GM-CSF and uPA are required for *Porphyromonas gingivalis*-induced alveolar bone loss in a mouse periodontitis model

Roselind S Lam¹, Neil M O'Brien-Simpson¹, John A Hamilton², Jason C Lenzo¹, James A Holden¹, Gail C Brammar¹, Rebecca K Orth¹, Yan Tan¹, Katrina A Walsh¹, Andrew J Fleetwood², Eric C Reynolds¹

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¹Oral Health Cooperative Research Centre, Melbourne Dental School, Bio21 Institute, The University of Melbourne, 720 Swanston Street, Victoria 3010, Australia.

²The University of Melbourne, Department of Medicine, The Royal Melbourne Hospital, Parkville, Victoria 3050, Australia.

Address for Correspondence: Professor Eric Reynolds, Melbourne Dental School, The University of Melbourne, 720 Swanston Street, Carlton, Victoria, 3010, Australia.

Tel +61 3 9341 1547, Fax +61 3 9341 1599, Email: e.reynolds@unimelb.edu.au

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ABSTRACT

Granulocyte-macrophage colony stimulating factor (GM-CSF) and urokinase-type plasminogen activator (uPA) can contribute to the progression of chronic inflammatory diseases with possible involvement of macrophages. In this study we investigated the role of both GM-CSF and uPA in *Porphyromonas gingivalis*-induced experimental periodontitis using GM-CSF^{-/-} and uPA^{-/-} mice. Intra-oral inoculation of wild type (WT) C57BL/6 mice with *P. gingivalis* resulted in establishment of the pathogen in plaque and a significant increase in alveolar bone resorption. The infected mice also exhibited a CD11b⁺ CD86⁺ macrophage infiltrate into the gingival tissue as well as *P. gingivalis*-specific pro-inflammatory cytokine and predominantly IgG2b antibody responses. In comparison, intra-oral inoculation of *P. gingivalis* did not induce bone resorption and there was significantly less *P. gingivalis* recovered from plaque in GM-CSF^{-/-} and uPA^{-/-} mice. Furthermore, *P. gingivalis* did not induce a macrophage gingival infiltrate or activate isolated peritoneal macrophages from the gene- deficient mice. Pro-inflammatory *P. gingivalis*-specific T-cell cytokine responses and serum IFN- γ and IgG2b concentrations were significantly lower in GM-CSF^{-/-} mice. In uPA^{-/-} mice, T-cell responses were lower but serum IFN- γ and IgG2b levels were comparable with WT mice levels. These results suggest that GM-CSF and uPA are both involved in the progression of experimental periodontitis, possibly via a macrophage-dependent mechanism(s).

INTRODUCTION

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth associated with a polymicrobial biofilm (subgingival plaque) accreted to the tooth which results in destruction of the tooth's supporting tissues including the alveolar bone.¹ Moderate to severe forms of the disease affect 1 in 3 adults² and periodontitis has been linked to an increased risk of cardiovascular diseases, certain cancers, pre-term and low birth weight and other systemic diseases related to the regular bacteraemia and chronic inflammation associated with the disease.^{3, 4}

Although chronic periodontitis is associated with a polymicrobial biofilm, specific bacterial species of the biofilm such as *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* have been closely associated with clinical measures of disease.^{5, 6} Recently the level of *P. gingivalis* in subgingival plaque above threshold levels (~10% of total bacterial cell load) has been demonstrated to predict imminent clinical attachment loss (disease progression) in a prospective clinical trial of periodontitis patients on a maintenance program.⁷ *P. gingivalis* is found as microcolonies in the superficial layers of subgingival plaque adjacent to the periodontal pocket epithelium⁸ which helps explain the strong association with underlying tissue inflammation and disease at relatively low proportions (10%) of the total bacterial cell load of the plaque.^{7, 9} Furthermore, it has been suggested from studies using the mouse periodontitis model that *P. gingivalis* is a keystone pathogen or pathobiont which dysregulates the host immune response and promotes dysbiosis and disease.¹⁰ This model has also been used to show that inflammation is essential to allow establishment of *P. gingivalis* at the levels in plaque (10% or greater of total bacterial cell load) necessary to produce dysbiosis and significant

alveolar bone resorption¹¹, which makes the model results consistent with those of human disease.^{7, 11}

The monocyte-macrophage system plays a crucial role in the innate immune system, and in the initiation of the adaptive immune response.¹² Macrophage cell number is increased in the gingival tissue of chronic periodontitis patients compared with healthy subjects¹³, where they may contribute to the elevated levels of pro-inflammatory cytokines, such as TNF α and IL-1, that are found in the gingival tissue and crevicular fluid of those with the disease¹⁴⁻¹⁶. In agreement with this we recently demonstrated that the dominant macrophage phenotype present in the gingival tissue in a mouse periodontitis model had features of a 'pro-inflammatory' M1 phenotype.¹¹ Importantly, as lineage precursors to bone resorbing osteoclasts, macrophages are also likely to play a central role in the irreversible bone and extracellular matrix (ECM) destruction observed in chronic periodontitis¹⁷⁻¹⁹. Consistent with this we previously found that ablation of macrophages protected mice from *P.gingivalis*-induced alveolar bone loss in a model of periodontitis.¹¹ These studies support an association between chronic periodontitis and macrophages.^{11-13, 20-22}

Monocyte/macrophage lineages differentiate and mature from haematopoietic cells in bone marrow upon *in vitro* stimulation by granulocyte macrophage-colony stimulating factor (GM-CSF) and macrophage-colony stimulating factor (M-CSF).^{23, 24} However, the steady-state development of myeloid cells is mostly unaffected by the absence of GM-CSF, except mainly for the compromised maturation of alveolar macrophages. GM-CSF is a potent activator of macrophage function and is produced, for example, by activated T-cells, fibroblasts and macrophages following stimulation, for example, by bacterial lipopolysaccharide (LPS) and tumor necrosis factor (TNF), and in various inflammatory/autoimmune conditions.²⁵⁻²⁹ GM-CSF

also acts as a pro-inflammatory cytokine, the depletion of which can have profound effects on disease severity and progression in models of arthritis, multiple sclerosis, lung inflammation and asthma.³⁰⁻³⁷ Initial data suggest that antibody blockade of the GM-CSF receptor or its ligand has therapeutic benefit in rheumatoid arthritis.^{38, 39} In the context of periodontitis GM-CSF has been detected in gingival crevicular fluid from diseased sites, where it is thought to reduce cellular apoptosis.⁴⁰ The above findings suggest a potential role for GM-CSF in periodontal inflammation, but as yet no study has evaluated its role in the murine *P. gingivalis*-induced periodontitis model.

