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# Assessment of the involvement of the macrophage migration inhibitory factor (MIF)-glucocorticoid regulatory dyad in MMP2 expression during periodontitis

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**ABSTRACT**

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine and counter-regulator of endogenous glucocorticoids. It is implicated in acute and chronic inflammatory diseases. This study investigated the role of the MIF-glucocorticoid (GC) regulatory dyad in MMP2 expression and release during periodontitis *in vivo* and *in vitro*. In a MIF knockout (KO) mouse model of ligature-induced periodontitis, gingival tissues and blood were collected and analyzed for levels of IL6, MIF, MMP2 and corticosterone. In addition, human gingival fibroblasts (HGF) were tested for production of IL6 and MMP2 after stimulation with hydrocortisone (HC), MIF, TNF-α or *Fusobacterium nucleatum*, a pathogen known to elicit immune responses during periodontitis. Wild type (WT) mice showed a local and systemic increase of MIF levels during inflammation, which was confirmed by increased local IL6 concentrations. Systemic GC were reduced in WT and MIF KO mice during inflammation with overall lower concentrations in MIF KO mice. *In vivo* and *in vitro*, MMP2 production was not dependent on MIF or inflammatory stimuli, but was inhibited by HC. Therefore, MIF does not appear to stimulate MMP2 expression in the gingival tissues, whereas GC upregulate MIF and downregulate MMP2. Our findings further suggest that MIF may regulate systemic GC levels.

**Keywords:** macrophage migration-inhibitory factors, glucocorticoids, periodontitis, inflammation, matrix metalloproteinase 2

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Macrophage migration inhibitory factor (MIF), a proinflammatory mediator, plays a critical role in chronic and acute inflammatory diseases. Importantly, MIF is known to be the main counterregulator of endogenous anti-inflammatory glucocorticoids (GC), which are produced by the adrenal glands. For this reason, MIF has been classified as both a hormone and a cytokine (1). T cells were first reported to be potent producers of MIF (2). In further studies, the pituitary gland was also found to be a source of systemic MIF in response to inflammation, such as sepsis (3). Later, other leukocytes and fibroblasts were found to produce MIF (4), which elicited a variety of proinflammatory responses in inflamed tissues by upregulating other proinflammatory cytokines like IL6 (5). In addition to this, MIF is also implicated in the progression of several systemic inflammatory diseases, including rheumatoid arthritis (RA), atherosclerosis and diabetes mellitus, by lowering systemic cortisol levels (6-10).

Periodontitis, triggered by pathogenic bacteria such as *Fusobacterium nucleatum* (*F. nucleatum*), is a chronic and destructive inflammatory disease of the tooth supporting tissues (11). In periodontitis, MIF was shown to promote osteoclastogenesis, thereby enhancing periodontal bone loss in an animal model (12). While MIF is known to be expressed in periodontital lesions, its impact on soft tissue degradation remains elusive. However, in other chronic inflammatory diseases like RA, MIF is known to upregulate matrix metalloproteinases (MMPs), which cause soft tissue breakdown (5). Several reports suggest that MMP2 promotes tissue destruction in periodontitis (13-16). More specifically, MMP2 was found to participate in the degradation of extracellular matrixes, such as type I and IV collagen, laminin-332 and versican, which play a pivotal role in connecting the junctional epithelium to the tooth surface (17-20).

To better understand the role of MIF in regulating MMP2-mediated tissue degradation, a mouse model of ligature-induced periodontitis as well as *in vitro* approaches using stimulated gingival fibroblasts (HGFs) were employed. As MIF and endogenous GC constitute a functional dyad, the contribution of GC to MMP2 release was also investigated.

## MATERIALS AND METHODS

### Animals

Six male C57BL/6J wild type (WT) mice and six male C57BL/6J MIF knockout (KO) mice were used in this study. WT animals were obtained from The Jackson Laboratories (Bar Harbor, ME, USA) (stock number 000664). *Mif* KO mice were produced as described previously (21) and generously provided by Dr. Abhay Satoskar (Ohio State University, Columbus, OH, USA) as homozygotes, backcrossed to a C57BL/6J background. The number of animals used was in accordance with, or exceeding, the number of animals employed in previously published studies using *Mif* KO mice in the context of periodontitis or MMP2 research (12, 22). All mice were kept as previously described (23) and were six weeks old when the experiments were conducted. All procedures involving mouse experiments were approved by the Institutional Animal Care and Use Committee at The Forsyth Institute, Cambridge, MA, USA.

### Model of ligature-induced periodontitis

For all animals, a 5-0 braided silk ligature (PERMA-HAND, Ethicon, Somerville, NJ, USA) was placed around the upper left secondary molar. Ligatures are thought to facilitate local accumulation of bacteria and thereby enhance bacteria-mediated inflammation and bone loss (24). Ligature placement was performed under general anesthesia. Ketamine-xylazine in sterile saline was injected once intraperitoneally at a dose of 100 mg/kg ketamine and 10

mg/kg xylazine. The ligature placement procedure took 5 to min per animal using a surgical microscope and metal clamps to keep the mouths open. While the right maxillary side was left untreated, ligatures where kept in place for 9 d. Eating and drinking behavior was monitored and did not change in the mice following ligature placement.

**Sample collection**

Blood was drawn at 7 pm on 0 d and 9 d in order to avoid the influence of circadian fluctuations of GC levels. Whole blood (0.5 ml) was collected from the retro-orbital area, allowed to clot for 20 min to obtain serum, and centrifuged at 2,000 x g for 10 min at 4°C. Supernatants were either assessed immediately or stored at -20°C until completion of the study. After 9 d of ligature placement, mice where euthanized with carbon dioxide gas by trained personnel. Subsequently, surrounding gingivae of the upper secondary molars of both sides were collected and immediately frozen in liquid nitrogen. Total ribonucleic acid (RNA) was extracted from gingival tissues using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) for polymerase chain reaction (PCR) or tissue samples were stored at -80°C for one week.

