΒΗΣ

- ΑΡΧΙΚΗ ΑΙΤΗΣΗ: 02/2013
- ΟΡΙΣΜΟΣ ΤΡΙΜΕΛΟΥΣ ΕΠΙΤΡΟΠΗΣ: 22/09/2014
- ΟΡΙΣΜΟΣ ΘΕΜΑΤΟΣ: 04/05/2015
- ΚΑΤΑΘΕΣΗ ΔΙΑΤΡΙΒΗΣ ΠΡΟΣ ΚΡΙΣΗ: 18/09/2020
- ΟΡΙΜΟΣ ΕΠΤΑΜΕΛΟΥΣ ΕΠΙΤΡΟΠΗΣ: 29/09/2020
- ΥΠΟΣΤΗΡΙΞΗ ΔΙΑΤΡΙΒΗΣ: 16/03/2021
- ΒΑΘΜΟΣ ¨ΑΡΙΣΤΑ¨ ΠΑΜΨΗΦΕΙ

ΤΡΙΜΕΛΗΣ ΕΠΙΤΡΟΠΗ

1. **ΓΚΑΓΚΑΡΗ ΕΛΕΝΗ**

Επίκουρη Καθηγήτρια Ιατρικής Σχολής Αθηνών, Διευθύντρια Στοματολογικού Τμήματος Νοσοκομείου Αφροδίσιων και Δερματικών Νόσων, Ανδρέας Συγγρός, Αθήνα ,Ελλάδα (Επιβλέπον Μέλος ΔΕΠ).

2. ΘΕΟΧΑΡΙΔΗΣ ΘΕΟΧΑΡΗΣ

Καθηγητής, Διευθυντής του Εργαστηρίου Μοριακής Ανοσοφαρμακολογίας & Ανακάλυψης Φαρμάκων στην Ιατρική Σχολή Tufts, Βοστώνη, ΗΠΑ.

3. ΑΝΤΩΝΙΟΥ ΧΡΙΣΤΙΝΑ

Ομότιμη Καθηγήτρια Δερματολογικής Κλινικής, Νοσοκομείο Αφροδίσιων και Δερματικών Νόσων, Ανδρέας Συγγρός, Αθήνα, Ελλάδα.

ΕΠΤΑΜΕΛΗΣ ΕΠΙΤΡΟΠΗ

1. **ΓΚΑΓΚΑΡΗ ΕΛΕΝΗ,** ΕΠΙΚΟΥΡΗ ΚΑΘΗΓΗΤΡΙΑ ΕΚΠΑ
2. **ΘΕΟΧΑΡΙΔΗΣ ΘΕΟΧΑΡΗΣ,** ΚΑΘΗΓΗΤΗΣ TUFTS, USA
3. **ΑΝΤΩΝΙΟΥ ΧΡΙΣΤΙΝΑ,** ΟΜΟΤΙΜΗ ΚΑΘΗΓΗΤΡΙΑ ΕΚΠΑ
4. **ΣΤΡΑΤΗΓΟΣ ΑΛΕΞΑΝΔΡΟΣ,** ΚΑΘΗΓΗΤΗΣ ΕΚΠΑ
5. **ΝΙΚΟΛΑΙΔΟΥ ΗΛΕΚΤΡΑ,** ΑΝΑΠΛΗΡΩΤΡΙΑ ΚΑΘΗΓΗΤΡΙΑ ΕΚΠΑ
6. **ΓΡΗΓΟΡΙΟΥ ΣΤΑΜΑΤΗΣ,** ΑΝΑΠΛΗΡΩΤΗΣ ΚΑΘΗΓΗΤΗΣ ΕΚΠΑ
7. **ΒΟΥΡΟΣ ΙΩΑΝΝΗΣ,** ΚΑΘΗΓΗΤΗΣ ΑΠΘ

Το πειραματικό κομμάτι της παρούσας διατριβής πραγματοποιήθηκε εξολοκλήρου στο Εργαστήριο Μοριακής Ανοσοφαρμακολογίας και ανάπτυξης φαρμάκων, Ιατρική σχολή Πανεπιστημίου Tufts, Βοστώνη, ΗΠΑ.

Η γλώσσα συγγραφής αποφασίστηκε να είναι η Αγγλική, μετά τη σύμφωνη γνώμη της Τριμελούς Επιτροπής.

Στην οικογενειά μου,

Στους Μέντορες και Δασκάλους μου,

για την συνεχή στήριξη

και έμπνεση

«Το μυαλό δεν είναι ένα δοχείο που πρέπει να γεμίσει

αλλά μια φωτιά που πρέπει ν' ανάψει»

Πλούταρχος

ΒΙΟΓΡΑΦΙΚΟ - CV

Name: Kassiani-Iro Palaska

EDUCATION

- **2014- present**: Doctor of Philosophy (PhD) candidate, Andreas Syggros Hospital of Dermatology and Venereology, Medical School, Kapodistrian University of Athens, Greece *(Supervision: Dr Gagari).*
- **2016-2017**: International Team for Implantology Scholar, Barts and the London School of Dentistry, Queen Mary University of London, UK *(Supervision: Professor Donos).*
- **2015-2016**: Postdoctoral Research Fellow, Applied Oral Sciences, Centre for Clinical and Translational Research, Forsyth Institute, Boston, MA, USA *(Supervision Professor Van Dyke Thomas).*
- **2012-2016***:* Postdoctoral Research Fellow, Molecular Immunopharmacology and Drug Discovery Laboratory, Department of Integrative Physiology and Pathobiology, Tufts University School of Medicine, Boston, MA, USA *(Supervision Professor Theoharides Theoharis)*
- **2012-2013**: Visiting Clinical Trainee, Department of Postgraduate Periodontology, Tufts University School of Dental Medicine, Boston, MA, USA.
- **2011-2012**: Clinical Trainee, Department of Oral Pathology, Andreas Sygros Hospital of Dermatology and Venereology, Athens, Greece.
- **2008-2011**: MSc Certificate/ (36-month duration) in Periodontology - Implantology, Dental School of Aristotle University of Thessaloniki, Greece, Overall Grade 9.5/10.
- **2002-2008:** Degree of Dental Surgery (DDS), Dental School of Aristotle University of Thessaloniki, Greece. Overall Grade 7.45/10 *(Best second graduation score).*
- **2002**: Apolytirio (Upper High School Graduation Diploma) 1st Lyceum of Karditsa, Graduation with honors (98.5%) of second level education before entering the Aristotle University of Thessaloniki, through national exams.

EMPLOYMENT

- **2017-present:** Clinical Research Fellow at the Centre for Oral Clinical Research, Barts & the London School of Medicine and Dentistry, Queen Mary University, London *(Professor Donos)*
- **2017-present:** Independent Contractor Private Practice, Self-Employed Clinical Provider with practice limited to Periodontics and Implant Surgery in private practice, UK**.**
- **2011-2012***:* Clinical Associate, University of Athens, Greece. Volunteering part-time participation in the activities of the Clinic of Oral Medicine at "Andreas Syggros" Hospital, Hospital for Dermatological and Venereal Diseases. Involvement in patient management, record keeping and administration.

SKILLS

Teaching Activities

- **2017**: ITI education week hosted by the Centre of Oral Clinical Research with roles in the organisation, coordination and presentation of lectures and live surgeries (Program Co-ordinators: Professor Nikos Donos and Dr. Nikos Mardas)
- **2009-2011**: Clinical instructor of Periodontology in the Undergraduate clinics of the Aristotle University of Thessaloniki, School of Dentistry (Chair: Professor Konstantinidis Antonis)

Research Activities

2013 - present

Clinical Trials (Role: Co-investigator)

- The Efficacy of Different Surgical Modalities in the Treatment of Periodontitis. A Single-Centre Randomised Controlled Trial (Chief Investigator: Professor Nikos Donos), Centre of Oral Clinical Research, Queen Mary University of London, UK
- Radiographic peri-implant alveolar bone changes in post-menopausal osteoporotic women. Prospective case series study (Chief Investigator: Professor Nikos Donos), Centre of Oral Clinical Research, Queen Mary University of London, UK
- The use of non-invasive thermal and geometrical surface imaging on postoperative healing patterns following routine surgical procedures used for the treatment of

periodontal disease: a single centre, randomised, single-blind, parallel-group clinical trial. Centre of Oral Clinical Research, Queen Mary University of London, UK

- Investigation of the Role of Microbiota in Arthritic Inflammation in DMARD-naïve Early Arthritis Patients. Prospective, Observational, Longitudinal clinical Trial (Chief Investigator: Professor Pitzalis), Centre of Oral Clinical Research, Queen Mary University of London, UK
- The Efficacy and Safety of Chlorhexidine Gluconate Chip (PerioChip@) in Therapy of Periimplantitis. Multicenter, randomized, single blind masking, parallel, two-arm clinical study. (Chief Investigator: P Professor Nikos Donos), Centre of Oral Clinical Research, Queen Mary University of London, UK

Animal studies: (Co-investigator)

- Correlation between arthritis and periodontal bone loss. A pilot study on the murine model. Collaboration of Centre of Oral Clinical Research, Queen Mary University of London & Department of Veterinary Science University of Parma, Italy.
- The link between experimental induced periodontitis and Alzheimer's disease on a murine model (experimental animal study). (Chief Investigator: Dr Kantarci Alpdogan), The Forsyth Institute, Boston, MA, USA.

Basic research study (Principal Investigator)

- The effects of *P. gingivalis* and *E. coli* LPS on the expression of pro-inflammatory mediators in human mast cells and their relevance to periodontal disease. Molecular Immunopharmacology and drug discovery Laboratory, Tufts Medical School, Boston, MA.

AWARDS - SCHOLARSHIPS

- International Team for Implantology. Recipient of the ITI scholarship to complete 1-year fellowship program at the Royal London Hospital of Medicine and Dentistry, Department of Periodontology, QMUL, London, UK, 2017-2018, *(Professor Nikos Donos)*.
- Recipient of the Honorary 2016 Fotis Mitsis (F. Mitsis) award presented by the Hellenic Society of Periodontology, Athens, Greece. The F. Mitsis award is a prestigious honorary award given every two years to distinguished young Greek Periodontists.

- Recipient of the 2015 Honorary Award presented by the New England Hellenic Medical and Dental Society, Boston, MA. USA
- Gerondelis Scholarship (2015), Gerondelis Foundation, Boston, MA, USA
- Recipient of the award of Excellence (2011), 1st G.P.A. score over entire length of postgraduate studies in Periodontology: "Excellent" 8.78/10
- Recipient of the Honorary Award for Second Best Graduation Score, Examination Period April for the academic year 2007-08 presented by the Graduate Alumni of Aristotle University Dental School, Thessaloniki, Greece (total G.P.A. over entire length of studies: Very good 7.41/10)
- Recipient of Best Student Award at the 4th year of undergraduate studies (2005) presented by the Dental School Aristotle University, Thessaloniki (G.P.A. 8.13/10)

PUBLICATIONS

- Donos N, **Palaska I,** Calciolari E, Shirakata Y, Sculean A. Regenerative therapy of furcation involvements in animal models diagnosis and treatment of furcation-involved Teeth, Chapter 6 in Diagnosis and Treatment of Furcation-Involved Teeth
- Kantarci A, **Palaska I**, Stephens D, Crabtree L, Benincasa C, Jenkins BG, Carreras I, Dedeoglu A.Combined administration of resolvin E1 and lipoxin A4 resolves inflammation in a murine model of Alzheimer's disease. Exp Neurol. 2018 Feb; 300:111120.
- **Palaska I**., Gagari E., Theoharides TC. Differential effects of LPS from *Escherichia coli* and *Porphyromonas gingivalis* on TNF, VEGF and MCP-1 production in human LAD2 mast cells. J Biol Regul Homeost Agents. 2016 Jul-Sep; 30(3):655-664.
- **Palaska I.**, Tsaousoglou P., Vouros I., Konstantinidis A., Menexes A. Influence of placement depth and abutment connection pattern on bone remodeling around 1-stage implants: a prospective randomized controlled clinical trial. *Clinical Oral Implant Research* 2016 Feb; 27(2): e47-56.
- Papathanasiou E., **Palaska I.**, Theoharides T.C. "Stress hormones regulate periodontal inflammation." Journal of Biological Regulators and Homeostatic Agents. 2013 Jul-Sep;27(3):621-6

- **Palaska I.**, Papathanasiou E., Theoharides T.C. “Use of polyphenols in periodontal inflammation.” European Journal of Pharmacology. 2013 Nov 15; 720(1-3):77-83. Epub 2013 Oct 31.
- Alhelal M., **Palaska I.**, Panagiotidou S., Letourneau R., Theoharides T.C. “Trigeminal nerve stimulation triggers oral mast cell activation and vascular permeability.” Annals of Allergy Asthma and Immunology 2014 Jan; 112(1):40-5.
- Η επίδραση του *P. Gingivalis* λιποπολυσακχαρίτη στην έκφραση και έκκριση προφλεγμονωδών διαμεσολαβητών από ανθρώπινα μαστοκύτταρα και η συνάφειά του με την περιοδοντική φλεγμονή: μελέτη *in vitro.*
  Ηρώ Παλάσκα, Ελένη Γκαγκάρη, Θεοχάρης Κ. Θεοχαρίδης. Περιοδοντολογικά Ανάλεκτα Τόμος 25 (2016).

MEMBERSHIPS

- General Dental Council, UK; membership number: GDC No. 266254
- International Team for Implantology
- Hellenic Society of Periodontology
- International Association for Dental Research
- Dental Association of Karditsa, Greece

LANGUAGES

- Greek (Native)
- English (Fluent)
- French

ΠΕΡΙΛΗΨΗ

Ανασκόπηση: Ως Περιοδοντική νόσος χαρακτηρίζεται η φλεγμονώδης αντίδραση των στηρικτικών περιοδοντικών ιστών, λόγω της συσσώρευσης μικροβιακής πλάκας, που έχει ως αποτέλεσμα την προοδευτική απώλεια πρόσφυσης και απώλεια φατνιακού οστού. Η βακτηριακή συσσώρευση και ο σχηματισμός πλάκας είναι απαραίτητες για την έναρξη της

νόσου. Ένα απο τα σημαντικότερα περιοπαθογόνα, που εμπλέκονται στην εξέλιξη της περιοδοντικής νόσου, είναι ο *Porphyromonas gingivalis* (*P. gingivalis*) του οποίου συστατικά του κυτταρικού του τοιχώματος, όπως οι λιπο-πολυσακχαρίτες (LPS), μπορεί να προκαλέσουν ενεργοποίηση του ανοσοποιητικού μηχανισμού μέσω συγκεκριμένων υποδοχέων στα αμυντικά κυταρρα, τους Toll-like receptors (TLR). Η διασταυρούμενη ομιλία μεταξύ μικροβιακής προσβολής και ανοσο-ανταπόκρισης προκαλείται από κυτοκίνες/χημειοκίνες που εκκρίνονται τοπικά από τα κύτταρα-ξενιστές. Στους σημαντικότερους μεσολαβητές που υπαγορεύουν το ρυθμό και την εξέλιξη της περιοδοντικής καταστροφής μέσω της ενεργοποίησης φλεγμονώδουςς αντίδρασης ανήκει ο παράγοντας νέκρωσης των όγκων (TNF-α), ο αυξητικός αγγειακός ενδοθηλιακός παράγοντας (VEGF) και η χημειοτακτική πρωτείνη των μονοκυττάρων (MCP-1). Πολλές αναφορές, κατά τη διάρκεια των τελευταίων 10 ετών, έχουν ρίξει φως στο ρόλο συγκεκριμένων κυττάρων που διαμένουν στον συνδετικό ιστό των ούλων και εμφανίζουν σημαντική συνεισφορά στην φλεγμονώδη απόκριση κατά την περιοδοντική νόσο, τα μαστοκύτταρα (MCs). Τα μαστοκύτταρα εμπλέκονται σε πολλές δραστηριότητες, από τον έλεγχο του αγγειακού συστήματος σε βλάβη ή επιδιόρθωση των ιστών, στην αλλεργική φλεγμονή μέχρι και στην άμυνα του ξενιστή. Η σημαντική συμβολή των μεσολαβητών των κυττάρων αυτών, στην διάδοση της φλεγμονώδους απόκρισης καθιστούν τον έλεγχο της δραστικότητας τους, ζωτικής σημασίας για τη διαχείριση πολλών φλεγμονωδών ασθενειών. Η αύξηση των μαστοκυττάρων *in vivo* στις περιοδοντικές βλάβες, ενισχύει την άποψη της συμμετοχής τους, στους πιθανούς μηχανισμούς άμυνας ή/και καταστροφής κατά την διάρκεια της περιοδοντικής φλεγμονής. Ακόμη και εάν πολλές μελέτες έχουν διεξαχθεί σχετικά με τον ποιοτικό και ποσοτικό καθορισμό των μαστοκυττάρων σε υγιείς ή περιοδοντικά προσβεβλημένους ιστούς, ελάχιστες μελέτες έχουν γίνει με σκοπό την μελετή της επίδρασης των περιοπαθογόνων βακτηρίων (όπως *P. gingivalis*) στην διέγερση και δράση των ανθρωπίνων μαστοκυττάρων.

Σκοπός: Η παρούσα ερευνητική εργασία είχε ως σκοπό να μελετήσει την επίδραση του *P. gingivalis* LPS, στην διέγερση ανθρωπίνων μαστοκυττάρων, (κυτταρικής σειράς LAD2) και την παραγωγή μεσολαβητών της φλεγμονής. Συγκεκριμένα μελετήθηκε, τόσο σε επίπεδο mRNA όσο και σε πρωτεινικό επίπεδο, η έκφραση και απελευθέρωση του παράγοντα νέκρωσης των όγκων (TNF-α), του αγγειακού ενδοθηλιακού αυξητικού παράγοντα (VEGF)

και της χημειοτακτικής πρωτεΐνης των μονοκυττάρων (MCP-1) από τα ανθρώπινα διεγειρμένα μαστοκύτταρα. Οι συγκεκριμένοι μεσολαβητές επιλέχθηκαν, καθώς έχουν ενοχοποιηθεί για τον ρόλο τους στην έναρξη και επιδείνωση της περιοδοντικής νόσου. Επιπρόσθετα, μελετήθηκε η ύπαρξη ή όχι, διαφοροποιημένης διέγερσης των κυττάρων και κατ'επέκταση παραγωγή των μεσολαβητών, υπό την επίδραση ιδίων συγκεντρώσεων λιποπολυσακχαριτών προερχόμενοι απο *P. gingivalis* και *E. Coli* βακτήρια. Επιπλέον, μελετήκε εάν και μέσω ποιού TLR υποδοχέα, έγινε η διέγερση των ανθρωπίνων μαστοκυττάρων απο το κάθε λιποπολυσακχαρίτη και συγκεκριμένα διερευνήθηκε ο λειτουργικός ρόλος των TLR4 και TLR2.

Υλικά και Μέθοδοι: Τα κύτταρα που χρησιμοποιήθηκαν ηταν της κυτταρικής σειράς LAD2 (ευγενική χορηγία του Δρ. Kirshenbaum, National Institutes of Health, Bethesda) και προήλθαν από ανθρώπινα μαστοκύτταρα ασθενούς με λευχαιμική μαστοκυττάρωση. Η καλλιέργειά τους έγινε σε μέσο StemPro34 (Invitrogen), συμπληρωμένο με 100 U/mL πενικιλλίνης-στρεπτομυκίνης και 100 ng/mL rhSCF (Swedish Orphan Biovitrum AB). Τα κύτταρα διατηρήθηκαν σε θερμοκρασία 37°C σε επωαστήριο με σύστημα ύγρανσης με 5% CO_2 . Όλα τα κύτταρα χρησιμοποιήθηκαν στο στάδιο λογαριθμικής ανάπτυξής τους. Χρησιμοποιήθηκε εμπορικά διαθέσιμο παρασκεύασμα λιποπολυσακχαρίτη από *P. gingivalis* (Invivogen) και από *E. coli* 0111: B4 (Sigma-Aldrich). Τα κιτ μεθόδου sandwich ELISA για TNF-α, VEGF και MCP-1 προήλθαν από την R&D Systems. Τα ανθρώπινα μονόκλωνικά αντισώματα TLR4 και TLR2 (100μg Mab-hTLR4, Mab-hTLR2) προήλθαν από την Invivogen και οι ιχνηθέτες Taqman για TNF-α, VEGF, και MCP-1 και το Taqman Master Mix απο την Applied Biosystems. Η δοκιμή έκκρισης β-εξοζαμινιδάσης (β-hex) πραγματοποιήθηκε, χρησιμοποιώντας φθοριομετρική δοκιμή ως δείκτη της απώλειας κοκκίων απο τα μαστοκύτταρα. Τα LAD2 ($0,5 \times 10^5$) διεγέρθηκαν με LPS η ουσία P (SP) για 30 λεπτά. Η SP (2 μmol/L) χρησιμοποιήθηκε ως ουσία θετικού ελέγχου. Με σκοπό να μετρηθεί η έκκριση *de novo* των μεσολαβητών υπό εξέταση, τα κύτταρα LAD2 (0.5×10^5) διεγέρθηκαν με δύο διαφορετικές συγκεντρώσεις LPS (1 ng/ml και 1 μg/ml) για 24 ώρες. Τα κύτταρα διεγέρθηκαν επίσης με την ουσία P (SP) (2 μM) η οποία χρησίμευσε ως ουσία θετικού ελέγχου και μή διεγερμένα κύτταρα ως ομάδα αρνητικού ελέγχου. Τα υπερκείμενα υγρά συλλέχθηκαν με φυγοκέντρηση (5 λεπτά, 150 x g), αποθηκεύτηκαν σε θερμοκρασία 20° C και υποβλήθηκαν σε δοκιμή για TNF-α, VEGF και MCP-1, χρησιμοποιώντας κιτ

ενζυμικής δοκιμής ανοσοπροσρόφησης ELISA. Για την έκφραση σε επίπεδο mRNA, τα LAD2 διεγέρθηκαν είτε με SP (2 μM) ή με LPS από *P. gingivalis* και *E. coli* (1 ng/mL) για 6 ώρες. Το συνολικό mRNA εξάχθηκε με κιτ RNeasy Mini. Χρησιμοποιήθηκε κιτ σύνθεσης cDNA iScript για την αντίστροφη μεταγραφή κάθε δείγματος. Η ποσοτική qPCR πραγματικού χρόνου ανιχνεύθηκε με δοκιμές γονιδιακής έκφρασης TaqMan για TNF-α (Hs99999043_m1), MCP-1 (Hs00234140_m1) και VEGF (Hs00900055_m1). Η γονιδιακή έκφραση mRNA κανονικοποιήθηκε σε ενδογενή έλεγχο του ανθρώπινου γονιδίου GAPDH. Για να αξιολογηθεί ο λειτουργικός ρόλος των TLR2 και TLR4 στην έκκριση διαμεσολαβητών, τα LAD2 επωάστηκαν με πολυκλωνικό αντίσωμα anti-TLR2 ή anti-TLR4 (2 μg/ml) για 1 ώρα πριν από τη διέγερση με LPS από *P. gingivalis* και *E. coli* (1 μg/ml). Τα υπερκείμενα υγρά συλλέχθηκαν και υποβλήθηκαν σε δοκιμή για διαμεσολαβητές με τη μέθοδο ELISA μετά από 24 ώρες. Οι ουσίες θετικού ελέγχου ήταν κύτταρα επωασμένα με LPS, αλλά χωρίς anti-TLR, ενώ οι ουσίες αρνητικού ελέγχου ήταν κύτταρα μή διεγερμένα.

Αποτελέσματα: Και τα δύο LPS που χρησιμοποιήθηκαν, δεν προκάλεσαν αποκοκκίωση των μαστοκυττάρων σε αντίθεση με την ουσία P, η οποία οδήγησε σε σταστιστικά σημαντική αποκκοκίωση των κυττάρων ($p < 0.05$). Η διέγερση των LAD2 κυττάρων με *P. gingivalis* LPS είχε ως αποτέλεσμα μια μικρή, αλλά στατιστικά σημαντική αύξηση, στην γονιδιακή έκφραση και για τους τρεις υπό μελετη μεσολαβητές της φλεγμονής ($p <0.05$). Τα ίδια αποτελέσματα παρατηρήθηκαν και για τον *E. Coli* LPS σχετικά με την έκφραση του γονιδίου TNF-α και VEGF ($p < 0.05$). Αντιθέτως, η γονιδιακή έκφραση για την MCP-1, ακόμη και αν υπήρχε μικρή αύξηση, δεν ήταν στατιστικά σημαντική από την ομάδα ελέγχου ($p > 0.05$). Όσον αφορά στα δύο διαφορετικά LPS που χρησιμοποιήθηκαν, δεν παρατηρήθηκε στατιστικά σημαντική διαφορά στην έκφραση γονιδίων. Η διέγερση των κυττάρων LAD2 με την ουσία P προκάλεσε την πιο ισχυρή αύξηση της γονιδιακής έκφρασης για όλους τους μεσολαβητές, στατιστικά σημαντική τόσο σε σχέση με την ομάδα ελέγχου όσο και με τις ομάδες LPSs. Όσον αφορά στην απελευθέρωση σε επίπεδο πρωτεΐνης, και οι δύο συγκεντρώσεις LPS που ελέγχθηκαν (1 ng / ml και 1 μg / ml) οδήγησαν σε *de novo* απελευθέρωση και των τριών μεσολαβητών που μελετήθηκαν, σε σύγκριση με τα μη διεγερμένα κύτταρα ($p < 0.05$). Τα επίπεδα απελευθέρωσης για TNF-α, VEGF και MCP-1 κινήθηκαν στα ίδια επίπεδα για τον *P. gingivalis* και για *E. coli* LPS, στην ίδια συγκέντρωση, καθώς δεν υπήρχε στατιστικά σημαντική διαφορά ($p > 0.05$). Ωστόσο, υπήρχε η τάση

υψηλότερων επιπέδων απελευθέρωσης για το *P. gingivalis* LPS και στις δύο υπό εξέταση συγκεντρώσεις σε σύγκριση με το *E. Coli* LPS. Η ύπαρξη μιας εξαρτούμενης σχέσης συγκέντρωσης - απόκρισης δεν παρατηρήθηκε και για τα δύο LPS στη μελέτη μας. Η ουσία P οδήγησε σε απελευθέρωση των TNF-α, VEGF και MCP-1 στις υψηλότερες συγκεντρώσεις, στατιστικά σημαντικές σε σχέση με την ομάδα ελέγχου και τις ομάδες LPS ($p < 0.05$). Τα επίπεδα των τριών μεσολαβητών που μελετήθηκαν, μειώθηκαν μετά την προ-θεραπεία με αντι-TLR4 και αντι-TLR2 αντισώματα και στις δύο ομάδες LPSs. Τα επίπεδα TNF-α μειώθηκαν σημαντικά στην ομάδα *P. Gingivalis*, μετά από επώαση με αντίσωμα αντι-TLR2 ενώ στην ομάδα *E. coli*, η μείωση ήταν πιο εμφανής μετά την επώαση με το αντίσωμα αντι-TLR4. Τόσο τα αντι-TLR4 όσο και τα αντι-TLR2 αντισώματα μείωσαν σημαντικά τα επίπεδα του VEGF μετά από διέγερση είτε με *P. gingivalis* είτε με *E. Coli* LPS ($p < 0.05$). Είναι ενδιαφέρον ότι η προ-επώαση κυττάρων LAD2 με αντισώματα αντι-TLR4 ή αντι-TLR2 δεν μείωσε, στατιστικά σημαντικά, τα επίπεδα MCP-1 σε καμία από τις ομάδες LPSs ($p > 0.05$).

Συμπεράσματα: Η παρούσα ερευνητική προσπάθεια δείχνει για πρώτη φορά ότι ο LPS από το κυριότερο περιοπαθογόνο βακτήριο, *P. gingivalis*, επιλεκτικά, χωρίς αποκοκκίωση, διεγείρει τα μαστοκύττρα της σειράς LAD2, τόσο σε γονιδιακό όσο και σε πρωτεινικό επίπεδο για την απελευθέρωση TNF-α, VEGF και MCP-1, μεσολαβητές που έχουν τεκμηριωθεί ότι παίζουν σημαντικό ρόλο στην έναρξη και την εξέλιξη της περιοδοντικής νόσου. Επιπρόσθετα, στην παρούσα μελέτη, δεν παρατηρήθηκε διαφορά του *P. gingivalis* LPS σε σύγκριση με το *E. coli* LPS στην ικανότητα διέγερσης και παραγωγής κυτοκινών. Επιπλέον, τεκμηριώσαμε ότι ο *P. gingivalis* LPS μπορεί να λειτουργεί τόσο μέσω του TLR2 όσο και του TLR4 στην συγκεκριμένη κυτταρική σειρά. Στη μελέτη μας χρησιμοποιήσαμε την ουσία P ως ουσία θετικού ελέγχου για όλα τα πειράματα. Η ουσία P είναι ένα νευροπεπτίδιο που εμπλέκεται στη νευρογενή φλεγμονή και είναι ένας σημαντικός νευρο-διαμεσολαβητής. Πρόσφατα, το νευρικό σύστημα έχει αναγνωριστεί ως κρίσιμος ρυθμιστής της φλεγμονής και στις περιοδοντικές νόσους. Τα μαστοκύτταρα είναι παρόντα στο ανθρώπινο σώμα και διαδραματίζουν σημαντικό ρόλο τόσο στις αλλεργικές αντιδράσεις όσο και σε φλεγμονώδεις διεργασίες, ιδιαίτερα εκείνες που επιδεινώνονται λόγω άγχους, όπως η ρευματοειδής αρθρίτιδα, η ατοπική δερματίτιδα, η πολλαπλή σκλήρυνση, η ψωρίαση και η περιοδοντική φλεγμονή. Η αναστολή της ενεργοποίησης των

μαστοκυττάρων μπορεί να οδηγήσει σε νέους θεραπευτικούς στόχους για τον έλεγχο της φλεγμονώδους αντίδρασης στους περιοδοντικούς ιστούς.

ABSTRACT

Background: Periodontal diseases are a group of common chronic infectious diseases associated with pathogenic microorganisms forming the dental biofilm, affecting the supporting structures of teeth, and leading to their progressive destruction and tooth loss. Periodontitis is the 6th most prevalent disease in the world and the primary cause for tooth loss in adults. Recently, the role of the immune system in the progression of periodontitis was highlighted, indicating that bacterial antigens can trigger an immunopathologic reaction and that the host response is an important factor in determining the extent and severity of the disease. Periodontal tissue breakdown is a result of the complex interplay between the pathogenic bacteria forming the biofilm and the host's immune responses. *Porphyromonas gingivalis (P. gingivalis*) is the species most highly associated with the chronic form of periodontitis and can be detected in up to 85% of the diseased sites. The major virulence factors produced by *P. gingivalis* are its lipopolysaccharides (LPS). LPS is an outer membrane component recognized by pattern recognition receptors on immune cells and specifically by the toll like receptors (TLR). TLR4 have been identified and characterized as the main pathogen sensor against LPS of most Gram-negative bacteria. During the past decade, the importance of mast cells (MCs) in the defense mechanism against bacterial infections has been increasingly recognized. MCs are multifunctional, immune system secretory cells, well known for their involvement in a wide variety of physiological and pathological processes, including mainly allergic and anaphylactic reactions, through the release of inflammatory mediators. Increasing evidence indicates that MCs are also critical for the pathogenesis of inflammatory diseases such as arthritis, atopic dermatitis, psoriasis, multiple sclerosis, and oral inflammation including periodontal diseases. The increase of MCs numbers, *in vivo*, in periodontally affected tissues, has drawn attention with respect to the possible participation of MCs in the defense mechanism and destructive events in periodontal disease.

Objectives: This *in vitro* study was designed to a) examine the effects of *P. gingivalis* LPS on the expression and release of inflammatory mediators from the LAD2 human MC line b) to compare the differential effects of *P. gingivalis* and *E. coli* LPS on the expression and release of these mediators from human MCs and c) to assess the functional role of TLR2 and TLR4 in mediator release from MCs stimulated with either LPS. . The mediators selected were the tumor necrosis factor (TNF-α), the vascular endothelial growth factor (VEGF) and the

monocyte chemoattractant protein (MCP-1) as they have been documented to be significantly implicated in the initiation and progression of periodontal disease,

Materials and Methods: LAD2 MCs (kindly supplied by Dr. Kirshenbaum, National Institute of Health, Bethesda, Maryland) derived from a human MC leukemic patient were cultured in StemPro-34 medium supplemented with 100 U/mL of penicillin–streptomycin and 100 ng/mL of Recombinant human stem cell factor (rhSCF). All cells were used during their logarithmic growth period. Substance P (SP) was diluted in Milli-Q water, and a stock solution (10mM) was prepared. Commercially available preparation of *P. gingivalis* LPS (Invivogen, CA) and *E. coli* 0111: B4 LPS (Sigma-Aldrich) was used. Bacterial LPS was purified by the supplier to be free from contaminating lipoproteins. SP and LPS were dissolved in sterile distilled water. ELISA kits for TNF-α, VEGF and MCP-1 were obtained from R&D Systems. Purified monoclonal antibodies to human TLR4 and TLR2 (100µg Mab-hTRL4, Mab-hTRL2) were obtained from Invivogen. TNF-α, VEGF, and MCP-1 Taqman probes and Taqman Master Mix were purchased from Applied Biosystems. Beta-hexosaminidase (β-hex) release was assayed as an index of MC degranulation. LAD2 cells (0.5×10^5) were stimulated with LPS (1 µg/ml) and SP (2 µmol/L) for 30 min. SP was used as the positive control and medium alone as the negative control. Results were expressed as the percentage of β-hex released over the total amount present in LAD2 cells. In order to examine the mediator expression on mRNA level, LAD2 cells were stimulated with SP (2 µM), *P. gingivalis* and *E. coli* LPS (1 ng/mL) for 6 h. Total mRNA was extracted with a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Relative mRNA abundance was determined from standard curves run within each experiment. An iScript cDNA synthesis kit (Bio-Rad Laboratories) was used for reverse transcription of each sample. Quantitative real-time PCR was performed with TaqMan gene expression assays (Applied Biosystem) for TNF (Hs99999043_m1), MCP-1 (Hs00234140_m1) and VEGF (Hs00900055_m1). Samples were run at 45 cycles by using a real-time PCR system (7300, Applied Biosystems). The mRNA gene expression was normalized to human GAPDH endogenous control (Applied Biosystems). To measure *de novo*–synthesized cytokine/chemokine release, LAD2 cells (1×10^5) were stimulated for 24 h, with a minimum and a maximum concentration of LPS (1 ng/ml and 1 µg/ml respectively). The cells were also stimulated with either SP (2 µM) serving as the positive control or media alone serving as the negative control for 24h. The supernatant fluids were collected by

centrifugation (5 min, 150 x g), stored at –20° and assayed for TNF-α, VEGF and MCP-1 release using enzyme-linked immunosorbent assay (ELISA) kits according to the protocol suggested by the manufacturer (R&D Systems). To assess the functional role of TLR2 and TLR4, MCs were incubated with either anti-TLR2 or anti-TLR4 polyclonal antibody (2 μg/ml) for 1 h before stimulation with *P. gingivalis* or *E. coli* LPS (1 μg/ml). Supernatants fluids were collected and assayed for mediators by Elisa after 24 h. The positive controls were MCs incubated with LPS, but without anti-TLRs, whereas the negative controls were unstimulated MCs. All experiments were performed in triplicates and repeated at least 3 times (n=3). The results are presented as means ± Standard Deviation. Data between different treatment groups were analyzed by using the unpaired 2-tailed Student's t test (GraphPad Prism 6). Mean values of the parameters were tested by means of the least significant difference test at significance level $p < 0.05$.

Results: Both LPSs used did not induce MCs degranulation in contrast to SP ($P<0.05$). The stimulation of LAD2 cells with P. *gingivalis* LPS resulted in a small, but statistically significant increase, in gene expression for all the three mediators *($p<0.05$).* The same results were observed for *E. coli* LPS regarding the TNF-α and VEGF gene expression *($p<0.05$).* On the contrary, for MCP-1 mRNA expression, even if there was a small increase, it was not statistically significant from the control group *($p>0.05$).* Regarding the two different LPSs used, no statistically significant difference in gene expression was observed. The stimulation of LAD2 cells with SP induced the most potent increase in gene expression for all mediators that was statistically significant from both the Control and the LPSs groups. Regarding the release on a protein level, both LPS concentrations tested (1 ng/ml, 1 μg/ml) led to *de novo* release of all the three mediators studied, compared to unstimulated cells (*$p<0.05$*). *P. gingivalis* LPS and *E. coli* LPS, at the same concentration, triggered approximately, the same amount of *de novo* release of TNF-α, VEGF and MCP-1, as there was no statistically significant difference (*$p>0.05$*). However, there was a tendency of higher levels of mediator release for *P. gingivalis* LPS in both tested concentrations compared to *E. coli* LPS. A dose response relationship could not be documented for both LPSs in our study. The release of TNF-α, VEGF and MCP-1 reached the highest concentrations, which were statistically significant in relation to the control and the LPSs Groups ($p<0.001$) when stimulated with SP *($p<0.05$).* The levels of the three mediators studied, were reduced after pre-treatment with anti-TLR4 and

anti-TLR2 antibodies in both LPSs Groups. TNF-α levels were significantly reduced in the *P. gingivalis* Group, after incubation with anti-TLR2 antibody whereas in the *E. coli* Group, the reduction was more prominent after incubation with the anti-TLR4 antibody. Both anti-TLR4 and anti-TLR2 antibodies significantly reduced VEGF levels after stimulation with either *P. gingivalis* or *E. coli* LPS *($p<0.05$).* Interestingly, pre-incubation of LAD2 cells with either anti-TLR4 or anti-TLR2 antibodies did not statistically significantly reduce the levels of MCP-1 in either of the LPSs Groups *($p>0.05$).*

Conclusions: The increase of MCs in periodontally affected tissues, has called attention with respect to the possible participation of MCs in the defense mechanism and destructive events in periodontal inflammation. Overall, we reported for the first time, that LPS from *P. gingivalis* selectively, i.e. without degranulation, stimulated MCs to generate and release mediators that have been documented to play an important role in the initiation and progression of periodontal disease, with the same potency as *E. coli* LPS, TNF-α, VEGF and MCP-1. In addition, we documented that *P. gingivalis* LPS could function through both TLR2 and TLR4 in MCs. It is undisputable that MCs play a crucial role in the development of inflammation during many pathological processes including allergic reactions as well as during bacterial infection. Therefore, our findings could indicate that MCs might be involved in the emergence of inflammatory processes evolved in response to *P. gingivalis* infection such as periodontal disease. In addition, the recent identification of a neurogenic component in periodontal disease and the existence of spatial interactions between nerves and MCs in a neural-immune network with the significant effect of SP on MCs, that was documented in our study as well, opens new possibilities for altering the function of these critical immune cells. In the future, it may be possible to develop novel approaches that influence the release of inflammatory molecules and neuropeptides to ameliorate MCs driven inflammation including periodontal disease.