GM-CSF *in vitro* can upregulate the formation of the serine protease, urokinase-type plasminogen activator (uPA) in monocytes/macrophages.⁴¹⁻⁴³ uPA catalyzes the cleavage of the zymogen (plasminogen) into the less specific serine protease, plasmin. Plasmin in turn can cleave a range of ECM proteins, degrade fibrin and activate latent matrix metalloproteinases (MMPs).⁴³⁻⁴⁸ These functions ensure that uPA is a central member of the extracellular proteolytic cascade that regulates a number of biological processes, including matrix remodeling, wound repair, inflammation and tumour cell invasion.⁴⁹⁻⁵² Deppe *et al.* (2010)⁵³ reported that there was a significant increase in the concentration of uPA in inflamed periodontal tissue compared with healthy tissue. Macrophages strongly express uPA⁵⁴ and a recent study highlighted the importance of uPA in promoting macrophage matrix degradation and motility.⁵⁵ Interestingly, uPA depletion (similar to the GM-CSF depletion mentioned above) protected mice in models of arthritis.^{56, 57} There was significantly less macrophage infiltration within tumours of uPA knockout (-/-) mice compared with wild-type mice.⁵⁸ Several studies have demonstrated uPA activity in human gingival tissue and in gingival crevicular fluid⁵⁹⁻⁶¹, and polymorphisms in

the uPA gene have been found to be associated with periodontal bone loss.⁶² Despite these findings, the role of uPA in a *P. gingivalis*-induced periodontitis model has not been studied.

Two distinct macrophage lineages, M1 and M2, have been proposed.⁶³ M1 macrophages, termed “classically activated”, are induced by priming with interferon-gamma (IFN- γ) and activating with a TLR ligand. M2 macrophages, termed “alternatively activated”, are induced by IL-4 or IL-13 cytokines and do not necessarily require TLR stimulation. GM-CSF is known to preferentially promote the development of M1-like macrophages characterised by the expression of the CD86/CD80 cell-surface markers that participate in T cell priming, produce IL-1, IL-12, IL-23, TNF- α , and produce bactericidal reactive oxygen intermediates (ROI) and nitric oxide (NO).^{29, 63} In contrast IL-4 promotes the development of M2 macrophages which are characterised by low production of NO and an increased in the production of the collagen precursor L-ornithine with anti-inflammatory humoral antibody production.^{12, 29, 63, 64} M2 macrophages have an immunoregulatory and tissue repair phenotype reflecting an anti-inflammatory response.⁶³⁻⁶⁵

We have previously shown that macrophages with some M1 features are the dominant gingival infiltrating macrophage in response to *P. gingivalis* oral infection in the mouse periodontitis model.¹¹ In addition, the transient depletion of macrophages from gingival connective tissue and submandibular lymph nodes using clodronate liposomes in the mouse model reduced alveolar bone resorption, pro-inflammatory antibody production and antigen-specific cytokine responses.¹¹ Macrophage depletion was also associated with reduced levels of *P. gingivalis* in plaque.¹¹ These findings suggest that macrophages play a role in the modulation of host inflammatory response to *P. gingivalis*.

Given that GM-CSF and uPA can act as pro-inflammatory mediators with evidence linking their expression, and given that depletion of each of them suppresses, for example, inflammatory arthritis severity, we investigated their respective roles in a *P. gingivalis*-induced periodontitis model.

RESULTS

***P. gingivalis* does not induce alveolar bone resorption and weakly colonises subgingival plaque in GM-CSF^{-/-} and uPA^{-/-} mice compared with wild type mice**

The role of GM-CSF and uPA in *P. gingivalis*-induced alveolar bone resorption was evaluated using GM-CSF^{-/-} and uPA^{-/-} mice, respectively. Figures 1A and 1B show that oral inoculation with *P. gingivalis* of wild type (WT) C57Bl/6 mice led to enhanced bone resorption which was not observed in either GM-CSF^{-/-} or uPA^{-/-} mice. There was no significant difference in the levels of alveolar bone loss between the uninoculated WT, GM-CSF^{-/-} or uPA^{-/-} mice.

DNA was extracted at the termination of the periodontitis model experiment from half maxillae of WT, GM-CSF^{-/-} and uPA^{-/-} mice and *P. gingivalis* infection was expressed as a percentage of total bacterial cell numbers recovered. *P. gingivalis* DNA was recovered from all of the mice (WT, GM-CSF^{-/-} and uPA^{-/-}) inoculated with the bacterium but not from the uninoculated animals (Figures 2A and 2B). Although GM-CSF^{-/-} and uPA^{-/-} mice inoculated with *P. gingivalis* were found to be positive for the presence of the bacterium there were significantly ($p < 0.001$) lower levels recovered in both strains compared with the inoculated WT mice (Figure 2).

***P. gingivalis*-specific T cell, cytokine and antibody responses in GM-CSF^{-/-} mice in the periodontitis model**

Submandibular T cells were isolated from the *P. gingivalis* inoculated and uninoculated groups in WT and GM-CSF^{-/-} mice at termination, and stimulated with *P. gingivalis* RgpA-Kgp complexes to determine the antigen-specific proliferation and cytokine responses. There was a significant ($p < 0.01$) reduction in the *P. gingivalis* specific T cell proliferative response in GM-

CSF^{-/-} mice compared with that in WT mice (Figure 3A). Maximal T cell proliferation in *P. gingivalis* inoculated WT mice was induced at 0.024 µg/mL RgpA-Kgp complexes whereas a higher dosage (0.391 µg/mL) was required to induce a maximal T cell proliferation in GM-CSF^{-/-} mice (Figure 3A). There was no specific T cell proliferative response to RgpA-Kgp complexes across all concentrations in the uninoculated WT and GM-CSF^{-/-} mice.