**Cell culture**

Primary human gingival fibroblasts (HGFs) were harvested from five periodontally healthy donors as described in DAMANKI *et al.* (25), after obtaining written informed consent and approval of the Ethics Committee of the University of Bonn (#043/11). HGFs were cultured separately in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% $CO_2$ atmosphere. All cell culture assays were performed five times as biological replicates for each experimental condition. For experimental procedures, the medium was changed to 1% FCS and cells were seeded into separate 24-well

tissue culture plates at a density of $5x10^5$ and allowed to adhere overnight. Next, MIF (20 ng/ml), hydrocortisone (HC, 100 µg/ml) (Sigma Aldrich, Schnelldorf, Germany) or TNF-α (20 ng/ml) were added to the experimental wells. TNF-α is a potent and ubiquitous stimulator of inflammation and therefore served as a positive control (26). As a negative control, cells incubated with PBS were used. The concentrations used were based on those commonly described in the literature and verified by prior assays determining concentration dependent release of MIF and MMP2 (27-29). To mimick bacterial infection, HGFs were inoculated with *F. nucleatum* (ATCC 10953) at a MOI of 1:10. *F. nucleatum* is a pathogenic anaerobe microorganism that is found in human periodontal pockets. It is associated with periodontitis and elicits a strong immune response in host cells (11). Human T cells (Jurkat) were used as a positive control, as they are known to release high levels of MIF (2). Cells were incubated for 12 h at 37°C in a 5% $CO_2$ atmosphere. Subsequently, supernatants were collected and stored at -20°C until further use, whereas adherent cells were lysed, and total RNA was obtained using TRIzol reagent (Life Technologies, Darmstadt, Germany). All cell culture experiments were performed at the University Hospital Bonn.

**Polymerase chain reaction**

Total RNA from mouse gingival tissues and HGFs was converted into complementary DNA (cDNA) (iScript cDNA synthesis kit, Bio-Rad, Hercules, CA, USA) using 1 µg of total RNA by addition of reverse transcriptase (0.5 µl) in a total of 20 µl solution. cDNA was analysed by quantitative PCR (qPCR) and reverse transcriptase PCR (RT-PCR), respectively. The custom primer sets used for murine tissues were company-designed and tested for *Actb* (β-actin), *Il6, Mif* (Qiagen QuantiTect, Valencia, CA, USA) and *Mmp2* (Real Time Primers, Elkins Park, PA, USA) mouse genes. qPCR was performed using a LightCycler 480 system with LightCycler 480 SYBR I Master mix (Roche Applied Science, Branford, CT, USA) for 50 cycles. The PCR cycling profile consisted of an initial denaturation step at 95°C for 5 min,

followed by 50 cycles of 1) denaturation at 95°C for 15s and 2) annealing/extension 58-60°C for 60s. Melting curve analysis was also performed using a single cycle of 95°C for 20s, 60°C for 20s, 40°C for 1s and 95°C prior to the final cooling. The primers applied to HGF cDNA for reverse transcriptase PCR (RT-PCR) were designed using the NCBI Primer-BLAST tool and purchased from Thermo Fisher (Darmstadt, Germany). Primer sequences for the *GADPH* reference gene (NCBI accession number: NM_002046) were forward (5' → 3') TCCCTGAGCTGAACGGGAAG and reverse (5' → 3') GGAGGAGTGGGTGTCGCTGT (annealing temperature 64°C) for a product length of 218 bp. The sequences for human *MMP2* (NCBI accession number: NM_001127891) were forward (5' → 3') ACTCCTGGCTCATGCCTTC and reverse (5' → 3') GCGTTCCCATACTTCACACG (annealing temperature 59°C) for a product length of 293 bp. Thirty-five cycles were run for each PCR, and after gel elctrophoresis, bands were visualized using ethidium bromide.

**Enzyme-linked immunosorbent assay (ELISA)**

Serum samples were assessed for corticosterone (murine cortisol) by competitive ELISA (Oxford Biomedical Research, Rochester Hills, MI, USA). MIF levels in serum samples and tissue lysates were measured by mouse MIF, DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). HGF supernatants were analyzed by ELISA for MMP2 and IL6 (Boster Bio, Pleasanton, CA, USA) and MIF (Cloud-Clone, Houston, TX, USA) levels. All ELISA procedures were carried out according to the protocols provided by the manufacturers.

**Gelatin zymography**

To assess whether MMP2 was secreted from HGFs as an active enzyme or as a proenzyme, gelatin zymography was conducted. Cell culture supernatatants were collected, and the total protein concentration was enhanced by filtering samples using Vivaspin 4 concentrators with a pore size of 10,000 MWCO (Sartorius Stedim Biotech, Gloucestershire, UK). Total protein

was measured by BCA assay (Life Technologies, Darmstadt, Germany). Sodium dodecyl sulfate gels containing 20 mg/ml gelatin (Merck Millipore, Darmstadt, Germany) were prepared, and polyacrylamide gel electrophoresis was performed under non-reducing conditions, adding 20 µg/ml of sample into each well. Subsequently, gels were stained with 0.05% Coomassie Brilliant Blue G250 (Merck Millipore, Darmstadt, Germany) for 2h and destained in deionized water containing 8% acetic acid and 4% methanol for 2h. Destained bands were recorded using a Gel Doc XR+ system (Bio Rad, Hercules, CA, USA).

**Image processing**

Images were acquired using Gene Tools image analysis software (Syngene, Frederick, MD, USA). Image J Fiji (National Insititutes of Health, Bethesda, MD, USA) was used for further image analysis and data quantification after performing PCR and gelatin zymography.

**Statistical analysis**

A post-hoc power analysis was conducted to determine the power (53%, $P$=0.05) of the MMP2-related outcomes in WT and KO animals for the observed effect size of d=1.3, using G*Power software (Version 1.3) (30). Relative gene expression was determined by applying the $\Delta\Delta C_T$ calculation method, using β-actin as a control. Unpaired unequal variance *t*-tests were employed to calculate statistically significant differences between two groups. Paired unequal variance *t*-tests were applied for comparison of the means of two related groups on the same dependent variable. Quantification of PCR and zymography bands was statistically evaluated using One-way ANOVA and post hoc Tukey's test. $P$ values of $\leq 0.05$ were considered statistically significant. All data are shown as mean values ± standard deviations (SD).

**RESULTS**

**Confirmation of MIF deficiency in MIF KO mice**

To confirm the MIF KO phenotype, DNA genotyping was performed. Moreover, gingival tissues were subjected to RT-PCR and analyzed for the presence of *Mif* mRNA, and serum was analyzed by ELISA for the presence of MIF protein. Genotyping, RT-PCR and ELISA showed no evidence of MIF in the KO animals (data not shown).

