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Loss of neutrophil homing to the periodontal tissues modulates the composition and disease potential of the oral microbiota.

A Hashim[1], A Alsam[2], MA Payne[2], J Aduse-Opoku[3], MA Curtis[3], S Joseph[3#]

[1]Department of Biomedical Sciences, College of Dentistry, King Faisal University, 31982 Al-Ahsa, Saudi Arabia

[2]Institute of Dentistry, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London E1 2AD

[3]Centre for Host-Microbiome Interactions, Faculty of Dentistry, Oral & Craniofacial Sciences, King's College London, Tower Wing, Guy's Hospital, London SE1 9RT, UK

[#]Address correspondence to Susan Joseph (susan.m.joseph@kcl.ac.uk)

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ABSTRACT:

Periodontal disease is considered to arise from an imbalance in the interplay between the host and its commensal microbiota, characterized by inflammation, destructive periodontal bone loss and a dysbiotic oral microbial community. The neutrophil is a key component of defence of the periodontium: defects in their number or efficacy of function predisposes individuals to development of periodontal disease. Paradoxically, neutrophil activity, as part of a deregulated inflammatory response, is considered to be an important element in the destructive disease process. In this investigation we examined the role the neutrophil plays in the regulation of the oral microbiota, by analysis of the microbiome composition in mice lacking the CXCR2 neutrophil receptor required for recruitment to the periodontal tissues. A breeding protocol was employed which ensured that only the oral microbiota of wild type ($CXCR2^{+/+}$) mice was transferred to subsequent generations of wild type, heterozygote and homozygote littermates. In the absence of neutrophils, the microbiome undergoes a significant shift in total load and composition compared to when normal levels of neutrophil recruitment into the gingival tissues occur, and this is accompanied by a significant increase in periodontal bone pathology. However, transfer of the oral microbiome of $CXCR2^{-/-}$ mice into germ free $CXCR2^{+/+}$ mice led to restoration of the microbiome to the wild type $CXCR2^{+/+}$ composition and the absence of pathology. These data demonstrate that the composition of the oral microbiome is inherently flexible and is governed to a significant extent by the genetics and resultant phenotype of the host organism.

40 **INTRODUCTION**

41 As is the case in many chronic inflammatory conditions, periodontal disease is considered to arise
42 from an imbalance in the interplay between the host and its commensal microbiota (1,2). In this
43 instance, the disease is characterised by a deregulated and injurious inflammatory response in the
44 periodontal tissues with consequential irreversible destruction of bone and major changes to the
45 total load and community structure of the subgingival microbiota, frequently referred to as dysbiosis
46 (3,4). Unsurprisingly, risk factors for periodontal disease include defects, or reductions in efficacy, in
47 the repertoire of antimicrobial host defence measures required for protection of the periodontal
48 tissues (5,6).

49 The role that the dysbiotic microbiota plays in the disease process is controversial. On the one hand,
50 it is evident that the changes in the hierarchy of the predominant organisms in the subgingival
51 domain are driven by the altered environmental conditions including the intensity of the
52 inflammatory response and the availability of nutrients for bacterial growth. These pressures exert
53 selective effects on the microbial community organisation resulting in enhanced proliferation of
54 inflammophilic organisms and reductions in those bacteria routinely associated with low levels of
55 inflammation (7). Furthermore, in some circumstances, alleviation of the stressed environmental
56 conditions through interventionist approaches designed to reduce the inflammatory response in
57 periodontal patients has been shown to lead to a reversal of dysbiosis and restoration of the
58 community structure associated with health (8,9).

59 Contrary to this inflammo-centric view of a bystander role for the microbiota in periodontal disease,
60 it is clear that organisms which predominate in subgingival dysbiosis have properties consistent with
61 a more direct influence in driving the destructive disease process. The secretion of a broad range of
62 hydrolytic enzymes and cytotoxic agents and employment of mechanisms which both subvert and
63 deregulate the inflammatory and immune response all provide some justification for considering
64 these disease-associated organisms as causative in the pathology of this condition (10).

65 Understanding the contribution of the microbial challenge to the development and progression of
66 periodontal disease has implications particularly in relation to the principles of treatment of this
67 condition: targeted to the control of the dysbiotic bacterial community or the inflammatory
68 response.

69 In studies using the murine model of periodontal bone loss, we previously demonstrated that
70 introduction of *Porphyromonas gingivalis* strain W50, a bacterium frequently associated with human
71 disease, into the oral cavity led to the development of dysbiosis of the murine commensal oral
72 microbiota and concomitant periodontal bone loss (1, 11). Critically, in the context of dysbiosis as a
73 cause or consequence of the disease, we showed that once the commensal microbiota was
74 transformed into a dysbiotic mode with elevated load and altered community organisation, the
75 structure of the community was stable to transfer into healthy animals and recapitulated the disease
76 experience of the donor mice (11). These data suggested that in this particular instance of
77 experimentally induced dysbiosis, there is an inherent resilience to perturbation of the altered
78 community: a reversal in the environmental conditions to those found in health, in this instance
79 vertical or horizontal transfer into non-diseased mice, did not lead to restoration of the health
80 associated microbiota nor abrogation of the pathological response. In addition, we have also
81 demonstrated in these studies and others, the inherently low diversity of the laboratory mouse oral
82 microflora which therefore makes laboratory culturing a convenient and effective way to represent
83 the microbial population in these mice (1, 11, 12).

84 In the present work we aimed to extend these findings to an alternative murine model which does
85 not rely upon the introduction of a human periodontal organism to provoke the disease process in
86 mice. We performed these experiments in the CXCR2$^{-/-}$ mouse which is compromised in its ability to
87 recruit neutrophils into the periodontal tissues because of the absence of the receptor which guides
88 these cells along chemokine (CXCL1 and CXCL2) gradients produced by the junctional epithelium at
89 the subgingival tooth surface. The neutrophil is regarded as a critical element of the defence of the

90 periodontium in humans and defects in either the number or efficacy of function of neutrophils
91 predisposes to the development of periodontal disease (13-15). Accordingly, we have previously
92 demonstrated that the absence of neutrophils in the periodontal tissues of the CXCR2$^{-/-}$ mouse is
93 correlated with a higher oral microbial load and more periodontal bone loss than wild type animals
94 (16). In this work we aimed to shed further light on the relationship of oral microbial dysbiosis to
95 the development of disease by analysis of the composition of the oral microbiota in CXCR2$^{-/-}$ and
96 wild type BALB/c mice and the potential stability of the CXCR2$^{-/-}$ microbiome and associated disease
97 phenotype to transfer into a wild type background.