ELISPOT assays were used to measure the frequency/number of antigen (RgpA-Kgp complexes) specific IFN-γ and IL-4-producing submandibular T cells from the *P. gingivalis* inoculated and uninoculated groups in WT and GM-CSF^{-/-} mice (Figure 3B). IFN-γ and IL-4 commonly indicate inflammatory (Th-1) and anti-inflammatory (Th-2) T cell responses, respectively. The T-cell ELISPOT assay showed that the T cell IFN-γ response in the *P. gingivalis*-inoculated WT and GM-CSF^{-/-} mice was significantly ($p < 0.01$) higher than that in the uninoculated groups of mice (Figure 3B), with that in the inoculated GM-CSF^{-/-} mice around four-fold lower than that in inoculated WT mice (Figure 3B). There was no IL-4 response to RgpA-Kgp complexes detected in *P. gingivalis* inoculated WT and GM-CSF^{-/-} mice (Figure 3B). There was also no IFN-γ or IL-4 response to the RgpA-Kgp complexes in T cells from the uninoculated WT and GM-CSF^{-/-} mice.

WT and GM-CSF^{-/-} mice were also bled at termination and serum IgG, IgG1, IgG2c, IgG2b, IgG3, IgA and IgM titres to the RgpA-Kgp complexes determined by ELISA (Figure 4A). The pre-dominant IgG subclass of antibodies directed to the RgpA-Kgp complexes was IgG2b in *P. gingivalis* inoculated WT mice, followed by IgG3 > IgG1 = IgG2c = IgM. The level of the IgG2b subclass antibody was significantly ($p < 0.05$) lower in the inoculated GM-CSF^{-/-} mice compared with the level in inoculated WT mice, with IgG1, IgG2c, IgG3, IgGA and IgM

not being detectable in the former. No specific IgG, IgA or IgM antibody responses to RgpA-Kgp complexes were detected in the uninoculated WT and GM-CSF^{-/-} mice.

The above serum samples were also analysed in a microbead array (Bio-plex) for eleven cytokines (IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17, GM-CSF, IFN- γ , TNF- α , and M-CSF). Only IL-2, IL-5, IL-10, IL-12p70, IL-13, IL-17, IFN γ and TNF α were consistently detected (Figure 4B) and of these only the levels of IL-12p70, IL-13, IFN- γ and TNF α were significantly ($p < 0.01$) higher in the *P. gingivalis* inoculated WT mice than in the uninoculated group. The levels of both IFN γ and TNF were lower in the *P. gingivalis* inoculated GM-CSF^{-/-} mice than in the corresponding WT mice (Figure 4B). Relatively low concentrations of IL-2, IL-10 and IL-17 were detected in both the *P. gingivalis* inoculated and uninoculated groups of WT and GM-CSF^{-/-} mice; however, there were no statistically significant differences between each group.

***P. gingivalis*-specific T cell, cytokine and antibody responses in uPA^{-/-} mice in the periodontitis model**

Submandibular T cells were isolated from the *P. gingivalis* inoculated and uninoculated groups of WT and uPA^{-/-} mice at termination, and stimulated as above with the RgpA-Kgp complexes to determine the antigen-specific proliferation and cytokine responses. Although the RgpA-Kgp complexes induced maximal T cell proliferation at 0.1 $\mu\text{g/mL}$ in the *P. gingivalis* inoculated groups of both WT and uPA^{-/-} mice, the maximal T cell proliferation was significantly ($p < 0.01$) higher in WT mice compared with that in uPA^{-/-} mice (Figure 5A). ELISPOT assays were used to measure the frequency/number of antigen (RgpA-Kgp complexes) specific IFN- γ and IL-4 producing submandibular T cells from the *P. gingivalis* inoculated and uninoculated groups in

WT and uPA^{-/-} mice (Figure 5B). The T-cell ELISPOT assay showed that the T cell IFN- γ response from the *P. gingivalis* inoculated groups of WT and uPA^{-/-} mice was significantly ($p < 0.01$) higher than the response from the uninoculated groups (Figure 5B) with that in the *P. gingivalis* inoculated uPA^{-/-} mice lower than the response in WT mice. There was no IL-4 response to RgpA-Kgp complexes detected in *P. gingivalis* inoculated WT and uPA^{-/-} mice. There were no specific cytokine response to the RgpA-Kgp complexes in T cells from the uninoculated groups of WT and uPA^{-/-} mice.

WT and uPA^{-/-} mice were also bled at termination and serum IgG, IgG1, IgG2c, IgG2b, IgG3, IgA and IgM titres to the RgpA-Kgp complexes again determined by ELISA (Figure 6A). There was a significant ($p < 0.001$) and predominant serum IgG2b specific antibody titre to the RgpA-Kgp complexes compared with the very low titres of the other IgG isotypes (IgG3, IgG1, IgG, IgG2c) in the *P. gingivalis* inoculated WT and uPA^{-/-} mice. Similar titres of IgG2b were detected in WT and uPA^{-/-} mice inoculated with *P. gingivalis*. There were no specific IgG, IgA and IgM antibody responses to the RgpA-Kgp complexes detected in the uninoculated WT and uPA^{-/-} mice.

The above serum samples were also analysed in a microbead array (Bio-plex) assay for the same eleven cytokines as above (Figure 6B). In this case, for the four cytokines (IL-12p70, IL-13, IFN γ and TNF α) that increased in the *P. gingivalis* inoculated WT mice there was a similar response in the corresponding inoculated uPA^{-/-} mice (Figure 6B). Interestingly, uninoculated uPA^{-/-} mice also exhibited a significantly ($p < 0.01$) higher level of IFN- γ and this level was unaltered in the uPA^{-/-} group upon inoculation with *P. gingivalis* (Figure 6B). There were detectable, although low, concentrations of IL-2, IL-4, IL-10 and IL-17 (with no significant

differences between each group) in both the *P. gingivalis* inoculated and uninoculated groups of WT and uPA^{-/-} mice.

