Mast cells contribute to *Porphyromonas gingivalis* induced bone loss

Jennifer Malcolm[1], Owain Millington[2], Emma Millhouse[3], Lauren Campbell[1], Ana Adrados-Planell[1], John Butcher[4], Catherine Lawrence[2], Kirsty Ross[2], Gordon Ramage[3], Iain McInnes[1], and Shauna Culshaw[3]

[1]Institute of Infection, Immunity and Inflammation, [3]Infection and Immunity Research Group, University of Glasgow Dental School, [1 & 3] College of Medical, Veterinary and Life Sciences, University of Glasgow. [2]Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow.

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ABSTRACT

Periodontitis is a chronic inflammatory and bone destructive disease. Development of periodontitis is associated with dysbiosis of the microbial community, which may be caused by periodontal bacteria, such as *Porphyromonas gingivalis*. Mast cells are sentinels at mucosal surfaces and are a potent source of inflammatory mediators, including TNF. The number of mast cells increases in the gingival tissues of patients with periodontal disease, although their role in the disease process remains to be elucidated. This study sought to determine the contribution of mast cells to local bone destruction following oral infection with *P. gingivalis*. Mast cell deficient mice ($Kit^{W\text{-}sh/W\text{-}sh}$) were protected from *P. gingivalis* induced alveolar bone loss, with a reduction in anti- *P. gingivalis* serum antibody titres compared with wild-type infected controls. Furthermore, mast cell deficient mice had reduced expression of *Tnfα*, *Il6* and *Il1b* mRNA in gingival tissues compared with wild-type mice. Mast cell engrafted $Kit^{W\text{-}sh/W\text{-}sh}$ mice infected with *P. gingivalis* demonstrated alveolar bone loss and serum anti- *P. gingivalis* antibody titres equivalent to wild-type infected mice. The expression of *Tnfα* mRNA in gingival tissues of $Kit^{W\text{-}sh/W\text{-}sh}$ mice was elevated following the engraftment of mast cells, indicating that mast cells contributed to the *Tnfα* transcript in gingival tissues. *In vitro,* mast cells degranulated and released significant TNF in response to oral bacteria; and neutralizing TNF *in vivo* abrogated alveolar bone loss following *P. gingivalis* infection. These data indicate that mast cells, and TNF, contribute to the immunopathogenesis of periodontitis and may offer therapeutic targets.

INTRODUCTION

Periodontitis is a chronic inflammatory disease that destroys the alveolar bone and connective tissues supporting the teeth. Periodontitis is one of the most prevalent chronic infectious conditions of humans and is the leading cause of tooth loss among adults {Kassebaum, 2014 #1}. The 'keystone' pathogen, *Porphyromonas gingivalis* can trigger the microbial dysbiosis characteristic of periodontitis {Hajishengallis, 2011 #2}, in parallel, the responding cells and cytokine milieu are central to the tissue destruction {Hajishengallis, 2014 #4959}{Cekici, 2014 #5147}.

Mast cells are sentinels at mucosal surfaces and as such, have a pivotal role in orchestrating both innate and adaptive immune responses against local microbial challenge {Gordon, 1990 #6}. *In vivo* studies have demonstrated that mast cells can bind to, phagocytose, and kill enterobacteria {Malaviya, 1994 #7}, and present bacterial antigens to T cells {Malaviya, 1996 #8}. Mast cells contain a vast array of mediators in their granules, including large amounts of TNF. In a model of acute septic peritonitis, TNF blockade suppressed the protective role of mast cells via reduced neutrophil influx {Malaviya, 1996 #10;Echtenacher, 1996 #11}.

Mast cells are integral to anaphylaxis and other allergic disorders but are also implicated in chronic inflammatory diseases, such as rheumatoid arthritis {Lee, 2002 #12}. Within inflamed periodontal tissues, increases in both mast cell numbers and the proportion of degranulated cells in periodontal tissues, associated with severity of periodontitis {Huang, 2014 #14;Lagdive, 2013 #15;Huang, 2013 #16}. Oral mucosal mast cells contain TNF in their granules. In inflammation, *Tnfα* mRNA up-regulation, in parallel with mast cell degranulation and depletion of intracellular TNF, is consistent with continued TNF synthesis and release by mast cells {Walsh, 1995 #17}. Thus, mast cells may contribute to protection from potentially invasive bacteria, and to the local and systemic pathological inflammation associated with periodontitis.

We hypothesised that mast cells, with their armamentarium of inflammatory mediators, could contribute to the local and systemic pathology associated with periodontitis. We sought to determine the contribution of mast cells and TNF to local bone destruction following oral infection with *P. gingivalis*.

MATERIALS AND METHODS

Multi-species oral biofilm model

Overnight cultures of *Streptococcus mitis* NCTC 12261, *Streptococcus intermedius* NCTC 11324, *Streptococcus oralis* NCTC 11427, *Aggregatibacter actinomycetemcomitans* ATCC 43718, *Veillonella dispar* NCTC 11831, *Actinomyces naeslundii* ATCC 19039, *Fusobacterium nucleatum* ATCC 10953, *F. nucleatum subspecies vincentii* ATCC 49256, *P.*

gingivalis W83 and *Prevotella intermedia* ATCC 25611 were standardized in artificial saliva and added sequentially to Thermanox™ coverslips as previously described to generate a multi-species oral biofilm {Millhouse, 2014 #19}.