98

99 **Material and Methods**

100 **Animal experiments and ethics**

101 Animal experiments were conducted in accredited facilities in accordance with the UK Animals
102 (Scientific Procedures) Act 1986 (Home Office license number 7006844). Specific pathogen free
103 (SPF) CXCR2$^{-/-}$ mice (strain C.129S2-B6) deficient in the receptor for the murine homologues of
104 human Interleukin-8 (CXCL1 and CXCL2) were derived from SPF BALB/c strain mice and purchased
105 from The Jackson Laboratory (Bar Harbor, ME, USA). SPF BALB/c wild type (BALB/c WT) mice were
106 purchased from Charles River Laboratories, UK. Mice were maintained in individually ventilated
107 cages (IVCs) at the animal care facilities of Queen Mary University of London (QMUL). Germ-free
108 (GF) C3H/Orl mice were bred and maintained under axenic conditions at the Royal Veterinary
109 College, University of London using founder GF C3H/Orl mating pairs purchased from Charles River
110 Laboratories, UK as previously described (1). The GF mice were bred and maintained in a separate
111 location to the SPF colonies due to the highly specialist nature of their housing requirements.

112

113 **Breeding and genotyping**

114 To unambiguously determine the influence of genotype on the oral microbiome and disease
115 phenotype, adult CXCR2 $^{-/-}$ males deficient in the receptor for the murine homologues of interleukin-
116 8 (CXCL1/CXCL2) were bred with SPF BALB/c WT females to produce the first generation (F1)
117 heterozygotes. After pregnancy was confirmed by daily examination of the females, the male CXCR2^{-}
118 $^{/-}$ mice were separated from the pregnant females' cages such that the resultant heterozygous
119 CXCR2$^{+/-}$ litters (F1) were only exposed to the maternal wildtype microbiome. Thereafter, the F1
120 mice were interbred (6 pairs) to produce segregating second generation (F2) litters of wild type
121 CXCR2$^{+/+}$ (F2 WT), heterozygotes CXCR2$^{+/-}$ (F2 HT) and homozygotes CXCR2$^{-/-}$ (F2 HM).
122 (Supplementary Figure 1).
123 The F2 mice were genotyped after weaning (3 weeks) by ear notches. Briefly, genomic DNA was
124 isolated from each ear notch and analyzed by amplification using the wild type and mutant primers;
125 oIMR0453 GGTCGTACTGCGTATCCTGCCTCAG (Wild type F), oIMR0454
126 TAGCCATGATCTTGAGAAGTCCATG (Wild type R) (17), oIMR6916 CTTGGGTGGAGAGGCTATTC
127 (Mutant F), oIMR6917 AGGTGAGATGACAGGAGATC (Mutant R) (The Jackson Laboratory). The
128 conditions for thermocycling were as follows: Step 1, 94°C for 4 min; step 2, 94°C for 20 sec, 64°C for
129 30 sec, and 72°C for 35 sec, for 12 cycles; step 3, 94°C for 20 sec, 58°C for 30 sec, and 72°C for 35 sec,
130 for 25 cycles; and step 4, 72°C for 2 min. Diagnostic mutant and wild type bands were 750 base pairs
131 (bp) and 900 bp in size, respectively, on 2.0% agarose gel electrophoresis (Supplementary Figure 1).
132

133 **Microbiota transfer into germ-free mice**

134 Eight-week-old GF mice were transferred under sterile conditions from the Royal Veterinary College,
135 University of London to the QMUL animal facility and co-caging was set up immediately on arrival
136 into IVCs. Oral swabs were collected from the GF mice before co-caging to confirm sterility and from
137 the BALB/c WT and CXCR2$^{-/-}$ mice to determine the microbiology at baseline. Two female donors and
138 4 recipient GF mice were co-housed in each cage at a ratio of 1:2 for six weeks. A total of 16 SPF and
139 16 GF mice were used in these experiments. Oral swabs were collected from all mice at week 1, 3

140 and 6 of co-caging. At the end of the experiment, all mice were euthanized by the CO_2 asphyxiation
141 method prior to assessment of periodontal bone loss.
142
143 **Cultural microbiology analyses**
144 The murine oral cavity was sampled for 30 seconds using sterile fine tip rayon swabs (VWR) and
145 placed in a tube containing 100 µl reduced John's transport medium as previously described (12).
146 Serial dilutions of the suspension were spread onto blood agar plates for aerobic and anaerobic (80%
147 nitrogen, 10% hydrogen and 10% carbon dioxide) growth for 48 hours at 37°C. The predominant
148 colony forming units (CFU) of cultivatable bacteria were counted, purified by subculture and
149 identified by full length 16S ribosomal RNA gene sequencing. On average, 4-6 different colony types
150 were identified on each blood agar plate. Genomic DNA for each isolated bacterial strain was
151 extracted by using a GenElute bacterial DNA kit (Sigma-Aldrich), following the Gram-positive
152 protocol according to manufacturer's instructions. The extracted DNA was used as a template for
153 PCR, followed by full length Sanger sequencing of the 16S rRNA gene and species level identification
154 based on the murine oral microbiome database, as described previously (12).
155
156 **Immunohistochemistry of neutrophils and IL-8**
157 All mice were sacrificed at the age of 16 weeks by carbon dioxide asphyxiation. 3-5 mice were
158 selected in each genotype group, their mandibles and maxillae were dissected and fixed in 4%
159 paraformaldehyde solution (w/v) at 4°C for 3 days. Tissues were processed according to the
160 standard histological procedures and embedded in paraffin. Each molar was sectioned in frontal
161 buccolingual orientation using a microtome (5 mm) for approximately 100 serial sections and
162 mounted on charged glass slides. Every tenth section was stained by haematoxylin and eosin, such
163 that 2-3 sections were available for each mouse and in each section, the neutrophils were counted
164 across the slide. The mean of the total neutrophil count per section was then calculated for each

genotype. Immunohistochemistry (IHC) was carried out following previously published methodology (16). Briefly, neutrophils were detected and counted using an anti- neutrophil elastase primary antibody (Abcam) and anti-rabbit secondary antibody (Abcam). CXCL2 (murine homologue of human IL-8) was detected using anti-CXCL2 antibody (Abcam) with biotinylated secondary anti-rabbit antibody (Abcam). The slides were developed using a peroxidase substrate DAB Kit (Vector Laboratories). The microscopy images were analyzed using the ImageJ software (NIH, USA) and scale bars were set to the global calibration setting.

