Study of the inflammatory activating process in the early stage of Fusobacterium nucleatum infected PDLSCs

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Article

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Abstract

Fusobacterium nucleatum (*F. nucleatum*) is the early pathogenic colonizer of periodontitis, while the host response to this pathogen infection remains unclear yet. In this study, we built the *F. nucleatum* infectious model with human periodontal ligament stem cells (PDLSCs) and showed that *F. nucleatum* could inhibit proliferation, facilitates apoptosis, ferroptosis, and inflammatory cytokines production in a dose-dependent manner. *F. nucleatum* adhesin FadA acted as a proinflammatory virulence factor and increased the expression of IL-1β, IL-6 and IL-8. Further study showed FadA could bind with PEBP1 to active Raf1-MAPK and IKK-NF-κB signaling pathways. Time-course RNA-sequencing analyses showed the cascade of gene activation process of PDLSCs with the increasing duration of *F. nucleatum* infection. NFκB1 and NFκB2 were up-regulated since 3 h of *F. nucleatum*-infection and time serially elevated the inflammatory related genes on the NF-κB signaling pathway. Using computational drug repositioning analysis, we predicted and validated that two potential drugs (piperlongumine and fisetin) could attenuate the negative effects of *F. nucleatum*-infection. Collectively, this study unveils the potential pathogenic mechanisms of *F. nucleatum* and the host inflammatory response at the early stage of *F. nucleatum*-infection.

Introduction

Periodontitis is a widespread chronic immunoinflammatory disease of periodontal tissues, affecting more than 60% of the global adult population[1,2]. The pathogenesis of periodontitis is convoluted, which involves microbial challenges, host genetic variations, and acquired environmental stressors[3,4]. Among the numerous risk factors, the increase of pathogenic microbes in subgingival plaque is widely accepted as a necessary prerequisite for the development of periodontitis. Among which, *Fusobacterium nucleatum* (*F. nucleatum*) is one of the most frequently detected pathogens and is arising more and more attention in recent years as the opportunistic pathogen of many systematic diseases, such as colorectal cancer[5], cardiovascular diseases[6], Alzheimer's disease[7], and adverse pregnancy outcomes[8], ect.

F. nucleatum is an invasive bacterium that can elicit a variety of host responses[9]. Clinical studies showed the prevalence of *F. nucleatum* increased with the severity and progression of periodontitis[10,11]. *F. nucleatum* could invade into various host cells such as epithelial and endothelial cells, monocytes and fibroblasts etc., to initiate a cascade of inflammation and elicit the secretion of pro-inflammatory chemokine IL-6 and IL-8[12,13]. Toxic protein is an important way for bacteria to exert pathogenicity, *F. nucleatum* expresses a variety of virulence factors to elicit various host responses[14]. For instance, RadD and Fap2 induce lymphocyte apoptosis[15], and FadA mediates host-cell binding and invasion in epithelial cells[16,17]. These evidences indicate that *F. nucleatum* might have different pathogenic mechanisms to deliver its pathogenic effect on different cell types.

As a main cell type of the periodontal ligament, periodontal ligament stem cells (PDLSCs) play an indispensable role in maintaining periodontal homeostasis[18]. According to emerging evidence, the

inflammatory environment caused by periodontitis leads to the dysfunction and pyroptosis of PDLSCs[19]. Zhao *et al*. demonstrated that treatment of secondary metabolite butyrate from periodontal pathogens could induce ferroptosis in periodontal ligament fibroblasts and regulate cell survival and death[20]. Nevertheless, the biological characteristic and gene regulation changes of PDLSCs caused by *F. nucleatum* still not fully clarify yet.

In this study, we explored the pathogenic effects of *F. nucleatum* and the host response of PDLSCs at early stage of infection. We evaluated the changes of biological activities in PDLSCs under *F. nucleatum* infection, and illuminated the virulent effect of *F. nucleatum* adhesin FadA. The time serial gene expression analysis was applied to reveal the gene regulation process in response to the infection of *F. nucleatum*. Lastly, the co-expression-based computational drug repositioning was used to identify the drug candidates to attenuate the pathogenic effects of *F. nucleatum* on PDLSCs.

Results

Human PDLSCs from healthy and young volunteers were successfully isolated and cultured as described in **Material and Methods**. The cultured PDLSCs exhibited the spindle-shaped fibroblast-like morphology (Fig. S1a). In the multi-differentiation assay, Alizarin Red–positive mineralized matrix and Oil Red O–positive lipid droplets were observed (Fig. S1b-c). Immunophenotype analysis showed that PDLSCs expressed MSC-specific surface markers, but not hematopoietic or endothelial cell-specific markers (Fig. S1d).