Bone marrow derived mast cell culture

Bone marrow was obtained from C57BL/6 mice and bone marrow derived mast cell (BMMCs) generated as previously described {Kuehn, 2010 #20}. After 4 weeks BMMCs were assessed by flow cytometry to determine purity and maturity. Cell suspensions were resuspended in Fc block (CD16/32, eBioscience) containing fluorochrome-labeled antibodies against FcεR1 and CD117, or similarly labeled isotype controls. Cells were analysed using a FACscalibur (BD Biosciences), and data analysed using Flowjo (Tree Star Inc., Oregon, USA). Cell viability, assessed by exclusion of 7-aminoactinomycin, was greater than 98%. All of the cultured cells were CD117 positive and > 90% were double positive for CD117 and FcεR1.

For co-culture experiments, mature BMMCs were seeded in 24-well plates at $5x10^5$ cells/well Dulbecco's modified eagle's medium. BMMCs were cultured adjacent to the biofilm-covered coverslip attached to a cell culture insert hanging basket as previously described for 2 h at 37°C in 5% CO_2.

Mast cell degranulation

β-hexosaminidase activity of cell culture supernatants and cell lysates was assessed as a proxy for mast cell degranulation using a previously described method {Kuehn, 2010 #20}. The percentage degranulation for each condition was calculated: 100 x (total supernatant content)/(supernatant + lysate).

ELISA

The concentration of TNF and IL-6 in the BMMC supernatants following co-culture, and of mast cell protease 1 in mouse serum were measured by ELISA using paired antibodies (eBioscience) according to the manufacturer's instructions.

Murine model of periodontal disease

Female BALB/c (Harlan, UK) or female and male C57BL/6 (bred in house at Strathclyde University) or age and sex-matched mast cell deficient C57BL/6 $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice (originally sourced from Jackson Laboratories, USA and subsequently bred in-house at University of Strathclyde (Grimbaldeston et al., 2005), were maintained before and during experiments in specific pathogen-free conditions with ad libitum food and water at the Universities of Strathclyde or Glasgow. All work was performed in accordance with UK Home Office regulations and after ethical approval from local research ethics committees and is reported according to ARRIVE guidelines. Mice were orally infected with 10^9 colony forming units (CFU) *P. gingivalis* W83 (ATCC, Middlesex, UK) in 2% carboxymethylcellulose (CMC) on 4 consecutive days as previously described {Baker, 1994 #22}. Sham-infected control mice

received CMC alone. At six weeks post-infection (PI), serum, gingival tissues and maxillae were collected under terminal general anaesthesia. In other experiments, mice received 0.1mg/mouse of anti-TNF antibody or isotype control antibody (both BioLegend), administered i.p on days -3, 0, 7 and 14 PI. $Kit^{W\text{-}sh/W\text{-}sh}$ mice received 5 x 10^5 mature BMMCs/mouse, administered by i.v. on days -28 and 0, as outlined in figure legends. The mast cells reconstitution was carried out based on previous studies. Following transfer, it takes 4 weeks to establish mast cells in the tissues, which remain readily detectable 12 weeks after transfer (Grimbaldeston et al).

Assessment of alveolar bone loss

The maxillae were separated from the skull and gingivae removed. Maxillae were defleshed and treated as previously described {Baker, 1994 #22}. Images were captured using an Olympus SZX7 stereo zoom microscope fitted with SC100 digital colour camera. Measurements of the distance between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC) were made using ImageJ software (National Institute of Health, Bethesda, MD, USA) to assess alveolar bone loss as previously described {Baker, 1994 #22}.

Anti- *P. gingivalis* ELISAs

Antibody titres to *P. gingivalis* in the serum samples were measured as previously described (Malcolm et al., 2015). Antibody titres were calculated as described previously {Gmur, 1986 #24}.

TaqMan® real-time PCR

RNA was extracted from periodontal tissues using the RNeasy® fibrous tissue kit (Qiagen), reverse transcribed with High Capacity RNA-to-cDNA (Applied Biosystems®, Life Technologies), then mRNA expression analysed by TaqMan® real-time PCR, using murine primers and fluorescent probe assays obtained from Applied Biosystems® (*Il6*: Mm00446190_m1, *Il1b*: Mm00434228_m1, *Tnfa:* Mm00443258_m1, *Il17*: Mm00439618_m1, *Mpo*: Mm01298424_m1, *Tpsab1*: Mm00491950_m1, *Mcpt4:* Mm00487636_g1). Analysis was performed in duplicate, gene expression normalised to 18S and relative expression of the gene of interest calculated by $2^{-\Delta CT}$. The data are presented as the fold change in expression of the gene of interest in the test population e.g. *P. gingivalis* infected test group normalized to the control (sham-infected) population.

Statistical analyzes

Data were analyzed by Student's *t* test or ANOVA with Tukey comparison, as indicated in the figure legends, using GraphPad Prism 6 (La Jolla, CA, USA).

RESULTS

Mast cell deficient mice are protected from *P. gingivalis* mediated alveolar bone loss

To elucidate the role of mast cells in *P. gingivalis*-induced inflammatory bone loss, wild-type (WT) or mast cell deficient mice ($Kit^{W\text{-}sh}/^{W\text{-}sh}$) were orally infected with *P. gingivalis* (experiment outline Figure 1A). In WT animals, alveolar bone loss was significantly greater following *P. gingivalis* infection compared with sham-infected controls ($p<0.001$). In contrast, *P. gingivalis* infection failed to elicit alveolar bone loss in $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice (Figure 1B [$p<0.05$ compared with *P. gingivalis*-infected WT]). Infection of WT mice with *P. gingivalis* induced a robust serum IgG anti-*P. gingivalis* antibody response (Figure 1Ci), which appeared dominated by IgG1. Anti-*P. gingivalis* IgG1 titres were significantly reduced in infected $Kit^{W\text{-}sh}/^{W\text{-}sh}$ compared with infected WT mice ($p<0.05$, Figure 1Cii). There was no statistically significant difference in the total IgG in serum of infected $Kit^{W\text{-}sh}/^{W\text{-}sh}$ compared with infected WT mice.