Periodontal bone loss determination

Mice were euthanized as above, mandible and maxillae were dissected, defleshed and assessed under a Stemi SVII dissecting microscope (Zeiss) and the captured images were analysed by ImageJ software (NIH, USA). The distance from the cemantoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured on 14 predetermined points on the buccal surface of the maxillary molars. In order to calculate bone loss for the 14 sites, total CEJ-ABC distance from each mouse was subtracted from the mean CEJ-ABC distance of the control mice (18). The results were expressed in mm and negative values indicate bone loss relative to the controls.

Statistical analyses

Bone levels between different experimental groups were compared using one-way analysis of variance (ANOVA) and unpaired Student's t-test between 2 comparison groups. Significance was expressed at the $p<0.05$ level. The statistical analyses and graphical visualization of the bone levels were performed using Graphpad Prism 7 (Graphpad Software Inc.). The differences in the composition of the overall microbiome between the treatment and control groups at each time point in each experiment were assessed by PERMANOVA analyses using the Adonis function in the Vegan package in R (19). Differences in the number of CFUs of individual bacterial genera between treatment and control groups were assessed by Welch's t-test using Graphpad Prism 7.

RESULTS

A modified host genotype (CXCR2$^{-/-}$) prevents neutrophil transmigration in oral tissues and leads to the development of a dysbiotic oral microbiome.

Immunohistochemical analysis of the F2 mice groups enabled examination of the influence of genotype on the transmigration of neutrophils and expression of chemokine signals (CXCL2) in the periodontal tissues. All of the second-generation mice demonstrated localised expression of CXCL2 in the junctional epithelium indicating that the absence of the CXCR2 gene had no effect on expression of this chemokine (Figure 1A). Neutrophil staining showed the expected high localisation in the junctional epithelium of wild type (WT) mice and lower levels in the oral epithelium (Figure 1B). Neutrophil staining was not detected in the junctional epithelium of homozygote CXCR2$^{-/-}$ animals (HM) consistent with the absence of the neutrophil receptor for junctional epithelium-derived chemokine gradients in these mice. Neutrophils were present in the junctional epithelium of heterozygote mice (HT) but at a lower level than their WT littermates. (Figure 1B). These visual observations were then confirmed by neutrophil quantitation. Neutrophils were counted and expressed as percentage of the total number in the entire tissue section in each of the junctional epithelium (JE), oral connective tissue (OT), oral epithelium (OE) and blood vessels (BV) (Figure 1C). In WT mice, 25% of neutrophils were present in the JE, 5.1% in the OE, 13.7% in the OT and the remainder (65%) within the vasculature (BV). In contrast, only 0.06% of neutrophils were located in the JE of HM mice and over 95% of cells were confined to the BV. In the HT mice, 7% of the neutrophils were found to be in the JE and 86% in the blood vessels. (Figure 1C). Hence in the absence of CXCR2, neutrophil transmigration to the gingival crevice is abolished and the oral microbiome in these animals develops in the absence of this element of the innate host defence.

214 We next examined the influence of mouse genotype on the oral commensal microbiota from 16-
215 week-old parents (BALB/c WT and CXCR2$^{-/-}$). The predominant organisms in the oral microbial
216 population of all mice groups belonged to the genus *Streptococcus*, with *Gemella species 2* and low
217 levels of *Actinobacillus muris* and *Enterobacteriaceae* members in Balb/c WT mice. The oral
218 microbiota of the CXCR2$^{-/-}$ mice on the other hand was substantially different, where apart from the
219 dominant *Streptococcus*, the remaining community was composed of *Actinobacillus muris* and
220 members of the *Enterobacteriaceae* family. *Gemella* species 2 MOT43 was recently identified as a
221 unique, yet to be named murine-specific species of the genus, found to be predominant in the oral
222 microbiome of certain SPF laboratory mice backgrounds (12). Comparative PERMANOVA analyses
223 showed a significant difference between the oral microbial compositions of the Balb/c WT and
224 CXCR2$^{-/-}$ mice ($p=0.026$; $R^2=0.73$; Figure 2A).

225 In addition to a different community structure, the CXCR2$^{-/-}$ mice also showed elevated total
226 microbial counts compared to the BALB/c WT mice (Mean 8.47 x 10^7 CFU vs 1.76 x10^7; $p<0.05$;
227 Figure 2B). Similarly elevated total microbial counts have been observed in previous studies of oral
228 microbial dysbiosis in both mouse and human periodontal investigations (1,11).

229 **Vertical transmission of the maternal CXCR2$^{+/+}$ oral microbiome into second generation CXCR2$^{-/-}$**
230 **mice demonstrates the direct influence of genotype on the composition of the oral microbiota.**

231 In order to verify a direct influence of mouse genotype on the oral microbiota we next determined
232 the quantitative composition of the oral microbiome of the F2 wild type CXCR2$^{+/+}$ (WT), heterozygous
233 CXCR2$^{-/+}$ (HT) and homozygous CXCR2$^{-/-}$ (HM) mice (Figure 2B). Importantly, as the CXCR2$^{-/-}$ mice in
234 the F0 breeding were removed prior to the birth of the F1 generation, the F1 generation and
235 subsequently F2 generations were only exposed to those bacteria present in the maternal CXCR2$^{+/+}$
236 (BALB/c WT) oral microbiome. The pattern of total microbial counts seen in the F2 generation mice
237 mirrored that of the original F0 breeders. The total microbial counts of the F2 HM mice were
238 significantly higher (mean 4.4 x 10^7 CFU) than both the F2 WT mice (mean 4.07 x 10^6 CFU; $p<0.0005$)

239 and the F2 HT mice (6.47 x 10^6 CFU; $p<0.0005$). Thus, there is an inverse correlation between dosage
240 of the *cxcr2* gene and total oral microbial counts in these mice.