**MIF is not involved in the upregulation of local IL6 in periodontitis**

To confirm inflammation, murine gingival tissues and *in vitro* cell cultures were assessed for IL6 gene expression and quantities of IL6, respectively, as this cytokine is known to be significantly enhanced in periodontal lesions (31, 32). Nine days after ligature placement, local tissue mRNA levels of *Il6* were markedly higher in inflamed tissues (0.006 ± 0.002 in WT animals and 0.005 ± 0.002 in KO animals) compared to those of the control non-ligatured sites in both animal groups (0.003 ± 0.001 in WT animals and 0.0005 ± 0.0002 in KO animals) (Fig. 1A,B), confirming the presence of inflammation. A more pronounced fold change was seen in *Mif* KO animals (10-fold) compared to WT mice (2-fold), indicating that IL6 was induced by other endogenous stimuli than MIF under inflammatory conditions. To further investigate the *in vivo* findings on the cellular level, an *in vitro* model of infected human gingival fibroblasts, the most abundant cell type in the gingival tissues, was employed. Increases in IL6 could also be observed in HGFs after stimulation with *F. nucleatum* (618.7 ± 220.2 pg/ml), compared with the negative control (115.7 ± 45.1 pg/ml) (Fig. 1C).

**MIF levels increased, corticosterone levels decreased during inflammation**

*Mif* mRNA expression in WT mice was increased two-fold in inflamed sites compared to healthy sites ($P$=0.03). MIF protein levels in serum and gingival tissues were significantly higher during inflammation (Table 1). Notably, we observed an overall higher expression of

the *Mif* gene in relation to *Actb* compared to *Il6*, indicating that *Mif* may be produced in larger quantities in response to local inflammation. The level of MIF protein released from HGF cells was inducible by HC (9.5 ± 0.26 ng/ml *vs.* 4.9 ± 0.66 ng/ml in unstimulated HGFs), but not by TNF-α or *F. nucleatum* (Fig. **2**), and was overall higher (nanogram range) than that of IL6 (picogram range), consolidating the observation that MIF may be produced in larger quantities than IL6. Corticosterone levels decreased during inflammation (136.1 ± 50.1 ng/ml on 9d *vs.* 186.5 ± 32.5 ng/ml on 0d in WT animals) and were overall lower in *Mif* KO animals (42.9 ± 27.7 ng/ml on day 9 *vs.* 91.7 ± 21.2 ng/m. on day 0) (Fig. **3**), suggesting that corticosterone release may be MIF-dependent.

**MMP2 expression in gingival tissues is independent of MIF**

As shown in Fig. 4A, *Mmp2* gene levels in both WT and *Mif* KO mice did not change significantly during periodontal inflammation (0.04 ± 0.03 in ligatured sites *vs.* 0.03 ± 0.02 in non-ligatured sites of WT mice and 0.04 ± 0.01 in ligatured sites *vs.* 0.05 ± 0.02 in non-ligatured sites of *Mif* KO mice). Also in cell culture experiments, MMP2 release by HGFs was not influenced by exogenous MIF (Fig. 4B), however, it was significantly suppressed by HC (0 ± 0.29 ng/ml *vs.* 8.3 ± 0.58 ng/ml in unstimulated HGFs). *MMP2* expression in HGFs in response to different stimuli was confirmed by RT-PCR (Fig. 5A,B), where the sample containing HC showed a lower intensity of the band, indicating its suppression of the *MMP2* gene (0.64 ± 0.03 AU *vs.* 1 ± 0.05 AU in unstimulated HGFs). Gelatin zymography confirmed that most MMP2 released was active (2-fold higher than pro-MMP2 in unstimulated controls) (Fig. 6A,B). Also, a more pronounced release of active MMP2 from cells stimulated with F. *nucleatum* could be observed compared to unstimulated HGFs (75.4 ± 5.7 AU *vs.* 61.6 ± 2.95 AU), whereas less activation was seen in the sample containg HC (44.5 ± 1.66 AU), supporting the findings from MMP2 ELISA.

## DISCUSSION

MIF is a key proinflammatory mediator released during local and systemic inflammation (1). Our experiments were aimed at investigating whether MIF has an influence on the expression and release of MMP2 in gingival tissues during inflammation. A *Mif* KO mouse model as well as cell culture experiments were employed using stimulated HGFs, which are the most abundant structural cells in gingival tissues (33). This study revealed a higher expression of MIF in inflamed periodontal tissue and in HGFs when compared to IL6, as well as increased systemically circulating MIF levels. However, we could not verify a correlation between MIF and MMP2 in periodontal inflammation. PAKOZDI and colleagues demonstrated a significant increase of MMP2 expression in synovial fibroblasts in response to MIF during experimental RA in mice previously (22). RA and periodontitis are thought to be co-related chronic inflammatory diseases, both developing in a progressive manner if left untreated (34). Interestingly, MMP2 may have a protective function in RA, which was shown by two independent studies (35, 36), suggesting that MMP2 could counterbalance MIF to prevent excessive immune response in RA.

Although the underlying mechanism for the protective role of MMP2 in RA has not been established, it is known that MMP2 can cleave the proinflammatory cytokine IL-1β and monocyte attractant protein 3 (37), thus exhibiting an anti-inflammatory effect. MMP2 in periodontitis, however, is known to be destructive to humans and rodents (15, 16). Although MIF was upregulated during periodontitis locally and systemically in our study, we found that it was not an influence factor for MMP2 expression *in vivo* and *in vitro*, suggesting that the effects of MIF on local MMP2 production are different in RA and periodontitis. Notably, HGFs released MMP2 without any external stimulus, but also upon stimulation, we observed

no change in the release of MMP2. This confirms the findings of other studies which reported that MMP2 is constitutively expressed by fibroblasts and other cell types, but is not upregulated during gingival inflammation (38, 39). A post-hoc power analysis was conducted in order to determine the power to detect differences between the MMP2 levels in the two animal groups and between healthy and inflamed tissues. Power was calculated with 53% for the observed effect size (d=1.3). Thus, we cannot completely rule out that a small or medium-sized effect of MIF deficiency on MMP2 levels remained undetected in our study. In order to identify medium-sized MIF-dependent alterations (d=0.7) of MMP2 production *in vivo* at a power of 80%, larger animal cohorts (n=34 per group) need to be included in future studies.