F. nucleatum **inhibits proliferation, facilitates apoptosis, ferroptosis, and inflammatory cytokines production in PDLSCs**

To determine the pathogenic effect of *F. nucleatum* on PDLSCs, we first evaluated the viability of PDLSCs exposed to different MOIs of *F. nucleatum*. The results showed that *F. nucleatum* significantly inhibited the proliferation of PDLSCs in a time- and dose-dependent manner ($p < 0.001$) (Fig. 1a-c). The proliferation was almost blocked at the MOIs of 200 and 400 Next, we evaluated the apoptosis ratios of *F. nucleatum*-infected PDLSCs. As shown in Fig. 1d and Fig. S2, *F. nucleatum* significantly increased the apoptosis rate of PDLSCs in a dose- and time-dependent manner ($p < 0.05$). Notably, early apoptosis mainly occurred at 6 h, while late apoptotic cells account for a substantial part of total apoptotic cells at 24 h and 48 h.

Ferroptosis, a novel described necrotic cell death pathway, is triggered by iron overload[21]. Perturbation of iron homeostasis is one of the major pathogenic strategies for bacterial infection[22]. To investigate whether *F. nucleatum* induced ferroptosis in PDLSCs, we compared the intracellular free iron levels between the normal and *F. nucleatum* infected PDLSCs. Fluorescence intensity of Fe^{2+} was significantly enhanced in the *F. nucleatum*-infected group ($p < 0.05$) (Fig. 1e and g). As iron overload led to mitochondrial dysfunction, which mainly manifested as mitochondrial membrane potential (MitoMP) depolarization[23,24]. Therefore, we evaluated the intracellular MitoMP of PDLSCs using JC-1 fluorescent

dye and observed that *F. nucleatum* treatment reduced the fluorescence intensity of JC-1 aggregates and enhanced the green fluorescence of JC-1 monomers (Fig. 1f-g). Quantitative analysis showed that the JC-1 monomers/aggregates intensity ratio was increased after *F. nucleatum* infection, indicate that *F. nucleatum*-induced iron overload may impair the mitochondrial function in PDLSCs (Fig. 1h). These results firstly showed *F. nucleatum* treatment could increase the intracellular labile iron levels and promote the MitoMP depolarization on host cells.

We next evaluated whether *F. nucleatum* could trigger inflammatory responses in PDLSCs by qRT-PCR and ELISA. As is shown in Fig. 1i, the gene expression levels of *IL-1β, IL-6* and *IL-8* were successively increased with the increase of stimulating time and to the greatest extent at 6 h in a dose dependent pattern, while regressed with the enduring of stimulating time. At the protein level, *IL-1β, IL-6* and *IL-8* were also consistently increased with the stimulating time at the early stage and reached to the top level at 12 h (Fig. 1j). These results suggest the potential immunomodulatory ability of PDLSCs under the stimulation of periodontal pathogens.

FadA activates NF-κB and MAPK signaling pathways via interaction with PEBP1

Fusobacterium adhesin A (FadA) has been reported as one of the most important adhesin and virulence factor of *F. nucleatum*[16]. To explore the molecular mechanism of *F. nucleatum* infection, we investigated the pathogenic effect of FadA on PDLSCs. We obtained recombinant histidine (His)-tagged FadA through an *E. coli* expression system (Fig. S3). Upon addition of 0.5mg/mL FadA protein, mRNA levels of *IL1β*, *IL6* and *IL8* were significantly increased than controls ($p < 0.01$) (Fig. 2a). On protein level, IL1β level was increasing immediately at 1 h, while IL6 and IL8 increased at 3 h after FadA stimulation (Fig. 2b).

Next, we isolated FadA-binding proteins by a His pull-down assay, and identified all pull-down proteins by mass spectrometry. Among the candidates (Table S3), phosphatidylethanolamine-binding protein 1 (PEBP1), also known as Raf1 kinase inhibitory protein (RKIP)[25–27], was proved to directly interact with FadA by Co-IP assay (Fig. 2c).