241 A strong negative correlation (R^2 = 0.7859) was also observed between the percentage of
242 neutrophils in the junctional epithelium (JE) and total oral microbial counts: BALB/c WT mice with
243 the highest proportion of JE associated neutrophils had the lowest bacterial counts and the
244 homozygote CXCR2$^{-/-}$ mice with a very low proportion of neutrophils in this epithelium had the
245 highest microbial burden (Figure 2C).

246 These experiments therefore demonstrate the flexibility of the oral microbiome of the CXCR2$^{+/+}$ mice
247 (BALB/c WT) in response to the genetic background of the host – in this case resulting in the
248 presence or absence of neutrophils in the periodontal tissues. In summary, the oral microbiome of
249 the BALB/c WT mice is inherently flexible, strongly governed by the genetic background of the host
250 organism and will form reproducible symbiotic and dysbiotic signatures upon vertical transfer into
251 either wild type or CXCR2$^{-/-}$ offspring respectively.

252

253 **Relationship between host genotypes on alveolar bone levels**

254 The influence of the genotype on the development of periodontal disease was assessed by
255 measuring the alveolar bone loss relative to the bone height of the F0 CXCR2$^{+/+}$ (BALB/c WT) animals.
256 (Figure 3). The F0 CXCR2$^{-/-}$ mice displayed significantly elevated bone loss (mean -0.11 ± 0.03 mm; $p<$
257 0.05) compared to BALB/c WT mice. This level of bone loss is similar to that observed in previous
258 studies following oral challenge with *Porphyromonas gingivalis* (1,11). Thus, it has been
259 demonstrated that the deletion of the CXCR2 gene predisposes these animals to periodontal bone
260 destruction. The second generation CXCR2$^{-/-}$ mice (F2 HM) also demonstrated similar reduced bone
261 levels as F0 CXCR2$^{-/-}$ mice (mean -0.11 ± 0.04 mm; $p< 0.05$). The F2 WT mice also showed higher
262 levels of bone loss (mean -0.067 ± 0.02; $p<0.05$) compared to the F0 WT animals. However, no

263 significant difference was observed in the bone levels compared with their F2 HM littermates (Mean
264 -0.06 ± 0.02 mm). This may reflect the continuous co-housing of these littermates of different
265 genotypes in excess of 8 weeks: we have previously suggested that co-caging of mice with markedly
266 different oral microbiomes and bone loss phenotypes can lead to cage-normalisation effects wherein
267 a dysbiotic oral microbiome in one group of animals may have an influence on the microbiome and
268 disease phenotype of co-caged animals with normal health associated oral microbiome (11).

269 **The dysbiotic microbiome from CXCR2$^{-/-}$ mice is not stable upon transfer to wild type mice and**
270 **does not lead to transfer of the bone loss phenotype into recipients.**

271 In previous work, we demonstrated the efficient transfer of the dysbiotic microbiome, generated
272 following oral gavage of SPF mice (C3H/Orl) with *Porphyromonas gingivalis*, into GF mice (C3H/Orl)
273 by co-caging donor and recipient mice in a 1:2 ratio for 6 weeks. Transfer and establishment of the
274 dysbiotic microbiota was accompanied by the development of periodontal bone loss in the formerly
275 GF mice equivalent to that observed in the original challenged mice (11). Here, we used the same
276 co-caging protocol to transfer the oral health-associated microbiome from BALB/c WT mice and the
277 dysbiotic microbiome from CXCR2$^{-/-}$ mice into C3H/Orl GF recipients. The oral microbiota of donor
278 and recipient mice was examined at 1, 3, and 6 weeks of co-caging.

279 The commensal oral microbiome from BALB/c WT mice transferred to GF mice, with no significant
280 differences to the qualitative or quantitative composition of the microbiota assessed by laboratory
281 culture at all three time points (Figures 4A and 4B). At the end of 6 weeks, the donor BALB/c WT
282 mice and recipient GF-BALB/c mice had a microbiota predominantly comprised of the *Streptococcus*
283 and *Gemella* species, both with mean total counts of 1.13 X 10^7 CFU.

284 In contrast, a different pattern of microbial transfer was observed using the CXCR2$^{-/-}$ mice as donors
285 into GF animals. This was particularly marked in the case of the total microbial counts. After one
286 week of co-caging, the mean oral bacterial counts of the recipient animals (GF-CXCR2$^{-/-}$) were 5.14
287 x10^7 CFU which was not significantly different to that of the donor CXCR2$^{-/-}$ mice at the same time

288 point (9.6 x 10^7 CFU; p =0.05). However, by 3 weeks and until the end of the experiment, the mean
289 total oral microbial counts of the recipient mice fell to significantly below the level of the CXCR2$^{-/-}$
290 donors (3 weeks: mean 1.02 x10^7 CFU vs donor mean 6.24 x10^7 CFU; p =0.0002; 6 weeks: mean 1.23
291 x 10^7 CFU vs donor mean 9.7 x 10^7 CFU; p = 0.0002). At both 3 weeks and 6 weeks, the mean total
292 oral counts of the GF-CXCR2$^{-/-}$ microbiota were equivalent to the mean counts of BALB/c WT mice
293 and the GF-BALB/c recipient mice. (Figure 4B). The microbial composition of the GF-CXCR2$^{-/-}$
294 recipient mice also reverted to represent the GF-BALB/c mice, comprised of *Streptococcus*, *Gemella*
295 and *Enterobacteriaceae* species. *Actinobacillus muris,* which was one of the major components of
296 the donor CXCR2$^{-/-}$ mice was conspicuously absent in the recipients after 6 weeks (Figure 4A). The
297 inter-individual variation in these mice groups has also been presented in Supplementary Figure 2 in
298 the form of box and whisker plots.