IL6, used as a local marker for inflammation in our study, showed a significantly higher fold change during inflammation in MIF KO mice compared to WT animals. It is possible that MIF deficiency leads to a compensatory upregulation of IL6 and other proinflammatory mediators during inflammation, as has been described previously (40, 41). Furthermore, HGFs were stimulated with F. *nucleatum*, a pathogenic anaerobe microorganism that is found in human periodontal pockets and elicits a strong immune response in host cells. We found that *F. nucleatum* could directly upregulate proinflammatory IL6 in HGFs, presumably via toll-like receptors 2 or 4 (38), both of which have been previously detected on the cell surface of HGFs (42). IL6 upregulation was observed in both animal model and cell culture experiments, indicating that oral bacteria accumulating in the area of ligature placement may contribute to IL6 release from murine HGFs via the TLR signalling cascade.

MIF is a key modulator of endogenous glucocorticoids (GC). According to CALANDRA & BUCALA, MIF is the first mediator to be identified that can directly counter-regulate the immune inhibitory effects of glucocorticoids and thus plays a critical role in the host control of inflammation and immunity (43). More specifically, MIF counteracts the anti-

inflammatory effects of GC on the cellular level, whereas GC can induce MIF release from the pituitary gland and from macrophages (44, 45). This process is concentration-dependent and is thought to control excessive or insufficient immune responses (46). In our experiments, a coherence between MIF and GC was detectable also: interestingly, in MIF KO animals significantly less systemic corticosterone was observed, and in both groups the levels decreased during inflammation. Although it is well known that stress, which may occur under experimental conditions, increases corticosterone levels in mice, it is also established that C57BL/6 animals are relatively stress-resistant (47, 48). Furthermore, all mice were handled and kept in an identical manner, yet these differences in systemic cortisol levels were highly significant. Moreover, the fact that corticosterone decreased over time indicates that stress was not a contributing factor. Taken together, these findings support that systemic cortisol induces MIF release, as described in numerous previous studies (1, 49), and that MIF may reversely be involved in glucocorticoid induction. An enhancement of corticosterone by MIF has, to our knowledge, not been reported in the literature, therefore this theory requires further investigation to confirm this putative function.

In our experiments, we stimulated HGFs with hydrocortisol (HC), an exogenous glucocorticoid, in order to assess its effects on MMP2 release. The results from MMP2 ELISA, RT-PCR and zymography showed a reduction of MMP2 on the protein and gene levels. These findings are supported by those of a previous study and corroborate the anti-inflammatory role of glucocorticoids, suggesting that HC may help to decrease local tissue destruction mediated by MMP2 (50). Compared to other GC, HC has properties very similar to those of endogenous cortisone. For this reason, HC was chosen for our cell culture experiments. Other GC such as Dexamethasone also feature immunosuppressive properties and were reported to decrease MMP2 release from host cells (51, 52). Several GC were

shown to affect MIF production, however, data are discordant regarding their ability to up- or downregulate MIF (45, 53-56).

It is important to note that ligature-induced periodontitis in mice does not perfectly correlate with the onset and long-term development of the disease in humans. However, our *in vivo* mouse model combined with an *in vitro* model of stimulated HGFs may help to provide a better understanding of the involvement of MIF in periodontal inflammation. Importantly, this study investigated the expression of MMP2, not other MMPs or tissue inhibitors of matrix metalloproteinases (TIMPs). These could be addressed in future studies in order to gain further insight into the role of MIF in tissue degradation during periodontitis. MMP2 is secreted into the extracellular environment, however, pro-MMP2 can be released or remain bound to membrane-type 1 MMP and membrane-type tissue inhibitor of metalloproteinases 2 (TIMP-2) (35, 57). Therefore, our zymography assays were confined to the measurement of released pro-MMP2 only. For a more accurate determination of the ratio between MMP2 and pro-MMP2, cell culture supernatants as well as cell membrane preparations should be used in future detection assays.

As a further limitation of this study, HGFs were infected with *F. nucleatum* only, whereas periodontal inflammation is caused by an array of microbial species living in a biofilm community. However, *F. nucleatum* is a dominant microorganism within human oral biofilms and found at increased levels in periodontal disease (11). Moreover, *F. nucleatum* was reported to induce more significant changes in cultured cells than other microbial inducers of periodontitis (58, 59) and is known to play a pivotal role in biofilm formation (60). Mice were not infected with *F. nucleatum*, therefore, the underlying pathobiological mechanisms of periodontitis induction may have been different. As *F. nucleatum* is naturally found in the murine oral cavity (61) and experimental periodontitis is thought to be induced by the local

accumulation of bacteria around the ligature (24), *F. nucleatum* may have been present in the periodontitis lesions of our mouse model.

Supernatants of *F. nucleatum*-stimulated HGFs showed an enhanced gelatinolytic activity in the zymography assays. The increased gelatin digestion by supernatants obtained from HGFs stimulated with *F. nucleatum* corresponded to the increased levels of released MMP2 in this study, and the proteolytic band coincided with the known MMP2 protein size. Although the potential of *F. nucleatum* proteases to degrade gelatin has been demonstrated (62), our own experiments using *F. nucleatum* only for gelatin zymography did not show detectable gelatinase activity. However, this may have been due to the low concentrations of bacteria ($5x10^6$ per well) used in this assay. Notably, *F. nucleatum was* reported to convert pro-MMP-9 into the proteolytically active form (63), thus, it is possible that *F. nucleatum* may have similar effects on pro-MMP2. Little is known about proteases expressed by *F. nucleatum* and their ability to degrade host tissues, therefore, future studies may be aimed at investigating these as a potential pathomechanism in periodontal tissue breakdown.

In conclusion, the results of this study indicate that MIF may be involved in the upregulation of systemic GC in mice, as knockout animals had overall lower levels of corticosterone. Furthermore, we demonstrated an inhibition of MMP2 production by GC. However, our findings do not support a modification of local MMP2 concentrations by MIF. Moreover, MMP2 production appears to be differentially regulated by MIF in RA and periodontitis.

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**Conflicts of Interest statement**

The authors declare that there are no conflicts of interest regarding the publication of this study.

