ROLE OF INTERCELLULAR INTERACTIONS BETWEEN
MAST CELLS AND GINGIVAL FIBROBLASTS IN
MEDIATING INFLAMMATION

by

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Abstract

The mechanisms that mediate acute exacerbations in chronic inflammatory diseases such as periodontitis are not understood. IL-8 is a potent chemoattractant for neutrophils in acute inflammatory lesions. We investigated the role of fibroblast-mast cell interactions on short-term IL-8 release. Human gingival fibroblasts were co-cultured with human mast cells (HMC-1). After co-culture, the concentration of IL-8 was measured by ELISA. HMC co-cultured with fibroblasts increased IL-8 secretion by >6-fold, which required intercellular contact and was blocked by the gap junction inhibitor BGA. Thapsigargin-induced elevations of intracellular calcium increased IL-8 levels by 15-fold. Chemotaxis of human neutrophils was significantly enhanced in response to conditioned medium from co-cultures. Calcein-dye transfer showed intercellular, gap junction communication between HMC and fibroblasts that was dependent in part on β1 integrins. We conclude that mast cells adhere to fibroblasts and promote IL-8 secretion, thereby enhancing neutrophil chemotaxis and possibly the perpetuation of the inflammatory response.
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Statement of the problem

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Chapter 1: Literature Review

1. Periodontal diseases

In general, periodontal diseases are divided into two main clinical categories, gingivitis and periodontitis. Gingivitis is the diagnosis that is provided for conditions in which there is inflammation of gingival tissue around teeth but without involvement of the attachment apparatus [1]. In contrast, periodontitis is the diagnosis for lesions in which inflammation extends into the periodontal ligament and is associated with bone loss [1]. Periodontitis is the second most prevalent infectious disease in North American human populations and has been sub-categorized into aggressive, chronic or systemic periodontitis. Aggressive periodontitis is characterized by rapid tissue destruction and periodontal attachment loss in which the severity of destruction is not consistent with the abundance of bacterial biofilms. In comparison to aggressive periodontitis, the rate of progression is slower for chronic periodontitis, which is the most common form of periodontitis and affects over 30% of the U.S. adult population [2], [3], [4].

   a. Periodontitis and systemic diseases

Over the past two decades increasing evidence has suggested positive associations between periodontal inflammation and several important systemic diseases. Periodontitis is positively associated with cardiovascular diseases, atherosclerosis, myocardial infarction and stroke [5], [6]. Notably, periodontal treatment can improve glycemic control in diabetic patients [7]. In pregnant women, periodontal diseases may be a risk factor for pre-term and/or low-birth weight infants [8]. Further, periodontitis may be associated with increased risk of respiratory diseases such as chronic obstructive pulmonary disease and nosocomial pneumonia [9], [10]. One longitudinal study showed that periodontal diseases were associated with a small but
significant increase in the overall risk of developing cancer; these associations were also found in patients who had never smoked [11].

b. Microbial challenge

Periodontitis is an infectious disease, but other genetic and environmental factors also affect its severity and outcome [12], [13]. Among the hundreds of bacterial species that reside within subgingival biofilms, A. actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythensis and Treponema denticola are particularly associated with progressive periodontitis [14]. Subgingival biofilms exhibit organizational and structural features of communities [15] and individual species often exhibit extensive collaborative strategies for survival and communication [16]. Bacterial behaviour in biofilms is markedly different from bacteria in the planktonic state; pathogenicity and virulence are often markedly increased in biofilms [16].

Within subgingival biofilms, some species facilitate the growth and survival of other species, which might explain the aggregation of certain specific species in complexes that are associated with progressive lesions [17], [18]. As periodontal pathogens are typically found in subgingival biofilms, the prevention and treatment of periodontitis by antibiotics is often hampered because the shielding effect of the biofilm surface layers enhances the resistance of bacteria to locally or systemically delivered antibiotics [16].

Biofilms resist attack by the innate immune response. For example, some bacteria within the biofilm (e.g. P. gingivalis) can impair neutrophil responses by inhibiting their adhesion capacity to bacteria [19], a process that increases bacterial survival. Physical disruption, for example by scaling and root planing, is one of the most effective approaches for treatment of bacterial biofilms in the periodontal environment [20] and the very large positive effect that is seen clinically after debridement is probably a result of the disruption of the structure and
therefore the pathogenic potential of the biofilm. A common observation in many microbiological studies of periodontal infections is that not all patients harbouring periodontal pathogens develop periodontitis. This observation underlines the important effect of host-modifying factors in the pathogenesis of periodontitis.

c. Early steps of the pathogenic process in periodontitis

One of the most important sites of initial confrontation between the host immune system and bacteria associated with pathogenic biofilms is in the gingival crevice. When the abundance or the pathogenic potential of the subgingival bacterial challenge is increased, microbial metabolites can affect the structural integrity and metabolism of the junctional epithelium and underlying lamina propria of the gingival connective tissue, thereby causing an increase in vascular permeability. One of the hallmarks of the resulting inflammatory process is the enhanced infiltration and migration of neutrophils into the gingival crevice in response to chemokines such as IL-8 [21], [22], [23], [24]. If the bacterial challenge is maintained, after a few days, signs of inflammation will be manifested as redness and swelling of the marginal gingiva. In many patients, host immune mechanisms may contain the growth and the maturation of the subgingival biofilm, thereby preventing its spread to deeper regions of the periodontium. At inflamed periodontal sites, the lesion is referred to as gingivitis. If the microbial challenge is not contained by the host response, microbial-induced inflammation can mediate tissue destruction within the periodontium, which may affect the supporting structures of the teeth. At these sites, the lesion is referred to as periodontitis [25]. Although gingivitis is thought to represent the initial phase in the natural history of periodontitis, one theory of disease progression posits that gingivitis is a separate entity and may be stable (the so-called “established lesion”) and may not progress [24]. Previous hypothesis papers focusing on the importance of the
inflammatory response have suggested that host response factors play the key role in the
pathogenesis of periodontitis while bacterial virulence may be responsible for only about 20% of
periodontal disease cases [15], [26], [27].

d. Mechanisms of tissue destruction in periodontitis

In response to bacterial lipopolysaccharides, which are thought to be important virulence
factors in periodontitis, macrophages may express and secrete inflammatory cytokines including
IL-1β, prostaglandins, TNFα and the important tissue degrading family of enzymes, the matrix
metalloproteinases (MMPs). IL-1β and TNFα can activate fibroblasts to enhance expression of
MMPs and prostaglandin E2 [28]. MMPs, which are also produced by other cell types including
neutrophils, fibroblasts, epithelial and endothelial cells, can mediate degradation of extracellular
matrix molecules like collagen [29] while PGE2 can enhance bone resorption [30]. One of the
effects of periodontal tissue destruction is that bacterial biofilms may extend further apically and
laterally into the gingival connective tissue, a process that can lead to deepening of the gingival
crevise and creation of periodontal pockets [31], [32]. As the disease progresses, inflammatory
infiltrates replace the normal structure of the lamina propria of the gingival and of the
periodontal ligament [24]. These progressive lesions are also associated with an increase in the
proportion of gram negative anaerobic bacteria within subgingival plaques [18]. Although some
putative periodontal pathogens (e.g. P. gingivalis) can release bacterial enzymes like collagenase
(bacterial collagenase), nearly all of the collagenases found in periodontally diseased tissues are
derived from host cells and not from bacteria [33], [34], [35].

Cytokines, prostaglandins and chemokines are thought to be important regulators of the
inflammatory responses that characterize periodontitis. Bacteria in subgingival biofilms probably
contribute to periodontal tissue destruction by attracting and activating host cells that produce
these inflammatory mediators [33], [36], [37]. Notably, patterns of cytokine abundance in gingival crevicular fluid may be markedly different between periodontally active and stable sites [38], [39], [40]. Conceivably, periodontal lesions can be perpetuated, blocked or possibly reversed as a result of the biological activity of these host modifying factors. Arising from these hypotheses and observations, some treatment approaches have been suggested that involve inhibition of the degradation of the extracellular matrix by pharmacological intervention as a viable therapeutic modality [29].

e. Natural history of periodontitis

Early models of the natural history of periodontitis suggested that the rate of destruction of bone and periodontal ligament was continuous over time [41]. Slowly progressive attachment loss was thought to occur throughout adult life, which in some cases may have lead to tooth loss if treatment was not provided. However, a series of longitudinal studies undertaken 25-30 years ago at the Forsyth Institute (Boston) suggested that the natural history of periodontitis may be more aptly characterized as a heterogeneous group of diseases in which recurrent episodes of acute exacerbation and tissue destruction alternate with longer periods of quiescence. During the acute exacerbations, there was thought to be increased rate of attachment loss [42]. The bursts of activity seemed to occur randomly at different sites while at the same time, most other periodontal sites in the same mouth showed no disease activity. The exact mechanism of disease destruction that may account for the observations from the Forsyth group are not defined, but, according to our current understanding of periodontitis, it may be explained by fluctuations in the efficacy of the host immune response. However, it should be noted that the Forsyth model of rare, site-specific, acute exacerbations of attachment loss could be artefactual and may be explained by the manner in which attachment levels were measured. Notably, because of errors
associated with clinical measurement of gingival attachment level, the investigators at the Forsyth used very high thresholds of attachment loss (>2.5 mm) to define whether or not attachment loss had actually occurred at each site during the repeated measurements, which were made monthly. Because the threshold for what represented “true” attachment loss was set reasonably high (i.e. >2.5 mm), continuous loss of attachment (e.g. increments of attachment loss of say, 0.5 mm) could not be detected using these measurement and statistical approaches. Further, the manner in which the Forsyth investigators calculated degrees of freedom and their assumptions of the independence of the behaviour of individual sites within the same subject, were not well-supported by conventional (parametric) statistical approaches based on Gaussian-distributed data sets. Almost certainly, there are systemic factors in each subject that affect multiple sites in a similar way (i.e. not all sites are indeed independent within the same subject). Accordingly, considerable caution should be taken when considering the Forsyth model and its relevance in understanding the natural history of periodontal disease progression.