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SPECIFIC AIMS

LITERATURE REVIEW

(ΓΕΝΙΚΟ ΜΕΡΟΣ)

1. BACKGROUND

Periodontal diseases are common chronic infectious diseases associated with pathogenic microorganisms forming the dental biofilm, that is implicated as the primary etiologic factor in their initiation, affecting the supporting structures of teeth and leading to progressive destruction and tooth loss (Uzel et al. 2011). Although more than 700 bacterial species can

colonize the oral cavity, only a handful of those are highly implicated in the periodontal disease (Aas et al. 2005, Paster et al. 2006).

Porphyromonas gingivalis (P. gingivalis) is a periodontopathogen bacteria that has been documented to be strongly associated with periodontal inflammation as it is present in up to 85% of the disease sites (Yang et al. 2004). The presence of *P. gingivalis* in a periodontal pocket may predict disease progression and a significant positive correlation is found between *P. gingivalis* numbers and pocket depths (van Winkelhoff et al. 2002, Kawada et al. 2004). The major virulence factors produced by *P. gingivalis* are its lipopolysaccharides (LPS). LPS is an outer membrane component recognized by the host that can trigger intracellular signalling events resulting in pro-inflammatory cytokine production, leading to chronic inflammation, destruction of the alveolar bone, and eventually tooth loss (Pathirana et al. 2010).

The role of the immune system in the evolution of periodontitis was highlighted, indicating that bacterial antigens can trigger an immunopathologic reaction and that the host response is an important factor in determining the extent and severity of the disease (Kirkwood et al. 2009, Mombelli et al. 2018)). The host response in periodontal inflammation, has traditionally been mediated mainly by neutrophils, monocytes/ macrophages, B and T lymphocytes. These are triggered to produce inflammatory mediators, including cytokines, chemokines, arachidonic acid metabolites and proteolytic enzymes, which collectively contribute to tissue degradation and bone resorption by activation of several distinct host degradative pathways (Birkedal-Hansen 1993, Hernandez et al. 2011). The increased number of mast cells (MCs) in periodontally affected tissues have shed new light on the possible implication of these cells in periodontal inflammation as they have been detected in both healthy and inflamed gingiva, in different numbers at various sites (Arzi et al. 2010).

MCs are multifunctional immune system secretory cells, well known for their involvement in allergic and anaphylactic reactions (Theoharides et al. 2012). They also participate in the regulation of the immune response by the release of chemical mediators, against inappropriate stimulus. Many reports have, during the last 15 years, focused on the MCs as pivotal cells in both innate and acquired immunity and in wound-healing processes (Theoharides et al. 2019). There is an increased awareness of the potential interactions between MCs and other components of the immune response, contributing to the

modulation of humoral and cellular events in host defence mechanisms against bacterial infection. Recent findings implicate MCs in inflammatory diseases as they are capable of responding to different stimuli, participating in a wide variety of physiological and pathological processes, and are responsible for the synthesis and release of numerous pharmacologically active products (Theoharides et al. 2007, 2012).

Taking the above into consideration, MCs can participate in the pathogenesis of periodontal disease as well. Even if a lot of studies have been conducted concerning the number of MCs in healthy or periodontally affected gingival tissues, little is known about the effect of periodontally involved Gram-negative bacteria (such as *P. gingivalis*) on MCs stimulation.

2. PERIODONTAL DISEASES

2.1. Introduction

Periodontal diseases include a wide variety of chronic multifactorial inflammatory conditions associated with dental plaque biofilms that affect the periodontal supporting structures including gingiva, periodontal ligament, and alveolar bone (Highfield 2009, Caton et al. 2018, Papapanou et al. 2018). The most common periodontal diseases, namely plaque-induced

gingivitis and chronic periodontitis, are infectious in nature and elicit an inflammatory response, clinically characterized by local redness, edema, bleeding on probing upon accumulation of bacterial plaque and formation of periodontal pockets (*Image 1*).

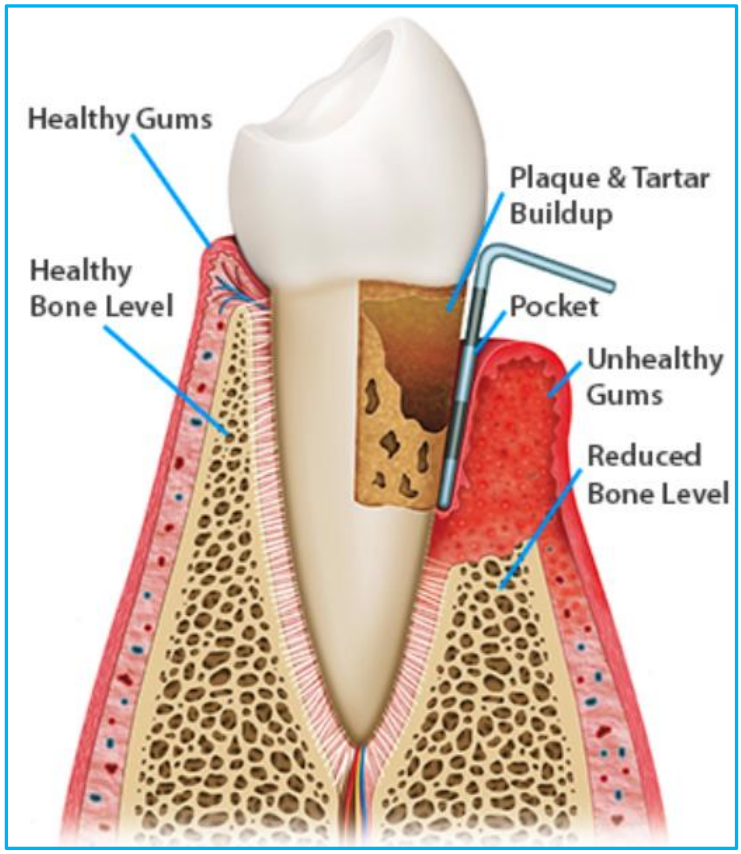


***Image 1**:* Development of gingivitis and periodontitis. Following dental plaque accumulation, the gingivitis lesion develops into a periodontitis lesion, which is characterized by formation of a pathogenic periodontal pocket, bone loss and destruction of periodontal tissues. (*Pathogenesis of Periodontal Disease by José Luis Muñoz-Carrillo, June 2019, open access chapter*)

Gingivitis is a reversible inflammatory reaction of the marginal gingival tissues to plaque accumulation, whereas periodontitis is a destructive, non-reversible condition resulting in bone loss, which ultimately leads to tooth loss, although it can be preventable and treatable in the majority of cases (Tonetti et al. 2005). The primary features of chronic periodontitis include the loss of periodontal tissue support, clinically assessed by attachment loss (CAL) presence of periodontal pockets and bleeding on probing and radiographically by alveolar bone loss, (Papapanou et al. 2018). Existing evidence indicates that gingivitis precedes the onset of periodontitis; however, not all gingivitis cases will proceed to periodontitis. The

reason for this is that accumulation of plaque bacteria is necessary but not sufficient by itself for the development of periodontitis: a susceptible host is also necessary (Hajishengallis 2014).

Periodontitis is a complex disease with multiple risk factors, some with their basis in genetics, some caused by epigenetic influences and others that are modifiable because they relate to patient behaviour, medications or environmental factors, all of which conspire to establish and propagate the periodontitis lesion. In addition to such 'patient-specific' risk factors, there are also 'site-specific characteristics which may favour the development of a periodontal lesion (Lang & Tonetti 2015).

Periodontal disease has dynamic states of exacerbation and remission and is characterized by episodes of activity, followed by periods of relative quiescence (Goodson et al. 1982). Longitudinal studies have indicated that progression of periodontitis, in terms of loss of periodontal attachment, is infrequent and episodic, and most progression occurs in smaller portion of highly susceptible individuals (Socransky et al. 1984, Lindhe et al. 1989, Hugosan & Laurell 2000).

2.2. Prevalence of Periodontal Disease

Periodontal disease is probably the most common disease in humans (Guinness World Records 2001). According to a detailed report by the World Health Organization, periodontitis leading to tooth loss affects 5–15% of most populations worldwide (Armitage 2004). The recent Global Burden of Disease Study (GBD, 1990-2010) indicated that: a) periodontitis is the 6th most prevalent disease, with an overall prevalence of 11.2% and b) the global burden of periodontal disease increased by 57.3% from 1990 to 2010 (Marcenes

et al. 2013, Kassebaum et al. 2014, Jin et al. 2016). The overall prevalence of periodontitis increases with age, and the incidence rises in adults aged 30 to 40 years (Kassebaum et al. 2014, Tonetti et al. 2017, Jepsen et al 2018).

In addition, it is now well documented that periodontal disease can affect the rest of the body by the haematogenous dissemination of both bacteria and their products originating in the dental biofilm (Hajishengallis et al. 2018). Considerable evidence points out that periodontitis has a bidirectional relationship with systemic diseases such as diabetes, atherosclerosis, rheumatoid arthritis, and pulmonary infections (Hasturk et al. 2015). The systemic inflammatory burden consequent to severe periodontitis may contribute to the pathogenesis of chronic non-communicable diseases (Jepsen et al. 2018).

Periodontal disease is a major public health problem due to its high prevalence, and since it may lead to tooth loss and disability, it significantly impairs quality of life. Preservation of periodontal health is a key component of oral and overall health and as such is a fundamental human right (Baehni & Tonetti 2010). Recognizing that periodontal health is a critical component of overall health, advocates the need and importance of additional research to elucidate deeper, the pathogenic mechanisms of periodontal diseases and discover novel therapeutic targets for the successful management of periodontitis.

2.3. Etiology of Periodontal Disease

2.3.1. Dental Bacterial Biofilm

For decades, periodontitis has been considered to be caused by specific bacteria or groups of bacteria. Classical experiments have demonstrated that inadequate oral hygiene and subsequent accumulation of bacterial plaque triggers host inflammatory responses in associated gingival tissues that play a major role in the pathological alterations and

ultimately destruction of periodontal structures (Loe et al. 1965). As a result, treatment protocols for periodontitis have been mainly “anti-infective” aiming at the reduction of the levels of pathogenic bacteria in periodontal pockets (Bartold et al. 2013).

The bacterial aetiology of periodontal disease has been explored for over 100 years, evolving along with technologic advances in bacteria identification and characterization. Although early studies indicated that periodontal disease occurred in response to plaque mass (non-specific plaque hypothesis), current thinking implicates specific microbial species in disease causation (specific plaque hypothesis) (Loesche et al. 1976, 2001). The bacterial aetiology of periodontal disease is strongly supported by clinical studies that have reported that mechanical and chemical antibacterial treatment can prevent or treat gingivitis and periodontitis (Theilade et al. 1996). The identification of specific causative species, or periodontopathogens, has been hampered by some of the unique features of periodontal diseases. The foremost of these features is that disease occurs in a site already colonized by a bacterial population. Thus, the ecologic plaque hypothesis supported by Marsh et al. 1990, unified the existing theories on the role of dental plaque in periodontal disease, presenting periodontitis as an “ecological catastrophe”. Both, the total amount of plaque (non-specific) and the specific microbial composition of plaque (specific) may contribute to the transition from health to disease Thus, host response can be disturbed by excessive accumulation of plaque, plaque independent host factors (immune disorder, hormonal changes), or environmental factors (smoking, diet).

Many methods have been used to study the composition of plaque bacteria (Socransky & Haffajee 2005). Initial studies were based on cultivation and microscopic visualization. Research studies, using detection systems based on specific antibodies, were useful in determining the presence and levels of species of interest (Laurenco et al. 2014). With the advent of molecular methods, however, the diversity of the oral flora has been extensively explored (Kuramitsu et al. 2007, Peters et al 2012). Studies have revealed that over half of the plaque accumulated is composed of heretofore uncultivated species. Culture-based studies have not been able to explore the diversity of this polymicrobial infection. Thus, it is possible that as-yet-undetected species are responsible for periodontal disease (Socransky et al. 1998).

Dental bacterial biofilm has been shown to be the primary etiological factor of periodontal disease (Haffajee et al. 1994, Socransky et al. 2005). The dental biofilm is an organised bacterial community which forms when a solid structure is placed in an aqueous environment. In the oral cavity, the solid surfaces are either teeth or restorative materials. Dental biofilms differ from biofilms on mucosal surfaces as they form on non-shedding surfaces, so stable communities can therefore become established. The gingival crevice and the periodontal pocket may harbor several hundred different bacterial taxa (Paster et al. 2001). Studies that investigated the periodontal microbiota have demonstrated that they are comprised by many different species (Socransky et al. 1998, Cugini et al. 2000, Madianos et al. 2005). Most of those taxa seem to be host-compatible species and only a subset of that consortium may contribute directly to the pathogenesis of periodontal disease (Socransky et al. 1998).

The biofilm is formed by more than 300 different bacterial species and their cell wall components can trigger immune activation. The most common forms of periodontitis are associated with large numbers of bacteria (up to and possibly exceeding 108 per site) living on or adjacent to the tooth root surface. *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia* (*T. forsythia*), collectively known as the red complex, were identified as major players in the initiation and progression of periodontitis (Socransky et al. 1988, Socransky et al. 2005). In fact, in 1996, *P.gingivalis* and *T. forsythia* were classified as periodontal pathogens by the American Academy of Periodontology, due to the extensive body of evidence available.

Subgingival biofilms exhibit organizational and structural features of communities and individual species often exhibit collaborative strategies for survival and communication (Zijnge et al. 2012). Bacterial behavior in biofilms is markedly different from bacteria in the planktonic state as pathogenicity and virulence are often markedly increased. Some of the properties of the biofilm include a) cell to cell communication, b) gene transfer, c) antimicrobial resistance, and d) regulation of gene expression (Tatakis et al. 2005). This explains the fact that the prevention and treatment of periodontitis by antibiotics is often hampered because of the shielding effect of the biofilm surface layers that enhance the resistance of bacteria to locally or systemically administered antibiotics (Ardila et al. 2010, Leszczyńska et al. 2011).

Interestingly, the presence of pathogens *per se* is required, but is not sufficient for disease initiation (Graves 2008). Periodontitis-associated microbial communities show synergistic interactions for enhanced colonization, nutrient procurement, and persistence in an inflammatory environment. These pathogens transform the normally symbiotic microbiota into a dysbiotic state that triggers a destructive change in the normally homeostatic host-microbial interplay in the periodontium. This destructive pattern was characterized by Hatjishengalis et al. (2014) as the Polymicrobial synergy and dysbiosis model (PSD).

The major component of soft- and hard- tissue destruction associated with periodontal disease is the result of activation of the host's immune-inflammatory response to the bacterial challenge (Salvi et al. 2005). Over the past two decades, there has been increased interest to investigate the molecular mechanisms of the host response underlying the periodontal tissue destruction and identify several host factors as possible diagnostic and therapeutic targets for the management of the periodontal inflammation.

2.4. Pathogenesis of Periodontal Disease

2.4.1. Host-microbe Homeostasis in Health and Disease

The pathogenesis of periodontitis has been gradually elucidated during the latter half of the 20th century. In the 1960s and 1970s, human and animal research showed that bacteria played a critical role in initiating gingivitis and periodontitis (Pihlstrom et al. 2005, Kornman

et al. 2008). Leading up to the 1980s, there were further advances within the field, and the pivotal role of the host inflammatory response in disease progression began to emerge (Ranney et al. 1991). The importance of hereditary factors was subsequently demonstrated in several studies, including those comparing monozygotic and dizygotic twins (Michalowicz et al. 2000). Systemic conditions and environmental factors such as smoking, and stress were also shown to greatly affect the disease onset and progression (Madianos et al. 2005).

The healthy oral cavity has a very specific microenvironment where millions of bacteria can live in harmony with the host defence mechanisms. Bacterial species co-evolve with the host resulting in a finely balanced system and health is preserved provided this bacterial–host balance is maintained by controlling the amount of bacterial load through regular oral hygiene practices (Marsh & Devine 2011). This balance, however, can be disturbed by either quantitative (higher bacterial load) or qualitative (growth of pathogenic species) changes in the biofilm involved in the pathways from health to disease and/or changes in the host defences resulting in the development of disease (Terheyden et al. 2014).

We now recognize that a pathogenic biofilm is a prerequisite for periodontitis to develop but, is insufficient to cause the disease (Hajishengalis et al. 2014). Periodontitis results from complex interactions between the biofilm and the inflammatory immune response, and it is the latter that is estimated to account for almost 80% of periodontal tissue damage (Grossi et al. 1994). The periodontitis phenotype is characterized by an exaggerated, yet poorly effective and non-resolving, inflammation of the connective tissues supporting the teeth that leads to tissue destruction, rather than a specifically targeted, effective, and self-resolving inflammatory immune response (Kinane et al. 2007, 2009).

2.4.2. Histopathology of the Periodontal Lesion

The initial inflammation in the periodontal tissues should be considered a physiologic defence mechanism against the microbial challenge, rather than pathology. The clinical findings of the disease at this stage include supragingival and subgingival plaque formation, which are usually accompanied by calculus formation and gingival inflammation (Page 1986).

If plaque is removed, there is resolution with return to homeostasis; if the lesion persists, it becomes pathology (Kinane et al. 2000). There are four well-known stages of gingivitis and periodontitis, described by Page & Schroeder, in 1976: the initial lesion, the early lesion, the established lesion, and the advanced lesion (Page & Schroeder 1976).

The initial lesion is the response of resident leukocytes and endothelial cells to the bacterial biofilm. At this stage, there are no signs of clinical inflammation, but the changes in the tissues can be observed histologically (Ekstein et al. 1993). The metabolic products of bacteria trigger junctional epithelium cells to produce cytokines and stimulate neutrons to produce neuropeptides, which cause vasodilatation of local blood vessels. Neutrophils leave the vessel and migrate toward the site of inflammation in response to chemokines (Cekici et al. 2014). The early lesion follows, with increased numbers of neutrophils in the connective tissue and the appearance of macrophages, lymphocytes, plasma cells and MCs. Complement proteins are activated as well. The epithelium proliferates to form rete pegs, observed histologically, and clinical signs of gingival inflammation, such as bleeding, can be seen. Gingival crevice fluid (GCF) flow is increased. The following stage is the established lesion. This can be considered as the period of transition from the innate immune response to the acquired immune response. Macrophages, plasma cells, and T and B lymphocytes are dominant, with IgG1 and IgG3 subclasses of B lymphocytes also present. Blood flow is impaired, and the collagenolytic activity is increased. There is also increased collagen production by fibroblasts. Clinically, this stage is a moderate to severe gingivitis with gingival bleeding and colour and contour changes. The final stage is the transition to periodontitis, the advanced lesion. Irreversible attachment loss and bone loss are observed histologically and clinically. The inflammatory lesion extends deeper, affecting the alveolar bone leading, if left untreated, to tooth loss (Hajishengallis et al. 2007).

2.4.3. The Role of Innate and Adaptive immunity in Periodontal Disease

Our body's immune system comprises multiple cell-types whose primary function is to fight invading oral pathogens to protect and maintain immune homeostasis. The innate immune system that is by nature non-adaptive, provides the most immediate (and often a completely

sufficient) response when cells encounter pathogens, and the adaptive immune system provides a delayed (but typically effective) sustained immunity (Berglundh & Donati 2005).

The role of innate immune cells is generally to detect microbes and maintain host microbial immune homeostasis and to induce antimicrobial defence mechanisms. Innate immune cells like epithelial cells, fibroblasts, dendritic cells, macrophages, and neutrophils act as the first line of defence against invading pathogens (Cekici et al. 2014). One of the most critical pathways in the initiation of the immune response is the recognition of the microbe-associated molecular patterns (MAMPs) by the pattern recognition receptors (PRRs) on the host cells (Silva et al. 2015). The innate immune system involves non-specific immune responses that require no prior exposure to the stimulus, aiming to provide immediate protection against infection. Early reactions to the bacterial challenge include secretion of vasoactive substances, like histamine and vascular endothelial growth factor (VEGF), mainly produced by MCs in the periodontium (Holgate, 2000), which increase the vascular permeability in gingival tissues. Vasodilation enhances the recruitment of phagocytic cells, like polymorphonuclear cells (PMNs), monocytes and macrophages. The periodontal tissues are infiltrated by many phagocytic cells, which activate a cascade of cellular and biochemical events in other periodontal cells by secreting several inflammatory mediators, like cytokines, chemokines, and prostaglandins (Van Dyke & Kornman 2008). These responses are intended to eliminate the microbial challenge but usually cause further periodontal tissue damage.

When the innate immunity is not able to cope with the bacterial challenge, the adaptive immune system, which is a secondary line of defence, is activated with the aim to improve the host's ability to recognize and combat the infection. The adaptive immune response is characterised by specificity and memory (Nicholson et al. 2016). Once recognition of microbial antigens has taken place by the appropriate receptor on macrophages or dendritic cells, cytokines are released which activate T and B cells, thereby engaging cell-mediated and humoral immune responses. B lymphocytes act as antigen-presenting cells (APCs), and together with dendritic cells, present the bacterial antigens to the immune-competent cells that consist of the T-cell subgroups. (Iwasaki et al. 2010, Terheyden et al. 2014). Antibodies, produced by plasma cells, aim to protect the host by enhancing the phagocytosis of the antigens by the PMNs and the macrophages, a process known as opsonization. However,

the antibodies' actions might be protective or further destructive, pending on their ability to eliminate the causative organisms (Gemmell et al. 2004).

We could say that innate and adaptive immune responses function together. The first response is predominantly innate, subsequently helping adaptive immune responses to develop. Unfortunately, the specific mechanism that connects innate to adaptive immune response is not fully understood in periodontal inflammation. The mechanisms involved in inflammation, resolution and wound healing include all compartments of the immune system that work in collaboration, even overlapping one another, to protect the periodontal tissues (Cekici et al. 2014).

2.5. Main Components of Immune Response in Periodontal Disease

The immune response in periodontal inflammation results from the interaction of resident immune cells with the dental bacterial biofilm forming and attached to the tooth surface. The inflammatory response consists of four main components: (a) the molecules named PAMPs which are derived from the bacteria forming the oral biofilm (b) the receptors on the surface of implicated immune cells that recognize these molecular patterns (PRR) such as Toll-like receptors (TLR), (c) the inflammatory mediators, such as cytokines, chemokines, growth factors and (d) the targeted immune cells implicated in periodontal inflammation (Silva et al. 2015). The inflammatory response is mainly characterized by four successive phases: (1) silent phase, where the cells synthesize and release pro-inflammatory mediators, (2) vascular phase, characterized by an increase in vascular permeability, (3) cellular phase, characterized by the infiltration of inflammatory cells at the site of infection, and (4) the resolution of the inflammatory response (Ebersole et al. 2013).

2.5.1. Pathogen-associated Molecular Patterns (PAMPs)

One of the mechanisms that the host uses to control the inflammatory response to different bacteria, is by recognition of selected bacterial structures knows as PAMPS. This concept, termed pattern recognition, was originally proposed by Janeway (Janeway CA 1992) and suggests that the host has developed receptors (termed pattern recognition receptors) that are able to recognize common structures found in various microbes. Lipopolysaccharides (LPS), peptidoglycans (PGNs), lipotechoic acids (LTA), fimbriae, proteases, heat-shock proteins (HSPs) and toxins are probably the most studied PAMPS that may be deleterious

for the host (Satoh et al. 2016). As these molecules can directly or indirectly induce periodontal destruction, it is very tempting to consider them as virulence factors but there is a difference between virulence factors and PAMPs (Wolf et al. 2018, Ugolini et al. 2019). Virulence factors are produced by pathogens to allow them to interact and survive within the host by assisting in invasion, colonization, adjusting to new nutrient sources and avoiding the ever surveillant host immune responses. On the other hand, PAMPs did not evolve to interact with the host immune system but are considered to perform essential physiologic functions for the bacteria.

LPS, a major constituent of the outer membrane of gram-negative bacteria, is the best characterized PAMP (Bainbridge et al. 2001). LPS is highly pathogenic and is capable of inducing the release of pro-inflammatory mediators and cytokines through TLR mediated activation on several host cells. By triggering intracellular signalling events, an LPS-activated cell becomes metabolically active and produces intracellular stores of oxygen, free radicals, and inflammatory mediators implicated in the process of inflammatory disease such as periodontitis (Bostanci et al. 2007, Hamedi et al. 2009).

2.5.2. Pattern Recognition Receptors (PRRs)

It is well known that innate immunity constitutes the first line of immunological defence. In the past, it was thought to involve a non-specific immune response, but lately it showed increased specificity or discrimination between host and pathogens, through a well orchestrating system based on PRRs and their related cellular signalling pathways. The role of PRRs is also critical for activation of adaptive response, at later stages of inflammatory processes. PRRs that have been identified and characterized as pathogen sensors, include TLRs, Nod-like receptors, RIG-like receptors and dectins (Medzhitov 2010). TLRs are among the most studied PRRs, due to their role in detecting various PAMPS (Medzhitov et al. 1998, Hans & Hans 2011). Recently, co-operation between different TLR and their cellular signalling pathways in recognizing oral pathogens was documented (Eskan et al. 2008, Wang et al. 2010, Song et al. 2016).

2.5.2.1. TLRs

TLR are transmembrane glycoproteins and together with the interleukin-1 receptors, form a receptor superfamily, known as the “Interleukin-1 Receptor/Toll-Like Receptor Superfamily”

(Narayanan et al. 2015). TLRs are so-called because of their similarity to the protein encoded by the Toll gene, which was identified in Drosophila in 1985 by Christiane Nüsslein-Volhard. The association of TLRs with innate immunity was recognized with the discovery of mouse TLR4, which acted as a receptor of bacterial LPS (Poltorak et al. 1998). To date, ten Toll-like receptors in humans and twelve Toll like receptors in mice have been described and are believed to detect a discrete collection of molecules of microbial origin and to signal the presence of infections (Beutler et al. 2004).

Each TLR resides in a specific part of the cell and is capable of sensing distinct PAMPs. Based on the composition of PAMPs that they recognize, TLRs can be classified into two categories. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 reside in the cell membrane and can sense PAMPs that are mainly composed of lipid or protein. In contrast, TLR3, TLR7, TLR8, and TLR9 are found in the endosomal membranes and can sense PAMPs that consist primarily of nucleic acids (Takeda & Akira 2005). All individual TLRs can identify numerous structurally unrelated ligands and some TLRs may require accessory proteins to recognize their ligands. The co-operation of different TLRs adds greater specificity and a broader range of ligand recognition capacity to the TLR proteins as well as enhancing their signal transduction capacity (Kurt Jones et al. 2002, Kawai et al. 2007). The TLR domain can bind four different adapter proteins and has the potential to induce the production of various cytokines through different signalling pathways including the Myeloid differentiation primary response protein 88-dependent pathway (Myd88), the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and the mitogen-activated protein (MAP) kinase pathway. TLR are predominantly expressed on cells of the innate immune system, which mediate the first line of defence, including neutrophils, MCs, monocytes/macrophages, and dendritic cells. The expression of different TLRs allows these cells to induce a wide variety of immune responses to specific pathogens. TLRs expressed by different categories of immune cells are listed in Table 1 (*adapted from Hans & Hans 2011*).

Table 1	**TLRs expressed by different categories of immune cells**
Monocytes/macrophages	TLR 1, 2, 4, 5, 6, 7, 8

Neutrophils	TLR 1, 2, 4, 5, 6, 7, 8, 9, 10
Myeloid dendritic cells	TLR 1, 2, 3, 4, 5, 6, 8, 10
B lymphocytes	TLR 1, 3, 6, 7, 9, 10
T lymphocytes (Th1/Th2)	TLR 2, 3, 5, 9
T lymphocytes (regulatory)	TLR 2, 5, 8

Recent research has indicated an essential role of TLRs, acting as a double-edged sword, in maintaining the periodontium in a healthy state, as well as in contributing to the pathogenesis of periodontal disease (Cekici et al. 2014). Since the gingiva is constantly exposed to microbes present in plaque biofilm, TLR signalling plays an important role in the innate immune response and maintenance of periodontal health. However, over-production of pro-inflammatory cytokines due to chronic stimulation of TLRs may lead to tissue destruction (Shang et al. 2019).

To date, 11 different TLR molecules have been identified in human periodontal tissues, and their expression, distribution and ligand specificities have been characterized (Takeda et al. 2003, Krutzik et al. 2004, Liu et al. 2010). TLR2 and TLR4 are the ones that predominate in periodontal tissues (Hatakeyama et al. 2003). TLR4 was the first characterized mammalian TLR and its main ligand is LPS from Gram negative bacteria (Medzhitov & Janeway 1997). On the other hand, TLR2 is involved in the recognition of microbial products like PGN and LTA from Gram-positive bacteria (Schwandner et al. 1999, Takeuchi et al. 1999), lipoproteins (Takeuchi et al. 2001), atypical LPS of *P. gingivalis* and components of spirochetes (Lien et al. 1999). Chronic stimulation of TLRs in periodontal tissues by bacterial PAMPs can lead to excessive production of pro-inflammatory mediators like IL-8, TNF-α, MMPs and biologic mediators responsible for bone resorption, resulting in tissue destruction (Hajishengallis et al. 2003, Hans & Hans 2011).

Numerous studies have shown that altered expression of TLRs was detected in periodontally affected patients (Ozturk & Yildiz 2011). The expression levels of TLR2 and TLR4 were significantly higher in patients diagnosed with severe periodontal disease than in individuals

with healthy periodontium (Becerik et al. 2011). Based on immunohistochemical analysis of the periodontal tissues, clinical studies have demonstrated that the levels of TLR2 and TLR4 in affected groups were significantly higher than the levels in control groups (Duarte et al. 2012, Beklen et al. 2014). Both human periodontal ligament fibroblasts and human gingival fibroblasts, purified from periodontally affected patients, expressed significantly higher levels of TLR4 and TLR2 than periodontally healthy subjects (Scheres et al. 2011).

However, there are still gaps in our knowledge of the mechanisms and specific signalling pathways by which TLRs maintain periodontal health and which imbalance leads to bacterial immune evasion and disease progression.

2.6. Inflammatory Mediators

Most of the cell–cell interactions between immune cells are mediated by signalling molecules released in response to different stimuli. These mediators are involved in both the activation and inhibition of different cell functions by regulating cell differentiation, normal turnover or fast migration into the injury site and reparation processes (Pan et al. 2019). In this respect, defective regulation of this network seems to play an important role in the pathogenesis of many diseases and clinical settings.

During periodontal disease, numerous immune and resident cells in the periodontium produce inflammatory mediators including growth factors, cytokines, chemokines and MMPs, collectively contributing to the destruction of soft and hard periodontal tissues (Silva et al. 2015). Numerous mediators have been detected in the GCF and in gingival tissues from periodontally affected patients. The vast array of signalling molecules participating in the complex cellular interactions can be categorized as pro-inflammatory and anti-inflammatory (Tatakis et al. 2005). In many instances, it is the balance between the two types of signals that determines the tissue response and the initiation or progression of disease (Hughes 1995).

In our research project we will be mainly focused on the role of specific inflammatory mediators: MCP-1, TNF-α and VEGF.

2.6.1. Cytokines

Cytokines are soluble proteins, secreted by cells involved in both innate and adaptive immunity and include interleukins, interferons, and TNF family (Ferreira et al 2018). They act as messenger molecules by binding to specific receptors on resident immune cells and through intra-cellular signalling cascades they result in altered gene regulation and phenotypic cell changes (Preshaw & Taylor 2011). They can be effective at low concentrations; they are produced transiently in the tissues and primarily act in the tissues in which they are produced (Striz et al. 2014). Cytokines can act in an autocrine or paracrine fashion and have pleiotropic effects on many cell types. Cytokines do not function in isolation, but rather in complex networks involving both pro-and anti-inflammatory effects (Preshaw & Taylor 2011). It has been recognized that under normal homeostasis, there is a balance between the pro- and anti-inflammatory activities. The balance between these

cytokines and regulation of their receptors and signaling pathways will determine the extent of periodontal tissue destruction (Kinane et al. 2011).

Interleukins are important members of the cytokine group and are primarily involved in the communication between leucocytes and other cells, such as epithelial cells and fibroblasts engaged in the inflammatory process. Several pro-inflammatory cytokines have been demonstrated to be involved in the pathogenesis of periodontitis (Graves & Cochran 2003, Madianos et al. 2005). Enhanced levels of these cytokines have been demonstrated in the GCF of patients with periodontitis, compared with healthy controls, and higher expression was reported in diseased gingival tissues when compared to healthy controls (Fitzsimmons et al. 2006, Deo et al.2010). Elevated cytokine levels, however, are not limited to the infected gingiva, and people with chronic periodontitis have been found to have increased levels of these cytokines in the circulation as well (Sorsa et al. 2006). Similarly, increased circulating systemic levels of these cytokines, decreased after non-surgical periodontal therapy resulting in clinical improvement of the overall periodontal status (Gamonal et al. 2003, Orozco et al. 2006).

- **TNF-α and its role in periodontal inflammation**

TNF-α is a pleiotropic pro-inflammatory cytokine released by a variety of different cell types in response to various stimuli and it is involved in systemic and local inflammation via different signal pathways (Parameswaran et al. 2010). Its deregulation is implicated in the pathogenesis of numerous diseases, including periodontal disease and it was shown to cause several biological processes (Noh et al. 2013, Kato et al. 2014). Specifically, TNF-α can a) induce upregulation of adhesion molecules on leukocytes and endothelial cells b) stimulate the production of chemokines which are needed to recruit circulating leucocytes, c) induce the expression of other mediators such as prostaglandins, that amplify or sustain the inflammatory response, d) induce the production of lytic enzymes such as MMPs capable of degrading the connective tissue e) enhance the bacterial killing and phagocytic activity of specific cells, f) enhance osteoclast formation and activity that leads to bone loss and g) stimulate apoptosis of matrix producing cells leading to limited repair capacity of periodontium (Graves & Cochran 2003, Genco et al 2005).

TNF-α is an essential mediator of the immune response in periodontitis. Studies evaluating chronic periodontitis in humans and in experimental animal models have demonstrated significantly higher levels of TNF-α in periodontal gingival tissues and GCF of affected individuals (Gomez et al. 2016, Afacan et al. 2019). These higher levels of TNF-α are commonly associated with an increase of multinucleated osteoclasts leading to reduction in alveolar bone volume and resulting loss of tooth support (Zhao et al. 2017). The evaluation of TNF-α receptors in periodontitis has demonstrated abundant receptor expression (both TNFr1 and TNFr2) by a variety of infiltrating cell types, including pre-osteoclastic monocytes/macrophages, fibroblast-like cells, and endothelial cells (Algate et al. 2015). Mean salivary levels of TNF-α were significantly higher in individuals with periodontal disease than in controls. Subjects with salivary TNF-α levels above a threshold of 5.75 pg/ml had significantly more sites with bleeding on probing and attachment loss ± 2 mm (Frodge et al. 2008).

2.6.2. Chemokines

Chemokines are chemotactic cytokines that play a very important role in the migration of phagocytic cells to the site of infection (Palomino et al. 2015). They are mainly secondary inflammatory mediators that are produced in response to external signals, growth factors, viral and bacterial infection (Rossi et al. 2000, Zlotnic at al. 2000). Chemokines can be synthesized by different immune cells including MCs, endothelial, epithelial, as well as leukocytes. They exert their effects on target cells by binding to specific receptors on the cell surface (Sallusto et al. 2000). Functionally, chemokines can be grouped as homeostatic or inflammatory (Moser et al. 2004). In addition to their cell-trafficking role, chemokines are involved in other processes including angiogenesis, cell proliferation, apoptosis, tumor metastasis host defence and bone metabolism (Rossi D et al. 2000, Rot et al. 2004, Zlotnik et al. 2004). They are essential signals for the trafficking of osteoblast and osteoclast precursors, and consequently potential modulators of bone homeostasis (Bendre et al. 2003, Wright et al. 2005).

Chemokines comprise a large family of proteins with low molecular weight (around 8 to 12 kd), and they are remarkably homogeneous in their primary amino acid sequences and are characterized by four conversed cysteine amino acid residues. (Bonecchi et al. 1998, D'Ambrosio et al. 1998, Gu L et al. 2000, Gemmell et al. 2001, Loetscher et al. 2001).

Chemokines can be divided into two subfamilies: C-X-C and C-C branches. Chemokines C-X-C generally attract and activate neutrophils, whereas chemokine C-C members are usually chemotactic for either monocytes or T lymphocytes (Baggiolini et al. 1998, 2001). However, it should be kept in mind that CXC and CC chemokines have the capacity to stimulate migration in other cell types besides neutrophils and monocytes, respectively (Van Damme et al. 1992, Ward et al. 1998). In patients with periodontitis, altered chemotactic behavior was noticed, highlighted that deficiency in the adhesion cycle during cell migration can lead to severe forms of periodontal disease (Del Fabbro et al. 2000, Sigusch et al. 2001).

- **MCP-1 and its role in periodontal inflammation**

MCP-1 is a C–C group chemokine that has chemotactic activity for lymphocytes and monocytes, and it is considered as the major signal for the chemotaxis of mononuclear leukocytes (Colotta et al. 1992). MCP-1 can be synthesized by various cells such as leukocytes, fibroblasts, MCs, keratinocytes, and endothelial cells due to different endogenous and exogenous stimuli. Specifically, it stimulates the chemotaxis of monocytes and several cellular events associated with chemotaxis, including Ca^{++} flux and the expression of integrins (Deshmane et al. 2009). It is also a weak inducer of cytokine expression in monocytes, and at very high concentrations, it elicits a respiratory burst, leading to the generation of oxygen radicals (Rollins et al. 1991, Jiang et al. 1992). MCP-1 expression has been detected in atherosclerosis, rheumatoid arthritis, and delayed-type hypersensitivity, reactions which are characterized with chronic inflammation (Nelken et al. 1991, Antoniades et al. 1992, Graves et al. 1992, Daly et al. 2003).