The expression of macrophage phenotypes in gingival tissue from mice intra-orally inoculated with *P. gingivalis* and in the peritoneal cavity following HK-W50 injection

Following intra-oral inoculation with *P. gingivalis* the gingival tissues were harvested and single cell suspensions produced. M1-like macrophages (defined here as CD11b⁺ CD86⁺ TCRβ⁻ B220⁻ Ly6G⁻) and non-M1-like macrophages (defined here as CD11b⁺ CD86⁻ TCRβ⁻ B220⁻ Ly6G⁻) were identified in the flow cytometric analysis of the infiltrate. This staining protocol has been reported previously as a reproducible method for the identification of macrophage populations^{66, 67}; however it should be noted that it does not necessarily exclude some sub-populations of dendritic cells. Flow cytometry data for the total number of CD11b⁺ cells (predominantly macrophages), as well as the CD11b⁺ CD86⁺ population (predominantly M1-like macrophages) and the CD11b⁺ CD86⁻ population (predominantly non-M1-like macrophages)⁶³ in WT, GM-CSF^{-/-} and uPA^{-/-} mouse gingival tissue are shown in Figure 7 (A-C). Prior to inoculation, the GM-CSF^{-/-} mice exhibited a higher number of CD11b⁺ gingival macrophages compared with WT mice, in contrast uPA^{-/-} mice had a lower number (Figure 7A). Upon inoculation with *P. gingivalis*, the number of gingival macrophages increased only in the WT mice (Figure 7A). When macrophage sub-populations were examined, in untreated mice the minor CD11b⁺ CD86⁺ population was increased in GM-CSF^{-/-} mice but unaltered in u-PA^{-/-} mice (Figure 7B) while the major CD11b⁺ CD86⁺ population was lower in both gene-deficient strains (Figure 7C). Following bacterial inoculation there was a dramatic increase in the CD11b⁺ CD86⁺ macrophage number in WT mice which was not apparent in the gene-deficient strains (Figure 7B), while for

the CD11b⁺ CD86⁻ population there was a reduction of their numbers only in WT mice (Figure 7C).

To acquire sufficient macrophage numbers for *in vitro* functional assays, peritoneal macrophages (defined as CD11b⁺ F4/80⁺ CD115⁺ TCRβ⁻ B220⁻ Ly6G⁻) from WT, GM-CSF^{-/-} and uPA^{-/-} mice were harvested on Day 6 post heat-killed *P. gingivalis* (HK-W50) injection and incubated with or without HK-W50 for 24 h ¹¹. We have previously shown day 6 to be the time of maximal infiltration of macrophages into the peritoneal cavity in response to HK-W50 injection ¹¹. Similar to the periodontitis model, HK-W50 administration led to a significantly higher number of macrophages in WT mice but not in GM-CSF^{-/-} or uPA^{-/-} mice (Figure 7D).

Supernatants were collected from macrophage cultures to determine the amount of NO produced, and the cells were also lysed to measure their arginase activity. Untreated WT peritoneal macrophages secreted more NO than both gene-deficient macrophages which was further enhanced upon *in vitro* incubation with HK-W50 only in the WT macrophages (Figure 7E). For urea expression (arginase activity), the untreated WT peritoneal macrophages had similar levels to uPA^{-/-} macrophages but higher than GM-CSF^{-/-} macrophages, with HK-W50 incubation enhancing only WT and GM-CSF^{-/-} macrophage expression (Figure 7F).

DISCUSSION

GM-CSF and uPA are known pro-inflammatory mediators which can have critical roles in chronic diseases where bone resorption is a clinical outcome.^{30, 32, 56, 57} Also, GM-CSF can enhance uPA expression in monocytes/macrophages.⁴¹⁻⁴³ In spite of this evidence to date very few studies have examined their role in periodontitis. Our analyses above indicate that there was significantly less alveolar bone resorption in *P. gingivalis* inoculated GM-CSF^{-/-} and uPA^{-/-} mice compared with that in *P. gingivalis* inoculated WT mice, indicating a major role for GM-CSF and uPA in alveolar bone resorption in the mouse periodontitis model.

GM-CSF has been shown to be involved in the pathogenic processes in a number of models of chronic inflammation and autoimmunity, including models of arthritis, lung inflammation, multiple sclerosis, and now for the first time in the *P. gingivalis*-induced periodontitis model.^{30, 32, 33, 36} Interestingly, we found that protection of GM-CSF^{-/-} mice from *P. gingivalis*-induced alveolar bone resorption was associated with a reduced proliferative T cell response in both magnitude and sensitivity, as evidenced by the 16-fold higher requirement of RgpA-Kgp as the antigen to reach maximum T cell proliferation relative to that observed in WT mice. Furthermore, T cells from GM-CSF^{-/-} mice had impaired IFN- γ production and these mice had lower serum IFN- γ levels and titres of total IgG and IgG2b isotype antibodies to RgpA-Kgp complexes, suggesting that the pro-inflammatory response was dampened. As T cells lack the GM-CSF receptor⁶⁸ these effects on the T cell response are not mediated directly, suggesting that GM-CSF plays a crucial role in supporting the RgpA-Kgp-specific Th-1 response via its ability to promote antigen-presenting cell (APC) function.^{28, 68-70}

Chronic periodontitis is characterised by a high rate of matrix turnover and bone loss which depends on the regulated expression of proteolytic enzymes.⁷¹ GM-CSF has been shown

to upregulate uPA⁴¹⁻⁴³ and matrix metalloproteinase (MMP) expression in monocytes/macrophages and can under some conditions lead to the development of precursors of bone resorbing osteoclasts.⁷² Recently, alveolar bone loss was found to involve the co-ordinated production of TNF- α by macrophages following *P. gingivalis*-induced TLR2 stimulation.¹⁷ GM-CSF is a potent priming agent that is able to augment macrophage cytokine production⁷³, including enhancing TNF- α production in response to TLR2 stimulation⁷⁴, as well as upregulating TLR2 expression on macrophages.^{74, 75} Notwithstanding its proposed role in supporting the adaptive immune response in *P. gingivalis*-induced periodontitis (discussed above), GM-CSF, given its pleotropic nature, is also likely to play a role in the pathogen-induced cytokine response that mediates bone loss in periodontitis.