In spite of our lack of understanding of how host immune responses regulate natural history of periodontitis, there are reasonably good data to indicate that multiple modifying factors can activate or inhibit the inflammatory response, and subsequently affect tissue homeostasis and repair. Accordingly, it is anticipated that there will be wide variations of tissue destruction both between and within patients over time if the host response varies. This contention may explain the considerable variation that exists among individuals in terms of their susceptibility to periodontitis, clinical manifestations, rate of disease progression and response to treatment. One of the key cell types that play a crucial role in the inflammatory response and in innate immunity is the neutrophil [43], [44].
2. Neutrophils

Neutrophils (polymorphonuclear leukocytes, PMN) are derived from the myeloid lineage and represent the first line of defence against microorganisms [43], [45]. They originate from multi-potential hematopoietic stem cells in bone marrow and differentiate in response to specific growth factors [46]. Neutrophils are the most abundant and fastest migrating leukocytes, and are crucially important cells of the innate immune system. Neutrophils are 12-15 µm in diameter with abundant cytoplasmic granules and a segmented nucleus. Neutrophils can chemically sense bacteria at a distance, move towards them, engulf and kill them [43], [45]. Although neutrophils are a key requirement for mammalian survival, these same cells contribute to the pathogenesis of several inflammatory diseases including inflammatory bowel disease [47], rheumatoid arthritis [48] and periodontal diseases [49], [50].

a. Function

In response to bacterial stimulation [43] and to obtain access to the microbial “threat”, neutrophils must exit the blood circulation between the intercellular junctions of small capillaries and post-capillary venules. There are five sequential stages in the responses of neutrophils to pathogens: (1) neutrophil rolling along capillary endothelium, (2) neutrophil adhesion to the endothelium, (3) trans-endothelial migration, (4) chemotaxis, and (5) phagocytosis and microbiocidal activity [51], [52], [53]. Neutrophil rolling and adhesion to endothelium is mediated in part by a large number of adhesion molecules that include L-selectin [51], [53]. For neutrophil adhesion to the endothelium, specific surface molecules arrest the rolling of neutrophils, such as activated integrins located on the inflamed endothelial surface [54]. For example, the beta 2 integrin (CD11/CD18), which is exclusively expressed by leukocytes, modulates neutrophil adherence. Endothelial ICAM-1 (CD54) is its major ligand [54]. Although
ICAM-1 is constitutively expressed by endothelial cells, its expression levels are significantly enhanced after stimulation with inflammatory mediators such as IL-1β, TNFα, INFγ or endotoxin [55], [56].

Shortly after adherence to endothelium, neutrophils migrate between endothelial cells to exit the vessel and reach the inflammatory site [57]. This process of neutrophil trans-endothelial migration (diapedesis), unlike the previous stages, is not reversible. Two mechanisms have been suggested that enable neutrophil trans-endothelial migration: (1) paracellular migration, which involves squeezing of neutrophils between tight junctions that attach endothelial cells to one another [58], [59] and, (2) Transcellular migration, a process by which neutrophils may migrate through the individual endothelial cells via a transcytotic pathway [60]. The relative prevalence of paracellular and transcellular migration is not yet defined in vivo.

Chemotactic migration of cells towards certain molecular gradients (e.g. chemotactic factors) within the tissue is controlled by the organized development and disassembly of adhesion complexes, which are tightly linked to actin filament assembly in the leading lamellipodium of the cell [61]. Neutrophils migrate to the site of injury in response to chemotactic factors that are receptor-dependent (e.g. IL-8, C5a, fMLP, substance P and platelet-activating factor) or receptor-independent (e.g. phorbol esters) [62], [63]. Most in vitro studies of neutrophil chemotaxis use the microbial peptide fMLP as a positive control. Chemokines are small proteins that promote and guide migration of cells, mainly leukocytes, to the site of inflammation [64]. Neutrophil responses to many chemokines involve hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), which will then lead to a wide variety of important signalling events that include synthesis of inositol triphosphate (IP3), mobilization of Ca2+ [65] and tyrosine phosphorylation of a large array of adhesion-associated proteins [66], [67], [68].
At the site of injury, neutrophils recognize pathogens by means of opsonins, which include either C₃b complement fragments or antibodies specific to that pathogen. This recognition process is followed by internalization of the bacteria and formation of the phagosome [45], [69], [70]. After the internalization phase of phagocytosis, there are 2 microbiocidal pathways by which the phagocytosed pathogens are killed: (1) oxidative and (2) non-oxidative pathways. The oxidative pathway is characterized by the respiratory burst that is initiated by activation of NADPH oxidase. This process results in generation of superoxides, hydrogen peroxide, hydroxyl radicals, hypochlorous acid and chloramines [71], [72]. The major components of the non-oxidative pathway are the hydrolase granules within the neutrophils that can degrade phagocytosed bacteria [71]. Simultaneously, extracellular killing of bacteria can occur via secretion of enzymes such as collagenase, elastase and free radicals [73]. The extracellular pathway may be particularly important in host cell-mediated tissue destruction in diseases such as periodontitis and rheumatoid arthritis [48], [73].

b. Role of neutrophils in periodontal diseases

Neutrophils are a key component of the host inflammatory response in most infectious diseases, including periodontitis. Neutrophils are the most abundant host cell type in the gingival crevice and represent the first line of defence against bacterial challenges [74]. Impaired neutrophil function in diseases like Chediak Higashi syndrome (defective degranulation), leukocyte adhesion deficiency and Wiskott Aldrich syndrome (impaired chemotaxis) can increase susceptibility to infections, including in aggressive periodontitis [75].

After emigration from periodontal blood vessels, neutrophils reach their microbial targets by migration through the collagen-rich extracellular matrix of the periodontium. This migration through the lamina propria of the gingiva is facilitated by the release of collagenase (MMP-8)
from neutrophils, which degrades extracellular matrix collagen [44]. Other contents of neutrophils, such as elastase and oxygen radicals, are released into the extracellular space to kill the bacteria, but these molecules can also be destructive to periodontal tissues [76], [77]. If neutrophil-mediated tissue destruction predominates over tissue repair, there will be net loss of periodontium, which is seen in periodontitis. In this instance, neutrophils are recruited to neutralize periodontal pathogens; however, at the same time they contribute to periodontal tissue destruction because of their release of degradative molecules [44]. In comparison to healthy subjects, patients with progressive periodontitis express higher levels of collagenase [50], oxygen radicals [78] and β–glucuronidase, which are associated with increased extracellular matrix degradation [79]. Further, neutrophil-mediated tissue destruction is associated with localized aggressive periodontitis [80]. Accordingly, disproportionate accumulation and activation of neutrophils within the periodontium, followed by their degranulation and release of matrix degrading enzymes, can lead to tissue destruction. This destruction is thought to be largely the result of the host immune response [50]. In the context of this thesis, elevated production and release of chemokines such as IL-8 may be one of the key host mechanisms that mediate recruitment of neutrophils into the inflamed periodontium.

3. Chemokines

a. Structure and nomenclature

As mentioned above, chemokines are low-molecular-weight proteins composed of 70-80 amino acids that can mediate chemotaxis in adjacent responsive cells [64]. Chemokines contain cysteines at well-conserved positions. Depending on the spacing of the first two cysteine residues, there are four subgroups of chemokines. C chemokines have only two cysteines while CC, CXC and CX3C chemokines have four to six cysteines (Fig. 1) [81].
For C chemokines, one cysteine is located at the N-terminal cysteine and the second cysteine is located towards the C-terminal [64], [81]. Two chemokines have been identified in this subgroup, XCL1 (lymphotactin-α) and XCL2 (lymphotactin-β). Both of these molecules induce migration of T-cell precursors to the thymus [82]. In CC chemokines, the two cysteines are adjacent to each other. A well known chemokine from this sub-group is monocyte chemoattractant protein-1 (MCP-1), which is a powerful chemoattractant for monocytes and T-lymphocytes [83]. In response to specific stimuli, many different cell types including monocytes, fibroblasts, smooth muscle cells, endothelial and epithelial cells can secrete MCP-1 [84]. At least one human mast cell line can express MCP-1 mRNA and protein [85].

The two N-terminal cysteines in CXC chemokines are separated by one amino acid (X). Based on the presence of a specific amino acid sequence (glutamic acid-leucine-arginine; ELR) before their first cysteine, CXC chemokines are further subdivided into ELR⁺ and ELR⁻ categories [81]. ELR⁺ CXC chemokines stimulate the migration of neutrophils and exhibit strong angiogenic activity [86], [87]. IL-8 is an example of an ELR⁺ CXC that induces neutrophil
chemotaxis [81]. Other CXC chemokines that lack the ELR motif tend to be chemotactic for T-helper 1 (Th1) lymphocytes and natural killer cells [88], [89], [90]. CXC chemokines bind to CXC chemokine receptors; seven have been identified so far and are designated as CXCR1-7 [91], [92].

CX3C chemokines are differentiated from the CXC subgroup by the presence of three amino acids between their cysteines [81]. The only member of the CX3C subgroup that has been defined so far is CX3CL1, also known as fractalkine. It exists in two forms, a secreted and a membrane-bound form. It can function as both an adhesion molecule and as a chemoattractant [93]. In its membrane-bound form fractalkine promotes retention of T-lymphocytes and monocytes at inflamed sites [94]. The secreted form possesses chemotactic activity for T-lymphocytes, monocytes and NK cells [93]. Recent studies have suggested important roles for CX3CL1 and its receptor CX3CR1 in affecting inflammatory and neoplastic diseases of the cardiovascular, pulmonary, hepatic, pancreatic, intestinal, renal and musculoskeletal systems [95].

A new nomenclature system has been proposed for chemokines and their receptors [96], [97]. In this system, chemokines are named based on their cysteine subgroup (C, CC, CXC, CX3C) followed by “L” for “ligand”. The terminal number corresponds generally to the same number used in the associated gene nomenclature. Because most chemokine receptors are restricted to a single chemokine sub-class, the nomenclature system of chemokine receptors is rooted by the chemokine sub-class specificity, followed by “R” for “receptor” and the number. Based on this new system, IL-8 is now named CXCL8 [96], [97].
b. **Biology of IL-8**

Interleukin-8 (IL-8) is a pro-inflammatory cytokine that promotes neutrophil chemotaxis [21] and was the first member of the chemokine family to be discovered [64]. The cDNA for IL-8 encodes a precursor, 99 amino acid protein, which is then cleaved into a 72 or 77-residue mature protein [98]. Further proteolytic processing of IL-8 at the NH₂ terminus leads to the formation of 69-, 70-, 71-, 72- and 77-amino acid proteins [99], [100], [101], [102]. The 77- and 72-amino acid acid forms of IL-8 are two major sub-types and there is a smaller, 69-amino acid protein. Among the three forms, the 69-amino acid protein is the strongest chemoattractant for neutrophils. The 77-amino acid form, quickly converts to 72-amino acid form in vivo [103]. X-ray crystallography and nuclear magnetic resonance spectroscopy have demonstrated that at concentrations >100 µM, IL-8 forms a homodimer with two identical subunits. However, at nanomolar concentrations, which are closer to pathophysiological levels, IL-8 occurs mainly in a monomeric form and which exhibits a maximal biological effect [104], [105], [106].

c. **IL-8 production**

IL-8 is produced by a variety of cells including leukocytes (neutrophils, monocytes, T-lymphocytes and natural killer cells) as well as fibroblasts, endothelial and epithelial cells [64], [107], [108], [109]. IL-8 synthesis can be stimulated by pro-inflammatory cytokines such as IL-1 and TNF-α [98], bacterial products like lipopolysaccharide [64] and viruses and viral products [110], [111], [112], [113]. Among environmental factors, low oxygen tension can induce IL-8 production by activation of the transcription factors NF-κB and activator protein-1 [114], [115]. NF-κB is activated by reactive oxygen intermediates, which then lead to IL-8 gene transcription [116]. In most cells, co-operative activation of NF-κB and activator protein-1 is required for initiation of IL-8 gene transcription [107].
d. IL-8 receptors and function

CXCR1 and CXCR2 are the two distinct, high affinity membrane-bound receptors for IL-8, and contain 350 and 360 amino acids respectively [117], [118]. They are comprised of seven, trans-membrane domains that in turn are functionally coupled to G proteins at the COOH-terminal portion and possibly the third intracellular loop [96], [119]. Differences in the structure of the NH2-terminal seem to account for different binding specificities.