It has been reported that monocyte functions are important for periodontal breakdown and MCP-1 concentration might be an important factor that influences the severity of the periodontal disease (Jiang et al. 1999, Garlet et al. 2003). Enhanced MCP-1 expression in the periodontal tissues of patients with periodontitis was demonstrated in different studies (Kabashima at al. 2002, Fokkema et al. 2003, Emingil et al. 2004). MCP-1 was mainly synthesized in inflamed gingiva by vascular endothelial cells and mononuclear phagocytes (Yu et al. 1993). The marked expression of MCP-1 was also reported in gingival biopsies and in the inflammatory infiltrates from diseased periodontal sites (Tonetti et al. 1994). A strong relationship between MCP-1 and host response was also suggested for the aggressive form

of periodontitis. Higher GCF levels of MCP-1 were found in patients with generalized aggressive periodontitis compared to healthy or chronic periodontal patients (Emingil et al. 2004, Kurtis et al. 2005). The marked expression of MCP-1 in gingival tissues was reported to be induced by cell components of *P. gingivalis*, as it was markedly induced in gingival fibroblasts treated with *P. gingivalis* LPS in a dose-and treatment time–related manner (Pradeep et al. 2009). It was also documented that *P. gingivalis* LPS could indirectly induce the production of MCP-1 *via* action of inflammatory cytokines induced by itself. Particularly, IL-1β and TNF-a markedly induced MCP-1 gene expression in human gingival fibroblasts (Hanazawa et al. 1993).

2.6.3. Growth factors

Growth factors are a class of natural biological mediators that regulate key cellular events in tissue repair including cell proliferation, chemotaxis, differentiation, matrix synthesis and bone resorption (Giannobile et al. 1996, Giannobile et al. 1997, Smith et al. 2015). Several growth factors are concentrated in the organic matrix of bone and released during bone resorption (Giannobile et al. 1997),and are therefore suggested to play a role in bone remodeling through regulation of the coupling process of bone resorption and formation (Smith et al. 2015). The role of various growth factors in periodontal tissue regeneration is an area of active research and their potential value as diagnostic markers of periodontal tissue inflammation and/or destruction has not been thoroughly investigated. There are several growth factors that are possibly implicated in periodontal disease including epidermal growth factor (EGF), transforming growth factor-a (TGF), platelet-derived growth factor (PDGF) and VEGF (Koidou et al. 2020)

- **VEGF and its role in periodontal inflammation**

The development of a neovascular supply, plays important homeostatic role as the blood vessels carry nutrients to tissues and organs and are responsible for the removal catabolic products (Apte et al. 2019). VEGF, a multifunctional angiogenic-osteogenic mediator, is one of the key regulators in angiogenesis and plays an important role in controlling vascular permeability, particularly in the case of inflammatory conditions (Chapple et al. 2000, Pickkers et al. 2005, Weis et al. 2005). VEGF expression is influenced by cytokines, or nitric oxide (NO), and is strongly upregulated in conditions characterized by increased

microvascular permeability and angiogenesis, such as physiologic wound healing, ischemia, tumor growth or acute inflammation (Boussat et al. 2000, Slevin et al. 2000, Ferrara et al. 2003). While VEGF mainly targets endothelial cells (where its name came from), it has been shown that this factor has multiple effects on additional cell types (Ferrara et al. 2003). It is synthesized and released by vascular smooth muscle cells, MCs, epithelial cells, platelets, leukocytes, and macrophages and can increase vascular permeability to fluid and proteins 50,000 times more, compared to histamine (Zachary et al. 2001, Cetinkaya et al. 2006, Sakallioglu et al. 2007).

As angiogenesis is important for periodontal tissues breakdown, VEGF may be crucial for the progression of gingivitis to periodontitis through its role in promoting the expansion of the vascular network observed in inflammation (Degidi et al. 2006, Prapulla et al. 2007). Higher concentrations of VEGF have been noted in inflamed periodontal tissues and in tissues at healing stages of chronic periodontal disease compared to healthy controls (Cetinkaya et al. 2006, Pradeep et al. 2011). It was shown that in case of periodontitis, GCF and serum VEGF levels were positively correlated with increased clinical periodontal parameters (Pradeep et al. 2011). Elevated VEGF levels in GCF of patients suffering from chronic periodontitis were found when compared with the gingivitis patients and healthy controls (Booth et al. 1998, Prapulla et al. 2007). In addition, increased VEGF protein expression has been shown in periodontally affected tissues when compared to gingivitis and healthy tissues (Gölz et al. 2015, Vasconcelos et al. 2016, Beral et al. 2018). These findings support the important role of VEGF in the regulation of periodontal microcirculation under pathological states.

3. *Porhyromonas. Gingivalis (P. gingivalis),* a "Keystone Pathogen"

As previously mentioned, the oral cavity is home to a menagerie of bacterial species. Over 300 different bacterial species have been cultivated from human subgingival plaque sample (Palmer 2004). Only some of these bacteria, either alone or in combination, have periodontopathic potential and can initiate periodontal disease when a critical concentration is reached (Paster 2001, Colombo et al. 2012). Overgrowth of periodontal pathogens may result from a deficiency in the host defence system or modification of the subgingival environment (Watanabe 1989). Periodontal diseases are associated with a consortium of organisms rather than individual pathogens at periodontally affected sites (Socransky et al. 1998, Haffajee et al. 2008).

Five microbial complexes repeatedly found together in the subgingival biofilm of subjects with and without periodontal disease have been defined: the green complex, the orange complex, the orange-associated complex, the *Aa* complex and the red complex. The red complex appears in later stages in biofilm development and comprises a consortium of three species: *Tannerella forsythia, P. gingivalis* and *Treponema denticola*. The red complex has been considered the most pathogenic microbial complex (Socransky et al. 1998, Holt et al. 2005). The features of the red complex bacteria are their proteolytic activity, the production of toxic metabolites, and their outer membrane vesicles. These bacteria produce a broad array of virulence factors that allow them to colonize subgingival sites, disturb the host defence system, invade and destroy periodontal tissues, and promote an immune destructive host response (O'Brien-Simpson et al. 2004, Holt & Ebersole 2005, Bodet et al. 2007). Due to these characteristics, they consider to be "periodontal-disease-associated bacteria", and they have received much attention regarding their role in periodontal disease progression and treatment outcomes (Socransy 1979).

P. gingivalis is the species most highly associated with the chronic forms of periodontitis and can be detected in up to 85.75% subgingival plaque of chronic periodontitis patients (Fiorillo et al. 2019). Prevalence of *P. gingivalis* is related to the severity of periodontal disease and has been identified as one of the major causative agents in the periodontal pathogenesis (How et al. 2016). Recently, the keystone pathogen theory postulates that even at low abundance, *P. gingivalis* can induce chronic periodontitis by remodelling the commensal bacterial community to promote a state of dysbiosis, which leads to disease (Olsen &

Hajishengallis 2017). *P. gingivalis* has been widely investigated, partially benefited from the well-characterized genomics of different *P. gingivalis* strains and the accessibility of a great variety of mutants (Nelson et al. 2003, Naito et al. 2008, Darveau et al. 2012). *P. gingivalis* can interact and co-aggregate with other bacteria in the subgingival plaque, event that benefits its destructive effects on periodontal tissues (Kuboniwa et al. 2017). It can also efficiently modify the host immune response and create an environment favourable to its own survival and other pathogens' continued persistence (Hajishengallis & Lambris 2011).

Colonization of *P. gingivalis* is associated with severe local inflammation in the underlying connective tissues and clinically, is strongly associated with increasing pocket depth and bleeding on probing in the affected sites (How et al. 2016). For this bacterium to be pathogenic, it needs, firstly, to have the ability to infect the periodontal tissues by attaching to the tooth surface or to the epithelial cell. Virulence factors of *P. gingivalis* enable the organism to invade the host cell, to induce microbe-host interactions (attachment) and to interfere with host defences. Virulence factors of *P. gingivalis* are responsible for tissue destruction, as its cell wall components are able to degrade the extracellular matrix of the gingival tissues and to activate osteoclastic resorption of alveolar bone, which directly or indirectly, leads to irreversible loss of periodontal supportive tissues (Travis et al. 1997). P. gingivalis has also evolved strategies to escape protective immunity, often by manipulating key components of innate immunity, such as the TLR and complement systems (Lambris et al. 2008, Flannagan et al. 2009). Such strategies enable these pathogens to disable the overall host response, since complement and TLRs play instructive roles in the development of adaptive immunity (Medzhitov et al. 2007, Ricklin et al. 2010).

In relation to periodontally infected sites, *P. gingivalis* is detected rarely or at low numbers in healthy sites. The presence of *P. gingivalis* in a periodontal pocket may predict imminent disease progression (van Winkelhoff et al. 2002) and a significant positive correlation is found between *P. gingivalis* numbers and increased probing pocket depths (Kawada et al. 2004). Following periodontal treatment, a reduction of *P. gingivalis* numbers is associated with resolution of inflammation at the affected sites (Haffajee et al. 1997, Fujise et al. 2002). On the contrary, high numbers are commonly encountered in sites that exhibit recurrence of disease or persistence of deep pockets, post-treatment (Mombelli et al. 2000, Fujise et al. 2002, Kawada et al. 2004). It was shown that experimental implantation of *P. gingivalis* in

animal models induced an inflammatory response that subsequently led to severe bone loss (Evans et al. 1992, Hajishengallis et al. 2011). Elevated levels of antibody to antigens of *P. gingivalis,* suggests that this species can gain access to the underlying tissues and may initiate or contribute to observed pathology. In addition, early studies documented the ability of *P. gingivalis* to invade human gingival epithelial cells, buccal epithelial cells and endothelial cells as it has been found in higher numbers on or in these cells recovered from affected periodontal sites in comparison to healthy sites (Rudney et al. 2001, Takahashi et al. 2006). Collectively, due to these properties *P. gingivalis* is considered an 'opportunistic pathogen', in line with the modified Koch's postulates for oral infections, such as periodontal diseases (Slots & Genco 1984, Dzink et al. 1985).

3.1. Structural and Growth Characteristics of *P. gingivalis*

P. gingivalis is a black-pigmented, rod-shaped, assaccharolytic, non-motile Gram-negative species that requires anaerobic conditions for growth, and the presence of heme or hemin and vitamin K in its nutrient milieu (Olczak et al. 2005). *P. gingivalis* gains its metabolic energy by fermenting amino acids. *P. gingivalis* is a late colonizer, and it is found in close proximity to, and interacts with the gingival tissues (Olczak et al. 2005, Kolenbrander et al. 2011, Zijnge et al 2011). The aggregation of heme on its cell surface is responsible for the black pigmentation of *P. gingivalis* colonies (Liu et al. 2004, Smalley et al. 2006). This property is connected to its ability to act as an opportunistic pathogen, as when grown in a heme-limited medium, it becomes less virulent (McKee et al. 1986).

Early interest in *P. gingivalis* arose primarily because of its essential role in certain experimental mixed infections (Socransy & Gibbons 1965) and the production of an unusually large array of virulence factors such as collagenase, gingipains, proteases, endotoxins, fatty acids etc. (Haffajee & Socranky 1994, Deshpande & Khan 1999, Holt & Ebersole 2005). Studies initiated in the late 1970s showed that *P. gingivalis* could inhibit migration of PMNs across an epithelial barrier (Madianos et al. 1997), could affect the production or degradation of cytokines by mammalian cells (Darveau et al. 1998, Fletcher et al. 1998, Sandros et al. 2000) and could produce extracellular vesicles that contributed to the loss of membrane-bound $CD14^{+}$ receptors on human macrophage-like cells (Duncan et al. 2004).

3.2. *P. gingivalis* Virulence Factors

P. gingivalis possesses several important virulence factors. The main virulence factors known as the survival weapons of *P. gingivalis* include: LPS, capsular polysaccharide (CPS), fimbriae and gingipains (Zeonobia et al. 2015). An interesting point is that whole viable *P. gingivalis* is differentially sensed by the host, compared with its released virulence factors, with the potential to activate distinctive intracellular pathways or differential cytokine production (Zhou et al. 2005, Pathirana et al. 2010).

- **The LPS**

LPS is a PAMP or more recently, identified as microbe-associated molecular pattern (MAMP) (Medzhitov 2007). LPS is a major component of the cell wall (i.e. outer cell membrane) of Gram-negative bacteria, including *P. gingivalis* (Darveau 2002). LPS was well known for its toxicity and the ability to cause unwanted host inflammation, which gave it the name endotoxin (Munford et al. 2008).

P. gingivalis LPS is one of the key virulent attributes, which is significantly involved in the pathogenesis of periodontal disease (Bainbridge & Darveau 2001, Lu et al. 2009). In general, bacterial LPS contains three major components including the outermost polysaccharide, core oligosaccharide regions and innermost lipid A structures (Dixon & Darveau 2005). Lipid A is biologically the most active part of LPS, and it is a phosphorylated glucosamine disaccharide attached with multiple fatty acids. Its structure differs greatly among Gram-negative bacterial species depending on the differences in composition of attached fatty acids, number of phosphorylation sites and substituted groups attached to the phosphate residues (Dixon & Darveau 2005).

During periodontal disease process, *P. gingivalis* releases copious amount of outer membrane vesicles containing LPS that penetrate periodontal tissues and mediate an immuno-inflammatory response (Darveau 2009). Recently, it was revealed that *P. gingivalis* could generate heterogeneous LPS lipid A structures by changing the lipid A moiety through hemin dependent modulation (Al-Qutub et al. 2006). The two isoforms LPS $_{1,435/1,449}$ and LPS $_{1,690}$ appear responsible for tissue-specific immune signalling pathways activation and increased virulence activity. The different isoforms may modulate cytokine production in

human cells by exerting opposite effects on the human TLR2 and TLR4 activation (Olsen et al. 2018).

It was documented that hemin concentration may exert conformational changes in *P. gingivalis* LPS via regulation of his hemin receptors (Al-Qutub et al. 2006). It was shown that *P. gingivalis* in high hemin conditions produces predominantly the isoform $LPS_{1435/1449}$, whereas in low hemin conditions *P. gingivalis* produces the isoform LPS_{1690} (Coats et al. 2009). High hemin concentration during inflammation may promote *P. gingivalis* to shift its LPS from the predominant penta-acylated lipid A structure towards more tetra-acylated one as it was shown in both laboratory and clinical studies using different isolates of *P. gingivalis* (Darveau et al. 2004, Al-Qutub et al. 2006, Reife et al. 2006). Due to its ability to transform the Lipid A structure and hence modulate the innate host response in a favourable way to gain access and multiply in gingival tissues under specific conditions, *P. gingivalis* LPS is considered a keystone bacterial pathogen component, strongly related to periodontal disease (Herath 2011).

3.3. Immune Responses Triggered by *P. gingivalis* LPS

P. gingivalis LPS is the major pathogenic component in its cell wall, acting as the prototypical endotoxin. Gram-negative bacteria LPS is known to elicit its virulent effects and activate the innate defence system through TLR, which are pattern recognition receptors that detect specific bacteria (Jain et al. 2008). These in turn can activate inflammatory pathways and induce inflammation via pro-inflammatory cytokine production (Hajishengallis & Kinane 2007, Takeuchi & Akira 2010). TLR4 is the major signal transducer for most types of LPS, while TLR2 is suggested to be a signal transducer for other bacterial components, such as peptidoglycans and lipoproteins (Hosino et al. 1999).

Interestingly, *P. gingivalis* LPS elicits an unusual host response when compared with the better-known activity of *E. coli* LPS. A well-characterized innate host recognition pathway has been documented for *E. coli* LPS, which starts with the initial binding of LPS to LPS-binding protein (LBP) and CD14 (Schumann et al. 1990, Wright et al. 1990, Takeuchi et al. 1999). Transfer of *E. coli* LPS by either mCD14 or sCD14 to a cell-associated TLR4 and MD-2 protein complex initiates host cell activation pathways, leading to innate host defence mediator production. Early studies with canonical *E. coli* LPS, have shown that it exclusively

binds to TLR4 (Muta et al. 2001). Although some studies claimed that *E. coli* LPS might bind to TLR2, later studies showed that this was a result of lipoprotein contamination in LPS, since TLR2 is known to mainly occupy the LPS ligand (Da Silva et al. 2001)

However, *P. gingivalis* has been shown to signal through TLR2 or/and TLR4 to activate different cell types to produce cytokines, such as macrophages (Hirschfeld et al. 2001), gingival fibroblasts (Wang and Ohura 2002), gingival epithelial cells (Sandros et al. 2000), and endothelial cells (Nassar et al. 2002). These discrepancies were hypothesized to be due to differences in LPS preparations, since differently acylated LPS Lipid A moieties induce different cellular effects or to lipoproteic contaminants (Darveau et al. 2004, Hashimoto et al. 2004, Reife et al. 2006). There are studies that showed that *P. gingivalis* LPS stimulation led to the upregulation of TLR2 expression and pro-inflammatory cytokine production *in vitro* (Papadopoulos et al. 2013). In addition, production of NO, TNF-a, and IL-6 by macrophages was increased after LPS-TLR2 activation (Holden et al. 2014).

Different studies showed that *P. gingivalis* LPS up-regulated the expression of both TLR-2 and TLR-4 in periodontal ligament cells and mesenchymal stromal cells. This stimulation led to overproduction of inflammatory cytokines, such as IL-1, IL-6 and TNF, which facilitated the recruitment of lymphocytes and the release of oxygen free-radicals, resulting in destruction of the surrounding tissues (Sun et al. 2010, Raicevic et al. 2010, Mealey 2008). In recent studies the use of highly purified LPS preparations, that contains different forms of the LPS lipid A moiety, was able to interact with both TLR2 and TLR4 (Darveau et al. 2004), thus inducing different cellular effects (Reife et al. 2006, Kumada et al. 2008). It is still unknown, whether *P. gingivalis* utilizes TLR4 or/and TRL2 to activate MCs.

Taking the above into consideration, *P. gingivalis* supports the keystone pathogen hypothesis proposed by Hajishengalis et al. (2012), as its effects are disproportionately large, relative to its abundance. Colonization by *P. gingivalis* elevates the virulence of the entire community leading to an impaired host immune surveillance eventually disrupting tissue homeostasis and causing destruction of periodontal tissues.

4. Mast Cells (MCs)

4.1. Introduction

MCs are multifunctional tissue-dwelling cells that have been traditionally associated with the allergic response. Recent studies suggest that these immune cells are implicated in several health and disease states (Theoharides et al. 2015, Krystel-Whittemore et al. 2016). It is increasingly evident that MCs maturation, phenotype, and function are a direct consequence of the local microenvironment and have a marked influence on their ability to specifically recognize and respond to various stimuli through the release of an array of biologically active mediators (Galli et al. 2011). The extensive tissue distribution and versatility of MCs endow them with the potential to not only act as first responders in harmful situations but also to react to environmental changes by communicating with a variety of other cells implicated in physiological and immunological responses (da Silva et al. 2014). In addition to their involvement in physiological processes such as tissue repair, wound healing, and angiogenesis, MCs are increasingly becoming accepted as having a crucial role in innate and adaptive immunity and inflammation (Theoharides et al. 2012, Shaik-Dasthagirisaheb et al. 2013). The capacity of MCs to promptly interact with the microenvironment and respond through the release of an array of biologically active mediators is a delicate balance where the inadequate regulation of MCs functions can result in devastating effects to the organism. Hence, MCs have been implicated in the pathogenesis of several chronic allergic/inflammatory disorders, autoimmune diseases, and cancers (Rao & Brown 2008, Theoharides et al. 2019).

The host response in periodontal inflammation, has traditionally been mediated mainly by neutrophils, monocytes/ macrophages, B and T lymphocytes. These are triggered to produce inflammatory mediators, including cytokines, chemokines, arachidonic acid metabolites and proteolytic enzymes, which collectively contribute to tissue degradation and bone resorption by activation of several distinct host degradative pathways (Birkedal-Hansen 1993, Hernandez et al. 2011). In recent years, the increased number of MCs in periodontally affected tissues have shed new light on the possible implication of MCs in periodontal inflammation.

4.2. Historical Development of MCs Research

Paul Ehrlich was the first researcher who showed the unique tinctorial properties of MCs. He described cells in connective tissue that were stained reddish–purple (referred to as metachromasia) with aniline dyes, calling them “mästzellen,” a term that may have referred to feeding or could be interpreted as “well-fed” based on their granule contents (Ehrlich 1878). Since their first description, MCs have been viewed, for the most part, as effectors of allergy, particularly in the early and acute phases of allergic reactions. Early research on these cells relied on morphological features to identify their distribution in physiological and pathological states. The following 15 years witnessed the establishment of a relationship between MCs, histamine, and anaphylaxis, which was supported by the discovery that histamine was present in MCs (Riley & West 1952) and was released, along with heparin, during anaphylactic shock (Rocha & Silva 1947). It was not until 1967 that Ishizaka & Ishizaka identified the “reaginic” antibody as being γE antibodies, subsequently recognized as IgE that was capable of mediating the release of histamine and another “slow reacting substance” from sensitized tissue MCs (Ishizaka et al. 1970). These discoveries paved the way for MCs to become famous for their role in Type I hypersensitivity. The unravelling of MCs functions, in addition to their established and extensively studied role in IgE-mediated reactions, has been the focus of MCs research in the past decades.

4.3. Origin and Differentiation of MCs

MCs derive from $CD34^{+}$ pluripotential hematopoietic progenitor cells located in bone marrow and spleen (Kirshenbaum et al. 1999), where they start their maturation under the influence of a variety of growth factors and cytokines such as SCF, interleukins (IL-3, IL-6), nerve growth factor, TGF-β, and thrombopoietin (Kitamura et al. 1981, Agis et al. 1993, Rottem et al. 1994, Okayama et al. 2006, Kirshenbaum et al. 2006, Gurish et al. 2006). Unlike other cells of hematopoietic origin, which differentiate and mature in the bone marrow before being released to the blood stream, MCs migrate as immature progenitor cells through the blood stream to peripheral tissues where they complete their maturation (Kitamura et al. 1993, Metcalfe 1997, Lantz et al. 1995, Kawakami et al. 2002, Hallgren & Gurish 2007). Mature MCs in peripheral tissues, then exert their effects, under the influence of SCF and other microenvironmental molecules including adhesion molecules (integrins and cadherins) and diverse chemokines (Okayama et al. 2006). During maturation, MC

precursors in bone marrow progressively lose expression of markers associated with early stages of differentiation. As they mature, MCs start to express proteins associated with the inflammatory response (e.g. FcεRI), cytoplasmic mediators (e.g. CPA, tryptase, chymase, and histamine), integrins (e.g. CD49b and CD49c), and immunomodulatory molecules (e.g. Siglec-6 and Siglec-8). Once in peripheral tissues, MCs acquire a specific phenotype, which is shared by all MCs independently of the tissue where they reside. The phenotype is characterized by strong expression of 3 different molecules: (1) the antigen CD117 (KIT), which is the receptor for SCF (2) FcεRI, the high-affinity serum IgE receptor and (3) intracytoplasmic tryptase, which is the most abundant protein stored in the granules of MCs (Shimizu et al. 2002, Yokoi et al. 2006).

4.4. The Role of SCF/c-KIT

SCF and its ligand, the CD117/c-Kit receptor, are essential for MCs survival and development. SCF has multiple biological effects on MCs, including modulating differentiation and homing, prolonging viability, inducing MCs hyperplasia, and enhancing mediator production (Iemura et al. 1994, Galli et al. 1995, Okayama & Kawakami 2006). The receptor for SCF which is known as c-KIT, a transmembrane glycoprotein that belongs to the type III TK family of receptors, is one of the most relevant receptors of MCs. Even though it is also present in hematopoietic precursor cells, in none of these cells are the levels of expression of c-KIT as high as those found in MCs (Zakut et al. 1993, Ashman et al. 1999). In contrast to other protein receptors expressed by MCs whose function is usually restricted to advanced stages of differentiation, the c-KIT receptor exerts its function throughout the development of MCs, playing a crucial role in their proliferation, differentiation, migration, and survival (Irani et al. 1992, Galli et al. 1995, Kitamura et al. 2006). The importance of c-KIT and the processes regulated by this receptor have been largely established based upon genetically modified animal models. The culture of mouse bone marrow enriched for hematopoietic progenitors with SCF in combination with IL-3 resulted in the surface expression of FcεRI on MCs and the initiation of secretory granule formation after 3 days of culture (Lantz & Huff 1995). *In vivo*, mouse strains bearing mutations in the genes for the c-KIT receptor (KitW/W-v and KitW-sh) or its ligand SCF (Sl/Sld), which are deficient in MCs, corroborate the significance of SCF for MSc survival and development (Huang et al. 1990, Kitamura 2000, Grimbaldeston et al. 2005). Mice with c-KIT–deficiency and SCF-deficiency, lack of mature MCs and suffer from

hypoplastic anaemia, hypopigmentation, and sterility (Kitamura et al. 1978). In humans, studies have demonstrated that mutations of c-KIT which lead to the constitutive activation of the c-Kit receptor, are associated with mastocytosis, a neoplastic disorder characterized by MCs expansion and accumulation in the skin and other tissues (Orfao et al. 2007, Theoharides et al. 2015). However, MCs that have been deprived of SCF undergo programmed cell death (PCD) or apoptosis (Mekori et al. 1995).

4.5. Adhesion of MCs

Adhesion molecules expressed on the surface of MCs are critically important, not only for the endothelial and tissue migration, but also for providing ongoing information about the local tissue microenvironment, influencing responses to external various biological stimuli (Harjunpää et al. 2019). MCs adhere to extracellular matrix proteins (EMP) including fibronectin, laminin and vitronectin through several integrins expressed on their surface (Hogaboam et al. 1998, Vliagoftis et al. 1997, Schoeler et al. 2003). In addition to adhesion to EMP, MSc can adhere to endothelial and mesenchymal cells, including fibroblasts, at least in part through c-kit receptor interacting with transmembrane SCF expressed by fibroblasts (Adachi et al. 1992). Human MC progenitors adhere on activated endothelial cells through interaction of P-selectin glycoprotein ligand-1 (PSGL-1) and α4 integrin, with E-selectin and VCAM-1 respectively expressed on endothelial cells (Boyce et al. 2002). Recently a new adhesion molecule, spermatogenic immunoglobulin superfamily (SgIGSF), has been identified on MCs that also mediates MCs adhesion to fibroblasts (Ito et al. 2003). A diagram of the functional consequences of MCs adhesion to extracellular matrix through various integrins is presented on *Image 2.*

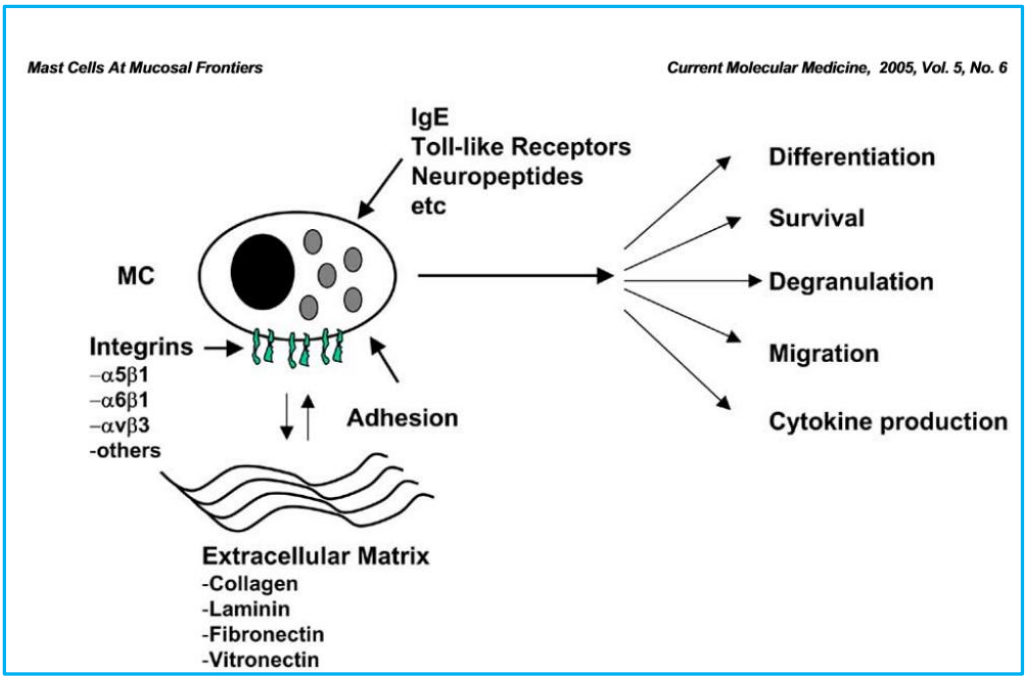


Image 2. A diagram of the functional consequences of MCs Adhesion. MCs adhesion to Extracellular matrix through various integrins acts as co-receptor system for MCs activation to induce and promote several biological events (*Vliagoftis & Befus 2005. MCs at Mucosal Frontiers Current Molecular Medicine Sep;5 :573-89*).

4.6. Apoptosis of MCs

Apoptosis is an important homeostatic mechanism that regulates cell numbers. MCs are long-lived cells in peripheral tissues (Elmore 2007). Once resident in tissues, MCs have the ability to live for many months and remain capable of mitotic division. CSF and IL-3 are some of the most important growth factors for the survival of MCs. These proteins induce survival of cultured human MCs through the increased expression of anti-apoptotic proteins like B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma-extra-large (Bcl-xL) and in their absence, apoptosis is noted (Mekori et al. 2001). On the other hand, bacterial products can prevent MCs apoptosis suggesting that TLR may be involved and underscoring the role that MCs may play in innate immunity. Indeed, activation of TLR4 on MCs surface by LPS induces Bcl-xL expression and prevents MCs apoptosis (Yoshikawa et al. 2003).

4.7. Distribution of MCs

MCs are distributed in connective tissue, particularly in sub-epithelial regions and in areas surrounding blood vessels, nerves, smooth muscle cells, mucus glands and hair follicles (Galli et al. 2005). The distribution of MCs is dependent on mechanisms of homing, enhanced recruitment, survival, and local maturation of MCs progenitors (Metcalfe et al. 1997, Galli et al. 2005, Jamur et al. 2005, Metcalfe & Boyce 2006). MCs have a widespread tissue distribution and are mainly found at the interface between the host and the external environment, at places of potential entry of pathogens or contact with harmful substances, such as skin, respiratory mucosa, gastrointestinal tract, conjunctiva, pulmonary airways and ventricles of the heart (Schulman et al. 1982, Dvorak et al. 1988, Irani et al. 1990, Baddeley et al. 1995) Specifically, they represent the 10% of the total amount of immune cells in the skin where they are often adjacent to blood vessels, nerves and lymphatics, with a density estimated at 7,000-20,000 MCs per mm^2 skin (Cowen et al. 1979). In addition, MSc are found in the choroidal plexus of the brain and in vascular structures of the meninges (Polyzoidis et al. 2015).

4.8. Phenotypic Heterogeneity of MCs

Alterations in phenotype can take place during all stages of MCs existence. Different subsets of mature MCs have been described based on their location and functional, structural, and biochemical characteristics *(Image 3).* Thus, it is the microenvironment encountered by MCs that ultimately determines their mature phenotype (Jamur & Oliver 2011).

According to the pattern of expression of cytoplasmic tryptase and other MCs proteases, three main phenotypically different subtypes of human MCs can be distinguished (Church et al. 1997, Schwartz et al. 2006) as follows: (1) MCs that only contain tryptase (MC_T), (2) MCs containing tryptase, chymase, carboxypeptidase A (CPA), and cathepsin G (MC_{TC}), and (3) MCs that contain chymase, CPA, and cathepsin G in the absence of tryptase (MC_C). MC_T predominate in mucosal sites, including the lining of the gut and respiratory tract often in close proximity to T cells. These T lymphocytes are especially of the T-helper 2-type (Th2 secreting IL-4 and IL-5). This subset usually is seen in increased numbers infiltrating the mucosa in patients suffering from allergic and parasitic disease. On the other hand, MC_{TC} tends to predominate in the gastrointestinal tract as well as in skin, synovium, and

subcutaneous tissues. Increased numbers of MC $_{TC}$ are seen in fibrotic diseases whereas its numbers are relatively unchanged in allergic or parasitic diseases (Goldstein et al. 1987, Irani et al. 1987). The third phenotype of MCs was recently described in the airway epithelium in asthmatic subjects and oesophageal samples of patients with eosinophilic esophagitis (Abonia et al. 2010, Dougherty et al. 2010).

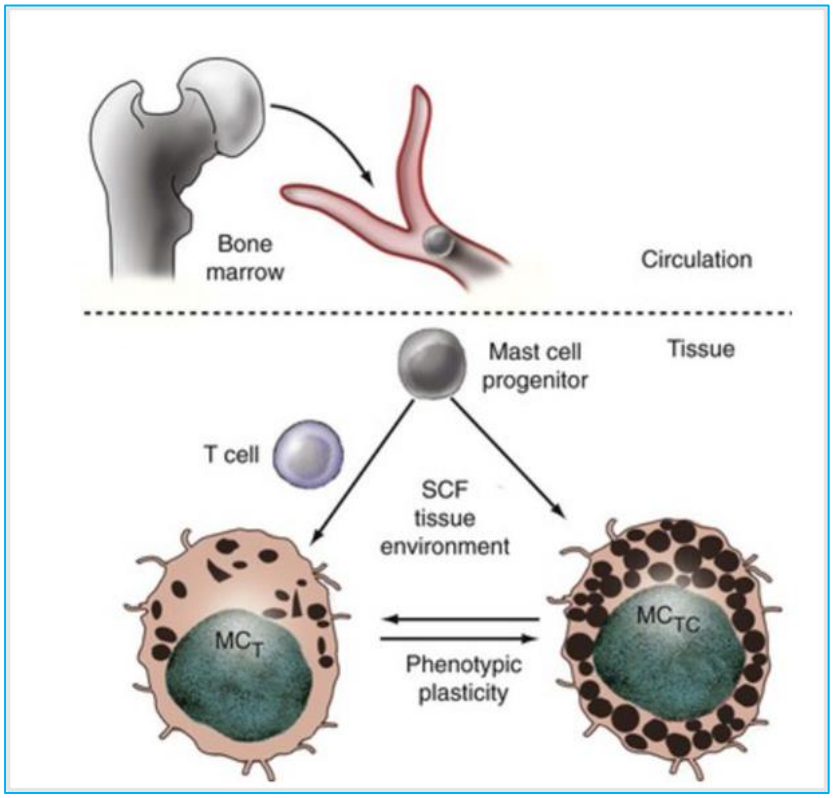


Image 3. MCs origin and differentiation. MCs arise in the bone marrow, circulate as committed progenitors, and differentiate into mature MCs upon entering tissue. Human MCs may be classified based on granule proteases into tryptase+ MCs (MC_T) and tryptase+/chymase+ MCs (MC_{TC}), with characteristic tissue localization and mediator production. SCF, stem cell factor. (*Adapted from Gurish MF, Austen KF: The diverse roles of MCs, J Exp Med 194: F1–F5, 2001. Illustration by Steven Moskowitz*.)

4.9. Isolation, Culture and Collection of MCs

During the past decades, the unravelling of MC functions in many laboratories has been in the focus of research. Nevertheless, one of the major limitations and challenges is the difficulty to obtain large quantities of primary MCs for *in vitro* and *in vivo* study purposes (Bischoff et al. 2007). The difficulty for their isolation lays on the fact that when MCs precursors enter in the bloodstream, they circulate for a very short time and is very difficult to be distinguished from other cell types as they do not possess specific characteristics of mature MCs that reside in the tissues (Derakhshan et al. 2018). Mature human MCs can be isolated from solid tissues and can be cultured with relatively complex enzymatic processes and techniques. "Immature" human MCs cells can be collected from human umbilical cord or from peripheral blood and grow in culture, although they differ significantly from MCs that are normally found in human tissues. Therefore, many studies that have been conducted in immortalized MSc lines, result in findings that must be interpreted cautiously due to activating mutations in key signalling components.

4.10. Human MCs Lines

Despite efforts to establish long-term human MCs cultures, at present there is a limited number of immortalized lineages. For more than 10 years the only established human MCs line was the so-called human MCs line-1 (HMC-1) (Yu et al. 2018). More recently, Kirshenbaum and co- workers isolated two MCs lineages (LAD-1 and LAD-2) that more closely resemble primary culture of $CD34^{+-}$ derived human MCs (Kirshenbaum et al. 2003). Culturing conditions for these cells however have prohibitive costs and therefore LAD-1 and LAD-2 are not the first choice for routine experiments. The latest entry among the human MCs lines cohort is the recently established LUVA line. LUVA cells arose spontaneously during culture of peripheral blood $CD34^{+}$ cells from a healthy donor. They have been reported to degranulate after IgE receptor cross-linkage and present a phenotype of fully mature MCs (Laidlaw et al. 2011).

- **HMC-1 and HMC-1 Sublines**

The HMC cell line was originally established from the peripheral blood of a patient with MCs leukaemia (Nilsson et al. 1994, Kirshenbaum et al. 2003, Sundström et al. 2003, Laidlaw et al. 2011). HMC cell line shows undoubted advantages in that it is a relatively easy-to-

cultivate and a great number of homogeneous cells can be obtained in a limited time frame. Its growth is independent from the presence of growth factors and this reduces subsequent handling costs. It has been demonstrated that HMC-1 cells express many of the typical MCs surface antigens. HMC-1 cells also show intracellular metachromatically staining granules containing histamine, β-tryptase, and heparin. On the other hand, HMC-1 cells lack functional FcεRI, α-tryptase, and chymases (Sundström et al. 2003)

- **LAD Cell Line**

In 2002, Kirshenbaum and co-workers characterized two novel human MCs lineages, namely LAD-1 and -2 (Kirshenbaum et al. 2003). These cell lines originate from bone marrow aspirates of a male patient affected by MCs sarcoma/leukaemia and, at present, the LAD-2 cell line is available. Unlike HMC-1, LADs do not express the activating mutation of Kit and therefore their growth is dependent on the presence of SCF in the culture medium (10 ng/ml). This characteristic makes LAD cells more similar to primary cultured MCs (Dahl et al. 2004).