In the periodontitis model, there was a significant increase in macrophages in the gingival tissue of WT mice intra-orally inoculated with *P. gingivalis* compared with that in the uninoculated group and this was predominantly a M1-like macrophage phenotype consistent with our recent findings.¹¹ These data fit with the concept that GM-CSF can act as a pro-survival factor for macrophages as well as activate them.^{29, 39, 73, 76} We are aware that caution needs to be exercised in employing the M1/M2 nomenclature to macrophage populations⁷⁷ as more characteristics of the macrophage populations appearing during *P. gingivalis*-induced periodontitis, in addition to the ones used above, need to be defined to fully categorize their functions. To generate sufficient numbers of macrophages for functional assays we injected *P. gingivalis* into the peritoneal cavity of mice. In the peritoneal cavity, as in the gingival tissue, there was no significant change in macrophage number in the GM-CSF^{-/-} mice following inoculation. This observation parallels that noted in antigen-induced peritonitis, but not in the thioglycolate-elicited model, highlighting again that the GM-CSF link with peritonitis is stimulus

dependent.⁷⁸ The peritoneal macrophages isolated from *P. gingivalis* inoculated WT mice expressed high NO and urea; cells from GM-CSF^{-/-} mice had lower NO but higher urea expression consistent with the ability of GM-CSF to preferentially promote the development of macrophages with some M1 features.^{28, 63} Interestingly, the baseline number of M1-like macrophages detected in the gingival tissue of GM-CSF^{-/-} mice was relatively high. However, unlike the response in wild type mice, there was no increase in M1-like macrophages upon *P. gingivalis* inoculation. Further, the M1-like macrophages in GM-CSF^{-/-} mice expressed low levels of NO and this did not increase in response to bacterial stimulation, indicating that M1-like macrophages in GM-CSF^{-/-} mice lack the ability to induce a classical inflammatory response.

Similar to GM-CSF, uPA has been implicated in the pathogenesis of chronic inflammation and autoimmunity.^{50, 56, 57, 79} Oral inoculation of *P. gingivalis* in uPA^{-/-} mice also resulted in significant reduction of alveolar bone resorption compared with that noted in *P. gingivalis*-inoculated WT mice. In the inoculated uPA^{-/-} mice gingival macrophage numbers and function (NO and urea expression) were both suppressed compared to those in the corresponding WT mice. Of possible relevance to our observations of reduced macrophage numbers and alveolar bone resorption, uPA can regulate not only macrophage adhesion and motility but also contribute to macrophage-mediated tissue matrix degradation including via MMP and cytokine activation.^{18, 44, 46, 55, 80-82} uPA has also been implicated in bone homeostasis.⁸³ Similar to GM-CSF deletion, the absence of uPA led to lower *P. gingivalis*-induced T cell proliferation and IFN γ production but, unlike GM-CSF deletion, the humoral response was not altered suggesting that T cell helper function was still intact.

The level of *P. gingivalis* infection (11-16 % of the recoverable bacterial cell load) in WT mice post *P. gingivalis* inoculation resulted in significant alveolar bone resorption (Figure 1 and Figure 2) which is consistent with previous findings.^{6, 7, 11, 84} This suggests that the lower level of infection in GM-CSF^{-/-} and uPA^{-/-} mice (4 - 7 % of the recoverable bacterial cell load) had not reached a threshold to induce dysbiosis and bone resorption and that GM-CSF and uPA were essential for the dysbiosis to occur.⁸⁵⁻⁸⁷

We have shown here for the first time that GM-CSF and uPA are both involved in the pathogenesis of *P. gingivalis*-induced alveolar bone resorption. Even though some aspects of the responses to oral *P. gingivalis* differ in the two gene-deficient strains, the respective actions of these two mediators might incorporate a common macrophage-dependent pathway given the similarities noted. However, such a mechanism requires substantiation and the possible contribution of other GM-CSF responsive myeloid cells, such as neutrophils, needs to be considered. Whatever the respective mechanisms of action our data suggest that both GM-CSF and uPA may be targets as part of an adjunctive therapy to conventional treatment. In support of this, the recent encouraging trials targeting GM-CSF and its receptor in rheumatoid arthritis (RA) patients arose out of similar GM-CSF depletion studies in murine models.^{38, 39} The association between periodontitis and RA^{88, 89} perhaps lends weight to this proposal.

METHODS

Animal Ethics

All animal experimental procedures were carried out in strict accordance with the recommendations in the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. The protocols for the experiments were approved by The University of Melbourne Ethics Committee for Animal Experimentation (Approval Number 081049).