In addition to CXCL8 (IL-8), CXCR2 binds CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL7 with high affinity, while CXCR1 binds to CXCL6 only and with a lower affinity than CXCL8 [96]. After binding to ligand, the receptor becomes internalized, which is followed by recycling and reappearance on the plasma membrane in about 60 minutes [120]. Activation of G proteins subsequent to ligand binding results in the generation of active phosphatidylinositol 3-kinase-γ which in turn leads to generation of PIP3 [119], [121]. PIP3 triggers protein kinase B and the activation of small GTPases, which induce directed cell migration [121]. Both PI3K-γ-dependent and -independent pathways play a role in mediating chemokine-induced chemotaxis [122], [123]. Using ICAM receptors to enhance adherence, neutrophils follow a concentration gradient of IL-8 during their chemotaxis within the tissue [124]. Several IL-8 analogues have been reported including NAP-2, GRO proteins (GROα, GROβ and GROγ) and an epithelial cell-derived neutrophil-activating protein, ENA-78. They all belong to the CXC family and are similar to IL-8 in that they promote neutrophil chemotaxis [125].

e. IL-8 in periodontitis

Interleukin-8 (IL-8) is present in gingival crevicular fluid [40], [126], [127], and IL-8 mRNA is expressed in inflamed gingiva [128], [129]. IL-8 function is particularly important in facilitating the transmigration of neutrophils and their accumulation at the surface of sub-
gingival biofilms [130]. The level of IL-8 increases following plaque accumulation during gingivitis [131]. Further, *Porphyromonas gingivalis*, a well-known periodontal pathogen, enhances IL-8 expression by human gingival fibroblasts, which in turn results in increased neutrophil chemotaxis [36]. Similar findings have been reported with *Tannerella forsythia*, another periodontal pathogen [37]. Further, IL-8 levels in gingival crevicular fluid are positively correlated with the number of periodontal pathogens [132] and may reflect the rapidity of periodontal destruction [40]. Gingival crevicular fluid levels of IL-8 increase in periodontitis lesions and decrease significantly after periodontal treatment [39], [133], [134]. Similarly, IL-8 levels in plasma are significantly decreased after periodontal scaling in subjects with periodontitis [135].

Elevated IL-8 production by stimulated gingival fibroblasts may lead to maintenance of gingival inflammation and promote continuous tissue destruction [136]. In this context and as noted above, IL-8 is produced by various cell types, including gingival fibroblasts [137]. Together with its receptors, CXCR1 and CXCR2, IL-8 is present within gingival epithelium [138]. The increased cellular expression of CD40 may contribute to IL-8 production by stimulated gingival fibroblasts [133].

4. Fibroblasts

Fibroblasts, mast cells, macrophages and lymphocytes comprise the main cell populations of gingival connective tissue [139]. In healthy periodontal tissues fibroblasts are the most abundant cell type and make up 65% of the total cell population [139]. The main functions of fibroblasts are the synthesis and remodelling of extracellular matrix molecules such as collagen, fibronectin, hyaluronic acid, tenascin and thrombospondin. Fibroblasts also regulate collagen degradation [140] and, in wound healing, fibroblasts make important contributions to this
process by virtue of their ability to degrade and phagocytose collagen [141], [142], [143]. In pathological states, there may be imbalances between degradation and formation of extracellular matrix by fibroblasts, which can contribute to tissue fibrosis [144].

Not only do fibroblasts secrete proteins like collagen, but in wound healing they also contract the wound edges via traction, a process that is enhanced when fibroblasts express α-smooth muscle actin (α-SMA). Indeed, under certain conditions, fibroblasts can differentiate into myofibroblasts, cells that are rich in α-SMA and exhibit enhanced contractile capacity [145], [146]. α-SMA is frequently expressed by fibroblasts from the periodontium [147], which appears to enhance collagen remodelling by traction [141].

Fibroblasts and macrophages are the main sources of tissue inhibitors of matrix metalloproteinases (TIMPs) as well as MMPs [29]. The signals received by these cells from their surrounding environment will affect important biological outcomes in inflamed sites [148], [149]. Thus in healthy periodontium, actively expressed genes in gingival fibroblasts include various collagens and TIMPs while the genes coding for MMPs increase expression during periodontitis [35], [149], [150], [151]. In response to bacterial lipopolysaccharides, human gingival fibroblasts secrete inflammatory cytokines such as IL-1β, IL-1α and IL-6 [152]. Further, fibroblasts are among the main producers of IL-8 within connective tissues [137], [153].

a. Intercellular adhesion

Early electron microscopic studies of human gingival biopsies showed intimate contact between damaged gingival fibroblasts and lymphocytes, suggesting that lymphocytes sensitized to bacterial plaque might exert a cytotoxic effect on human gingival fibroblasts [154]. Fibroblasts can function as antigen-presenting cells within the connective tissue and interact with T-lymphocytes [155], [156], [157]. Indeed, direct adhesion of activated lymphocytes to fibroblasts
has been reported [158]. Intercellular adhesion can be induced following pre-treatment of fibroblasts with IFN-γ [159]. ICAM-1 and LFA-1 can mediate adhesive interactions between fibroblasts and lymphocytes [160]. Further, adhesion of T lymphocytes to human gingival fibroblasts is increased in the presence of inflammatory cytokines such as IL-1, IFN-γ and TNF-α. Adhesion may be enhanced by a combination of ICAM-1/LFA-1, the CD44/hyaluronic acid pathway and VLA integrins [161], [162], [163]. Co-culture of human gingival fibroblasts with lymphoid cells induces the expression of IL-1β, IL-1α and IL-6 mRNA plus hyaluronic acid, which may enhance direct interactions between the two cell types [162], [164]. Conceivably, direct contact between fibroblasts and lymphocytes may not only stimulate the inflammatory response but may also increase the production of extracellular matrix proteins by fibroblasts [164]. Indeed, histological studies of fibrotic diseases and asthma demonstrated that fibroblasts are frequently in close proximity to mast cells and that these interactions play an important role in the fibrotic process [165], [166], [167].

5. Mast cells

a. Origin and physiology

Mast cells are of the myeloid lineage and are derived from CD34+ pluripotential progenitor cells in bone marrow [168]. Mast cell differentiation occurs partially in the bone marrow under the effect of specific growth factors, primarily stem cell factor (SCF). Differentiation also occurs in the blood circulation, while final maturation takes place in connective tissues [169], [170].

Mast cells are widely distributed throughout many connective tissues. As part of host defence systems, mast cells are strategically located close to the surface epithelium for optimal interaction with environmental triggers [168], [171]. Typically, there are ~20,000 mast cells/mm³
in the intestinal lamina propria and this cell density increases in response to inflammation [172]. In the oral cavity mast cells are found throughout the gingival connective tissue and are often in close proximity to endothelial cells; but they can also be found within the epithelium [172], [173]. Based on their proteinase content, mast cells are divided into two main groups. Those mast cells that only contain tryptase and reside in mucosa are designated as MCₜ. Another type of mast cell which contains both tryptase and chymase are found in connective tissue (MCₜ𝑐) [174].

b. Mast cell mediators

Mast cell tryptase is a tetrmeric trypsin-like serine protease (molecular mass of 134 kDa) and is the major secretory component of human mast cells [175]. Tryptase is comprised of four monomers, which are non-covalently bound together. The active sites of these monomers face the central pore of the enzyme molecule [176]. α-tryptase and β-tryptase are the two main types of mast cell tryptases, which are further divided into α1, 2 and βI, II and III subtypes respectively [177], [178]. Tryptase is stabilized by heparin, which prevents it from being converted to inactive monomers. β-tryptase substrates include fibronectin [179], fibrinogen [180], pro-urokinase [181], pro-matrix metalloprotease-3 (proMMP-3) [182], protease-activated receptor-2 [183] and complement component C₃ [184].

MCₜ𝑐 mast cells produce and secrete chymase, which is a chymotrypsin-like protease [185]. Chymase converts angiotensin I to angiotensin II [186], cleaves type I pro-collagen to collagen fibrils [187] and digests several cytokines [188]. Histamine is a very well-known mast cell mediator and is primarily secreted during immune responses such as allergy. Histamine plays a variety of roles including vasodilation, bronchoconstriction and increased vascular permeability [189]. Histamine is stored in granules (100 mM concentration) [190], [191]. After
release, histamine is rapidly catabolised by oxidation and methylation. Four histamine receptors have been identified so far: HR1, HR2, HR3 and HR4 [189].

Heparin and chondroitin sulfate are two of the most abundant proteoglycans found in mast cells. These molecules function as stabilizers for some other pre-formed mediators, including proteinases. Further, they possess anti-coagulant and anti-complement effects [191]. Mast cells also contain metabolites of both cyclooxygenase and lipooxygenase pathways including prostaglandins (PG), thromboxanes (TX), leukotrienes (LT) and 5-, 12-HETEs (hydroxy-eicosatetraenoic acids). PGD2, LTB4 and LTC4 are among the most abundant lipid-derived mediators in mast cells [191].

As noted above, matrix metalloproteinases are among the host-related molecules that are associated with rapid tissue destruction in periodontal tissues. MMP-1 is present in gingival mast cells [192] and mast cells also produce MMP-1 and MMP-8 (about half of mast cells can express MMP-2 and TIMP-1). Only a small fraction of mast cells express TIMP-2 [192], [193]. These findings suggest that mast cells should be considered as important cells in mediating the host-response in periodontitis.

In addition to the molecules described above, mast cells can produce and release a large number of cytokines including IL-1β, -3, -4, -5, -6, -8, -10, -12 (only the p35 chain), -13, -16, TNF-α, GM-CSF, TGF-β, IFN-γ and VEGF [194], [195]. The level of maturation affects the ability of mast cells to synthesize cytokines (Fig. 2) [196].
c. Mast cell proliferation and activation

As described above, mast cells differentiate from bone marrow-derived, hematopoietic progenitors. The exact mechanism by which these cells exit the bone marrow and migrate to connective tissues is not fully understood, but earlier studies showed that P-selectin may play a role in the initial migration of mast cells from the bone marrow [197]. Adhesion molecules are believed to be important in localization and accumulation of mast cells within tissues [198]. Some of the surface adhesion molecules that have been identified in mast cells include integrins such as VLA-3, VLA-4 and VLA-5, suggesting that mast cell localization to connective tissues may require interactions with collagen, fibronectin and laminin [199], [200].