To study whether FadA induce the inflammatory response by interaction with PEBP1, we firstly evaluated the phosphorylation state of PEBP1, Fig. 2d showed the binding of FadA-PEBP1 phosphorylated PEBP1 at S153. As the devitalization of PEBP1 could activate Raf1 and IKK, we hypothesized that FadA promotes the production of proinflammatory cytokines via activation of NF-κB and MAPK signaling pathways through binding to PEBP1. Western blot showed Raf1 and IKK were both significantly activated, ERK-JNK-p38 MAPKs and NF-κB-p65 were subsequently activated significantly in PDLSCs ($p < 0.01$) (Fig. 2d-e). These findings suggest that FadA act as a pathogenic effectors of *F. nucleatum* and can initiate the intracellular immune signal transduction in PDLSCs.

F. nucleatum **-infection induces the dynamic gene activating process in PDLSCs**

At present, the gene regulation process in oral cells at the early stage of *F. nucleatum* infection is unknown. We performed a time serial RNA-seq analysis of the PDLSCs under the *F. nucleatum* infection for 1h, 3h, 6h and 12h. Principal component analysis (PCA) showed that transcriptomes of the control groups maintained a stable gene expression state, while the *F. nucleatum*-infected groups changed continuously in a particular pattern (Fig. S4). Gene expression profiles of control group and experimental group at 1, 3, 6 and 12 hours were compared by DEseq2, and identified 25, 271, 495 and 619 differentially expressed genes (DEGs) at each time point, respectively (Fig. 3a). Among which, 18, 235, 415, and 495 genes were up-regulated, while 7, 36, 80, and 124 genes were downregulated at each time point, respectively (Fig. 3b). Of note, the Venn's diagram showed that 4 DEGs were consistently up-regulated in *F. nucleatum* stimulated group across the four time points (Fig. 3a and Fig. S5a). Among which, *CXCL1* and *CXCL2* are two vital neutrophil chemo-attractants. The continuous up-regulation of these two chemokines indicated the key role of PDLSCs in recruiting immune cells during *F. nucleatum* infection.

To reveal the gene expression patterns related to the cytological phenotypes altered by *F. nucleatum* stimulation mentioned before, we annotated all the DEGs in KEGG pathway database and obtained 80 proliferation-related, 66 apoptosis-related, 101 ferroptosis-related and 183 inflammation-related DEGs, and respectively clustered into 6, 4, 4 and 8 expression patterns by Mfuzz analysis ($p < 0.01$).

As showed in Fig. 3c, the expression pattern of proliferation-related DEGs could be classified into 3 types: continuous upregulation (clusters 1 and 2, including proliferation-inhibition genes *IFIT3, ING1*, and *PML*), gradual downregulation (cluster 6, including *GDF5* and *SPRY1*), and firstly rising then descending (clusters 3, 4, and 5). Apoptosis-related gene clusters could be classified into 3 types with similar trends to proliferation genes according to the expression patterns. Some apoptosis-induction genes showed a consistently increasing trend, such as *IFI27*, *PML* and *LGALS9*. There were three expression patterns of ferroptosis-related DEGs: continuous upregulation (clusters 1 and 2), gradual downregulation (cluster 4), and gradually decreasing then rising (cluster 3). Notably, genes related to iron metabolism (such as *TFRC*, *ELAVL1*, *FTH1* and *TF*) had similar expression patterns in cluster 1 and 2, while those associated with ROS detoxification (such as *GPX4* and *HMOX1*) had similar expression patterns in cluster 3, suggest that *F. nucleatum* could induce ferroptosis by aggravating intracellular iron overload and inhibiting lipid hydroperoxides detoxification. In respect of inflammation-related DEGs, various proinflammatory cytokines were classified into cluster 1–4 (such as *IL1β*, *IL6*, *CXCL6*, and*CCL2*), which showed a gradual upregulated trend. The expression levels of these DEGs were gradually increased during the infection. Part of the DEGs were validated by qRT-PCR, and the expression were consistent with the RNA-Seq analysis (Fig. S6).

The inflammatory genes are expressed sequentially in response to F. nucleatum-**infection**

To explore the intracellular cascade induced by *F. nucleatum*-infection, we further analyzed the co-expressed genes of each two adjacent time points. As showed in Fig. 4a, 9 members of CXC chemokine subfamily, 11 members of CC chemokine subfamily, and some proinflammatory cytokines formed a co-expression network. Interestingly, all the chemokines in this network are inflammatory chemokines and

are mainly involved in the recruitment of leukocytes to inflamed tissues[28]. Besides, *CCL11* and *CCL20* also have homeostatic functions and act as dual-function chemokines. Inflammatory chemokines *CXCL1*, *CXCL2*, *CCL3L1* and *CCL3L3*, and inflammatory cytokines TNF were first released in response to *F. nucleatum* stimulation. With lastingness of infection, the types and expression levels of chemokines increased gradually, which suggested the recruitment potential of PDLSCs on immune cells.

