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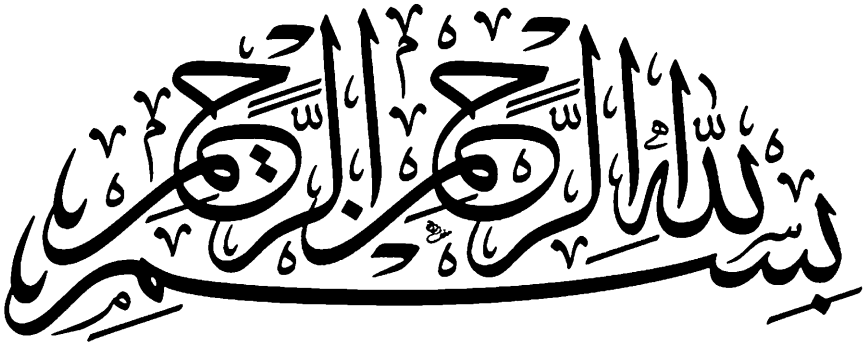

“In the name of God, Most Gracious, Most Merciful"

To my dear parents
To my beloved husband Ahmed
To my lovely kids, Yakoot and Lulu

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ABSTRACT

Cyclic dinucleotides, cyclic di-adenosine monophosphate (c-di-AMP) and cyclic di-guanosine monophosphate (c-di-GMP) are bacterial signaling molecules essential for regulating various bacterial cellular processes. Host cells can recognize cytosolic nucleic acids and cyclic dinucleotides through a stimulator of interferon genes (STING). Such recognition can stimulate the immune response by activating the production of interferons and other pro-inflammatory cytokines. Nevertheless, the impact of bacterial cyclic dinucleotides on the cellular response of gingival cells remains unknown.

The objectives of this PhD study series were to 1) investigate the regulatory roles of bacterial cyclic dinucleotides and *Porphyromonas gingivalis* (*Pg*) lipopolysaccharides (LPS) on the cellular response of human gingival keratinocytes (HMK) and human gingival fibroblasts (HGFs), 2) evaluate cyclic dinucleotide-mediated activation of STING/TANK-binding kinase1 (TBK1)/interferon regulatory factor 3 (IRF3) pathway in HMK cells, and 3) examine the effects of cyclic dinucleotides solely or combined with *Pg* LPS on gingival fibroblasts' proteome response by using global proteomics analysis.

In the experiments, HMK and HGF cells were stimulated with (100, 10, 1 μM) of c-di-AMP and c-di-GMP. Cytokine expressions of HMK cells and cytokine and matrix metalloproteinase expressions of HGF cells were evaluated by the Luminex technique. Expressions of mitogen-activated protein kinases and STING/TBK1/IRF3 pathway activation in HMK cells and HGFs were measured with western blot. The gingival fibroblasts' proteome response against bacterial cyclic dinucleotides was studied using proteomics analysis.

Our results showed that cyclic dinucleotides can either enhance or inhibit LPS-regulated HMK cytokine response, and this variation depends on the type of cytokine. Moreover, STING/TBK1/IRF3 pathway operates within HMK cells and can be regulated by bacterial cyclic dinucleotides. In HGFs, cyclic dinucleotides interacted with *Pg* LPS to control the early cellular response, while *Pg* LPS predominantly influenced the late cellular response. Bacterial cyclic dinucleotides solely or with *Pg* LPS upregulated innate immunity-related and interferon signaling-related proteins in HGFs, in addition to controlling various other critical processes.

The results of this PhD project suggest that bacterial cyclic dinucleotides, which are previously unstudied components of periodontal bacteria, can regulate initial cellular response of gingival cells through STING/TBK1/IRF3 pathway. A thorough description of cyclic dinucleotide-regulated cellular responses in periodontium may allow us to design cyclic dinucleotide analogues that can function as STING-agonists, antagonists, and potential therapeutics.

KEYWORDS**:** cyclic dinucleotides, cyclic di-adenosine monophosphate, cyclic di-guanosine monophosphate, lipopolysaccharides, *Porphyromonas gingivalis*, STING/TBK1/IRF3, human gingival keratinocytes, human gingival fibroblasts, cellular response, proteomic analysis, interferon signaling.

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TIIVISTELMÄ

Sykliset dinukleotidit, syklinen di-adenosiinimonofosfaatti (c-di-AMP) ja syklinen di-guanosiinimonofosfaatti (c-di-GMP) ovat bakteerien signalointimolekyylejä, jotka ovat olennaisia monenlaisten bakteerien soluprosessien säätelyssä. Isäntäsolut kykenevät tunnistamaan sytosoliset nukleiinihapot ja sykliset dinukleotidit interferonigeenien stimulaattorin (STING) kautta. Tällainen tunnistus voi stimuloida immuunivastetta aktivoimalla interferonien ja muiden proinflammatoristen sytokiinien tuotantoa. Bakteerien syklisten dinukleotidien vaikutus iensolujen soluvasteeseen on kuitenkin edelleen tuntematon.

Tämän väitöskirjatutkimuksen tavoitteina oli tutkia bakteerien syklisten dinukleotidien ja *Porphyromonas gingivalis* bakteerin lipopolysakkaridien (*Pg*-LPS) säätelytehtävää ihmisen ienkeratinosyyttien (HMK) ja ienfibroblastien (HGF) soluvasteessa sekä arvioida syklisen dinukleotidin välittämää STING/TANK-sitovan kinaasi1 (TBK1)/interferonin säätelytekijä 3 (IRF3) -reitin aktivointia HMK-soluissa. Näiden lisäksi tutkittin syklisen dinukleotidin vaikutusta joko yksinään tai yhdessä *Pg*-LPS:en kanssa ienfibroblastien proteomivasteeseen käyttäen globaalia proteomistaanalyysiä.

Kaikissa kokeissa HMK- ja HGF-soluja stimuloitiin c-di-AMP- ja c-di-GMP- dinukleotideillä (pitoisuudet 100, 10 ja 1 μM). HMK-solujen sytokiini-ilmentymiä ja HGF-solujen sytokiini- ja matriisimetalloproteinaasi-ilmentymiä mitattiin Luminex-tekniikalla. HMK- ja HGF-solujen mitogeenien aktivoimia proteiinikinaaseja ja STING/TBK1/IRF3-reitin aktivointia mitattiin immunobloteilla. Ienfibroblastien proteomivastetta bakteerien syklisiä dinukleotideja vastaan tutkittiin proteomisella analyysillä.

Tulokset osoittivat, että syklisten dinukleotidien tyypistä riippuen ne kykenevät joko lisäämään tai estämään LPS:en sytokiinivastetta. Sen lisäksi osoittautui, että STING/TBK1/IRF3-reitti toimii HMK-soluissa ja sitä voivat säädellä bakteerien sykliset dinukleotidit. HGF-soluissa sykliset dinukleotidit vuorovaikuttivat *Pg*-LPS:en kanssa kontrolloiden varhaista soluvastetta, kun taas yksinään *Pg*-LPS vaikutti pääasiassa myöhäiseen soluvasteeseen. Bakteerien sykliset dinukleotidit yksinään tai yhdessä *Pg*-LPS:en kanssa säätelevät synnynnäiseen immuniteettiin ja interferonisignalointiin liittyviä proteiineja sekä kontrolloivat useita muita kriittisiä prosesseja HGF-soluissa.

Tämän väitöskirjatutkimuksen mukaan aiemmin tutkimattomat parodontaalibakteerien komponentit, sykliset dinukleotidit, voivat säädellä iensolujen alkuvaiheen soluvastetta STING/TBK1/IRF3-reitin kautta. Perusteellinen kuvaus syklisten dinukleotidien säätelyvaikutuksista iensolujen soluvasteeseen saattaa mahdollistaa syklisten dinukleotidianalogien suunnittelun. Tämänkaltaiset analogit voisivat toimia STING-agonisteina, -antagonisteina ja mahdollisina terapeuttisina aineina.

AVAINSANAT: sykliset dinukleotidit, syklinen di-adenosiinimonofosfaatti, syklinen di-guanosiinimonofosfaatti, lipopolysakkaridit, *Porphyromonas gingivalis*, STING/TBK1/IRF3, ihmisen ienkeratinosyytit, ihmisen ienfibroblastit, soluvaste, proteominen analyysi, interferonisignalointi.

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Abbreviations

AMP	adenosine monophosphate
ANOVA	analysis of variance
B2M	Beta-2-microglobulin
c-di-AMP	cyclic di-adenosine monophosphate
c-di-GMP	cyclic di-guanosine monophosphate
cGAMP	cyclic GMP-AMP
cGAS	cyclic guanosine monophosphate adenosine monophosphate synthase
DGC	diguanylate cyclases
DGK	diacylglycerol kinase
DMSO	dimethyl sulfoxide
dsDNA	double-stranded DNA
ECM	extracellular matrix
ENSA	α-endosulfine
ER	endoplasmic reticulum
ERKs	extracellular signal-regulated kinases
FILIP1L	filamin A interacting protein 1 like
G3PP	glycerol-3-phosphate phosphatase
GMP	guanosine monophosphate
GNG12	guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12
h	hour
HGFs	human gingival fibroblasts
HLA-A	MHC class I antigen
HMK	human gingival keratinocytes
HRP	horseradish peroxidase
IFIT	interferon-induced protein with tetratricopeptide repeats
IFNs	interferons
IKK	IκB kinase
IL	interleukin
IPA	ingenuity pathway analysis
IRF3	interferon regulatory factor 3

ISG15	ubiquitin-like protein ISG15
JNKs	c-Jun amino terminal kinase
LFQ	label-free quantitation
LPS	lipopolysaccharides
MAMPs	microbe-associated molecular patterns
MAPKs	mitogen-activated protein kinases
MCP-1	monocyte chemoattractant protein1
MHC	major histocompatibility complex
MMPs	matrix metalloproteinases
MX	interferon-induced GTP-binding protein
NFKB2	NF-kappa-B p100 subunit
NF-κB	nuclear factor-κB
NK	natural killer cell
OAS3	2'-5'-oligoadenylate synthetase 3
PBS	phosphate-buffered saline
PCA	principal component analysis
pg	picogram
Pg	*Porphyromonas gingivalis*
PLS-DA	partial least squares discriminant analysis
PRRs	pattern recognition receptors
RECON	aldo-keto reductase domain of oxidoreductase
SAMHD1	deoxynucleoside triphosphate triphosphohydrolase
SERPINB2	plasminogen activator inhibitor 2
SOD2	superoxide dismutase
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
TAP1	transporter associated with antigen processing 1
TBK1	TANK-binding kinase1
TIMPs	tissue inhibitors of metalloproteinase
TLRs	toll-like receptors
TNF	tumor necrosis factor
TNFRSF11B	tumor necrosis factor receptor superfamily member 11B
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
UBE2L6	ubiquitin conjugating enzyme E2L6
ULK1/ATG1	UNC-51-like kinase
VEGF	vascular endothelial growth factor
VIP	variable importance plot
°C	degree of Celsius

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Elmanfi S**, Zhou J, Sintim HO, Könönen E, Gürsoy M, Gürsoy UK. Regulation of gingival epithelial cytokine response by bacterial cyclic dinucleotides. *Journal of Oral Microbiology* 2018; 11(1):1538927.

II **Elmanfi S**, Sintim HO, Zhou J, Gürsoy M, Könönen E, Gürsoy UK. Activation of gingival fibroblasts by bacterial cyclic dinucleotides and lipopolysaccharide. *Pathogens* 2020; 9(10):792.

III Onyedibe KI, **Elmanfi S**, Aryal UK, Könönen E, Gürsoy UK, Sintim HO. Global proteomics of fibroblast cells treated with bacterial cyclic dinucleotides, c-di-GMP and c-di-AMP. *Journal of Oral Microbiology* 2021; 14(1):2003617.

IV **Elmanfi S**, Onyedibe KI, Aryal UK, Könönen E, Sintim HO, Gürsoy UK. Activation of cellular responses by cyclic dinucleotides and *Porphyromonas gingivalis* lipopolysaccharide: A proteomic study on gingival fibroblasts. *Journal of Oral Microbiology* (in press).

Additionally, some unpublished data are presented.

1 Introduction

Gingiva, among the initial lines of defense in the oral cavity, is a barrier against continuous microbial exposure (Moutsopoulos & Konkel, 2018; Takahashi et al., 2019). Gingival cells participate in periodontal tissue repair and regeneration, maintain homeostasis, and modulate immune response by sensing the shifts in microbial imbalance (Lee &Yilmaz, 2021; Ahangar et al., 2020). Gingival cells can recognize bacteria and their by-products by pattern recognition receptors (PRRs). These receptors initiate the innate immune response by activating the expression of pro-inflammatory cytokines and chemokines (Song et al., 2017).

The stimulator of interferon genes (STING) protein is a PRR, which is a critical mediator for modulating immune responses. Through STING, host cells can recognize cytosolic nucleic acids and cyclic dinucleotides (Guimarães et al., 2021; Danilchanka & Mekalanos, 2013). Cyclic dinucleotides are composed of cyclic di-adenosine monophosphate (c-di-AMP), cyclic di-guanosine monophosphate (c-di-GMP), and 3′,3′-cyclic GMP-AMP (3′3′ cGAMP) (Moradali et al., 2022), and regulate various central bacterial processes, such as bacteria signal transduction, bacterial homeostasis, biofilm formation and virulence (Guey & Ablasser, 2019; Yan & Chen, 2021). Recognition of cyclic dinucleotides by STING receptor stimulates the immune response by activating the production of interferons (IFNs) and other pro-inflammatory cytokines (Yan & Chen, 2021). Recent studies suggested that the immunostimulatory function of these molecules might allow them to be used as potential molecular adjuvants to induce innate and adaptive immune responses (Gogoi et al., 2020; Yan & Chen, 2021).

The current PhD work aimed to investigate the regulatory roles of cyclic dinucleotides in the cellular response of human gingival keratinocytes (HMK) and human gingival fibroblasts (HGFs), study the combined effect of cyclic dinucleotides and *Porphyromonas gingivalis* (*Pg*) lipopolysaccharides (LPS) on the protein expression of HGFs profiles, and examine the role of STING in gingival cells in terms of microbe-associated molecular patterns (MAMPs).

2 Review of the Literature

2.1 Structure of gingiva

2.1.1 Gingival epithelium and epithelial cells

Periodontium comprises four tissue types: gingiva, periodontal ligament, alveolar bone, and cementum (Koller & Sapra, 2023). The gingiva comprises epithelial and connective tissues (Groeger & Meyle, 2019). The stratified squamous epithelium of gingiva is the initial line of defense against continuous bacterial exposure and plays an essential function in initiating and remission of periodontal immune responses (Takahashi et al., 2019; Fujita et al., 2018). Indeed, uncontrolled and chronic cellular inflammatory responses of gingiva can lead to aggravation of periodontal disease (Koller & Sapra, 2023). The stratified squamous epithelium of gingiva consists of three different structures: junctional epithelium, sulcular epithelium, and oral gingival epithelium (Takahashi et al., 2019). Sulcular epithelium can be orthokeratinized or parakeratinized stratified squamous epithelium and not attached to the tooth surface (Gibbs et al., 2019). The junctional epithelium is a non-keratinized epithelium that attaches directly to the tooth surface at the bottom of the gingival sulcus via hemidesmosomes and forms a barrier to protect periodontal tissues from mechanical trauma and bacterial infections (Gibbs et al., 2019). The gingival epithelium is a keratinized epithelium and acts as a barrier against microbial challenge. Gingival keratinocytes are interconnected by intercellular bonds known as cell-cell junctions. These cell-to-cell junctions are classified as tight, adherens junctions, and gap junctions (Groeger & Meyle, 2019). Tight junctions contain various proteins, including occludin, claudins, and zonula occludens, and have semipermeable properties to transport ions, water, and solutes between the cells (Takahashi et al., 2019). Adherens junctions can initiate and stabilize cell-cell adhesion and regulate the actin cytoskeleton, transcriptional regulation, and intracellular signaling (Hartsock & Nelson, 2008; Campbell et al., 2017). Gap junctions are a group of intercellular channels connecting two adjacent cells through the cytoplasm to mediate intercellular communication and allow the permeability of various molecules, such as small metabolites, ions, and intracellular signaling molecules (Zappitelli & Aubin, 2014; Liu et al., 2020). In addition, gap junctions

play pivotal roles in various physiological processes, including homeostasis, regeneration, and developmental processes (Peiris & Oviedo, 2013).

2.1.2 Connective tissue and fibroblasts

The gingival connective tissue (lamina propria) consists of three components: collagen fibers comprising 60%, ground substance comprising 35%, and cells comprising 5% (Koller & Sapra, 2023). Collagen is involved in the integrity of gingival connective tissue. The most prominent type of collagen in gingiva is type I collagen. Other than that, type III collagen is mainly located underlie the gingival epithelium, and type IV collagen is a supplier to the gingival mucosa associated with the gingival basement membranes and the blood vessels (Koller & Sapra, 2023). Connective tissue proteoglycans regulate the flow and diffusion through the tissue matrix. Conversely, glycoproteins keep the structural integrity of connective tissue, both form the ground substance of gingival connective tissue (Koller & Sapra, 2023). Gingival connective tissue cells include fibroblasts, mast cells, macrophages, endothelial cells, and inflammatory cells (Koller & Sapra, 2023). Gingival fibroblasts are prevalent cells and the main laborer in connective tissues (Naruishi, 2022). Fibroblast functions include forming collagen fibers and ground substances in connective tissue, contraction, locomotion, and synthesis and degradation of the extracellular matrix (ECM) (Plikus et al., 2021; Bautista-Hernández, 2017). Gingival fibroblasts secrete various pro-inflammatory cytokines, including interleukins ((IL)-1β, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α) (Makkar et al., 2022). Synthesis and regulation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) by gingival fibroblasts have a role in maintaining ECM remodeling and homeostasis (Herath et al., 2013).

2.2 Role of gingival cells in periodontal health and diseases

2.2.1 Regenerative ability of gingival cells

The epithelium acts as a barrier to protect the underlying tissues from environmental stress, to encounter the vascularized connective tissue responsible for the support and nutrients to the gingiva, and to regenerate in response to injuries or damage (Groeger & Meyle, 2019; Gibbs et al., 2019). Indeed, the epithelium controls the communication between mucosal immune cells and microbes of the outer environment. In inflammatory diseases or mechanical injury, epithelium gets damaged, and a mucosal barrier is disrupted (Leoni et al., 2015). Distinct biological pathways are activated following tissue injury to prevent infection and stimulate

repair. Immune cells, endothelial cells, keratinocytes, and fibroblasts are recruited during wound healing with alternation in their gene expression, cell proliferation, differentiation, and migration (Smith et al., 2015). Gingival wound healing includes homeostasis, cell migration, proliferation, and ECM deposition and remodeling (Alfonso García et al., 2020).

Human gingival epithelial cells are highly proliferating cells. Cells of basal layers of gingival epithelium proliferate rapidly, followed by differentiation and keratin expression of keratinocytes. Afterward, keratinocytes move upwards, change to flattened cells, and lose their nuclei (Takahashi et al., 2019; Gibbs et al., 2019; Groeger & Meyle, 2019). The response of epithelial cells to damage starts with the migration of keratinocytes over the wound, as epithelial cells change their morphology to elongated migrating keratinocytes. Basal epithelial cells adjacent to migrating cells start proliferating and providing new cells to repair the wound (Smith et al., 2015). Indeed, various factors in the oral cavity, including saliva, crevicular fluid, moisture, temperature, and commensal oral biofilms, support keratinocyte proliferation, stratification, and expression of host defense proteins (Gibbs et al., 2019). Beneficial bacteria can maintain the gingival epithelial barrier by enhancing the synthesis of antimicrobial peptides and stimulating the immune response (Takahashi et al., 2019).

Gingival fibroblasts are the most common cell type in gingival tissue, maintain the integrity of periodontal tissue, and participate in wound healing and tissue regeneration (Alfonso García et al., 2020; Wielento et al., 2023). Circulating molecules, such as hormones, growth factors, and cytokines influence the behavior and control the migration of gingival fibroblasts (Uitto & Larjava, 1991). Various proteins, such as platelet-derived growth factor, transforming growth factor beta-1, vascular endothelial growth factor (VEGF), insulin-like growth factor-1, and epidermal growth factor, that are produced by gingival fibroblasts can accelerate the process of tissue regeneration (Dereka et al., 2006; Anitua et al., 2005; Anitua et al., 2008). MMPs are also involved in the remodeling of ECM components that are necessary for tissue regeneration (Kandhwal et al., 2022). ECM components are essential for tissue regeneration and wound healing. Of these components, fibronectin mediates cell adhesion, proliferation, and motility (González-Tarragó et al., 2017). After wounding, due to fiber differentiation, the lamina propria of gingiva regenerates promptly compared to specific cells (Melcher, 1976). Communication of gingival fibroblasts with adjacent cells, immune cells, or other gingival tissue components regulates the regenerative response (Alfonso García et al., 2020). Interaction between the cells and ECM is essential for epithelial cell migration by subjecting keratinocytes to a group of ECM components such as type I collagen, polymerized fibrin, and plasma fibronectin (Smith et al., 2015).

2.2.2 Homeostasis between gingival cells and oral bacteria

The healthy oral cavity contains over 700 oral bacterial species that perform various indispensable functions for the host (Deo & Deshmukh, 2019). In health, the host-microbial interface should be stable to maintain gingival homeostasis (Deo & Deshmukh, 2019).

Maintaining homeostasis requires crosstalk between oral bacteria, immune cells, and the epithelium (Lin et al., 2021). Barrier immunity is not achieved in the absence of bacteria. On the contrary, healthy host-microbe interaction dictates the host immune system to keep the para-inflammatory state to respond to pathogenic insult and restore homeostasis (Medzhitov, 2008; Chen & Xu, 2015; Fine et al., 2016).

Even though the oral cavity is continuously exposed to environmental stressors, epithelial tissues can keep homeostasis between the host tissues and microbes (Lamont et al., 2018). The constant transit of neutrophils contributes to host protection and homeostasis by monitoring the quality and quantity of dental biofilm (Tonetti et al., 1998; Darveau, 2009). Moreover, epithelial antimicrobial peptides can maintain homeostasis by inhibiting the growth of pathogens (Luong et al., 2020).

Commensal bacteria such as *Streptococcus sanguinis, Streptococcus cristatus, Streptococcus salivarius, Streptococcus mitis, Actinomyces naeslundii, Lactococcus lactis,* and *Haemophilus parainfluenzae* can also sustain oral health (Sedghi et al., 2021). Although epithelial cells preserve a balanced relationship with resident microbiota, some microorganisms can create adaptive strategies to colonize and permeate to deeper tissues (Ribet & Cossart, 2015; Lee & Yilmaz, 2021). The epithelial barrier can be affected by the bacteria and their by-products that change its gene and protein expressions that are related to its barrier and immune response-regulating functions (Takahashi et al., 2019).

2.2.3 Recognition of bacterial components by gingival cells

The first line of defense against invading pathogens is stimulating cellular immune response through pathogen recognition and activating signaling pathways and effector mechanisms (Bautista-Hernández et al., 2017). Host cells stimulate host defense against pathogens' invasion by recognizing MAMPs mediated by PRRs (Hajishengallis & Lambris, 2011). This recognition is the base of the early host defense against infections that stimulate the production of IFNs and inflammatory cytokines, followed by triggering the adaptive immune response (Li & Wu, 2021). MAMPs can be distributed on bacterial cell surfaces as peptidoglycans, lipoproteins, and LPS and can be intracellular as specific motifs of RNA and DNA derived from microbes (Uehara & Takada, 2007). Gingival cells (immune and non-immune cells) express toll-like receptors (TLRs) to recognize microbes and their by-products. TLR-

induced inflammatory response activates the expression of inflammatory cytokines (Akira et al., 2006).

Microbial nucleic acids can be detected by various receptors based on their location, and this recognition is classified into two groups: TLR-dependent and TLR-independent pathways. TLRs include 13 transmembrane receptors that can be found in the plasma membrane, endoplasmic reticulum (ER), lysosomes, and endosomes of immune cells (including monocytes, macrophages, dendritic cells, and B cells) and non-immune cells (such as keratinocytes, epithelial cells, and fibroblasts) (Pandey et al., 2014; Kawasaki et al., 2011; Wicherska-Pawłowska et al., 2021; Kong et al., 2023; Crump & Sahingur, 2016). TLR stimulation triggers the production of cytokines, major histocompatibility complex (MHC), and other costimulatory molecules to induce the immune response (Duan et al., 2022). TLRs can also stimulate innate and adaptive immune responses by binding to endogenous molecules from degenerated tissues (Yu et al., 2010). MAMP recognition by TLRs activates subfamilies of mitogen-activated protein kinases (MAPKs). MAPKs include extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal (JNKs), and p38 kinases (Duan et al., 2022). MAPK, serine/threonine protein kinases biosynthesis of proinflammatory cytokines and chemokines important for the immune response to counter infections and kill pathogens (Kirk et al., 2020; Li et al., 2012).