299 We next examined the levels of periodontal bone loss in donor and recipient mice. The
300 conventionalized recipients GF-BALB/c, of the BALB/c WT mice microbiome did not show any
301 differences in the alveolar bone levels when compared to the donors (Figure 4C). As anticipated,
302 CXCR2$^{-/-}$ donor mice showed significantly more bone loss compared to the BALB/c WT mice of the
303 same age. (- 0.122 mm, p = <0.005). However, the recipient GF-CXCR2$^{-/-}$ mice did not demonstrate
304 increased alveolar bone loss compared to GF-BALB/c mice after 6 weeks of co-housing (Figure 4C).
305 Hence these transfer experiments demonstrated that the oral microbiota of CXCR2$^{-/-}$ mice is not
306 stable to transfer into a wild type mouse background and co-caging does not lead to transfer of the
307 disease phenotype of these mice.

308 **Discussion**

309 In this study, we aimed to determine the influence of reduced effectiveness of the innate defences
310 operative within the periodontal tissues – in this instance abrogation of neutrophil transmigration -
311 on the nature and stability of the oral microbiota. We have demonstrated how the absence of the
312 murine CXCR2 gene led to an almost complete loss of neutrophil homing to the periodontal tissues
313 which both reinforced the crucial role played by this receptor and its two murine ligands, CXCL1 and
314 CXCL2 in recruitment of this cell type into the periodontal tissues of the mouse. It also provided the
315 opportunity to determine the impact of a significant loss of periodontal defence function on the oral
316 microbiota in this model.

317 The human leucocyte adhesion deficiency (LAD) group of inherited disorders present a similar
318 pattern of reduced neutrophil extravasation and their recruitment to sites of infection/inflammation
319 and the periodontium. LAD patients have defects in the expression or function of the leukocyte-
320 restricted β2 integrins or other adhesion molecules. Consequently, circulating neutrophils of LAD
321 patients do not adhere to vascular endothelial cells and hence are unable to leave the blood vessels
322 and enter the tissues (20). In all the different variations of the condition, LAD patients are found to
323 be highly susceptible to periodontal disease (21). However, because of the rarity of these monogenic
324 diseases there have been few comprehensive studies on the microbiology of these patients,
325 although LAD-1 patients are reported to harbour more bacterial biomass than control subjects (22)
326 and Gram- staining of extracted teeth and surrounding tissues from LAD-I patients has demonstrated
327 very significant microbial colonization of tooth surfaces, although not of the underlying diseased
328 gingival tissue (23).

329 Mouse models of reduced neutrophil trafficking to the oral tissues provide an alternative means to
330 address the question of the influence of defects in the innate host defences on the oral microbiota
331 (24). However, there are few such comparative studies of the oral microbiology of wild type
332 compared to appropriate homozygous gene knockout mice which have properly controlled for

333 animal husbandry/cage effects. When mice of different genotypes are sourced and reared
334 separately, it becomes problematic to differentiate the effects of variations to the microbiota caused
335 by differences in mouse genotype from variations caused by different environmental exposures
336 during rearing. In the present study we developed a breeding protocol (Supplementary Figure 1) in
337 which second generation F2 WT, HT and HM mice were only exposed to a microbiome which had
338 originated from the original maternal BALB/c WT F0 generation. Differences in the oral microbiota of
339 the F2 mice can therefore be ascribed reasonably to the influence of genotype.

340 We have previously demonstrated that the oral microbiota of laboratory (and wild) mice exhibits low
341 diversity compared to human studies, is frequently dominated by a small number of microbial taxa
342 and a very low proportion of uncultivable species (11,12). Culture therefore provides an appropriate
343 means of analysis of the community structure in addition to providing a direct read out of total
344 microbial load, with congruence being observed with the 16S rRNA gene amplicon-based population
345 analysis as well (11,12). As seen in previous mouse studies, similarly low diversity oral microbiomes
346 were observed in the current study (Figure 2A). Analysis of the oral microbiota of F2 littermates
347 revealed significant differences in the total oral bacterial counts inversely proportional to the dosage
348 of the CXCR2 allele:: $CXCR2^{-/-} > CXCR2^{+/-} > CXCR2^{+/+}$ and the elevated bacterial numbers present in
349 the F2 HM mice were similar to the counts in the original F0 $CXCR2^{-/-}$ founder mice. The total
350 bacterial counts of the F2 WT mice were higher than those in the F0 wild type mice which we
351 speculate may be a consequence of the prolonged co-caging of the former with their F2 HT and HM
352 littermates (Figure 2B).

353 These findings accord with accumulating evidence on the modifying effects of host genetics on the
354 human oral microbiome particularly derived from studies on mono- and dizygotic twins (25,26). An
355 analysis of over 750 twin pairs demonstrated that, independent of cohabitation status, the beta-
356 diversity of monozygotic twins is significantly lower than for dizygotic or unrelated individuals. In this
357 population, a number of microbiome phenotypes were more than 50% heritable, consistent with the

358 hypothesis that human genes influence oral microbial communities (27). Similarly, in a study of 485
359 monozygotic and dizygotic twins (28), the similarity of the oral microbiome increased with shared
360 genotype and, although most of the variation in the microbiome was determined by environmental
361 factors, highly hereditable oral taxa were also identified. More recently, however, Mukherjee and
362 colleagues (29) determined the influence of host genetics on acquisition of the oral microbiome by
363 comparing the oral microbiota of 55 biological versus 50 adoptive mother-child dyads. Only children
364 adopted immediately at birth and unrelated to the adoptive family were included to minimize
365 transmission of bacteria from the biological mother. They discerned no difference in how closely oral
366 bacterial community profiles matched for adoptive versus biological mother-child pairs, and from
367 this concluded that there is little, if any, effect of host genetics on the fidelity of transmission. The
368 apparent contradictory finding of a significant effect of genotype on the acquisition of the oral
369 microbiome that we report in the current study using the CXCR2$^{-/-}$ mouse is likely to reflect the very
370 different phenotypes of the wild type versus homozygote knockout animals with respect to
371 neutrophil extravasation into the periodontal tissues.