Additionally, several transcription factors were differentially expressed at 3 h and regulated a series of target genes among DEGs at 3 h, 6 h and 12 h. Notably, NFKB1 and NFKB2, two central activators of genes involved in inflammation and immune function, were significantly up-regulated at 3 h post *F. nucleatum*-infection and sustained activation at 6 h and 12 h. The elevated NFKB1 and NFKB2 sequentially regulated downstream target genes on the NF-κB signaling pathway, such as *MAP3K8*, *NFKBIA*, *REL*, *et al* (Fig. 4b).

To improve the understanding of the biological functions of DEGs, we further performed Metascape analysis and displayed the top 20 enriched clusters in Fig. 5a. The regulation of cytokine production, regulation of MAPK cascade, apoptotic signaling pathway and negative regulation of cell proliferation were highlighted in the network. Consistent with these results, the GO analysis of the DEGs indicated that they were involved in the inflammatory response process (GO:0006954) and chemokine-mediated signaling pathway (GO:0070098) during the whole *F. nucleatum*-infection process. Immune-related processes were changed at the initial phase of infection. With lastingness of infection, the genes were enriched in the apoptotic process (GO:0006915) and negative regulation of cell proliferation (GO:0008285) (Fig. 5b).

The top 10 abundant KEGG pathways displayed in Fig. 5c showed that *F. nucleatum* stimulation mainly resulted in the changes of immune-related pathways. DEGs at 1 h were mainly enriched in cytokine receptor pathway, while at the three other time points, the IL-17, TNF and NF-κB signaling pathways were prominent. Pathview analysis showed the activated genes in TNF, IL-17 and NF-κB signaling pathways. Most of the significantly altered genes were connected with survival and inflammation (Fig. S7). These findings implied that the reactions of PDLSCs to *F. nucleatum* infection were inclined to the recognition of bacterial surface epitopes by host receptors at 1 h, followed by the activation of host defense system within 12 hours after infection. These results collectively indicated that *F. nucleatum* could induce a pro-inflammatory response in PDLSCs associated with the activation of NF-κB signaling pathway and production of inflammatory chemokines.

Screening of miRNAs and transcription factors, and constructing the gene regulatory network of PDLSCs

Next, we sought to identify the miRNAs and their target mRNAs specifically expressed during *F. nucleatum*-infection as described in **Material and Methods**. The differentially expressed miRNA at each time points were listed in Table 1. After matching the differentially expressed miRNA with their predicted target genes, we constructed networks containing 3 up-regulated miRNAs and 1 down-regulated miRNA associated with a total of 22 target genes at 6 h, and 3 down-regulated miRNAs associated with 23 target

genes at 12 h (Fig. 6a). Notably, target genes of miR-4257 were significantly enriched in cysteine-type endopeptodase activity involved in apoptotic process; target genes of miR-4696 were significantly enriched in iron ion homeostasis and positive regulation of MAPK cascade (Fig. S8).

Table 1
Differentially expressed miRNAs at each time points of *F. nucleatum* infected PDLSCs

Time points	Differentially expressed miRNA
1h	hsa-miR-3118, hsa-miR-96-5p
3h	hsa-miR-6747-5p, hsa-miR-5089-3p, hsa-miR-3940-5p, hsa-miR-1181, hsa-miR-12127, hsa-miR-4804-5p, hsa-miR-6853-3p, hsa-miR-6089
6h	hsa-miR-200a-5p, hsa-miR-4257, hsa-miR-4696, hsa-miR-4472, hsa-miR-12127
12h	hsa-miR-4696, hsa-miR-4745-3p, hsa-miR-12127, hsa-miR-3192-5p, hsa-miR-2278

Next, we clustered the DEGs by the similarity of expression pattern to investigate the gene regulation process with the duration of *F. nucleatum* infection. As shown in Fig. 6b, all DEGs were group into 5 clusters, which were named as Module 1- Module 5 (M1-M5): (1) genes in Module 1 were continuously down-regulated during the *F. nucleatum* infection; (2) genes in Module 2 were up-regulated at early stages of *F. nucleatum* infection and down-regulated at following time points; (3) genes in Module 3 was gradually up-regulated within 6 hours after infection; (4) genes in Module 4 were up-regulated after 1 h; (5) and genes in Module 5 was down-regulated between 1 h and 3 h and recover after 3 h (Fig. 6c). In order to reveal the overall regulatory relationships of the *F. nucleatum*-infected PDLSCs, we constructed a regulatory network between modules using high-dimensional ordinary differential equations[29] and showed in Fig. 6d.