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## FIGURE LEGENDS

*Fig. 1.* Levels of proinflammatory IL6 *in vivo* and *in vitro*. A and B: Local tissue *Il6* mRNA levels in healthy and inflamed gingivae nine days after ligature placement are shown as relative expressions in relation to β-actin and as fold change (compared to healthy sites). **P*=0.05, ***P*=0.006 for healthy *vs*. inflamed tissues in WT and MIF KO mice and ***P*=0.01 for inflamed sites in WT *vs*. MIF KO animals. Grey bars: WT mice (n=6), white bars: MIF KO mice (n=6). Results are shown as mean values ±SD, each sample was run in duplicate. C: Levels of IL6 released from HGFs after stimulation with HC, MIF or *F. nucleatum* (**P*≤0.05 compared to negative control). As a positive control, TNF-α, which is known to induce IL6 release (64) was employed. Results are shown as mean values ±SD and represent five different donors; each sample was run in triplicate.

*Fig. 2.* MIF protein release from HGFs. MIF release was inducible by hydrocortisol (HC), but not by TNF-α or F. *nucleatum* (**P*≤0.05 compared to negative controls). Jurkat T cells, which are known to release high levels of MIF (65), were used as a positive control. Results are shown as mean values ±SD and represent five different donors; each sample was run in triplicate.

*Fig. 3.* Local and systemic corticosterone levels during ligature-induced periodontitis. Corticosterone levels at baseline and after induction of inflammation in MIF KO and WT animals. Asterisks represent intergroup (WT *vs.* MIF KO animals) or intragroup (0d *vs.* 9d) differences in corticosterone levels (*P**=0.05 ***P*≤0.004, ****P*=0.0003). Blood was withdrawn from all animals at 7pm on both days from the retro-orbital area; each sample was run in triplicate ELISA wells.

*Fig. 4.* MMP2 levels in murine gingival tissues and HGF supernatants. A*: Mmp2* mRNA levels in WT and MIF KO mice in healthy and inflamed gingival sites shown as relative expressions in relation to β-actin and as fold change (compared to healthy sites). B: MMP2 release by HGFs from five different donors in cell culture experiments in response to hydrocortisol (HC), MIF and *F. nucleatum* (***$P$=0.0003 compared to negative controls), each sample was run in triplicate wells.

*Fig.5. MMP2* gene expression in HGFs. Confirmation of *MMP2* gene expression (RT-PCR) by HGFs in response to the stimuli shown and in comparison to *GAPDH* reference gene expression (A). Bands were quantified using ImageJ software (***$P$=0.0004 compared to the negative control) (B), gene expression was assessed in three independent experiments.

*Fig. 6.* MMP2 activity. MMP2 activity was assessed by gelatin zymography, white bands indicate gelatinolytic activity (A). Quantification was performed using ImageJ software (B). Asterisks represent differences between pro-MMP2 and active MMP2 or differences between stimulated active MMP2 release and the negative control (**$P\leq0.005$ and ***$P\leq0.0009$). Gelatinolytic activity was assessed in three independent experiments. All bar graphs show results as mean values ±SD.

*Fig. 7.* Summary of the putative roles of MIF, GC and MMP2 in periodontal inflammation. It is well established that MIF enhances inflammation (A), while glucocorticoids (GC) dampen the immune response (B). However, GC may evoke MIF release (C) (1 and 45), which then counteracts these antiinflammatory effects of GC (D). The findings of the present study support a proinflammatory role of MIF in periodontal inflammation. However, a direct relationship between MIF and MMP2, which is thought to have a destructive role in periodontitis (E), could not be detected (F). Instead, MIF seemed to increase systemic GC

levels in mice (G) and HC, an exogenous GC, was able to diminish MMP2 release (H). We hypothesize that systemic MIF and GC can promote the release of one another, but that MIF may not play a relevant role in MMP2 induction in periodontal inflammation.

Table 1

*Local and systemic presence of macrophage migration inhibitory factor (MIF) in vivo* and *in vitro*

| | Healthy | SD | Inflammed | SD | P value |
|---|---|---|---|---|---|
| Relative Mif gene expression (ratio to ß-actin) | 1.47 | 0.81 | 3.03 | 0.73 | 0.03 |
| Mif gene expression as fold changes (ratio to ß-actin) compared to healthy sites | 1.00 | 0.55 | 2.10 | 0.49 | 0.03 |
| MIF tissue levels (ng/mg) | 0.47 | 0.24 | 1.99 | 1.00 | 0.02 |
| MIF serum levels (ng/mL) | 8.8 (day 0) | 3.17 | 12.70 (day 9) | 2.78 | 0.04 |

Tissues of five wildtype animals per condition were used, and ELISA was run in duplicate wells. Systemic elevations of MIF in serum were observed after 9 d of ligature placement. All data are presented as means and standard deviations (SD).