In the context of chemokine receptors, immature mast cells express CXCR2, CXCR4, CCR3 and CCR5. But after reaching maturity, mast cells only express CCR3 [201]. The c-kit ligand stem cell factor (SCF) induces mast cell proliferation both in vitro and in vivo [202], [203], [204]. The SCF effect is augmented in the presence of IL-3 [205]. Mast cells undergo apoptosis after withdrawal of IL-3, but this process can be prevented if cells are treated with SCF [206], [207]. IL-4 also promotes mast cell proliferation and may function synergistically with IL-
3 [208], [209]. Other activators of mast cells include complement fragments C$_3$a, C$_4$a and C$_5$a, which induce degranulation and anaphylactoid reactions [210], [211].

d. Immune function

Mast cells express surface receptors for the Fc portion of IgE (FccRI) and play a role in IgE-associated immune responses such as type I hypersensitivity. However, mast cells can become activated and exert their immunomodulatory functions through other IgE-independent mechanisms [212], [213], [214]. Mast cells are capable of phagocytosis and can function as antigen-presenting cells [215], [196] and contribute to wound healing [194]. Recent studies have suggested a role for mast cells in the pathophysiology of chronic inflammatory disorders such as cardiovascular diseases and atherosclerosis through their impact on regulating lymphocyte and macrophage function [216], [217], [218].

Mast cell populations increase during the development of periodontitis and in sites with progressive attachment loss [219], [220], [221], [222]. On the other hand, not all the functions of mast cells are pro-inflammatory. Despite the fact that mast cells are key cells in IgE-associated immune responses such as asthma, β-tryptase, which is a mast cell protease, is able to cleave IgE. Conceivably, this process may limit the allergic response. This contention has been supported by the finding of IgE degradation products in allergic inflammatory sites [223]. Further, mast cells produce IL-10, which is an anti-inflammatory cytokine and limits leukocytic infiltration [224].

e. Angiogenesis

Mast cells are in close proximity to blood vessels of connective tissues [225], [226]. Activation of mast cells in adult mammalian tissues can induce angiogenesis [227], [228]. Mast cell secretions increase vascularity by stimulating the proliferation of endothelial cells and their migration [229], [230]. Angiogenesis in mast cell-deficient animals occurs at a reduced rate but
is restored by local reconstitution of mast cells [231]. Among mast cell mediators, histamine has the most potent angiogenic effect [228] while TNF-α has both stimulatory and inhibitory effects [232]. Heparin potentiates the effect of fibroblast growth factor, a key angiogenic factor, perhaps by increasing its affinity to its receptors [233]. Further, mast cells can degrade extracellular matrix to provide space for newly formed vessels [233]. During wound healing fibroblast growth factor, VEGF and PDGF promote mast cell chemotaxis to sites of neovascularisation [234].

f. Mast cells and T-lymphocytes

By electron microscopy, direct intercellular adhesion between mast cells and T-cells has been shown; this is a key requirement for antigen-presenting functions [235]. Mast cells produce and secrete the CXC chemokine IL-8, which is a potent chemokine for both neutrophils and lymphocytes [236], [237], [238]. Further, mast cells secrete lymphotactin and IL-16, which are chemotactic factors for CD8+ and CD4+ lymphocytes respectively [239], [240]. Mast cell expression of macrophage inflammatory protein (MIP)-1β positively affects T-cell emigration from the circulation into lymph nodes during the inflammatory response [241]. In addition to attracting lymphocytes, mast cells can migrate towards these cells by means of integrins [241], [242],[243]. A list of mast cell receptors is shown in Figure 3.

Figure 3 Mast cell surface receptors [196]
**g. Mast cells and fibroblasts**

Previous histological studies have shown that fibroblasts are frequently in close proximity to mast cells and that their interactions may be important for many cellular processes [165], [166], [167]. Studies of fibrosis have suggested the possibility that gap junctions can form between these two cell types [244], [245]. Fibroblasts produce and secrete stem cell factor (SCF), the ligand of the c-kit proto-oncogene product, which is a major regulator of human mast cells and promotes their proliferation and differentiation. SCF enhances mast cell maturation and directly stimulates release of mast cells mediators such as histamine [246]. Fibroblast-derived SCF also upregulates the expression of MCP-1 and eotaxin (a potent eosinophil specific C-C chemokine) in mast cells [247], [248].

Conversely, mast cells also affect fibroblasts in multiple processes. The expression of SCF by fibroblasts, which can activate mast cells, is upregulated in response to TNF-α, a mast cell mediator [249]. Tryptase promotes fibrosis by stimulating human fibroblast chemotaxis [250] and by increasing collagen synthesis [165], [251]. Tryptase also induces the proliferation of fibroblasts via protease-activated receptor-2 signalling, which may account for the hyperplasia seen in asthma [251], [252]. Collagen gel contraction studies have shown that in co-culture, mast cells increase fibroblast contractility and may induce their differentiation into myofibroblasts [253], [254], [255], [256].

Mast cells express fibroblast growth factor-2 (FGF-2) and are able to stimulate FGF-2 secretion (via tryptase) and FGF-7 secretion (via histamine) by human fibroblasts [257]. Mast cells and their products can also promote the expression of inflammatory cytokines by fibroblasts. Tryptase can enhance expression of IL-8 in synovial fibroblast-like cells and also induce their proliferation [258]. Interactions between human lung fibroblasts and mast cells,
which were studied in co-culture, showed that mast cells induce IL-6 expression by fibroblasts [166]. Studies of lung fibroblasts demonstrated that CD40, a membrane glycoprotein, is a crucial factor in fibroblast activation by mast cells [259]. Expression of CD40 has been reported in human gingival fibroblasts [260]. Ligation of CD40 receptors promotes synthesis of inflammatory cytokines such as IL-1, IL-6, IL-8, PGs and hyaluronate (an ECM protein) [260], [261], [262]. Several cell types including mast cells express CD40 ligand (CD40L) [259]. Therefore, hypothetically, mast cells may potentially activate fibroblastic CD40 receptors and cause an increase in inflammatory cytokine production. Evidently, mast cells may collaborate with fibroblasts in a wide range of metabolic activities. These findings underline their potentially interactive roles in connective tissue remodelling in inflammatory diseases such as periodontitis.
Statement of the problem:

Periodontitis is the second most prevalent infectious disease of North Americans and affects >30% of the US adult population [3]. Periodontitis is associated with increased risk of various systemic diseases including diabetes, myocardial infarction and stroke [5], [7]. Host responses to periodontal pathogens contribute to the pathogenesis of inflammatory systemic diseases and play a central role in the progression of periodontitis [26], [27], [263]. Due to our lack of knowledge of how host response mechanisms control episodic exacerbations in periodontitis [42], current clinical management of periodontitis cannot always be optimized to improve the clinical course.

Neutrophils are critical cells in the innate immune response and are the first line of defence against periodontal pathogens [74]. Neutrophils release proteolytic enzymes, which mediate periodontal tissue destruction in acute inflammatory episodes [50]. Neutrophil chemotaxis into inflamed sites is promoted by interleukin-8 (IL-8; or CXCL-8) [21], [40],[126], a pro-inflammatory chemokine. IL-8 is expressed by several cell types including gingival fibroblasts [137]. IL-8 levels in gingival crevicular fluid are increased in periodontitis and are reduced after periodontal treatment [133], [134].

Fibroblasts are the most abundant cells of gingival connective tissue and contribute to the synthesis and degradation of periodontal extracellular matrices [264]. Fibroblasts are often located in close proximity to inflammatory cells, including mast cells. Indeed, their interactions with mast cells and other immune cells may be important for intercellular signal transduction pathways that mediate the host response [162], [164], [165], [166], [167]. Mast cells are of the myeloid series and are normal residents of periodontal connective tissues. The abundance of mast cells increases during periodontitis and in sites with progressive attachment loss [219],
[220], [221], [222]. Notably, fibroblasts form gap junctions with mast cells in fibrotic lesions [244], [245] but it is not known whether direct interactions between mast cells and fibroblasts affect fundamental regulation of the inflammatory response.

**Hypothesis:** Mast cells interact with fibroblasts to regulate IL-8 release and perpetuation of inflammation.

**Objectives:**

1) Measure IL-8 levels in co-cultures of fibroblasts with mast cells.
2) Study fibroblast-mast cell co-cultures to assess their impact on neutrophil chemotaxis.
3) Examine intercellular communication between fibroblasts and mast cells.
4) Identify molecules that mediate IL-8 production in fibroblast-mast cell co-cultures.
Chapter 2: Role of intercellular interactions between mast cells and gingival fibroblasts in mediating inflammation

Introduction

Periodontitis is a chronic inflammatory disease that is characterized by recurrent episodes of acute exacerbation and increased destruction of bone and soft connective tissues [42]. While the mechanisms that initiate these acute exacerbations are not defined, host immune responses to the pathogenic microbiota that colonize the periodontium likely contribute to tissue destruction [263]. Neutrophils are key components of the inflammatory response and are the most abundant cell type in the gingival crevice, where they are the first line of defence against colonizing bacteria [74]. Degranulation and release of matrix-degrading enzymes from neutrophils can lead to excessive tissue destruction in acute exacerbations of periodontitis [50]. Notably, neutrophil migration into inflammatory sites is induced by chemokines [61]. One of the most potent chemokines that promotes neutrophil chemotaxis is interleukin-8 (CXCL-8) [21]. The levels of IL-8 in gingival crevicular fluid are temporally related to periods of periodontal destruction [40], [126] and are decreased after treatment [133], [134]. IL-8 is produced by various cell types within the periodontium, including gingival fibroblasts [137].

Fibroblasts are the most abundant cells of gingival connective tissue and play key roles in the synthesis, degradation and remodelling of the extracellular matrix of the periodontium [264]. Gingival fibroblasts (HGF) also contribute to the control of inflammation by secretion of inflammatory mediators such as IL-1β, IL-1α, IL-6 and IL-8 [137], [152], [153]. Notably, fibroblasts interact with immune cells including lymphocytes and mast cells. Co-culture of
Human gingival fibroblasts with T-lymphocytes induces the expression of inflammatory cytokines by fibroblasts as a result of direct interactions between these two cell types [164]. Further, fibroblasts are frequently in close proximity to mast cells in vivo [165], [166], [167].