Bacterial strains and growth conditions

P. gingivalis strain W50 (ATCC 53978) was obtained from the culture collection of the Oral Health Cooperative Research Centre, The Melbourne Dental School, University of Melbourne, Australia. This strain was selected for the study as it has been shown to be virulent in murine periodontitis models⁹⁰⁻⁹² and because W50/W83-like strains, based on polymorphisms in the ribosomal internal spacer region, have been closely associated with periodontitis in humans.⁹³ *P. gingivalis* W50 was grown and harvested as described previously.⁹⁴⁻⁹⁶

Preparation of heat-killed *P. gingivalis* W50 (HK-W50) and purification of the RgpA-Kgp proteinase-adhesin complexes (RgpA-Kgp complexes)

HK-W50 was prepared as previously described.¹¹ The protein extraction and purification of the RgpA-Kgp complexes (from strain W50) were performed as previously described.⁹⁷

Mouse periodontitis model and measurement of alveolar bone resorption in mouse maxillae

Mice (6-8 weeks) of C57BL/6J, granulocyte-macrophage colony-stimulating factor (GM-CSF) knockout (-/-) and urokinase-type plasminogen activator (uPA) knockout (-/-) backgrounds were

obtained from Department of Medicine, Royal Melbourne Hospital, The University of Melbourne, Victoria, Australia. The uPA^{-/-} mice were originally provided by Dr P Cameliot (University of Leuven, Belgium). The GM-CSF^{-/-} and uPA^{-/-} mice have been backcrossed onto C57BL/6J strain for >11 generations. The mouse periodontitis model was adapted from that previously described.^{11, 91, 98} Briefly, 12 mice (C57BL/6 and GM-CSF^{-/-} or uPA^{-/-} mice) per group were treated with kanamycin (Sigma-Aldrich, Castle Hills, New South Wales, Australia) at 1 mg/mL in deionised water *ad libitum* for seven days followed by a three day antibiotic-free period. Mice were inoculated with an oral inoculation regime on Day 0 and Day 20 with each regime consists of four doses of *P. gingivalis* W50 [1.0×10^{10} viable *P. gingivalis* W50 cells suspended in 20 μ L PG buffer (50 mM Tris-HCL, 150 mM NaCl, 10 mM MgSO₄ and 14.3 mM Mercaptoethanol, pH 7.4) containing 2 % w/v carboxymethylcellulose (CMC, Sigma-Aldrich, Castle Hills, New South Wales, Australia)], given two days apart. On Day 54, mice were bled for sera analysis from the retrobulbar plexus then killed. Maxillae were removed and halved through the midline, with 12 halves used to determine alveolar bone resorption and 12 halves used to enumerate *P. gingivalis* W50 colonization of subgingival plaque by real-time PCR. Sera were used to determine the antibody and cytokine profile using ELISA and Bioplex assays, respectively. Submandibular lymph nodes and spleens were also removed for T-cell proliferation and ELISPOT assays.

Determination of *P. gingivalis* infection using real-time PCR

Maxillae obtained at the termination of the mouse periodontitis model for real-time PCR analysis were stored in RNA Later (Qiagen Pty. Ltd., Chadstone, Victoria, Australia) at -70 °C until

processed and determined using similar methodology as described⁹⁹ with modifications as described.¹¹

Determination of T cells proliferation against the RgpA-Kgp complexes using T-cell proliferation assay

T cell proliferation assays were performed as previously described.¹⁰⁰ Concanavalin A (ConA) (3 µg/mL per well) was used as a positive control. T cell proliferation data were presented as the stimulatory index (S.I.) $\pm$ SD, where S.I. is the counts per min (cpm) divided by the negative control (no antigen) cpm. An S.I. of ≥ 2 was considered a positive result.

Determination of cytokines expressed by T cell using ELISPOT assay

ELISPOT assays were performed as previously described.⁹¹

Determination of subclass antibody in sera using Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs were performed to evaluate subclass antibody in sera as described previously⁹⁹ in triplicate using (5 µg/mL) of RgpA-Kgp complexes in 0.1 M PBS (pH 7.4) to coat wells of flat-bottom polyvinyl microtiter plates (Microtiter; Dynatech Laboratories, McLean, VA) (16 h, 4 °C). The dilutions of antibodies were modified and IgG2a antibody was replaced with IgG2c as described previously.¹¹

Determination of cytokines in sera using Bio-plex assay

The concentrations of the following cytokines; IL-2, IL-4, IL-5, IL-10, IL-12(p70), IL-13, IL-17, GM-CSF, IFN- γ , TNF- α and M-CSF were determined in sera from mice used in the mouse periodontitis model by Bio-Plex mouse cytokine panel and plates read on Bio-Plex Array Reader (Bio-Plex 200 System and Bio-Plex Manager Version 4.0, Bio-Rad Laboratories, Inc., Victoria Road, Gladesville, New South Wales, Australia) according to manufacturer's instructions as previously described.¹¹

Reagents used in flow cytometry and cell culture assays

Antibodies to the following mouse antigens (Ag) were purchased from BD Biosciences (Franklin Lakes, NJ) CD86-PeCy7 conjugate (clone GL1), CD11b-APC conjugate (clone M1/70), CD45R/B220-FITC conjugate (clone RA3-6B2), Ly-6G-FITC conjugate (clone 1A8), CD11b-PE conjugate (clone M1/70); eBioscience (San Diego, CA) TCR β -FITC conjugate (clone H57-597), CD115(CSF-1R)-PE conjugate (clone AF598), F4/80-PECy7 conjugate (clone BM8). All cell culture reagents were obtained from Sigma-Aldrich Pty. Ltd. (Castle Hills, New South Wales, Australia) unless otherwise specified.

Determination of macrophage phenotypes in gingival tissue from mice intra-orally

inoculated with *P. gingivalis* W50

Mice (female C57BL/6, GM-CSF^{-/-} and uPA^{-/-}, 6-8 weeks old, 6 mice/group), were intra-orally inoculated with eight doses of *P. gingivalis* with each dose consisting of 1.0×10^{10} viable *P. gingivalis* W50 cells suspended in 20 μ L PG buffer as described above. Three negative control groups of C57BL/6, GM-CSF^{-/-} and uPA^{-/-} mice (uninoculated) were sham- inoculated with PG buffer. The digestion of whole maxillae, preparation of single cell suspensions and surface

cellular markers staining were prepared as previously described¹¹ with the following modifications. The phenotypic analysis was carried out by negative gating on T cells, B cells and neutrophils (TCR β , B220 and Ly6G markers respectively) followed by positive gating identification of M1 macrophages (defined here as CD11b⁺/CD86⁺) and non-M1 macrophages (defined here as CD11b⁺/CD86⁻).