On functional level, GO analysis confirmed that genes in M1 were involved in signal transduction, genes in M5 enriched in activating G-protein coupled receptor signaling pathway, and genes in M4 were involved in defense response to virus and innate immune response. To decipher the inter-module regulatory relationships, we integrated the regulatory linkages between the TFs and their target genes, and constructed regulatory networks (Fig. 6e and Fig. S9). Consistent with previous results, NFKB1 was predicted in all the modules. Taken together, these results lent support to the pivotal role of NFKB1, and revealed the gene regulation process at different time points of *F. nucleatum* infection.

Identify the therapeutic target to attenuate the negative effects of F. nucleatum **infection**

We used cogena[30] to perform co-expression analysis and divided the DEGs into three clusters (Fig. 7a). KEGG pathway enrichment analysis for co-expressed genes showed that genes in clusters 1 and 2 were highly enriched in immune-related pathways, while genes in cluster 3 were enriched in calcium signaling pathway and inositol phosphate metabolism (Fig. 7b). Considering the major pathological changes of *F. nucleatum*-infected PDLSCs, we further performed drug repositioning analysis for clusters 1 and 2 to identify potential drug candidates. The list of drug candidates targeting co-expressed genes in clusters 1 and 2 was shown in Fig. 7c. Pathway enrichment analysis of the target genes of each candidate drug was performed to narrow the field of candidates (Fig. S10). Based on the enrichment results, we ultimately selected six drugs and assessed the therapeutic value of these six candidates.

Cytotoxicity assays helped us to select the drug concentrations that cells could tolerant for the follow-up experiments (Fig. S11). *F. nucleatum* infection of PDLSCs after 12 h significantly elevated the mRNA expression of *IL1β*, *IL6* and *IL8* ($p < 0.01$), and all the six candidates significantly reduced the expression level of these inflammatory genes ($p < 0.01$) (Fig. 8a). These results validated the efficacy of our predicted agents.

Among the drugs investigated, piperlongumine and fisetin exhibited the best attenuating effect, this prompted us to further assess their potency in FadA-induced inflammation. Similarly, piperlongumine and fisetin could also significantly decrease the FadA-induced pro-inflammatory cytokines production($p < 0.01$) (Fig. 8b-c). Considering the pro-ferroptotic effects of *F. nucleatum* on PDLSCs, we further examined the effects of piperlongumine and fisetin on ferroptosis. As shown in Fig. 8d-g, piperlongumine and fisetin reversed the trend, reduced the level of intracellular Fe^{2+}, and restored the impaired mitochondrial function. As the IL-17 signaling pathway and NF-κB signaling pathway were enriched in the KEGG analysis, we used molecular docking to simulate the combination of piperlongumine or fisetin with key protein targets of IL-17 and NF-κB signaling pathways. The hub targets predicted of piperlongumine were displayed the 3D results in Fig. 8h.

Discussion

Recent studies indicate that PDLSCs play a crucial role in the maintenance of periodontal homeostasis[31].Normal periodontal milieu is in dynamic equilibrium of cell proliferation and apoptosis[32], and the invasion of periodontal pathogens could impair the self-renewal function of PDLSCs[33]. In our study, we demonstrated that *F. nucleatum* inhibited the cell proliferation and promoted the apoptosis of PDLSCs, and firstly found that *F. nucleatum* could induce ferroptosis through intervening iron metabolism in PDLSCs. As a bacterial virulence strategy, programmed cell death in response to bacterial infection is a complex process, involving in apoptosis, pyroptosis, necroptosis and ferroptosis[34]. Further studies still need to illuminate the intersections between apoptosis and ferroptosis, or with other programmed cell death pathways.

FadA was reported to launch the pathogenic effect of *F. nucleatum* on colorectal cancer cells[16]. In this study, we showed FadA acted as a virulence factor and increased the expression levels of inflammatory

cytokines in PDLSCs. We firstly identified PEBP1 as a FadA-interacted protein and illuminated that binding with FadA could deactivate PEBP1 to activate IKK-NF-κB and Raf1-MAPK signaling pathways. It has been reported that PEBP1 is involved in inflammation-related diseases[35], including autoimmune diseases[36] and antivirus innate immune responses[37]. Our findings further confirmed the pivotal inflammation-regulatory function of PEBP1, and revealed a previously unrecognized molecular mechanism of *F. nucleatum* pathogenicity.