Mast cells, which are of the myeloid lineage, are normal residents of periodontal connective tissues. The numbers of mast cells increases during periodontitis and at sites with progressive attachment loss [219], [220], [221], [222]. Mast cells and fibroblasts can interact to form gap junctions [244], [245], which may be important in the development of fibrosis. Direct interactions between mast cells and lung fibroblasts can enhance the expression of IL-6 [166]. As IL-8 is produced by gingival fibroblasts [137], we examined interactions between mast cells and gingival fibroblasts to define their potential effects in regulating acute inflammatory response through IL-8 release.
Materials and methods

Reagents

Alpha-thioglycerol, β-glycyrrhetic acid (BGA), bovine serum albumin (BSA), brefeldin A (BFA), fMLP, HEPES, Percoll, protease inhibitor cocktail, rhodamine B isothiocyanate dextran (10 kDa) and thapsigargin (TSGN) were purchased from Sigma-Aldrich (Oakville, ON). Ionomycin was from Calbiochem-EMD (Mississauaga, ON). Calcein-AM and FITC-dextran (70 kDa) were obtained from Molecular Probes (Eugene, OR). HBSS, α-MEM and IMDM were purchased from GIBCO®, Invitrogen (Burlington, ON). Purified bovine collagen solution was obtained from PureCol®, Advanced BioMatrix Inc. (San Diego, CA). The 4B4 IgG1 monoclonal antibody was purchased from Beckman Coulter (Mississauga, Ontario). The 2-µm diameter polystyrene and protein-A conjugated microspheres were obtained from Polybead®, Polysciences Inc. (Warrington, PA). E-Lyse was purchased from Cardinal Associates (Phoenix, AZ). Normal mouse serum and human cytokine antibody array #5 were obtained from Cedarlane (Burlington, ON). Human CXCL8/IL-8 QuantiGlo ELISA kits were purchased from R&D Systems (Minneapolis, MN).

Cell Cultures

Human gingival fibroblasts (HGF) were derived from primary explant cultures as described [265]. Cells from passages 4–10 were grown as monolayers in T-75 flasks. Full growth medium consisted of α-minimal essential medium (α-MEM), antibiotics (0.017% v/v penicillin G; Ayerst, Montreal, Canada; 0.01% v/v gentamycin sulfate in α-MEM) and 10% (v/v) heat-inactivated fetal bovine serum (ICN Biomedicals, Costa Mesa, CA). The cells were grown to 80-100% confluence prior to all experiments. Fibroblasts were passaged with 0.01% trypsin.
The human mast cell line (HMC-1) was kindly provided by Dr. J.H. Butterfield (Mayo Clinic, NY). Mast cells were grown in suspensions in T-75 flasks according to a previously described protocol [266]. Mast cell growth media contained Iscove's modified Dulbecco's medium (IMDM) supplemented with 1.2 mM alpha-thioglycerol, 10% (v/v) heat-inactivated calf serum, 100 U/ml penicillin and 100 U/ml streptomycin. For passaging, cell suspensions were centrifuged for 8 minutes at 400xg. Both types of cells were grown at 37°C in a humidified incubator containing 5% CO₂ and were counted electronically (Beckman Coulter).

**Cytokine array**

Fibroblasts were grown on 6-well cell culture plates (Falcon, Becton Dickinson, Mississauga, ON). When 80% confluent (200,000 cells per well), growth media were replaced with serum-free α-MEM. For co-cultures, 100,000 mast cells were incubated on monolayers of fibroblasts at a 1:2 ratio. Conditioned media from these co-cultures or from cultures of fibroblasts or HMC alone were collected after 8 hours. Cytokine expression was analyzed with a human cytokine antibody (array #5; RayBiotech, Inc) according to the manufacturer’s instructions. Briefly, each cytokine array membrane was incubated with blocking buffer at room temperature for 30 minutes and then incubated overnight with 1 ml of conditioned media at 4°C. After washing, the membranes were incubated with primary antibody for 1.5 hours at room temperature followed by another wash and incubation with HRP-conjugated streptavidin secondary antibody for 2 hours at room temperature. After the final wash, detection buffer was applied on membranes for 2 minutes and arrays were exposed to x-ray film (Kodak, Canada).

**IL-8 quantification**

HGFs were grown on 6-well plates to reach 80% confluence (200,000 cells/well). Growth media were replaced with serum-free α-MEM unless otherwise indicated and HMC-1 cells were added
at a 1:2 ratio (100,000 mast cells per well). HGFs were co-cultured with HMC-1 cells for 8 hours except for time-course experiments, which were performed from 1-8 hours both in the presence and absence of serum. Pure cultures of either HGF or HMC-1 were used as controls. For examination of dose-response effects, fixed ratios of HGF:HMC (2:1, 1:1, 1:2, 1:4) were examined after 8 hours. Following co-incubation, supernatants were collected, samples were sedimented (5 minutes at 9300×g) to remove particulates from the conditioned medium, and the media were stored at -20ºC for later quantification of IL-8 by ELISA.

For IL-8 quantification, human CXCL8/IL-8 QuantiGlo ELISA kits (R&D Systems, Minneapolis, MN) were used. Briefly, previously stored samples were thawed at room temperature and 50 µl samples were loaded into each well of 96-well IL-8 ELISA plates. Following 2 hour incubations at room temperature on a shaker, wells were aspirated and washed four times with wash buffer (400 µL), incubated with 200 µl of IL-8 antibody conjugate, incubated for 3 hours at room temperature, washed and incubated with 100 µl of Working Glo Reagent for 5 - 20 minutes at room temperature while being protected from light. The relative luminescence of each well was measured with a luminometer (FLUOstar OPTIMA, BMG Labtech, Germany). The average of duplicate readings was collected and the concentration of IL-8 was estimated from a standard curve of known IL-8 concentrations.

**Integrin and cadherin ligation**

We determined whether co-culture-mediated IL-8 release involves adhesion molecules. HGFs were incubated with either BSA or type I bovine dermal collagen [267] or N-cadherin [268] - coated 2-µm polystyrene beads. The N-cadherin-Fc fusion protein was expressed in HEK-293 cells and collected as described [269]. Beads were counted with a hemocytometer.
The effect of BSA-coated beads on IL-8 expression was compared to mast cells. In this experiment 50,000 HGFs at 100% confluence on 24-well cell culture plates (Falcon) were co-incubated with either 50,000 BSA-coated latex beads or 50,000 HMCs in serum containing fibroblast growth media. Samples of conditioned media were collected at 0, 1, 2 and 4 hours. For comparison of the effect of ligation of adhesion receptors, 40,000 HGF were grown on 24-well plates and were incubated for 4 hours with either BSA-coated, collagen-coated or N-cad-Fc protein-coated beads. For each condition, 240,000 beads were incubated with cells at a 1:6 ratio of HGF:Beads. For comparison, co-cultures of HGF:HMC and HGF:HGF (both at 1:1 ratios) were established. Conditioned media was collected after 4 hours. Samples were prepared, stored and IL-8 was quantified by ELISA.

*Intercellular contact and IL-8 expression*

We assessed the importance of direct cell-cell contact on IL-8 release from fibroblasts. HGFs were grown on 6-well plates as described above (200,000 cells per plate) and then 100,000 mast cells were plated on 25 mm diameter Cyclopoire® membranes (Whatman Inc, NJ; pore size-0.45 µm) which were inserted into the fibroblast cultures. This approach physically separated the mast cells from HGFs during co-culture but allowed free passage of small molecules to exchange between fibroblasts and mast cells (Fig. 4).

In a separate experimental approach, the effect of mast cell sonicates on HGFs was examined. Mast cell suspensions in αMEM were sonicated (Branson model 185; 5 times for 10 seconds each; output setting of 5.0) on ice in the presence of protease inhibitors. A volume of sonicate from 100,000 HMC was added into each well of a 6-well plate containing HGFs. Following incubation at 37ºC for 8 hours, IL-8 levels were measured.
We examined a possible role for gap junctions in IL-8 release. HMCs and HGFs were pre-incubated with the gap junction inhibitor, β-glycyrrhetinic acid (BGA; 100 µM; Sigma-Aldrich, ON) for 1 hour. Cells were co-cultured at a 2:1 ratio (HGF:HMC). BGA was present throughout the 8-hour co-incubation period. Samples were collected at 8 hours for quantification of IL-8. Flow cytometry was performed to quantify the effect of BGA on gap junction formation. HMC-1 (donor) cells were incubated with calcein/AM (5 µg/ml) for 1 hour at 37°C. HGFs and HMCs were treated with 20 µM BGA for 1 hour and were then co-cultured in the presence or absence of BGA. After 1 and 3 hours, media were aspirated and co-cultures were rinsed with PBS to remove unbound HMCs. Cells were detached from culture plates with 0.01% trypsin. After sedimentation, cells were re-suspended in PBS and analyzed by flow cytometry (Beckman Coulter Altra, Mississauga, ON). Laser excitation was 488 nm. To minimize measurement of fluorescence attributable to HMCs, side and forward scatter gates were set to maximize measurement of HGFs. Calcein fluorescence was measured in 10,000 HGFs in each samples from BGA and control groups after 1 and 3 hours of co-culture. In some experiments, since cold
temperature inhibits gap junction formation in vitro [270], co-cultures were conducted at 4°C in α-MEM and supplemented with 25 mM HEPES buffer to stabilize the pH.

**IL8 secretion**

We determined whether changes of IL-8 levels were due to protein secretion. Cells were treated with the protein secretion inhibitor, brefeldin A (BFA) at 10 µg/ml for 30 minutes prior to co-culture. BFA was present throughout the co-culture period. Samples were collected after 8 hours of co-culture for IL-8 quantification.

**Role of calcium**

As calcium is an important second messenger that may be involved in intercellular signalling events between fibroblasts and mast cells, in some experiments we stimulated cells with thapsigargin (TSGN), a sesquiterpene lactone that inhibits Ca\(^{2+}\) uptake by the endoplasmic reticulum and causes a net increase of intracellular free ionic calcium levels \([\mathrm{Ca}^{2+}]_i\). In other experiments we used ionomycin, a calcium ionophore, to increase \([\mathrm{Ca}^{2+}]_i\). HGFs were grown on 6-well plates as described above and pure cultures of either HGFs or HMCs were treated with TSGN (500 nM) for 30 minutes prior to co-culture. Treated cells were rinsed twice with phosphate buffered saline (PBS) to remove any remaining TSGN prior to co-culture (HGF:HMC=2:1) for 8 hours. For experiments using ionomycin, both cell populations were incubated with ionomycin (2 µM) 30 minutes prior to co-culture. HMCs were added to HGF, similar to the TSGN experiment except that ionomycin was present throughout the 8 hour co-culture period.