Isolation of mouse peritoneal macrophages for nitric oxide and arginase functional assays

Peritoneal cells from three groups of mice (female C57BL/6, GM-CSF^{-/-} and uPA^{-/-} of 8-10 weeks old, 6 mice/group) elicited on day 6 post intra-peritoneal (i.p.) injection with HK-W50 (100 μ g in 100 μ L PBS/mouse) were isolated for two functional assays; nitric oxide and arginase assays as previously described.¹¹ The sorted macrophage cells (CD11b⁺/F4/80⁺/CD115⁺ and TCR β ⁻/B220⁻/Ly6G⁻) were seeded 1.0×10^5 cells/180 μ L in complete DMEM in 24-well plate in triplicate and incubated in the absence or presence of 2.0×10^2 HK-W50 [1: 500 multiplicity of infection (MOI)] for 24 h. Supernatants from these macrophage cultures were collected for nitric oxide assay and the cell lysates used in the arginine assay. Nitric oxide concentration was determined using the Greiss reagent kit (Life Technologies, Tullamarine, Victoria, Australia) according to the manufacturer's instructions as described previously.¹¹ The arginase assay was performed as described previously.¹¹

Statistical analysis

Homogeneity of variances was assessed using Levene's test (SPSS for Windows, version 12). The alveolar bone resorption (mm²) and real time PCR data were statistically analysed using a one-way classification ANOVA and Dunnett's post-hoc multiple comparison (SPSS for

Windows, version 12). The serum IgM and IgG subclass antibody titres, the concentration of cytokines in serum, the cell number of M1 and non-M1 macrophages in gingival tissue, the cell number of peritoneal macrophages and, urea and nitric oxide concentrations in peritoneal macrophage culture supernatants were statistically analysed using Student's t-test (SPSS for Windows, version 12). Effect sizes, represented as Cohen's d , were calculated using the effect size calculator provided online by Evidence-Based Education U.K. website (<http://cem.dur.ac.uk/ebeuk/research/effectsize/>).¹⁰¹ According to Cohen, a small effect size is $d \geq 0.2$ and < 0.5 , a moderate effect size is $d \geq 0.5$ and < 0.8 , and a large effect size is $d \geq 0.8$. Flow cytometric data was analysed using Kaluza Flow Cytometry software V1.1 (Beckman Coulter Australia Pty. Ltd., Gladesville, New South Wales, Australia).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. *P. gingivalis* inoculation induced less alveolar bone resorption in GM-CSF^{-/-} and uPA^{-/-} mice compared with that of wild type (WT) C57BL/6 mice. GM-CSF^{-/-} (A) and uPA^{-/-} (B) mice were pre-treated with antibiotics and orally inoculated with a total of eight doses of 1.0×10^{10} *P. gingivalis* W50 or inoculated with PG buffer containing 2 % CMC alone (uninoculated group as negative control). *P. gingivalis*-induced bone resorption was determined as described in the Material and Methods section for each group (n = 12), and the data are expressed as the mean $\pm$ standard deviation in mm² and were analysed using a one-way ANOVA and Dunnetts T3 post-hoc test. * indicates data that are significantly different ($p < 0.001$) from the data for *P. gingivalis* inoculated WT group.

Figure 2. Enumeration of *P. gingivalis* cells as a percent of total bacterial cells recovered per half-maxillae. *P. gingivalis* cells enumerated in half maxillae of wild type (WT) C57BL/6 mice and GMCSF^{-/-} (A) and uPA^{-/-} (B) mice by real PCR, using *P. gingivalis*-specific and Universal bacteria 16S rRNA forward and reverse primers. Data are expressed as the percentage of *P. gingivalis* cells of total bacterial cells recovered per half maxilla mean $\pm$ standard deviation (n = 12) and were analysed using a one-way ANOVA and Dunnetts T3 post-hoc test. * indicates data that are significantly different ($p < 0.001$) from the value for the uninoculated group.

Figure 3. *P. gingivalis* –specific T cell proliferation and IFN γ production are reduced in GM-CSF^{-/-} mice. (A) T cell proliferation. T cells were isolated from submandibular lymph nodes following the termination of the mouse periodontitis model. The concentrations of the

stimulatory RgpA-Kgp antigen ($\mu\text{g}/\text{well}$) required for the maximal T cell proliferation (^3H -thymidine incorporation) (Materials and Methods) is shown above each bar. The cell proliferation data are expressed as stimulatory index (SI), where SI is the counts per minutes (cpm) divided by the negative control (no antigen) cpm. Data represent the mean $\pm$ standard deviations of three biological replicate assays and were statistically analysed using a one-way analysis of variance and Dunnett's T3 test. **(B)** T cell cytokine formation from inoculated or *P. gingivalis* inoculated mice Lymph node T cells were stimulated with 1 $\mu\text{g}/\text{mL}$ of RgpA-Kgp complexes for 48 h. RgpA-Kgp complexes and specific cytokine T cells were expressed as spot-forming cells per million. Data represented the mean $\pm$ standard deviation of three biological replicate assays and were statistically analysed using a one-way ANOVA and Dunnetts T3 post-hoc test. * indicates data that are significantly different ($p < 0.01$) from data for the uninoculated group.

Figure 4. Serum antibody and cytokine profile were modulated in GM-CSF $^{-/-}$ mice during *P. gingivalis*-induced periodontitis. At termination, wild type (WT) C57BL/6 and GM-CSF $^{-/-}$ mouse sera were collected from uninoculated groups and *P. gingivalis* intra-orally inoculated groups. **(A)** The sera were used to determine the antibody titre in response to RgpA-Kgp proteinase-adhesin complexes. Antigen-specific antibody titres are expressed as the serum dilution that corresponds to double the ELISA OD_{415nm} of the ELISA assay control. Data are expressed as the mean $\pm$ standard deviation ($n = 12$) and were analysed using Student's t-test and are representative of two independent experiments. * indicates data that are significantly different ($p < 0.001$) from the data for the uninoculated groups. **(B)** Cytokine levels in WT and GM-CSF $^{-/-}$ mouse sera from the mouse periodontitis model experiments were determined using

an 11-plex kit for Bioplex analysis. Cytokine levels that were significantly ($p < 0.01$) different from the uninoculated group are shown. Data are representative of two independent experiments and are expressed as the mean $\pm$ standard deviation ($n = 6$) and were analysed using Student's t-test. * indicates data that are significantly different ($p < 0.01$) from the data for uninoculated group.