TLR-independent pathways that can detect nucleic acids in the cytoplasm include nucleotide-binding domain and leucine-rich-repeat-containing receptors, RNA polymerase III, a retinoic acid-inducible gene I, melanoma differentiation-associated gene 5, mitochondrial antiviral signaling protein, absent in melanoma 2, cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS), and STING (Cui et al., 2014; Elmanfi et al., 2021).

2.2.4 Contribution of gingival cells to immune-inflammatory process

Periodontitis-associated pathogens contribute to the disturbance of the gingival barrier with their virulence factors, e.g., LPS, gingipains, and fimbriae (Xu et al., 2020). These factors disturb the intact epithelial barrier and induce cellular inflammatory responses (Xu et al., 2020; Takahashi et al., 2019). Epithelial cells are essential transporters of signals between the internal and external environment. They can recognize these environmental cues and regulate intracellular events by secreting immune effectors such as cytokines, chemokines, and defensins to respond to any invasion and to strengthen and resist the tissues underlying the epithelial layer against any potential risk (Lee & Yilmaz, 2021). For example, gingival epithelial cells can release antibacterial β-defensins, cathelicidin, and calprotectin, as well as

IL-8, to stimulate the migration of neutrophils and to stop the penetration of bacteria through the epithelial barrier and maintain gingival health (Hans & Hans, 2011; Pöllänen et al., 2012). Nevertheless, some bacteria, for example, *Pg*, can escape from immune surveillance by disturbing the gene transcriptions related to immune and inflammatory responses via controlling the epithelial cell signaling cascades (Aleksijević et al., 2022; Bi et al., 2023). Gingival fibroblasts, on the other hand, may mediate a secondary immune response by producing chemokines and cytokines that chemoattract and induce inflammatory cells, such as IL-1β, IL-6, IL-8, IL-10, and monocyte chemoattractant protein1 (MCP-1). These cells also degrade the ECM by MMP-1 and MMP-3 (Alfonso García et al., 2020).

2.3 Stimulator of interferon genes (STING)/TANK-binding kinase1 (TBK1)/interferon regulatory factor 3 (IRF3) pathway

2.3.1 STING receptor

STING (known as MPYS, MITA, ERIS, and TMEM173) is a transmembrane PRR accompanying the ER. STING protein contains 379 amino acids and is recognized at 42 kDa (Zhang et al., 2022). STING comprises two parts: the transmembrane part, including four helices, and the C-terminal domain. STING is present in immune cells as macrophages, dendritic cells, T and B lymphocytes, and non-immune cells as epithelial and endothelial cells (Couillin & Riteau, 2021; Decout et al., 2021).

STING, a cytoplasmic DNA sensing pathway, is essential in modulating the cellular response and defense against pathogens or aberrant double-stranded DNA (dsDNA) in the cell cytosol (Decout et al., 2021). STING is activated by binding to bacterial signaling molecules (c-di-GMP, c-di-AMP, 3′3’-cGAMP). Moreover, 2'3'-cGAMP, produced by cGAS after dsDNA activation, can also activate STING (Yan & Chen, 2021; Zhang et al., 2022; Liu et al., 2022). The dsDNA derived from a pathogen or host that escaped from the nucleus or mitochondria of injured cells with a length of more than 70 bp in the cytosol is defined as ubiquitous danger-associated molecules (Ahn & Barber, 2019; Decout et al., 2021). This dsDNA can activate cGAS and synthesize 2'3'-cGAMP (Ahn & Barber, 2019; Guimarães et al., 2021; Barber, 2015).

Following STING's activation and conformational change, STING will move from ER to the perinuclear compartment to phosphorylate TANK-binding kinase1 (TBK1). The complex form of STING and TBK1 will reach perinuclear Golgi and release TBK1, phosphorylating interferon regulatory factor 3 (IRF3) and producing nuclear factor-κB (NF-κB). Stimulation of IRF3 and NF-κB signaling pathways will promote the transcription of pro-inflammatory cytokines and type I IFNs in the

nucleus and stimulate the immune response against the invading pathogens (Ahn & Barber, 2019; Guimarães et al., 2021) (Figure 1). After that, STING is stopped by a negative feedback mechanism by being subjected to modification by degradation to prevent a prolonged and unlimited stimulation of innate immunity to keep the host homeostasis and avoid autoinflammatory diseases (He et al., 2024).

STING modification is promoted by many factors that act as negative feedback regulators. Of these, E3 ubiquitin ligase, which regulates the cGAS activity, prevents continuous stimulation of immune gene expression and production of type I IFNs by STING through a proteasome-dependent pathway (Li et al., 2024; Zhou et al., 2023; Abe & Shapira, 2019; Wang et al., 2014; Wang et al., 2015). One of the pivotal mechanisms governing the turnover and levels of STING is the most conserved branch of ER-associated degradation, the suppressor of lin-12-like–HMG-CoA reductase degradation 1 complex by ubiquitinating and directing fledgling STING protein for proteasomal degradation (Ji et al., 2023). Serine/threonine UNC-51-like kinase (ULK1/ATG1) regulates subjecting STING to lysosomal degradation when separated from its receptor AMP-activated protein kinase (Konno et al., 2013). Cyclic dinucleotides stimulate ULK1/ATG1 and lead to phosphorylation of STING at site S366 (Konno et al., 2013). Therefore, cyclic dinucleotides, besides activation of STING, can also control this activation by negative feedback mechanisms to block the continuous innate immune response and avoid inflammatory diseases (Konno et al., 2013).

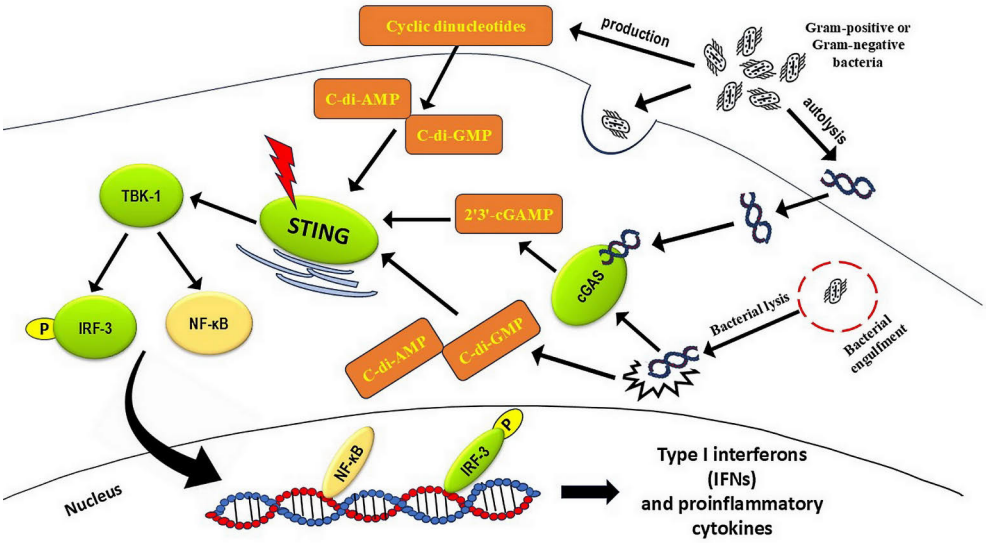

Figure 1. Cyclic dinucleotides modulate immune response through stimulator of interferon genes (STING).

2.3.2 STING/TBK1/IRF3 pathway in inflammatory diseases

STING/TBK1/IRF3 pathway activates innate and adaptive immunity by producing pro-inflammatory cytokines and type I IFNs to protect against pathogen infection (Ou et al., 2021). The binding of cGAS to cytosolic DNA or leaked self-DNA, regardless of DNA sequence, can stimulate STING/TBK1/IRF3 pathway (Kim et al., 2023). Under pathological conditions, self-DNA can be accumulated in the cell's cytosol from many sources, such as nuclear damage, mitochondrial stress, infection, cell death, cellular stress, tissue damage, and DNA damage (Hong et al., 2022).

Unrestricted accumulation of the inducers of STING pathway causes excessive and continuous STING activation, leading to overproduction of type I IFNs and robust immune response, causing inflammatory and autoimmune diseases (Hong et al., 2022). Type I IFNs act as critical cytokines produced by anti-pathogen immunity. These cytokines consist of interferon-α and interferon-β, and subtypes as IFN-ε, −κ, −τ, and –ζ (Li et al., 2017). Another cause that leads to dysregulation in immunity, resulting in inflammatory and autoimmune diseases is a mutation in STING, as polymorphisms make STING over-stimulated (Barber, 2015).

Over-activation of STING/TBK1/IRF3 pathway has been demonstrated in various inflammatory diseases, such as cancer, lung diseases, myocardial infarction, liver disease, and pancreatitis (Ma et al., 2020; Hong et al., 2022). STING pathway contributes to cancer development by stimulating cellular proliferation, survival, and angiogenesis through cytokines, chemokines, and growth factors (Barber, 2015). In addition, high levels of STING activation lead to several autoimmune diseases. In systemic lupus erythematosus, STING induction elevates the level of cGAMP and type I IFNs (An et al., 2017; Kato et al., 2018). The connection between STING and Alzheimer's disease, Parkinson's disease, as well as amyotrophic lateral sclerosis has also been observed (Decout et al., 2021).

2.4 Bacterial cyclic dinucleotides

2.4.1 Origin and functions of bacterial cyclic dinucleotides

Cyclic dinucleotides are bacterial signaling molecules essential for regulating many bacterial cellular processes and detecting/responding to environmental stimuli (Zaver & Woodward, 2020). Cyclic dinucleotides include c-di-GMP, c-di-AMP, and 3′3′-cGAMP. The first identified cyclic dinucleotide was c-di-GMP in 1987. In 2008, c-di-AMP was also demonstrated to be produced by bacteria, followed by cGAMP in 2012 (Yan & Chen, 2021; Aline et al., 2020).

Cyclic dinucleotides are synthesized by cyclases or nucleotidyltransferases in heterocyclic configuration, and the regulated action of phosphodiesterase controls

their signaling (Zaver & Woodward, 2020; Yan & Chen, 2021) (Figure 2). C-di-GMP is synthesized from two GMP molecules linked by two 3′-5′ phosphodiester bonds through the diguanylate cyclases (DGC) (Banerjee et al., 2021). C-di-AMP is synthesized from two molecules of AMP by diadenylate cyclase enzymes (Pathania et al., 2021).

C-di-AMP is produced by Gram-positive bacteria, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Bacillus subtilis*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*. Some Gram-negative bacteria, such as *Chlamydia trachomatis* and *Borrelia burgdorferi*, also produce c-di-AMP (Opoku-Temeng et al., 2016; Fahmi et al., 2017). C-di-AMP is involved in critical bacterial cellular processes, such as cell wall metabolism, maintenance of DNA integrity, cell division, cell-wall homeostasis, potassium ion channel expression and function, diverse gene expression, biofilm formation, bacterial growth, and antibiotic resistance (Aline et al., 2020).

C-di-GMP regulates bacterial motility, virulence, stress survival, metabolism, biofilm formation, and differentiation (Danilchanka & Mekalanos, 2013; Römling et al., 2013) and has been identified in Gram-negative bacteria, including *Pseudomonas aeruginosa, Caulobacter crescentus, Escherichia coli, Salmonella typhimurium*, and *Vibrio cholerae*, and a few Gram-positive bacteria, such as *Bacillus subtilis* and *Listeria monocytogenes* (Opoku-Temeng et al., 2016). C-di-GMP has also been detected in periodontitis-associated bacteria, including *Porphyromonas gingivalis, Treponema denticola*, and *Selenomonas noxia* (Frederick et al., 2011; Bian et al., 2013; Chaudhuri et al., 2014; Gursoy et al., 2017). In addition, these bacterial signaling molecules also regulate the oxidative response, extracellular polysaccharide matrix production, and cariogenic *Streptococcus mutans* biofilm formation (Yan et al., 2010; Cheng et al., 2016).

The impact of cyclic dinucleotides on bacterial physiology is determined according to their type and concentration (Aline et al., 2020). Bacterial behaviors can be regulated for faster response by regulating protein function or slow response when affecting gene transcription or protein translation (Aline et al., 2020). Bacteria have different cyclic dinucleotide receptors that regulate a wide range of bacterial behaviors; although they are specific to only one type of cyclic dinucleotide, receptors bind cyclic dinucleotides with similar conformation (Aline et al., 2020). Some of these receptors are STING proteins, proteins containing PilZ domains, degenerate EAL domains, allosteric sites of DGC enzymes containing GGDEF domains, and Aldo-keto reductase domain of oxidoreductase (RECON) proteins (Aline et al., 2020).

Bacterial cyclic dinucleotides, classified as MAMPs, are potent stimulators of innate immune response (Danilchanka & Mekalanos, 2013; Ahn & Barber, 2019); both c-di-AMP and c-di-GMP can bind STING to stimulate innate immune

responses and regulate the production of type I IFNs (IFN-α and IFN-β) (Danilchanka & Mekalanos, 2013; Zhou et al., 2023). In addition to STING, another cytosolic receptor, RECON, can bind to cyclic dinucleotides and modulate the activation of inflammatory genes by inhibiting STING and NF-κB (McFarland et al., 2017). Bacterial cyclic dinucleotides inhibit the oxidoreductase RECON, and loss of RECON activity leads to increased NF-κB activation and reduced bacterial survival to support the cellular response toward an antibacterial reaction (McFarland et al., 2017).

2.4.2 Cyclic dinucleotide-mediated STING pathway in cell response regulation

Cyclic dinucleotides, as immune stimulators via STING pathway, have essential roles in modulating the cellular response and defense against pathogens or dsDNA in the cell cytosol (Decout et al., 2021). Such a role opens the door to be used as immunotherapeutic agents for treating infections, cancer, and other immunological diseases (Aline et al., 2020). Cyclic dinucleotides activate immune cascades that significantly impact the host cellular microenvironment in two ways. First, they enhance macrophages' maturation, polarisation, antigen presentation, and cytokine secretion ability. Second, in a paracrine way, they activate antigen presentation that mediates innate immune responses that could promote T lymphocyte recruitment, proliferation, and differentiation to mediate adaptive immune response and trigger the generation of type I IFNs and pro-inflammatory cytokines (Ou et al., 2021).

Bacterial cyclic dinucleotide-mediated STING activation has been demonstrated in macrophages and dendritic cells, and also observed in healthy and diseased periodontal tissues (Paludan & Bowie, 2013; Abe & Barber, 2014; Deng et al., 2022). For example, in macrophages, bacterial cyclic dinucleotides take part in bacterial elimination through phagocytosis (Aryal et al., 2020). In dendritic cells, activation of STING/cGAS pathway and recognition of cytoplasmic DNA is followed by TBK1/IRF3 and NF-κB pathway activation (Ou et al., 2021).

The expression of cluster of differentiation CD80, CD86, and CD83, MHC class II, IL-12, IL-8, IL-1β, IFN-gamma, MCP-1, and TNF, as well as type 1 T helper and chemokine (C-C motif) ligand 5 have been increased in cultured human immature dendritic cells stimulated by c-di-GMP (Karaolis et al., 2007a). In a mouse model, c-di-GMP facilitates the induction of chemokines and the pulmonary recruitment of neutrophils for the host defense (Zhao et al., 2011). C-di-AMP induces natural killer cell (NK) mediated tumor rejection by activation of NK cells by type I IFN-enhanced expression of IL-15 and its receptors (Nicolai et al., 2020). Moreover, cyclic dinucleotides are absorbed into the bone marrow and inhibit osteoclastogenesis via STING pathway, leading to inhibited osteoporosis (Kwon et al., 2019; Sun et al.,

2020), and stimulating innate immune response against infection with pathogenic bacteria, such as *Bordetella pertussis, Acinetobacter baumannii, Staphylococcus aureus, Klebsiella pneumoniae, Clostridium perfringens*, and various serotypes of *Streptococcus pneumoniae* (Yan & Chen, 2021).

3 Aims

Gingiva is one of the first responders against oral bacterial challenges and is constantly exposed to the action of multiple MAMPs. However, there is a gap in the research on the regulatory effect of cyclic dinucleotides on the cellular response of gingival cells. The research purpose of the present PhD thesis was to study the extent and magnitude of the effect of cyclic dinucleotides on gingival cells' behavior in terms of cellular response and protein expression, either alone or together with *Pg* LPS. The current project hypothesized that 1) gingival cells produce a STING-dependent cellular reaction (keratinocytes and fibroblasts) against bacterial cyclic dinucleotides and that 2) *Pg* LPS and cyclic dinucleotides induce antagonistic effects on gingival cells as LPS-dependent TLR and cyclic dinucleotide-dependent STING pathways may compete with each other. To test the hypotheses, the following specific aims were set:

1. To evaluate the impact of bacterial-derived cyclic dinucleotides (c-di-AMP and c-di-GMP) alone or in the presence of *Pg* LPS on epithelial cytokine production and MAPK activation (Study **I**).

2. To study the independent and combined impact of bacterial-derived cyclic dinucleotides (c-di-AMP and c-di-GMP) and *Pg* LPS on cytokine and MMP response of HGFs (Study **II**).

3. Using a global proteomics approach to demonstrate the full proteome response of HGFs against bacterial-derived cyclic dinucleotides (c-di-AMP and c-di-GMP) (Study **III**).

4. To evaluate the effect of the simultaneous exposure of bacterial-derived cyclic dinucleotides and *Pg* LPS on the global proteome of HGFs by proteomics analysis (Study **IV**).

5. To demonstrate bacterial-derived cyclic dinucleotides-regulated STING/TBK1/IRF3 pathway activation in HMK cells (Unpublished data).

4 Materials and Methods

4.1 Materials

4.1.1 Cyclic dinucleotides

Cyclic dinucleotides were developed in Purdue University's laboratory by our collaborator Herman Sintim and his group according to the protocol described by Gaffney et al. (2010) and Gaffney et al. (2013). In brief, cyclic dinucleotides were developed by an integrated set of reactions, and deprotection was carried out as a one-flask procedure, with crystallization allowing the separation of the final products from the reaction mixture.

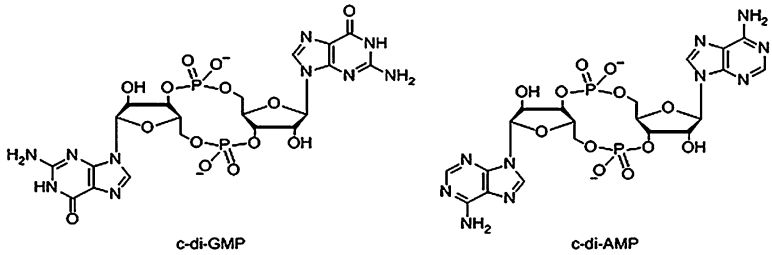

Figure 2. The structure of c-di-GMP and c-di-AMP in bacteria. (Source: Kalia et al., 2013)

According to the protocol by Gaffney et al. (2010), a stock solution of cyclic dinucleotides was prepared by dissolving the disodium form of cyclic dinucleotides in sterile water. Molarity was calculated by diluting 2 µl of stock solution 1000 times and heating for 1 h at 45°C using a heating block. Then, the solution was cooled down for 1 h at room temperature. The UV absorption of the stock solution was measured with a UV spectrophotometer at 253 nM for c-di-GMP and 259 nM for c-di-AMP, followed by molarity calculation for each sample by molarity = absorbance/molar absorptivity.

Among all studies, the stock solutions of cyclic dinucleotides were divided into small tubes at -20°C. The stock solution was prepared by dimethyl sulfoxide (DMSO) and further dilution to reach the final molarity (100 µM, 10 µM,

and 1 μM) by culture media. To avoid the unknown effect of the freeze/thaw cycle, the rest of the thawing tube was discarded after the experiment. In this PhD project, test concentrations of cyclic dinucleotides were selected based on their effective ranges on the viability and cellular response of mammalian cells, as well as on bacterial survival and biofilm formation, as described in previous studies (Karaolis et al., 2005a; Tosolini et al., 2015; Wang et al., 2017; Gries et al., 2016; Karaolis et al., 2005b).

4.1.2 Lipopolysaccharides of *Porphyromonas gingivalis* (*Pg* LPS)

With endotoxin-free water supplied by the company, ultrapure *Pg* LPS (Invivogen, San Diego, CA, USA) was dissolved to prepare the stock solution at a concentration of 1 mg/ml. Furthermore, it is stored at -20°C in small aliquots to avoid the freeze/thaw cycle.

4.1.3 STING inhibitor H-151

Commercial STING inhibitor H-151(10 mg) (Invitrogen, Carlsbad, CA, USA) was dissolved in DMSO to get a stock solution (1 mg /ml). The stock solution was maintained at -20ºC in small aliquots. Further dilution was carried out on the day of the experiment using the culture media.

4.2 Cell culture

4.2.1 Human gingival keratinocytes (HMK) (I and unpublished data)

Spontaneously immortalized non-tumorigenic gingival keratinocyte cell lines, HMK cells, were extracted from a human gingival biopsy sample of a periodontally healthy patient (Mäkelä et al., 1999). Cells were cultured in keratinocyte-serum-free medium with human recombinant epidermal growth factor, bovine pituitary extract (Gibco, Paisley, Scotland), and antibiotics (100 IU/ml penicillin with 100 μg/ml streptomycin) (Gibco, Bethesda, Maryland, USA) at 37°C and 5% CO_2. Cell cultures were passaged after cells reached the 80–90% confluence; until that, culture media were changed three times weekly. The cell passage number used in the experiment was from 17 to 22.

4.2.2 Human gingival fibroblasts (HGFs) (II, III, and IV)

HGFs were obtained from the extracted wisdom teeth of periodontally healthy individuals (Oksanen & Hormia, 2002). Cells cultured in Dulbecco's modified eagle medium supplemented with fetal bovine serum (10%) (Gibco BRL, Life Technologies), antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin), and 1% non-essential amino acid (Gibco BRL, Life Technologies) and incubated at 37°C and 5% CO_2. Changes in culture media were done three times weekly, while passaging was done when cells reached 80–90% confluence. The cell passage numbers used in the experiment were 9 and 10. All patients signed an informed consent, and permission for tissue biopsies was given on 19 November 2002, and the number of study case was §262. The experimental protocol was approved by the Ethics Committee of the Hospital District of South-West Finland and the Ethical Committee of the Dentistry, University of Helsinki.

4.2.3 Incubation of cells with cyclic dinucleotides (I, II, and unpublished data)

HMK cells and HGFs were trypsinized and centrifuged at 10000 g for 5 min. Then, cells were cultured at (300000/well) density in 12-well plates and incubated at 37ºC and 5% CO_2 for 24 h. After three times washing with phosphate-buffered saline (PBS), HMK cells (I) and HGFs (II and III) were co-cultured with cyclic dinucleotides (c-di-GMP or c-di-AMP) at three different test concentrations (100 µM, 10 µM, 1 µM), either with or without *Pg* LPS (1 µg/ml).

To study the TBK1 and IRF3 responses' dependency on STING (unpublished data), HMK cells were co-cultured with commercial STING inhibitor H-151 (0.5 µM or 5 µM) (Invitrogen, Carlsbad, CA, USA) and two concentrations (100 µM, 10 µM) of c-di-GMP or c-di-AMP for 24 h.

For all studies, the experiment's control group consisted of cells incubated in pure culture media without adding *Pg* LPS, cyclic dinucleotides, or STING inhibitor H-151.

After 24 h of incubation of HMK cells (I and unpublished data) and two-time points of HGFs incubation, 2 h and 24 h (II and III), culture media of wells were collected and kept at −70°C to use for analysis extracellular cytokine levels (I and II). Cells were lysed with lysis buffer (300 µl) (50 mM Tris-Cl, 150 mM NaCl, and 1% Triton X-100) and scraped mechanically, then collected. After cell lysate was sonicated for 10 s, Bradford Assay (Bio-Rad, Hercules, CA, USA) determined the protein concentration in each cell lysate. Then, the lysate was used to analyse intracellular cytokine levels (I) and to run the western blot (I, III, and unpublished data).

4.2.4 Preparation of cell culture pellet for proteomic analysis (III and IV)

HGFs (700000/Petri dish) were cultured in 3 ml of Dulbecco's modified eagle medium with c-di-GMP or c-di-AMP at 100 μM (III) or with c-di-GMP or c-di-AMP at 100 μM with *Pg* LPS (1 μg/ml) (IV) at 37°C and 5% CO_2. After 24 h of incubation and following trypsinization of the cells, the pellet was collected for proteomics analysis.

4.3 Cell proliferation (Unpublished data)

To study the effect of cyclic dinucleotides on cell viability, two sets of HMK cells and HGFs were cultured at a density of (50000/well) in 96-well plates with c-di-GMP and c-di-AMP (100 μM, 10 μM, 1 μM) and incubated at 37ºC and 5% CO_2 for 24 h. After washing three times with PBS buffer, plates were incubated again at the same conditions for 2 h with proliferation kit assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay, Promega, WI, USA). Then, absorbance values were read at 492 nm by using a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer.