Cytokine secretion is the first wave of host immune response to periodontal pathogens challenge[38]. Our study showed that *F. nucleatum* infection stimulates the secretion of IL-1β, IL-6, and IL-8 at the very early stage. Results of RNA-seq also proved that ample cytokines and chemokines were released at the initial stage of *F. nucleatum* infection. These findings proved that PDLSCs have immunoregulative capacity, and *F. nucleatum* could aggravate periodontal inflammation through impairing the immunosuppressive function of PDLSCs. However, the prolongation of *F. nucleatum* infection could not lead to the continuous secretion of these inflammatory cytokines. This is probably because the limitation of the *in vitro* cell model as it cannot perfectly replicate the conditions found within living organisms. Besides, *F. nucleatum* has limited survival time in aerobic environment[39], which may also limits its pathogenicity.

Drug repositioning is a tool for exploring new uses of approved or investigational drugs. The findings of coexpression-based drug repositioning prediction and experimental validation suggest that piperlongumine and fisetin could be considered as candidate drugs to treat *F. nucleatum*-infected PDLSCs. Our results are of significance as an attempt to identify drugs to treat periodontal pathogen infection, but the evaluation of drug effects requires more profound and systematic research.

In summary, our study provides more specific evidence of the host's early immune response to *F. nucleatum* infection and shows a novel insight into the pathogenic mechanism of periodontitis.

Material And Methods

Bacteria and Cell Culture

This study was approved by the Ethics Committee of Stomatology Hospital of Shandong University (Protocol Number: 20170303) and all volunteers signed the informed consent before providing the oral tissue samples. *Fusobacterium nucleatum* ATCC 25586 was provided by the Shandong Key Laboratory of Oral Tissue Regeneration (Jinan, China). Human PDLSCs obtained from healthy premolars and third molars were freshly extracted as described in the previous study[40].

Cell Phenotype Analysis and Multilineage Differentiation Assays

To identify the cell phenotype of PDLSCs, BD Stemflow^{TM}hMSC Analysis Kit (BD Biosciences, California, USA) was implemented according to the manufacturer's instructions. For multilineage differentiation

assays, PDLSCs were cultivated in 6-well plates at 2×10^5 cells/well. At 80–90% density, the corresponding differentiation medium was replaced to assess osteogenesis and adipogenesis. After 21 days, the cells were stained by Alizarin Red (Sigma-Aldrich, Missouri, USA) to observe the mineralization. After 28 days, the adipogenesis was detected by Oil Red O (Sigma) staining.

Cell Viability Assays and Cell Apoptosis Analysis

The number of cells was counted using Countstar (Shanghai, China). The proliferation rate was detected using an EdU Apollo DNA *in vitro* kit (RiboBio, Guangzhou, China) and observed by a fluorescent microscope. Cell viability was estimated by Cell Counting Kit-8 (Boster, Wuhan, China). In accordance with the manufacturer's instructions, Annexin V-FITC/PI double staining kit (Dojindo, Kumamoto, Japan) was used to detect apoptosis.

Enzyme-Linked Immunosorbent Assay (ELISA)

The proinflammatory cytokine concentrations were evaluated using the specific ELISA kits (Biolegend, California, USA). The optical density values were measured at 450 nm and 570 nm by a microplate reader.

Real-time quantitative PCR Analysis

Total RNA was isolated with TRIzol reagent (CWBIO, Beijing, China), and cDNA was reverse transcribed using a HiFiScript cDNA Synthesis kit (CWBIO). Real-time quantitative PCR (qRT-PCR) was performed using UltraSYBR Mixture (CWBIO). The relative mRNA expression levels were analyzed by the $2^{\wedge(-\Delta\Delta ct)}$ method and normalized by the GAPDH level. The sequences of the primers used in the experiment are listed in **Table S1**.

Western Blot Analysis

Cells were lysed in a RIPA lysis containing PMSF (Solarbio, Beijing, China). Protein concentration was measured with a BCA Protein Assay Kit (Cwbio). Equivalent amounts of proteins were loaded onto SDS-PAGE gels and transferred to PVDF membranes (Millipore, Massachusetts, USA). After blocked with 5% milk in TBST and incubated with primary antibodies listed in **Table S2** overnight, the membranes were incubated with HRP-conjugated secondary antibodies (Proteintech, Indiana, USA). The immunoreactive bands were visualized by an Immobilon Western HRP Substrate (Millipore) and determined using ImageJ gel analysis software.