In the absence of infection, there was greater expression of *Tnfα* and *Il1b* in the gingival tissues of WT mice compared with $Kit^{W\text{-}sh/W\text{-}sh}$. *P. gingivalis* infection of WT mice resulted in increased expression of *Il6, Tnfα* and *Il1b* mRNA compared with WT sham-infected controls (Figure 1D); the difference was statistically significant for *Il6* only (Figure 1Dii). After infection with *P. gingivalis,* expression of *Il6* and *Tnfα* in the gingival tissues of $Kit^{W\text{-}sh/W\text{-}sh}$ mice remained significantly reduced compared with *P. gingivalis*-infected WT mice ($p<0.05$). Overall, there appeared to be a reduction in the expression of *Tnfα, Il1b and Il6* in the gingival tissues from $Kit^{W\text{-}sh/W\text{-}sh}$ mice compared with normal WT mice, suggesting that mast cells may be a significant source of the cytokine transcripts in gingival tissues in both health and disease. Expression of *Il17* was evaluated in the gingival tissues and no significant differences were found in any of the groups (data not shown).

BMMC engraftment restores alveolar bone loss in *P. gingivalis*-infected Kit-W mice

The $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice have compromised kit signalling. Kit (stem cell ligand) is essential for mast cell development but is also expressed on all hematopoietic stem cells. As a result, kit mutant mice have a number of immune alterations {Grimbaldeston, 2005 #21}. Although these are relatively mild in the $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice compared with other kit mutants, we sought to investigate whether the reduced alveolar bone loss observed in the $Kit^{W\text{-}sh}/^{W\text{-}sh}$ was mediated by mast cells and no other phenotypic abnormality of $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Mast cells were engrafted to reconstitute $Kit^{W\text{-}sh/W\text{-}sh}$ mice with BMMCs prior to infection with *P. gingivalis* as outlined (Figure 2A). Engrafted BMMCs were greater than 90% double positive for CD117 (c-kit) and FcεR1 (Figure 2B). As indicators of mast cell engraftment, we assessed serum mast cell protease 1 (MCPT1), which was readily detectable in WT mice and undetectable in the serum of $Kit^{W\text{-}sh/W\text{-}sh}$ mice but could be detected, albeit at a low level, in the serum of $Kit^{W\text{-}sh/W\text{-}sh}$ mice engrafted with BMMCs, indicating some mast cell engraftment in $Kit^{W\text{-}sh/W\text{-}sh}$ mice

at 6 weeks post-BMMC transfer (Figure 2C). Next, we sought to determine whether mast cells were engrafted within the gingival tissues of $Kit^{W\text{-}sh/W\text{-}sh}$ mice following BMMC transfer. Mast cell tryptase 1 (TPSAB1) and mast cell protease 4 (MCPT4) were expressed in WT mice but were either undetectable (TPSAB1) or at the limit of detection (MCPT4) in $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Figure 4Di and ii). In BMMC-engrafted $Kit^{W\text{-}sh/W\text{-}sh}$ mice, both TPSAB1 and MCPT4 mRNA transcripts were detected in the gingival tissues indicating that mast cells had engrafted to the gingival tissues in these mice. $Kit^{W\text{-}sh/W\text{-}sh}$ mice demonstrate neutrophilia {Grimbaldeston, 2005 #21}, and therefore we quantified the level of myeloperoxidase expression in gingival tissues as a surrogate measure of neutrophil infiltration. MPO mRNA was significantly elevated in the gingival tissues of $Kit^{W\text{-}sh/W\text{-}sh}$ infected mice, compared with infected BMMC-engrafted $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Figure 2Diii). There was no difference in the level of MPO mRNA in the gingival tissues of BMMC transferred $Kit^{W\text{-}sh/W\text{-}sh}$ mice compared with WT mice.

We next sought to investigate the alveolar bone loss in BMMC-engrafted $Kit^{W\text{-}sh/W\text{-}sh}$ mice. As before, *P. gingivalis* infection of WT mice induced alveolar bone loss and this phenotype was attenuated in $Kit^{W\text{-}sh/W\text{-}sh}$ infected mice (Figure 2E [$p<0.05$]). The disease phenotype was recapitulated in BMMC-engrafted, *P. gingivalis*-infected $Kit^{W\text{-}sh/W\text{-}sh}$ mice, with similar alveolar bone loss to that observed in WT-infected mice ($p<0.01$ [Figure 2E]). Similarly, the predominantly IgG1 serum antibody response to *P. gingivalis* was restored following BMMC engraftment of $Kit^{W\text{-}sh}/^{W\text{-}sh}$ infected mice (Figure 2F).

The expression of *Tnfα* mRNA was greater in the gingival tissues of infected $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice following BMMC engraftment compared with infected $Kit^{W\text{-}sh}/^{W\text{-}sh}$ that had not received mast cells ($p<0.05$ [Figure 2G]). These data indicate that mast cells are a significant source of *Tnfα* transcript in gingival tissues. We next examined the ability of BMMCs to produce and release TNF protein in response to oral bacteria.