4.4 Cellular response profile (I and II)

Cell lysates and culture media were collected after HMK and HGF cells were incubated with cyclic dinucleotides. The concentrations of IL-1β, IL-8, IL-1Ra, MCP-1, and VEGF were detected from cellular lysates of HMK (intracellular cytokine level) and HMK growth media (extracellular level) (I). In addition, the extracellular concentrations of IL-8, IL-10, IL-34, MMP-1, MMP-2, and MMP-3 were measured from the growth media of HGFs (II). Cytokine and enzyme concentrations were analysed with the Luminex technique (Bio-Rad, Santa Rosa, CA, USA) using commercially optimized Bio-Plex kits (pro-human cytokine group I assay; Bio-Rad, Santa Rosa, CA, USA). The experiment protocol was followed according to the manufacturer's instructions. The limit of detection of cytokines concentrations in study I is as follows: IL-1β (0.6 pg/ml), IL-8 (1.0 pg/mL), IL-1Ra (5.5 pg/ml), MCP-1 (1.1 pg/ml), and VEGF (3.1 pg/ml). In study II, IL-8 (2.7 pg/ml), IL-10 (0.6 pg/ml), IL-34 (51.9 pg/ml), MMP-1 (33.7 pg/ml), MMP-2 (39 pg/ml), and MMP-3 (28.5 pg/ml). The amount of each cytokine and enzyme per 1 μg of protein was calculated for the data presentation.

4.5 Analysis of levels of mitogen-activated protein kinases (MAPKs) of HMK cells and levels of STING TBK1-IRF3 of HGFs and HMK cells by western blot technique (I, III, and unpublished data)

Western blot was used to analyse levels of ERK 1/2, JNK, and p38 protein kinases in HMK cells (I) and STING, TBK1, and IRF3 protein levels in HGFs (III) and HMK cells (unpublished data). Samples were mixed with Laemmli buffer (4X) at (3:1) and heated at 95°C for 5 min, followed by separation according to the molecular weight by 10% for (TBK1), 12% for (STING and IRF3), and 15% for (ERK 1/2, JNK, and p38 protein kinases) of sodium dodecyl sulfate-polyacrylamide gels. Afterwards, gels were transferred to membranes (Trans-Blot® Turbo™ Transfer System, Bio-Rad, Hercules, CA, USA). Then, membranes were incubated overnight with primary antibodies. The different primary antibodies are illustrated in Table 1.

On the next day, secondary antibodies were added to the membranes (Table 1). Novex® ECL chemiluminescent substrate reagent kit (Invitrogen, Carlsbad, CA, USA) was used to detect horseradish peroxidase (HRP). Visualization of bands on the membranes was performed by the ChemiDoc™ MP imaging system (Bio-Rad, Hercules, CA, USA).

4.6 Proteomic analysis (III and IV)

Proteomics analysis was performed as described previously (Aryal et al., 2020; Sooreshjani et al., 2018) at the Purdue Proteomics Facility. Before subjecting the cells to high-pressure lysis by Barocycler (Pressure Bioscience Inc., Easton, MS, USA) and determining the protein concentration by using bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL, USA), cells were suspended in 100 mM ammonium bicarbonate. Proteins were extracted using acetone precipitation, and the resulting protein pellets were resolubilised in 8M urea dissolved in deionized water for reduction, alkylation, and trypsin/LysC digestion, following the methods described by Sooreshjani et al. (2018) and Opoku-Temeng et al. (2019). LC-MS/MS data were collected on a Thermo Q Exactive Orbitrap HF mass spectrometer equipped with a Dionex UltiMate 3000 HPLC system, using a 120-minute LC gradient. One microgram (1 µg) of digested peptides was loaded onto a trap column (200 µm ID × 5 mm) packed with 5 µm 100 Å PepMap C18 medium and then separated on an Acclaim PepMap 100 Å analytical column (75 µm ID × 50 cm) packed with 2 µm 100 Å C18 (Thermo Fisher Scientific, Waltham, MA, USA).

Mobile phases (A and B) had 0.1% formic acid in water and 0.1% formic acid in 80% acetonitrile, respectively, and the temperature was kept at 50°C. The mass spectrometer operation used a standard data-dependent MS/MS scan method. For

MS/MS fragmentation, the full scan MS spectra were acquired by a Top20, followed by collection in the 350-1600 m/z with a maximum injection time of 100 m/s and a resolution of 120 000 at 200 m/z.

For fragmentation of precursor ions, high-energy C-trap dissociation and normalized collision energy of 27 eV were used, and the MS/MS scans were at the resolution of 15,000 at m/z 200. At the beginning of the experiment, the instruments were optimized and calibrated, and their performance was assessed by Hela digest (Pierce). This process was repeated every three days. The dynamic exclusion of identical peptides was set at 20 s. For protein identification and label-free quantitation (LFQ), LC-MS/MS data in MaxQuant (version 1.6.3.3) against the UniProt human protein database was used (Cox & Mann, 2008).

After the collection of LC-MS/MS data under three biological replicates for each treatment group, Venny software (Venny. 2.1) and Perseus software were used to plot the Venn diagrams and initial bioinformatics analysis, respectively (Tyanova et al., 2016). For further study, proteins were only recognized in at least two of three treatment replicates and with at least two MS/MS counts and LFQ intensities. With the exception if otherwise mentioned, LFQ intensities were Log2 transformed, filtered, and a student's t-test with permutation-based false discovery rate (5%). MetaboAnalyst software Version 5.0 (R package) was used for the PLS-DA analysis.

OriginPro 2020 software (OriginLab, MA) acquired volcano plots and principal component analysis (PCA) plots. Hierarchical clustering and heat map analysis in Morpheus software and MetaboAnalyst were performed using auto-scale normalized data. At the same time, a correlation between replicates was determined using scatter plots and correlation plots. The pathway enrichment and graphics were generated using the ingenuity pathway analysis (IPA) functional network core analysis (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis).

4.7 Statistical analysis

All experiments in the current project were run in triplicate and were repeated at least two independent times. The IBM SPSS software (version 23, IBM, Armonk, New York, USA) was used for statistical analysis. In all bar charts, results are presented as means and standard deviations. For normal data distribution, one-way analysis of variance (ANOVA) and Bonferroni's post-hoc test were used to analyse inter-group differences between the treatment groups. The Kruskal-Wallis and Mann-Whitney U post-hoc test was used for non-normally distributed data. (p-value < 0.05 was defined as statistically significant).

Table 1. A list of primary and secondary antibodies used in the original publication. (I, III, and unpublished data).

Antibody	Dilution	Company
STING polyclonal antibody	1:1000	Thermo Fisher, Rockford, USA
TBK1 polyclonal antibody	1:1000	Thermo Fisher, Rockford, USA
IRF3 polyclonal antibody	1:1000	Thermo Fisher, Rockford, USA
ERK1/2 antibody	1:500	Thermo Fisher, Rockford, USA
p38 MAPK alpha polyclonal antibody	1:500	Thermo Fisher, Rockford, USA
JNK pan-specific antibody	1:1000	R&D Systems, a Bio-Techne Brand, Minneapolis, MN, USA
Phospho-ERK1/ERK2 (Thr185, Tyr187) polyclonal antibody	1:1000	Thermo Fisher, Bengaluru, India
Phospho-p38 MAPK alpha (Thr180, Tyr182) polyclonal antibody	1:1000	Thermo Fisher, Rockford, USA
Phospho-JNK (T183, Y185) antibody	1:1000	R&D Systems, a Bio-Techne Brand, Minneapolis, MN, USA
β-actin antibody	1:10000 1:5000	Thermo Fisher, Waltham, USA
Goat anti-Rabbit IgG (H+L) secondary antibody, horseradish peroxidase (HRP) conjugate	1: 10000 1:2000 1:1000	Thermo Fisher, Rockford, USA
Goat anti-mouse IgG (H + L) secondary antibody, HRP conjugate	1:4000	Thermo Fisher, Rockford, USA

5 Results

5.1 Impact of cyclic dinucleotides and *Pg* LPS on the cellular response of gingival cells

5.1.1 Effect of cyclic dinucleotides and *Pg* LPS on intracellular levels of cytokine expression of HMK cells (I)

Intracellular IL-1β levels of HMK cells after incubation with cyclic dinucleotides, alone or combined with *Pg* LPS, are presented in Figure 3. Incubation of HMK cells with *Pg* LPS negatively affected the production of IL-1β ($p < 0.001$). The same effect was shown in the presence of c-di-AMP alone (100 μM $p < 0.001$, 10 μM $p = 0.001$, and 1 μM $p < 0.001$) or with c-di-AMP and *Pg* LPS (100 μM $p < 0.001$, 10 μM $p < 0.001$, and 1 μM $p < 0.001$). This inhibition in the level of IL-1β appeared also when cells were cultured with c-di-GMP together with *Pg* LPS (100 μM $p < 0.001$, 10 μM $p = 0.001$, and 1 μM $p < 0.001$). None of the MAMPs (LPS, c-di-GMP, or c-di-AMP) significantly affected the intracellular IL-8 levels of HMK cells (Figure 4). The significant upregulation of c-di-AMP either alone or in the presence of *Pg* LPS (100 μM $p < 0.001$ or $p = 0.002$, respectively) on the intracellular level of IL-1Ra is shown in Figure 5. In contrast, the lowest concentration of c-di-AMP increased the production of intracellular MCP-1 level ($p = 0.025$) (Figure 6). Moreover, the significant increase in intracellular levels of VEGF was only in the presence of the highest concentration of c-di-AMP ($p = 0.039$) (Figure 7).

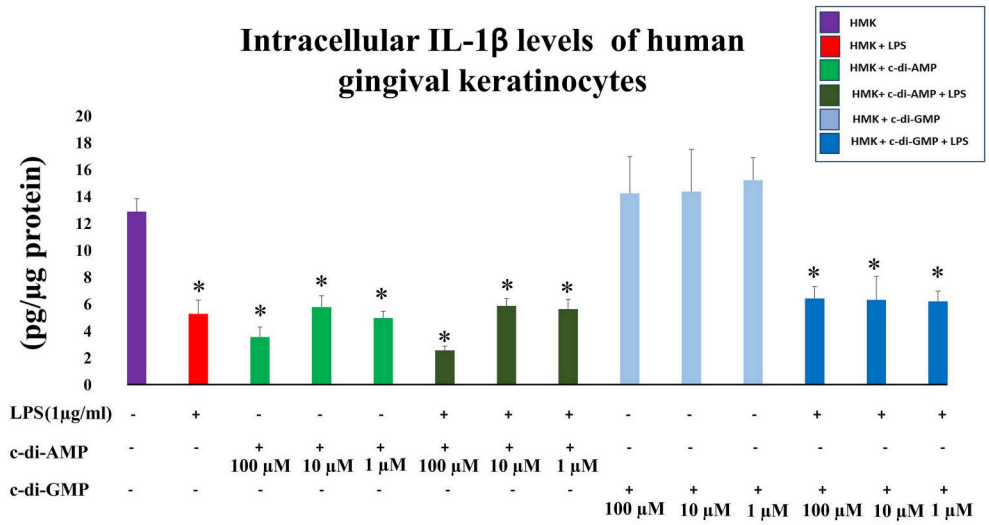

Figure 3. Intracellular IL-1β levels of human gingival keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication I).

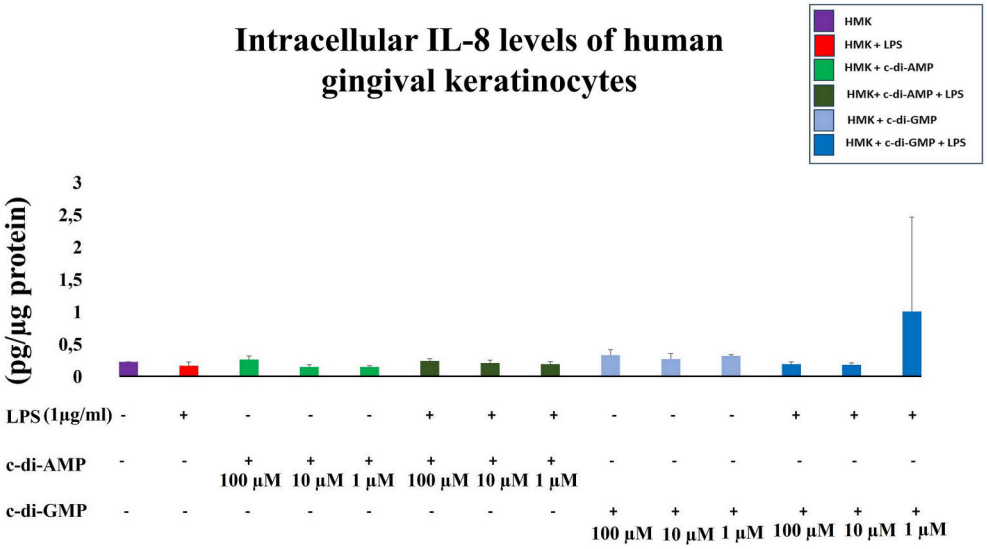

Figure 4. Intracellular IL-8 levels of human gingival keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. (Modified from original publication I).

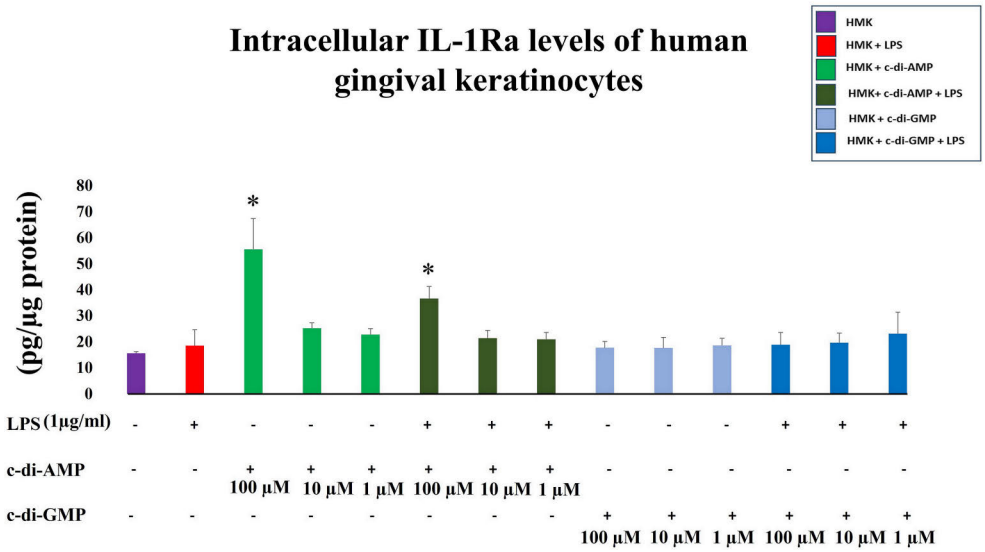

Figure 5. Intracellular IL-1Ra levels of human gingival Keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication I).

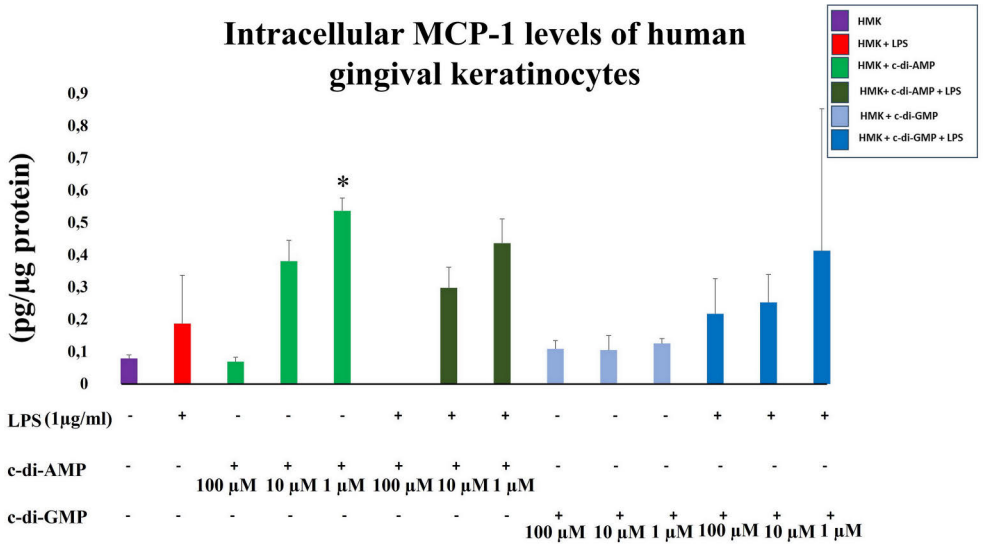

Figure 6. Intracellular MCP-1 levels of human gingival keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication I).

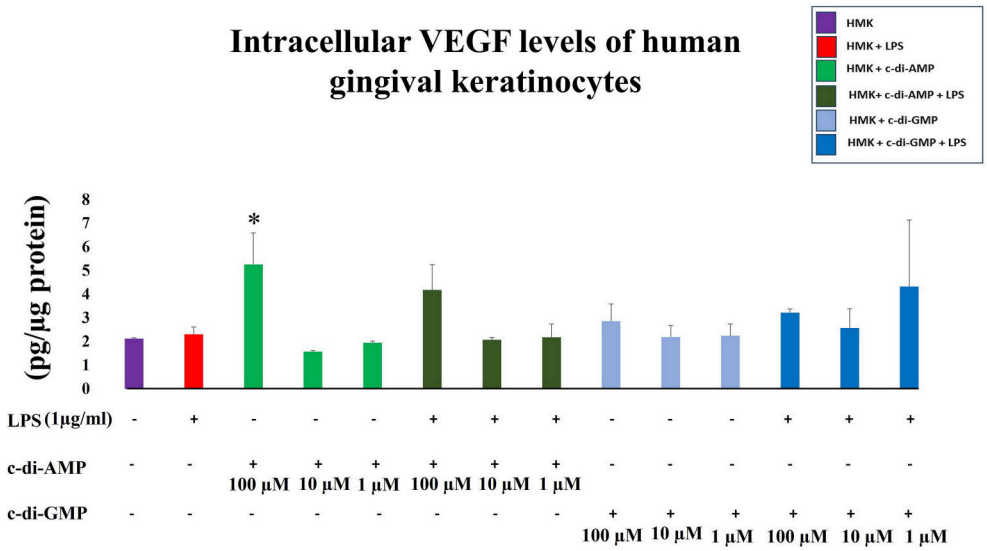

Figure 7. Intracellular VEGF levels of human gingival Keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication I).

5.1.2 Effect of cyclic dinucleotides and *Pg* LPS on extracellular levels of cytokine expression of HMK cells (I)

C-di-AMP at 100 µM elevated the extracellular levels of IL-1β ($p = 0.033$) significantly (Figure 8). In contrast to the intracellular levels, incubation of HMK cells with *Pg* LPS alone ($p = 0.015$) or *Pg* LPS and 100 µM ($p = 0.024$), 10 µM ($p = 0.006$), and 1 µM of c-di-GMP ($p = 0.037$) significantly suppressed the extracellular levels of IL-8. At the same time, IL-8 levels were brought to the same level of control when cells were incubated with c-di-AMP and *Pg* LPS. Significant elevation in IL-8 level was observed in the presence of 100 µM of c-di-AMP alone ($p = 0.017$) (Figure 9). The highest and lowest concentrations of c-di-AMP only (100 µM $p < 0.001$ and 1 µM $p = 0.020$) and of c-di-AMP at the highest concentration in the presence of *Pg* LPS ($p = 0.007$) increased extracellular level of IL-1Ra (Figure 10). At 10 µM and 1 µM of c-di-AMP alone ($p < 0.001$ and $p < 0.001$) or combined with *Pg* LPS ($p = 0.022$ and $p < 0.001$), respectively, increased the extracellular MCP-1 levels significantly (Figure 11). Extracellular levels of VEGF in HMK cells incubated with c-di-GMP at 100 µM combined with *Pg* LPS ($p = 0.024$) or incubated with 100 µM of c-di-AMP alone ($p = 0.005$) showed a significant elevation (Figure 12).

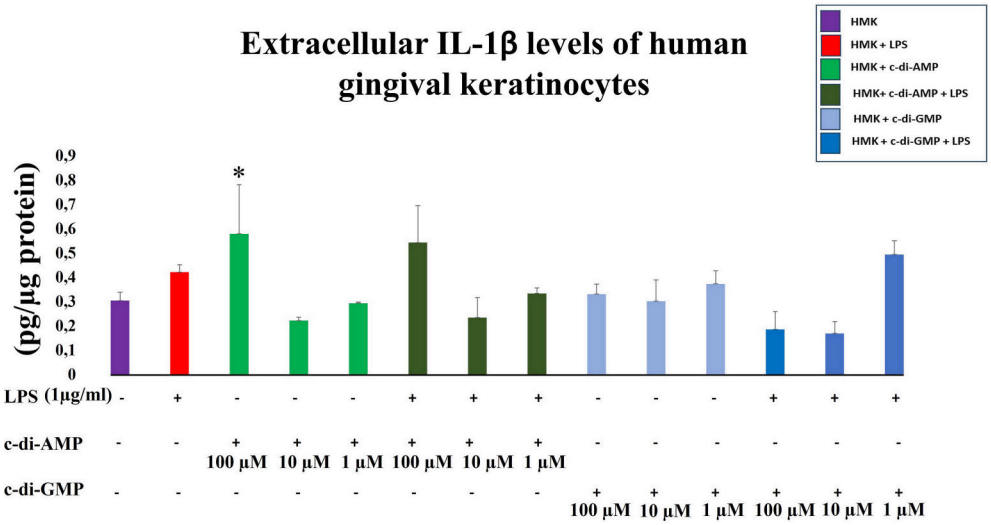

Figure 8. Extracellular IL-1β levels of human gingival Keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication I).

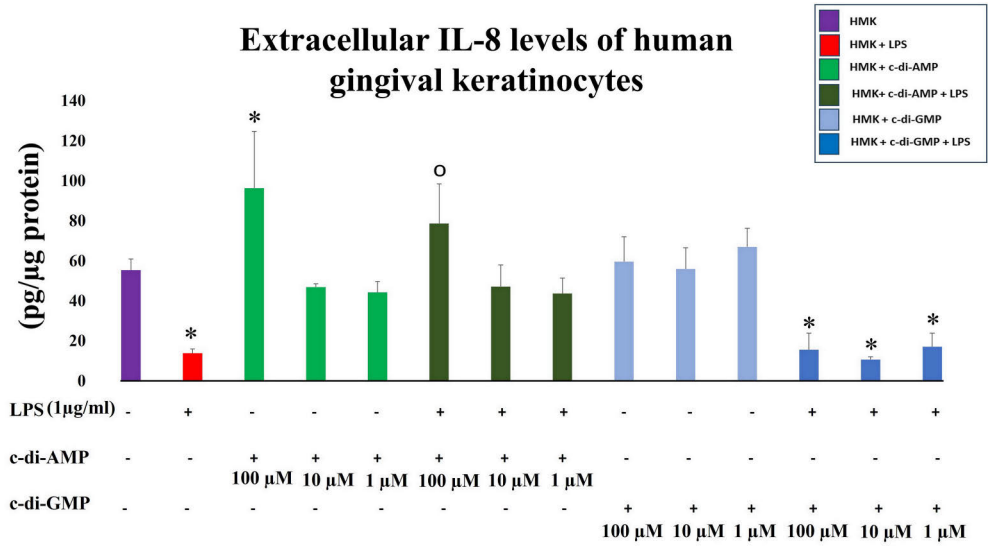

Figure 9. Extracellular IL-8 expression in human gingival Keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP), and ○ indicates a statistical difference with *Pg* LPS alone. (Modified from original publication I).

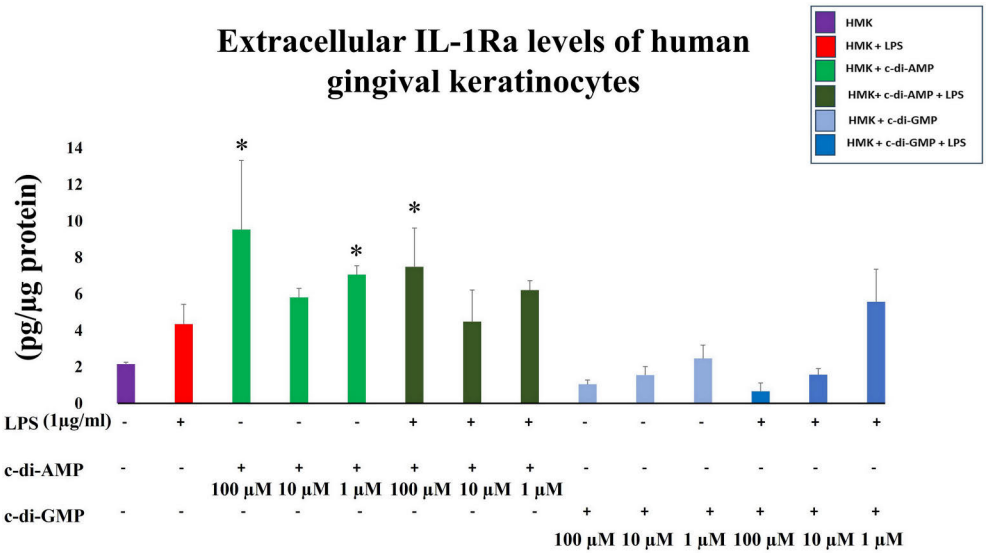

Figure 10. Extracellular IL-Ra expression in human gingival Keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication I).