The LAD-2 cell line expresses ultrastructural features of matured MCs such as CD surface markers and intracytoplasmic histamine, tryptase, and chymase. LAD cells also express functional FcεRI and FcγRI and they can degranulate following IgE-mediated receptor cross-linking (Guhl et al. 2010). LAD-2 cells can be used as a tool to investigate IgE-FcεRI binding and subsequent downstream events, MCs signalling pathways, and the effect of novel drugs. In addition, they can express functional $CD14^{+}$ and TLRs (Mc Curdy et al. 2001). Some groups, however, have reported a decreased ability of LAD cells to release cytokines compared to other lineages and primary MCs. LAD cells represent one of the most powerful tools in MCs research but, due to the high cost of rhSCF, LAD cells are not often the first choice as MCs model. Additionally, LAD-2 cells also show a slow doubling time (approximately 2 weeks) which limits their applicability when large numbers of cells are required (Gibbs et al. 2014).

- **LUVA Cell Line**

The LUVA lineage is a recently established MCs line derived from a culture of non-transformed hematopoietic progenitor cells (Laidlaw et al. 2011). LUVA cells spontaneously differentiated from $CD34^{+}$-enriched mononuclear cells from a donor with aspirin exacerbated respiratory disease but without any clonal MCs disorder. It has been reported

that LUVA cells can degranulate following FcεRI cross-linking. Metachromatic intracellular granules resulted positive for tryptase, chymase, cathepsin G, and carboxypeptidase A3 (CPA3). Although LUVA cells do not show KIT mutations they can survive and proliferate in culture without the presence of SCF in the culture media. LUVA cell line represents a new tool for future studies as it is a fully functional MCs line without some of the aberrant characteristics that limit the applicability of other MCs lineages.

- **KU812 Cell Line**

KU812 is an immature pre-basophilic cell line obtained from a male patient affected by chronic myeloid leukaemia and it shows phenotypic features of basophil precursors (Kishi et al. 1985). Characteristic of this cell line is the presence of scarce metachromatic granules containing low amounts of histamine and tryptase. KU812 cells express low levels of FcεRI and no FcεRII (Blom et al. 1992).

4.11. Imaging of MCs

A variety of technologies have been developed over the years that facilitate the visualization of MCs both at the microscopic and ultrastructural level.

In morphological terms, MCs are normally round to ovoid in shape mononuclear cells, with an off-central round nucleus and numerous granules filling the cytoplasm, often hiding the nucleus completely. They usually show phenotypic diversity regarding the morphology of their cytoplasm and their diameter varies in size up to 25 µm. The most distinct feature of MCs is their numerous, electron-dense secretory granules, ranging in size from 0.2 to 0.8 micrometres, which occupy a large proportion of the MCs cytoplasm and upon activation, can be discharged rapidly within seconds to minutes.

These granules contain high amounts of anionic proteoglycans (Metcalfe et al 1979, Thompson 1988) that result in the characteristic metachromatic staining when visualized with cationic dyes such as Toluidine blue. The major constituent of granules is MCs specific proteases, which are tightly complexed with sulfated proteoglycans such as heparin and chondroitin sulfate, the specific combinations of which vary with MCs phenotype. MCs granules are also rich sources of biogenic amines, the most notorious of which is histamine, the compound responsible for many of the symptoms of allergic inflammation. The neurotransmitter, serotonin, is also highly expressed in rodent MCs but less, in human MCs. In addition, as part of their unique function, MCs also store preformed cytokines and growth factors within granules, such as TNF and VEGF (Wernersson et al. 2014).

- **Histological staining of MCs**

Since the early years of investigation, MCs have been assessed using staining dyes that allow for the identification within tissues as well as the assessment of their morphology and intracellular structures. MCs can be identified on tissue sections using simple histochemical stains including hematoxylin & eosin (H&E), May- Grünwald Giemsa, toluidine blue or by using more specific reagents such as antibodies against enzymes (tryptase or chymase) or the stem cell factor receptor c- kit (CD117) and FcεR1 (Tikoo et al. 2018).

For histochemical staining of MCs, toluidine blue, a metachromatic stain is the method of choice. The biologically active mediators present within the MCs granules are acidic in nature

and bind basic dyes like toluidine blue. As a result, the metachromatic granules stain red-purple and the orthotropic background stains blue.

➢ **Electron Microscopic Imaging Modalities**

Although decades of histochemical staining have been crucial for identifying the role of MSc and their secretory granules under various pathological conditions, this methodology is inept at providing insights into granule secretion at a subcellular resolution. Therefore, electron microscopy has been employed to investigate the cellular and subcellular structure of MSc in the steady state and post- activation.

- **Light Microscopy**

Under light microscopy, a dense granular cytoplasm is seen, often obscuring the nucleus and other organelles. When it can be visualized, the nucleus is central, and the cell is mononuclear. The secretory granules vary in size with a range of 0.3 µm to 0.8 µm (*Image 4*)

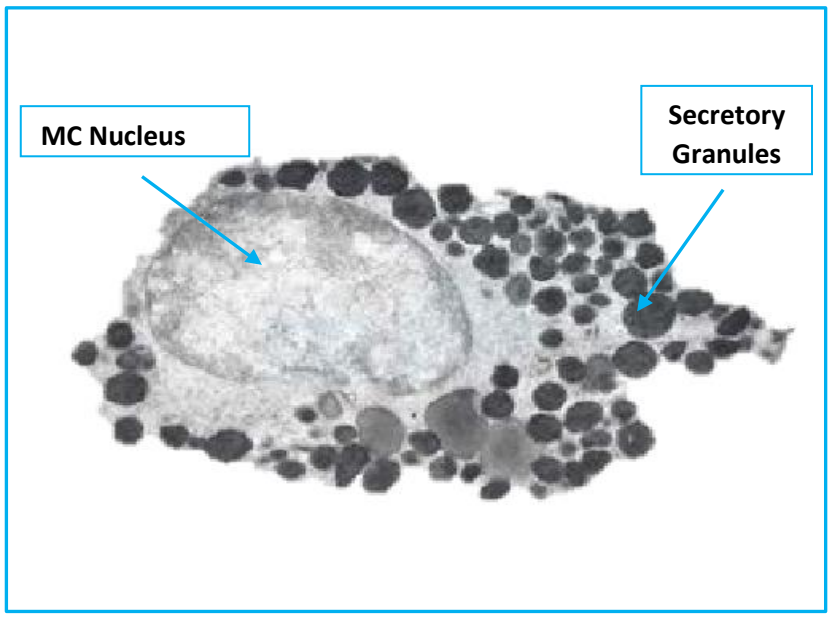


Image 4. Presentation of morphological characteristics of MCs under Light Microscopy (*Courtesy of Mariana Castells, MD PhD, Lawrence B Schwartz, MD, and Shirley Craig, PhD*).

- **Electron Microscopy**

MCs are characterized by their cytoplasmic granules, which may also be described as secondary lysosomes. Finger-like pseudopods projecting out of the cell membrane can be detected and the round nucleus is centrally located, with densely packed peripheral chromatin. The granules are surrounded by a lipid membrane and deep invaginations of the cell membrane can be noted. When degranulating, the cell form channels with its plasma membrane, allowing granules deep within the cell to be exposed to the extracellular environment (*Image 5*)

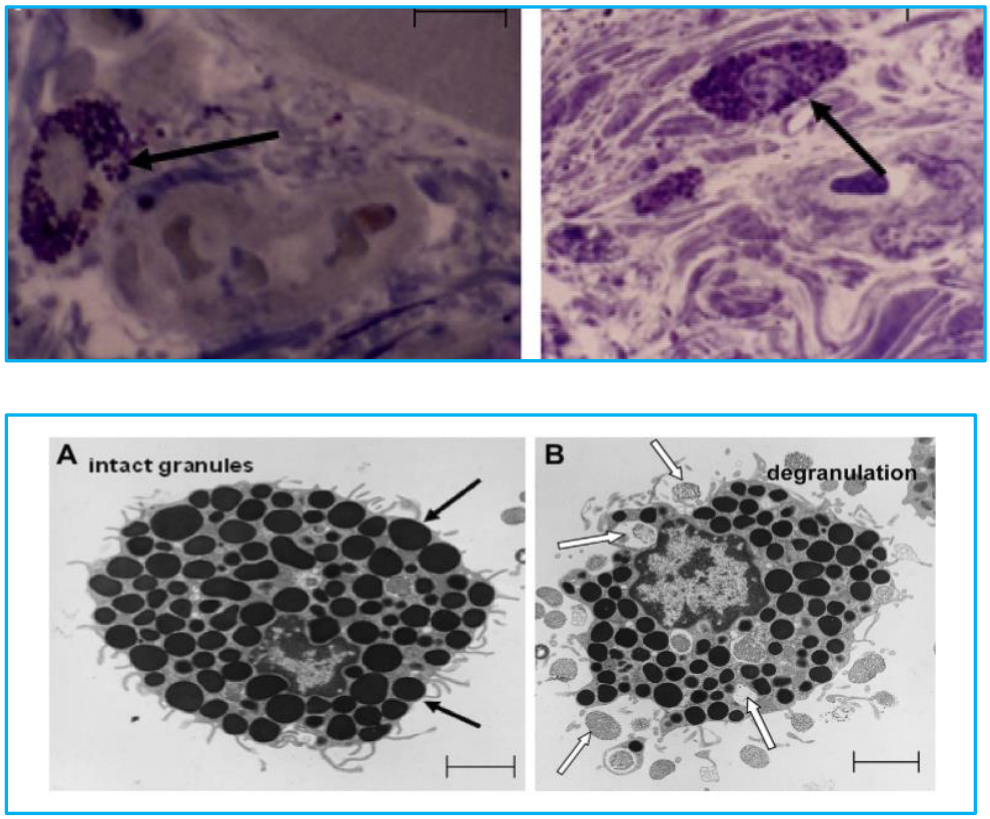


Image 5. Transmission electron microscopic photomicrographs of the buccal mucosa showing MCs degranulation. Intact (black arrows) and depleted (white arrows) secretory granules are displayed. Magnification bar ¼ 2 mm (*Images were obtained at Dr Theoharides Lab and* presented at *Alhelal et al. 2014. Ann Allergy Asthma Immunol 112 40e45*).

5. MCs Activation

MCs can be activated by several and different stimuli that exert their action through the numerous receptors on their surface, influenced by intrinsic and microenvironmental factors (Galli et al. 2005, Theoharides et al. 2000, Metcalfe et al. 2009). MCs can participate in multiple cycles of activation and can be differentially activated to release distinct mediators or cytokines, depending on the type and strength of the stimuli (Gonzalez Espinosa et al. 2003, Theoharides et al. 2012). The most studied method of MCs activation is the allergic reaction, an immune response mediated by the high affinity IgE receptor on the MCs surface (Galli et al. 2005). The kinetics of the secretory response and the amount and type of mediators released, represent the net result of positive and negative signalling events that take place at the cell surface as well as intracellularly (Turner et al. 1999, Blank et al. 2004, Galli et al. 2005).

As innate immune cells, MCs are designed for early and rapid sensing of invading microorganisms such as bacteria, parasites, fungi, and viruses. These pathogens display conserved molecular structures called PAMPs, which are recognized by PRRs, such as TLRs, on the MCs surface. This induces direct MCs activation and selective mediator release (Moreno et al. 2003). In addition, MCs can be activated by several other stimuli such as neuropeptides, cytokines, growth factors, toxins, basic compounds, complement, immune complexes, certain drugs, as well as physical stimuli such as pressure and heat (Moreno et al. 2003, Tkaczyk et al. 2004, Gilfillan et al. 2009, de Almeida Buranello et al. 2010). Although, the early events of the signalling cascades initiated by these receptors are different, they converge downstream in order to provide the necessary signals for mediator release (Gilfillan & Tkaczyk 2006).

5.1 Molecules and Receptors Implicated in MCs Activation

5.1.1. IgE and its High Affinity Receptor FcεRI

FcεRI-mediated activation is the most studied and best characterized pathway for MCs activation (Galli et al. 2005). Type I allergic reactions are mediated through FcεRI, which is highly expressed on the MCs surface and are positively regulated by increased IgE concentrations (Yamaguchi et al. 1997, Kawakami & Galli 2002). It is estimated that there are 10^3 to 10^6 FcεRI molecules on the surface of MCs (Havard et al. 2011). For MCs activation, FcεRI must be engaged by IgE and then be exposed on the cell surface to encounter antigen that will cross-link with two or more receptors. The final events of this signalling cascade culminate in MCs degranulation, lipid mediator and cytokine production (Ozawa et al. 1993, Razin et al. 1994, Metcalfe et al. 1997, Ahamed et al. 2004). Another important concept is that the amount of antigen available can modify the activation pattern induced by FcεRI signalling (Gonzalez-Espinosa et al. 2003). Stimulation of MCs with low amounts of antigen is sufficient to initiate the cascade leading to allergic inflammation. Higher amounts of antigen induce stronger stimulation and can be associated with release of mediators that can alter subsequent immune and inflammatory responses.

5.1.2. IgG and FcγR Receptors

MCs can be positively or negatively regulated by IgG multimeric receptors (FcγR) (Tkaczyk et al. 2004). These receptors enable MCs to participate in humoral defence but also endow them with the capacity to act in antibody-induced pathologies (Nigrovic & Lee 2005). The IgG-induced MCs activation provides a possible link between MCs and inflammatory diseases such as scleroderma, inflammatory bowel disease, fibrosis, vasculitis, and synovial inflammation (Woolhiser et al. 2001). Crosslinking of FcγRI and FcγRIII by IgG immune complexes induces MCs degranulation and the subsequent generation of several lipid mediators (Katz et al. 1990, Katz et al. 1992). In contrast to FcγRI and FcγRIII, which activate MCs in a similar way as FcεRI, FcγRIIB receptors, exert an inhibitory effect on MCs actions (Bochner et al. 2009).

5.1.3. Complement Factors

The anaphylatoxins C3a and C5a, products of complement activation, can cause degranulation and chemotaxis of human MCs via C3aR and C5aR receptors, respectively (Hartmann et al. 1997, Ali 2010). They induce degranulation mainly in MC_{CT} such as skin MCs, but not in lung MCs since MC_T are the predominant cell type present in the lung (Ali 2010). In addition, C3a can exert synergic effects on IgG mediated degranulation of human PDMCs and induce chemokine release from LAD2, such as MCP-1 and RANTES (Woolhiser et al. 2004, Venkatesha et al. 2005).

5.1.4. Antimicrobial Peptides

Antimicrobial peptides (AMPs), including human β-defensins, except their microbicidal activities (Niyonsaba et al. 2001), they can participate in inflammatory responses by recruiting and activating MCs. Data from both murine and human studies showed that AMPs can induce MCs prostaglandins and various cytokines production in a G protein-dependent pathway, mainly in the skin (Niyonsaba et al. 2001, 2010, Chen et al. 2007, Schiemann et al. 2009).

5.1.5. Cytokines

SCF is an essential cytokine in supporting MCs proliferation, differentiation, and survival by interacting with the c-kit receptor (CD117). It has also been widely studied as a potential activator of MCs as it was demonstrated that SCF alone could induce serotonin release from mouse peritoneal MCs (Coleman et al. 1993) and histamine release from human lung MCs (Lewis et al. 2013).

IL-9 is a pleiotropic cytokine mainly derived from activated T cells and MCs and one of its main functions is to promote MCs proliferation (Wiener et al. 2004, Shiohara & Koike 2005). A recent study showed that IL-9 alone can stimulate human LAD2 to secrete VEGF without degranulation or the release of other mediators (Sismanopoulos et al. 2012). As IL-9 and its receptor are significantly elevated locally in patients with atopic asthma and atopic dermatitis, these results suggest a pathological link between IL-9 and MCs in the development of atopic diseases (Sismanopoulos et al. 2012).

IL-33, that belongs to the IL-1 superfamily, acts as an alarmin in both host defence and allergic responses (Saluja et al. 2015). IL-33 modulates various aspects of MCs function, including adhesion, survival, maturation, and activation, through the MCs surface receptor ST2 (Gross et al. 2019, Bawazeer et al. 2019). Other mediators such as IL-5, IL-6, have also been demonstrated to augment the SCF-dependent proliferation of human cord blood derived MCs (hCBMC) (Bischoff & Sellge 2002).

5.1.6. Neuropeptides

Corticotropin-releasing hormone (CRH) is typically secreted from the hypothalamus under stress, but it can also be secreted locally from nerve endings, where it exerts pro-inflammatory effects (Alysandratos et al. 2012). Human MCs express CRH receptors, through which CRH leads to selective secretion of VEGF by HMC1 and hCBMC. (Cao et al. 2005). In addition, CRH also induces expression of FcεRI on LAD2 and hCBMC and augments IgE-induced release of VEGF (Asadi & Theoharides 2012). The CRH-induced MCs activation may contribute to the worsening of various inflammatory conditions by stress, such as multiple sclerosis, atopic dermatitis, autism spectrum disorders, psoriasis and coronary artery disease (Esposito et al. 2002, Alysandratos et al. 2012, Asadi & Theoharides 2012, Alevizos et al. 2014).

Substance P (SP) is a neuropeptide that belongs to the tachykinin family of peptides and is secreted by nerves and inflammatory cells. It has been reported to stimulate both degranulation and chemokine production from MCs (Kulka et al. 2008) and it is reported to possibly be involved in the pathogenesis of various neuro-inflammatory diseases, such as psoriasis and multiple sclerosis (Nicoletti et al. 2012). In addition, SP can enhance the innate immune response to bacterial infections by upregulating TLR2 expression on LAD2 cell line (Tancowny et al. 2010). SP can also downregulate FcεRI expression on human MCs (LAD2 and hPDMC), implying its role in the modulation of IgE-mediated allergic diseases (McCary et al. 2010).

Neurotensin is another neuropeptide secreted locally under stress. It can trigger degranulation and VEGF release from LAD2 and hCBMC human cell lines, which can be enhanced by CRH (Alysandratos et al. 2012). Interestingly, neurotensin induces expression of CRH receptor-1 on LAD2 cells and *vice versa*, CRH increases NTR expression on MCs

(Alysandratos et al. 2012). The mutual interaction between neurotensin and CRH in MCs may contribute to allergy symptoms that worsen with stress, such as in autism spectrum disorders (Theoharides 2012).

5.1.7. PRRs

MCs have the ability to express on their surface, PRRs that are able to recognize bacterial, viral, fungal, or parasitic components and host-derived molecules, called damage-associated molecular patterns (DAMPs). The PRRs are categorized in five families including: TLRs, C-type lectin-like receptors (CLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and recently designated absent-in-melanoma (AIM)-like receptors (ALRs). (Agier et al. 2018).

As previously mentioned, TLRs are the best studied and described molecules among other PRRs. They comprise a large group of transmembrane receptors expressed in various types of cells. Several TLRs have been identified to be expressed on MC surface and the type of MC and its environmental history can influence its TLR repertoire (McCurdy et al. 2001, Applequist et al. 2002). Recent data revealed that nearly every TLR might be expressed either in MC plasma membrane or inside the cell (Agier et al. 2016, 2018). In most cases, TLR activation does not lead to MCs degranulation, but distinct TLR ligands stimulate differential release of MCs-derived mediators and cytokine production (Marshall 2004). The expression of TLRs in MCs has been confirmed both at mRNA and protein level in a wide range of MCs (murine and human lines). However, it should be noted that expression of TLRs in human MCs is distinct among different locations (e.g. cord-blood, peripheral blood, skin, and lung), as some can only be detected at the mRNA level (Sandig & Bulfone-Paus 2012).

TLRs are expressed by murine MCs, with expression of TLR1-4 and TLR6-9 identified at least at the mRNA level (McCurdy et al. 2001, Supajatura et al. 2001, Masuda et al. 2002, Ikeda & Funaba 2003, Matsushima et al. 2004, Li et al. 2009, Mrabet-Dahbi et al. 2009). On the other hand, expression of TLR5 has not been shown on murine MCs (McCurdy et al. 2001, Supajatura et al. 2001, Ikeda & Funaba 2003, Matsushima et al. 2004). Expression of TLR1-10 except from TLR8 has been documented in human MCs (Marshall et al. 2003, Okumura et al. 2003, Varadaradjalou et al. 2003). TLR expression on the MCs lines LAD2, HMC-1 and

MC-9 has been assessed with varied results in different studies (McCurdy et al. 2001, Masuda et al. 2002, Kulka et al. 2004, Kubo et al. 2007, Yoshioka et al. 2007).

- **TLR2 and TLR4 ligands**

It has been reported that TLR2 and TLR4 ligands can cause differential release of mediators in both murine and human MCs. By using TLR-deficient mice, it has been demonstrated that both *in vivo* and *in vitro,* murine MCs respond to LPS through TLR4 and to bacterial peptidoglycan through TLR2 (McCurdy et al. 2001, Supajatura et al. 2001, 2002).

While PGN (a TLR2 ligand) causes mouse bone marrow-derived MCs (BMMCs) to release TNFα, IL-6, IL-4, IL-5 and IL-13, but not IL-1β, LPS (a TLR4 ligand) stimulates BMMCs to produce TNFα, IL-6, IL-13 and IL-1β, but not IL-4 and IL-5 (Supajatura et al. 2002). Furthermore, PGN can cause degranulation in both murine and human MCs, while LPS does not have this property (Supajatura et al. 2002, Varadaradjalou et al. 2003). However, human cord-blood derived MCs (hCBMC) require the priming of IL-4 and soluble CD14 to release TNF-α when stimulated by LPS (Varadaradjalou et al. 2003). Furthermore, different TLR2 stimuli have also been shown to induce release of distinct mediators by hCBMC. Both TLR2 and TLR4 can interact synergistically with FcεRI to augment activation of murine MCs, suggesting infection may contribute to allergic diseases via TLR signalling (Qiao et al. 2006). It has been observed that oxidized low-density lipoprotein (ox-LDL), via TLR4, can cause the human MCs line HMC-1 to secrete cytokines (TNF-α, MCP1 and IL-6), showing a novel pathological link between TLR4 and MCs in atherosclerosis (Meng et al. 2013). Currently, we lack direct evidence that TLR mediated signalling is responsible for lipid mediator production from MCs in response to known TLR activators, although both degranulation and cytokine production from MCs has been shown to be TLR dependent in murine (McCurdy et al. 2001, Supajatura et al. 2001, 2002) and human models (Varadaradjalou et al. 2003, Okumura et al. 2003).

Interestingly, there are limited studies examining the effect of periodontopathogenic bacteria like *P. gingivalis* on TLR activation and production of mediators, in human MCs lines.

6. MCs Secretory pathways

Constitutive exocytosis occurs in the absence of discernible stimuli and can occur throughout the lifetime of a cell. Regulated exocytosis occurs after a clearly defined stimulus, either through changes in the extracellular environment or ligation of a cell surface receptor (Spiliotis et al. 2004).

The terms degranulation, secretion, and exocytosis are often used interchangeably but have subtle variations in meaning. Degranulation refers to the loss of or release of granules and is most often associated with MCs and basophils, both of which are characterized by their large intracellular granules (Klein et al. 2019). Secretion involves the release of a substance from one place of containment to another whereas exocytosis is a process of cellular secretion in which substances contained in vesicles are discharged from the cell by fusion of the vesicular membrane with the outer cell membrane. MC exhibit all forms of these release events, but they are best known for their rapid secretion of granules (degranulation) that contain large stores of pre-formed mediators.

MCs activation can induce release of pre-formed mediators from their granules, as well as release of *de novo* synthesized lipid mediators, cytokines, and chemokines that play diverse roles, not only in allergic reactions but also in numerous physiological and pathophysiological responses. Indeed, MC release their mediators in a discriminating and chronological manner, depending upon the stimuli involved and their signalling cascades (Moon et al. 2014).

6.1. Mechanisms of Secretion of Preformed Mediators - Degranulation

Two types of degranulation have been described for MCs, piecemeal degranulation and anaphylactic degranulation (Image 6). Both occur *in vivo*, *ex vivo*, and *in vitro* in human and mice derived MCs (Dvorak et al. 1991, 1992, 1994, 1997, Ebihara et al. 2008). Anaphylactic degranulation is the explosive release of prestored granule contents or entire granules to the outside of cells after granule-to-granule and/or granule-to-plasma membrane fusion. This is the best studied degranulation mechanism that occurs after cross-linking of FcεRI receptors with IgE and relevant antigen in allergic reactions (Moon et al. 2014). It can involve a high proportion of the granules in the cell or can be more subtle. Granules exhibit signs of swelling soon after activation of the cell, then fuse with adjacent granules and ultimately

fuse with the plasma membrane and create openings through which granule contents are released into the environment.

In contrast, piecemeal secretion involves selective release of only certain mediators, and not the total spectrum of mediators stored in large granules, but the mechanisms underlying this selectivity are unknown. During piecemeal degranulation, vesicles, containing a given portion of the granular content, bud from the granule's membrane (Burgoyne et al. 2003). This is followed by transportation through the cytoplasm (without granule-to granule fusion) leading to fusion with the plasma membrane and ultimate release of mediators (Crivellato et al. 2010). Piecemeal degranulation is the most prevalent form of granule loss identified *in situ* in MCs during pathological processes in allergy (Hugle et al. 2014), Crohn's disease (Eriefalt et al. 2001), urticaria (Criado et al. 2013), chronic inflammation (Crivellato et al. 2003) or malignant tumours (Demitsu et al. 2002). PMD seems to also play a role in communication of MCs with other cell types.

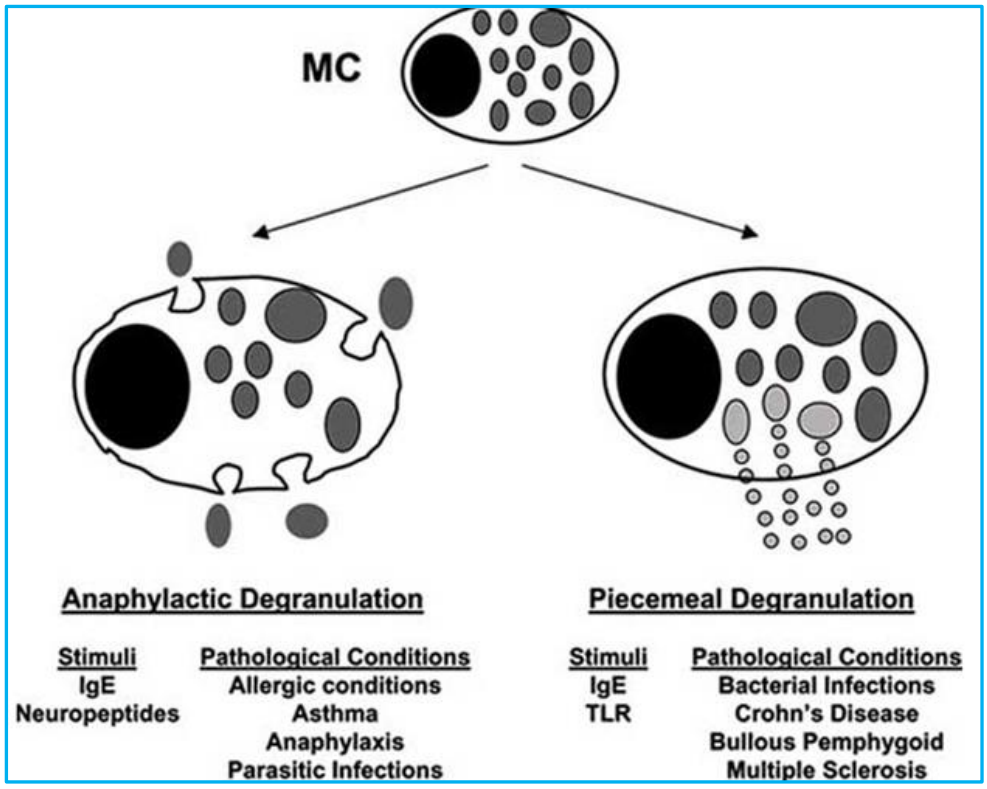


Image 6. Mechanisms of MCs Mediator Release. The 2 main mechanisms, anaphylactic and Piecemeal Degranulation are presented along with the common stimuli for each mechanism. Pathologic conditions related to each mechanism are also presented (*Image taken from*

Vliagoftis & Befus (2005) MCs at Mucosal Frontiers, Current Molecular Medicine Sep;5(6):573-89).

6.2. Selective *De Novo* Secretion of Mediators

Given the potential of MCs to synthesize many mediators following activation, it is not surprising that MCs are capable of differential secretion of selected mediators depending on the stimulus. This selectivity was originally demonstrated by the surprising observation that histamine and serotonin could be differentially released (Theoharides 1982, Vilagoftis et al. 1990), even though they are apparently both stored in the same granules. More recent studies have emphasized the ability of MCs to release only selected cytokines, chemokines, and arachidonic acid metabolites depending on the activation stimulus. This differential mediator secretion may act at the level of signalling pathways that induce gene expression and/or synthesis of a specific cytokine, chemokine, or metabolite, as many of these are not stored, but synthesized *de novo* following cell activation. Some excellent examples of this kind of differential mediator secretion by MCs have been demonstrated using among others, different TLR ligands, c-kit-mediated MC activation (Gagari et al. 1997) and IL-1-mediated activation (Kandere Grzybowska et al. 2003).

6.3. MCs Recovery Following Degranulation

One of the characteristics of MCs activation, shared with basophils and distinguishes MCs from many other granule-containing immune cells, is the fact that they can recover after release of their granule-associated mediators (Dvorak et al. 1987). During this process, MCs re-synthesize granules and their components. The recovery may take one to several days, and the mechanisms that initiate this process following activation instead of apoptotic pathways are still not fully understood (Xiang et al. 2001).

7. MCs Mediators

The result of MCs activation is the release of a wide variety of pro-inflammatory and vasoactive substances into the extracellular environment. These include mediators constitutively stored inside the cytoplasmic granules of MCs (primary MC mediators), mediators synthesized *de novo* upon MC activation (secondary MC mediators), and diverse cytokines which results in a broad spectrum of clinical manifestations (Castells et al. 2006, Ogawa et al. 2007) (Table 2 adapted from Theoharides et al. 2012).

The release of preformed mediators from MCs occurs in the early phase of the immune response, a few seconds, or minutes after the contact with the antigen. These preformed mediators include biogenic amines, proteases, proteoglycans, and inflammatory cytokines. This phase is followed by the release of mediators newly synthesised which include prostaglandins, leukotrienes (LTs), and platelet activating factor (PAF), as well as a variety of cytokines and chemokines that facilitate the activation and recruitment of other cells of the immune system(González-de-Olano et al. 2018).

7.1. Preformed Mediators

MCs store an extensive variety of preformed mediators in their secretory granules. Preformed granules of MCs contain biogenic amins, proteoglycans, proteases, lysosomal enzymes, cytokines, and growth factors (Wernersson et al. 2014).

- **Biogenic Amins**

Histamine and serotonin are the most important vasoactive mediators released from human MCs. Histamine has high diffusion capacity once it is secreted and fulfils different biological functions after binding to specific receptors (H1, H2, H3, and H4). The most relevant effects of histamine are commonly associated with allergic and inflammatory reactions (Lundequist & Pejler 2011). They include contraction of smooth muscle tissue, vasodilation, increased vascular permeability, nerve stimulation, and increased glandular secretion (Repka-Ramirez et al. 2003, O'Mahoni et al. 2011). The presence of serotonin in human MCs was demonstrated in human peripheral blood, where its levels are elevated in patients with mastocytosis (Kushnir-Sukhov et al. 2007, Theoharides & Tsilioni 2019).

- **Proteoglycans**

MCs glycosaminoglycans/proteoglycans include heparin, chondroitin and serglycin are responsible for the metachromatic staining of MCs (Abrink et al. 2004). The main function of proteoglycans stored in the secretory granules of MCs such as heparin and chondroitin sulphate is to form stable complexes with other MC mediators, thus facilitating their storage and their transportation through the lymphatic vessels (Serafin et al. 1986).

- **Proteases**

MCs proteases are stored within MCs granules as active enzymes and constitute approximately 25% of MCs protein content (Huang et al. 2000). Chymases, tryptases, and carboxypeptidase A are exclusively expressed by MCs with tryptase being the most abundant MCs protease. Measurements of total tryptase in human serum has been proven to be a useful tool in the diagnostic work-up of both anaphylaxis and mastocytosis (Schwartz et al. 1994). The main biological effects of proteases on the human body include contraction of smooth muscle (Johnson et al. 1997), degradation of neuropeptides (Caughey et al. 1988, Tam et al. 1990), activation of collagenase (Birkedal-Hamsen et al. 1976), proliferation of fibroblasts, generation of C3a and bradykinin (Imamura et al. 1996, Fukuoka et al. 2008), and inactivation of fibrinogen (Prieto-Garcia et al. 2014). They are also capable of promoting the recruitment of other immune cells, actively participates in remodelling processes and angiogenesis, and exerts a protective function against the potential damaging effect of substances generated during the inflammation process, such as neurotensin and endothelin (Blair et al. 1997, Douaiher et al 2014)

- **Lysosomal Enzymes**

β-hexosaminidase is the best characterized of these enzymes and is ubiquitous to all MCs subtypes in all species. Quantification of the released β-hexosaminidase activity is often used as a measure of MC degranulation (Lundequist & Pejler 2011).

- **Cytokines-Growth Factors**

MCs are the only cells known to store preformed TNF-α in their cytoplasmic granules and release it upon activation (Olszewski et al. 2006, 2007). Among the large number of cytokines and chemokines released after MCs activation β-FGF, IL-4, and SCF are also known to be pre-

stored in MC granules and can be released by regulated exocytosis (Wilson et al. 2000, Dvorak et al. 2001). These molecules are involved in the activation of other cell types, in induction of inflammation, in the development of immune and adaptive response during infection, in pathogenesis of allergy and in development of autoimmune diseases (Metz et al. 2007, Sutherland et al. 2011,).

7.2. *De Novo* Synthesized MCs Mediators

In the late phase of the immune response that typically occurs between 2 and 6 hours after exposure to a trigger, diverse mediators newly synthesised from membrane phospholipids as well as a variety of cytokines and chemokines that facilitate the activation and recruitment of other cells of the immune system are released from MCs.

- **Lipid mediators (Eicosanoids), Cytokines, Chemokines**

MCs activation induces the synthesis and further release of pro-inflammatory lipid mediators such as eicosanoids and PAF. The process of synthesis of these mediators begins with the activation of phospholipase A2, which promotes the generation of arachidonic acid (AA) from phospholipids present on the MCs membrane (Fujishima et al. 1999, Nakatani et al. 2000). Once generated, AA can be metabolized by the action of 2 enzymes, cyclooxygenase (COX) and lipoxygenase (LO), resulting in the production of prostaglandins (PGs) and leukotrienes (LTs), respectively (Schleiner et al. 1985). Prostaglandin D2, E2 (PGD2, PGE2), leukotriene B4, C4 (LTB4, LTC4) and PAF are the most common mediators in this category (Boyce 2005, 2007).

MCs released eicosanoids participate in the regulation of vascular permeability, smooth muscle contraction and recruitment of immune effector cells. Specifically, prostaglandins contribute to increased vascular permeability, leukocyte recruitment, mucus production, and nerve cell activation (Galli et al. 2005, Weller et al. 2007). PGD2, the main mediator generated upon activation of MCs, has a potent vasodilatory effect, increases vascular permeability (Morrow et al. 1994), and promotes chemotaxis of eosinophils (Hirrai et al. 2001, Fujitani et al. 2002). Leukotrienes function locally on the vascular endothelium by promoting rolling and recruitment of neutrophils and eosinophils, which contribute to host defence against bacterial infections (Malaviya and Abraham 2000, Carlos et al. 2011). LTB4 is secreted in small quantities by activated MCs and has an important role in the recruitment

of neutrophils, eosinophils, and effector T lymphocytes (Goodarzi et al. 2003, Carlos et al. 2011).

PAF is a potent mediator capable of acting at low concentrations and has a very short half-life. It is implicated directly, in a variety of symptoms such as bronchoconstriction, mucous secretion, vasodilation, increased vascular permeability, and platelet aggregation (Korth et al. 1986). Besides these effects, PAF indirectly participates in the inflammatory response through the activation and chemotaxis of leukocytes and through the induction of release of other mediators by MCs and platelets (Spina et al. 1989).

Like other cells of the immune system, MCs produce a wide variety of cytokines and chemokines, which are synthesized *de novo* after activation of MCs and are further released into the extracellular medium (Mukai et al. 2018). These mediators are synthesized after transcriptional activation, as the result of MCs activation. Their regulation depends on the type of stimuli as well as the specific receptor involved in the activation. MCs synthesize and release both pro-inflammatory and anti-inflammatory cytokines (Gessner et al. 2005, Kohno et al. 2005, Hsu et al. 2010, Nam et al. 2011).

The following Table, summarizes the mediators, preformed and *de novo* synthetized, that are released from MCs after their stimulation (adapted from Theoharides et al. 2013).

Table 2	**MCs Mediators**	
Preformed	Biogenic Amins	Histamine, Serotonin, Dopamine, Polyamines
	Proteases	Chymase, Tryptase, Carboxypeptidase A, Cathepsin G, Granzyme B, Matrix metalloproteinases, Renin
	Proteoglycans	Serglycin, Heparin, Chondroitin sulphate
	Lysosomal Enzymes	β-hexosaminidase, β-glucuronidase, β-D-galactosidase
	Cytokines	TNF, IL-4, IL-15
	Growth Factors	TGF-β, bFGF-2, VEGF, NGF, SCF
***De novo* Synthesis**	Phosholipid Metabolites	PGD2, PGE2, LTB4, LTC4, PAF
	Cytokines	IL-33, IL-10, IL-12, IL-17, IL-5, IL-13, IL-1, IL-2, IL-3, IL-4, IL-6, IL-8, IL-9, IL-16, Type I and Type II IFN, TNF-α, MIP-2β
	Chemokines	CCL2, CCL3, CCL4, CCL5, CXCL8
	Growth Factors	SCF, GM-CSF, β-FGF, NGF, PDGF, TGF-β, VEG

8. MCs Functions

MCs are present throughout the body and they play important roles in the maintenance of many physiological functions as well as in the pathophysiology of diseases. MCs are involved in the regulation of vasodilation, angiogenesis, bacterial, and parasite elimination, inflammatory process (Maurer et al. 2004, Gali et al. 2005, 2010, Abraham et al. 2010), as well as in autoimmunity (Rottem et al. 2005). In addition, MCs regulate functions of many cell types, such as dendritic cells, macrophages, T cells, B cells, fibroblasts, eosinophils, endothelial cells, and epithelial cells. Since, MCs generate and release multi-potent molecules, such as histamine, proteases, prostanoids, leukotrienes, heparin, and many cytokines, chemokines, and growth factors, they have the capacity to be involved in regulating the functions of many organs and tissues (Krystel-Whittemore et al. 2015). One of the mostly studied functions of the MCs is its role in vascular and bronchial homeostasis. MCs also play a significant role in the regulation of bone growth, remodelling, and mineral homeostasis (Weller et al. 2006).