372 Following on from earlier studies in this laboratory, which indicated that a dysbiotic oral microbiome
373 generated by oral gavage with the human periodontal organism *P. gingivalis* is stable to transfer
374 both horizontally and vertically, and transmits the disease phenotype into recipient animals, we
375 examined whether the CXCR2$^{-/-}$ dysbiotic oral microbiome shared similar properties. When CXCR2$^{-/-}$
376 mice were co-housed with GF wild type mice (Figure 4 A & 4B), we observed transmission of the
377 dysbiotic oral microbiota to a similarly raised bacterial count after 1 week. However, by week 3 and
378 till the end of the experiment, the level of bacterial colonisation was reduced to the level of the total
379 counts routinely observed in wild type mice. The temporary nature of the initially high counts and
380 their subsequent reduction may reflect temporal adaptation of the GF mice to microbial exposure. It
381 is well established that the response to microbes of mice reared under germ free conditions differs
382 from conventionally reared animals and can lead to a delay in the inflammatory response (30).
383 Specifically in relation to the oral cavity, Fukuhara and colleagues (31) reported that following

384 administration of lipopolysaccharide from *P. gingivalis* to the gingiva, GF mice show decreased
385 numbers of CD4+ cells in the periodontal tissues and lower expression of tumour necrosis factor-α
386 and fork head box protein p3 than SPF mice of similar age. Hence, in the current work, the initial
387 exposure to the high levels of bacteria in the CXCR2$^{-/-}$ mice may have led to immediate colonisation
388 at the same level in the recipients (GF- CXCR2$^{-/-}$), but, following a period of maturation of the
389 immune system in these mice, colonisation levels reduced to the level seen in wild type animals. In
390 this regard, it is perhaps noteworthy that the levels of bacteria in GF-BALB/c mice housed with
391 BALB/c WT animals were also at their highest, albeit not significantly, at week 1. The overall
392 community composition of the microbiota of the recipients was significantly different to the CXCR2$^{-/-}$
393 donor mice at all time points. Conversely, no differences in either total microbial load or
394 composition of microbiota was observed between wild type donor and recipient mice.

395 As might be predicted based on these microbiological findings, recipients of the CXCR2$^{-/-}$ microbiota
396 experienced similar levels of bone loss compared to wild type animals and far lower than the donor
397 mice (Figure 4C). These findings contrast with many reports regarding the transfer of dysbiotic
398 microbiomes into GF recipients and associated transmission of the host phenotype. For example,
399 several experimentally generated inflammatory conditions, such as obesity, diabetes, heart disease,
400 autoimmune disorders and cancer have been shown to be transmissible from diseased donors into
401 GF mice through either co-housing or direct transfer of the associated gut dysbiotic microbiota
402 (32,33). Mutations in the genes for leptin production have been targeted to develop the ob^{-}/ob^{-} or
403 obese mice genotypes that have often been used in such microbiome studies. Using a similar
404 experimental protocol described in the our current report, transfer of the gut microbiota of ob^{-}/ob^{-}
405 mice into wild type GF animals led to the development of obesity in the recipients - the resilience of
406 the dysbiotic ob^{-}/ob^{-} microbiota appearing to be sufficient to enable transfer into a +/+ lean genetic
407 background (34). In this current work, transfer of the dysbiotic microbiome from CXCR2$^{-/-}$ donor mice
408 into mice with a properly functioning neutrophil recruitment into the periodontal tissue is
409 accompanied by transformation of the microbiota to that routinely present in wild type animals.

410 Thus, the neutrophil function affected by the mouse host genotype appears to be the deciding factor
411 in this case, for the development of dysbiosis and disease.

412 In summary, these data suggest that the genetic background of the host can have an impact on the
413 composition, amount and disease potential of the murine oral microbiome. The oral microbiota
414 thereby demonstrates significant flexibility in composition and expression of virulence potential.
415 Such flexibility is a significant factor in the bi-directional relationship between the microbiota and
416 the host response (35) which is a distinguishing characteristic of current concepts of the
417 susceptibility to and progression of periodontal disease.

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Author Contributions

A Hashim contributed to design, data acquisition and analysis, and critically revised the manuscript; M Payne contributed to design, data acquisition, analysis, and critically revised the manuscript; A. Alsam contributed to design, data acquisition and analysis and critically revised the manuscript; J. Aduse-Opoku contributed to data analysis and critically revised the manuscript; MA Curtis contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript; S. Joseph contributed to design, data analysis and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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435 **References**

436 1. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskan MA, McIntosh ML, Alsam A,
437 Kirkwood KL, Lambris JD, Darveau RP, Curtis MA. 2011. Low-Abundance Biofilm Species
438 Orchestrates Inflammatory Periodontal Disease through the Commensal Microbiota and
439 Complement. Cell Host Microbe 10(5):497-506.

440 2. Moore WE, Holdeman LV, Smibert RM, Hash DE, Burmeister JA, Ranney RR. 1982.
441 Bacteriology of severe periodontitis in young adult humans. Infection and Immunity.
442 38(3):1137-48.

443 3. Darveau RP. 2010. Periodontitis: a polymicrobial disruption of host homeostasis. Nature
444 Reviews Microbiology. 8(7):481-90.

445 4. Kirst ME, Li EC, Alfant B, Chi YY, Walker C, Magnusson I, Wang GP. 2015. Dysbiosis and
446 alterations in predicted functions of the subgingival microbiome in chronic periodontitis.
447 Applied and Environmental Microbiology. 81(2):783-93.

448 5. Burns E, Bachrach G, Shapira L, Nussbaum G. 2006. Cutting Edge: TLR2 is required for the
449 innate response to Porphyromonas gingivalis: activation leads to bacterial persistence and
450 TLR2 deficiency attenuates induced alveolar bone resorption. The Journal of Immunology.
451 177(12):8296-300.