**Neutrophil chemotaxis**

We examined the effect of co-culture conditioned medium on neutrophil chemotaxis. Mouse neutrophils were isolated as previously described [271]. Briefly, following euthanasia, femurs
and tibias were removed and bone marrow was isolated. After lysing erythrocytes (E-Lyse), discontinuous Percoll gradients of 82%/65%/55% were used to layer the remaining bone marrow [271]. Mature neutrophils were recovered at the 82%/65% interface. Wright-Giemsa staining has identified over 85% of cells isolated using this protocol as neutrophils [272]. For analysis of chemotaxis, neutrophils were suspended in HBSS and 1% gelatine and this suspension (1 x 10^6/ml) was allowed to attach to BSA-coated glass coverslips (22 x 40 mm) at 37°C for 20 minutes. Coverslips were inverted on to Zigmond chambers and 100 µL HBSS media was added to the left chamber with 100 µL HBSS media containing fMLP [10^{-6}] as positive control. Samples of conditioned media were added to the right chamber. Time-lapse video microscopy was used to quantify neutrophil migration. Images were captured at 20-second intervals with a Nikon Eclipse E1000 microscope. Cell-tracking software (Retrac version 2.1.01 Freeware) was used to analyse the captured images and to measure the speed and distance travelled by neutrophils [272]. All procedures were conducted in accordance with the Guide for the Humane Use and Care of Laboratory Animals and this protocol had been approved by the University of Toronto Animal Care Committee.

Microscopy

We observed the spatial relationship between mast cells and fibroblasts by phase contrast light microscopy. HGFs were grown on 35-mm-diameter culture dishes (Falcon, Becton Dickinson, Mississauga, ON) in HGF growth media. At 80% confluence, mast cells were added (HMC:HGF=2:1 ratio). Following 24 hours of co-culture, medium was aspirated and cultures were jet-washed [273] with phosphate buffered saline (PBS) to remove unbound or loosely attached cells. A light microscope (Leitz Wetzlar, Germany) and camera (PixeLINK PL-A642) were used to obtained images of the attached cells.
Intercellular adhesion

To study intercellular communications between mast cells and gingival fibroblasts in our simplified model [274], HGF were grown overnight to 80% confluence in normal growth media. HMC-1 (donor cells) were fluorescence-labeled with calcein-AM (5 µg/ml) for 1h at 37°C, followed by three washes with α-MEM. These cells were then plated onto the established HGF monolayer (acceptor cells). Cells were co-cultured in HGF growth media supplemented with 25 mM HEPES to stabilize pH. Dye transfer from HMC-1 (donor) into HGF (acceptor) was monitored via live imaging (0–360 min) and recorded at specific time points with a laser-scanning confocal microscopy (Leica TCS SL, Heidelberg, Germany). Calcein was imaged with excitation set at 488 nm and emission was collected with a 530/20-nm barrier filter. Cells were imaged with a 40x 1.25 oil-immersion lens. Transverse optical sections were obtained from the level of cell attachment at the substratum of the acceptor cell to the dorsal surface of the donor cell (as verified by phase-contrast microscopy).

Role of integrins in HGF-HMC attachment

HGFs (15,000) were grown on 8-well chamber slides (Falcon, Becton Dickinson, Mississauga, ON). HGFs and HMC cultures were incubated separately overnight at 37°C with 2 mg/ml FITC Dextran and 1 mg/ml Rhodamine B isothiocyanate dextran respectively. The next day, HGFs were rinsed twice with PBS. Growth medium containing an attachment inhibitory antibody against β1 integrins (4B4 at 1:20 dilution) was added to wells (15 µl of 4B4 mAb in 300 µl of growth media per chamber). For controls, normal mouse serum at 1:100 dilution was used instead of 4B4. After incubation for 1 hour, 7500 mast cells were added on to HGFs. Chamber slides were rinsed with PBS at 1, 3 and 6 hours following co-culture in all three groups and the
ratio of attached HMC to HGF was calculated by counting the cells under the fluorescent microscope (Leica, Heidelberg, Germany) using FITC and RITC filters.

Statistical Analysis

For cell culture experiments, biological triplicates and technical duplicates were used. Means between 2 groups were compared using the unpaired Student’s $t$-test. $p<0.05$ was considered to be statistically significant.
**Results**

We used an antibody array to assess the release of inflammatory cytokines into the medium after co-culture of fibroblasts with mast cells. After 8 hours of fibroblast-mast cell co-culture there were increased levels of several inflammatory cytokines including IL-8, GRO (IL-8-related chemokine), IL-6 and MCP-1 (CCL2) compared to control fibroblasts, which were cultured in the absence of mast cells (Fig. 5A). Based on previous reports showing enhanced IL-8 release in response to ligation of CD40 on fibroblasts by the CD40 ligand that is also expressed by mast cells [259], [260], [262] and because of the importance of IL-8 in promoting neutrophil recruitment to inflammatory sites [43], we focussed subsequent investigations on IL-8.

**Effect of fibroblast-mast cell co-culture on IL-8**

We quantified IL-8 levels in serum-free media of fibroblast-mast cell co-cultures using an ELISA. In initial experiments that examined cells after 8 hours of co-culture, there were significantly increased IL-8 levels ($p<0.001$) in conditioned media compared to fibroblasts plated alone (Fig. 5B). While fibroblasts alone secreted measurable levels of IL-8, the amount of IL-8 secreted by mast cells was negligible (0.02±0.01 pg/ml).

We determined whether there was a dose-response effect between the relative numbers of mast cells and levels of IL-8 by adjusting the ratios of mast cells to fibroblasts in co-cultures. The levels of IL-8 were increased 30-fold when the ratios of mast cells to fibroblast were increased 8-fold (from 1:2 to 4:1; Fig. 5C; $p<0.001$).

We assessed whether there was a time-dependent increase of co-culture-induced IL-8. Within 2 hours there was a statistically significant difference between fibroblasts alone and fibroblasts cultured with mast cells ($p<0.05$; Fig. 5D) and by 8 hours there was 50% more IL-8 in the co-cultures than fibroblasts alone.
We determined whether the stimulatory effect of co-culture on IL-8 release was affected by serum. In comparison to serum-free conditions, co-culture of cells in the presence of serum showed 50-fold higher levels of IL-8 and in mast cell-fibroblast co-cultures compared to fibroblasts alone, IL-8 levels were >40-fold higher (Fig. 5E).

**Role of adhesion molecules**

We examined whether co-culture-driven increases of IL-8 were mediated by ligation of specific adhesion molecules expressed by fibroblasts. HGFs were incubated with collagen or N-cadherin-coated beads in serum-containing medium; BSA-coated beads were used as controls and IL-8 was measured after 0-4 hours. In contrast to time-dependent increases of IL-8 in mast cell-fibroblast co-cultures, incubation of fibroblasts with BSA-coated beads did not significantly increase IL-8 levels (Fig. 6A). Similarly, there were no increases of IL-8 levels when HGFs were incubated with N-cadherin or collagen-coated beads (Fig. 6B). Further, we incubated single cell suspensions of fibroblasts on to previously spread, homotypic fibroblast monolayers and found that after 4 hours there were no statistically significant differences of IL-8 levels in conditioned media between homotypic fibroblast co-cultures and fibroblast monolayer cultures (p>0.05).

**Intercellular adhesion**

The effect of serum on co-culture-mediated-IL-8 release suggested that attachment and spreading of mast cells on to fibroblasts may be important for the enhanced IL-8 release. We assessed intercellular adhesion between fibroblasts and mast cells after 24 hours of co-culture in serum-containing media by light microscopy, which showed that even after vigorous rinsing with PBS (Fig. 7 A,B), large numbers of mast cells remained attached to the fibroblasts. Notably, as mast cells normally grow in suspension and do not tend to adhere to plastic culture surfaces [266], we
considered that mast cell attachment to fibroblasts and the potential transfer of intercellular signals may be important for the induction of IL-8 release.

**Role of integrins**

In separate culture containers, fibroblasts and mast cells were loaded overnight with FITC-dextran and rhodamine B isothiocyanate dextran, respectively, a procedure [274] that selectively marks these two cell populations. On the next day, fibroblasts were pre-treated with a β1 integrin blocking antibody (4B4) or control IgG and then mast cells were added to fibroblast monolayer cultures. The ratios of the numbers of attached mast cells to fibroblasts were determined at 1, 3 and 6 hours after incubation by enumerating the two, differentially labelled populations by fluorescence microscopy (Fig. 7C). For all three time points, pre-treatment with the β1 integrin inhibitory antibody significantly reduced the ratio of attached mast cells to fibroblasts (p<0.001).

We studied intercellular communication between mast cells and fibroblasts by loading mast cells with FITC-calcein/AM, a small (MW=995 Da) fluorescent molecule that exchanges between attached cells through gap junctions [275]. Calcein-loaded mast cells were incubated on unstained fibroblasts, confocal imaging was performed and images were recorded every 30 minutes for six hours. Within 60 minutes of co-incubation, there was detectable dye transfer from mast cells to the fibroblasts, which increased over prolonged incubation (Fig. 7D).

We examined the role of gap junctions by pre-treatment of cells with the gap junction inhibitor, BGA. At defined periods after co-cultures were started, single cell suspensions were prepared and flow cytometry was used to quantify the relative number of fibroblasts that exhibited above-threshold calcein fluorescence. Forward and side scatter were used to distinguish fibroblasts from mast cells. BGA reduced calcein fluorescence in fibroblasts
compared to untreated controls after 1 hour of co-culture \((p<0.05; \text{Fig. 8A})\) but there was no statistically significant difference at 3 hours \((p>0.05)\).

We studied the role of direct physical contact between fibroblasts and mast cells in IL-8 release. Mast cells were physically separated from fibroblasts by Cyclopore® membranes during 8 hour co-cultures. IL-8 levels in conditioned media were significantly reduced compared to conventional mast cell-fibroblast co-cultures \((p<0.01; \text{Fig. 8B})\). Similar results were observed when fibroblasts were incubated in the presence of sonicated mast cells \((p<0.01)\). Pre-treatment of mast cell-fibroblast co-cultures with the gap junction inhibitor, BGA, also blocked IL-8 release \((p<0.05)\).

Since cold temperature inhibits gap junction formation \([270]\), IL-8 was quantified in co-cultures conducted at 4°C. There was a very large reduction of IL-8 release when co-cultures were conducted at cold temperature \((p<0.001; \text{Fig. 8C})\).

**IL-8 secretion**

When fibroblasts and mast cells were pre-treated with brefeldin A to block protein secretion, IL-8 levels were reduced by >50-fold in both fibroblast monolayer controls and in media derived from co-cultures \((p<0.01; \text{Fig. 9A})\).