8.1. Angiogenesis

Angiogenesis, i.e., the formation of new vessels from pre-existing ones – such as capillaries and post-capillary venules – plays a pivotal role during embryonic development (Ribatti et al. 2006). Later, in adult life, angiogenesis occurs in several physiological and pathological conditions, such as tumour growth and chronic inflammation, where it may contribute to the progression of disease (Ribatti et al. 2013). Under physiological conditions, angiogenesis is dependent on the balance of positive and negative modulators within the vascular microenvironment and requires the functional activities of a number of molecules, including angiogenic factors, extracellular matrix proteins, adhesion receptors, and proteolytic enzymes (Hanahan et al. 1996). MCs can produce a large spectrum of pro-angiogenic factors such as VEGF, FGF, TGF, TNF-a, and IL-8 and can modulate angiogenesis in physiological conditions (Theoharides et al. 2012)

Pathological angiogenesis is linked to a switch in the balance between positive and negative regulators and mainly depends on the release by inflammatory or neoplastic cells, of specific growth factors, that stimulate the growth of the host's blood vessels (Ribatti et al. 2008). There is increasing evidence to support the view that angiogenesis and inflammation are

mutually dependent. During inflammatory reactions, immune cells including MCs, synthesize and secrete pro-angiogenic factors that promote neovascularization. On the other hand, the newly formed vascular supply contributes to the perpetuation of inflammation by promoting the migration of inflammatory cells to the site of inflammation (Ribatti et al. 2009). MCs have the ability to release proteases and histamine that induce the permeability of the microvasculature and lead to further angiogenesis. Following IgE-dependent activation, MCs release several pro-angiogenic mediators stored in their granules, such as VEGF and FGF-2 (Kranzhöfer et al. 1999), that promote angiogenesis even in the early phase of allergic inflammation. There is also evidence that the link between MCs and tumour growth in cancer, is based on the ability of MCs to release potent pro-angiogenic factors (Ribatti et al. 2012).

8.2. Homeostasis

Due to their location in the skin and mucosa, MCs serve as first line of defence against pathogens entering the body (Bulfone-Paus et al. 2015). MCs are especially important in the homeostasis of the commensal bacteria of the gut (Goto et al. 2015). The digestive system is constantly exposed to different pathogenic stimuli, such as and food antigens. The epithelial cells that line the digestive system serve as a barrier to these antigens. The contributions of MCs to mucosal barrier control are exerted through mechanisms that control and modulate the epithelial function and the innate and adaptive defensive responses (Bayo et al. 2019)

In addition, it was documented that MCs are important in the homoeostasis of organs that undergo continuous growth and remodelling such as hair follicles and bone. MCs derived IL-1, TGF-β, IL-6 and histamine can influence the recruitment and development of osteoclastic cells. Recently, MCs were found to be able to produce OPN, a secreted glycoprotein that controls bone metabolism and has a role in immune responses (Nagasaka et al. 2008).

8.3. Wound healing

MCs are involved in wound healing starting from the initial inflammatory response, followed by re-epithelialization and revascularization of the affected tissue and finally resulting in the resolution phase characterized by deposition of collagen and re-modelling of the matrix (Abe at al. 2000, Noli et al. 2001). Recently, it was demonstrated that MCs deficient mice exhibit

impaired wound healing, conclusively proving the role of these cells in the maintenance of tissue homeostasis (Weller et al. 2006).

8.4. Host Defence against Pathogens

MCs play crucial role in host defence by modulating both innate and adaptive immune responses (Abraham et al. 1997, Galli et al. 1999, Henz et al. 2001). Increasing amount of recent evidence has demonstrated that MCs may play a critical role in host immune defence against bacteria (Malaviya et al. 1996, Abraham et al. 2010). The earliest evidence came from studies on MCs deficient mice (W/Wv mice), which showed increased mortality after intra-peritoneal injection of *E. coli* than normal counterparts (Mannel et al. 1996). This led to the question how MCs recognize the invading Gram-negative bacteria. It has been well documented that MCs recognize invading pathogens basically through two distinct mechanisms, one which is opsonin-dependent (indirect recognition) and the other which does not require opsonin (direct recognition) through implementation of both innate and adaptive immunity (Trivedi et al. 2013).

8.4.1. Innate Immune Responses

Direct evidence of the role for MCs in host defence against bacterial pathogens did not become available until the mid-1990s after studies indicated that MCs could produce cytokines in response to LPS without degranulation (Leal-Berumen et al. 1994). As resident cells within the tissues that interface with the external environment, such as the skin, airways, and intestine, MCs are well placed to initiate and enhance early responses to a variety of challenges adding to the concept that the MCs serves as a critical mobilizer of innate immune responses through early mediator production (Galli et al. 1991). One of the most significant roles of MCs in innate immunity is the ability to rapidly recruit neutrophils and other cell types to the site of infection by releasing histamine, TNF, VEGF and proteases that contribute to increased local vascular permeability and oedema at the site of infection (Boesiger et al. 1998, Gordon et al. 1990). MCs are likely to be particularly effective in aiding cell recruitment in view of their strategic association with blood vessels, an anatomic relationship that occurs throughout the body. MCs secrete a wide variety of chemo-attractants and cytokines that promote leukocyte trafficking to sites of infection, where they

can contribute to pathogen clearance or amplify inflammation by secretion of pro-inflammatory cytokines (Marshall et al. 2004).

One of the most important cytokines secreted is TNF-α, which plays an important role in the trafficking of neutrophils to sites of MCs activation in order to initiate bacterial clearance (Malaviya et al. 1996). Few minutes after MCs exposure to certain types of bacteria, TNF-α, histamine, and proteases are released leading to enhanced vascular permeability, increase adhesion molecule expression and recruitment of both neutrophils and eosinophils to sites of inflammation (Schmidlin et al. 2001). MCs production of TNF, initially through limited preformed stores and then rapidly newly generated, plays a critical role in this process (Malaviya et al. 1996). MCs derived TNF has been reported to promote neutrophil recruitment in bacterial peritonitis models and other inflamed tissues (Huang et al. 1998, Sutherland et al. 2008). Even in the absence of degranulation, certain bacteria, have been demonstrated to induce production of multiple cytokines and chemokines from human MCs either in the early stages of response or later on (over a longer time course of 24 to 48 hours) associated with the long-term recruitment of immature dendritic cells and T cells (Mukai et al. 2018). Chemokine production by MCs has been implicated in the recruitment of other participants in the inflammatory response, including eosinophils and natural killer (NK) cells, through CC-chemokine ligand 11 and IL-8 respectively (Burke et al. 2008)

MCs also synthesize LTs, such as LTB4 and LTC4, in response to bacterial challenge, which upregulate P-selectin on endothelial cells, enhancing the rolling phase of the leukocyte adhesion cascade (Kanwar et al. 1995). The activity of leukotrienes has been demonstrated to facilitate neutrophil recruitment and bacterial clearance in an infectious peritonitis model (Malaviya et al. 2000).

Another function of MCs in innate host defence is their direct antimicrobial activity based on the production of antimicrobial peptides. Cathelicidins and defensins are major families of antimicrobial peptides and their function is to break the integrity of the microbial membrane by binding to negatively charged microbes. The human cathelicidin (LL-37) and the murine cathelicidin-related antimicrobial peptide (CRAMP) are both expressed by MCs cells. LPS activation of MCs has been shown to induce expression of CRAMP mRNA more than 6-fold, as well as to increase its secretion at the protein level (Di Nardo et al 2003).

In addition, MCs are able to endocytose enterobacteria, such as *E. coli*, through the cell surface molecule CD48 (Malaviya et al. 1994, 1999). Endocytosed bacteria are then killed through oxidative burst as MCs produce compounds to aid bacterial killing after phagocytosis, including reactive oxygen species (Abraham et al. 1997). There is also evidence that MCs, like neutrophils, can trap and kill bacteria via extracellular trap formation (MCs extracellular traps) (von Kockritz-Blickwede et al. 2008). However, due to their low numbers at sites of infection, relative to the large numbers of recruited professional phagocytes and other immune cells, it is unlikely that MCs contribute significantly to direct bacterial killing *in vivo*.

8.4.2. Adaptive Immune Responses

After stimulation, MCs shape the inflammatory milieu and control the activation state of many cells crucial for adaptive immunity (Cardamone et al. 2016). Regarding T cells, MCs promote their migration by directly releasing chemotactic factors (i.e. MCP-1, MIP-1, RANTES, and LTB4) (Nakajima et al. 2002, Lin et al. 2003, Ott et al. 2003), or indirectly by up regulating expression of adhesion molecules on endothelial cells (Metcalfe et al. 1997, Mekori & Metcalfe 1999, Marshall 2004). Also, MCs can affect B cells by supporting their survival, proliferation and immunoglobulin A synthesis (Merluzzi et al. 2010).

Another property of MCs is to act as antigen presenting cells as they can express major MHC molecules. *In vitro* studies showed that MCs could directly interact with T cell receptors to induce antigen-specific clonal expansion of T cell populations along with their polarization towards Th1, Th2 immune responses (Mekori & Metcalfe 1999, Henz et al. 2001). MHC class I molecules are expressed on mouse BMCMCs and on human MCs from lung, liver, uterus, and skin (Henze et al. 2001).

Together with local responses to pathogens, MCs have long-distance and long-term effects in the host by modulating draining lymph nodes and promoting the development of adaptive immunity to pathogens. MCs secretion of TNF recruits T cells and DCs to draining lymph nodes within 24 h after bacterial infection (McLachlan et al. 2003). Interestingly, peripherally located MCs can also direct distal responses in lymph nodes by the release inflammatory mediators. These insoluble particles drain via the lymphatics to local lymph nodes, transporting MCs-derived TNF where it is slowly released (Kunder et al. 2009). MCs may also

induce DC migration to the lymph node in a TNF-independent histamine and IL-6-dependent manner when stimulated with bacterial peptidoglycan (Dawicki et al. 2010). Thus, following infection at peripheral sites, MCs serve to kick-start adaptive immunity by promoting the coordinate trafficking of professional APCs from infection site into the draining lymph nodes and simultaneously sequestering T lymphocytes to facilitate optimal antigen presentation.

8.5. MCs and Nervous System

A major component of the regulatory function of MCs is their role in connecting the nervous and immune system (Theoharides et al. 2017, 2019). MCs can be activated by neuropeptides allowing neural control of innate and adaptive immunity. Conversely, MCs secrete inflammatory mediators including neurotransmitters and neurotrophic factors that directly influence nerves, causing acute activation and/or long-lasting changes in excitability and phenotype (Theoharides 1990, Forsythe 2019).

MCs are located around nerve endings in various tissues including skin, intestinal mucosa, lung, and the central nervous system. Specifically, MCs are located perivascular, in close proximity to neurons in the leptomeninges and hypothalamus where they contain most of the brain histamine (Rozniecki et al. 1999, Alstadhaug 2014). In fact, they are located adjacent to corticotropin-releasing factor (CRF)-positive neurons in the rat median eminence and could contribute to neuroinflammatory diseases (Theoharides et al. 1995, Theoharides & Cochrane 2004). This localization and, more importantly, the mediators released by both MCs and neurons collaborate in the establishment of a neuroimmune interaction between these cells.

The somatosensory system is a prime example of the MC–nerve functional unit in action. MCs are important first responders in protective pain responses that provoke withdrawal from noxious environmental stimuli. The activation of MCs, in response to immunologic, chemical, or physical stimuli and the consequent release of molecules that actuate/sensitize nociceptors directly contribute to pain sensation (Kulka et al. 2008).

MCs express receptors for a range of neurotransmitters (such as SP, neurotensin), and consequently can be modulated by them (Masini et al. 1985). Neural stimulation of MCs is an important component of neurogenic inflammation where often released neurotransmitters, in addition to directly stimulating vasodilation and plasma extravasation,

cause MCs degranulation. The released granule-derived mediators contribute to the inflammatory response while also activating nociceptors causing further neuropeptide release (Vincent et al. 2013).

Psychological stress can also induce MCs degranulation particularly in the diencephalon and cerebellum where they are most abundant, an effect mediated largely by CRH and/or substance P, and results on stress induced exacerbation of inflammatory conditions (Theoharides et al. 2004, Theoharides & Konstantinidou 2007). Substance P is the most comprehensively studied mediator of neural communication and serves as an exemplar of neurotransmitter signalling in MCs. MCs activation induced by substance P is proposed to be critical to neurogenic inflammation associated with a range of disorders including allergic diseases (e.g., asthma, rhinitis, atopic dermatitis), rheumatoid arthritis (Okamura et al. 2007), and atherosclerosis (Bot et al. 2010). In addition to triggering degranulation (Piotrowski & Foreman et al. 1985, van der Kleij et al. 2003), substance P has also been reported to induce differential production and secretion of cytokines including TNF, IL-6 and lipid mediators such as prostaglandin D2 and leukotriene C4 in the absence of degranulation (Karimi et al. 2004, Azzolina et al. 2003). At concentrations much lower than those required to induce degranulation, substance P can 'prime' MCs, lowering the threshold for activation in response to subsequent stimuli (Janiszewski et al. 1999)

However, while interactions between MCs and all branches of the nervous system have been documented for decades, the full extent to which such interactions influence health and disease is still obscure. There is still much to be learned, for instance, about how combinations of neurotransmitters encode distinct MCs responses to regulate innate and adaptive immunity.

8.6. MCs and Oral Inflammation

Increasing evidence indicates that MCs are critical for the pathogenesis of inflammatory diseases (Theoharides et al. 1996, 2004) such as arthritis (Wooley 2003), atopic dermatitis, psoriasis (Harvmima et al. 1993, Ozdamar et al. 1996), multiple sclerosis (Theoharides 1990) and oral inflammation including periodontal diseases (Walsh 2003, Kamal et al. 2015).

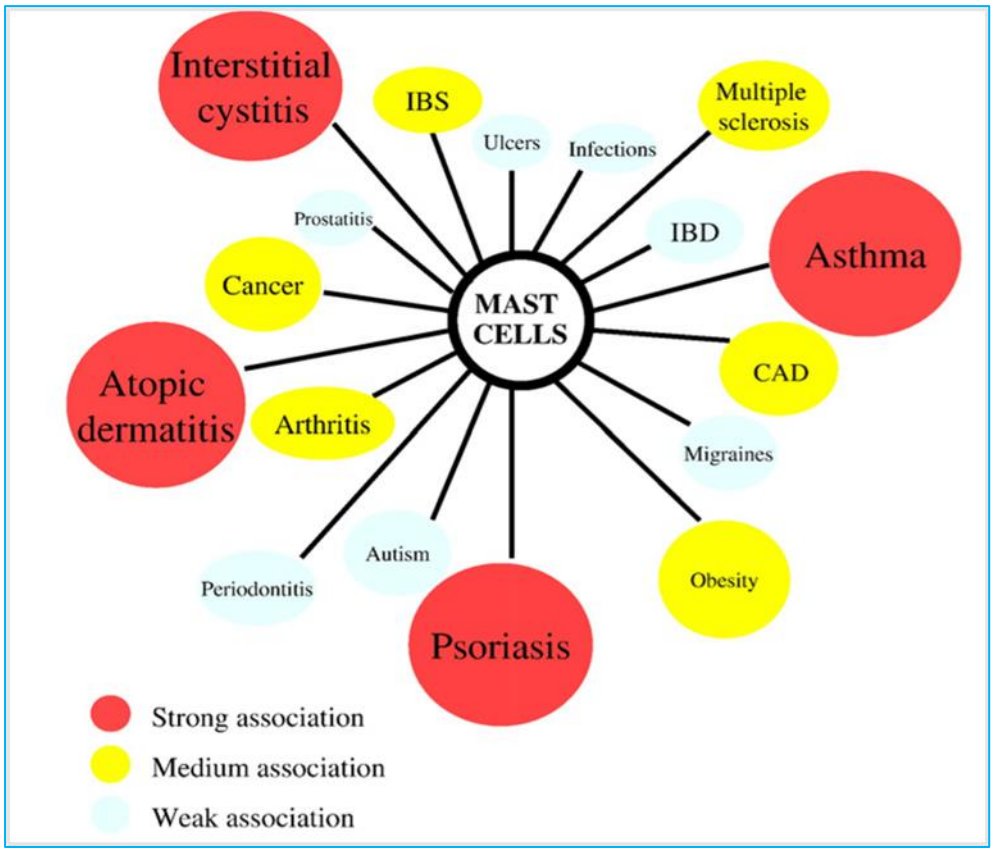


Image 7. MCs involvement in inflammatory diseases. Increasing evidence indicates MCs are involved in many diseases. Colors indicate the strength of the association (red = strongest, white = weakest). CAD, coronary artery disease; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome (*Theoharides TC et al, 2012. MCs and Inflammation. Biochimica et Biophysica Acta 1822L; 21–33*)

MCs are found in the majority of the tissues in the oral cavity, including the dental pulp and the periodontal tissues (Kamal et al. 2015). Mucosal MCs together with T cells and other cells constitute a network that can orchestrate protective immunity or harmful inflammation, processes known to be modulated by the nervous system (Cekici et al. 2014). MCs are seen scattered throughout gingival connective tissues, often in close association with endothelial

cells, but are also found sub- and intra-epithelially (Lin & Befus 1999, Steinsvoll et al. 1999, Walsh et al. 1995). They are distributed preferentially around the microvascular bed, close to the basement membrane of blood vascular endothelial cells and nerves (Mekori & Metcalfe 1999, Walsh et al. 1990).

An increase in numbers of MCs was found in reactive lesions such as fibroma, peripheral giant cell granuloma and inflammatory fibrous hyperplasia compared with normal gingival tissues (Farahani et al. 2010). In addition, MCs were found to be increased in periapical cysts and were more numerous in regions of active inflammation (Kamal et al. 2020). Moreover, MCs numbers were increased in recurrent aphthous ulcers of the oral mucosa and in premalignant lesions and conditions such as leucoplakia as they showed signs of activation or degranulation suggesting active involvement in their pathogenesis (Sudhakar et al. 2005).

MCs are widely distributed in periodontal tissues in close apposition to immune cells including leucocytes and plasma cells as well as to cells of the connective tissue such as fibroblasts (Batista et al. 2005). MCs have been reported to reside close to T cells, to phagocytose and process bacterial antigens and to initiate acquired immune responses by presenting antigens to T cells (Nakae et al. 2005). In addition, MCs can secrete both Th1-type and Th2-type cytokines influencing the differentiation of T cells *in vitro*. It is known that Th1 cells are associated with the stable lesions of periodontitis whereas Th2 cells are associated with the progressive ones (Yamazaki et al. 2003). MCs express both MHC class I and class II molecules and the CD80, CD86 and CD54 molecules that serve as the second signal for T lymphocyte activation during antigen presentation (Frandji et al. 1996). The resultant T-cell activation would activate MCs, leading to both degranulation and cytokine release contributing to further periodontal tissue destruction. Another interesting finding is the intercellular interactions between MCs and fibroblasts. Fibroblasts are the most abundant cells of soft periodontal connective tissues. While their principal roles are the synthesis and remodelling of extracellular matrix proteins, they also participate in the inflammatory response through their production of inflammatory-regulating cytokines. It was shown that MCs through the formation of gap junctions with fibroblasts could increase the expression of molecules like IL-8, which enhances neutrophil chemotaxis and promotes the inflammatory response (Termei et al. 2013).

Recently, it was found that MCs strongly express MMPs that play important role in cell migration, wound healing and tissue remodelling, and have pathogenic roles in infectious diseases such as periodontitis (Steinsvoll et al. 2004, Su-Jin Ahn et al. 2014). MMPs are key enzymes in degradation of periodontal tissues by degrade periodontal ligamental attachment and bone matrix proteins (Reynolds 2000). Gingival MCs were found to strongly express MMP-1 and MMP-8 while about half expressed MMP-2 (Girolamo & Wakefield 2000). In addition, strong experimental evidence exists for a relationship between MCs and pathogenesis of bone turnover. MCs deficiency is associated with a low remodelling state, while their excess is associated with accelerated bone loss, property associated with periodontal destruction (Cekici et al. 2014).

The nervous system has been identified as a critical regulator of inflammation in periodontal disease (Castro et al. 2020, Papathanasiou et al. 2013). With the identification of neuropeptides in GCF, it is becoming increasingly evident that periodontitis and other orofacial inflammatory disorders may be modulated by imbalances in certain neuropeptides (Cekici et al. 2014). As MCs reside close to neurons and when activated they produce a vast array of neuropeptides, it is evident that they may play an important role in modulating neuro-inflammation related to periodontal disease.

A lot of studies have been undertaken to identify and quantify the presence of MCs in different stages of human periodontal disease using histochemical (toluidine blue) and immunohistochemical (tryptase-positive MCs) techniques. So far, there have been contradictive reports about MCs numbers in human periodontitis; some reported that MCs numbers are decreased in chronic periodontitis compared with healthy/gingivitis lesions (Robinson et al. 1972) whereas others reported that they are increased in periodontitis (Batista et al. 2005)

Initial attempts to find quantitative relationship between MCs and gingival health, reported increased number of MCs in inflamed gingiva when compared to healthy tissues (Zachrisson et al. 1968, Zachrisson et al. 1969). In more recent studies, the variations, and alterations in MCs densities in human samples of dental plaque-induced gingivitis and localized chronic periodontitis were analysed. It was noticed that MCs densities (cells per mm^2) were significantly increased in chronic periodontitis/gingivitis lesions compared with clinically healthy gingival tissues. Interestingly, MCs were distributed specifically in close apposition

to mononuclear cells (Batista et al. 2005). In similar studies, MCs numbers and their density were significantly increased in human moderate and advanced periodontitis compared to healthy controls. The numbers of MCs were equal to and often surmounting the numbers of macrophages in the inflamed periodontal lesion (Arzi et al. 2010, Huang et al. 2013, 2014, 2018).

Based on these results, the increase of MCs *in vivo* has called attention with respect to the possible participation of MCs in the defence mechanism and destructive events in periodontitis, as well as the possible functional relationship between MCs and immuno-competent cell populations in periodontal lesions.

Even if a lot of studies have been conducted, concerning the number of MCs in healthy or periodontally affected gingival tissues, little is known about the effect of periodontally involved Gram-negative bacteria (such as *P. gingivalis*) on MCs stimulation. To the best of our knowledge this is the first *in vitro* research study focusing on the differential effects of *P. gingivalis* LPS on MCs stimulation and release of mediators implicated in periodontal disease progression.

SPECIFIC AIMS

1. BACKGROUND

The laboratory techniques used in our study to measure MCs mediator release on a mRNA and protein level, from different samples where the Enzyme-Linked Immunosorbent Assay (Sandwich ELISA) and the Quantitative Polymerase Chain Reaction (qPCR).

1.1. Enzyme-Linked Immunosorbent Assay (ELISA)

- **Definition**

Elisa is a quantitative analytical method that shows antigen–antibody reactions through the colour change obtained by using an enzyme-linked conjugate and enzyme substrate. It serves to identify the presence and concentration of molecules in biological fluids. It was first described in 1971 (Engvall & Perlmann 1971) and has been used as a diagnostic tool in medicine, plant pathology, and biotechnology, as well as a quality control check in various industries.

- **Principle**

In ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measurable product. Light absorption of the product formed after substrate addition is measured and converted to numeric values. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

1.2. Quantitative Polymerase Chain Reaction (qPCR)

- **Definition**

PCR is a method to spectacularly amplify a desired DNA fragment (piece of DNA) to increase the target DNA to detectable levels (Kary Mullis 1983). Using PCR, copies of very small amounts of DNA sequences are exponentially amplified in a series of cycles of temperature changes. The majority of PCR methods rely on thermal cycling.

1.3. Reverse Transcription- Quantitative Polymerase Chain Reaction (RT-qPCR)

In many applications of PCR, the starting template material is not DNA, but indeed RNA. As RNA is single-stranded and very unstable, it makes it difficult to work with. Since it cannot serve as a template for PCR, reverse transcription is combined with PCR to make RNA into a

complementary DNA (cDNA) suitable for PCR. The combination of techniques is colloquially referred to as RT-PCR. In our study, the RT-qPCR was used.

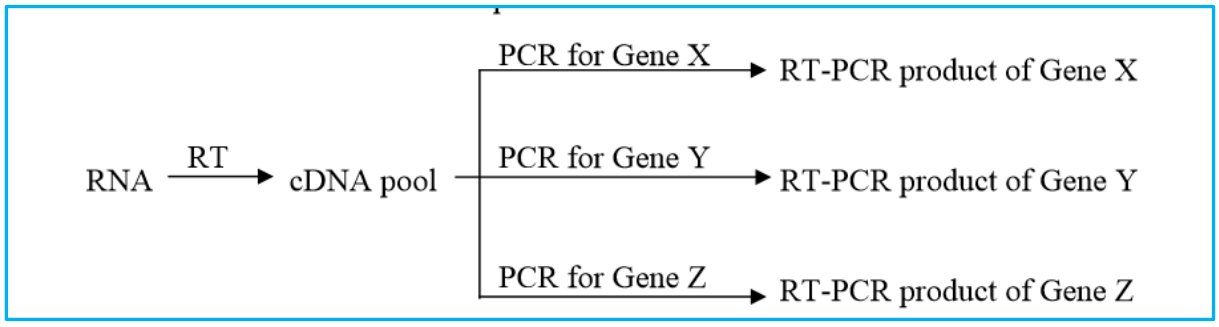
The RT-qPCR can be performed by using 2 different protocols: the one-step and two-step protocol.

- **One-step protocol**

The components of RT and PCR are mixed in a single tube at the same time. The one-step protocol generally works well for amplifying targets that are reasonably abundant.

- **Two-steps protocol**

The two-step protocol is usually more sensitive than the one-step method; yields of rare targets may be improved by using the two-step procedure (this protocol was used in our study).



2. MATERIALS AND METHODS

2.1. Culture of Human MCs

➢ **LAD2 Cell Line**

The cell line that was used for all the experiments was the human LAD2 cell line. These cells were derived from a human MC leukemia patient and were kindly supplied by Dr. Kirshenbaum from the National Institute of Health, Bethesda, Maryland, USA.

The cells were cultured in specific cell culture flasks and were maintained at 37°C in a humidified incubator at 5% CO_2. StemPro-34 medium (Invitrogen, Carlsbad, California, USA) supplemented with 100 U/mL of penicillin and 100 U/mL streptomycin, LBP was added to the cultured cells. In addition, a growth factor, the recombinant human stem cell factor (100 ng/mL rhSCF) also named mast cell growth factor or *kit*-ligand, responsible for mast cell growth and survival (Swedish Orphan Biovitrum AB, Stockholm, Sweden) was added to the cultured cells (Grabe at al. 1994). All cells were used for the experiments, during their logarithmic growth period.

➢ **Substances used to trigger/stimulate the LAD2 cells**

- SP, Sigma-Aldrich (St. Louis, MO, USA), was diluted in Milli-Q water, and a stock solution (10mM) was prepared.
- Commercially available standard LPS preparation of *P. gingivalis*, concentration of 1 mg/mL (Invivogen, San Diego, CA).
- Commercially available *E coli* 0111: B4 LPS from Sigma-Aldrich, concentration of 1 mg/mL (St. Louis, MO, USA)
- Sandwich Elisa kits were obtained from R&D Systems (Minneapolis, USA)

✓ DuoSet Elisa Development System for human TNF-α

✓ DuoSet Elisa Development System for human MCP-1

✓ DuoSet Elisa Development System for human VEGF

- Purified monoclonal antibodies to human TLR-4 and TLR-2 (100μg Mab-hTLR4, Mab-hTLR2) were obtained from Invivogen, (San Diego, CA)
- Human GAPD (GAPDH) Endogenous Control probe in concentration of 50 μmol/L, (VIC® ⁄TAMRA Probe, Primer Limited).

- Probes for targeted genes: Taqman TNF (Probe ID: Hs99999043_m1), Taqman MCP-1 (ID probe: Hs00234140_m1) and Taqman VEGF (ID probe: Hs00900055_m1), concentration of 50 µmol/L, (ID probe: Applied Biosystems, MA, USA).

➢ **Preparation of Stock Solutions**

- SP (SP) was diluted in Milli-Q water, and a stock solution (10mM) was prepared.
- *P. gingivalis* LPS: It was reconstituted by adding 1 ml of endotoxin-free water (provided) and homogenization. Aliquots of stock solution were prepared and stored at -20°C. Further dilutions could be prepared using Milli-Q water.
- *E. coli* LPS: It was reconstituted by adding 1 ml of cell culture medium to a vial (1 mg) and swirling gently until the powder was dissolved. Aliquots of stock solution were prepared and stored at -20°C Solutions could be further diluted to the desired working concentration with additional cell culture media.
- Purified monoclonal antibodies to human TRL-4 and TRL-2: They were reconstituted by adding 1 ml of sterile Milli-Q water to obtain a concentration of 0.1 mg/ml. Re-suspended reagents were stored at -20 °C.

2.2. Cell viability

The viability of the cells used in the experiments was tested with the use of blue tryptane, a vital stain. Cells were triggered with LPS from *P. gingivalis* and *E. coli* at the highest concentration (1 µg/ml) and with SP at a concentration of 2µM. Cell viability was assayed by means of trypan blue exclusion at the highest LPS concentration (1 µg/ml) and SP. More than 98% of MCs were viable after 24 hours of incubation with either stimulus.

2.3. Degranulation assay

Beta-hexosaminidase (β-hex) release was assayed using a fluorometric assay as an index of MC degranulation. LAD2 cells (0.5×10^6) were stimulated with LPS or SP for 30 min. SP (2 µmol/L) was used as the positive control and medium alone as the negative control. Supernatant fluids were collected, and cell pellets were lysed with 1% Triton X-100. Supernatant fluids and cell lysates were incubated in the reaction buffer (p-nitrophenyl-N-acetyl-β-D-glucosaminide from Sigma) for 1.5 h and then 0.2 mol/L glycine was added to

stop the reaction. Absorbance was measured at 405 nm. Results are expressed as the percentage of β-hex released over the total amount present in LAD2 cells.

2.4. Enzyme-Linked Immunosorbent Assay, ELISA

2.4.1. Stimulation of LAD2 MCs for Enzyme-Linked Immunosorbent Assay, ELISA

LAD2 cells (1×10^5) were stimulated for 24 hours to measure *de novo* synthesized cytokine/chemokine release. The cells were stimulated with different concentrations of LPS (1-1000 ng/ml) but only the maximum (1µg/ml) and minimum (1ng/ml) are presented as there was no dose-response relationship. As a positive control, cells were stimulated with SP (2 µM) for 24 h. As a negative control, media alone was used. The supernatant fluids were collected by centrifugation (5 min, 150 x g) stored at –20° and assayed for TNF, VEGF and MCP-1 release using enzyme-linked immunosorbent assay (ELISA) kits according to the protocol suggested by the manufacturer (R&D Systems) *(Table 3).*

Table 3

Trigger	Volume used
SP	2 µM
P. gingivalis LPS min	1 ng / ml
P. gingivalis LPS max	1 µg /ml
E. coli LPS min	1 ng / ml
E. coli max	1 µg / ml
Media alone	-

2.4.2 Blocking tests using anti-TLRs Antibodies

To assess the functional role of TLR-2 or TLR-4 in mediator release, MCs were incubated with either anti-TLR2 or anti-TLR4 polyclonal antibody at a concentration of 2 µg/ml for 1 h before stimulation with *P. gingivalis* or *E. coli* LPS (max concentration was used 1 µg/ml). Supernatants fluids were collected and assayed for mediators by Enzyme-Linked Immunosorbent Assay (Elisa) after 24h. The positive controls were MCs incubated with LPS, but without anti-TLRs, whereas the negative controls were MCs without both LPS and anti-TLRs.

- **Materials-Reagents**

- Antibody-coated 96-well microplate
- PBS (10 mM NaH2PO4, 140mM NaCl), pH: 8
- Wash buffer – 0.05% Tween 20 in PBS, pH 7.2-7.4, dilution in deionized water 1/4
- Reagent Diluent (1% BSA in PBS, pH 7.2-7.4)
- Capture Antibody (Mouse Anti-Human MCP-1, TNF and VEGF Capture Antibody)
- Detection Antibody (Biotinylated Goat Anti-Human MCP-1, TNF and VEGF Detection Antibody)
- Recombinant Standard
- Streptavidin conjugated to horseradish-peroxidase (Streptavidin-HRP)
- Substrate Solution: 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine)
- Stop Solution: 2 N H2SO4

- **Plate Preparation**

This procedure is the same for the three mediators measured.

1. Capture Antibody was diluted to the working concentration in PBS without carrier protein. Immediately a 96-well microplate was coated with 100 µL per well of the diluted Capture Antibody. The plate was sealed and incubated overnight at room temperature.
2. Each well was aspirated and washed with Wash Buffer, repeating the process two times for a total of three washes. Each well was washed by using Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or auto washer. Complete removal of liquid at each step was essential for good performance. After the last wash, removal of any remaining Wash Buffer by aspirating or by inverting the plate was completed and blotting it against clean paper towels.
3. Plates were blocked by adding 300 µL Reagent Diluent to each well and were incubated at room temperature for a minimum of 1 hour.
4. The aspiration/wash was repeated as in step 2. The plates were ready for sample addition.

- **Standards Preparation**

Standard controls are positive replicates with predetermined analyte concentrations. A careful design of standard samples results in calibration curve analysis, which can subsequently offer detailed validation of the assay.

- **Recombinant Human TNF-α Standard**: A seven-point standard curve using 2-fold serial dilutions in Reagent Diluent is recommended. This reconstitution produces a stock solution of 10,000 pg / mL. Starting from concentration of 1000 pg / ml, 6 consecutive dilutions are performed, the next one contained half the concentration than the previous one (in polypropylene vials). The 1000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL). Assay Range 15.6 - 1,000 pg/mL.
- **Recombinant Human MCP-1 Standard**: Same steps were followed, as above.
- **Recombinant Human VEGF Standard:** A seven-point standard curve using 2-fold serial dilutions in Reagent Diluent is recommended. Starting from concentration of 2000 pg / ml, 6 consecutive dilutions are performed, the next one contained half the concentration than the previous one (in polypropylene vials). The 2000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL). Assay Range: 2000pg/mL-31.3 pg/mL.

- **Assay Procedure (same for all mediators)**

1. 100 µL of sample or standards were added in Reagent Diluent, per well (100 µl / cell, 3 cells for each sample, 2 cells for each standard and control). The microplate was covered with an adhesive strip and incubated for 2 hours at room temperature.
2. The aspiration/wash was repeated as in step 2 of Plate Preparation.
3. 100 µL of the Detection Antibody was added, diluted in Reagent Diluent, to each well. Coverage with a new adhesive strip and incubation for 2 hours at room temperature performed.
4. The aspiration/wash was repeated as in step 2 of Plate Preparation.
5. 100 µL of the working dilution of Streptavidin-HRP was added to each well. Coverage of the plate and incubation for 20 minutes at room temperature repeated.
6. The aspiration/wash was repeated as in step 2 of Plate Preparation.

7. 100 µL of Substrate Solution was added to each well. Incubation for 20 minutes at room temperature repeated by avoiding the placement of the plate in direct light.
8. 50 µL of Stop Solution was added to each well.
9. The optical density of each well was determined immediately, using a microplate reader set to 450 nm or 570 nm.

- **Calculation of the results**

The Average of the triplicate readings for each standard, control, and sample was calculated and the average zero standard optical density (O.D.) was subtracted. A standard curve was created by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve through the points on the graph (x, y) was drawn. Then, the values for each unknown sample were calculated. The values of the unknown samples that are smaller of the levels of the trusted part of the curve were considered undetectable.

2.5. Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR)

2.5.1. Stimulation of LAD2 MCs for Quantitative Polymerase Chain Reaction

LAD2 cells were stimulated with either SP (2 µM) or *P. gingivalis* LPS and *E. coli* LPS with 1ng/mL for 6 h. Total mRNA was extracted with a RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Relative mRNA abundance was determined from standard curves run with each experiment. An iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) was used for reverse transcription of each sample. Quantitative real-time PCR was performed with TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for TNF (Hs99999043_m1), MCP-1 (Hs00234140_m1) and VEGF (Hs00900055_m1). Samples were run at 40 cycles by using a real-time PCR system (7300, Applied Biosystems, Foster City, CA). The mRNA gene expression was normalized to human GAPDH endogenous control (4310884E, Applied Biosystems).

2.5.2. RNA Isolation

Obtaining high-quality RNA is the first, and often the most critical, step in performing many molecular techniques such as reverse transcription real-time PCR (RT-qPCR).

- **Materials-Reagents**

- Buffer RLT. It is a lysis buffer for lysing cells and tissues prior to RNA isolation and simultaneous RNA/DNA/Protein isolation (RNeasy Mini Kit, Qiagen, Valencia, CA, USA).
- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M)
- Buffer RW1 for washing membrane-bound RNA when following RNeasy procedure (RNeasy Mini Kit, Qiagen, Valencia, CA, USA).
- Ethanol (100% and 70%) diluted in distilled water.
- Buffer RPE (55 ml). It is a concentrated buffer for washing membrane-bound RNA when using RNeasy. Addition of 100% of ethanol before use is mandatory (RNeasy Mini Kit, Qiagen, Valencia, CA, USA).
- RNase free water (RNeasy Mini Kit, Qiagen, Valencia, CA, USA)

- **Protocol followed for RNA Isolation.**

A. Preparation of Cell Lysate

1. In a sterile centrifuge tube, cell suspension (concentration 5 x 10^5 / ml) was centrifuged and collection of the supernatants was performed. Centrifugation at 500rpm (revolutions per minute) for 5 minutes.