Mast cells release TNF and IL-6 in response to oral bacteria

In vitro exposure of BMMC to a periodontitis associated biofilm model induced significant mast cell degranulation ($p<0.05$ compared with media control [Figure 3A]), and significant TNF and IL-6 release ($p<0.001$, compared with media control [Figure 3B and 3C]), demonstrating that mast cells can be stimulated to release TNF in response to oral bacteria. Given that mast cells appear to be a significant source of the *Tnfα* transcript *in vivo*, we hypothesised that TNF release from mast cells may contribute to alveolar bone loss in the murine model of periodontitis. The role of TNF in periodontitis has long been recognised {Kinane, 2011 #54} and TNF blockade described in primates {Graves, 1998 #26}. We next sought to evaluate TNF blockade in this murine model of *P. gingivalis*-induced alveolar bone loss.

TNF blockade reduces the severity of *P. gingivalis*-induced alveolar bone loss

Anti-TNF or isotype control antibodies were administered to BALB/c mice orally infected with *P. gingivalis* (Figure 4A). *P. gingivalis*-infected isotype control-treated mice demonstrated significant alveolar bone loss compared with sham-infected controls ($p<0.001$, [Figure 4B]). Anti-TNF treatment significantly reduced the severity of alveolar bone loss in *P. gingivalis*-infected animals compared with infected isotype-control mice ($p<0.05$). However, this anti-TNF treatment regime failed to completely attenuate alveolar bone loss, which remained significantly greater than the sham-infected mice ($p<0.05$). A robust serum IgG anti-*P. gingivalis* antibody response was induced following infection with *P. gingivalis* (Figure 4C), and these were reduced in mice treated with anti-TNF antibody.

DISCUSSION

These data show that mast cell deficiency is associated with protection against the local tissue destruction of periodontitis. Mast cell deficient $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice were completely protected from *P. gingivalis*-induced periodontitis and mast cells contributed to the expression of *Tnfα* in gingival tissues.

To our knowledge, this is the first study to use a mast cell deficient mouse to investigate the role of mast cells in periodontitis. Previous studies have demonstrated changes in the mast cell number and degranulation using immunohistochemistry {Huang, 2013 #16;Huang, 2014 #14;Gemmell, 2004 #28}. Moreover, treatment of beagle dogs with lodoxamide ethyl, an inhibitor of mast cell degranulation reduced the level of alveolar bone loss compared with untreated controls over a 1 year period {Jeffcoat, 1985 #29}, suggesting therapeutic targeting of mast cells may be beneficial to prevent periodontal disease. Our data provide evidence that mast cells and their products are directly involved in periodontal destruction, further confirmed by engrafting cultured mast cells into $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice and restoring the disease phenotype.

Oral administration of *P. gingivalis* induced a robust serum anti-*P. gingivalis* antibody response in WT mice, dominated by Ig1, characteristic of a primarily Th2-mediated response {Spellberg, 2001 #49}. Th2 immunity is generally associated with mast cell degranulation, and mast cells have been shown to directly support B cell antibody class-switching {recently reviewed by \Bulfone-Paus, 2015 #50}. Moreover, $Kit^{W\text{-}sh}/^{W\text{-}sh}$ show compromised IgG responses following challenge with mucosal adjuvants {Fang, 2010 #52}. Thus, whilst the trend to reduction in total IgG response and the significant reduction in the IgG1 and IgG2C anti-*P. gingivalis* antibody observed in $Kit^{W\text{-}sh}/^{W\text{-}sh}$ infected mice may be a failure of bacterial colonisation and invasion, data from other studies suggests an important role of mast cells in driving humoral immunity.

Mast cells, although forming a relatively small proportion of the cellular infiltrate in health or disease, are widely distributed throughout tissues and constitutively express a broad spectrum of immune mediators. Based on the reduction in *Tnf, Il1 and Il6* transcripts in healthy gingival tissue in kit-w mice compared with wild type, in these studies, mast cells may be speculated to constitutively generate some cytokines as previously documented {Okayama, 1995 #5155}, or respond to the commensal flora with a baseline elevation of cytokine profile. Rheumatoid arthritis shares similar immunopathogenesis with periodontitis {Culshaw, 2011 #53}. *In vivo* studies of mast cells in arthritis have revealed intriguing inconsistencies dependent on the nature of the mast cell deficiency and subtleties of the model of disease. Mast cells are redundant for the development of serum-induced arthritis in $Kit^{W\text{-}sh}/^{W\text{-}sh}$ mice {Zhou, 2007 #31}. This was confirmed using $Cpa3^{Cre}$ mice which are mast cell deficient and have a reduction in the numbers of basophils but are otherwise

immunologically normal {Scholten, 2008 #33}. In other studies, mast cells were redundant in the collagen-induced arthritis model in $Kit^{W\text{-}sh/W\text{-}sh}$ {Pitman, 2011 #34} but $Mcpt5^{Cre}$-iDTR mice (another kit-independent model) were in part protected from collagen-induced arthritis {Schubert, 2014 #35}. These data highlight the complexities of different models of disease and different models of mast cell deficiency. It would, therefore, be useful to explore the murine model of periodontitis in a mast cell deficient model that does not rely on impaired kit signalling.