452 6. Ricklin D, Hajishengallis G, Yang K, Lambris JD. 2010. Complement: a key system for immune
453 surveillance and homeostasis. Nature immunology. 11(9):785-97.

454 7. Hajishengallis G. 2014. The inflammophilic character of the periodonti- tis-associated
455 microbiota. *Mol Oral Microbiol*. 29(6):248-257.

456 8. Hajishengallis G, Chavakis T, Lambris JD. 2020. Current understanding of periodontal disease
457 pathogenesis and targets for host-modulation therapy. Periodontology 2000. 84(1):14-34.

458 9. Maekawa T, Krauss JL, Abe T, Jotwani R, Triantafilou M, Triantafilou K, Hashim A, Hoch S,
459 Curtis MA, Nussbaum G, Lambris JD. 2014. Porphyromonas gingivalis manipulates

460 complement and TLR signaling to uncouple bacterial clearance from inflammation and
461 promote dysbiosis. Cell Host & Microbe. 15(6):768-78.

462 10. Van Dyke TE. 2008. The Management of Inflammation in Periodontal Disease. J
463 Periodontol. 2008 Aug; 79(8 Suppl): 1601–1608.

464 11. Payne MA, Hashim A, Alsam A, Joseph S, Aduse-Opoku J, Wade WG, Curtis MA. 2019.
465 Horizontal and vertical transfer of oral microbial dysbiosis and periodontal disease. Journal
466 of Dental Research. Dec;98(13):1503-10.

467 12. Joseph S, Aduse-Opoku J, Hashim A, Hanski E, Streich R, Knowles SC, Pedersen AB, Wade
468 WG, Curtis MA. 2021. A 16S rRNA Gene and Draft Genome Database for the Murine Oral
469 Bacterial Community. mSystems. Feb 23: 6(1).

470 13. Deas DE, Mackey SA, McDonnell HT. 2003. Systemic disease and periodontitis:
471 manifestations of neutrophil dysfunction. Periodontol. 2000. 32, 82–104.

472 14. Hajishengallis E and Hajishengallis G. 2014. Neutrophil Homeostasis and Periodontal Health
473 in Children and Adults. J Dent Res 93(3) 2014.

474 15. Moutsopoulos NM, Konkel J, Sarmadi M, Eskan MA, Wild T, Dutzan N, Abusleme L, Zenobia
475 C, Hosur KB, Abe T, Uzel G. 2014. Defective neutrophil recruitment in leukocyte adhesion
476 deficiency type I disease causes local IL-17–driven inflammatory bone loss. Science
477 translational medicine. 6(229):229ra40-.

478 16. Zenobia C, Luo XL, Hashim A, Abe T, Jin L, Chang Y, Jin ZC, Sun JX, Hajishengallis G, Curtis MA,
479 Darveau RP. 2013. Commensal bacteria-dependent select expression of CXCL 2 contributes
480 to periodontal tissue homeostasis. Cellular microbiology. 15(8):1419-26

481 17. Lindner M, Trebst C, Heine S, Skripuletz T, Koutsoudaki PN, Stangel M. 2008. The chemokine
482 receptor CXCR2 is differentially regulated on glial cells in vivo but is not required for
483 successful remyelination after cuprizone-induced demyelination. Glia. 56(10):1104-13.

484 18. Baker PJ, Dixon M, Roopenian DC. 2000. Genetic control of susceptibility to Porphyromonas
485 gingivalis-induced alveolar bone loss in mice. Infect Immun. 68(10):5864-5868.

19. Dixon P. 2003. VEGAN, a package of R functions for community ecology. J Veg Sci. 14(6): 927-930.
20. Anderson DC, Springer TA. 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150, 95 glycoproteins. Annual Review of Medicine. 38(1):175-94.
21. Hajishengallis G, Moutsopoulos NM, Hajishengallis E, Chavakis T. 2016. Immune and regulatory functions of neutrophils in inflammatory bone loss. In Seminars in immunology (Vol. 28, No. 2, pp. 146-158). Academic Press.
22. Hanna and Etzioni. 2012. Leukocyte adhesion deficiencies. Ann. N.Y. Acad. Sci. 1250 (2012) 50–55.
23. Hajishengallis G, Moutsopoulos NM. 2016. Role of bacteria in leukocyte adhesion deficiency-associated periodontitis. Microbial pathogenesis. 94:21-6.
24. Niederman R, Westernoff T, Lee C, Mark LL, Kawashima N, Ullman-Culler M, Dewhirst FE, Paster BJ, Wagner DD, Mayadas T, Hynes RO. 2001. Infection-mediated early-onset periodontal disease in P/E-selectin-deficient mice. Journal of clinical periodontology. 28(6):569-75.
25. Kurushima Y, Tsai PC, Castillo-Fernandez J, Alves AC, Moustafa JS, Le Roy C, Spector TD, Ide M, Hughes FJ, Small KS, Steves CJ. 2019. Epigenetic findings in periodontitis in UK twins: a cross-sectional study. Clinical epigenetics. 11(1):27.
26. Oliveira NF, Damm GR, Andia DC, Salmon C, Nociti Jr FH, Line SR, De Souza AP. 2009. DNA methylation status of the IL8 gene promoter in oral cells of smokers and non-smokers with chronic periodontitis. Journal of clinical periodontology. 36(9):719-25.
27. Demmitt BA, Corley RP, Huibregtse BM, Keller MC, Hewitt JK, McQueen MB, Knight R, McDermott I, Krauter KS. 2017. Genetic influences on the human oral microbiome. BMC Genomics. 18(1):1-5.