2. Supernatant was removed and discarded.

3. Cells were washed with added ice-cold and sterile D-PBS. Centrifugation at 300 x *g* for 5 minutes was performed. Removal as much of residual supernatant as possible.

4. Lysis of cells with addition of RNA Lysis Buffer, RLT solution (500 µl for 5 x 10^5 / ml) and mild Mixing by pipetting up and down 7 - 10 times.

B. RNA Isolation

1. Cell lysate was loaded onto the spin column homogenizer (QIAshredder) and the column was placed in a collection tube and centrifuged for 2 min. The homogenized lysate was then collected (sediment).
2. Addition of equal volume of 70% ethanol to the sediment and stirring well with a pipette performed.

3. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to a RNeasy spin column placed in a 2ml collection tube (supplied). Centrifugation for 1 min at 1300 rpm. Flow-through was discarded.

4. Addition of 700 µl Buffer RW1 to the RNeasy spin column. Centrifugation for 1 min at 1300 rpm to wash the spin column membrane. Flow-through was discarded.

5. Addition of 500 µl Buffer RPE to the RNeasy spin column. Centrifugation for 1 min at 1300 rpm, Flow through was discarded.

6. Addition of 500 µl Buffer RPE to the RNeasy spin column. Centrifugation for 2 min at 1300 rpm, flow through was discarded.

7. The RNeasy spin column was placed in a new 1.5 ml collection tube (supplied). Addition of 30–50 µl RNAse-free water directly to the spin column membrane was performed. Centrifugation for 1min at 1300rpm. The tube was discarded, and the remaining sediment contained the clear total RNA.

- **Calculation of the results**

The method used for assessing RNA concentration and purity was UV spectroscopy. The spectrophotometer used was the Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). The principle is based on the measurements of the absorbance of a diluted RNA sample at 260 and 280 nm. The RNA concentration is calculated using the Beer-Lambert law* which predicts a linear change in absorbance with concentration. The A260/A280 ratio is used to assess RNA purity. An A260/A280 ratio of 1.8 to 2.1 is indicative of highly purified RNA.

** A = εbC where A is absorbance at a wavelength, ε is the extinction of coefficient (for RNA is 0.025 (µg/ml) $^{-1}$ cm^{-1}), b is the path length of the sample expressed in cm (typically 1 cm) and C is the concentration of nuclei acid.*

2.5.3. Reverse Transcription (RT), Polymerase Chain Reaction, RT-PCR

The protocol followed for RT-PCR was completed in two steps. Firstly, the reverse transcription step was processed and then the PCR step completed. The two-step protocol is usually more sensitive than the one-step method; yields of rare targets may be improved by using the two-step procedure.

Specifically, the procedure was based on following diagram:

A. 1st Step, Reverse Transcription (RT)

➢ **Materials-Reagents**

- 5x iScript Reaction Mix. It is a blend of oligo(dT) and random hexamer primers, (volume for 25 reactions, 100 µl), (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA).
- iScript reverse transcriptase (volume for 25 reactions, 25 µl), (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA).
- RNase free water (volume for 25 reactions, 1.5 µl), (iScript cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA)

➢ **Protocol followed:**

This step was based on the use of the thermocycler (PTC 200 thermal cycler, Bio-Rad) and the use of iScript cDNA Synthesis Kit, (Bio-Rad, Hercules, CA, USA).

MicroAmp® sterile Reaction Tubes were used for each reaction per condition (Microamp Optical sterile tubes and 8-cap strips, Applied Biosystems, Foster City, CA, USA).

The following **reaction mix** presented below *(table 4)* was prepared. The reaction mix was then placed for Incubation in the thermocycler:

- cDNA synthesis: 10min at 230°C, 50min at 420°C
- Inactivation of reverse transcriptase: 5min at 950°C

Table 4

Component	Volume per Reaction
5x iScript Reaction Mix	4 µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	5µl
RNA template	10 µl
Total Volume	**20 µl**

B. 2nd Step, quantitative PCR, (qPCR)

➢ Materials-Reagents

- Taqman Fast Advanced master mix contains AmpliTaq Fast DNA Polymerase, uracil-N-glycosylase (UNG), dNTPs with dUTP, ROX dye (passive reference), and optimized buffer components. It is supplied at a 2X concentration (Applied Biosystems).
- Human GAPD (GAPDH) Endogenous Control probe in concentration of 50 µmol/L, (VIC® ⁄ TAMRA Probe, Primer Limited).
- Probes for targeted genes: TNF (Probe ID: Hs99999043_m1), MCP-1 (Probe ID: Hs00234140_m1) and VEGF (Probe ID: Hs00900055_m1), concentration of 50 µmol/L, (Applied Biosystems).
- Nuclease-free water

➢ Protocol followed:

- Dilution 1:10 in dH20 of cDNA produced by the RT reaction (final concentration 100 ng/ml).
- For each condition to be tested, for the creation of the standard curve and for the DNA free reference samples (controls), the mixing of specific reagents was performed in specific tubes in triplicates presented at the following *Table 5.*

Table 5

Tested Conditions		**Standard Curve**		**Reference Samples**	
Component	**Volume**	**Component**	**Volume**	**Component**	**Volume**
Taqman Master Mix	10 µl	Taqman Master Mix	10 µl	Taqman Master Mix	10 µl
Probes for Targeted Gene (TNF, MCP-1, VEGF)	1 µl	Probe for Targeted Gene	1 µl	Probe for Targeted Gene	1 µl
Control Probe GAPDH	1 µl	Control Probe GAPDH	1 µl	c DNA	9 µl
c DNA	8 µl	c DNA (dilutions (10x, 100x, 1000x)	8 µl		
Total Volume 20 µl					

- Placement in the thermocycler (PTC 200 thermal cycler, Bio-Rad). The thermal cycles are described at the table *6.* The process was completed by determining the threshold cycle (C_T) for each sample.

Table 6

Cycles	Temperature	Time
40	50 °C	2 min
	95 °C	10 min
	95 °C	15 min
40	60 °C	1 min

➢ **Calculation of the results**

A standard curve was created by plotting the C_T-values against the logarithm of the dilution factors (DF). In the useable concentration range, there should be a linear relationship between Ct and log (DF). The standard curve is the regression line through these points. From this curve, the number of cells expressing the standard and target genes could be calculated. The following equation was applied: $R= 2^{- (\Delta Ct\ sample - \Delta Ct\ control)}$ [377].

3. Statistical Analysis

For the statistical analysis and the creation of the representative graphs, the scientific 2D graphing and statistics software GraphPad Prism 6 (GraphPad Prism 6) was used.

The performance for the qPCR machine was 1.8, based on the 1 / (1.8 ^ Ct) equation. The values for each sample were calculated and the results of each sample gene expression were based on GAPDH for the analysis.

All experiments were performed in triplicates and repeated at least 3 times (n=3). The results are presented as means ± Standard Deviation. Data between different treatment groups were analyzed by using the unpaired 2-tailed Student's t test (GraphPad Prism 6). Mean values of the parameters were tested by means of the least significant difference test at significance level $p < 0.05$. On the graphs, the horizontal line indicates the mean value (mean) and the vertical line the existing deviation (SD).

4. RESULTS

4.1 Effects of *P. gingivalis* LPS, *E. coli* LPS and SP on LAD2 MCs Degranulation

Beta-hexosaminidase (β-hex) release was assayed as an index of MCs degranulation. LAD2 cells (0.5×10^5) were stimulated with LPS (1 ng/ml) of the 2 different bacteria and SP (2 μmol/L) for 30 min. SP was used as the positive control and medium alone as the negative control. Results are expressed as the percentage of β-hex released over the total amount present in LAD2 cells. The experiment was performed in triplicates and repeated 3 times (n=3) for each different condition.

The stimulation of MCs with *P. gingivalis* and *E. coli* LPS resulted in 4.17 ± 1.38 and 6.53 ± 1.34 β-hex release, respectively. In the control group the amount of degranulation noted was 3.99 ± 1.15 and in the SP group was 19.01 ± 0.29 respectively. There was no statistically significant difference between the control group and the LPS groups in the amount of degranulation (*p>0.05).* In contrast, there was statistically significant difference between the LPS and the SP group (*p< 0.05*). Neither bacterial LPS stimulated degranulation of MCs (*Fig. 1*).

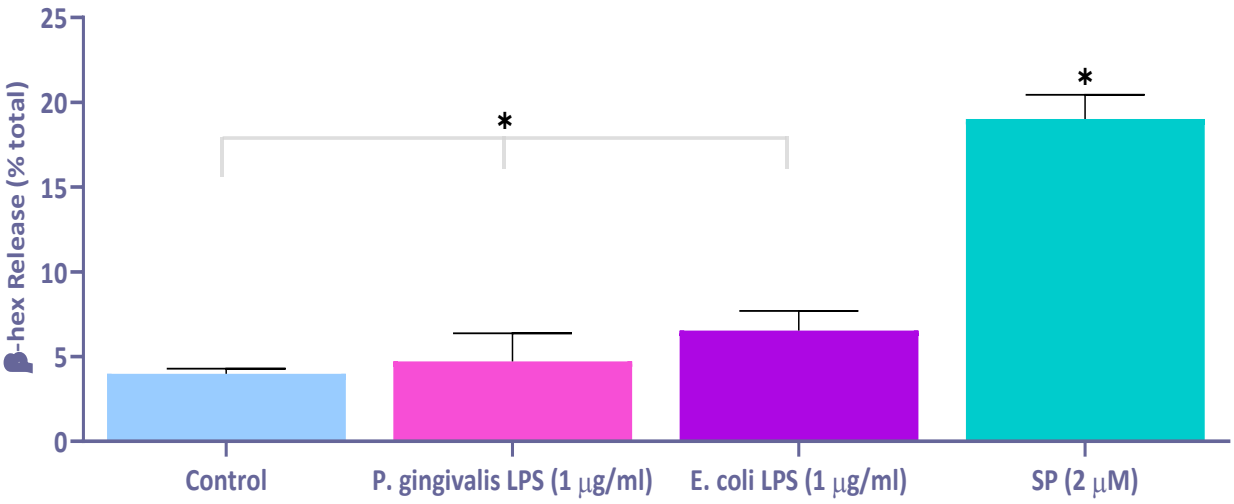


Figure 1. Beta-Hexosaminidase release. Supernatant fluids and cell pellets were collected 30 min after stimulation with LPS and SP. Statistically significant differences between the LPS groups and SP group *(p<0.05, unpaired t-test). (n=3, mean ± SD).*

4.2. Effects of *P. gingivalis* LPS and *E. coli* LPS on mediator gene expression from LAD2 MCs.

LAD2 cells were stimulated with either SP (2 µM) or *P. gingivalis* and *E. coli* LPS (1 ng/mL) for 6 h. Total mRNA was extracted with a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Relative mRNA abundance was determined from standard curves run within each experiment. An iScript cDNA synthesis kit (Bio-Rad Laboratories) was used for reverse transcription of each sample. Quantitative real-time PCR was performed with TaqMan gene expression assays (Applied Biosystem) for TNF (Hs99999043_m1), MCP-1 (Hs00234140_m1) and VEGF (Hs00900055_m1). Samples were run at 45 cycles by using a real-time PCR system (7300, Applied Biosystems). The mRNA gene expression was normalized to human GAPDH endogenous control (Applied Biosystems).

4.2.1. TNF-α gene expression

For TNF- α gene expression, MCs were stimulated for 6h, with *P. gingivalis* and *E. coli* LPS (1 ng/ml) and with SP (2Mm). Unstimulated cells served as the Control group (*Fig. 2*)

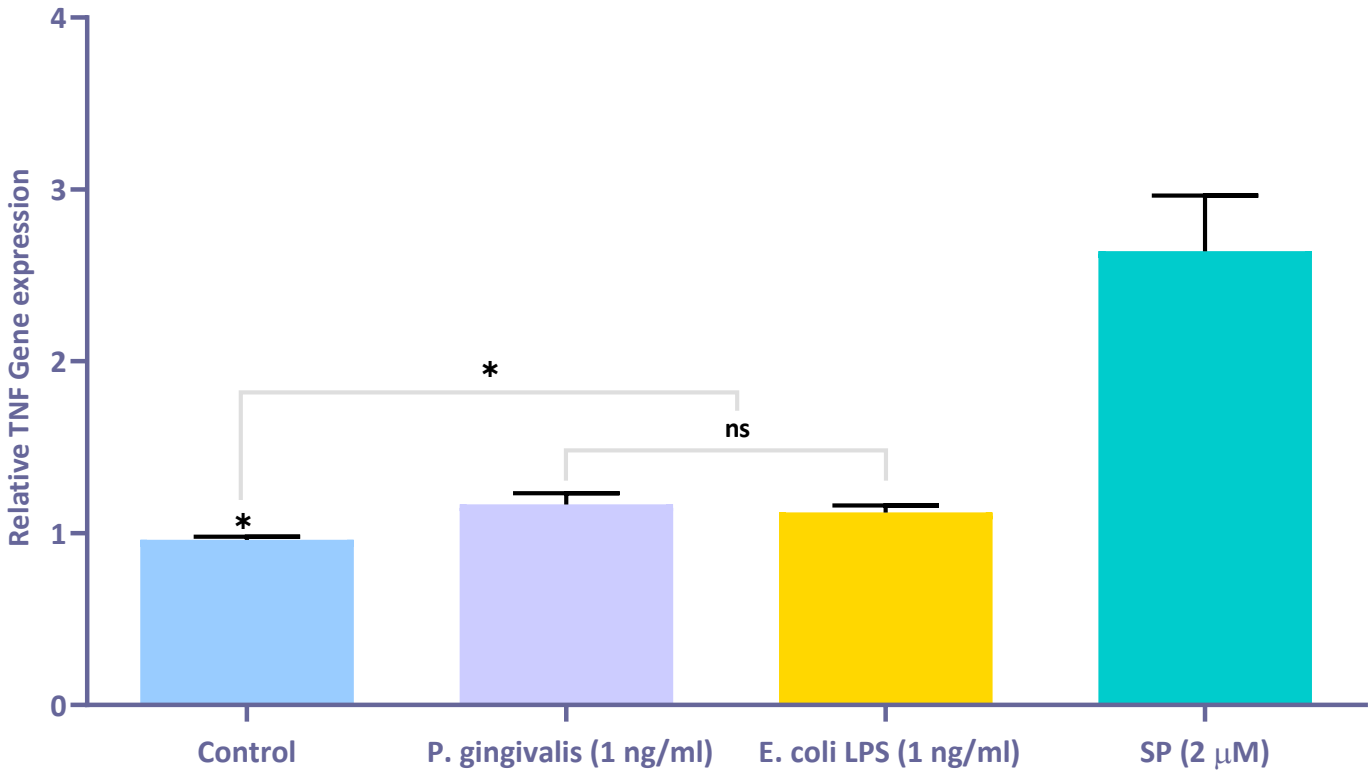


Figure 2. Relative TNF-α mRNA expression after stimulation of LAD2 cells with *P. gingivalis* LPS, *E. coli* LPS (1 ng/mL) and SP (2 μM) for 6 hours. *(* p<0.05, unpaired t-test). (n=3, mean ± SD).*

The relative TNF-α mRNA expression was 0.96 ± 0.03 for the Control group. For the LPS groups, a small, but statistically significant increase of TNF- α gene expression was noticed (*p<0.05*). The relative expression was 1.16 ± 0.11 and 1.12 ± 0.07 for *P. gingivalis* and *E. coli* LPS, respectively. There was no statistically significant difference on the TNF-α gene expression between the two different LPSs groups (*p>0.05*). On the contrary, the SP group induced a potent, statistically significant increase in the gene expression of TNF-α, compared to the other three groups, and was calculated at 2.64 ± 0.5 (*p<0.01*)

4.2.2. VEGF gene expression

For VEGF gene expression, MCs were stimulated for 6h, with *P. gingivalis* and *E. coli* LPS (1ng/ml) and with SP (2Mm). Unstimulated cells served as the Control group (*Fig. 3*).

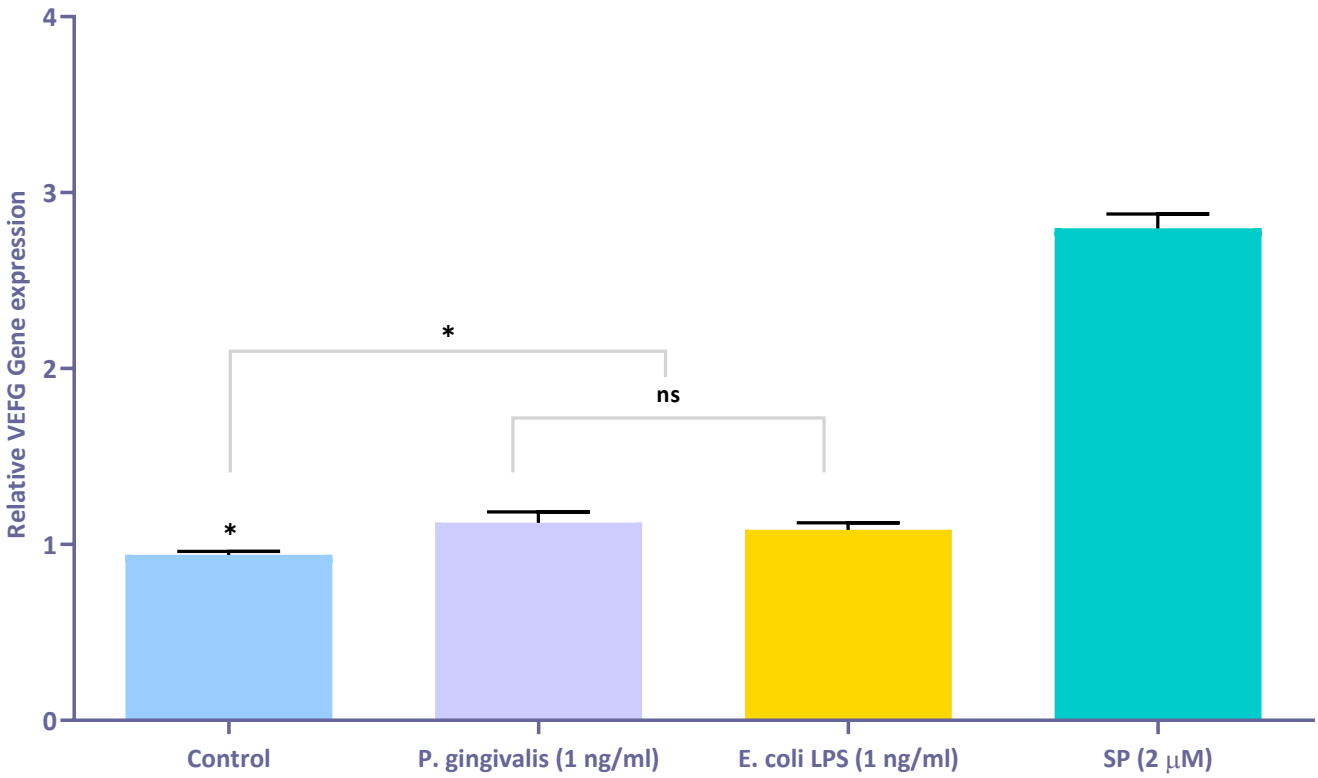


Figure 3. Relative VEGF mRNA expression after stimulation of LAD2 cells with *P. gingivalis* LPS, *E. coli* LPS (1 ng/mL) and SP (2 μM) for 6 hours. *(n=3, mean ± SD * p<0.05, **ns**: no significance).*

The relative VEGF mRNA expression was 0.96 ± 0.05 for the Control group. For the LPS groups, a statistically significant, slight increase of VEGF gene expression was noticed (*p<0.05*). The relative expression induced was 1.12 ± 0.1 and 1.08 ± 0.06 for *P. gingivalis* and *E. coli* LPS, respectively. There was no statistically significant difference on the gene expression between the two different LPS groups (*p>0.05*). As previously, the SP group induced a potent increase in the gene expression of VEGF, statistically significant compared to the other three groups (2.79 ± 0.14), (*p<0.01*)

4.2.3. MCP-1 gene expression

For MCP-1 gene expression, LAD2 were stimulated for 6h, with *P. gingivalis* and *E. coli* LPS (1 ng/ml) and with SP (2Mm). Unstimulated cells served as the Control group (*Fig. 4*).

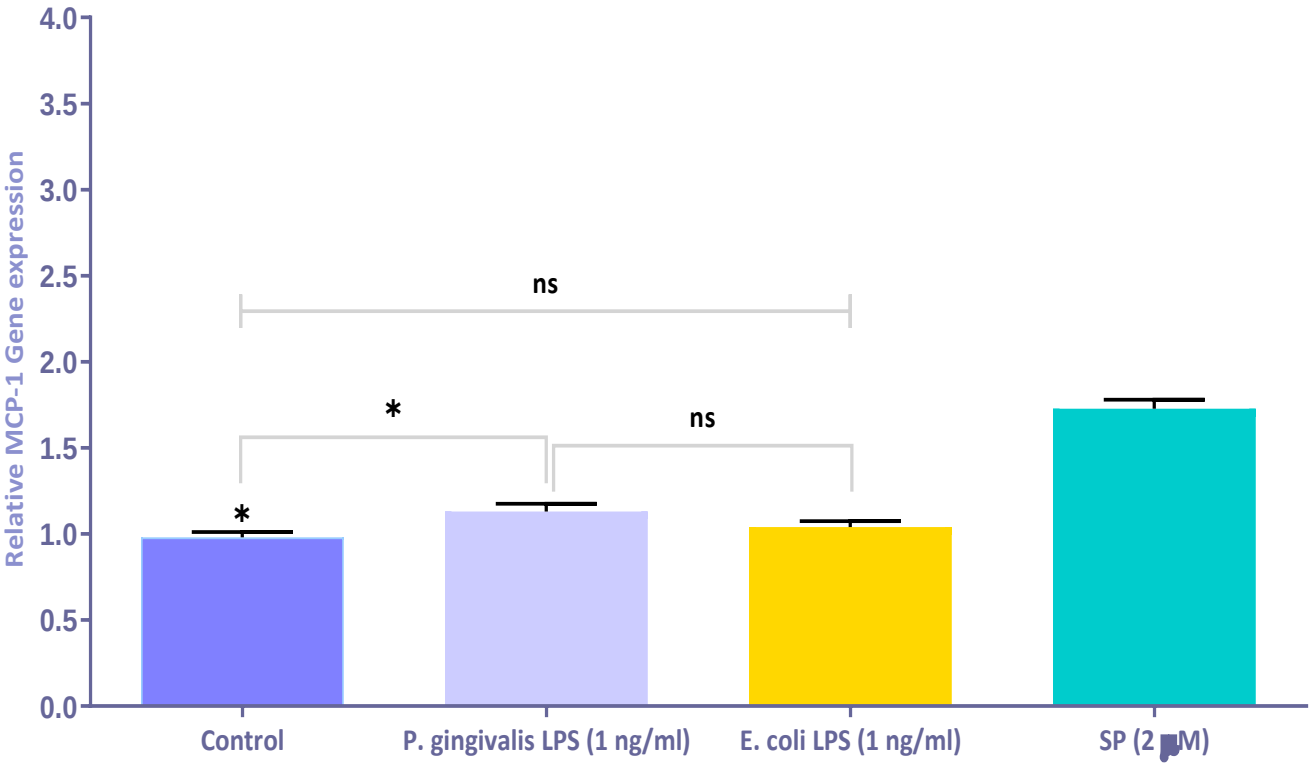


Figure 4. Relative MCP-1 mRNA expression after stimulation of LAD2 cells with *P. gingivalis* LPS, *E. coli* LPS (1 ng/mL) and SP (2 μM) for 6 hours. (*n=3, mean ± SD, * p<0.05, ** p<0.05, **ns**: no significance*).

The relative MCP-1 mRNA expression was 0.98 ± 0.06 for the Control group. The relative change in MCP-1 gene expression induced by P. *gingivalis* LPS was 1.13 ± 0.07, statistically significant from the Control group (*p<0.05*). In contrast, *E. coli* LPS, although induced a slight increase in MCP-1 gene expression (1.04 ± 0.08), it was not statistically significant from the Control group (*p>0.05*). SP induced an increase in MCP-1 gene expression, statistically significant compared to the other three groups (1.72 ± 0.09, *p<0.01*).

4.2.4. Conclusions

Taking into consideration the above, the stimulation of LAD2 cells with P. *gingivalis* LPS induced a slight but statistically significant increase in gene expression for all the three mediators studied (TNF-α, VEGF, MCP-1). The same results were observed for *E. coli* LPS regarding the increase in TNF-α and VEGF gene expression (statistically significant). On the contrary, as far as the relative MCP-1 mRNA expression is concerned, even if there was a small increase, it was not statistically significant from the control group.

Regarding the two different LPSs used, no statistically significant difference in gene expression was observed for the three mediators studied, implying that both LPSs had the potency to induce approximately the same amount of relative gene expression of the three mediators studied.

The stimulation of LAD2 cells with SP induced the most potent increase in gene expression for all mediators that was statistically significant from both the Control and the LPSs groups. The extent of TNF-a and VEGF gene expression was the same on average (2.64 ± 0.5 and 2.79 ± 0.14 respectively), with MCP-1 gene expression to be slightly lower but still significant (1.72 ± 0.09).

4.3. Release assays for TNF-α, VEGF, MCP-1

To measure *de novo*–synthesized cytokine/chemokine release, LAD2 cells (1×10^5) were stimulated for 24 h, with the minimum and maximum concentration of LPS from both bacteria (1 ng/ml and 1 μg/ml respectively). The cells were also stimulated with SP (2 μM) serving as the positive control or media alone serving as the negative control for 24h. The supernatant fluids were collected by centrifugation (5 min, 150 x g), stored at –20° and assayed for TNF-α, VEGF and MCP-1 release using enzyme-linked immunosorbent assay (ELISA) kits according to the protocol suggested by the manufacturer (R&D Systems).

4.3.1. TNF-α Release

The effect of *P. gingivalis* and *E. coli* LPS (1 ng/ml) on TNF-α release from LAD2 cells is shown in *Fig. 5* and the respective release when the concentration of 1 μg/ml was used, is shown in *Fig. 6.*

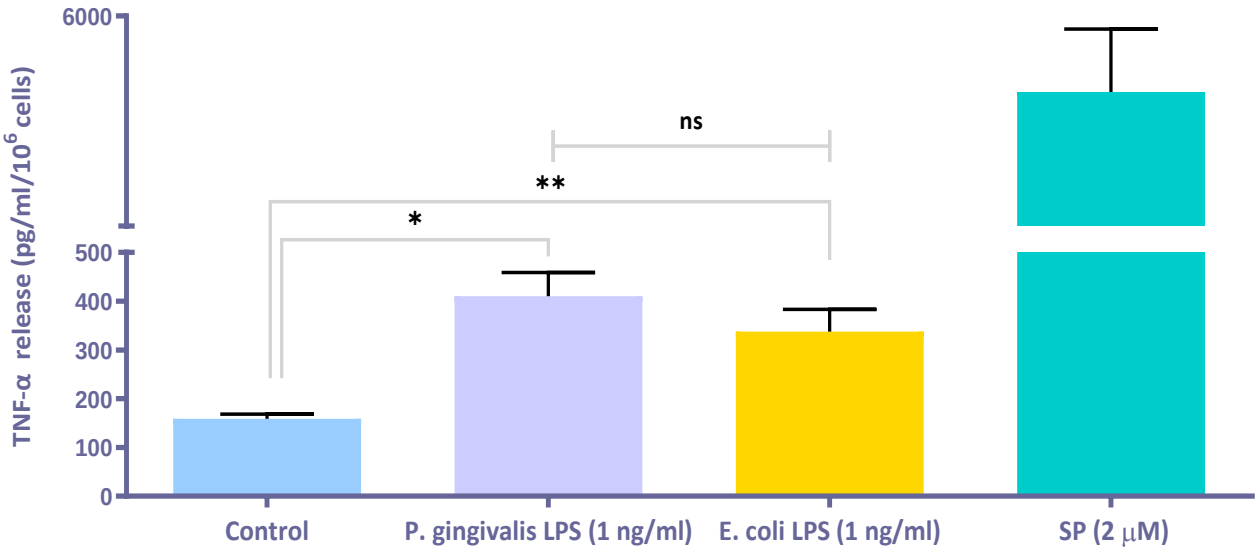


Figure 5. TNF-α release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 ng/ml** of LPS and assayed by ELISA (n=3, mean ± SD, ***** *p<0.05*, ****** *p<0.05*, ***ns**: no significance*)

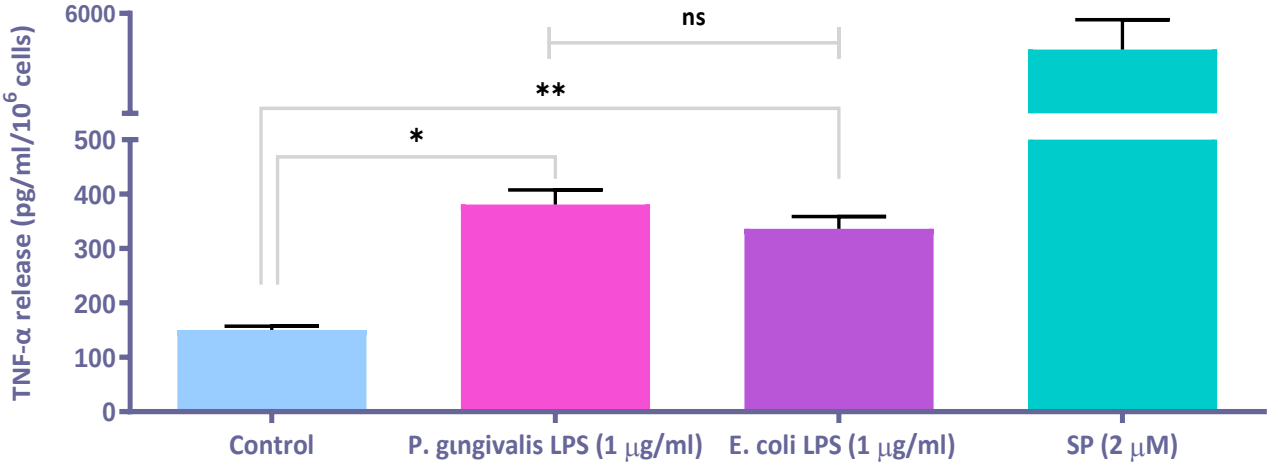


Figure 6. TNF-α release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 μg/ml** of LPS and assayed by ELISA (n=3, mean ± SD, **** p<0.05***, ***** p<0.05***, ***ns**: no significance*).

TNF-α release observed in unstimulated LAD2 was 150.3 ± 6.7 pg/ml/10^6 cells, serving as the negative control and its release after stimulation with SP (2 μM), was increased to 4000 ± 965 pg/ml/10^6, serving as the positive control. SP triggered the highest amount of TNF-α release that was statistically significant from the control and the LPSs groups (*p<0.001*).

When LAD2 cells were stimulated with the minimum concentration (1 ng/ml) of *P. gingivalis* and *E. coli* LPS, TNF-α release was increased to 410.0 ± 48.69 pg/ml/10^6 and 337.4 ± 45.87 pg/ml/10^6 respectively, difference statistically significant from the Control Group (*p<0.05*). Even if *P. gingivalis* LPS induced a higher release of the specific mediator, it was not statistically significant from the amount released after stimulation with *E. coli* LPS (p>0.05).

When LAD2 cells were stimulated with the maximum concentration (1 μg/ml) of *P. gingivalis* and *E. coli* LPS, TNF-α release was also increased to 380.7 ± 26.9 pg/ml/10^6 and 336.4 ± 22.43 pg/ml/10^6 respectively, difference statistically significant from the Control Group (*p<0.05*). Between the two groups, there was no statistically significant difference on TNF-α release (p>0.05).

Interestingly, when the minimum (1 ng/ml) and maximum (1 μg/ml) concentrations of each different LPSs were compared, there was no dose-response relationship as the difference

was not statistically significant for the two different concentrations used (*p>0.05*) as shown in *Fig 7.*

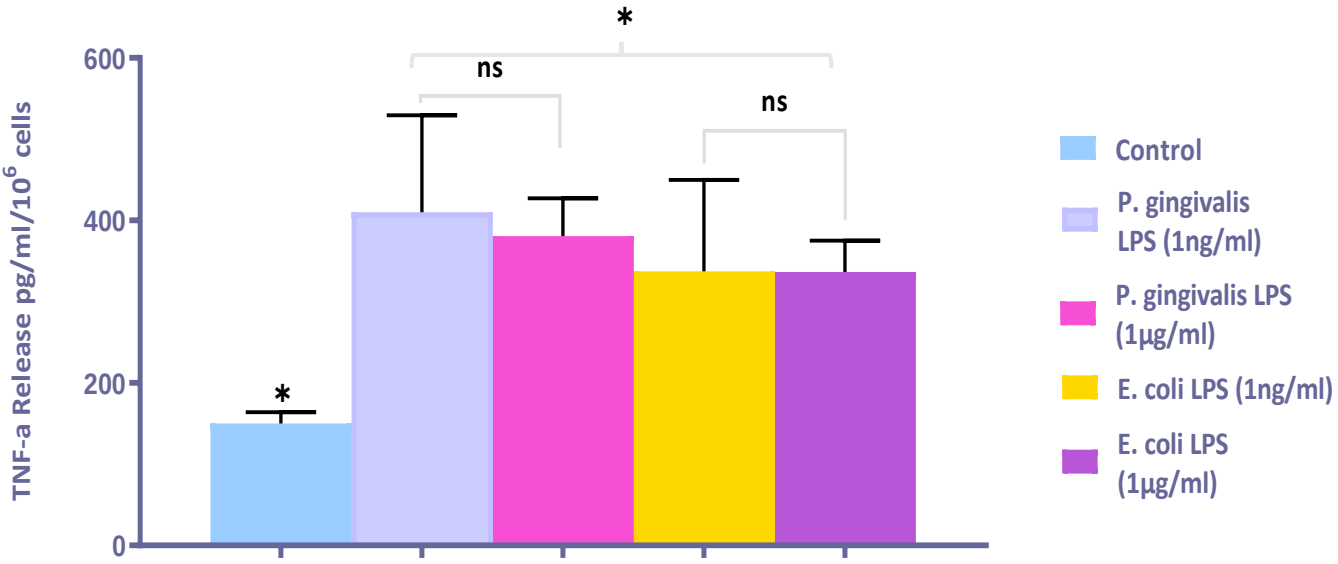


Figure 7. TNF-α release from LAD2 cells. All concentrations presented in total (n=3, mean ± SD, ** p<0.05, **ns**: no significance*)

- **Observations**

Both LPSs are potent inducers of TNF-α release from LAD2 cells. Stimulation of MCs with *P. gingivalis* and *E. coli* LPS led to a statistically significant increase in TNF-α release for both concentrations used (1 ng/ml, 1 µg/ml). Even if the observed TNF-α release was slightly higher for *P. gingivalis* LPS (for both concentrations), there was no statistically significant difference from the one noted for *E. coli* LPS. In addition, no dose-response relationship was noted for the two different concentrations used. The highest amount of TNF-α release was documented after stimulation of LAD2 with the neuropeptide SP.

4.3.2. VEGF Release

The effect of *P. gingivalis* and *E. coli* LPS (1 ng/ml) on VEGF release from LAD2 cells is shown in *Fig. 8* and the respective release when the concentration of 1 μg/ml was used, is shown in *Fig. 9.*

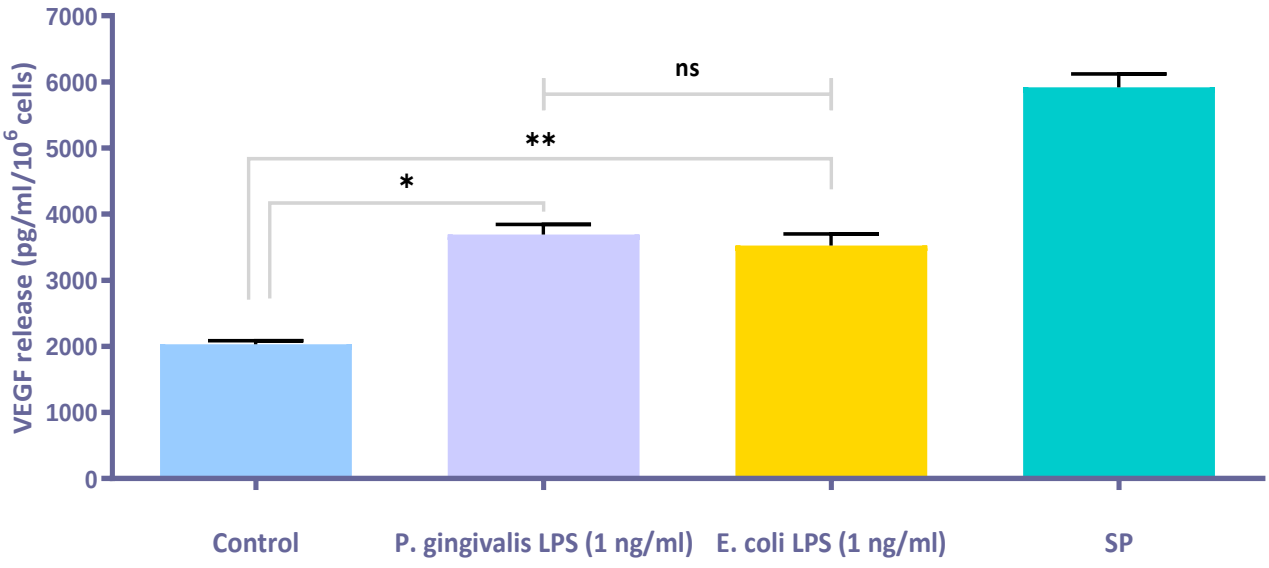


Figure 8. VEGF release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 ng/ml** of LPS and assayed by ELISA (n=3, mean ± SD, ***** *p<0.05*, ****** *p<0.05*, ***ns**: no significance*)

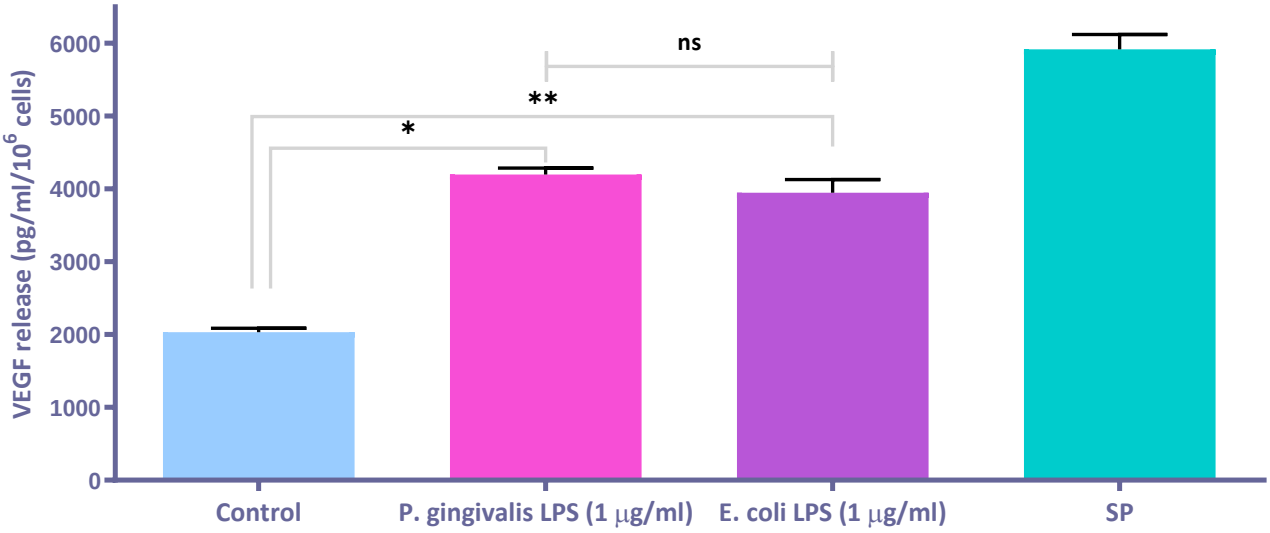


Figure 9. VEGF release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 μg/ml** of LPS and assayed by ELISA (n=3, mean ± SD, ***** *p<0.05*, ****** *p<0.05*, ***ns**: no significance*).