Our data above indicated that there may be an intercellular signal that drives fibroblast secretion of IL-8. Further, as gap junctions are known to provide a conduit for intercellular calcium signalling \([276]\), and as previous reports have indicated that IL-8 release from mast cells can be mediated by perturbation of calcium signalling \([277]\), we considered that IL-8 secretion may be mediated by calcium signals. Accordingly, we treated cells with thapsigargin to acutely release \(\text{Ca}^{2+}\) from intracellular calcium stores. Treatment of fibroblasts or mast cells for 30 minutes with thapsigargin prior to initiating co-cultures, markedly increased IL-8 levels in the
co-culture media compared to untreated groups ($p<0.001$; Fig. 9B). A similar increase was seen ($p<0.001$) when cells were co-cultured and treated with ionomycin, a calcium ionophore. The effect of thapsigargin was significantly reduced when mast cells were separated from thapsigargin-treated fibroblasts by Cyclopo® membranes ($p<0.01$) and also when fibroblasts were treated with brefeldin A ($p<0.01$; Fig. 9C), which blocks the protein release pathway in cells.

*Effect of co-culture medium on neutrophil chemotaxis*

The data described above showing enhanced IL-8 release into the medium in mast cell-fibroblast co-cultures, suggested that interactions between these cell types may affect neutrophil chemotaxis. Accordingly, we determined whether media derived from co-cultures would affect neutrophil chemotaxis. Assays were conducted using mouse neutrophils that were exposed to 24-hour conditioned media from either single fibroblast populations or from mast cell-fibroblast co-cultures. Neutrophil chemotaxis was significantly enhanced in response to co-culture media compared to media derived from single fibroblast population controls ($p<0.01$; Fig. 10A). Notably, the enhanced chemotaxis was significantly reduced when fibroblasts and mast cells were pre-treated with the gap junction inhibitor, BGA ($p<0.001$).
Discussion

Release of chemoattractants such as IL-8 can exacerbate inflammation by enhancing neutrophil migration into the lesion [43]. Our principal finding is that direct interactions between human mast cells and gingival fibroblasts mediate rapid release of IL-8, which promotes neutrophil chemotaxis [21]. These findings suggest a mechanism by which mast cells and fibroblasts cooperatively modulate the inflammatory response (Fig. 10B). Their interactions may generate episodic cycles of remission and acute exacerbation that characterize many types of inflammatory diseases such as chronic obstructive pulmonary disease [278], inflammatory bowel diseases [279], arthritis [280] and periodontitis [42].

Fibroblasts are the most abundant cells of soft periodontal connective tissues [139]. While their principal roles are the synthesis, degradation and remodelling of extracellular matrix proteins [141], [142], fibroblasts also participate in the inflammatory response through their interactions with lymphoid and myeloid cells and their production of inflammation-regulating cytokines [152], [153], [162], [164], [166]. As periodontitis is a high prevalence inflammatory disease [3] that is characterized by bursts of active tissue destruction followed by periods of remission and repair [42], we considered that interactions between human gingival fibroblasts and other normal residents of connective tissues, namely mast cells, may be informative of how fibroblasts regulate the inflammatory response. Notably, the relative numbers of mast cells in gingival connective tissues increase during active episodes of periodontitis [222] suggesting that mast cells may play a crucial role in regulating tissue homeostasis during acute inflammatory episodes.
**Mast cell co-culture with fibroblasts increases IL-8 expression**

Our data from cytokine arrays showed that human mast cells in co-culture with fibroblasts induce increased expression of MCP-1, IL-6, GRO and IL-8. MCP-1 is a strong chemoattractant for monocytes and T-lymphocytes [83] while IL-6 is a pleiotropic inflammatory mediator that can induce the synthesis of acute phase proteins and enhance B-lymphocyte differentiation and maturation [281] [282]. Our finding of increased IL-6 levels is consistent with earlier data from human lung fibroblast-mast cell co-cultures [166]. Two other cytokines were detectably increased in the antibody arrays, IL-8 and GRO, a member of the IL-8 family. Because neutrophil chemotaxis is a key feature in acute inflammation, and as IL-8 and GRO are both potent neutrophil chemoattractants [43], [125], we focused further experiments on how fibroblasts and mast cells may cooperate to induce IL-8 expression.

We found that IL-8 levels in co-culture-conditioned media were positively associated with both the duration of co-incubation and the proportion of mast cells that were incubated on the fibroblasts. Incubation of co-cultures in serum-containing media increased IL-8 levels up to 50-fold compared to serum-free co-cultures, which may be explained by the presence of vitronectin (serum spreading factor) an abundant serum protein [283] that facilitates mast cell adhesion and spreading over fibroblasts [284].

While low baseline levels of IL-8 were detected in HGF cultures, mast cells alone expressed virtually no IL-8 as measured by ELISA and qRT-PCR (data not shown). Therefore, we consider that HGFs are the most likely source of the observed, co-culture-induced elevation of IL-8. Although determining the actual source of IL-8 may be helpful, it should be taken into consideration that the significance of our findings is in the combined pro-inflammatory effect of mast cells and fibroblasts in co-culture. Analysis of mRNA by qRT-PCR on flow cytometry-
sorted cells following co-culture or immunostaining for IL-8 could be used to identify the cell population that is the source for the IL-8 released into the medium.

**Role of adhesion molecules in co-culture-induced IL-8 expression**

Physical separation of mast cells from fibroblasts using Cyclopore® membranes blocked the release of IL-8 into the medium. These membranes allow free passage of molecules between cells but prevent direct intercellular contact. In parallel experiments, incubation of fibroblasts with mast cell sonicates did not mediate increased IL-8 levels. Collectively, these data suggested that the enhanced IL-8 release that we observed was dependent on intercellular adhesion and possibly intercellular communication between mast cells and fibroblasts. Previous reports showed that intercellular contact is essential for co-culture-associated IL-6 expression by HMC-1 mast cells and human lung fibroblasts [166] and that direct interactions between human gingival fibroblasts and T-lymphocytes induce the expression of IL-1β, IL-1α and IL-6 mRNA [164].

We determined whether IL-8 release involves ligation of fibroblast adhesion receptors. Fibroblasts were incubated with collagen or N-cadherin-coated polystyrene beads or BSA-coated beads as controls. Previous analyses of human gingival fibroblasts have established that receptors for fibrillar collagen [267] and N-cadherin [274] are abundantly expressed by these cells. None of these molecules enhanced IL-8 release by fibroblasts, suggesting that mechanisms other than ligation of adhesion receptors may mediate this process. Further, the failure of homotypic fibroblast co-cultures to increase IL-8 levels supported the specificity of the mast cell-dependent effect and motivated us to examine in more detail the spatial relationships between fibroblasts and mast cells in co-cultures.

We studied the strength of intercellular adhesions between fibroblasts and mast cells with a fluid shear flow assay in later stage cultures [273]. After 24 hours of co-culture phase contrast
microscopy showed that mast cells localize and spread over fibroblasts. These cells remained attached to mast cells following jet washing with buffer at estimated maximum shear stress of 3.5 pascals [273], suggesting that mast cells in co-culture form robust adhesions with gingival fibroblasts. Notably, histological studies on fibrosis and asthma have demonstrated that mast cells are located in close proximity to fibroblasts [165], [166], [167] and intimate contacts between gingival fibroblasts and lymphocytes have been observed in vivo by electron microscopy [154].

Integrins are heterodimeric glycoproteins that mediate cell attachment to the extracellular matrix and to other cells [285]. We found that a blocking antibody against β1 integrins significantly reduced the number of mast cells that attached to fibroblasts at all time points. Taken together with the collagen bead binding data described above, β1 integrins evidently play an essential role in intercellular adhesion but that β1 integrin ligation alone is not sufficient for IL-8 release. β1 integrins are involved in intercellular attachment between T-lymphocytes and human gingival fibroblasts [162] and in particular, the ICAM-1/LFA-1 pathway is required for T-cell attachment to fibroblasts [160]. As human gingival fibroblasts express ICAM-1 [162] and HMC-1 cells express LFA-1[286], our findings are consistent with the possibility that integrins may mediate fibroblast-mast cell attachments.

Role of gap junctions

Gap junctions are gated membrane channels that allow intercellular passage of small molecules between attached cells [287]. Several cell types, including gingival and dermal fibroblasts, establish direct communication and coordinate cellular functions through gap junctions [274], [288], [289]. Passage of molecules through gap junctions is determined in part by the size of the molecules [287]. Gap junctions typically permit passage of small molecules such as Ca^{2+}, IP₃,
cAMP, and, in our experiments, calcein (molecular mass of <1000 Da), but block passage of proteins or nucleic acids [275], [287]. The gap junction channel is made of two connexon structures, for which each cell contributes one connexon. The connexon is composed up of six connexin proteins embedded within the plasma membrane [290]. Fibroblasts mainly express connexin 43 (Cx43) [274] while mast cells express Cx43 and Cx32 [291].

Since the elevated co-culture-induced IL-8 was observed within 2 hours of co-culture and as phase contrast microscopy showed mast cell attachment to fibroblasts, we examined intercellular communication between these cells by confocal microscopy immediately after mixing the cells. Fibroblasts were incubated with mast cells that had been labelled previously with calcein. Live imaging showed gradual dye transfer from mast cells to fibroblasts, which was detected within 60 minutes after initiation of co-cultures. Analysis of live images in different “z” sections did not show internalization of mast cells by fibroblasts. Further, to ensure that no free calcein was present in the mast cell culture media that could have been taken up by the fibroblasts, as a negative control, we removed mast cells from the medium by filtration and the filtered medium was then added to fibroblasts. These control cells showed no evidence of dye uptake after 6 hours of incubation, confirming that there was insufficient free calcein/AM remaining in the mast cell media (prior to adding them to the fibroblasts) to cause artefactual staining of the fibroblasts. Therefore, the observed dye in fibroblasts after co-culture indeed originated from mast cells and not from the medium. As calcein is a small molecule (MW=995 Da) that readily traverses gap junctions [274] [275], we conclude that gap junctions were involved in intercellular signalling between mast cells and fibroblasts.

We investigated the function of gap junctions between mast cells and fibroblasts using the gap junction inhibitor BGA. Calcein dye transfer from mast cells into fibroblasts was studied
by flow cytometry, which showed that BGA significantly reduced the total number of calcein-positive cells compared to controls after 1 hour of co-culture but not significantly after 3 hours. This latter result may have been due to the limitation of flow cytometry in differentiating between fibroblasts and mast cells based on their sizes. While separation of these cell types according to forward and side scatter gating was possible at early time periods, by 3 hours it was difficult to separate the cells with trypsin and to measure their fluorescence separately. Because of this same challenge in sorting, we were not able to sort and analyze individual cell populations after the co-culture for qRT-PCR to study IL-8 expression (data not shown).