Figure 5. *P. gingivalis* –specific T cell proliferation and IFN γ production are reduced in uPA $^{-/-}$ mice. (A) T cell proliferation. T cells were isolated from submandibular lymph nodes following the termination of the mouse periodontitis model experiments. The concentrations of the stimulatory RgpA-Kgp complexes ($\mu\text{g}/\text{well}$) required for the maximal T cell proliferation (^3H -thymidine incorporation) is shown above each bar. The cell proliferation data are expressed as stimulatory index (SI), where SI is the counts per minutes (cpm) divided by the negative control (no antigen) cpm. Data represent the mean $\pm$ standard deviation ($n = 6$) of independent experiments and were statistically analysed using a one-way analysis of variance and Dunnett's T3 test. **(B) Specific T cell cytokine formation** Lymph node T cells from uninoculated or *P. gingivalis*-innoculated mice were stimulated with 1 $\mu\text{g}/\text{mL}$ of RgpA-Kgp complexes for 48 h. RgpA-Kgp complexes and specific cytokine T cells were expressed as spot-forming cells per million. Data represented the mean $\pm$ standard deviation ($n = 6$) of two independent experiments and were statistically analysed using a one-way ANOVA and Dunnetts T3 post-hoc test. * indicates data that are significantly different ($p < 0.01$) from data for the uninoculated group.

Figure 6. Serum antibody and cytokine profile in uPA $^{-/-}$ mice during *P. gingivalis*-induced peritonitis. At termination wild type (WT) C57BL/6 and uPA $^{-/-}$ mouse sera were bled from

uninoculated groups and *P. gingivalis* intra-orally inoculated groups (**A**). The sera were used to determine the antibody titre in response to RgpA-Kgp proteinase-adhesin complexes. Antigen-specific antibody titres are expressed as the serum dilution that corresponds to double the ELISA OD_{415nm} of the ELISA assay control. Data are expressed as the mean $\pm$ standard deviations (n=12) and were analysed using Student's t-test and are representative of two independent experiments. * indicates data that are significantly different ($p < 0.001$) from the data for the uninoculated groups. (**B**) Cytokine levels in WT and uPA^{-/-} mouse sera from the mouse periodontitis model were determined using a 11-plex kit for Bioplex analysis. Cytokine levels that were significantly ($p < 0.01$) different from uninoculated group are shown. Data are representative of two independent experiments and are expressed as the mean $\pm$ standard deviation (n = 6) and were analysed using student's t-test. * indicates data that are significantly different ($p < 0.01$) from the data for uninoculated group.

Figure 7. The expression of macrophage phenotypes in gingival tissue from mice intra-orally inoculated with *P. gingivalis* and in peritoneal cavity following HK-W50 injection. Wild type (WT) C57BL/6, GM-CSF^{-/-} and uPA^{-/-} mice were intra-orally inoculated with *P. gingivalis* W50 and the total number of (**A**) gingival macrophages [CD11b⁺ TCR β ⁻B220⁻Ly6G⁻] in the infiltrate were determined using flow cytometry. These gingival macrophages were further gated to obtain (**B**) CD11b⁺CD86⁺ TCR β ⁻B220⁻Ly6G⁻ and (**C**) CD11b⁺CD86⁻ TCR β ⁻B220⁻Ly6G⁻ populations. Data are expressed as the mean $\pm$ standard deviation (n = 6) from two independent experiments. Data were analysed using Student's t-test. # indicates uninoculated GM-CSF^{-/-} or uPA^{-/-} mice significantly different ($p < 0.01$) to uninoculated WT. * indicates WT inoculated with *P. gingivalis* significantly different ($p < 0.01$) to GM-CSF^{-/-} and uPA^{-/-} mice

inoculated with *P. gingivalis*. **(D - F)** Activation profile of *P. gingivalis* primed peritoneal macrophages in WT, GM-CSF^{-/-} and uPA^{-/-} mice **(D)** Peritoneal macrophages [CD11b⁺F4/80⁺CD115⁺ TCRβ⁻B220⁻Ly6G⁻] from WT, GM-CSF^{-/-} and uPA^{-/-} mice were isolated on day 6 post-intraperitoneal (i.p.) injection with heat-killed *P. gingivalis* W50 (HK-W50). Data are representative of two independent experiments and are expressed as the mean ± standard deviation (n = 6) and were analysed using Student's t-test. # indicates data that are significantly different ($p < 0.01$) to uninoculated group. * indicates data that are significantly different ($p < 0.01$) to inoculated WT. These isolated peritoneal macrophages were then incubated for 24 h in the presence or absence of HK-W50 and the levels of nitric oxide (NO) **(E)** and urea **(F)** secreted were then determined. Data are representative of two independent experiments and are expressed as the mean ± standard deviation (n = 6) and were analysed using Student's t-test and Cohen's effect size (d). # indicates data that are significantly different ($p < 0.01$) to uninoculated group. * indicates data that are significantly different ($p < 0.01$) to the NO concentration data from uninoculated and HK-W50 stimulated WT groups, respectively. * indicates data that are significantly different ($p < 0.05$) to the urea concentration data from uninoculated and HK-W50 stimulated WT groups, respectively.

Figure 1.

A.

B.

Figure 2.

Figure 3.

A.

B.

Figure 4.

A.

B.

Figure 5.

A.

B.

Figure 6.

A.

B.

Figure 7.

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Author/s:

Lam, RS;O'Brien-Simpson, NM;Hamilton, JA;Lenzo, JC;Holden, JA;Brammar, GC;Orth, RK;Tan, Y;Walsh, KA;Fleetwood, AJ;Reynolds, EC

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