In the Control Group, the VEGF release was 2032 ± 52.96 /ml/10^6 cells, serving as the negative control. When LAD2 cells were stimulated with SP (2 μM), VEGF release increased significantly up to 5917 ± 117 pg/ml/10^6 (*p<0.001*).

When LAD2 cells were stimulated with the minimum concentration (1 ng/ml) of *P. gingivalis* and *E. coli* LPS, VEGF release was increased to 3691 ± 154.0 pg/ml/10^6 and 3525 ± 173.1 pg/ml/10^6 respectively, difference statistically significant from the Control Group for both LPS Groups (*p<0.05*). Comparing the two different LPSs, there was no statistically significant difference on VEF release (*p>0.05*).

When LAD2 cells were stimulated with the maximum concentration (1 μg/ml) of *P. gingivalis* and *E. coli* LPS, VEGF release was also increased to 4199 ± 86.17 pg/ml/10^6 and 3947 ± 181.9 pg/ml/10^6 respectively, difference statistically significant from the Control Group (*p<0.05*). Between the two groups, there was no statistically significant difference on VEGF release (p>0.05).

The comparison of the minimum (1 ng/ml) and maximum (1 μg/ml) concentration for each LPS is presented in *Fig. 10*. Regarding the *E. coli* LPS, there was no statistically significant difference for the two different concentrations used (*p>0.05*). In contrast, regarding *P. gingivalis* LPS, VEGF release, in the concentration of 1 μg/ml, was statistically higher compared to the one when the concentration of 1 ng/ml was used (*p<0.05*).

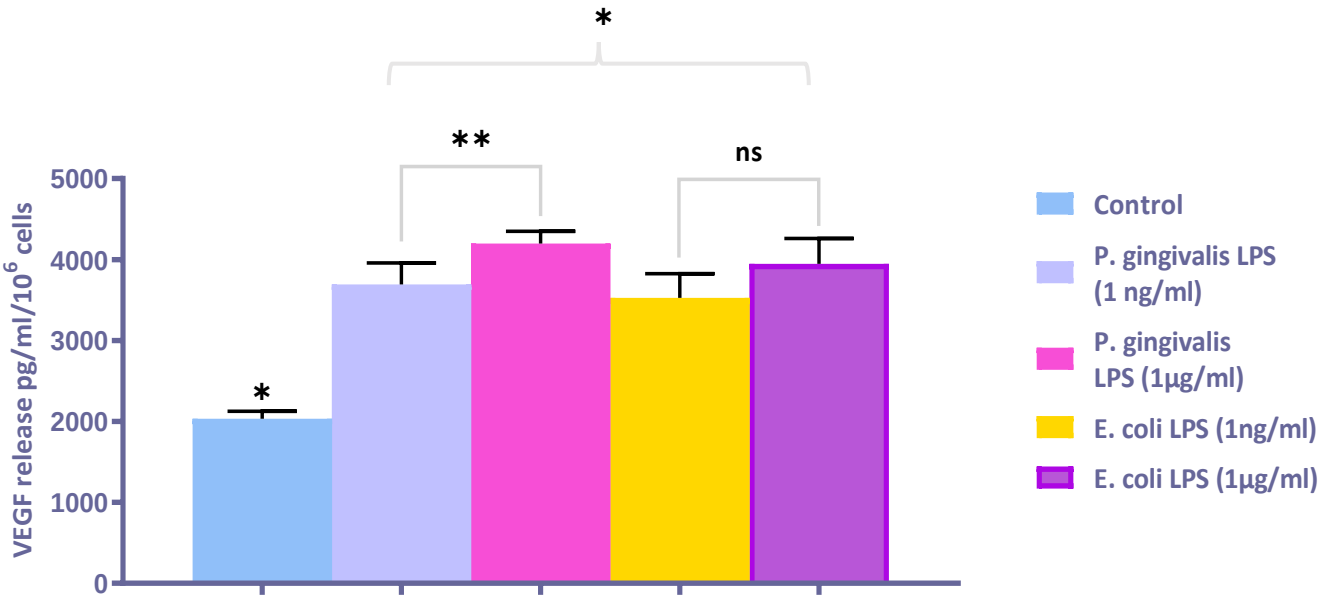


Figure 10. VEGF release from LAD2 cells. All concentrations presented in total (n=3, mean ± SD, ***** *p<0.05*, ****** *p<0.05*, ***ns**: no significance*).

- **Observations**

Both LPSs proved to be potent inducers of VEGF release from LAD2 cells. When *P. gingivalis* and *E. coli* LPS were compared in the same concentration (1 ng/ml or 1 µg/ml), VEGF release was observed to be higher in *P. gingivalis* LPS groups, but not statistically significant from *E. coli* LPS Groups.

When the two different concentrations of the same LPS (*E. coli* or *P. gingivalis*) were studied, *E. coli* LPS (1 µg/ml) stimulated higher but not statistically significant release of VEGF compared to the concentration of 1 ng /ml (no dose-response relationship noted). On the other hand, regarding *P. gingivalis* LPS, VEGF release was statistically significantly increased when the concentration of LPS used increased from 1 ng/ml to 1 µg/ml.

As previously noted, the highest amount of VEGF release was documented after stimulation of LAD2 with the neuropeptide SP and it was statistically significant compared to the Control and LPSs Groups.

4.3.3. MCP-1 Release

The effect of *P. gingivalis* and *E. coli* LPS (1 ng/ml) on MCP-1 release from LAD2 cells is shown in *Fig. 11* and the respective release when the concentration of 1 μg/ml was used, is shown in *Fig. 12.*

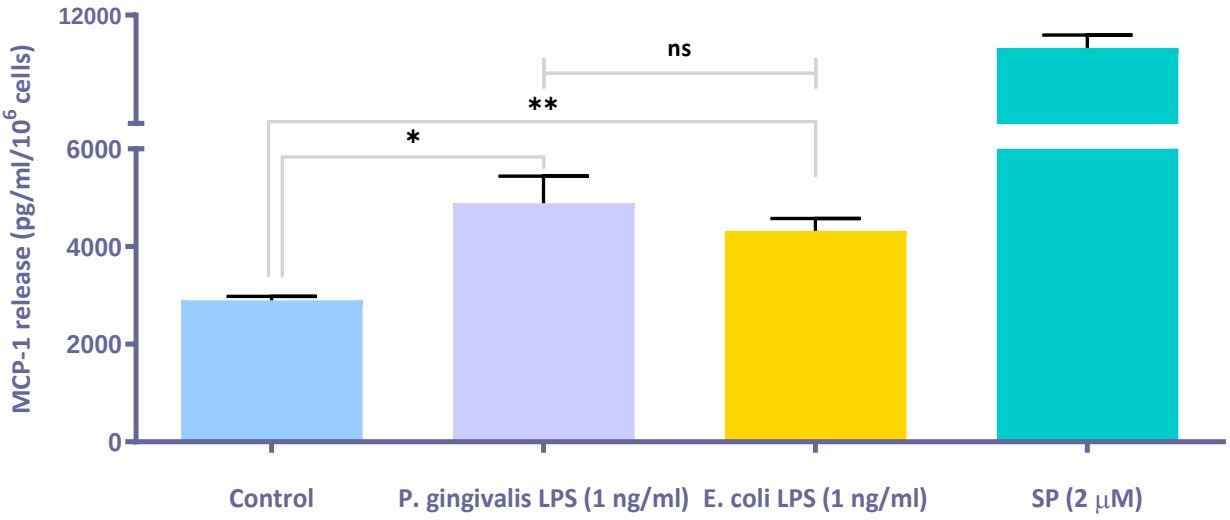


Figure 11. MCP-1 release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 ng/ml** of LPS and assayed by ELISA (n=3, mean ± SD, ** p<0.05*, *** p<0.05*, *ns: no significance*)

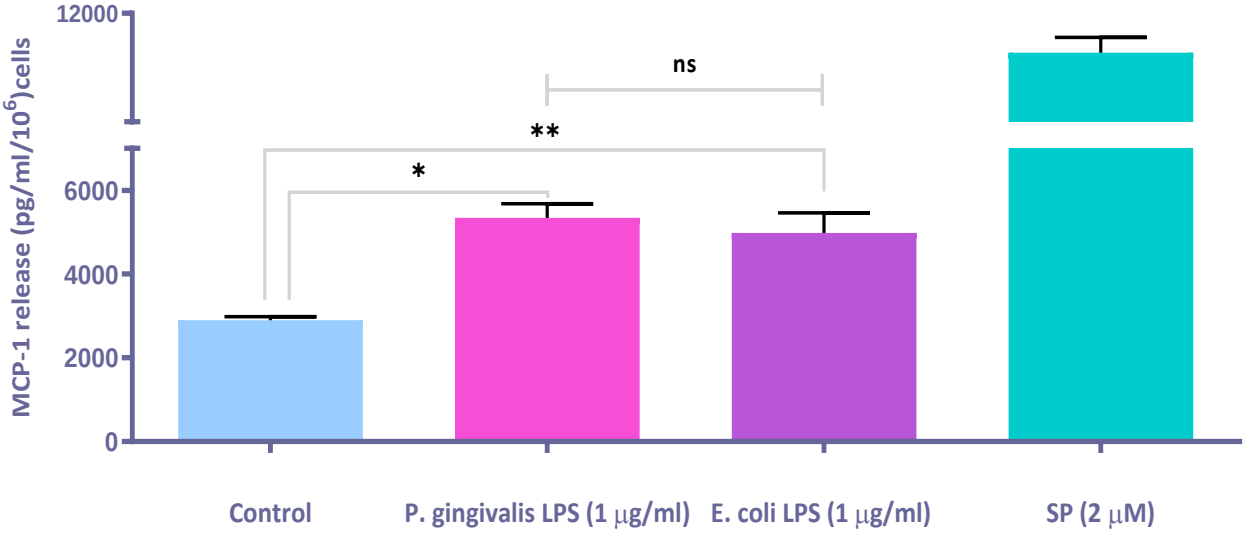


Figure 12. MCP-1 release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 µg/ml** of LPS and assayed by ELISA (n=3, mean ± SD, ** p<0.05*, *** p<0.05*, *ns: no significance*).

MCP-1 release observed in unstimulated LAD2 was 2899 ± 82.12 pg/ml/10^6 cells, serving as the negative control and its release after stimulation with SP (2 Mm), was increased to 9281 ± 423.4 pg/ml/10^6 cells, serving as the positive control. SP triggered the highest amount of MCP-1 release that was statistically significant from the control and the LPSs groups *(p<0.001).*

P. gingivalis and *E. coli* LPS in the same concentration (1 ng/ml) stimulated LAD2 to release 4886 ± 554.6 pg/ml/10^6 cells and 4320 ± 254.2 pg/ml/10^6 cells of MCP-1, respectively (statistically significant compared to Control Group, *p<0.05*). Even if the amount of released MCP-1 observed for *P. gingivalis* was higher, it was not statistically significant from *E. coli* LPS Group (*p>0.05*).

For the maximum concentration used (1 µg/ml), the production of MCP-1 increased to 5343 ± 337.4 pg/ml/10^6 cells and 4985 ± 478 pg/ml/10^6 for *P. gingivalis* and *E. coli* LPS, respectively. As noted above, there was a tendency for higher release of MCP-1 in *P. gingivalis* Group, but it was not statistically significant from that observed in *E. coli* Group (*p>0.05*).

Interestingly, when the minimum (1 ng/ml) and maximum (1 µg/ml) concentration of each different LPS Groups were compared, there was no dose-response relationship as the difference was not statistically significant for the two different concentrations used (p>0.05) as shown in *Fig. 13.*

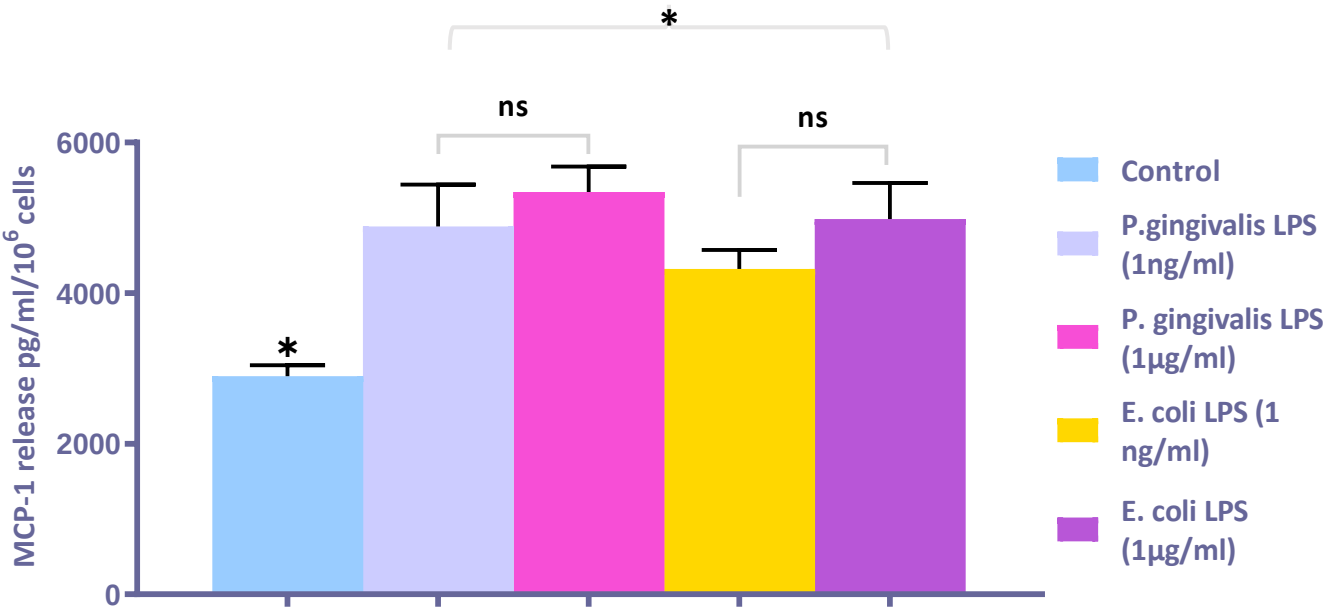


Figure 13. MCP-1 release from LAD2 cells. All concentrations presented in total (n=3, mean ± SD, ***** *p<0.05*, ***ns**: no significance*).

- **Observations**

Both LPSs are potent inducers of MCP-1 release from LAD2 cells. Stimulation of MCs with *P. gingivalis* and *E. coli* LPS led to a statistically significant increase in MCP-1 release for both concentrations used (1 ng/ml, 1 µg/ml). Even if the observed MCP-1 release was slightly higher for *P. gingivalis* LPS (for both concentrations), there was no statistically significant difference from the one noted for *E. coli* LPS. In addition, no dose-response relationship was noted for the two different concentrations used for both LPS Groups. The highest amount of MCP-1 release was documented after stimulation of LAD2 with the neuropeptide SP.

4.3.4. Conclusions

- The stimulation of LAD2 cells with *P. gingivalis* and *E. coli* LPS resulted in increased levels of TNF-α, VEGF and MCP-1 compared to unstimulated cells (*p<0.05*). Both the minimum and the maximum LPS concentrations tested (1 ng/ml, 1 μg/ml) led to *de novo* release of all the three mediators studied.

- *P. gingivalis* LPS (1 μg/ml *vs* 1 ng/ml): Comparing the two different concentrations used, even if there was a tendency for an increase on the levels of all the mediators when the maximum concentration was used, the difference was only statistically significant for VEGF release (*p<0.05*). Regarding the TNF-α and MCP-1 release, there was no dose-response relationship as the difference was statistically insignificant (*p>0.05*).

- *E. coli* LPS (1 μg/ml *vs* 1 ng/ml): Even if the levels of all the mediators where higher when the maximum concentration was used compared to the minimum one, there was no statistically significant difference in the release of TNF-α, VEGF and MCP-1 (*p>0.05*).

- *P. gingivalis* LPS vs *E. coli* LPS in the same concentration triggered approximately, the same amount of *de novo* release of TNF-α, VEGF and MCP-1, as there was no statistically significant difference (*p>0.05*). However, there was the tendency of slightly higher levels of mediator release for *P. gingivalis* LPS in both tested concentrations compared to *E. coli* LPS.

- SP has proven to be a potent stimulator of LAD2 cells as the release of TNF-α, VEGF and MCP-1 reached the highest concentrations, statistically significant in relation to the control and the LPSs Groups ($p<0.001$).

4.4. Blocking tests using anti-TRLs antibodies

To assess the functional role of TRL2 and TRL4 in TNF-α, VEGF and MCP-1 release, MCs were incubated with either anti-TLR2 or anti-TLR4 polyclonal antibody (2 µg/ml) for 1 h before stimulation with *P. gingivalis* or *E. coli* LPS (1 µg/ml). Supernatants fluids were collected and assayed for mediators by Elisa after 24 h. The positive controls were MCs incubated with LPS, but without anti-TLRs, whereas the negative controls were unstimulated MCs.

4.4.1. Blocking Effect on TNF-α Release

The effect of anti-TLR antibodies on the release of TNF-α is shown in *Fig. 14* for *P. gingivalis* LPS and in *Fig. 15* for *E. coli* LPS.

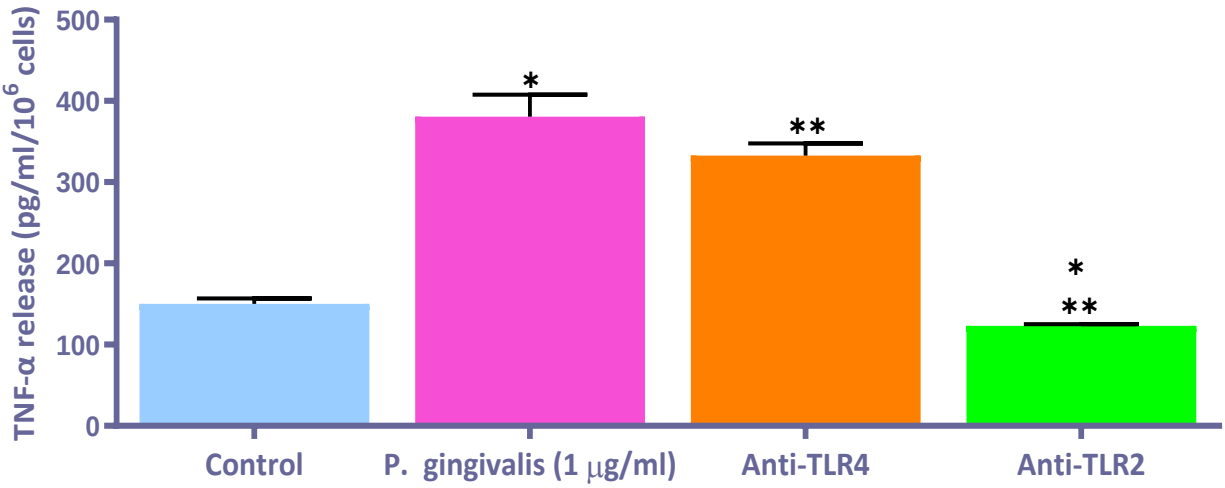


Figure 14. TNF-α release from LAD2 cells stimulated with *P. gingivalis* LPS (1µg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 µg/ml) for 1h. (n=3, mean ± SD, * *p<0.05*, ** *p<0.05*).

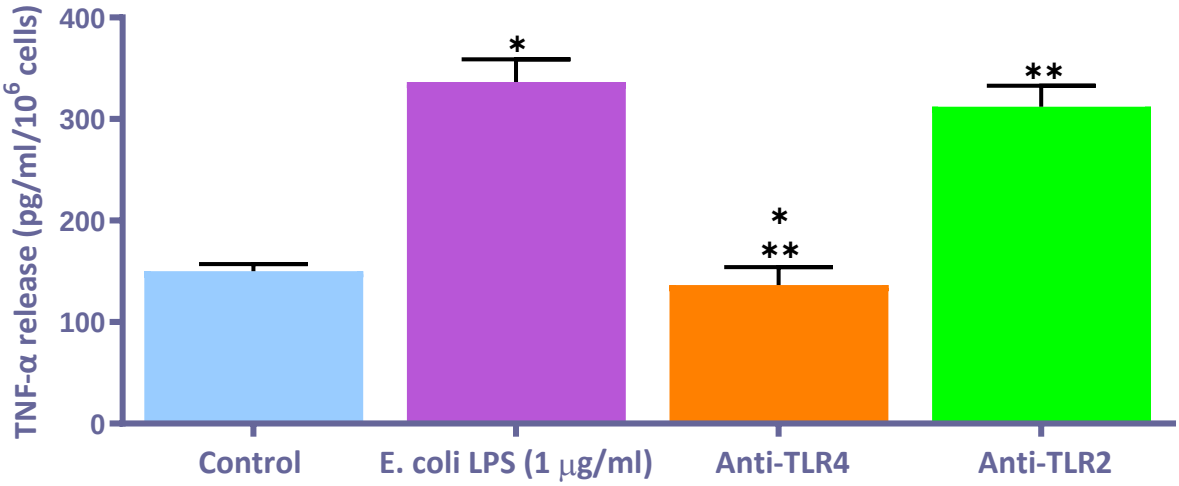


Figure 15. TNF-α release from LAD2 cells stimulated with *E. coli* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h. (n=3, mean ± SD, * *p<0.05*, ** *p<0.05*).

When LAD2 cells were stimulated with *P. gingivalis* LPS (1 μg/ml), TNF-α release increased to 380.7 ± 46.65 pg/ml/10^6 cells, compared to unstimulated cells where the release was limited to 150.3 ±13.56 pg/ml/10^6 cells (*p<0.05*). When LAD2 cells were pre-treated with anti-TLR4 antibody, the levels of TNF-α release were reduced at 332.7 ± 26.15 pg/ml/10^6, a change not statistically significant from the LPS Group (*p>0.05*). On the contrary, when the anti-TLR2 antibody was used, the levels of TNF-α release dropped to 142.9 ± 6.03 pg/ml/10^6, reduction statistically significant compared to both LPS and anti-TLR4 Groups (*p<0.05*).

When LAD2 cells were stimulated with *E. coli* LPS (1 μg/ml), TNF-α release increased to 336.4 ± 38.85 pg/ml/10^6 cells, compared to unstimulated cells where the release was limited to 151.2 ± 13.56 pg/ml/10^6 cells (*p<0.05*). When LAD2 cells were pre-treated with anti-TLR4 antibody, the levels of TNF-α were statistically significantly reduced at 136.6 ± 30.25 pg/ml/10^6 cells, compared to the LPS and anti-TRL2 Groups (*p<0.05*). On the contrary, when the anti-TLR2 antibody was used, the levels of TNF-α release were 312.3 ± 35.6 pg/ml/10^6 cells, change not statistically significant from the LPS Group (*p<0.05*).

- **Observations**

TNF-α release was statistically significantly increased after LAD2 cells were stimulated with both *P. gingivalis* and *E. coli* LPS compared to controls (*p<0.05*), findings in accordance with previous results. Pre-treatment with anti-TLRs had differential effects on TNF-α release from LAD2 cells stimulated with the two different LPSs. Specifically, only the anti-TRL2, but not the anti-TLR4 antibody, significantly reduced TNF-α release from MCs stimulated with *P. gingivalis* LPS. In contrast, pre-treatment with anti-TLR4 and not anti-TLR2, resulted in significant reduction of the levels of TNF-a in the *E. coli* LPS Group.

4.4.2. Blocking Effect on VEGF Release

The effect of anti-TLR antibodies on the release of VEGF is shown in *Fig. 16* for *P. gingivalis* LPS and in *Fig. 17* for *E. coli* LPS.

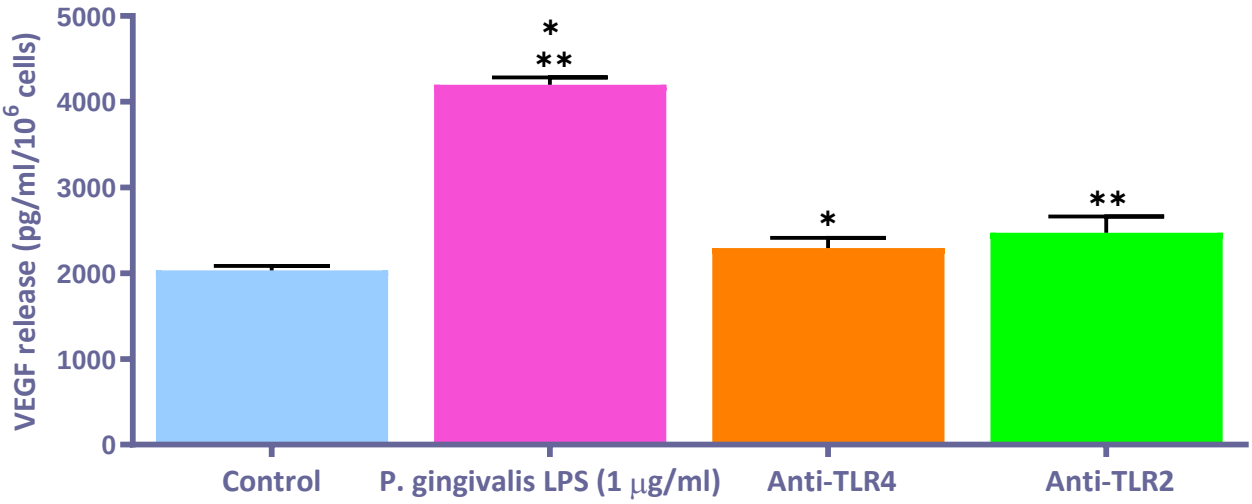


Figure 16. VEGF release from LAD2 cells stimulated with *P. gingivalis* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h. (n=3, mean ± SD, * *p<0.05*, ** *p<0.05*).

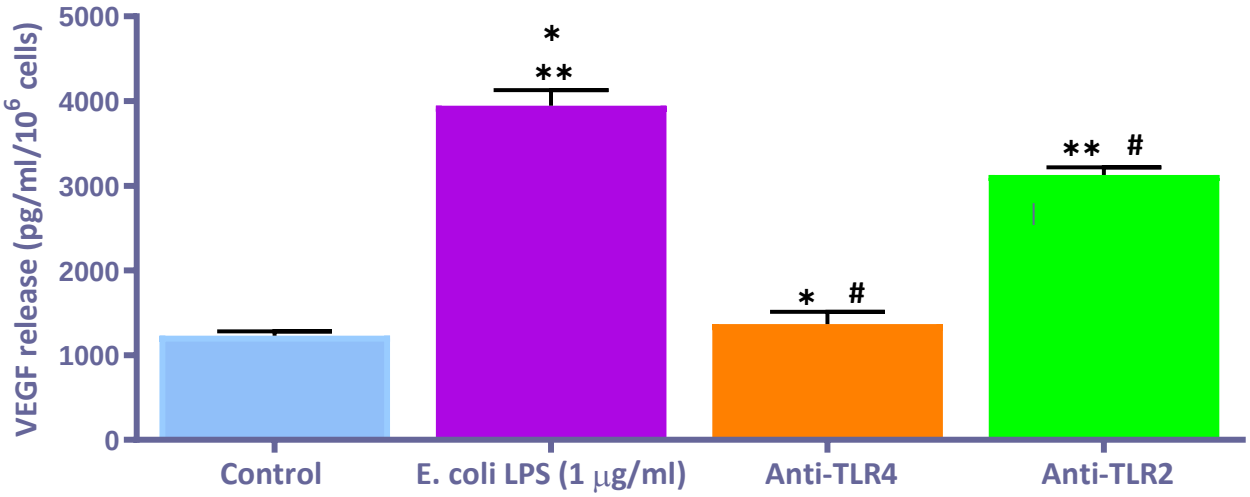


Figure 17. VEGF release from LAD2 cells stimulated with *E. coli* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h. (n=3, mean ± SD, * $p<0.05$, ** $p<0.05$, # $p<0.05$).

When LAD2 cells were stimulated with *P. gingivalis* LPS (1 μg/ml), VEGF release increased to 4199 ± 149.3pg/ml/10^6 cells, compared to unstimulated cells where the release was limited to 2032 ± 91.74 pg/ml/10^6 cells ($p<0.05$). When LAD2 cells were pre-treated with anti-TLR4 antibody, the levels of TNF-α release were reduced at 2292 ± 212.2 pg/ml/10^6, a change statistically significant from the LPS Group ($p<0.05$). In a similar manner when the anti-TLR2 antibody was used, the levels of VEGF release dropped to 2474 ± 327.9 pg/ml/10^6, reduction statistically significant compared to LPS Group ($p<0.05$). No statistically significant difference was noted for the two different antibodies used (anti-TLR2 *vs* anti-TLR4) ($p>0.05$).

When LAD2 cells were stimulated with *E. coli* LPS (1 μg/ml), VEGF release increased to 3947 ± 315.1 pg/ml/10^6 cells, compared to unstimulated cells where the release was limited to 1232 ± 82.29 pg/ml/10^6 cells ($p<0.05$). When LAD2 cells were pre-treated with anti-TLR4 antibody, the levels of VEGF release were statistically significantly reduced at 1369 ± 245.5 pg/ml/10^6 cells, compared to the LPS Group ($p<0.05$). When the anti-TLR2 antibody was used, the levels of VEGF were slightly but statistically significantly reduced to 3128 ± 159.6 pg/ml/10^6 cells compared to LPS Group. Even if both antibodies induced a reduction on VEGF levels, for the anti-TLR4 Group the reduction was higher and statistically significant in comparison to anti-TLR2 Group ($p<0.05$).

- **Observations**

VEGF release was statistically significantly increased after LAD2 cells were stimulated with both *P. gingivalis* and *E. coli* LPS compared to controls (*p<0.05*), findings in accordance with previous results. Both anti-TRL2 and anti-TRL4 antibodies significantly reduced VEGF release after stimulation with either *P. gingivalis* or *E. coli* LPS. Pre-treatment of LAD2 cells stimulated with P. *gingivalis* LPS with both antibodies, resulted in the same extent of reduction in the levels of VEGF. For the *E. coli* LPS Group, even if both antibodies resulted in reduction in VEGF levels, the reduction was more pronounced for the anti-TLR4 Group.

4.4.3. Blocking Effect on MCP-1 Release

The effect of anti-TLR antibodies on the release of MCP-1 is shown in *Fig. 18* for *P. gingivalis* LPS and in *Fig. 19* for *E. coli* LPS.

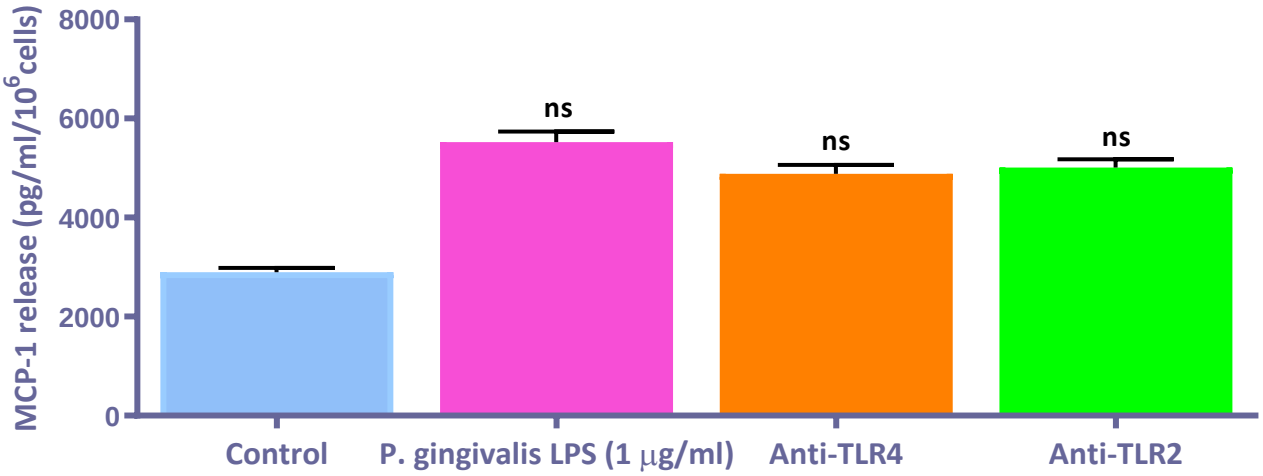


Figure 18. MCP-1 release from LAD2 cells stimulated with *P. gingivalis* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h. (n=3, mean ± SD, **ns**: no significance*, p>0.05*).

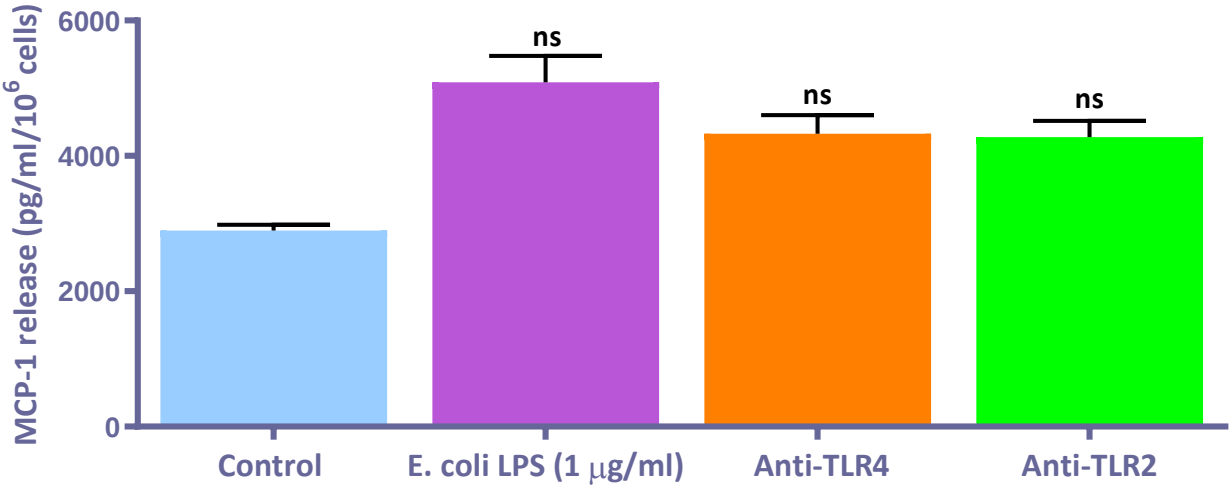


Figure 19. MCP-1 release from LAD2 cells stimulated with *E. coli* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h. (n=3, mean ± SD, **ns**: no significance*, p>0.05*).

When LAD2 cells were stimulated with *P. gingivalis* LPS (1 μg/ml), MCP-1 release increased to 5527 ± 361.0 pg/ml/10^6 pg/ml/10^6 cells, compared to unstimulated cells where the release was limited to cells 2899 ± 142.2 pg/ml/10^6 cells (*p<0.05*). When LAD2 cells were pre-treated with anti-TLR4 antibody, the levels of MCP-1 release were slightly reduced at 4886 ± 306.2 pg/ml/10^6, a change not statistically significant from the LPS Group (*p>0.05*). In a similar manner when the anti-TLR2 antibody was used, the levels of MCP-1 slightly dropped to 5012 ± 290.8 pg/ml/10^6, reduction statistically insignificant compared to LPS Group (*p>0.05*). No statistically significant difference was noted for the two different antibodies used (anti-TLR2 vs anti-TLR4) (*p>0.05*).

When LAD2 cells were stimulated with *E. coli* LPS (1 μg/ml), MCP-1 release increased to 5085 ± 681.5 pg/ml/10^6 cells, compared to unstimulated cells where the release was limited to 2899 ± 142.2 pg/ml/10^6 cells (*p<0.05*). When LAD2 cells were pre-treated with anti-TLR4 antibody, the levels of MCP-1 release were statistically insignificantly reduced at 4325 ± 476.1 pg /ml/10^6 cells, compared to the LPS Group (*p>0.05*). When the anti-TLR2 antibody was used, the levels of MCP-1 were slightly reduced to 4276 ± 415.5 pg/ml/10^6 cells, statistically insignificant compared to LPS Group (*p>0.05*). No statistically significant difference was noted for the two different antibodies used (anti-TLR2 vs anti-TLR4) (*p>0.05*).