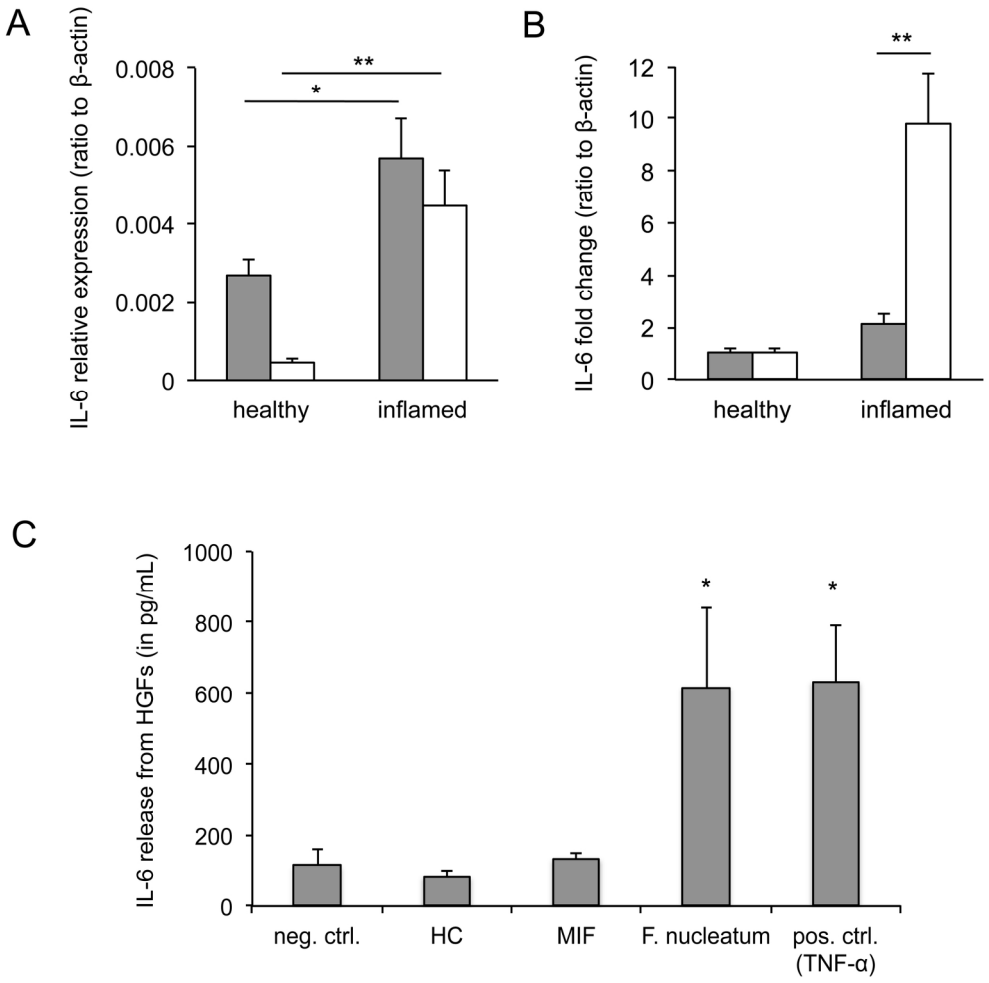


**Figure 1. Levels of proinflammatory IL-6 in vivo and in vitro.** A and B: Local tissue IL-6 mRNA levels in healthy and inflamed gingivae nine days after ligature placement are shown as relative expressions in relation to β-actin and as fold change (compared to healthy sites). *p=0.05, **p=0.006 for healthy vs. inflamed tissues in WT and MIF KO mice and **p=0.01 for inflamed sites in WT vs. MIF KO animals. Grey bars: WT mice (n=6), white bars: MIF KO mice (n=6). Results are shown as mean values ±SD, each sample was run in duplicate. C: Levels of IL-6 released from HGFs after stimulation with HC, MIF or F. nucleatum (*p≤0.05 compared to negative control). As a positive control, TNF-α, which is known to induce IL-6 release (64) was employed. Results are shown as mean values ±SD and represent five different donors; each sample was run in triplicate.

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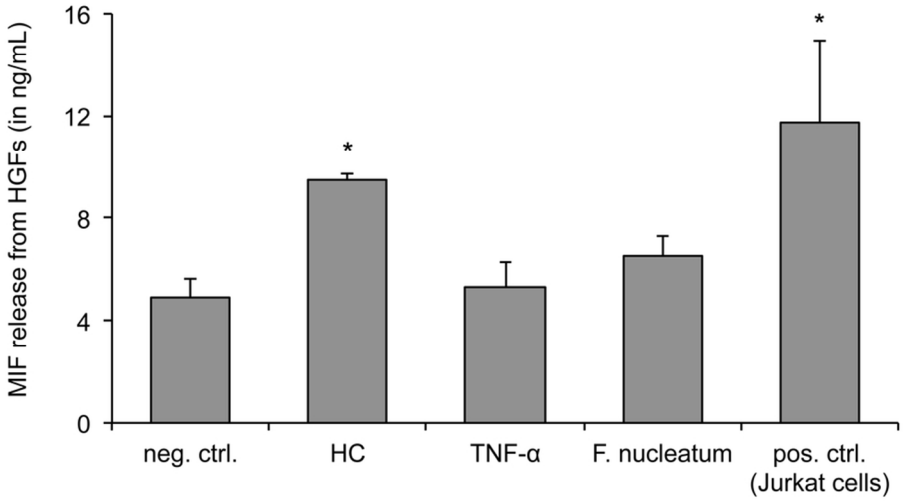


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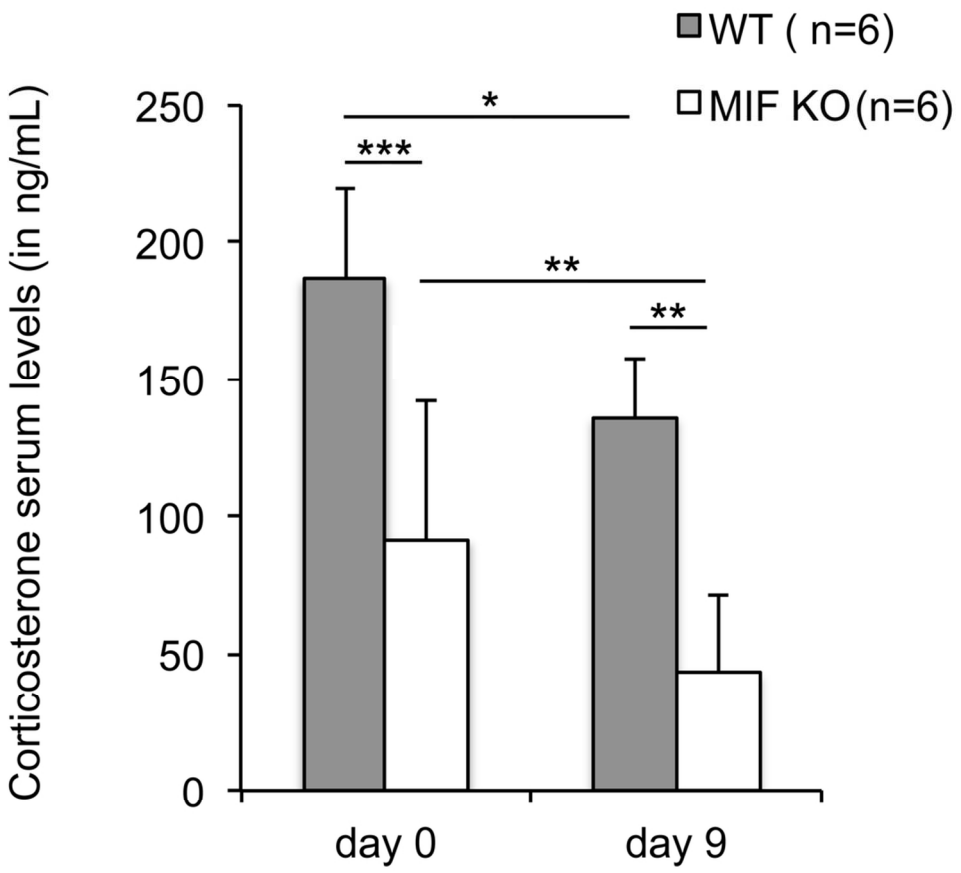