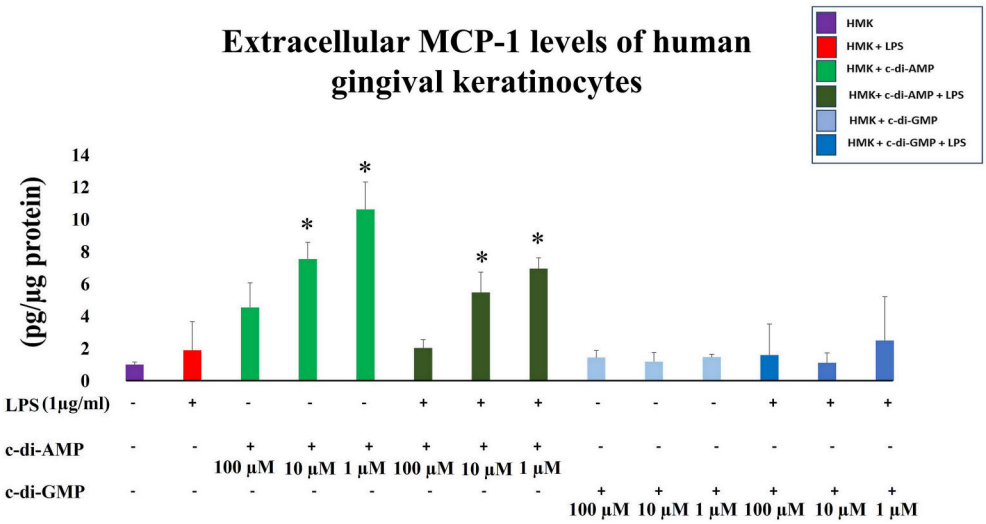

Figure 11. Extracellular MCP-1 expression in human gingival Keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication I).

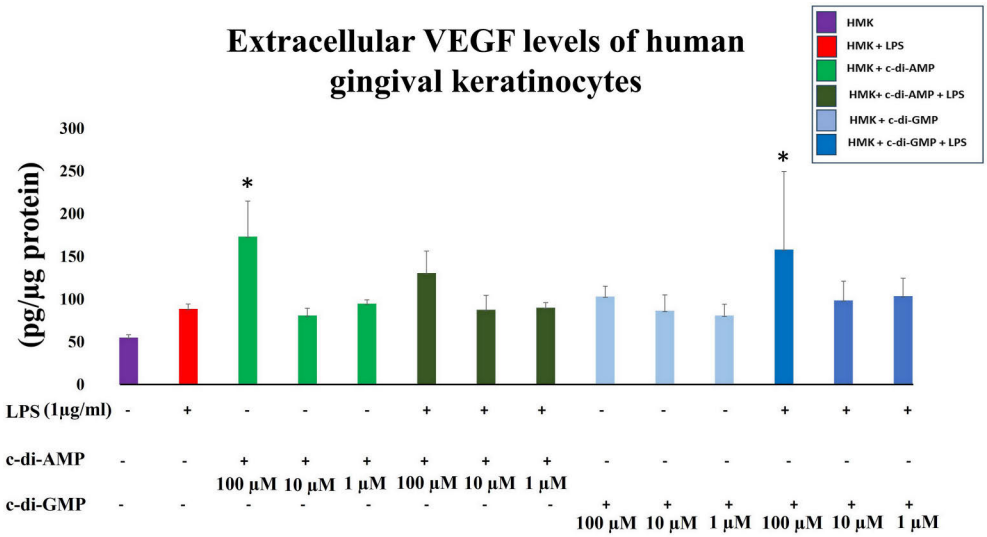

Figure 12. Extracellular VEGF expression in human gingival Keratinocytes (HMK). HMK cells were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication I).

5.1.3 Impact of cyclic dinucleotides and *Pg* LPS on the early cellular response of HGFs (II)

The IL-8 expression of HGFs was increased significantly when cells were incubated with *Pg* LPS alone ($p < 0.001$), *Pg* LPS and c-di-AMP ($p < 0.001$ at 100 µM), or *Pg* LPS and c-di-GMP ($p < 0.001$ at 100 µM, 10 µM, and 1 µM) for 2 h (Figure 13). The expression of IL-10 was significantly increased when HGFs were incubated with 10 µM and 1 µM of c-di-GMP combined with *Pg* LPS for 2 h (at 10 µM and 1 µM $p < 0.001$) (Figure 14). IL-34 concentrations of HGFs were not subjected to any significant changes when cells were cultured with cyclic dinucleotides and *Pg* LPS individually or combined (Figure 15).

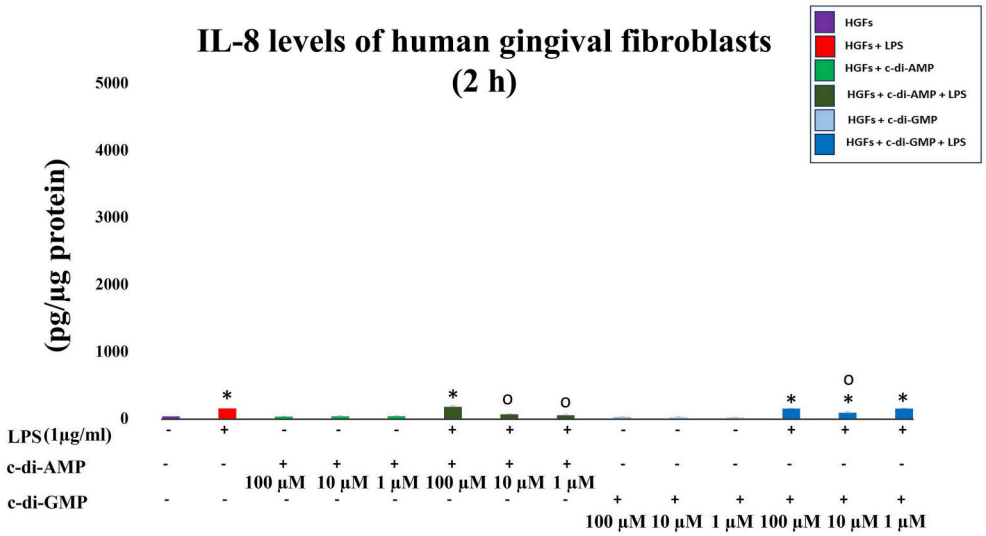

Figure 13. Early extracellular IL-8 expression in human gingival fibroblasts (HGFs). HGFs were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 2 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP), and ○ indicates a statistical difference with *Pg* LPS alone. (Modified from original publication II).

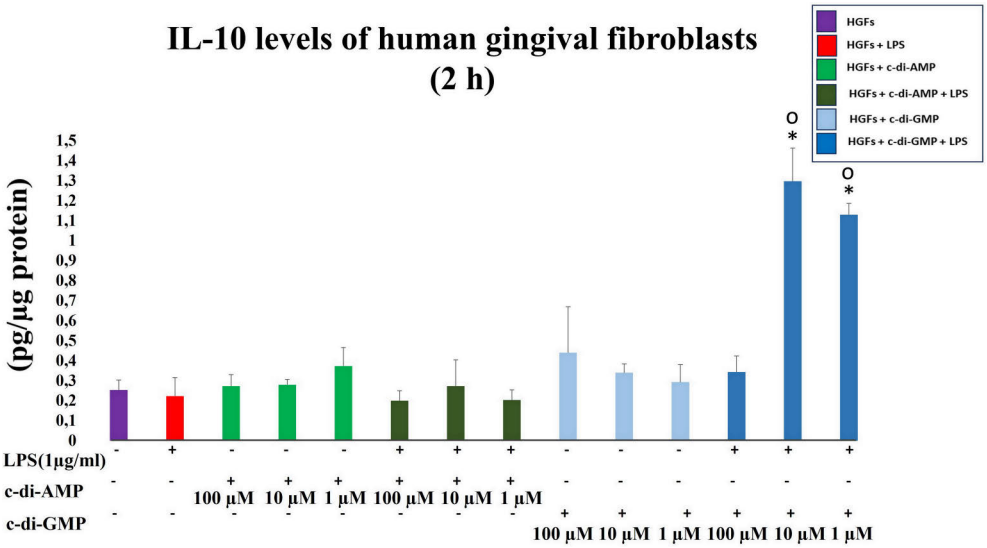

Figure 14. Early extracellular IL-10 expression in human gingival fibroblasts (HGFs). HGFs were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 2 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP), and ○ indicates a statistical difference with *Pg* LPS alone. (Modified from original publication II).

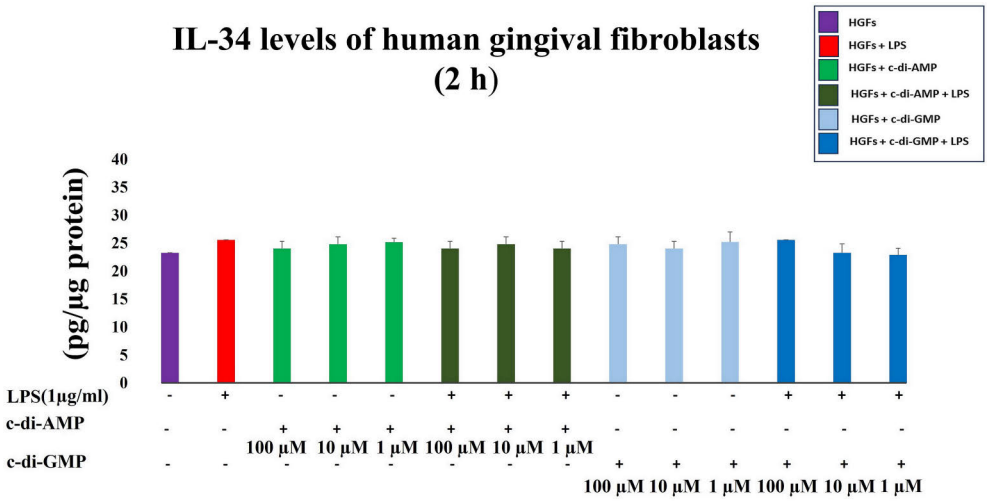

Figure 15. Early extracellular IL-34 expression in human gingival fibroblasts (HGFs). HGFs were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 2 h. Bars express the mean ± standard deviation for triplicate tests. (Modified from original publication II).

Although MMP-1 concentrations followed the same pattern as IL-34 and did not show any significant alteration when HGFs were incubated with any of the MAMPs (Figure 16), HGF MMP-2 expression was increased in the presence of 10 µM of c-di-GMP and *Pg* LPS ($p = 0.003$) (Figure 17). HGF MMP-3 expression enhanced significantly when cells were cultured with c-di-GMP and *Pg* LPS (10 µM and 1 µM $p < 0.001$) (Figure 18).

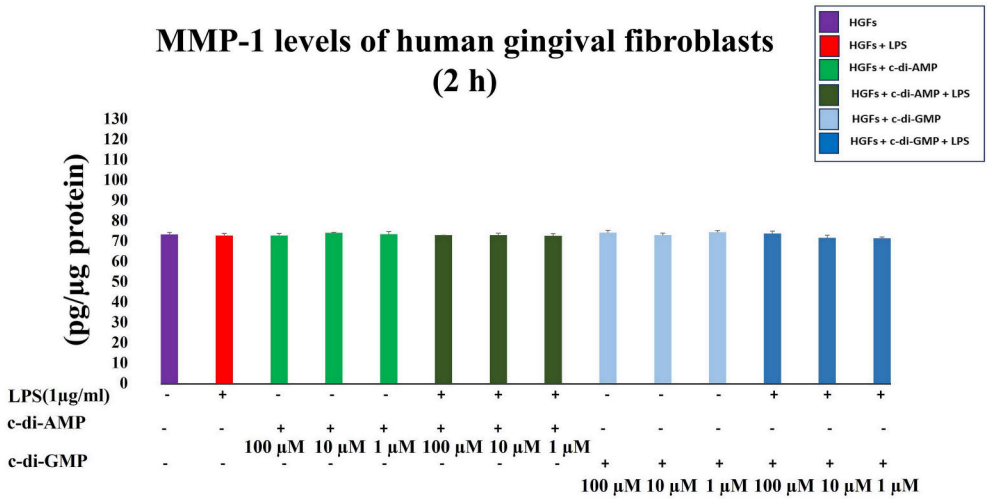

Figure 16. Early extracellular MMP-1 expression in human gingival fibroblasts (HGFs). HGFs were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 2 h. Bars express the mean ± standard deviation for triplicate tests. (Modified from original publication II).

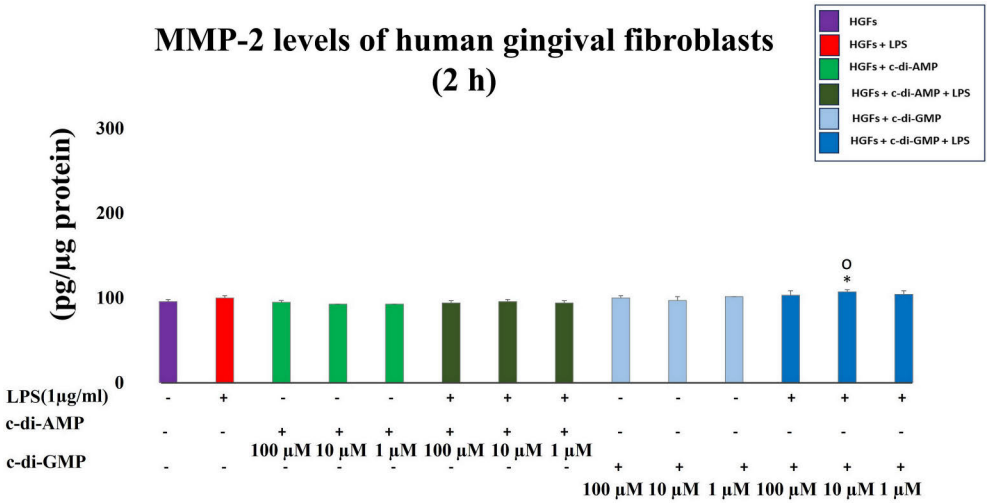

Figure 17. Early extracellular MMP-2 expression in human gingival fibroblasts (HGFs). HGFs were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 2 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP), and ○ indicates a statistical difference with *Pg* LPS alone. (Modified from original publication II).

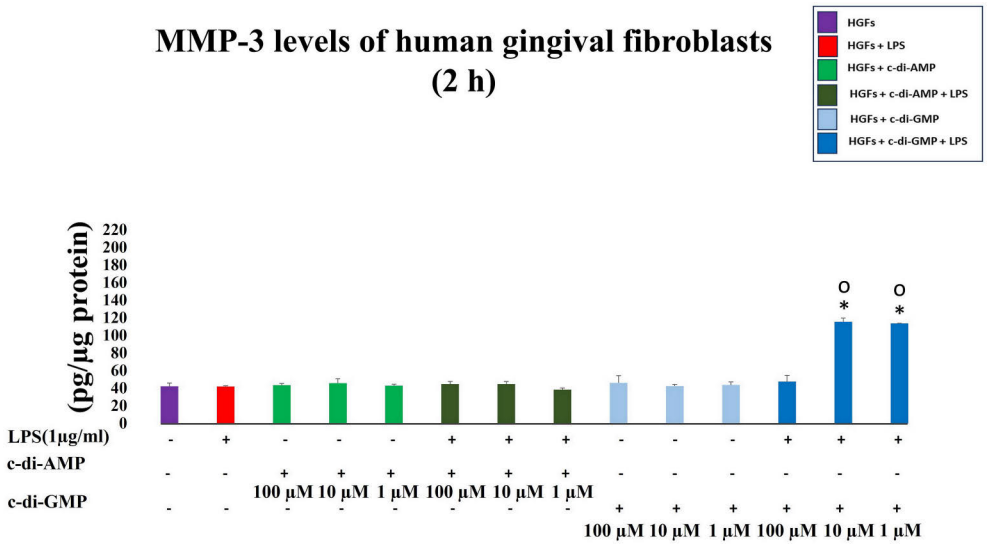

Figure 18. Early extracellular MMP-3 expression in human gingival fibroblasts (HGFs). HGFs were incubated with three concentrations of c-di-GMP and c-di-AMP alone or with *Pg* LPS for 2 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP), and ○ indicates a statistical difference with *Pg* LPS alone. (Modified from original publication II).

5.1.4 Impact of cyclic dinucleotides and *Pg* LPS on the late cellular response of HGFs (II)

HGF IL-8 concentrations elevated markedly in the presence of *Pg* LPS alone ($p < 0.001$) or combined with c-di-GMP ($p < 0.001$ at 100 µM and 10 µM, and $p = 0.002$ at 1 µM) or combined with any of the three c-di-AMP concentrations ($p < 0.001$) (Figure 19). When HGFs were cultured with c-di-GMP alone, IL-10 concentrations showed a marked increase ($p = 0.001$ at 100 µM) (Figure 20). Increasing the incubation time to 24 h did not enhance the expression of IL-34, and it stayed at the same unchanged level (Figure 21). Regarding the expression of MMP enzymes after prolonged incubation, MMP-1 levels increased significantly under the effect of *Pg* LPS and c-di-AMP (100 µM and 10 µM $p = 0.01$) (Figure 22). In contrast, more prolonged incubation decreased the expression of MMP-2 secretion of cells incubated with 1 µM of c-di-AMP ($p = 0.04$), 1 µM c-di-GMP ($p = 0.004$), and c-di-GMP with *Pg* LPS (100 µM and 10 µM $p = 0.01$, and 1 µM $p < 0.001$) (Figure 23). Exposure of HGFs to c-di-GMP and *Pg* LPS for 24 h enhanced significantly MMP-3 levels (100 µM $p = 0.009$, 10 µM $p = 0.03$) (Figure 24).

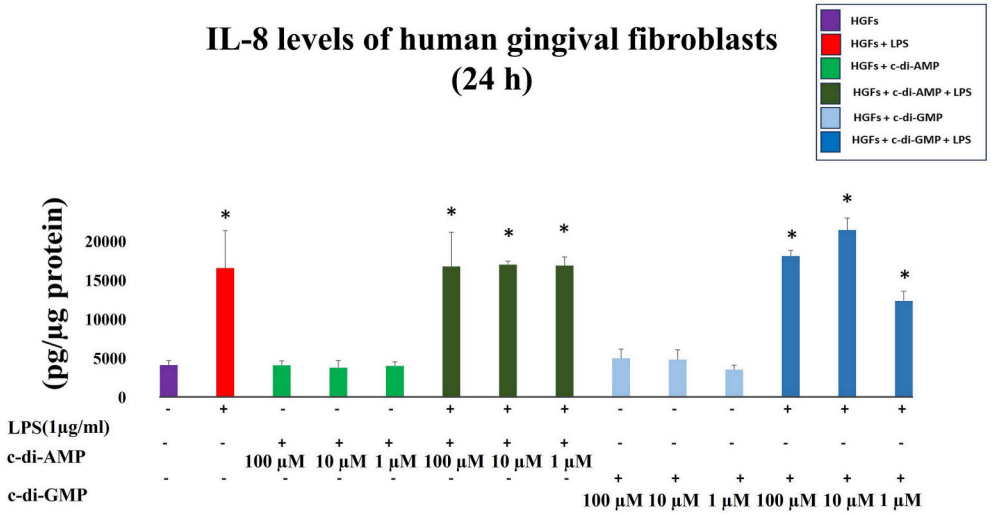

Figure 19. Late response of human gingival fibroblasts (HGFs) expression of extracellular IL-8. HGFs were incubated with three test concentrations of c-di-GMP and c-di-AMP either alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication II).

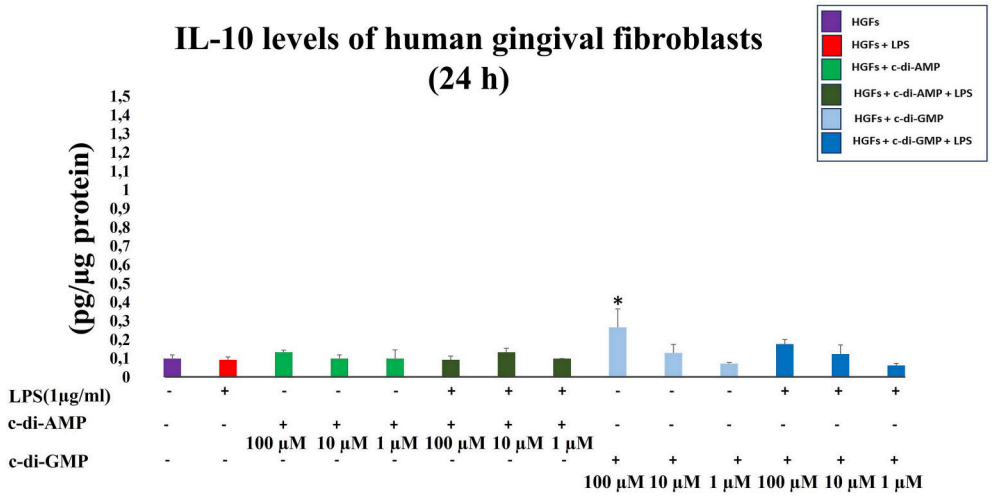

Figure 20. Late response of human gingival fibroblasts (HGFs) expression of extracellular IL-10. HGFs were incubated with three test concentrations of c-di-GMP and c-di-AMP either alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication II).

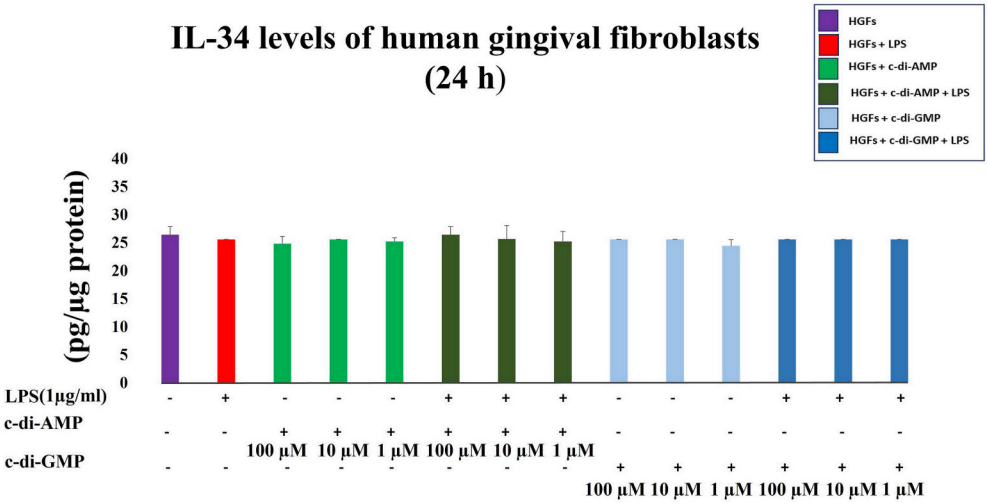

Figure 21. Late response of human gingival fibroblasts (HGFs) expression of extracellular IL-34. HGFs were incubated with three test concentrations of c-di-GMP and c-di-AMP either alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. (Modified from original publication II).