Although multiple cells implicated in periodontitis can release TNF in response to bacterial stimulation, mast cells contain particularly abundant preformed TNF {Gordon, 1990 #6}. Our observation of reduced *Tnfα* in mast cell deficient tissues support the hypothesis that mast cells in periodontal tissues likely contribute to elevated TNF. In periodontitis, TNF can play a tissue destructive rather than anti-bacterial protective role: in a rat ligature model, administration of human recombinant TNF exacerbated the inflammatory cell infiltrate and alveolar bone resorption {Gaspersic, 2003 #36}. Local administration of neutralising anti-TNF antibodies reduced alveolar bone loss in a primate model of periodontitis {Assuma, 1998 #37;Graves, 1998 #26}. Similarly, p55TNFR-1-KO mice demonstrated less severe bone loss and reduced inflammation in periodontitis induced by *Aggregatibacter actinomycetemcomitans,* although the bone loss was not fully ameliorated {Garlet, 2007 #38}. In the present study, we observed a significant reduction in the serum antibody response to *P. gingivalis* following anti-TNF treatment, possibly due to loss of TNF induction of cell migration, as was reported in p55TNF-1-KO mice. Alveolar bone loss was completely abrogated in *P. gingivalis*-infected mast cell deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice, but neutralising TNF, as in other studies {Garlet, 2007 #38}, only partially prevented the bone loss. Accurately defining the contribution of mast cell derived TNF could be achieved by reconstitution of $Kit^{W\text{-}sh/W\text{-}sh}$ mice with TNF knock out mast cells. TNF has often been considered a master regulator within the cytokine network. TNF also synergises with other cytokines, such as IL-17, amplifying its impact on inflammation {Griffin, 2012 #5152}. In addition to TNF, mast cells contain an array of pro-inflammatory cytokines in their granules, including IL-1β and IL-6 {Steinsvoll, 2004 #41}, both of which are elevated in periodontitis {Reis, 2014 #43} {Graves, 1998 #26;Graves, 2003 #44}. In the present study, there was marked release of both TNF and IL-6 from mast cells *in vitro* following stimulation with periodontal bacteria. Moreover, there was reduced expression of IL-1β and IL-6 in the gingival tissues of $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Mast cell deficient mice reconstituted with mast cells demonstrated significantly elevated *Tnfα* in their gingival tissues. Surprisingly, the *IL1b* and *Il6* expression were not significantly influenced by mast cells reconstitution. Whether the mast cell derived IL-1β and IL-6 functions downstream of the mast cell derived TNF remains to be determined. In rheumatoid arthritis, mast cells expression of IL-17 has been documented in rheumatoid arthritis

{Hueber, 2010 #3917}. IL-17 drives neutrophil recruitment to the inflamed periodontium {Yu, 2007 #5051}{Eskan, 2012 #4709}. Surprisingly, there were no consistent changes in *Il17* expression in the gingival tissues of irrespective of presence of mast cells or infection. Recently, mast cell derived CXCL1 has been implicated in neutrophil recruitment to sites of inflammation {Wezel, 2015 #5151}. In addition to cytokines and chemokines, mast cells exert their effects via serine proteases released from mast cell granules. In humans, mast cell tryptase expression correlated with the degree of inflammatory infiltrate and the severity of periodontal disease {Huang, 2013 #16}. These serine proteases are important for the recruitment of neutrophils to sites of infection {Huang, 1998 #45;Tani, 2000 #46}. This is particularly pertinent to periodontal diseases in which both hypo- and hyper-recruitment of neutrophils is associated with bone loss {Hajishengallis, 2014 #47}. Delineating the hierarchy of the neutrophil and mast cell responses is complex, particularly as mice with mutations in CD117, such as the Kit-W, show a tendency to neutrophilia ({Grimbaldeston, 2005 #4077}, and would therefore be more accurately investigated in models of inducible mast cell deficiency such as the Mcpt5^{Cre}-iDTR mice {Schubert, 2014 #35}.

Mast cell proteases are important during tissue repair but can also contribute directly to the destruction of extracellular matrix through degradation of fibrinogen and collagen and indirectly through the activation of host matrix metalloproteases (MMPs), leading to attachment loss, propagation of inflammation and exacerbation of bone loss {Steinsvoll, 2004 #41}. Mast cells present in gingival tissues are also reported to directly express MMPs {Naesse, 2003 #48}. Thus, mast cells have the ability to regulate cellular infiltration and tissue destruction in periodontal disease by numerous mechanisms.

Our results provide evidence to indicate that mast cells are a key cell type in the immunopathogenesis of PD, and these cells contribute to the expression of TNF, a key mediator in PD. These new data provide further basis for exploring strategies aimed at preventing and treating PD. Targeting mast cells is of particular interest given the breadth of existing therapies, targeting both mast cells and TNF, which are already in clinical use for treating a range of allergic and inflammatory conditions respectively.

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FIGURE LEGENDS

Figure 1: Mast cell deficient mice are protected from *P. gingivalis*-induced alveolar bone loss.

WT or Kit$^{W\text{-}sh/W\text{-}sh}$ mice were orally infected with *P. gingivalis* (Pg) or vehicle only (Sham) and were euthanized 42 days post-infection. (A) Overview of experimental model. (B) Bone loss was measured on defleshed maxillae; values indicate alveolar bone level (ABL) in mice relative to sham-WT control. (C) Serum anti-*P. gingivalis* antibody was assessed by ELISA at the end of the experiment (i) total IgG, (ii) IgG1 isotype and (iii) IgG2c isotype. (D) RNA was extracted from gingival tissues of WT or Kit$^{W\text{-}sh/W\text{-}sh}$ mice at the end of the experiment. Expression of (i) *Tnfa,* (ii) *Il6* and (iii) *Il1b* were assessed by real time PCR. Data shown are mean ± SD (n = 7-8 mice/group) * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ by ANOVA with Tukey comparison.

Figure 2: BMMC engraftment restores alveolar bone loss in *P. gingivalis*-infected Kit$^{W\text{-}sh/W\text{-}sh}$ mice.