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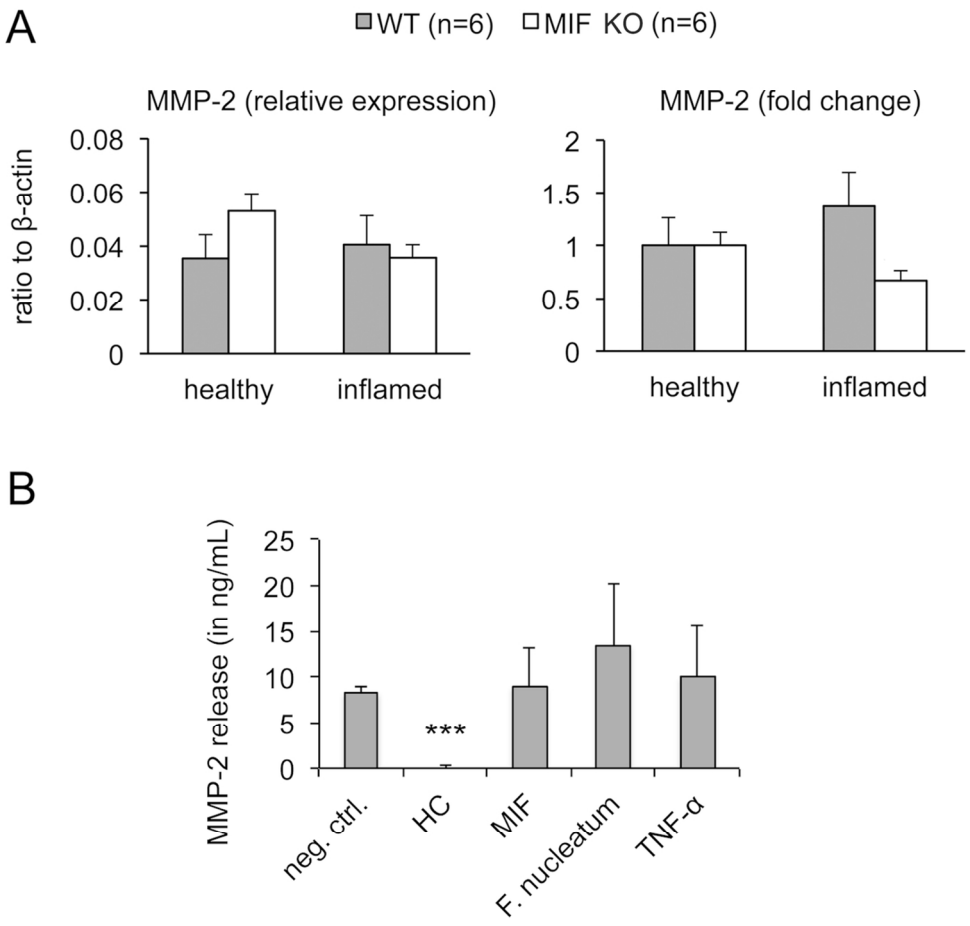


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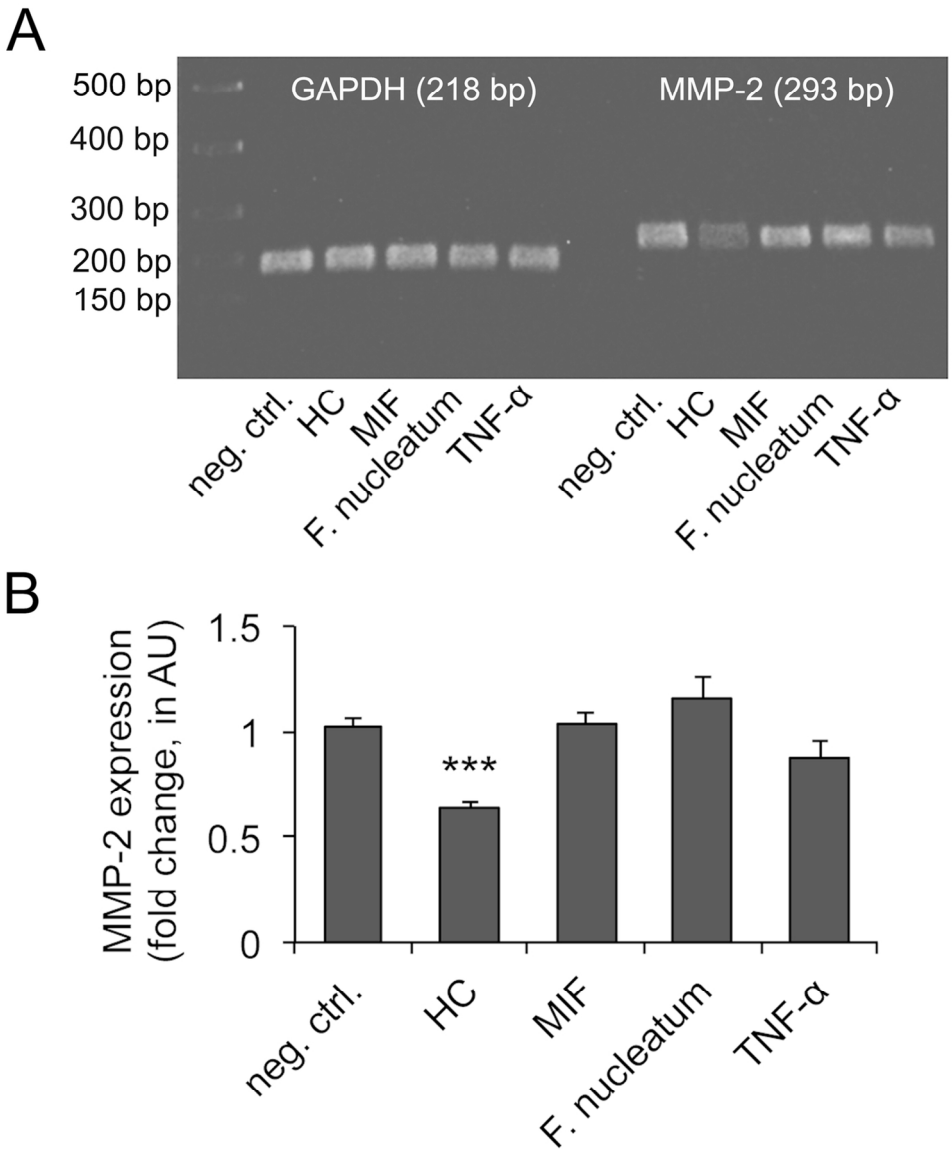
119x113mm (300 x 300 DPI)