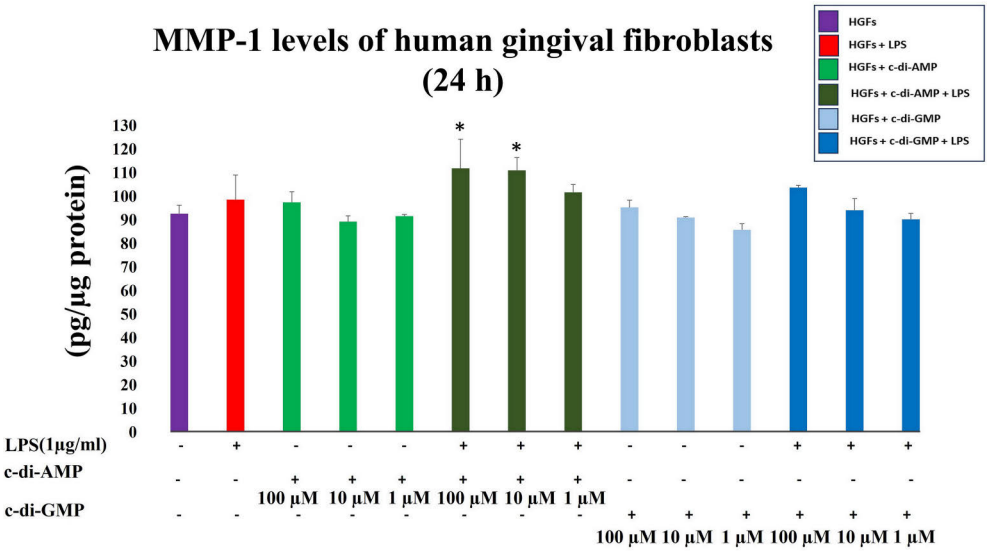

Figure 22. Late response of human gingival fibroblasts (HGFs) expression of extracellular MMP-1. HGFs were incubated with three test concentrations of c-di-GMP and c-di-AMP either alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication II).

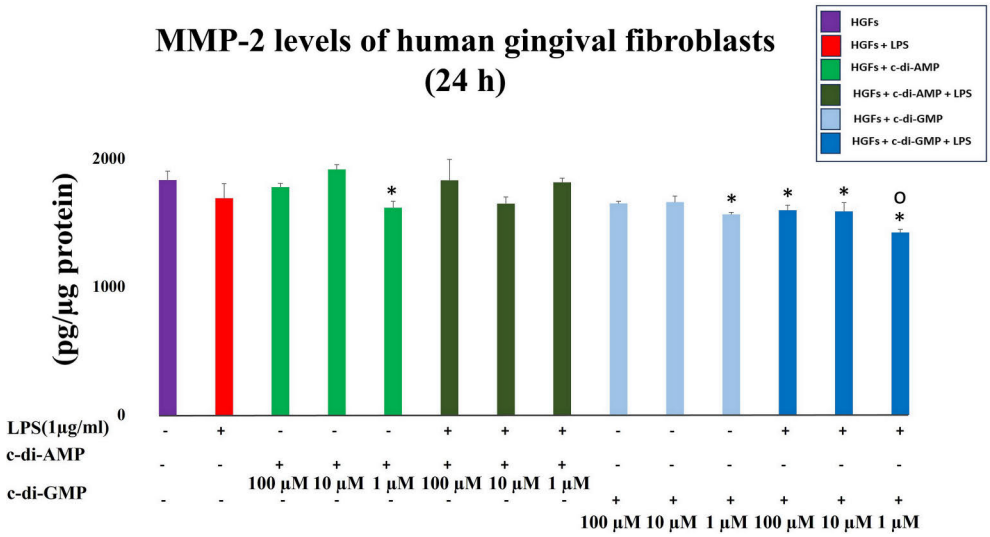

Figure 23. Late response of human gingival fibroblasts (HGFs) expression of extracellular MMP-2. HGFs were incubated with three test concentrations of c-di-GMP and c-di-AMP either alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP), and ○ indicates a statistical difference with *Pg* LPS alone. (Modified from original publication II).

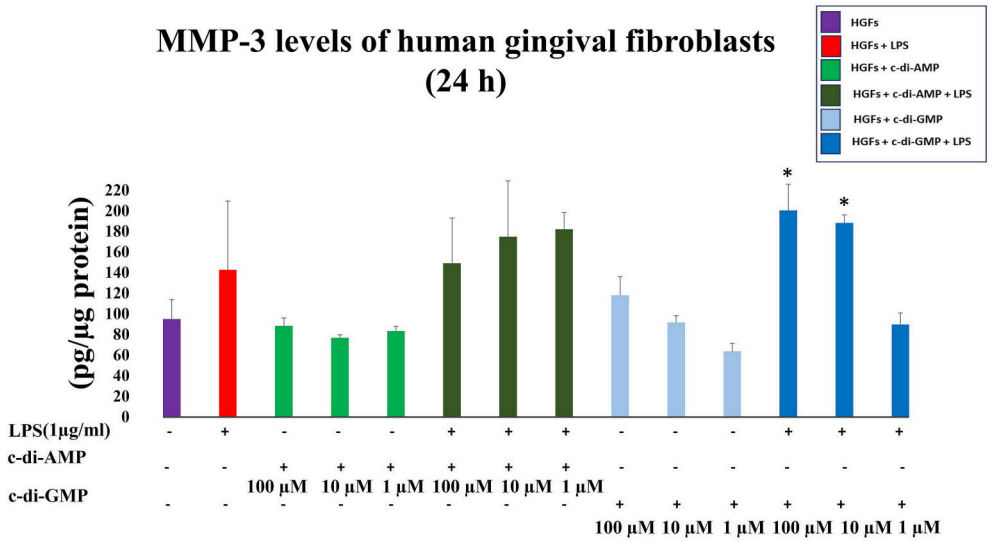

Figure 24. Late response of human gingival fibroblasts (HGFs) expression of extracellular MMP-3. HGFs were incubated with three test concentrations of c-di-GMP and c-di-AMP either alone or with *Pg* LPS for 24 h. Bars express the mean ± standard deviation for triplicate tests. ∗ Indicates a statistical difference with the control (no *Pg* LPS, c-di-GMP, or c-di-AMP). (Modified from original publication II).

5.2 Measurement of protein expression levels

5.2.1 Analysis of MAPKs in HMK cells (I)

Using image J software, western blot images were analysed to convert the intensity of protein bands to numbers, and significant correlations and bar charts were created using SPSS software. ERK1/2, phospho-ERK1/2, p38, and phospho-p38 were detected at ~45kDa, while JNK and phospho-JNK were detected at a range from 46 to 54 kDa in HMK cells. *Pg* LPS caused an increase in phospho-ERK1/2 levels and a significant decrease in phospho-p38 levels in HMK cells. C-di-AMP at 100 µM enhanced p38 and JNK kinases phosphorylation. In contrast, c-di-AMP and c-di-GMP at 1 µM inhibited p38 phosphorylation. Moreover, ERK1/2, JNK, and p38 phosphorylations were decreased in HMK cells incubated with c-di-GMP combined with *Pg* LPS. Most of the tested concentrations of c-di-AMP and c-di-GMP inhibited the phosphor-p38 level (Figure 25).

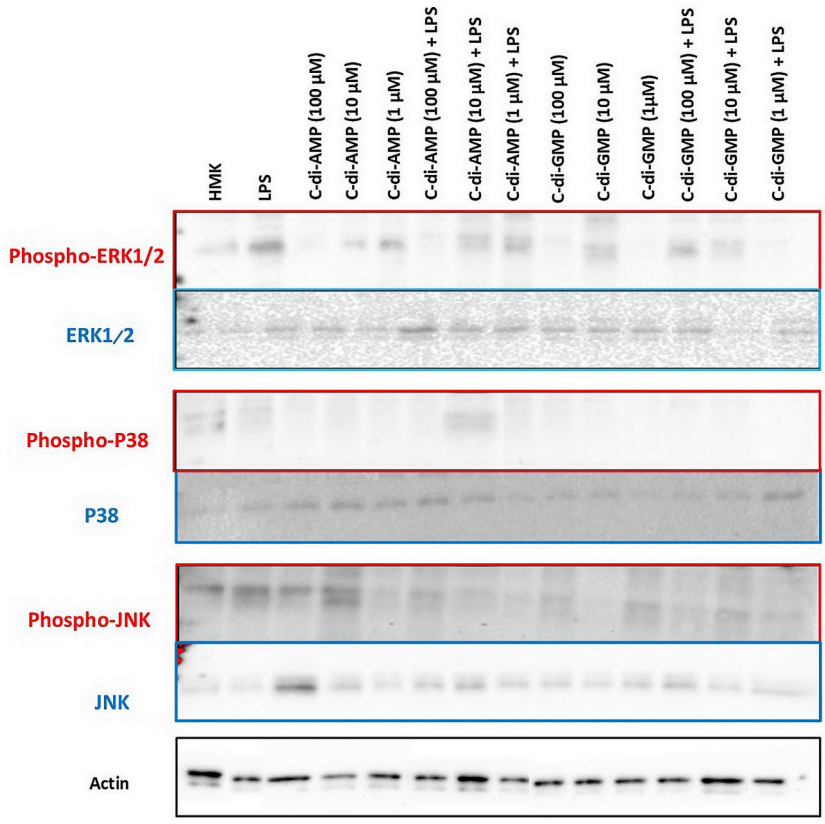

Figure 25. Effect of MAMPs on phosphorylated and non-phosphorylated forms of mitogenic-activated protein kinase pathways (MAPK) in cultured human gingival keratinocytes (HMK). (Modified from original publication I).

5.2.2 Analysis of STING, TBK1, and IRF3 protein levels in HGFs (III)

In response to stimulation of HGFs by three different concentrations of c-di-AMP and c-di-GMP, the presence of STING (at 42 kDa), TBK1 (at 84 kDa), and IRF3 (at 47 kDa) was detected at two time points of incubation (2 h and 24 h) by western blot assays (Figure 26).

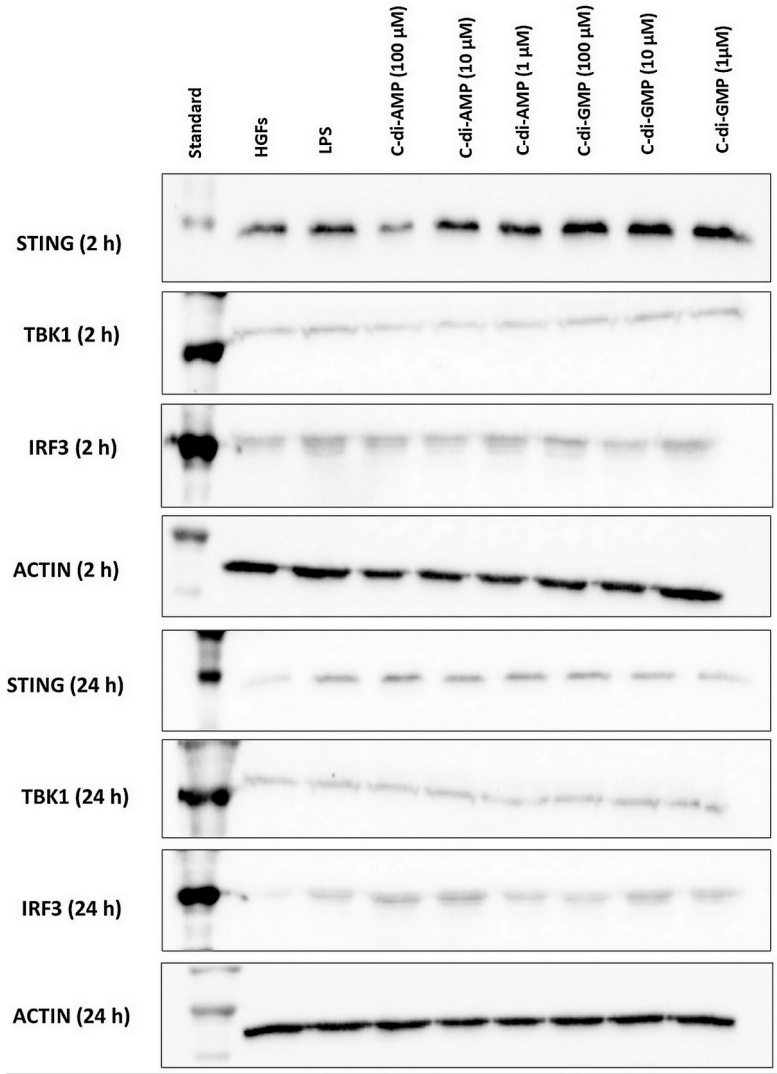

Figure 26. Expression of STING/TBK1/IRF3 proteins in human gingival fibroblasts (HGFs) at two time points of incubation (2 h and 24 h). (From the supplementary data of the original publication III).

5.2.3 Expression of STING, TBK1, and IRF3 in HMK cells (Unpublished data)

Figure 27 A displays the expression of STING, TBK1, and IRF3 proteins in cultured HMK cells with cyclic dinucleotides. By western blot, STING, TBK1, and IRF3 levels were detected. To confirm the non-specific bands of STING expression shown in the western blotting results, the same experiment was repeated by another cell line (human monocytic cell line (THP-1)); STING protein was detected at ~45 kDa by using a different STING polyclonal primary antibody (Novus biological, Abingdon) (Figure 27 B). A level of STING protein was detected at one specific band in HGFs at ~45 kDa, as visualized in Figure 26.

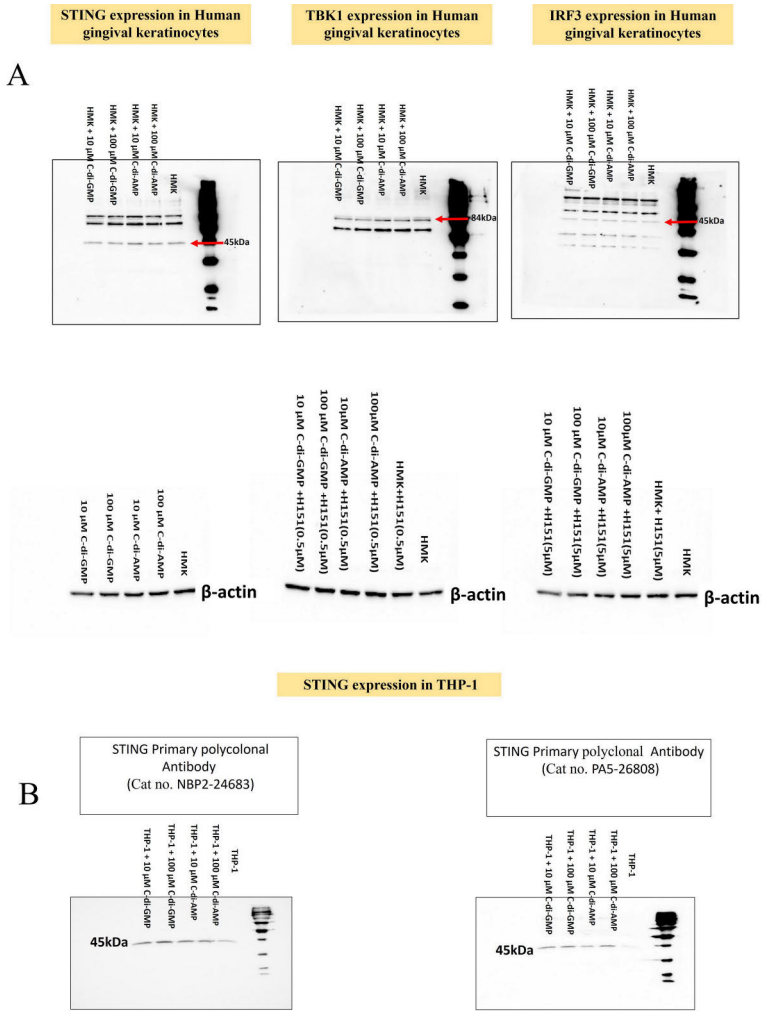

Figure 27. (A) Expression of STING, TBK1, and IRF3 in human gingival keratinocytes (HMK). (B) STING expression in a human monocytic cell line (THP-1). (Unpublished data).

5.2.4 Effect of cyclic dinucleotides and STING inhibitor H-151 on STING protein level in HMK cells (Unpublished data)

After analysis of the western blot images by image J software, this experiment showed that c-di-AMP and c-di-GMP at 100 µM (p =0.028 and p =0.048), respectively, decreased STING protein level of HMK cells. However, this effect disappeared when a low concentration of STING inhibitor H-151 (0.5 µM) was added to the cultured cells in the presence or absence of cyclic dinucleotides. Increasing the concentration of STING inhibitor H-151 to 5 µM decreased the expression of STING protein significantly again, not only when cells were cultured with STING inhibitor H-151 alone but also in the presence of c-di-AMP (at 100 µM p = 0.043 and 10 µM p = 0.007) or c-di-GMP (at 10 µM p =0.020) (Figure 28).

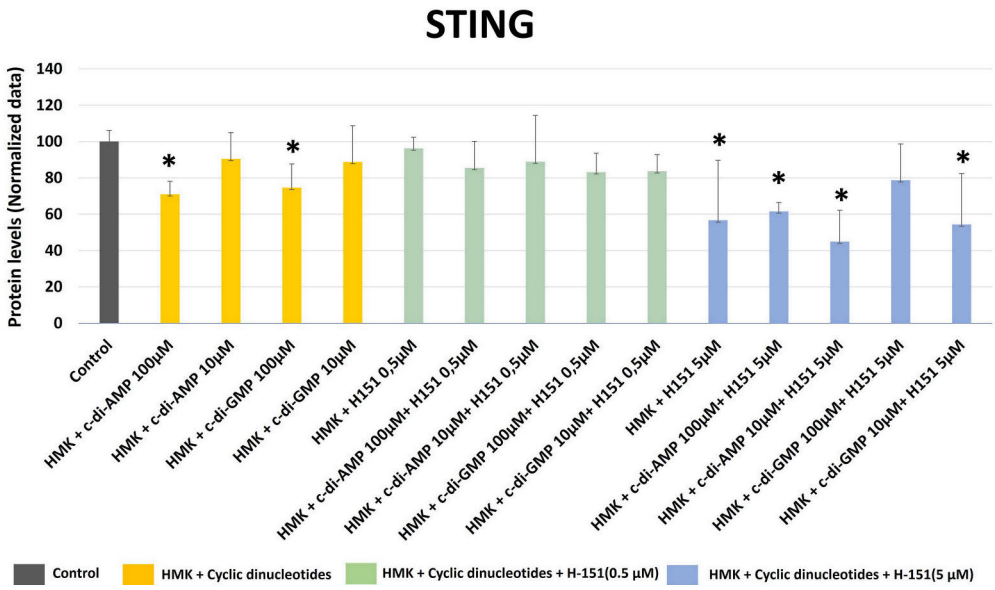

Figure 28. Level of stimulator of IFN genes (STING) in human gingival keratinocytes (HMK), HMK cells were cultured with cyclic dinucleotides (c-di-GMP and c-di-AMP), cyclic dinucleotides (c-di-GMP and c-di-AMP) with H-151 (0.5 µM), and cyclic dinucleotides (c-di-GMP and c-di-AMP) with H-151 (5 µM). The analysis was done on the raw data. (Unpublished data).

5.2.5 Effect of cyclic dinucleotides and STING inhibitor H-151 on TBK1 protein level in HMK cells (Unpublished data)

TBK1 protein levels showed inhibition in its expression in HMK cells cultured with c-di-AMP (at 100 µM p =0.038) and c-di-GMP (at 100 µM p 0.034 and 10 µM

p =0.039). Although the incubation of HMK cells with STING inhibitor H-151 (0.5 µM) did not represent any effect on protein expression, the level of TBK 1 decreased when STING inhibitor H-151 (5 µM) either individually or with 100 µM (p =0.008) and 10 µM (p =0.016) of c-di-AMP or 100 µM of c-di-GMP (p =0.016) added to cultured cells (Figure 29).

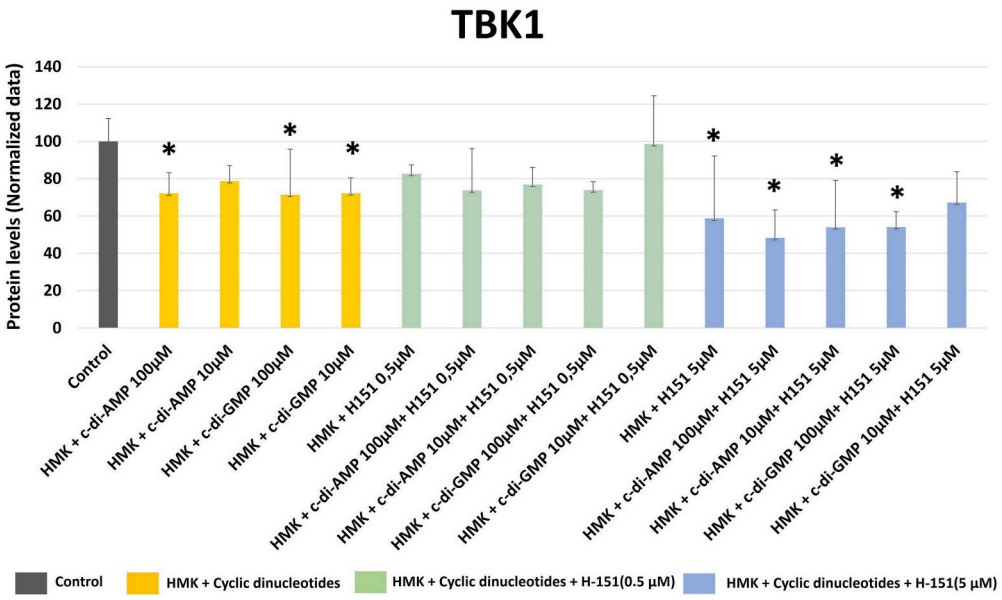

Figure 29. Level of TANK-binding kinase1 (TBK1) in human gingival keratinocytes (HMK). HMK cells were cultured with cyclic dinucleotides (c-di-GMP and c-di-AMP), cyclic dinucleotides (c-di-GMP and c-di-AMP) with H-151 (0.5 µM), and cyclic dinucleotides (c-di-GMP and c-di-AMP) with H-151 (5 µM). The analysis was done on the raw data. (Unpublished data).

5.2.6 Effect of cyclic dinucleotides and STING inhibitor H-151 on IRF3 protein level in HMK cells (Unpublished data)

Incubation of HMK cells with cyclic dinucleotides in the presence or absence of STING inhibitor H-151 (0.5 µM or 5 µM) did not affect the level of IRF3 expression (Figure 30).

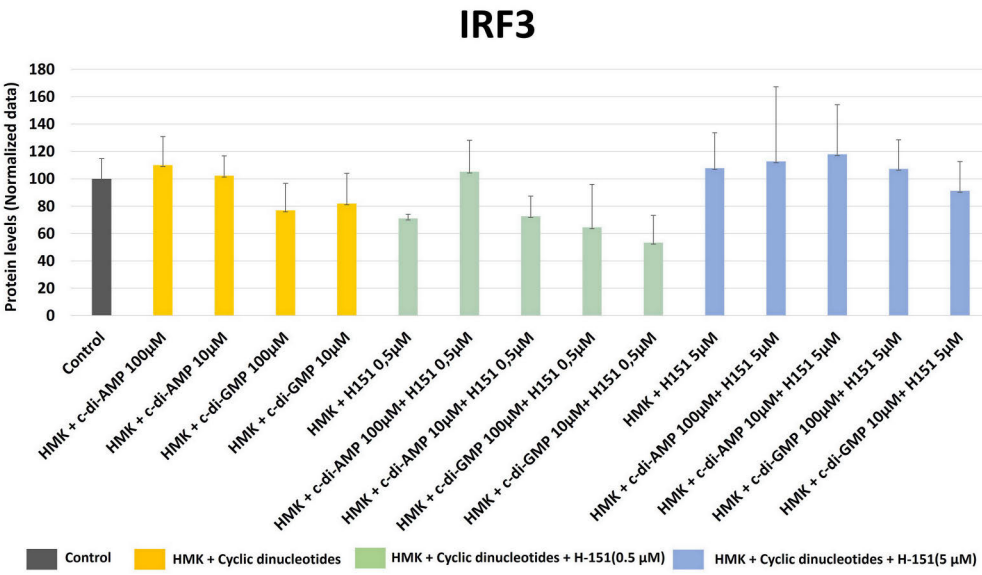

Figure 30. Level of interferon regulatory factors (IRF3) in human gingival keratinocytes (HMK). HMK cells were cultured with cyclic dinucleotides (c-di-GMP and c-di-AMP), cyclic dinucleotides (c-di-GMP and c-di-AMP) with H-151 (0.5 µM), and cyclic dinucleotides (c-di-GMP and c-di-AMP) with H-151 (5 µM). The analysis was done on the raw data. (Unpublished data).

5.3 An unbiased global proteomics analysis for HGFs (III and IV)

5.3.1 Number and percentage of regulated proteins in HGFs (III and IV)

The LC-MS/MS data analysis excluded proteins identified in only one biological replicate, while proteins detected in two or more biological replicates were included. Among these, the number of proteins were identified in the control fibroblasts without treatment (2,690) or treated with 100 µM of c-di-AMP (2,407) or with c-di-GMP (2,397). The percentage of total protein distribution among 100 µM of c-di-AMP, 100 µM of c-di-AMP, and control groups was 83% of the total proteins. The number and percentage of proteins recognized in fibroblasts treated with 100 µM of c-di-AMP, 100 µM of c-di-AMP, and in control fibroblasts are presented in Figure 31A.