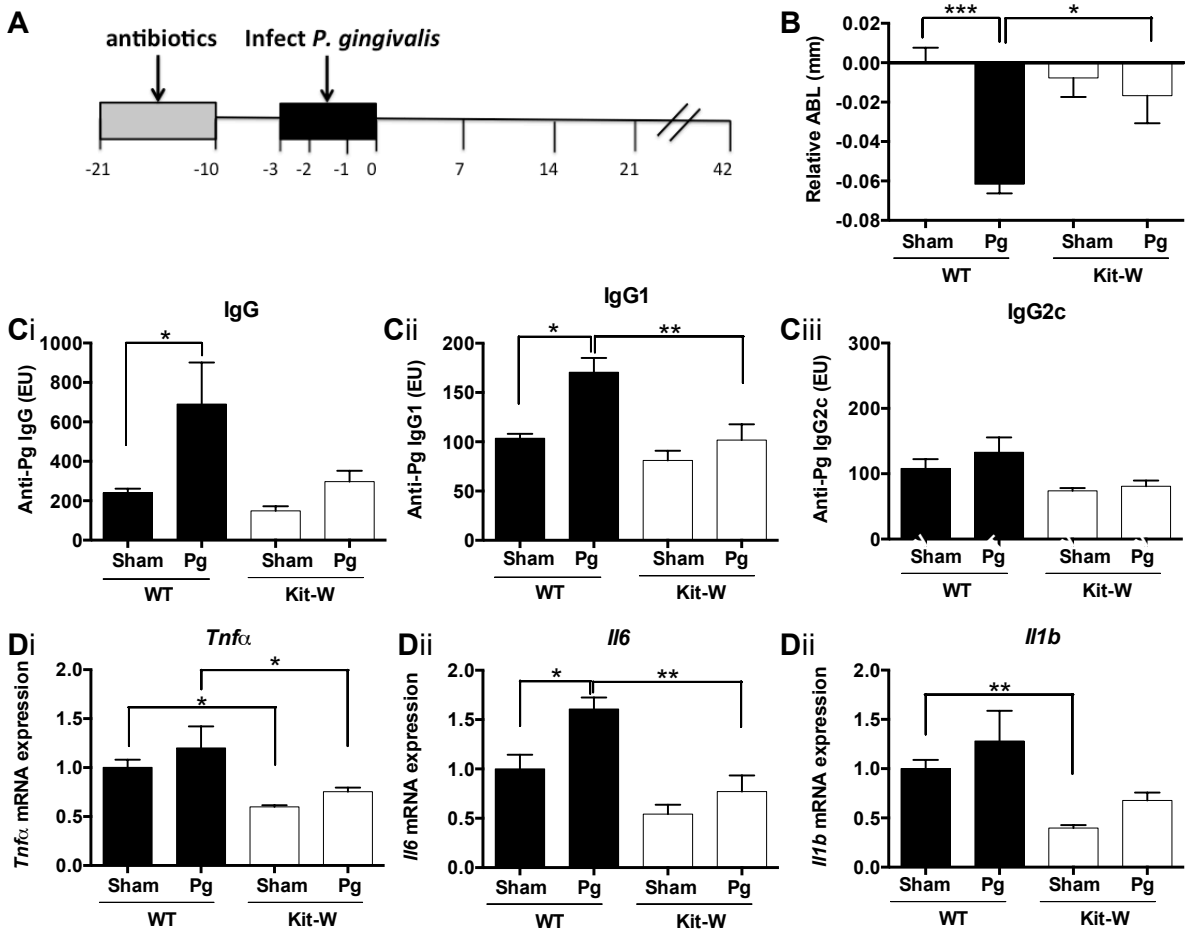
BMMCs were engrafted into Kit$^{W\text{-}sh/W\text{-}sh}$ mice orally infected with *P. gingivalis* (Pg) or vehicle only (Sham) and were euthanized 42 days post-infection. (A) Overview of experimental model. (B) Representative FACs plots showing surface expression of CD117 (c-kit) and FcεR1 of BMMCs used for engraftment into Kit$^{W\text{-}sh/W\text{-}sh}$ mice. (C) Serum concentrations (pg/ml) of mast cell protease 1 (MCPT1) assessed by ELISA. (D) RNA was extracted from gingival tissues of WT or Kit$^{W\text{-}sh/W\text{-}sh}$ mice at the end of the experiment and expression of (i) mast cell tryptase 1 (*Tpsab1*), (ii) mast cell protease 4 (*Mcpt4*) and (iii) myeloperoxidase assessed by real-time PCR. (E) Bone loss was measured on defleshed maxillae; values indicate alveolar bone level (ABL) in mice relative to sham-WT control. (F) Serum anti- *P. gingivalis* antibody was assessed by ELISA at the end of the experiment (i) total IgG, (ii) IgG1 isotype and (iii) IgG2c isotype. (G) Expression of (i) *Tnfa,* (ii) *Il6* and (iii) *Il1b* from the gingival tissues of Kit$^{W\text{-}sh/W\text{-}sh}$ mice were assessed by real time PCR at the end of the experiment. Data shown are mean ± SD (n = 4-7 mice/group). *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$ by ANOVA with Tukey comparison.

Figure 3: Mast cells undergo degranulation and TNFα release following co-culture with oral bacterial biofilms.

BMMC were cultured for 2 hours with biofilms of oral bacteria or medium only control. (A) *In vitro* mast cell degranulation by assessment of β-hexosaminidase activity of cell culture supernatants and cell lysates (B) TNFα and (C) IL-6 release into supernatants assessed by ELISA. Data were analysed by Student's t test. *$p<0.05$, ***$p<0.001$.

Figure 4: TNFα blockade reduces the severity of *P. gingivalis*-induced periodontal bone loss.