Detection of intracellular Fe2 + amount and Mitochondrial Membrane Potential (MitoMP) Assessment

Intracellular Fe^{2+} levels were examined using FerroOrange (Dojindo) according to the manufacturer’s instructions. Mitochondrial membrane potential was detected by a MitoMP assay Kit with JC-1 (Solarbio). The fluorescent intensity was measured using the EnVision multimode microplate reader (PerkinElmer, Massachusetts, USA). The fluorescence images were obtained by Dragonfly 200 high speed confocal microscope (Andor Technology, Belfast, UK).

Recombinant Protein Production and Purification

FadA was purified as previously described[17,41]. The entire *fadA* gene of *F. nucleatum* ATCC 25586 was synthesized by Sangon Biotech (Shanghai, China). After verification, the prokaryotic expression vector was transformed into *E. coli* BL21(DE3). *E. coli* was grown in LB medium to an OD_{600} of 0.6. Then the cultures were induced by 0.5 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) (Aladdin, Shanghai, China) for 2.5 h. An His-tag Protein Purification Kit (Byotime, Shanghai, China) was used for FadA purification, and Amicon® Ultra-15 Centrifugal Filters (Millipore) were used for desalting, diafiltration and concentrated.

Co-immunoprecipitation (Co-IP) Assay

To check whether FadA/PEBP1 interaction occurs *in vivo*, PDLSCs were preincubated with FadA. Total protein from PDLSCs was extracted using NP-40 solution (Boster) containing 1mM PMSF. 1,000 μg of cell lysate was incubated with 5 μg anti-His antibody (Proteintech) or IgG (Santa Cruz Biotechnology, Texas, USA) at 4 ℃ overnight. The protein complex was captured overnight by Protein A/G agarose (Santa Cruz Biotechnology) at 4 ℃. The beads were collected by centrifugation at 12,000×g, followed by 3 washes and Western blot analysis.

RNA-Sequencing Analysis

45 samples from 5 individuals co-cultured without or with *F. nucleatum* at an MOI of 100) for 0, 1, 3, 6 and 12 hours were analyzed by RNA-Sequencing (RNA-seq) at LC-BIO (Hangzhou, China). The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021)[42] in National Genomics Data Center (Nucleic Acids Res 2022)[43], China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-human: HRA002672) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human. R package DESeq2 (version)[44] was used for screening differential expression genes by setting statistical significance value (*P*-value) < 0.01 and absolute value of $\log_2$ (fold change) > 1. We used R package Mfuzz (version 2.50.0)[45] to classify the gene expression clusters. DAVID[46] and R package clusterProfiler (version 3.18.1)[47] were used for GO and KEGG enrichment analysis. R Pathview (version 1.30.1)[48] was used to visualize significant KEGG signaling pathways.

Construction of miRNA-mRNA Network and Regulatory Network

The has.gff3 annotation files were downloaded from the miRbase database, and the microRNA expression was obtained using FeatureCounts. R package DESeq2 (version) was used for screening differential expression miRNAs by setting statistical significance value (*P*-value) < 0.05. The potential target genes of miRNAs were predicted by miRWalk2.0. The miRWalk, miRanda, miRMap and Targetscan database were added to help predicting supposed target genes.

The module regulatory relationships were calculated using the reported method[29].We used TRRUST[49] to explore the TF targets and used RegNetwork[50] to construct a TF-miRNA-gene regulatory network.

Computational Drug Repositioning Analysis

The drug repositioning for the coexpressed genes were performed using the cogena package (version 1.24.0)[30]. The SwissTargetPrediction database was used to predict potential effector targets[51]. The SMILES format of candidate drugs obtained from ZINC15 was inputted into this database to obtain the potential effector targets of the candidate drugs.

We performed molecular docking using the program AutoDock Vina (version 1.1.2)[52]. The 3D structure of the candidate drugs was obtained from the ZINC15 and the structures of target proteins were obtained from PDB database or uniport database. AutoDockTools (version 1.5.6) was used to process the ingredients and protein structures. PyMOL (version 4.6.0) was used to visualize the combinations.

Statistical Analysis

All experiments were repeated independently at least three times with cells from three volunteers, and the data were plotted as mean ± standard deviation (SD). Data were analyzed using GraphPad Prism (version 8.0). Differences among multiple groups were assessed using one-way or two-way ANOVA followed by Tukey's honestly significant difference comparison test. Differences were considered statistically significant at $p < 0.05$.

Declarations

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Conflicts of interests

All authors declare that they have no competing interests.