28. Gomez A, Espinoza JL, Harkins DM, Leong P, Saffery R, Bockmann M, Torralba M, Kuelbs C, Kodukula R, Inman J, Hughes T. 2017. Host genetic control of the oral microbiome in health and disease. Cell Host & Microbe. 22(3):269-78.
29. Mukherjee C, Moyer CO, Steinkamp HM, Hashmi SB, Beall CJ, Guo X, Ni A, Leys EJ, Griffen AL. 2021. Acquisition of oral microbiota is driven by environment, not host genetics. Microbiome. 9(1):1-3.
30. Costa MC, Santos JR, Ribeiro MJ, de Freitas GJ, Bastos RW, Ferreira GF, Miranda AS, Arifa RD, Santos PC, dos Santos Martins F, Paixão TA. 2016. The absence of microbiota delays the inflammatory response to *Cryptococcus gattii*. International Journal of Medical Microbiology. 306(4):187-95.
31. Fukuhara D, Irie K, Uchida Y, Kataoka K, Akiyama K, Ekuni D, Tomofuji T, Morita M. 2018. Impact of commensal flora on periodontal immune response to lipopolysaccharide. Journal of periodontology. 89(10):1213-20.
32. Hu B, Elinav E, Huber S, Strowig T, Hao L, Hafemann A, Jin C, Wunderlich C, Wunderlich T, Eisenbarth SC, Flavell RA. 2013. Microbiota-induced activation of epithelial IL-6 signaling links inflammasome-driven inflammation with transmissible cancer. Proceedings of the National Academy of Sciences. 110(24):9862-7.
33. Vrieze A, Van Nood E, Holleman F, Salojärvi J, Kootte RS, Bartelsman JF, Dallinga–Thie GM, Ackermans MT, Serlie MJ, Oozeer R, Derrien M. 2012. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology. 143(4):913-6.
34. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature. 444(7122):1027-31.
35. Curtis MA, Diaz PI, Van Dyke TE. 2020. The role of the microbiota in periodontal disease. Periodontology 2000. 83(1):14-25.

536

537 **Legends:**

538 **Figure 1. Detection of CXCL2 and neutrophils in oral tissues of of BALB/c CXCR2$^{+/+}$, CXCR2$^{+/-}$ and**
539 **CXCR2$^{-/-}$ mice**

540 (A) CXCL2 staining in the F2 generation wild type (WT), homozygotes (HM) and heterozygotes
541 (HT) mice (x20 magnification). CXCL2 was detected in blood vessels and JE of all the three
542 genotypes.
543 (B) Neutrophil staining of F2 generation wild type (WT), homozygotes (HM) and heterozygotes
544 (HT) mice (x20 magnification). Most neutrophils were localised to the junctional epithelium
545 (JE) of WT mice with reduced staining in the JE of HT mice and minimal detection in HM.
546 (C) Quantification of neutrophils in the junctional epithelium, oral connective tissue, oral
547 epithelium and blood vessels was performed by counting their numbers in tissue sections of
548 F2 genotypes and were expressed as percentage of the overall cell numbers present. Data
549 are means ± SD. (WT n=5, HT n=4, HM n=2)

550 **Figure 2. Effect of the mouse genotype on the oral microbiota and total microbial counts of F0**
551 **parents and F2 offsprings**

552 (A) Composition of the cultivable oral microbiome of 16-week-old BALB/c WT mothers and the
553 CXCR2$^{-/-}$ fathers (F0 generation) expressed as relative abundance in percentages (Y axis). The
554 different coloured segments represent bacterial species based on the mean colony-forming
555 units (CFUs) of each microorganism in each group. Statistical significance in the differences
556 between the microbial communities of the two mice groups was determined by
557 PERMANOVA analysis and has been indicated.
558 (B) Total oral bacterial counts in 16-week-old BALB/c WT mothers and homozygous CXCR2 $^{-/-}$
559 fathers (F0 generation) and second-generation wild type CXCR2$^{+/+}$ (F2 WT) homozygous
560 CXCR2$^{-/-}$ (F2 HM); heterozygous CXCR2$^{+/-}$ (F2 HT) mice; expressed as $\log_{10}$ of total CFUs. Each
561 data point represents an individual mouse. (**P < 0.05, ***P < 0.005, ****P < 0.0005)

562 (C) Linear regression analysis of oral bacterial counts against percentage of neutrophils in the
563 junctional epithelium in the various F2 mice genotype groups.

564 **Figure 3. Effect of the mouse genotype on alveolar bone levels in F0 parents and F2 generation**
565 **offsprings**

566 Alveolar bone levels in 16 week old BALB/c WT mothers and CXCR2$^{-/-}$ fathers (F0
567 generation); homozygous CXCR2$^{-/-}$ (F2 HM); heterozygous CXCR2$^{+/-}$ (F2 HT) and wild-type CXCR2$^{+/+}$
568 (F2 WT). Bone loss was expressed as negative values relative to the BALB/c WT mothers, the donors
569 of the maternal microbiome (baseline). Each point represents the mean bone level for an individual
570 mouse with horizontal lines representing the mean bone levels per group ± SD (**P < 0.05).

571 **Figure 4. Transfer of the CXCR2$^{-/-}$ oral microbiota to germ-free mice is not to the same total**
572 **microbial load and does not induce bone loss.**

573 **(A**) Bacterial composition of the cultivable oral microbiome of BALB/C WT and CXCR2$^{-/-}$ mice and
574 after transmission into initially germ-free C3H/Orl mice (GF-BALB/C & GF-CXCR2$^{-/-}$) sampled at 1, 3
575 and 6 weeks. The sizes of the pie-charts are indicative of the variations in the total oral bacterial
576 counts in the different groups. The graphs have been plotted using the observed number of colony-
577 forming units (CFUs) of each microorganism in each group. Statistical significance in the differences
578 between the microbial communities of groups was determined by PERMANOVA analysis (**P < 0.05,
579 ***P < 0.005, ****P < 0.0005)

580 (B) Total oral bacterial counts in the different groups of mice at weeks 1, 3 and 6 expressed as $\log_{10}$
581 of total CFUs. Each data point represents an individual mouse. (**P < 0.05, ***P < 0.005)

582 (C) Alveolar bone levels in BALB/c WT and CXCR2$^{-/-}$ mice and conventionalized germ free C3H/Orl
583 mice (GF-BALB/c & GF-CXCR2$^{-/-}$) after 6 weeks. Bone loss was expressed as negative values relative
584 to the BALB/c WT donors. Each point represents the mean bone level for an individual mouse with
585 horizontal lines representing the mean bone levels per group ± SD (**P < 0.05, ***P < 0.005).