BALB/c mice were orally infected with *P. gingivalis* (Pg) or vehicle only (Sham), and injected i.p. with 0.1 mg/ml anti-TNFα or isotype control antibody. Mice were euthanized 42 days post-infection. (A) Overview of experiment design. (B) Bone loss was measured on defleshed maxillae; values indicate alveolar bone level (ABL) in mice relative to sham-WT control. (C) Serum anti-*P. gingivalis* antibody was assessed by ELISA at the end of the experiment (i) total IgG, (ii) IgG1 isotype and (iii) IgG2c isotype. Data shown are mean ± SD (n = 5 mice/group). *$p<0.05$, **$p<0.01$ ***$p<0.001$ by ANOVA with Tukey comparison.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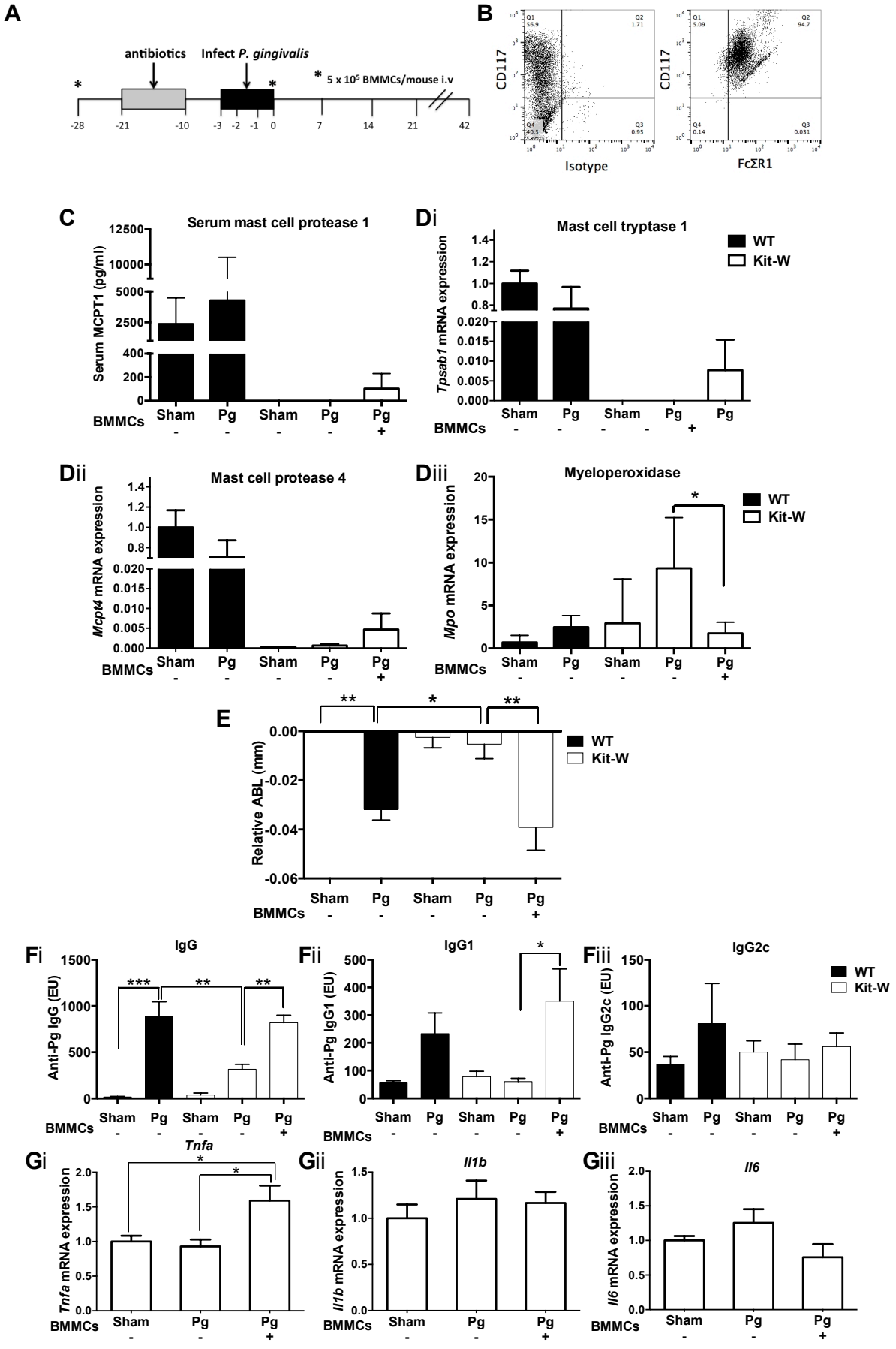
Kit-W
Ci
IgG
Anti-Pg IgG (EU)
Cii
IgG1
Anti-Pg IgG1 (EU)
Ciii
IgG2c
Anti-Pg IgG2c (EU)
Di
Tnfα
Tnfα mRNA expression
Dii
Il6
Il6 mRNA expression
Dii
Il1b
Il1b mRNA expression