Authors' contribution

Q.F. and X.X. designed and supervised this study. X.M.M. and S.S. performed the sample collection. Y.S.W., Z.X.L., X.F.G. and A.P. performed experiments. L.H.W. and T.Y.S. analyzed the data and plotted Figures. Y.S.W. and Q.F. wrote and edited the manuscript. All the authors reviewed the manuscript.

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Figures

Figure 1

F. nucleatum inhibits proliferation, facilitates apoptosis, ferroptosis, and inflammatory cytokine production in PDLSCs

(a) Cell-counting assay of PDLSCs cocultured with or without *F. nucleatum*. (b-c) Cell proliferation rate of PDLSCs detected by EdU assay. Scale bar: 100 μm. (d) Cell apoptosis examined using Annexin V/PI staining. (e) Intracellular Fe^{2+} detected by FerroOrange. Scale bar: 20 μm. (f) Confocal images of JC-1 in PDLSCs. Scale bar: 20 μm. (g) Quantitative assessment of FerroOrange and JC-1 fluorescence. (h) The ratio of JC-1 monomers/aggregates. (i) The mRNA expression detected by qRT-PCR. (j) Protein levels measured by ELISA. Data were expressed as mean ± SD. (n=3) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared with the control group).

Figure 2

F. nucleatum adhesin FadA promotes inflammatory response via interaction with PEBP1

(a) The mRNA expression detected by qRT-PCR. (b) Protein levels measured by ELISA. (c) Co-IP analysis showed the interaction between FadA and PEBP1. (d) Effects of FadA on the phosphorylation of Raf1, IKK, PEBP1, p38, JNK, ERK, and NF-κB p65 detected by Western blot analysis. Quantitative analysis of altered protein expression of these proteins used ImageJ. (e) Schematic diagram of the FadA-PEBP1-Raf1-MAPK pathway and FadA-PEBP1-IKK-NF-κB pathway. Created with BiorRender.com. Data were expressed as mean ± SD (n=3) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared with the control group).

Figure 3

Dynamic gene expression patterns of PDLSCs under F. nucleatum Infection

(a) Venn diagram depicted the extent of overlap between DEGs at different time points in PDLSCs. (b) The histograms of upregulated (red) and downregulated (blue) DEGs at each time point. (c) Mfuzz analysis clustered (1) proliferation-related, (2) apoptosis-related, (3) ferroptosis-related and (4) inflammation-related DEGs into different classes.

Figure 4

Gene regulation process in response to *F. nucleatum* infection in PDLSCs

(a) Co-expression network of DEGs showed the intracellular cascade induced by *F. nucleatum*-infection. (b) Transcription factors NFKB1 and NFKB2 up-regulated multiple DEGs.

Figure 5

GO and KEGG pathway enrichment analysis of differentially expressed genes.

(a) Network of enriched terms colored by cluster identity. (b) GO and (C) KEGG pathway analysis of DEGs of 1 h, 3 h, 6 h, and 12 h.

Figure 6

Construction of miRNA network and transcription factors network

(a) miRNA-mRNA regulatory network. (b) K-means method clustered the DEGs into 5 modules. (c) Gene expression trends in 5 modules. Red arrows represent positive regulation, and blue arrows represent negative regulation. (d) The functional linkages between the modules. (e) Regulatory networks of module 1 and module 5.

Figure 7

Drug repositioning based on transcriptome revealed candidate drugs

(a) K-means method clustered the DEGs of 1 h, 3 h, 6 h and 12 h into 3 clusters. (b) KEGG pathway analysis for coexpressed genes generated by cogena. (c) Computational drug repositioning for the coexpressed genes. Drug candidates target DEGs in (1) cluster 1 and (2) cluster 2.

Figure 8

Experimental validation of the candidate drugs

(a) The anti-inflammation effect of candidate drugs detected by qRT-PCR. (b) QRT-PCR and (c) ELISA showed piperlongumine and fisetin suppressed the expression of inflammatory cytokines. (d) Piperlongumine and fisetin reversed the increased intracellular Fe^{2+} induced by *F. nucleatum* stimulation. Scale bar: 20 μm. (e) Effect of piperlongumine and fisetin on the mitochondrial membrane potential. Scale bar: 20 μm. (f) Quantitative assessment of FerroOrange and JC-1 fluorescence. (g) The ratio of JC-1 monomers/aggregates. (h) Molecular docking between piperlongumine and main targets. Data were expressed as mean ± SD. (n=3) ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, compared with the control group; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$).

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