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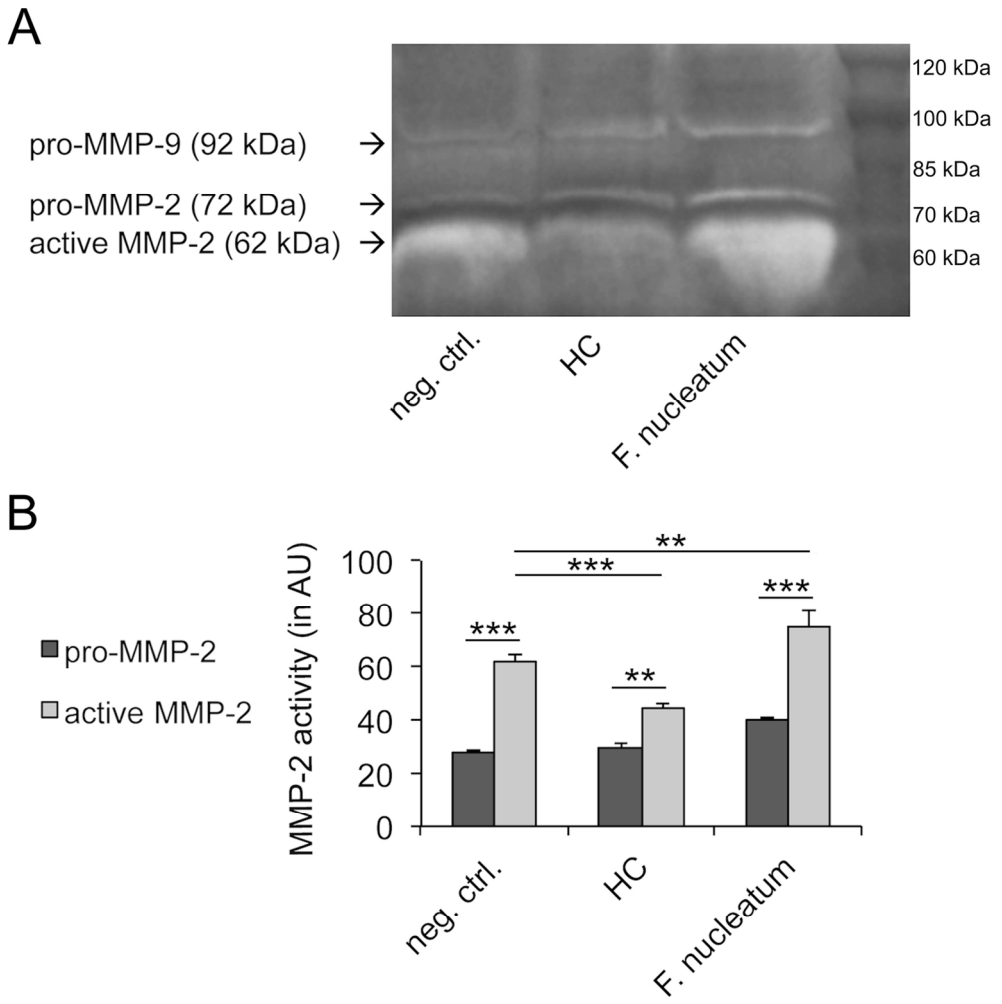


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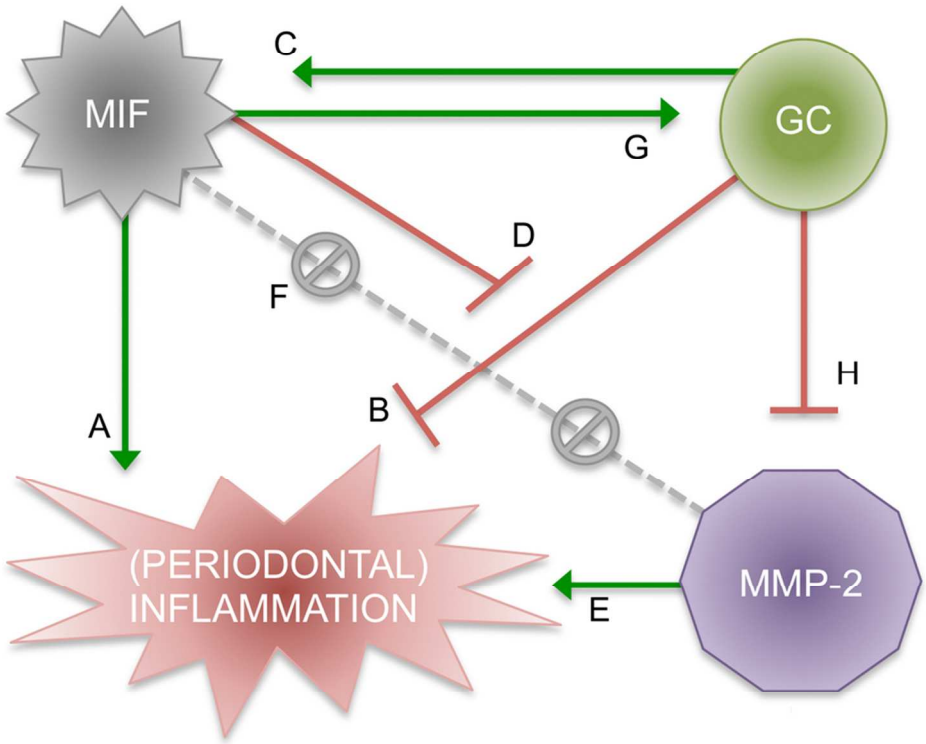


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90x76mm (300 x 300 DPI)