1

2 figure 1

3

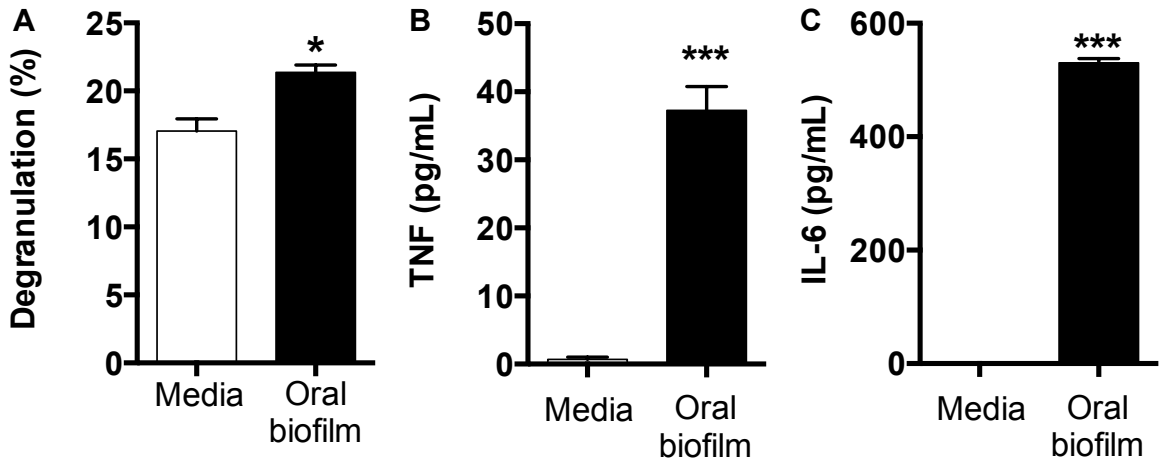

A
antibiotics
Infect P. gingivalis
5 x 10⁵ BMMCs/mouse i.v
-28
-21
-10
-3
-2
-1
0
7
14
21
42
B
CD117
Isotype
CD117
FcεR1
C
Serum mast cell protease 1
Serum MCPT1 (pg/ml)
BMMCs
Sham
Pg
Sham
Pg
Pg
Di
Mast cell tryptase 1
Tpsab1 mRNA expression
WT
Kit-W
Dii
Mast cell protease 4
Mcpt4 mRNA expression
Diii
Myeloperoxidase
Mpo mRNA expression
E
Relative ABL (mm)
Fi
IgG
Anti-Pg IgG (EU)
Fii
IgG1
Anti-Pg IgG1 (EU)
Fiii
IgG2c
Anti-Pg IgG2c (EU)
Gi
Tnfa
Tnfa mRNA expression
Gii
Il1b
Il1b mRNA expression
Giii
Il6
Il6 mRNA expression

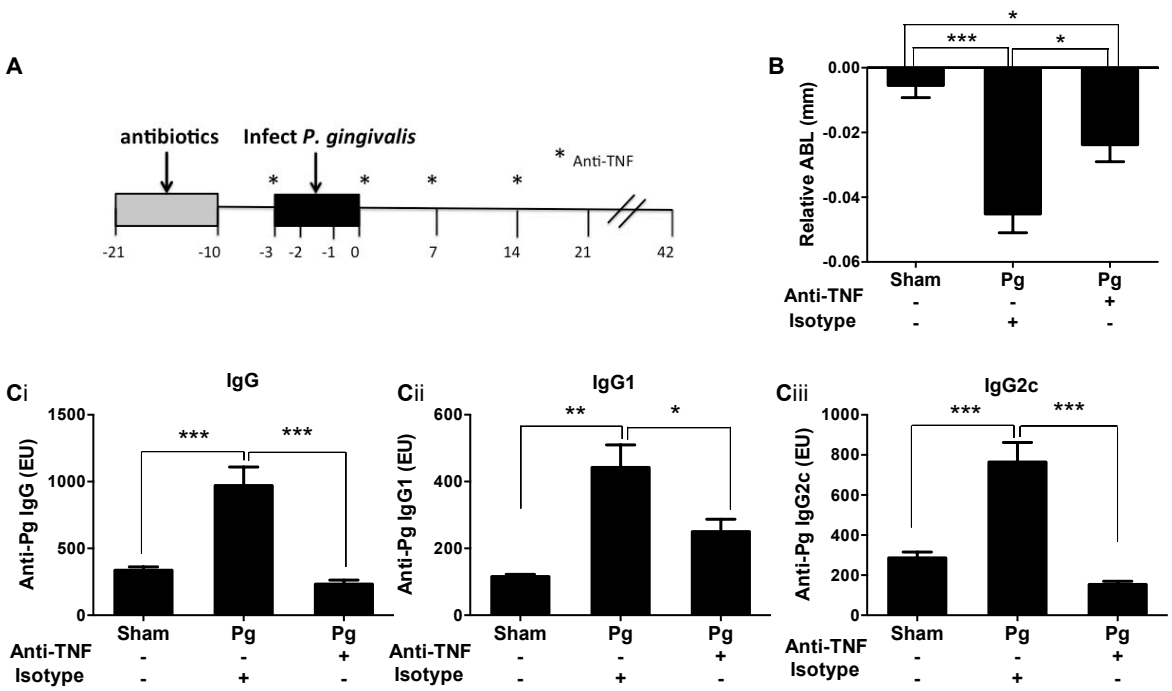
1

1 figure 2

2

figure 3


A
Degranulation (%)
25
20
15
10
5
0
*
Media
Oral biofilm
B
TNF (pg/mL)
50
40
30
20
10
0
***
Media
Oral biofilm
C
IL-6 (pg/mL)
600
400
200
0
***
Media
Oral biofilm


A
antibiotics
Infect P. gingivalis
* Anti-TNF
-21
-10
-3
-2
-1
0
7
14
21
42
B
Relative ABL (mm)
0.00
-0.02
-0.04
-0.06
*
***
*
Sham
Pg
Pg
Anti-TNF
Isotype
Ci
IgG
Anti-Pg IgG (EU)
1500
1000
500
0
***
***
Cii
IgG1
Anti-Pg IgG1 (EU)
600
400
200
0
**
*
Ciii
IgG2c
Anti-Pg IgG2c (EU)
1000
800
600
400
200
0
***
***

1 figure 4

2