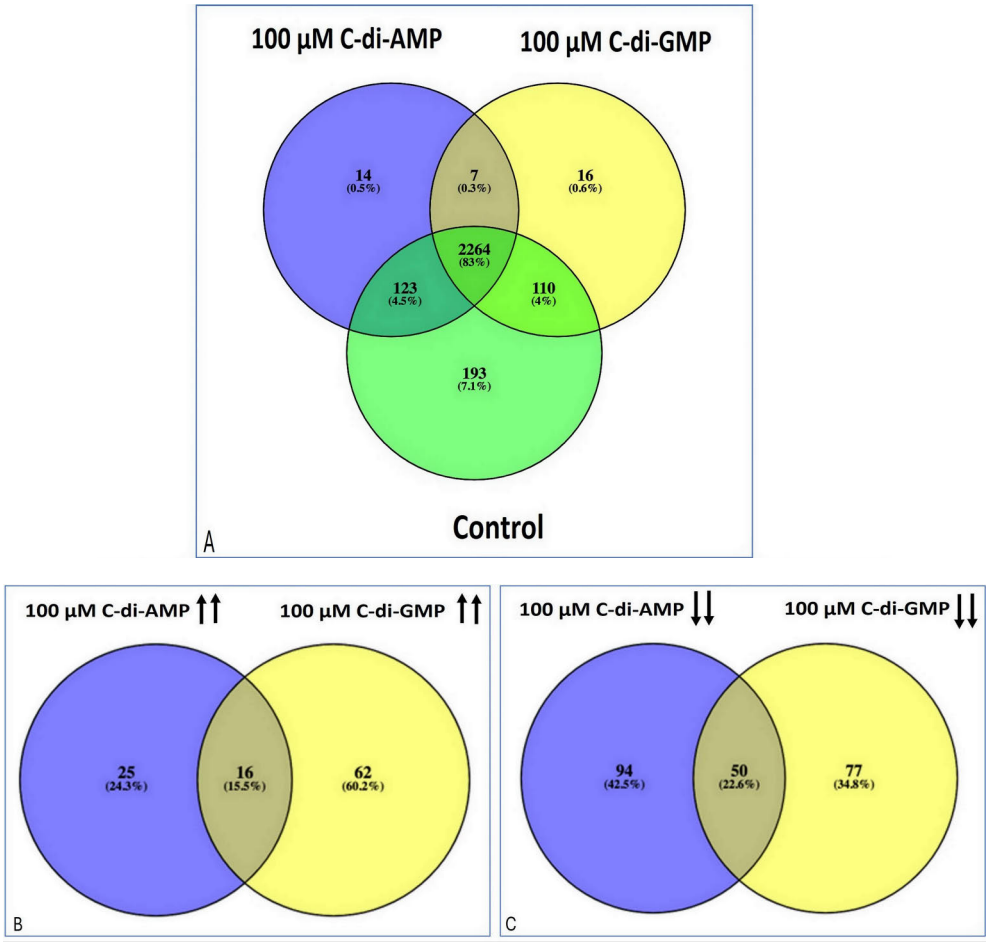

Figure 31. Venn diagram showing (A) number and percentages of proteins identified in the control and 100 µM of c-di-AMP and c-di-GMP treated human gingival fibroblasts (HGFs), (B) upregulated expressed proteins by c-di-AMP and c-di-GMP in HGFs, (C) downregulated proteins by c-di-AMP or c-di-GMP in treated HGFs. (A: From original publication III. B and C: unpublished data).

Figures 31 B and 31 C show the number and percentage of upregulated and downregulated proteins in HGFs treated by c-di-AMP and c-di-GMP, while Figures 32 A and 32 B present upregulated and downregulated proteins by c-di-AMP+*Pg* LPS, c-di-GMP+*Pg* LPS, and *Pg* LPS alone.

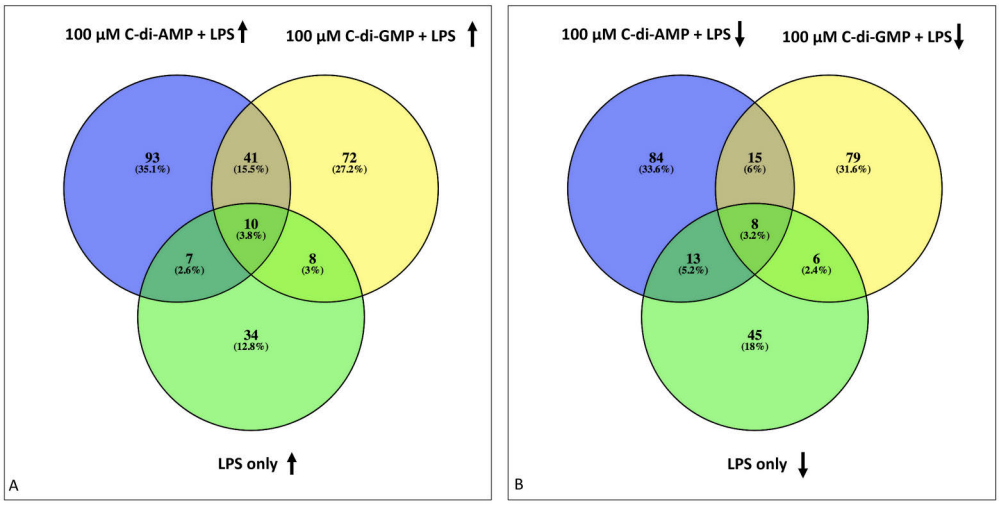

Figure 32. Venn diagram showing (A) number and percentages of upregulated proteins identified in 100 µM of c-di-AMP+*Pg* LPS, 100 µM c-di-GMP+*Pg* LPS, and *Pg* LPS treated human gingival fibroblasts (HGFs), (B) downregulated proteins (number and percentages) by c-di-AMP+*Pg* LPS, 100 µM c-di-GMP+*Pg* LPS, and *Pg* LPS in treated HGFs. (From original publication IV).

Scatter plots and correlation plots illustrate a good correlation between the biological replicates (Figure 33 A-C) and between samples (Figure 33 D and 33 E). In control and cyclic dinucleotides treated fibroblast samples, proteins were clustered into three distinct groups as hierarchical clustering (Figure 34). Volcano plots at significant p-value (< 0.05) demonstrated several upregulated and downregulated proteins by 100 µM of c-di-AMP and 100 µM of c-di-AMP (Figure 35).

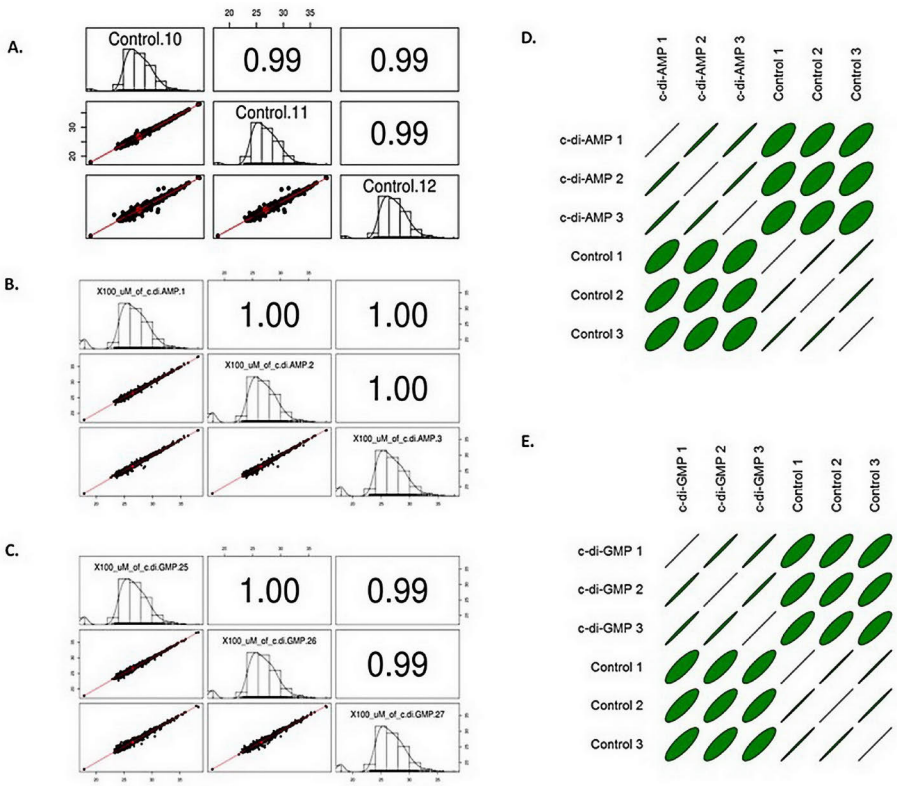

Figure 33. Scatter plots (A-C) illustrate the good correlation among the biological replicates. Correlation plots (D and E) present good correlation between samples. (From the supplementary data of the original publication III).

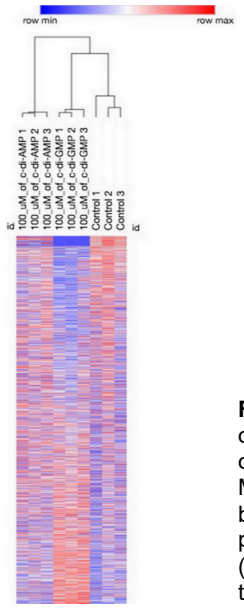

Figure 34. Heatmap showing hierarchical clustering of proteins into three groups (control and the c-di-AMP or c-di-GMP treatment) according to the intensities. Maximum intensities = red, and lower intensities = blue. Hierarchical clustering and heatmaps were plotted with Morpheus, Version 2020 software (https://software.broadinstitute.org/morpheus). (From the supplementary data of the original publication III).

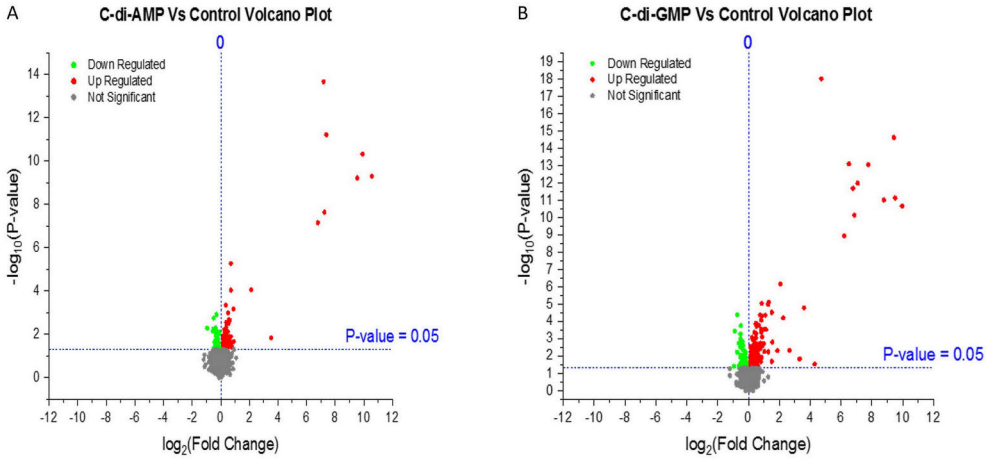

Figure 35. Volcano plots show significantly upregulated proteins (red dots) and downregulated proteins (green dots) in (A) 100 µM of c-di-AMP and (B) 100 µM of c-di-AMP groups compared to controls. The horizontal blue line represents the Log10 (p-value) cutoff. Volcano plots were plotted using the Origin (Pro) Version 2020 software (OriginLab Corporation, Northampton, MA). (From original publication III).

5.3.2 Analysis of differentially expressed proteins in HGFs treated with cyclic di-adenosine monophosphate (c-di-AMP) and cyclic di-guanosine monophosphate (c-di-GMP) (III)

Regarding the significantly upregulated proteins in both studies III and IV, proteins that were not identified in control fibroblasts but were identified in two biological replicates or more in each of the tested groups were recognized as significantly upregulated ones according to their treatment condition, in addition to upregulated proteins at ($p \leq 0.05$ and Log2 fold change ≥ 0.5). In contrast, proteins were considered significantly downregulated when identified in the control group but not in each treatment group and differentially downregulated proteins with $p \leq 0.05$ and Log2 fold change ≥ -0.5 (a measurable fold change) by the respective treatment.

Heatmap identified the top 50 differentially expressed proteins by c-di-AMP and c-di-GMP (Figures 36 A and 36 B). Of these, proteins related to the interferon signaling and innate immune response as ISG15 (ubiquitin-like protein ISG15), SAMHD1 (deoxynucleoside triphosphate triphosphohydrolase), STAT1 (signal transducer and activator of transcription 1-alpha/beta), STAT2, HLA-A (MHC class I antigen), MX1 (interferon-induced GTP-binding protein 1), IFIT1 (interferon-induced protein with tetratricopeptide repeats1), and IFIT3. Moreover, individual stimulation of c-di-GMP or c-di-AMP to HGFs upregulated other important proteins that stimulate immunity and kill the pathogen as SOD2 (superoxide dismutase), immunity related GTPase Q, and NFKB2 (NF-kappa-B p100 subunit) that were

upregulated by c-di-GMP. More examples of upregulated proteins by c-di-GMP include TNFRSF11B (tumor necrosis factor receptor superfamily member 11B), B2M (Beta-2-microglobulin), and MX2. In the same context, c-di-AMP upregulated IRF9 and interferon-induced 35 kDa protein.

Among the top 10 upregulated significant proteins with a measurable fold change, ISG15, SAMHD1, and 2'-5'-Oligoadenylate synthetase 3 (OAS3) were common upregulated proteins in cyclic dinucleotides treated fibroblasts compared to controls (Figures 37 A and 37 B).

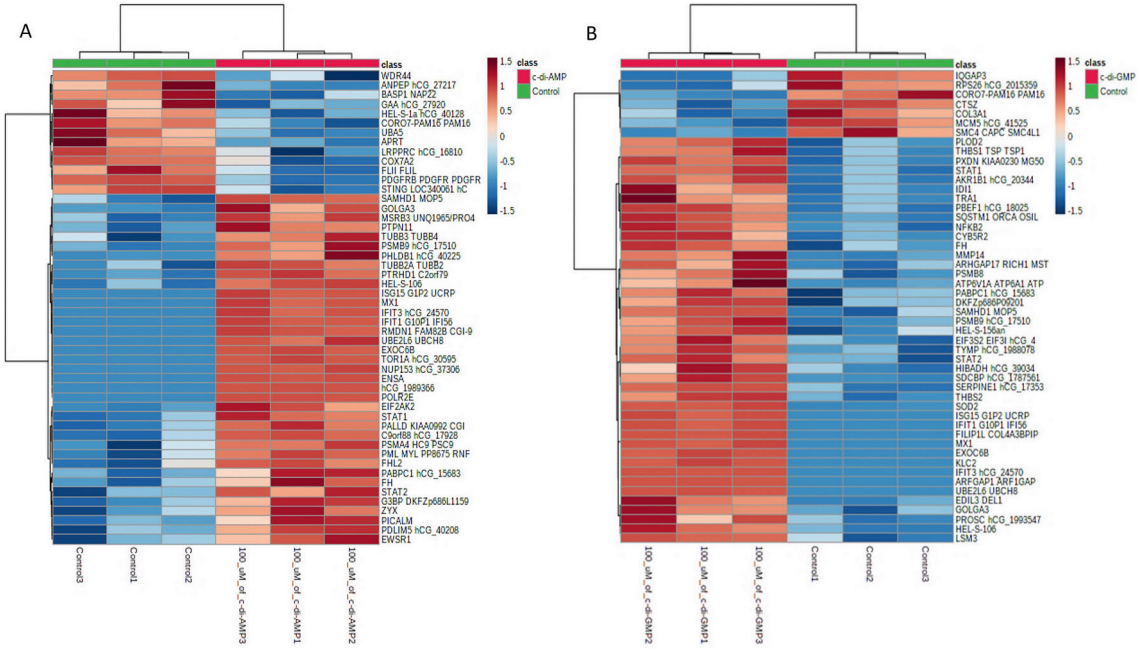

Figure 36. Heatmap illustrating the top 50 differentially expressed proteins by (A) c-di-AMP, (B) c-di-GMP, identified as (blue = downregulated, oxblood/red = upregulated). Heatmaps were plotted on the MetaboAnalyst software Version 5.0 with auto-scale normalized data. (From original publication III).

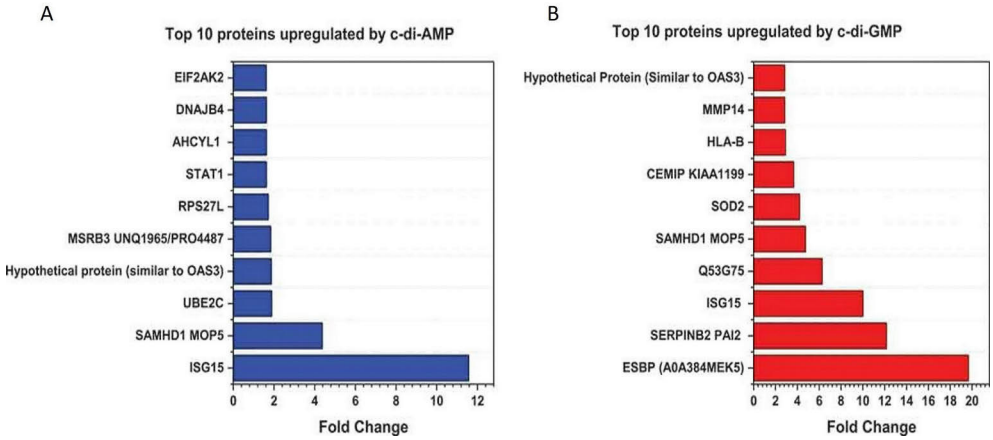

Figure 37. Top 10 proteins upregulated with a measurable fold change by **(**A**)** c-di-AMP (blue bars), (B) c-di-GMP (red bars) in human gingival fibroblasts (HGFs). Charts were plotted using the Origin (Pro) Version 2020 software (OriginLab Corporation, Northampton, MA). (From original publication III).

5.3.3 Analysis of significantly upregulated proteins in HGFs treated with cyclic dinucleotides combined with *Pg* LPS (IV)

Important innate immunity proteins were among the total 41 common proteins upregulated in either c-di-AMP+*Pg* LPS or c-di-GMP+*Pg* LPS treated HGFs as ISG15, SAMHD1, STAT1, HLA-A, MX1, MX2, IFIT1, IFIT3, B2M, OAS3, SERPINB2 (plasminogen activator inhibitor 2) and interferon gamma-inducible protein 16. More proteins that are important for host defense and play a role as anti-inflammatory agents were upregulated in the c-di-AMP+*Pg* LPS treatment group. Of these 2′,3′-cyclic nucleotide 3′-phosphodiesterase, E3 ubiquitin-protein ligase RNF213, and D-dopachrome tautomerase. In the same context, other proteins involved in immune and inflammatory responses were upregulated in HGFs exclusively by a combination of c-di-GMP+*Pg* LPS, such as NFKB2, interferon-induced guanylate-binding protein 1, MAPK protein, thrombospondin 1 and 2, and TNFRSF11B. Regarding proteins that are not primary inflammatory proteins, such as guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12 (GNG12), programmed cell death protein 8, protein phosphatase 1, adenylate kinase 2, and DNA repair protein XRCC1 were profiled as upregulated proteins in HGFs treated with c-di-AMP+*Pg* LPS.

Individual stimulation of HGFs by *Pg* LPS upregulated the expression of proteins that regulate immunity and inflammatory response, and it was not stimulated in combined groups (*Pg* LPS and cyclic dinucleotides), including NF-κB essential modulator and ATP-dependent RNA helicase. In the three tested groups, c-

di-AMP+*Pg* LPS, c-di-AMP+*Pg* LPS, and *Pg* LPS alone, the top 50 differentially expressed proteins are demonstrated by heatmaps (Figure 38 A-C).

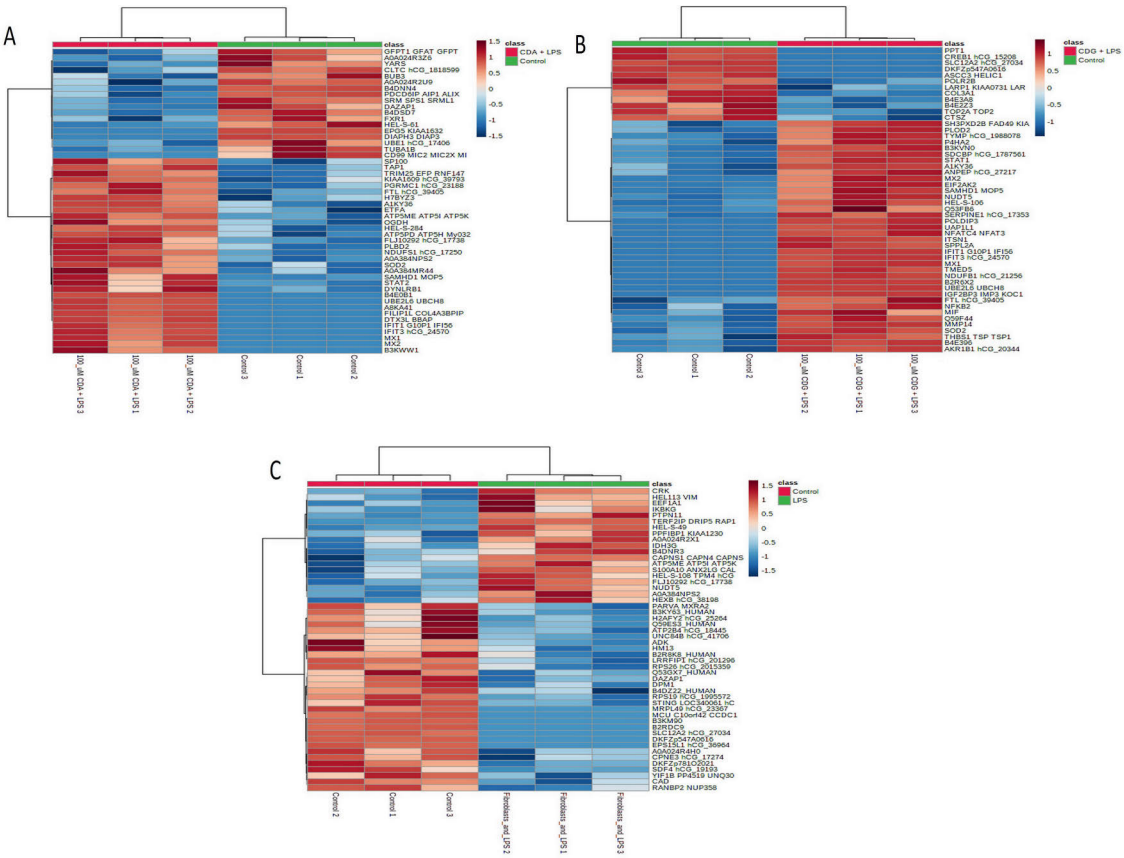

Figure 38. Heatmap illustrating the top 50 differentially expressed proteins after treatment of human gingival fibroblasts (HGFs) by (A) c-di-AMP+*Pg* LPS, (B) c-di-GMP+*Pg* LPS, (C) *Pg* LPS. Proteins are identified as (blue = downregulated, oxblood/red = upregulated). MetaboAnalyst software Version 5.0 with auto-scale normalized data was used to plot the heat maps. (From original publication IV).

Figure 39 (A-C) shows the top 10 significantly upregulated proteins in three treatment groups (c-di-AMP+*Pg* LPS, c-di-AMP+*Pg* LPS, and *Pg* LPS alone). In c-di-AMP+*Pg* LPS and c-di-GMP+*Pg* LPS treated HGFs, ISG15, OAS3 like, SAMHD1, and SERPINB2 were common upregulated proteins. Among the top 10 upregulated proteins by c-di-AMP+*Pg* LPS, ISG15 was the highest upregulated (Log2 fold change > 4).