- **Observations**

As previously reported, MCP-1 release was statistically significantly increased after LAD2 cells were stimulated with both *P. gingivalis* and *E. coli* LPS compared to controls ($p<0.05$). Interestingly, pre-incubation of LAD2 cells with either anti-TLR2 or anti-TLR4 antibodies, resulted in a minimal reduction in MCP-1 release, effect that was not statistically significant in both LPSs Groups. No difference was noted for the two antibodies used in each different LPS Group (*$p>0.05$*).

4.4.4. Conclusions

The levels of the three mediators studied, were reduced (either slightly, either pronounced) after pre-treatment with anti-TLR4 and anti-TLR2 antibodies in both LPSs Groups.

TNF-α levels were significantly reduced in the *P. gingivalis* LPS Group, after incubation with anti-TLR2 antibody whereas in the *E. coli* LPS Group, the reduction was more prominent after incubation with the anti-TLR4 antibody.

Both anti-TLR4 and anti-TLR2 antibodies significantly reduced VEGF levels after stimulation with either *P. gingivalis* or *E. coli* LPS. The maximum reduction in VEGF levels was noted in the *E. coli* LPS Group after incubation with anti-TLR4 antibody.

Interestingly, pre-incubation of LAD2 cells with either anti-TLR4 or anti-TLR2 antibodies didn't statistically significantly reduce the levels of MCP-1 in either of the LPSs Groups.

5. DISCUSSION

To our knowledge, this is the first study that aimed to investigate the effect of *P. gingivalis* LPS on MCs stimulation and to compare its potency with *E. coli* LPS, that is used as the standard LPS in the majority of the studies. The influence of periodontopathic bacterial cell wall components on synthesis and release of cytokines by MCs was so far, rarely studied. Although there was a broad scientific knowledge about the pathogenesis of periodontal disease, additional studies were needed for a better understanding of the biologic events involved in this pathology, especially those related to MCs. In the context of infectious diseases, it is likely that this will not only expand the scope of our knowledge of the role of MCs in innate immunity but might provide new therapeutic targets to control the inflammatory response in gingival and periodontal tissues against the pathogenic bacteria.

MCs are involved in numerous activities ranging from control of the vasculature to tissue injury and repair, allergic inflammation, and host defence (Tsilioni et al. 2019, Batista et al. 2005). Recently it was shown that MCs have important effector functions against pathogens, and they play a pivotal role in orchestrating both innate and adaptive immune response against local microbial challenge (Galli et al. 1993). MCs synthesize and secrete a wide variety of mediators, modulating the functions of nearby cells and initiate complex physiological changes. Increasing evidence implicates MCs in inflammatory diseases, especially those exacerbated by stress such as periodontal inflammation (Papathanasiou et al. 2013).

It was documented that MCs can accumulate at inflammatory sites of periodontal tissues in patients with chronic periodontitis and produce TGF, MMPs, TIMPs and IL-17, leading to destruction of periodontal tissues (Myint et al. 2002, Naesse et al. 2003, Parachuru et al. 2018). Moreover, it was shown that MCs release TNF-α and IL-6 in response to *P. gingivalis* infection and induce alveolar bone loss in mice (Malcolm et al. 2016). These findings imply that MCs derived mediators may play a role in exacerbation of inflammation in periodontal tissues. Other studies have shown that MCs tryptase, the major secretory product by human MCs, was positively correlated with the degree of infiltration of inflammatory cells and the severity of periodontal disease (Huang et al. 2013).

In our study, the LAD2 cell line was used. LAD2 MCs are known to be highly maturated human MCs and they are the first reported human MC line which closely resemble primary cultures of CD34+-derived human MCs. They respond to rhSCF and they have functional FcεRI and FcγRI receptors although their growth rate is very slow. They are also characterized by the expression of functional CD14 and TLRs (Kirshenbaum et al. 2003).

It was documented that LAD2 cells can express, on both mRNA and protein level, the TLR2 and TLR4 receptors (Mc Curdy et al. 2001). The mechanism implicated in TLR4 activation involves the membrane associated CD14 that is bound to LPS-LBP complex and subsequently transfers them to TLR4/MD2. Stimulation of TLR4/MD2 by the complex finally causes cells to generate pro-inflammatory cytokines such as TNF-α (Pallson-McDermott et al. 2004, Kubo et al. 2016). Regarding TLR2, it was demonstrated that exposure of human MCs to TLR2-activated microorganisms led to suppression of the acute degranulation of MCs mediated by FcεRI activation. This observation suggested that activation of TLR2 on MCs might direct their functions from being allergic responders towards being sentinel effectors in the host innate immune response (Yu et al. 2014). This was verified in our study, as the use of anti-TLR2 and anti-TLR4 antibodies resulted with different potency, in reduction of the amounts of the tested mediators released.

In this study we decided to assess the expression, on both mRNA and protein level, of three mediators that have been documented to be implicated in the pathogenesis and progression of periodontal disease. TNF-α, VEGF and MCP-1 exhibit a wide variety of biologic effects related to the destruction of periodontal tissues (Gravest et al 2008). Elevated levels of these mediators in the oral environment are important risk factors for periodontal disease progression (Garlet et al. 2003, Kurtis et al 2005). High levels of these mediators have already been detected in periodontal tissues, gingival crevicular fluid and saliva of periodontally affected patients compared to healthy controls or periodontally treated patients, as well as in the serum of patients presenting with periodontal disease (Petkoviz et al. 2010, Pradeep et al. 2011, Ozer et al. 2015).

We reported that *P. gingivalis* and *E. coli* LPS induced the expression and release of TNF-α, MCP-1 and VEGF by LAD2 MCs, 24h after stimulation with two different concentrations chosen. In addition, we showed that this effect was partially but significantly decreased by

anti-TLR2 or anti-TLR4 antibodies. We based the selection of the minimum (1 ng/ml) and the maximum (1 μg/ml) concentration of LPS used for our experiments on similar studies where LPS ranging between 1ng/ml to 1000 ng/ml was used to investigate their effects on different cell types (Kocgozly et al. 2009). Incubation of MCs with either LPS did not affect MCs viability, as was determined by trypan blue exclusion, findings in accordance with previous studies tested viability of MCs after LPS stimulation (Konopka et al. 2010).

We showed that stimulation of MCs with either type of LPS did not induce degranulation of MCs but instead, *de novo* synthesis and release of TNF-α, VEGF and MCP-1 was reported. It has been proven that most pathogens appear to not induce MCs degranulation but instead induce secretion of lipid mediators and *de novo* synthesis and release of cytokines. For example, it was shown that LPS from *E. coli*, PGN from *Staphylococcus aureus* and LTAs from *S. aureus* and *Bacillus subtilis* did not induce mature rat MCs degranulation (Leal-Berumen et al. 1994, Supajatura et al. 2001, Konopka et al. 2010) These are in accordance with our findings where stimulation of LAD2 cells did not induce their degranulation.

The last decade, more studies were focused on the role of MCs in inflammation. It is now well established that some bacterial cell wall components can stimulate both mature and immature MCs to synthetize many different cytokines, *de novo*. Diverse effects of bacterial wall components including LPS (mainly enterobacteria originated) on MCs degranulation and mediator release is documented in the literature. It has been shown that murine BMMCs activated by LPS, produced, and released TNF-α, IL-1, IL-5, IL-6, IL-10, and IL-13 (Supajatura et al. 2001, Masuda et al. 2002). Murine mature connective tissue MCs were able to produce IL-6, IL-10 and TNF-α, whereas human MCs released IL-8 and IL-12, in response to activation with LPS (Kirshenbaum et al. 2008, Mrabet-Dahbi et al. 2009). The effect of *E. coli* LPS on rat peritoneal MCs degranulation and histamine release was examined in an animal study. *E. coli* LPS in concentrations ranging from 1 ng/ml to 1000 ng/ml, was not able to induce degranulation and histamine release from MCs, results in accordance with our findings. In addition, on the same study, activation of MCs with different concentrations of LPS resulted in generation and release of cysteinyl leukotriene (cysLT) in a dose response manner (Wierzbicki et al. 2009).

In contrast, the influence of periodontopathic bacterial cell wall components on synthesis and release of mediators implicated in periodontal inflammation, by MCs was, so far, rarely studied. Limited studies examined the effect of *P. gingivalis* LPS on different MCs lines and they were mainly focused on the release of cysLT. This is an important pro-inflammatory mediator influencing microvascular permeability, causing leukocyte recruitment, especially the influx of neutrophils, and enhancing phagocytosis and killing of bacteria. Thus, LTs are responsible for initiating the inflammation as well as for sustaining the inflammatory periodontal process.

Specifically, Konopka et al. (2010) observed by using rat peritoneal MCs that *P. gingivalis* LPS (in a concentration of 1 ng/ml) stimulated MCs to generate and release significant amounts of cysLTs and that the extent of mediator release was 4-fold greater than in response *to S. enteritis* LPS stimulation. They also noted that *P. gingivalis* LPS did not stimulate MCs migration. At the same study it was shown that *P. gingivalis* LPS upregulated the expression of TLR2 and TLR4 expression on MCs surface indicated that these cells could use both receptors to stimulate cell response, findings in accordance with our study even if a different mediator was studied (Konopka et al. 2010).

Babolewska et al. (2012) presented the same results by examining the potency of different bacterial antigens in inducing cysLT generation by mature rat MCs. They showed *that P. gingivalis* LPS exhibited the highest potency in synthesis and release of cysLTs at very low concentration (10-4 ng/ml) in comparison to *E. coli* LPS that caused the same release at a higher concentration (up to 106- fold). Anti-TLR2 and anti-TLR4 antibodies as nuclear factor κB (NF- κB) inhibitor significantly diminished cysLT generation in response to *P. gngivalis* and *E. coli* LPS, results similar with the previous study.

In our study we showed that on the mRNA level, the stimulation of LAD2 cells with *P. gingivalis* LPS induced an increase in gene expression for all the three mediators studied (TNF-α, VEGF, MCP-1). The same results were observed for *E. coli* LPS regarding the increase in TNF-α and VEGF gene expression but not for MCP-1 where there was minimal change. Regarding the two different LPSs used, no statistically significant difference in gene expression was observed for the three mediators studied, implying that both LPSs had the potential to induce the same amount of relative gene expression of the three mediators

studied. Regarding the expression on the protein level, *P. gingivalis* LPS and *E. coli* LPS in the same concentration triggered approximately, the same amount of *de novo* release of TNF-α, VEGF and MCP-1. However, there was the tendency of slightly higher levels of mediator release for *P. gingivalis* LPS in both tested concentrations compared to *E. coli* LPS. Interestingly, in our study, a dose-response relationship was not shown, findings that are not in agreement with previous studies, where a dose-response relationship was documented after stimulation of different cells lines with *P. gingivalis* or *E. coli* LPS (Steffen et al. 2000, Wang & Ohura 2002). This difference may be due to the different behaviour of the cells used such as fibroblasts and monocytes in comparison to MCs. In our study, as both concentrations stimulated MCs at the same extent, this might imply the existence of an "on-off" response of MCs to this specific stimulus but of course further research to confirm this hypothesis is needed.

Some studies have suggested that *P. gingivalis* LPS has an ability to stimulate cytokine production comparable to that of *E. coli*, whereas others reported a much lower potency of *P. gingivalis* LPS in various cells (Reife et al. 1995, Darveau et al. 1995, Ogawa et al. 1996, Roberts et al. 1997, Shapira et al. 2000). In the present study, we did not observe any difference in their potency to induce release of TNF-α, MCP-1 and VEGF even if there was a tendency for higher release after stimulation with *P. gngivalis*. This is possibly related to *P. gingivalis* strain used, the LPS extraction method, the selection of the times for detecting cytokine levels and the target cells used. Different strains of the same bacterium or different extraction methods have considerable effect on the purity and especially the activity of LPS. Our findings are in accordance with previous studies where it was shown that there was a more potent increase in TNF-α and MCP-1 release from human monocytes and gingival fibroblasts respectively, when both cell lines were stimulated with *P. gingivalis* LPS compared to *E. coli* LPS (Agarwal et al. 1995, Doyle et al. 2014). Specifically, on a study following same methodology, the maximum concentrations of IL-1, IL-6 and TNF-α induced by *P. gingivalis* LPS were similar to those of *E. coli* LPS on a concentration of 1 μg/ml (Zhang et al. 2008). On the other hand, LPS from *P. gingivalis* (1 μg/ml) had no effect on IL-6 production from periodontal ligament cells compared to that of *E. coli* LPS, implying that *E. coli* was a more powerful stimulator (Nebel et al. 2013). Differential potency on MCP-1 release was also documented in mice cell lines, with *E. coli* LPS to induce stronger response

in gingival fibroblasts whereas *P. gingivalis* LPS to induce a stronger chemokine response on macrophages (Jones et al. 2010). Unfortunately, there are no studies yet conducted in MCs to directly compare the effects of LPS on the specific cytokines we used in our study.

As TLRs are critical for cellular responses to a variety of bacterial products and MCs might act as sentinels' cells in host defence, we tried to examine their role in MCs activation. We were based on previous studies that were conducted to examine the expression of TLR2 and TLR4 in different animal and human MCs. McCurdy et al. (2001) noted that BMMCs were able to express TLR2, TLR4 and TLR6 in their surface. In addition, stimulation of these cells with *E. coli* LPS on a concentration ranging from 0.05 to 5 μg/ml, resulted in synthesis and release of IL-6 and TNF-α, release that was completely dependent on functional TRL4 with no significant LPS response observed in its absence (McCurdy et al. 2001). In a similar study, using human CBMC, it was found that both *E. coli* LPS and PGN induced significant release of not only TNF-α but also IL-5, IL-10 and IL-13 and this stimulation was mediated through interactions with TLR4 and TRL2 (Varadaradjalou et al. 2003). On a later *in vitro* study, the expression of TLR4 in two different human MCs lines was investigating. After stimulation of HMC-1 and LAD2 cell lines with LPS (1 ng/ml), the expression of TLR4 on both mRNA and protein level was increased. LAD2 cells pre-treated with LPS for 8 hours resulted in a 2-fold increased TNF-α secretion after LPS stimulation (Kubo et al. 2007).

It is well known that TLR4 is the major signal transducer for most types of LPS while TLR2 was suggested to be a signal transducer for other bacterial components such as PGNs and lipoproteins (Takeuchi et al. 1999). A well characterized innate host recognition pathway is that for *E. coli* LPS. On the other hand, *P. gingivalis* usage is very controversial. It has been shown to signal through TLR2 or TLR4 to activate different cell types to produce cytokines such as macrophages, gingival epithelial cells, gingival fibroblasts, and endothelial cells (Nassar et al. 2002, Wang & Ohura et al. 2002). These discrepancies were hypothesized to be due to differences in LPS preparations, since differently acylated LPS Lipid A moieties induce different cellular effects or they were explained by trace amounts of lipoprotein contamination in native *P. gingivalis* LPS preparations (Hashimoto et al. 2004, Reife et al. 2006). However, in this study, we used a commercial *P. gingivalis* LPS preparation purified from lipoprotein contaminants that non-specifically activate TLR2. Our selection was based

on previous studies that used the same preparation to stimulate different cell lines including MCs (Hirschfeld et al. 2000, Konopka et al. 2010, Babolewska et al. 2012).

Therefore, we sought to find the functional involvement of TLR4 and TLR2 in LPS induced cytokine responses by LAD2 MCs. Both anti-TLR2 and anti-TLR4 antibodies significantly reduced VEGF release in response to *P. gingivalis* LPS. Anti-TLR4 resulted in reduced amounts of VEGF after stimulation with *E. coli* LPS in comparison to anti-TLR2 effect. Interestingly, *E. coli* LPS-stimulated VEGF release was influenced by anti-TLR2 too. Although ligand recognition shows specificity for each receptor, the downstream signaling pathways activated by TLRs have some redundancy, generating the potential for signaling cross talk. Intriguingly, cross talk between TLR2 and TLR4 has been reported in previous studies (Lee et al. 2007). TNF-α release was significantly reduced after pre-treatment with anti-TLR2 in response to *P. gingivalis* LPS in comparison to anti-TRL4. On the contrary, pre-treatment with anti-TRL4 led to significant reduction of TNF-α in response to *E. coli* LPS. MCP-1 release, on the other hand, was not affected by the pre-treatment of MCs with either anti-TRL2 or anti-TLR4. This may imply that MCP-1 release is controlled by a different pathway in MCs. Recently it was shown that MCP-1 release was stimulated through TLR9 in macrophages (Lee et al. 2008). Emerging trends emphasize the significance of TLR9 in host immune responses (Janeway et al. 2002) and especially in periodontitis as it was shown that TLR9-mediated inflammation triggered alveolar bone loss in experimental periodontitis (Kim et al. 2015).

In our study, we noticed that *P. gingivalis* LPS can utilize TLR4 and/ or TLR2 on MCs surface. Our findings are in agreement with previous studies conducted in different cell lines. Kirikae et al. (1999) stated that *P. gingivalis* LPS exhibited activity in mice macrophages, which were deficient for TRL4. Moreover, it was reported that *P. gingivalis* LPS stimulated human gingival fibroblasts to secrete IL-1 and IL-6 via TLR4 (Tabeta et al. 2000, Maezawa et al. 2006). In THP-1 cells, *P. gingivalis* could utilize both TLR2 and TLR4 (mainly through TLR2) to induce the production of cytokines, while *E. coli* LPS stimulated cytokine production via TLR4 (Zhang et al. 2008). In addition, it has been reported that TNF-α production in human macrophages induced by *P. gingivalis* LPS was blocked by anti-TLR2 antibody, but not anti-TLR4 antibody, findings in agreement with our results (Martin et al. 2001). Mori et al. showed that human gingival tissues in periodontitis, expressed TLR2 and TLR4 and the ratio of TLR2 positive cells was higher than that of TLR4 (Mori et al. 2003). In addition, *P. gingivalis* LPS-induced TNF-α

production was predominantly driven by TLR2 pathway in macrophages and endothelial cells (Wang et al. 2002, Hajishengalis et al. 2006, Maekawa et al. 2014). The stimulation of oral epithelial cells induced a 2.5fold upregulation of TNF-α release which was strongly inhibited by the use of anti-TLR2 antibody (Kocgozlu et al. 2009). In an *in vitro* study, the production of IL-6 and MCP-1 was increased in gingival fibroblasts stimulated with *E. coli* LPS, in contrast to *P. gingivalis* LPS that stimulated macrophages instead, to produce the same cytokines (Jones et al. 2015). In addition, it was shown that monocytes were activated mainly through TRL4 when triggered with *E. coli* LPS while *P. gingivalis* LPS activated the same cells through TRL2 (Barksby et al. 2009). Both TRL2 and TRL4 were required for monocytes derived dendritic cells maturation by *P. gingivalis* LPS. DCs stimulated by *P. gingivalis* LPS were prone to induce a stronger Th2 cell response mainly through TLR2 whereas same cells stimulated by *E. coli* LPS were prone to induce a stronger Th1 cell response through TLR4 pathway (Su et al. 2015).

In our study we used SP as positive control and it was shown to be the most potent inducer of TNF-α, MCP-1 and VEGF release from LAD2 MCs. In addition, SP was able to cause MCs degranulation as shown on β-hex release experiment. Our findings agree with previous studies where it was reported that SP could induce gene expression and secretion of VEGF from human LAD2 MCs and human umbilical cord blood-derived cultured MCs (hCBMCs). SP could also stimulate TNF-α and MCP-1 chemokine transcription in LAD2 cells *in vitro* (Gibbs et al. 2001, Castellani et al. 2009, 2010, Theoharides et al. 2010). Recently it was shown that SP in collaboration with IL-33 could stimulate MCs to release significant amounts of VEGF, TNF-α and IL-1β (Theoharides et al. 2010, Fux et al. 2014, Taracanova et al. 2017, 2018).

SP is a multifactorial neuropeptide that is involved in neurogenic inflammation and is an important neuromodulator (Nicoletti et al. 2012). It was reported that SP controls MCs physiopathology in several inflammatory diseases, neurological dysfunctions, and stress such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, multiple sclerosis, mastocytosis and fibromyalgia syndrome (Theoharides et al. 2015). Independent reports have shown that SP, IL-33, and TNF-α contribute to the inflammatory processes in these diseases (Theoharides et al. 2012, Tsilioni et al. 2016).

It is well known that Inflammatory responses in various organs have been reported to exhibit a neurogenic component. Recently, the nervous system has been identified as a critical regulator of inflammation in periodontal disease (Cekici et al. 2013, Preeja et al. 2013). During the innate immune response to periodontal pathogens, another element of the human defence system is also activated. Neurons generate electric impulses in response to chemical or mechanical stimuli, conduct the impulse and translate the electrical activity into a chemical signal. Alternatively, peptide neurotransmitters – neuropeptides – can be secreted into the extracellular fluid, where they act locally through receptors on other neurons or immune cells. Most neuropeptides act on nonneuronal targets, such as receptors for substance P and calcitonin gene-related peptide, found on immune cells, suggesting that the paracrine action of neuropeptides has important immunomodulatory roles (Lundy et al. 2004).

Neuropeptides are biologically active peptides generated primarily in neurons. Sensory neuropeptides play important role in neurogenic inflammation, including vasodilation, plasma extravasation, and recruitment of immune cells (Masaghi et al. 2016). However, a more extensive function for neuropeptides in the regulation of immune cells activity has also been proposed. During inflammation, there is a sprouting of peptidergic peripheral fibers and an increased content of neuropeptides. Peptide-containing nerve fibers are often seen close to immune cells, particularly macrophages and MCs (Kabashima et al. 2002). It was documented that immune cells present with functional neuropeptide receptors on their surface, implying their role in neuroimmunomodulation. (Akcali et al. 2007). The contribution of the nervous system to inflammation is not limited to vasodilatation and immune cell recruitment. Cytokines and other products of immune cells can modulate the action, differentiation, and survival of nerve cells, while neuropeptides released from neurons play pivotal roles in influencing the immune response. The interaction relies on the receptor-sensitizing characteristics of the cytokines. As MCs reside close to the sensory nerves, they play a significant role in orchestrating neuroinflammation that is exacerbating under psychological stress as noticed in periodontal inflammation (Akcali et al. 2007).

SP has been detected in gingival tissues and in GCF of healthy and periodontally affected patients, being elevated on the affected ones (Gupta et al. 2013). It was shown that SP levels in GCF were positively corelated with the degree of periodontal inflammation. Interestingly,

it was documented that resolution of inflammation, as a result of effective periodontal treatment was associated with a reduction in the levels of SP and with an improvement in the periodontal clinical parameters (Lundy et al. 2000, 2004, Haririan et al. 2018).

SP is important in initiating and sustaining inflammation, by regulating the proliferation, migration, and activation of immune cells. SP can act, not only by augmenting the production of other inflammatory mediators from immune cells, but also it can be produced from cells triggered with LPS or other cytokines (Praddeep et al. 2008). Cytokine-neuropeptide interactions are bidirectional—that is, cytokines and other products of the immune cells can modulate the action, differentiation, and survival of neuronal cells, while neuropeptides released from neurons play pivotal roles in influencing the immune response. Several studies have shown that SP exerts certain regulatory functions such as increasing vascular permeability, affecting vasodilation and increasing angiogenesis, actions important for the initiation and progression of periodontal disease (Dantzer et al. 2018).

Recently it has been stated that SP enhances the inhibition of osteoblastic cell differentiation induced by *P. gingivalis* LPS (Niedermair et al. 2018) and stimulates the resorption activity of osteoclasts (Azuma et al. 2004). This effect may be related to pathological bone metabolism in periodontal disease under conditions of stress. In addition, SP showed significant correlation with mediators of the host response such as IL-1, TNF-α and MCP-1. Specifically, SP induced the release of IL-1 and IL-6 in human monocytes under presence of plaque-related LPS in an *in vitro* model (Lieb et al. 1996). In addition, SP in low concentrations, was able to upregulate the release of TNF-α in gingival fibroblasts, thus further exacerbating tissue destruction and bone resorption (Yan et al 2020).

There have been many reports linking stress related disorders as a risk indicator for periodontal disease (Genco et al. 2013). Several environmental risk factors, including smoking, diabetes mellitus and psychological stress, may modify the host response, and hence disease progression, severity, and outcome of periodontal treatment (Peruzzo et al. 2007). Stress has also been known to be an important predisposing factor in the development of necrotizing ulcerative gingivitis for over 40 years (Decker et al. 2020).

Emotional stress is associated with the activation of a variety of neuro-immune-endocrine systems. MCs are the major link between neurons and neuroinflammation (Tore & Tuncel

2009). Under stress, activated MCs generate cytokines/chemokines which together with neuropeptides such as SP are involved in the development of several neuropathological processes, and are implicated in inflammation of the central and peripheral nervous system.

There is considerable evidence that stress worsens allergic diseases through MCs activation as they participate in the brain-skin axis (Paus et al. 2006) as targets of CRH and related peptides (Theoharides et al. 2004, 2012, Wright et al. 2005). CRH is typically secreted from the hypothalamus under stress and activates the hypothalamic-pituitary-adrenal (HPA) axis. MCs are located close to CRH-positive nerve endings. It was documented that SP increased the expression of receptors for CRH on MCs, implying its role on neuroinflammation (Asadi et al. 2012).

The mechanisms explaining the possible relationship between stress and increased susceptibility to periodontal disease remain poorly understood. Several studies showed that stress can alter immune response by specific mechanisms (Peruzzo et al. 2007). In a recent work that we published, we tried to explain the mechanism involved in stress induced periodontal disease (Papathanasiou et al. 2013). One of them is based on the idea that stressful events stimulate the HPA axis, promoting the release of CRH from the hypothalamus and glucocorticoid hormones, including cortisol, from the adrenal cortex. CRH may modulate eventually the immune and inflammatory responses via two pathways: an anti-inflammatory one operated by centrally released CRH, but also a pro-inflammatory one, through direct action of peripherally released CRH. Moreover, a cascade of several inflammatory cytokines, including TNF-α, IL-1, IL-6, released during immune system activation can stimulate the HPA axis and cause increased secretion of CRH. Another stressed-induced response mechanism is based on the induction of the release of neuropeptides such as SP from sensory nerve fibres and resident immune cells that could promote vasodilation, immune cell recruitment and amplification of inflammatory cytokine responses (Papathanasiou et al. 2013). In our study, we showed that SP-stimulated MCs produced significant amounts of TNF-α, MCP-1 and VEGF, findings that not only justify the existence of a neurogenic component in periodontal inflammation, but also, shed light on the potential implication of MCs on the reported association between stress and periodontitis.

Overall, we reported for the first time that LPS from *P. gingivalis* selectively, i.e. without degranulation, stimulated MCs to generate and release with the same potency as *E. coli* LPS, TNF-α, VEGF and MCP-1, mediators that have been documented to play an important role in the initiation and progression of periodontal disease. In addition, we documented that *P. gingivalis* LPS could function through both TLR2 and TLR4 in MCs. It is undisputable that MCs play a crucial role in the development of inflammation, in the course of many pathological processes including allergic reactions as well as during bacterial infection. Therefore, our findings could indicate that MCs might be involved in the emergence of inflammatory processes evolved in response to *P. gingivalis* infection such as periodontal disease.

It should be stressed, however, that our experiments were performed in LAD2 cell line. This model provides valuable tool for studying MC function *in vitro*, however it has important limitations to extrapolate data to human *in vivo* situations. Even if it closely resembles primary cultures of human MCs, it might display a different behaviour in comparison to MCs obtained directly from periodontal tissues. In addition, as in our study we used specific concentrations and time points (24h), more studies are needed to elucidate if there is a dose or time response relationship, as we were unable to document such correlations. Therefore, further experiments are needed to clarify the effect of LPS from *P. gingivalis* on human MCs *in vivo.*

Conclusions

MCs are essential tissue resident immune cells that are found in most tissues of the body, particularly abundant in locations that are in close contact with the external environment such as skin, airways and intestines, typically located adjacent to vessels and near the epithelium. Once released into circulation, undifferentiated precursors complete maturation and differentiation after being recruited to specific tissues, a process that is influenced by environmental factors, cellular origin, bacterial products, and cytokines (Steinvoll et al. 2004).

In this study, we established a possible role of MCs as the first line of defense against periodontopathic bacteria. The major function of MCs in antibacterial defence is to induce and control the development of inflammation, by *de novo* secretion and action of MCs derived pro-inflammatory mediators, cytokines and chemokines released in response to

bacteria and their products (Marshall et al. 2004, 2003, Kinet et al. 2007). The significant contribution of MCs mediators to tissue damage and the propagation of inflammatory response makes control of MCs function vital to the management of many inflammatory diseases such as periodontal disease.

An appreciation of the multiple interactions among MCs, nerves and other cells of the immune system will provide a basis for therapies for targeting MCs responses. The recent identification of a neurogenic component in periodontal disease and the existence of interactions between nerves and MCs in a neural-immune network opens new possibilities for altering the function of these critical immune cells. In the future it may be possible to develop novel approaches that influence the release of inflammatory molecules and neuropeptides to ameliorate MCs driven inflammation including periodontal disease.

6. LITERATURE

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7. ABBREVIATIONS

- Aa: Aggregatibacter actinomycetemcomitans
- AMPs: Antimicrobial peptides
- APCs: Antigen Presenting Cells
- Bcl: B-cell Lymphoma
- BMMC: Bone Marrow Derived
- C: Complement
- CAL: Clinical attachment loss
- CB: Cord Blood
- CCL: Chemokine (C-C motif) Ligand
- CD: Cluster of Differentiation
- CLRs: C-Type Lectin-like Receptors
- COX: Cyclooxygenase
- CPS: Capsyle polysacharide
- CRAMP: Cathelicidin-Related Antimicrobial Peptide
- CRH: Corticotropin-releasing hormone.
- DAMPs: Damage-associated molecular patterns
- DC: Dendritic Cells
- EGF: Epidermal Growth Factor
- EMPs: Extracellular Matrix Proteins
- FcγR: Fc region of IgG
- FcεRI: Fc region of antigen-specific immunoglobulin E
- FGF: Fibroblast Growth Factor
- FS: Fetal Skin
- GCF: Gingival Crevicular Fluid
- HSP: Heat sock proteins
- ICAM: Intercellular Adhesion Molecule 1
- Ig: Immunoglobulin
- IL: Interleukin
- INF: Interferon

- LBP: Lipopolysaccharide binding protein
- LO: Lipoxygenase
- LPS: Lipopolysaccharide
- LTA: Lipoteichoic acid
- LTB4: Leukotriene B4
- LTs: Leukotrienes
- MAMPs: Microbe-associated molecular pattern
- MAP: Mitogen-Activated Protein Kinase Pathway
- MCP-1: Monocyte Chemotactic Protein
- MCs: Mast Cells
- MHC: Major histocompatibility complex
- MIP-2β: Macrophage Inflammatory Protein 2α
- MMPs: Metalloproteinases
- Myd88: Myeloid Differentiation Primary Response Protein 88-dependent Pathway
- NF-κB: Nuclear factor Kappa-Light-chain-enhancer of Activated B cells Pathway
- NGF: Nerve Growth Factor
- NO: Nitric Oxide
- NODs: Nucleotide-binding Oligomerization Domain Like Receptors
- OPN: Osteopontin
- ox-LDL: Oxidized Low-density Lipoprotein
- PAF: Platelet-activating factor
- PB: Peripheral Blood
- PC: Peritoneal
- PCD: Programmed Cell Death
- PDGE2: Prostaglandin
- PDGF: Platelet Derived Growth Factor
- PF-4: Platelet Factor
- PGNs: Peptidoglycanes
- PMNs: Polymorphonuclear Leukocytes White Blood Cells
- PRR: Pattern Recognition Receptors
- PSGL-1: P-selectin glycoprotein ligand-1

- RANTES: Chemokine (C-C motif) ligand 5
- RIG-I: Retinoic acid-inducible gene I-like Receptors
- ROS: Reactive Oxygen Species
- SCF: Stem Cell Factor
- SgIGSF: spermatogenic immunoglobulin superfamily
- SP: Substance P
- TGF: Transforming Growth Factor
- TLR: Toll Like Receptors
- TNF: Tumour Necrosis Factor
- VCAM: Vascular cell adhesion protein
- VEGF: Vascular Endothelial Growth Factor

8. APPENDIX

8.1. Images

- **Image 1***:* Development of gingivitis and periodontitis. (*Open access peer-reviewed chapter* Pathogenesis of Periodontal Disease by José Luis Muñoz-Carrillo, 2019).
- **Image 2**. A diagram of the functional consequences of MCs Adhesion. (*Vliagoftis & Befus 2005. MCs at Mucosal Frontiers Current Molecular Medicine Sep;5 :573-89*).
- **Image 3.** MCs origin and differentiation. (*Adapted from Gurish MF, Austen KF: The diverse roles of MCs, J Exp Med 194: F1–F5, 2001. Illustration by Steven Moskowitz*).
- **Image 4.** Presentation of morphological characteristics of MCs under Light Microscopy (*Courtesy of Mariana Castells, MD PhD, Lawrence B Schwartz, MD, and Shirley Craig, PhD*).
- **Image 5.** Transmission electron microscopic photomicrographs of the buccal mucosa showing MCs degranulation (*Images were obtained at Dr Theoharides Lab and* presented at *Alhelal et al. 2014. Ann Allergy Asthma Immunol 112 40e45*).
- **Image 6.** Mechanisms of MCs Mediator Release. (*Image taken from Vliagoftis & Befus (2005) MCs at Mucosal Frontiers, Current Molecular Medicine Sep;5(6):573-89*).
- **Image 7.** MCs involvement in inflammatory diseases. Increasing evidence indicates MCs are involved in many diseases (*Theoharides TC et al, 2012. MCs and Inflammation. Biochimica et Biophysica Acta 1822L; 21–33*).

8.2. Tables

- **Table 1**: TLRs expressed by different categories of immune cells (Adapted from *Hans & Hans 2011*).
- **Table2**: Summary of MCs mediators (*Adapted from Theoharides et al. 2013*).
- **Table 3**: TNF, VEGF and MCP-1 release using enzyme-linked immunosorbent assay (ELISA). kits according to the protocol suggested by the manufacturer (R&D Systems).
- **Table 4**: Reaction Mix for Reverse Transcription (RT).
- **Table 5**: Reaction mix for qPCR.
- **Table 6**: Description of thermal cycles.

8.3. Figures

- **Figure 1**. Beta-Hexosaminidase release.
- **Figure 2.** Relative TNF-α mRNA expression after stimulation of LAD2 cells with *P. gingivalis* LPS, *E. coli* LPS (1 ng/mL) and SP (2 μM) for 6 hours.
- **Figure 3.** Relative VEGF mRNA expression after stimulation of LAD2 cells with *P. gingivalis* LPS, *E. coli* LPS (1 ng/mL) and SP (2 μM) for 6 hours.
- **Figure 4.** Relative MCP-1 mRNA expression after stimulation of LAD2 cells with *P. gingivalis* LPS, *E. coli* LPS (1 ng/mL) and SP (2 μM) for 6 hours.
- **Figure 5.** TNF-α release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 ng/ml** of LPS and assayed by ELISA.
- **Figure 6.** TNF-α release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 μg/ml** of LPS and assayed by ELISA.
- **Figure 7.** TNF-α release from LAD2 cells. All concentrations presented in total.
- **Figure 8.** VEGF release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 ng/ml** of LPS and assayed by ELISA.
- **Figure 9.** VEGF release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 μg/ml** of LPS and assayed by ELISA.
- **Figure 10.** VEGF release from LAD2 cells. All concentrations presented in total.
- **Figure 11**. MCP-1 release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 ng/ml** of LPS and assayed by ELISA.
- **Figure 12.** MCP-1 release from LAD2 cells. Supernatant fluids were collected 24 hours after stimulation with **1 μg/ml** of LPS and assayed by ELISA.
- **Figure 13. MCP-1** release from LAD2 cells. All concentrations presented in total.
- **Figure 14.** TNF-α release from LAD2 cells, assayed by ELISA, stimulated with *P. gingivalis* LPS (1μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h.
- **Figure 15.** TNF-α release from LAD2 cells, assayed by ELISA, stimulated with *E. coli* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h.
- **Figure 16.** VEGF release from LAD2 cells, assayed by ELISA, stimulated with *P. gingivalis* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h.
- **Figure 17.** VEGF release from LAD2 cells, assayed by ELISA, stimulated with *E. coli* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h.

- **Figure 18.** MCP-1 release from LAD2 cells, assayed by ELISA, stimulated with *P. gingivalis* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h.
- **Figure 19.** MCP-1 release from LAD2 cells, assayed by ELISA, stimulated with *E. coli* LPS (1 μg/ml), pretreated with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml) for 1h.

9. PUBLICATIONS RELATED TO THIS THESIS

- The effects of *P. gingivalis* and *E. coli* LPS on the expression of proinflammatory mediators in human mast cells and their relevance to periodontal disease.
  Palaska I, Gagari E, Theoharides TC. J. Biol Regul Homeost Agents. 2016 Jul-Sep;30 (3):655-664. *PMID: 27655482*

- Η επίδραση του *P. Gingivalis* λιποπολυσακχαρίτη στην έκφραση και έκκριση προφλεγμονωδών διαμεσολαβητών από ανθρώπινα μαστοκύτταρα και η συνάφειά του με την περιοδοντική φλεγμονή: μελέτη *in vitro.*
  Ηρώ Παλάσκα, Ελένη Γκαγκάρη, Θεοχάρης Κ. Θεοχαρίδης. Περιοδοντολογικά Ανάλεκτα Τόμος 25 (2017).

- Trigeminal nerve stimulation triggers oral mast cell activation and vascular permeability. Alhelal MA, **Palaska I**, Panagiotidou S, Letourneau R, Theoharides TC. Ann Allergy Asthma Immunol. 2014 Jan;112(1):40-5. *PMID: 24331392*

- Stress hormones regulate periodontal inflammation.
  Papathanasiou E, **Palaska I**, Theoharides TC. J. BIol Regul Homeost Agents. 2013 Jul-Sep;27(3):621-6. *PMID: 24152831*

- Use of polyphenols in periodontal inflammation.
  Palaska I, Papathanasiou E, Theoharides TC. Eur. J Pharmacol. 2013 Nov 15, 720(1-3):77-83. Review. *PMID: 24184667*