When we pre-treated mast cells and fibroblasts with BGA, IL-8 levels were significantly reduced compared to vehicle treated co-cultures. We also found that when cells were co-incubated at 4°C, a method that inhibits gap junction formation in vitro [270], IL-8 levels were virtually undetectable. Although the results from experiments at 4°C per se do not conclusively indicate the presence of gap junctions, these findings were in agreement with the notion that gap junctions play a central role in fibroblast-mast cell interactions that lead to IL-8 release. The presence of gap junctions between mast cells and fibroblasts was first demonstrated in the developing avian eye [292] and in vitro studies have shown that rat mast cells can establish gap junctions with human dermal fibroblasts [244]. Heterotypic gap junctions between mast cells and dermal fibroblasts can form in collagen lattices but not in monolayer cultures [245]. Our results in co-cultures indicate that intercellular attachment between fibroblasts and mast cells, and the subsequent establishment of gap junctions, is required for increased IL-8 release in co-culture. Previous reports have described a significant increase in IL-6 production 12 to 72 hours following co-culture of mast cells and fibroblasts [166]. Our data showed a much more rapid response in which a significant rise of IL-8 levels was detected as early as 2 hours after initiation
of co-culture. Further, pre-incubation of cells with brefeldin A, which inhibits protein secretion, significantly reduced IL-8 levels both in control fibroblasts and in mast cell-fibroblast co-cultures. Collectively, these data indicate that it is protein secretion and not synthesis that likely plays a role in the co-culture-induced IL-8 release.

Role of calcium

Gap junctions provide a conduit for intercellular Ca\(^{2+}\) signalling [276] and for transfer of IP\(_3\) between cells [293]. IP\(_3\) binding to intracellular stores causes Ca\(^{2+}\) release. Since Ca\(^{2+}\) is an important second messenger that may be involved in intercellular signalling events between fibroblasts and mast cells, we treated cells with either thapsigargin or ionomycin. Thapsigargin is a sesquiterpene lactone that inhibits Ca\(^{2+}\) uptake by the endoplasmic reticulum via blocking Ca\(^{2+}\)-ATPase, which will then lead to increased intracellular Ca\(^{2+}\) [Ca\(^{2+}\)]\(_i\) [294]. Ionomycin is a calcium ionophore which directly enhances calcium entry through the plasma membrane, thereby increasing [Ca\(^{2+}\)]\(_i\) [295]. A previous report showed that intercellular adhesion between pairs of homotypic fibroblasts causes increased calcium concentration at contact sites [296]. Our findings showed that in fibroblast-mast cell co-cultures, both thapsigargin and ionomycin strongly increased IL-8 release (up to 15-fold of untreated co-cultures). Therefore, we speculate that intercellular adhesion and increased communication between mast cells and fibroblasts may enhance IL-8 release by stimulating increased [Ca\(^{2+}\)]\(_i\), which then leads to IL-8 release.

Mast cell-fibroblast co-cultures produce soluble factors that enhance neutrophil chemotaxis

IL-8 is a pro-inflammatory cytokine that contributes to acute inflammatory responses by selectively enhancing neutrophil chemotaxis [21]. We examined the biological implications of our findings by measuring neutrophil chemotaxis of mouse neutrophils in Zigmond chambers [272]. We found that neutrophil chemotaxis was significantly increased in response to mast cell-
fibroblast co-culture media compared to controls. This effect was significantly inhibited when the co-cultures were pre-treated with BGA. These data suggest that mast cells, possibly through the formation of gap junctions with fibroblasts, increase the expression of molecules like IL-8, which can enhance neutrophil chemotaxis.

*Future directions*

Studies of lung fibroblasts have shown that CD40, a membrane glycoprotein, is a crucial factor in fibroblast activation [259]. Ligation of CD40 receptors promotes synthesis of inflammatory cytokines such as IL-1, IL-6, IL-8, PGs and hyaluronate (an ECM protein) [260], [261], [262]. Expression of CD40 expression has been reported in human gingival fibroblasts [260] and several cell types including mast cells express CD40 ligand (CD40L) [259]. Conceivably during co-culture, mast cells may activate gingival fibroblasts through CD40-CD40 ligand interactions, thereby enhancing IL-8 expression. Further experiments may be appropriate to examine the possible involvement of CD40 in fibroblast-mast cell-associated IL-8 release. Improved understanding of how a major mast cell mediator such as histamine may interact with fibroblasts could be helpful in understanding HMC/HGF pro-inflammatory interactions. Ultimately, after the actual mechanism for this phenomenon is identified, a clinical study on targeting pro-inflammatory interactions between mast cells and fibroblasts in patients with chronic inflammatory diseases such as refractory periodontitis would be important.
Figure 5

### A

**RayBio® Human Cytokine Antibody Array 5 Map**

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### B

![Graph showing IL-8 levels in HGF Control, HMC Control, and HGF/HMC Co-culture](image)

- **HGF Control**
- **HMC Control**
- **HGF/HMC Co-culture**

### C

![Graph showing IL-8 levels in different HGF/HMC ratios](image)

- **HGF + HMC RATIO = 2:1**
- **HGF + HMC RATIO = 1:1**
- **HGF + HMC RATIO = 1:2**
- **HGF + HMC RATIO = 1:4**

### D

![Graph showing IL-8 levels over hours of incubation](image)

- **0 hr**
- **1 hr**
- **2 hr**
- **4 hr**
- **6 hr**
- **8 hr**

### E

![Graph showing IL-8 levels in different conditions](image)

- **HGF in αMEM+FBS**
- **HGF & HMC in αMEM+FBS**

**Figure 5**
Fig. 5. (A) Human antibody cytokine array to assess inflammatory cytokines in HGF/HMC co-culture. After 8 hours of co-culture there were increased levels of several inflammatory cytokines including IL-8, GRO (IL-8-related chemokine), IL-6 and MCP-1 (CCL2) compared to control fibroblasts, which were cultured in the absence of mast cells. (B) IL-8 quantification of HGF/HMC co-culture in serum-free media for 8 hours. IL-8 was quantified by ELISA. * indicates $p<0.001$ different than fibroblasts and mast cells control groups. (C) Dose-response effect between relative numbers of mast cells to fibroblasts and levels of IL-8 in HGF/HMC co-culture. * indicates $p<0.001$, ** indicates $p<0.05$ and *** indicates $p<0.01$ when comparing co-cultures with different ratios of mast cells to fibroblasts. (D,E) The effect of co-incubation time on IL-8 levels in serum-free (D) and serum-containing (E) media. * in (D) indicates $p<0.01$ and * in (E) indicates $p<0.001$ different than IL-8 concentration in HGF control at same time point.
Figure 6

A

IL-8 pg/ml

0 hr 1 hr 2 hr 4 hr

Hours of incubation

B

IL-8 levels after 4 hours of co-incubation

IL-8 pg/ml

HGF  HGF+ BSA BEADS  HGF+ N CAD BEADS  HGF+ Collagen BEADS  HGF+ HGF  HGF+ HMC

*
**Fig. 6.** Role of adhesion molecules in co-culture-induced IL-8 release. (A) IL-8 concentration in HGF/HMC co-culture was compared to HGF control and HGF co-incubated with BSA-coated beads at 0, 1, 2 and 4 hours. * indicates $p<0.001$ different than HGF control and HGF + BSA coated beads. (B) IL-8 concentration in HGF/HMC co-culture was compared to those in HGF control, HGF co-incubated with BSA, N-cadherin or collagen coated beads and homotypic HGF/HGF co-culture at 4 hours. * indicates $p<0.001$ different than all other groups.
Figure 7

C: Role of Integrins in HMC/HGF attachment

D
Fig. 7. (A,B) Phase contrast microscopy: HGF (A) and HGF/HMC co-culture (B) after incubation for 24 hours, followed by jet wash with PBS. (C) Role of integrins in HGF/HMC attachment examined with 4B4 antibody to block β1 integrins. Cells were labelled with fluorescein dextran. Fluorescence microscopy was used to estimate the ratio of attached HMCs to HGF after rinse with PBS at 1, 3 and 6 hours of co-culture. * indicates $p<0.05$ and ** indicates $p<0.01$ different than the 4B4 untreated co-culture control. (D) Live imaging with laser-scanning confocal microscopy: HMC-1 (donor) were loaded with calcein/AM (green) and were plated on to established HGF monolayer (acceptor). Over 6 hours, there was gradual dye transfer from HMCs into HGFs.
Figure 8

A. Effect of BGA on dye transfer

B. IL-8 pg/ml

C. IL-8 pg/ml
Fig. 8. (A) Flow cytometry for analyzing effect of gap junction inhibitor (BGA) on dye transfer between HMCs and HGFs after 1 or 3 hours of co-incubation. * indicates $p<0.05$ different than untreated co-cultures at same time point. (B) Role of direct cell-cell contact and gap junctions between HMC-1 and HGF on IL-8 expression. For the first group, HGF/HMC co-culture was used as control. In the second group, HMCs were physically separated from HGFs by Cyclospore® membranes. For the third group, HGFs were incubated with HMC-1 sonicates. For the fourth group, co-cultures were established in the presence of gap junction inhibitor (BGA). * indicates $p<0.01$ and ** indicates $p<0.05$ different than HGF/HMC control group with regards to IL-8 concentration in conditioned media. (C) Effect of cold as an inhibitor of gap junction formation. IL-8 concentrations measured in regular cultures at 37°C compared to cultures at 4°C. * indicates $p<0.001$ different than all other groups.
Figure 9

A

B

C

IL-8 pg/ml

IL-8 pg/ml

IL-8 pg/ml
Fig. 9. (A) Effect of protein secretion block on IL-8 levels. Cells were treated with brefeldin A (BFA). IL-8 levels were measured after 8 hours of co-culture. * indicates $p<0.01$ different than BFA untreated cells. (B) Role of calcium in co-culture-induced IL-8 expression. Cells were treated with thapsigargin (TSGN) to release calcium from intracellular stores or with ionomycin to allow calcium entry from the extracellular space. * indicates $p<0.001$ different than untreated HGF/HMC co-cultures. (C) Role of direct HGF-HMC contact and protein secretion in calcium-mediated IL-8 expression in co-culture. In the first group (control) HGFs were treated with TSGN followed by co-incubation with HMC-1. In the second group, mast cells were separated from HGFs by Cyclopore® membranes. For the third group, HGFs and HMCs were treated with BFA. * indicates $p<0.01$ different than control group.
A

Neutrophil Chemotaxis Assay

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<tr>
<td>HMC</td>
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<tr>
<td>HMC+HGF</td>
<td>6.5 ± 0.7 *</td>
</tr>
<tr>
<td>fMLP</td>
<td>5.0 ± 0.6 **</td>
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* Untreated
** BGA treated

B

Proposed model for mast cell-fibroblast interactions that regulate the acute inflammatory response

** Figure 10 **
Fig. 10. (A) Effect of HGF/HMC co-culture medium on neutrophil chemotaxis. Migratory speed of neutrophils was significantly enhanced in response to HGF/HMC co-culture media compared to media derived from homotypic cell population controls. This effect was inhibited when co-cultures were incubated with BGA. * indicates $p<0.01$ different than HGF and HMC controls. ** indicates $p<0.001$ different than BGA untreated HGF/HMC co-culture group. (B) Proposed model for mast cell-fibroblast interactions in regulation of inflammation.
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