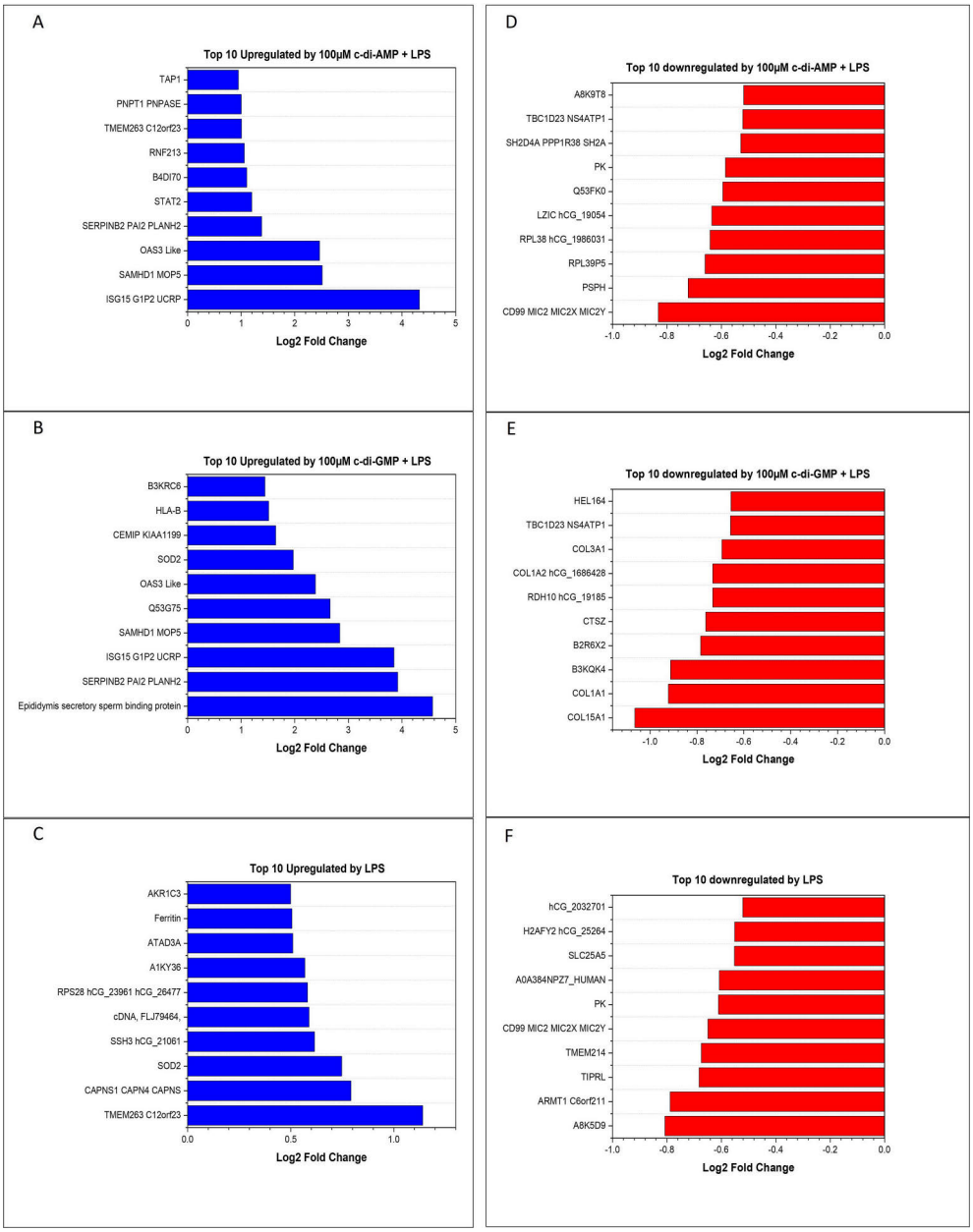

Figure 39. Top 10 significantly upregulated (A-C) and downregulated (D-F) proteins with a measurable fold change in three treatment groups (c-di-AMP+*Pg* LPS, c-di-AMP+*Pg* LPS, *Pg* LPS alone). Charts were plotted using the Origin (Pro) Version 2020 software (OriginLab Corporation, Northampton, MA). (From original publication IV).

5.3.4 Analysis of significantly downregulated proteins in HGFs treated with c-di-AMP and c-di-GMP (III)

Downregulated proteins in HGFs treated by c-di-AMP and c-di-GMP are seen in Figures 36 A and 36 B. Examples of downregulated proteins by both c-di-AMP and c-di-GMP include queuosine salvage protein and E3 ubiquitin-protein ligase, in addition, essential kinases such as uridine-cytidine kinase 2 and serine/threonine-protein kinase N2 were also downregulated by both c-di-GMP and c-di-AMP. While c-di-AMP downregulated E3 ubiquitin-protein ligase RNF 181 as well as other critical kinases such as MAPK, diacylglycerol kinase (DGK), and glycerol kinase. Moreover, other proteins were recognized as downregulated proteins exclusively by c-di-GMP, as a phosphatase, glycerol-3-phosphate phosphatase (G3PP), as well as a phosphatase inhibitor, cAMP-regulated phosphoprotein 19.

5.3.5 Analysis of significantly downregulated proteins in HGFs treated with cyclic dinucleotides combined with *Pg* LPS (IV)

Common downregulated proteins in c-di-AMP+*Pg* LPS or c-di-GMP+*Pg* LPS treated HGFs were nuclear pore complex protein Nup88, proliferating cell nuclear antigen, and IQ motif containing GTPase activating protein 3. However, c-di-AMP+*Pg* LPS downregulated serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit, phosphoserine phosphatase, and Ras-related protein R-Ras2. In comparison, G3PP was downregulated by only c-di-GMP+*Pg* LPS. Whereas when HGFs stimulated by *Pg* LPS, STING and guanine nucleotide-binding protein G(i) subunit alpha (G(i) alpha-3) that has a regulatory role in many pathologic processes (Li et al., 2015) were downregulated, and the highest measurable downregulated protein was A8K5D9 which is highly similar to homo sapiens anillin. Figure 39 (D-F) shows the top 10 significantly downregulated proteins in the three treatment groups (c-di-AMP+*Pg* LPS, c-di-AMP+*Pg* LPS, and *Pg* LPS alone).

5.3.6 Multivariate analysis of proteins identified in HGFs stimulated by cyclic dinucleotides (III)

For multivariate analysis, proteins included in this analysis are initially identified as significant proteins (Log2 fold change $\geq \pm 0.5$ and $p \leq 0.05$) or only identified in either control or treated HGFs. The PCA was used to determine if cyclic dinucleotides in treated HGF samples have different effects from control samples by illustrating the mapping of all the significant proteins in all tested groups (Figure 40 and Figure 41).

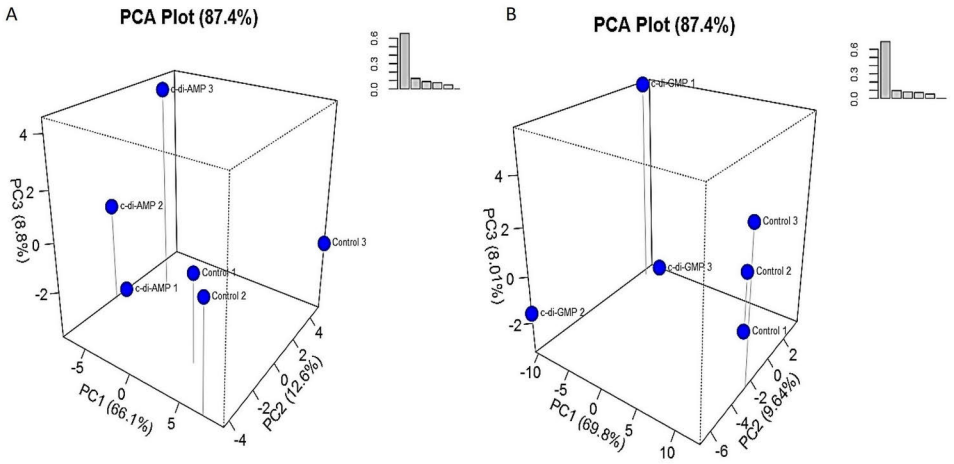

Figure 40. Principal component analysis (PCA) showing the mapping of all the significant proteins in (A) 100 µM c-di-AMP, (B) 100 µM c-di-GMP treated human gingival fibroblasts (HGFs). (From the supplementary data of the original publication III).

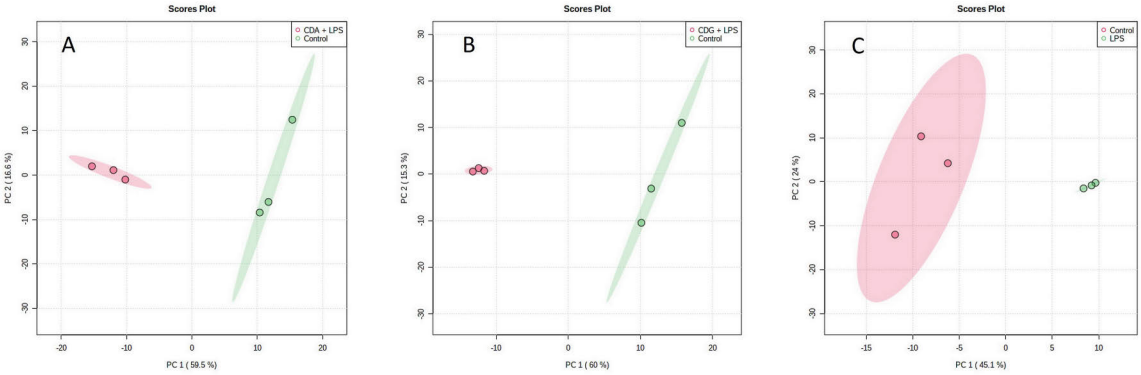

Figure 41. Principal component analysis (PCA) shows the relation between all significant proteins in human gingival fibroblasts (HGFs), following three treatment conditions: (A) C-di-AMP+*Pg* LPS, (B) C-di-GMP+*Pg* LPS, (C) *Pg* LPS. Metaboanalyst software was used to plot the PCA. (From the supplementary data of original publication IV).

In fibroblasts treated with c-di-AMP or c-di-GMP, principal components (PC) 1 and 2 account for more than 99% of upregulated or downregulated significant proteins (Figure 42). In addition, similar significantly upregulated proteins (MX1, IFIT1, IFIT3, SAMHD1, ubiquitin conjugating enzyme E2L6 (UBE2L6)) were found in the upper PC2 axis of component mapping in c-di-AMP and c-di-GMP biplots, and they were included in the 17 common proteins upregulated by both cyclic dinucleotides. For instance, proteins only upregulated by c-di-GMP as filamin A interacting protein 1 like (FILIP1L) and SOD2 appeared in the upper section at the PC2 and PC1 regions of the c-di-GMP biplot (Figure 42 B). Variable importance plots (VIP) scores were identified by a supervised classification method (Partial least

squares discriminant Analysis, PLS-DA) to score the concentrations of all upregulated and downregulated proteins in each treatment group. Both c-di-AMP and c-di-GMP upregulated common proteins including IFIT1, IFIT3, ISG15, UBE2L6, MX1, as well as STAT1 (Figure 43). By T-test ($p < 0.0001$), IFIT1, IFIT3, ISG15, and MX1 were the topmost significant upregulated common proteins, as shown in Figure 44 and Figure 45. Furthermore, IFIT1, IFIT3, ISG15, MX1, α-endosulfine (ENSA), SAMHD1, STAT1, SOD2, and FILIP1L, shown by PCA biplots, were also detected by the heatmaps among the top 50 common proteins (Figures 36 A and 36 B).

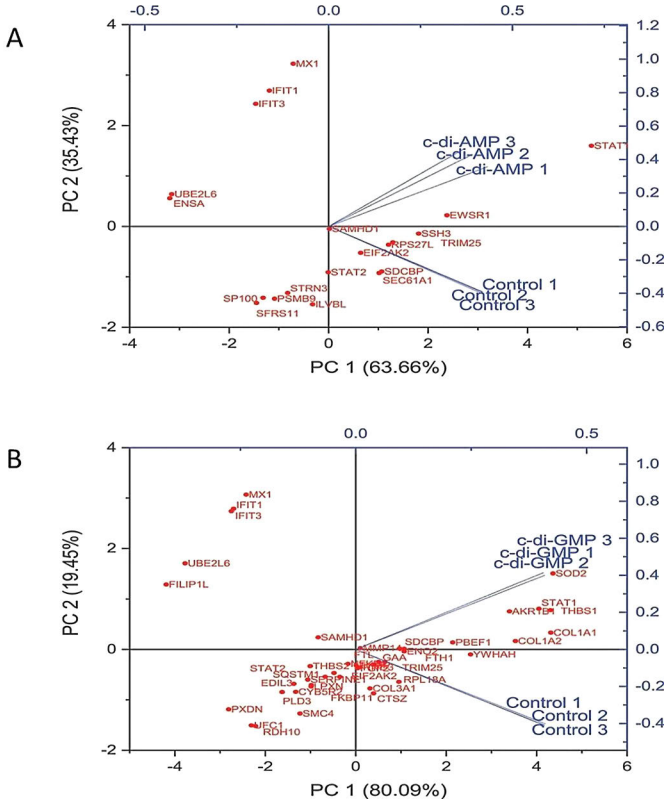

Figure 42. Multivariate analysis reveals a similar pattern of protein expression modulation by both c-di-AMP and c-di-GMP. PCA analysis and biplots were created in the Origin (Pro), Version 2020 software (originlab corporation, northampton, MA). (Modified from original publication III).

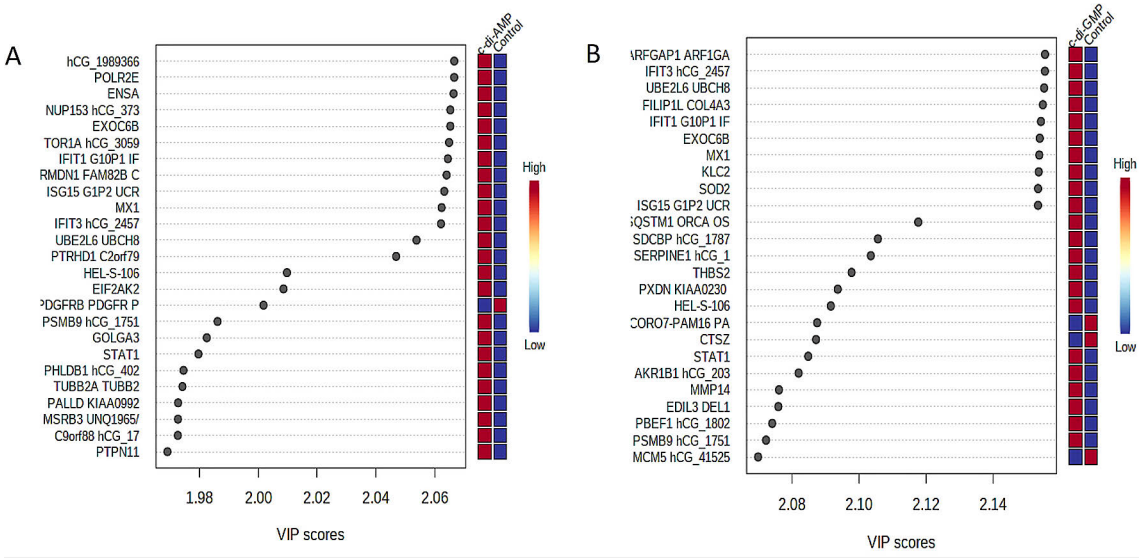

Figure 43. Variable importance plots (VIP) indicate the relative concentrations of the corresponding protein in (A) c-di-AMP (B) c-di-GMP by colored boxes on the right (red = upregulated, blue = downregulated). (From the supplementary data of the original publication III).

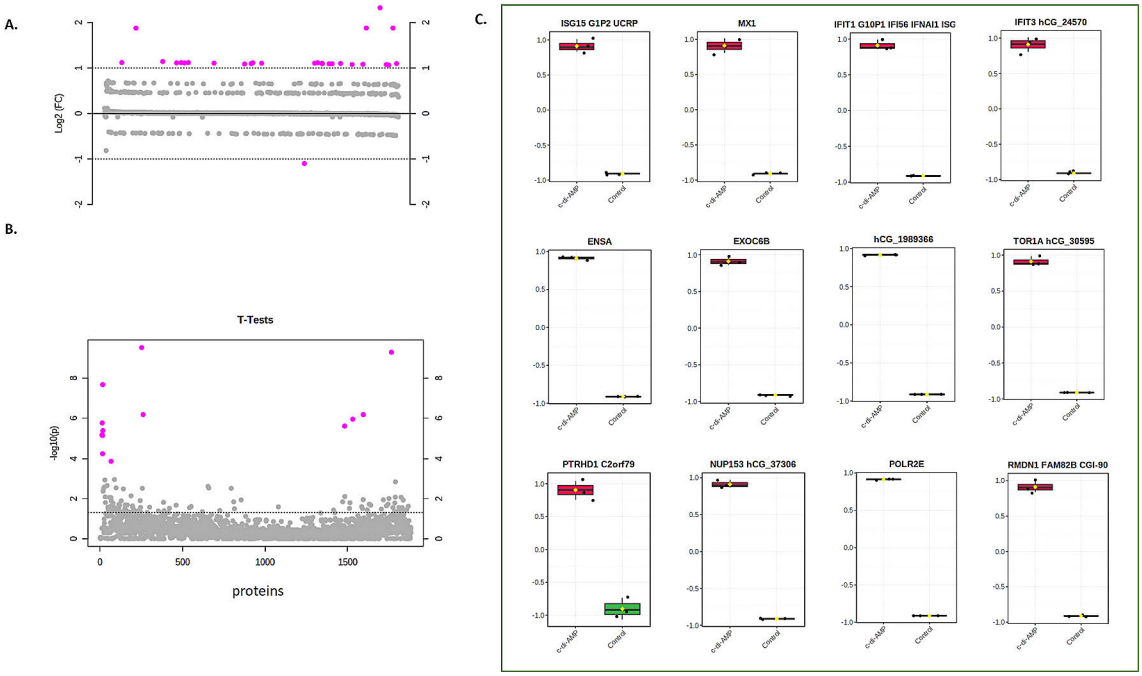

Figure 44. Statistical analysis of 100 µM c-di-AMP treatment group. (A) The most affected proteins with fold change analysis Log2 fold change threshold of 1 (2 fold increase). (B) T-test with p-values transformed by –Log10 so that most significant proteins (with smaller p-values) are seen higher on the graph (magenta dots). (C) Graphs of the most significant proteins from magenta dots from (B). (From the supplementary data of the original publication III).

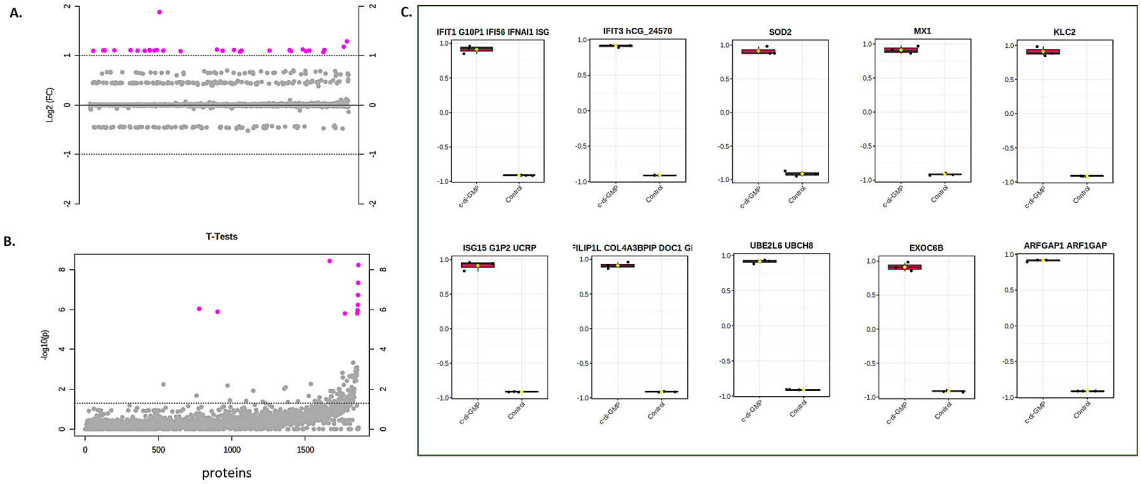

Figure 45. Statistical analysis of 100 µM c-di-GMP treatment group. (A) Magenta indicates the most affected proteins with fold change analysis Log2 fold change threshold of 1 (2 fold increase). (B) The most significant proteins (with smaller p-values) with T-test with p-values transformed by –Log10 are seen higher on the graph. (C) Graphs of the most significant proteins (magenta dots from (B)). (From the supplementary data of the original publication III).

5.3.7 Multivariate analysis of proteins identified in HGFs treated with cyclic dinucleotides combined with *Pg* LPS (IV)

The VIP scores showed that treatment of HGFs with c-di-AMP+*Pg* LPS or c-di-GMP+*Pg* LPS upregulated proteins, such as IFIT1, IFIT3, UBE2L6, MX1, and MX2 (Figure 46), and were presented by T-test ($p < 0.0001$) on the top of statistically significant upregulated proteins in same groups as demonstrated in Figure 47 and Figure 48. Moreover, by heatmaps, these proteins were among the top 50 upregulated proteins by c-di-AMP+*Pg* LPS or c-di-GMP+*Pg* LPS (Figures 38A and 38 B).

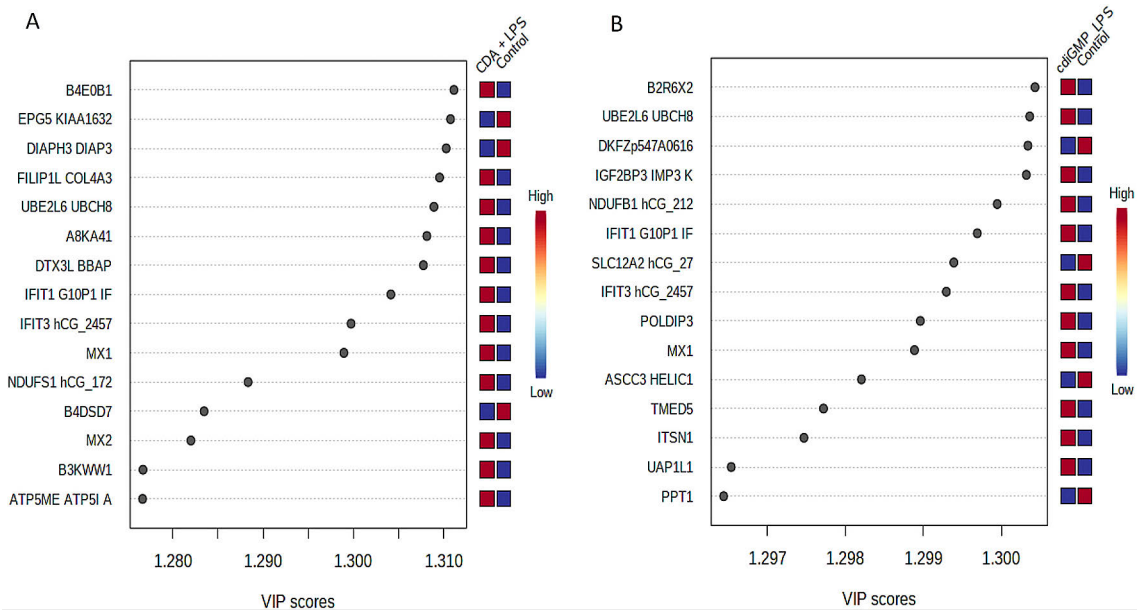

Figure 46. Variable importance plots (VIP) indicate the relative concentrations of the corresponding protein in (A) c-di-AMP+*Pg* LPS and (B) c-di-GMP+*Pg* LPS by colored boxes on the right (red = upregulated, blue = downregulated). (From original publication IV)

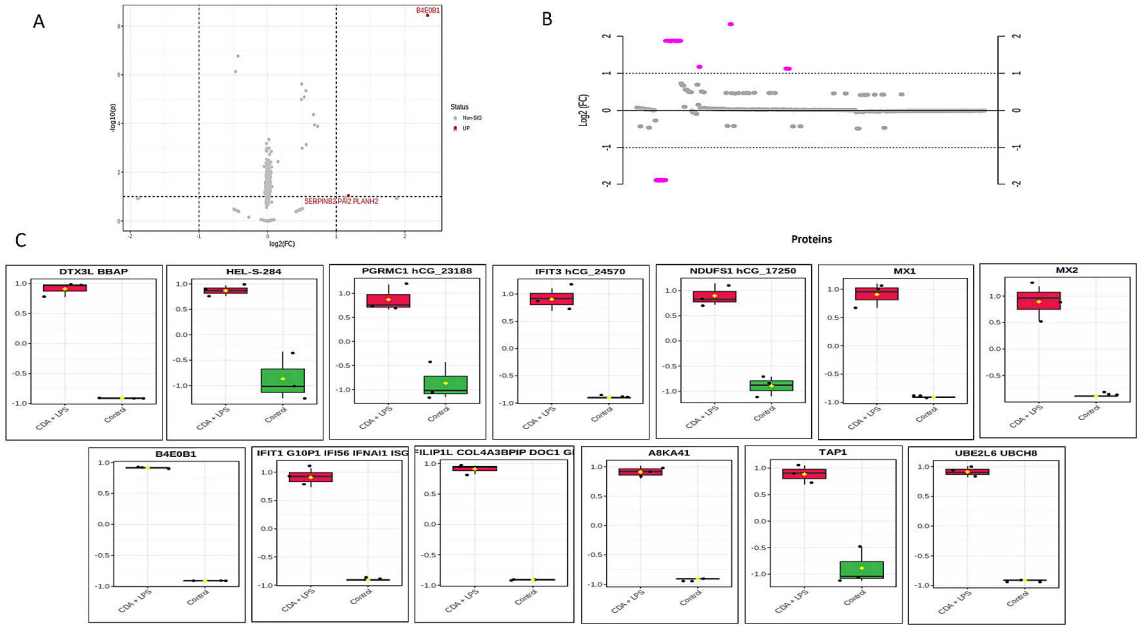

Figure 47. Statistical analysis of 100 µM c-di-AMP+*Pg* LPS treatment group. (A) Magenta indicates the most affected proteins with fold change analysis Log2 fold change threshold of 1 (2 fold increase). (B) The most significant proteins (with smaller p-values) with T-test with p-values transformed by –Log10 are seen higher on the graph. (C) Graphs of the most significant proteins (magenta dots from (B)). (From the supplementary data of the original publication IV).

