DOI:
10.1128/IAI.00309-21

Document Version
Peer reviewed version

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

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Running title: Oral dysbiosis and periodontal bone loss in CXCR2$^{-/-}$ mice

Abstract word count: 248 words

Text word count: 5221 words

No. of tables/figures: 4 figures

Keywords: periodontitis, oral microbiome, dysbiosis, bone loss, neutrophils, CXCR2

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.

INTRODUCTION

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

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

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

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

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

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

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

Material and Methods

Animal experiments and ethics

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

Breeding and genotyping

To unambiguously determine the influence of genotype on the oral microbiome and disease phenotype, adult CXCR2 $^{-/-}$ males deficient in the receptor for the murine homologues of interleukin-8 (CXCL1/CXCL2) were bred with SPF BALB/c WT females to produce the first generation (F1) heterozygotes. After pregnancy was confirmed by daily examination of the females, the male CXCR2$^{-/-}$ mice were separated from the pregnant females' cages such that the resultant heterozygous CXCR2$^{+/-}$ litters (F1) were only exposed to the maternal wildtype microbiome. Thereafter, the F1 mice were interbred (6 pairs) to produce segregating second generation (F2) litters of wild type CXCR2$^{+/+}$ (F2 WT), heterozygotes CXCR2$^{+/-}$ (F2 HT) and homozygotes CXCR2$^{-/-}$ (F2 HM). (Supplementary Figure 1).

The F2 mice were genotyped after weaning (3 weeks) by ear notches. Briefly, genomic DNA was isolated from each ear notch and analyzed by amplification using the wild type and mutant primers; oIMR0453 GGTCGTACTGCGTATCCTGCCTCAG (Wild type F), oIMR0454 TAGCCATGATCTTGAGAAGTCCATG (Wild type R) (17), oIMR6916 CTTGGGTGGAGAGGCTATTC (Mutant F), oIMR6917 AGGTGAGATGACAGGAGATC (Mutant R) (The Jackson Laboratory). The conditions for thermocycling were as follows: Step 1, 94°C for 4 min; step 2, 94°C for 20 sec, 64°C for 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, for 25 cycles; and step 4, 72°C for 2 min. Diagnostic mutant and wild type bands were 750 base pairs (bp) and 900 bp in size, respectively, on 2.0% agarose gel electrophoresis (Supplementary Figure 1).

Microbiota transfer into germ-free mice

Eight-week-old GF mice were transferred under sterile conditions from the Royal Veterinary College, University of London to the QMUL animal facility and co-caging was set up immediately on arrival into IVCs. Oral swabs were collected from the GF mice before co-caging to confirm sterility and from the BALB/c WT and CXCR2$^{-/-}$ mice to determine the microbiology at baseline. Two female donors and 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 16 GF mice were used in these experiments. Oral swabs were collected from all mice at week 1, 3

and 6 of co-caging. At the end of the experiment, all mice were euthanized by the CO_2 asphyxiation method prior to assessment of periodontal bone loss.

Cultural microbiology analyses

The murine oral cavity was sampled for 30 seconds using sterile fine tip rayon swabs (VWR) and placed in a tube containing 100 µl reduced John's transport medium as previously described (12). Serial dilutions of the suspension were spread onto blood agar plates for aerobic and anaerobic (80% nitrogen, 10% hydrogen and 10% carbon dioxide) growth for 48 hours at 37°C. The predominant colony forming units (CFU) of cultivatable bacteria were counted, purified by subculture and identified by full length 16S ribosomal RNA gene sequencing. On average, 4-6 different colony types were identified on each blood agar plate. Genomic DNA for each isolated bacterial strain was extracted by using a GenElute bacterial DNA kit (Sigma-Aldrich), following the Gram-positive protocol according to manufacturer's instructions. The extracted DNA was used as a template for PCR, followed by full length Sanger sequencing of the 16S rRNA gene and species level identification based on the murine oral microbiome database, as described previously (12).

Immunohistochemistry of neutrophils and IL-8

All mice were sacrificed at the age of 16 weeks by carbon dioxide asphyxiation. 3-5 mice were selected in each genotype group, their mandibles and maxillae were dissected and fixed in 4% paraformaldehyde solution (w/v) at 4°C for 3 days. Tissues were processed according to the standard histological procedures and embedded in paraffin. Each molar was sectioned in frontal buccolingual orientation using a microtome (5 mm) for approximately 100 serial sections and mounted on charged glass slides. Every tenth section was stained by haematoxylin and eosin, such that 2-3 sections were available for each mouse and in each section, the neutrophils were counted 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.

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

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

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

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

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

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

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

Relationship between host genotypes on alveolar bone levels

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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.

Acknowledgements

This work was supported by the Medical Research Council (Grant Award Nos. MR/P012175/1 and MR/P012175/2). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Legends:

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

(A) CXCL2 staining in the F2 generation wild type (WT), homozygotes (HM) and heterozygotes (HT) mice (x20 magnification). CXCL2 was detected in blood vessels and JE of all the three genotypes.

(B) Neutrophil staining of F2 generation wild type (WT), homozygotes (HM) and heterozygotes (HT) mice (x20 magnification). Most neutrophils were localised to the junctional epithelium (JE) of WT mice with reduced staining in the JE of HT mice and minimal detection in HM.

(C) Quantification of neutrophils in the junctional epithelium, oral connective tissue, oral epithelium and blood vessels was performed by counting their numbers in tissue sections of F2 genotypes and were expressed as percentage of the overall cell numbers present. Data are means ± SD. (WT n=5, HT n=4, HM n=2)

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

(A) Composition of the cultivable oral microbiome of 16-week-old BALB/c WT mothers and the CXCR2$^{-/-}$ fathers (F0 generation) expressed as relative abundance in percentages (Y axis). The different coloured segments represent bacterial species based on the mean colony-forming units (CFUs) of each microorganism in each group. Statistical significance in the differences between the microbial communities of the two mice groups was determined by PERMANOVA analysis and has been indicated.

(B) Total oral bacterial counts in 16-week-old BALB/c WT mothers and homozygous CXCR2 $^{-/-}$ fathers (F0 generation) and second-generation wild type CXCR2$^{+/+}$ (F2 WT) homozygous CXCR2$^{-/-}$ (F2 HM); heterozygous CXCR2$^{+/-}$ (F2 HT) mice; expressed as $\log_{10}$ of total CFUs. Each data point represents an individual mouse. (**P < 0.05, ***P < 0.005, ****P < 0.0005)

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

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

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

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

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

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

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