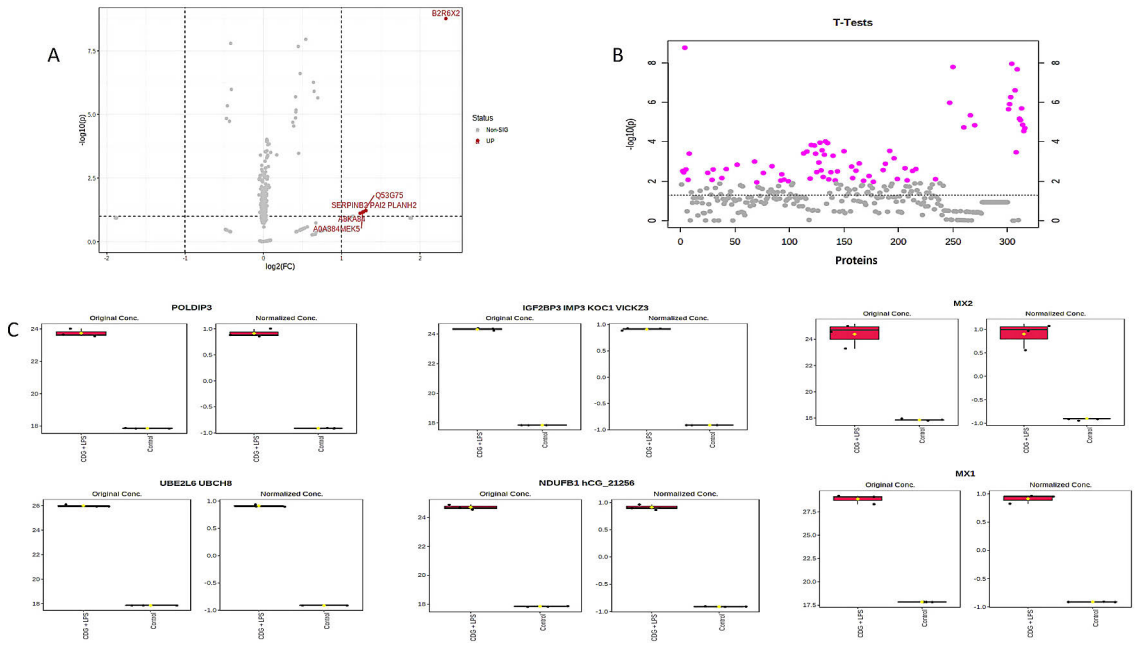

Figure 48. Statistical analysis of 100 µM c-di-GMP+*Pg* LPS treatment group. (A) Magenta indicates the most affected proteins with fold change analysis Log2 fold change threshold of 1 (2 fold increase). (B) The most significant proteins (with smaller p-values) with T-test with p-values transformed by –Log10 are seen higher on the graph. (C) Graphs of the most significant proteins (magenta dots from (B)). (From the supplementary data of the original publication IV).

5.3.8 Functional analysis of proteins modulated by cyclic dinucleotides (III)

Using IPA functional pathway analysis, the effect of MAMPs, either individually or combined, on various biological signaling pathways in HGFs treated by one of the following treatments (c-di-AMP, c-di-GMP, c-di-AMP+*Pg* LPS, c-di-GMP+*Pg* LPS, and *Pg* LPS) was investigated.

Signaling pathways were regulated differently according to treatment conditions in HGFs. C-di-GMP regulated 150 ingenuity canonical pathways (p-value ≤ 0.05), while c-di-AMP regulated 31 pathways. In comparison, 21 common pathways with z-scores ≥ 2 identified were regulated by c-di-AMP and c-di-GMP (Figure 49 A) and Table 2. Among the various canonical pathways, interferon signaling was the most significantly regulated in HGFs treated by c-di-GMP (a -log p-value of 9.49) and c-di-AMP (a -log p-value of 11.0), with a positive activation z-score > 2 in both treatment groups. For c-di-AMP and c-di-GMP treatment conditions, the top 10 most significantly regulated pathways based on $p < 0.05$ are shown in Figures 49 B and 49 C.

A clear illustration of specific proteins that were involved in various biological pathways by activation or inhibition is shown in the graphical summary (Figure 50). The difference in the molecules participating in the interferon signaling network between treatment groups, c-di-GMP and c-di-AMP, can be seen in Figure 51 (Table 2). For instance, IRF9 and transporter associated with antigen processing 1 (TAP1) played significant roles in the interferon signaling network, but the first one, IRF9, was involved only in the c-di-AMP interferon signaling but not in the c-di-GMP network. In contrast, the second, TAP1, appeared visibly in the c-di-GMP interferon signaling without a noticeable role in the c-di-AMP network (Figure 51)

Table 2. Pathways significantly regulated by c-di-AMP and c-di-GMP with -log p > 1.3 (p < 0.05).

S/No	Ingenuity Canonical Pathway	c-di-GMP (-log p-value)	c-di-GMP (z-score)	Molecules in c-di-GMP treated fibroblasts	c-di-AMP (-log p-value)	c-di-AMP (z-score)	Molecules in c-di-AMP treated fibroblasts
1	Interferon signaling	9.49	2.828	IFI35,IFIT1,IFIT3,ISG15,MX1,STAT1, STAT2,TAP1	11.0	2.646	IFI35,IFIT1,IFIT3,IRF9, ISG15,MX1,STAT1, STAT2
2	Hypercytokinemia/ hyperchemokinemia in the pathogenesis of influenza	6.39	2.828	EIF2AK2,IFIT3,ISG15,MX1,NFKB2, PYCARD, STAT1,STAT2	6.52	2.646	EIF2AK2,IFIT3,IRF9, ISG15,MX1,STAT1, STAT2
3	Coronavirus pathogenesis pathway	6.48	1.265	ACE,CASP3,FAU,NFKB2,PYCARD, RPS26,SERPINE1,STAT1,STAT2, TRIM25	3.9	−1.633	IRF9,RPS26,RPS27L, STAT1,STAT2,TRIM25
4	Activation of IRF by Cytosolic Pattern Recognition Receptors	2.81	1	ISG15,NFKB2,STAT1, STAT2	3.51	1	IRF9,ISG15,STAT1, STAT2
5	Role of PKR in interferon induction and antiviral response	4.37	2.449	BID,CASP3,EIF2AK2,NFKB2,PYCARD, STAT1, STAT2	3.44	1.342	EIF2AK2,IRF9,PDGFRB, STAT1,STAT2
6	Antigen presentation pathway	3.61	N	HLA-A,HLA-B,PSMB9,TAP1	2.98	N	CANX,HLA-B,PSMB9
7	Phagosome maturation	2.89		CTSZ,HLA-A,HLA-B,RAB7A,TAP1, TUBG1	2.96	N	ATP6V0C,CANX,DCTN4, HLA-B,VPS18
8	Necroptosis signaling pathway	2.8	1.633	EIF2AK2,PYCARD,STAT1,STAT2, TNFRSF11B, TOMM20	2.88	1.342	EIF2AK2,IRF9,STAT1, STAT2,TOMM20
9	Glycogen degradation III	2.28	N	GAA,TYMP	2.64	N	GAA,TYMP
10	Protein ubiquitination pathway	2.22	N	HLA-A,HLA-B, PSMB9,PSME2,TAP1, UBE2L6,UCHL5	2.54	N	HLA-B, PSMB9, PSME2, THOP1,UBE2C, UBE2L6
11	EIF2 signaling	2.69	−0.447	EIF2AK2,FAU,RALB,RPL18,RPL18A, RPL7,RPS26	2.22	N	EIF2AK2,EIF2S3, RPLP1, RPS26,RPS27L
12	Role of JAK1, JAK2 and TYK2 in interferon signaling	3.06	N	NFKB2,STAT1,STAT2	2.18	N	STAT1,STAT2
13	Insulin secretion signaling pathway	1.35	0.447	DLAT,PLCB4,SEC61A1, STAT1,STAT2	2.07	0.447	EIF2S3,SEC61A1,SSR4, STAT1,STAT2
14	PDGF signaling	1.51	N	EIF2AK2,RALB,STAT1	2.01	N	EIF2AK2,PDGFRB, STAT1
15	T cell exhaustion signaling pathway	1.9	N	HLA-A,HLA-B, RALB,STAT1,STAT2	1.90	N	HLA-B, IRF9, STAT1, STAT2
16	Systemic Lupus Erythematosus in B cell signaling pathway	2.8	2.121	IFIT3,IL6ST,ISG15,MAP4K4,NFKB2, RALB,STAT1,STAT2	1.86	2.236	IFIT3,IRF9,ISG15,STAT1, STAT2
17	Sumoylation pathway	2.04	N	NFKB2,PML,RND3, SP100	1.80	N	PML,RFC2,SP100
18	NER pathway	1.32	N	CETN2,POLR2A,TOP2A	1.80	N	CETN2,POLR2A,RFC2
19	Heme degradation	1.51	N	HMOX1	1.69	N	HMOX1
20	Eumelanin biosynthesis	1.41	N	DDT	1.60	N	MIF
21	Iron homeostasis signaling pathway	2.33	N	ACO1,FTH1,FTL,HMOX1,TFRC	1.47	N	ATP6V0C,HMOX1, PDGFRB

N = z-score was not determined/indeterminate by IPA.

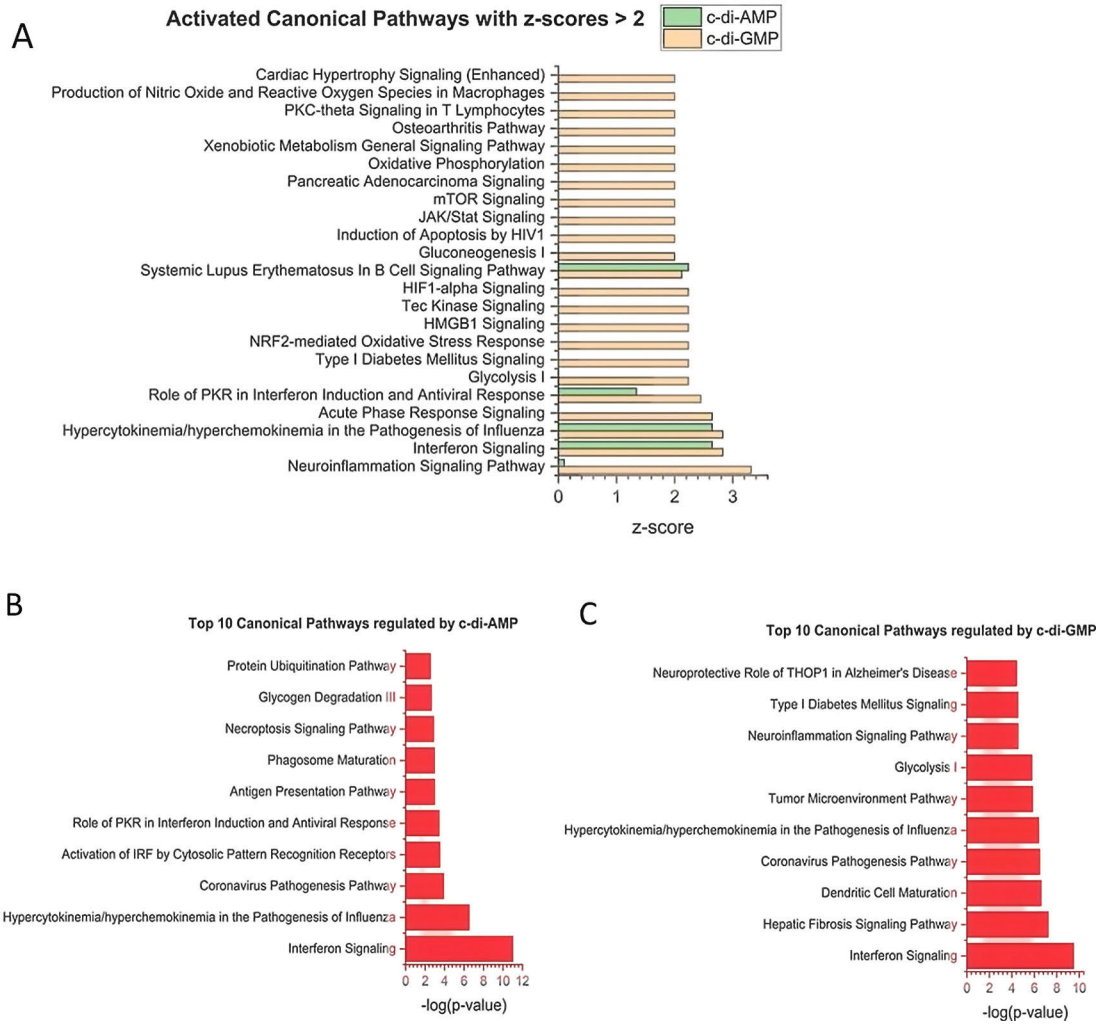

Figure 49. Enrichment analysis of ingenuity canonical pathways that c-di-GMP and c-di-AMP regulate. (A) Activated canonical pathways by c-di-AMP and c-di-GMP with z-scores ≥ 2. A positive z-score of 2 represents two standard deviations above the mean. Top 10 ingenuity canonical pathways significantly regulated by (B) c-di-GMP, (C) c-di-AMP. The functional analysis and enrichment were generated by IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). P-values, represented as -log10 (p-values) below the graph, represent the magnitude of changes in the entire network of all identified proteins. Data were plotted using the Origin (Pro), Version 2020 software (OriginLab Corporation, Northampton, MA). (Modified from original publication III)

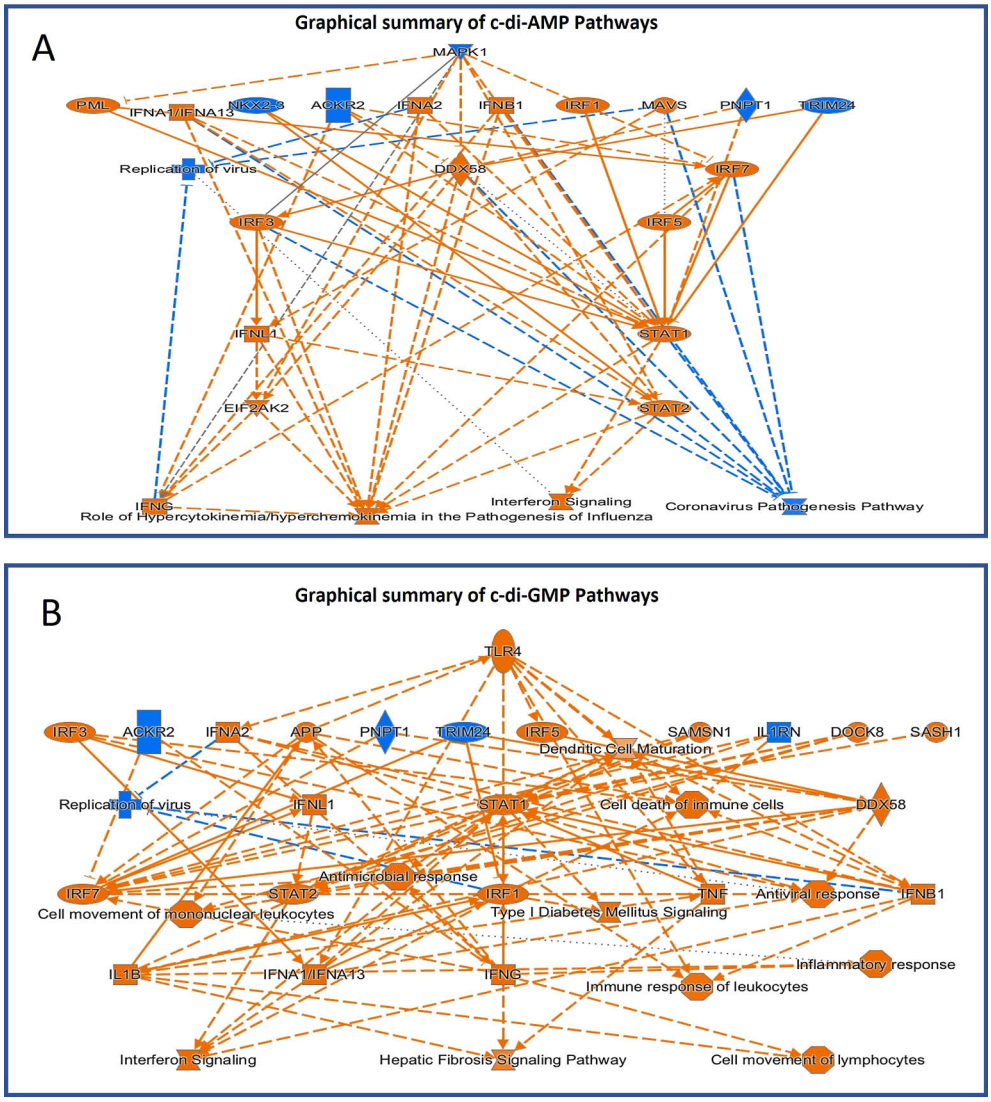

Figure 50. Summary of enrichment analysis of ingenuity pathways regulated by (A) c-di-AMP, (B) c-di-GMP. Direct relationships are represented by solid lines, while indirect relationships are represented by dotted lines. Activation is indicated by orange lines or shapes, and inhibition is denoted by blue lines or shapes inhibition. The functional analysis and enrichment were generated using IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). (From the supplementary data of the original publication III).

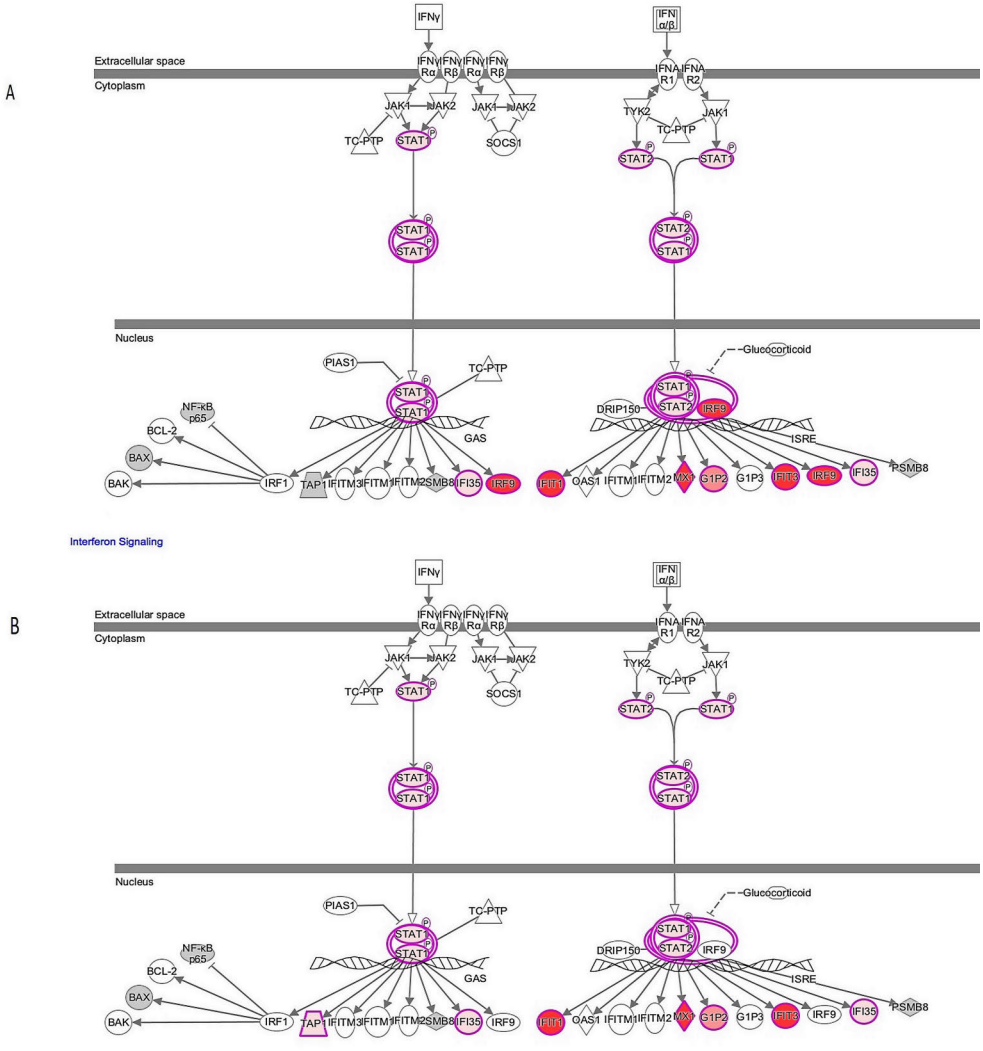

Figure 51. Deeper analysis of Interferon Signaling Pathway in fibroblasts following (A) 100 µM c-di-AMP treatment, (B) 100 µM c-di-GMP treatment. Colored nodes indicate proteins enriched from our data, intensity of the red nodes signifies the relative level of expression of the upregulated proteins. The functional analysis and enrichment were generated by IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). (From the supplementary data of the original publication III).

5.3.9 Functional analysis of proteins modulated by cyclic dinucleotides and *Pg* LPS (IV)

With -log p > 1.3 ($p < 0.05$), c-di-AMP+*Pg* LPS, c-di-GMP+*Pg* LPS, and *Pg* LPS alone significantly regulated 10, 30 and 8 pathways, respectively. C-di-AMP+*Pg* LPS and c-di-GMP+*Pg* LPS regulated common 5 pathways (Table 3), and the most

regulated considerably canonical pathway was the interferon signaling with a -log p-value of 8,16 and 7,78, respectively, and a z-score greater than 2 in c-di-AMP+*Pg* LPS and c-di-GMP+*Pg* LPS groups (Table 3). Figure 52 displays the highest 10 significantly regulated pathways by c-di-AMP+*Pg* LPS, c-di-GMP+*Pg* LPS, and *Pg* LPS alone (-log $p < 0.05$). Exclusively, c-di-AMP+*Pg* LPS regulated five pathways, such as the SPINK1 pancreatic cancer pathway and cell cycle: G1/S checkpoint regulation pathway. Among the regulated pathways, 23 pathways were exclusively regulated when HGFs were treated by c-di-GMP+*Pg* LPS, such as glucocorticoid receptor signaling, superoxide radicals degradation, and hepatic fibrosis signaling. Similarly, six pathways were regulated only by *Pg* LPS alone. The summary of enrichment analysis of commonly regulated pathways by cyclic dinucleotides and *Pg* LPS treated fibroblasts is illustrated in Figure 53. Moreover, the participating molecules in these commonly regulated pathways are shown in Table 3.

Table 3. Pathways significantly regulated by c-di-AMP+*Pg* LPS and c-di-GMP+*Pg* LPS with -log $p > 1.3$ ($p < 0.05$).

	Ingenuity Canonical Pathway	C-di-AMP +*Pg* LPS (-log p-value)	c-di-AMP +*Pg* LPS (z-score)	Molecules in c-di-AMP+*Pg* LPS treated fibroblasts	c-di-GMP +*Pg* LPS (-log p-value)	c-di-GMP +*Pg* LPS (z-score)	Molecules in c-di-GMP+*Pg* LPS treated fibroblasts
1	Interferon Signaling	8,16	2,236	IFIT1 IFIT3 IRF9 ISG15 MX1 STAT2	7,78	2,236	IFIT1 IFIT3 IRF9 ISG15 MX1 STAT2
2	Role of Hypercytokinemia/ hyperchemokinemia in the Pathogenesis of Influenza	4,57	2,236	IFIT3 IRF9 ISG15 MX1 STAT2	4,27	2,236	IFIT3 IRF9 ISG15 MX1 STAT2
3	Activation of IRF by Cytosolic Pattern Recognition Receptors	2,62	N	IRF9 ISG15 STAT2	2,44	N	IRF9 ISG15 STAT2
4	Hypoxia Signaling in the Cardiovascular System	1,41	N	UBE2A UBE2L6	1,3	N	CREB1 UBE2L6
5	Coronavirus Pathogenesis Pathway	1,3	N	IRF9 SMAD3 STAT2	1,81	0	IRF9 NPC1 SERPINE1 STAT2

N = z-score was not determined/indeterminate by IPA.

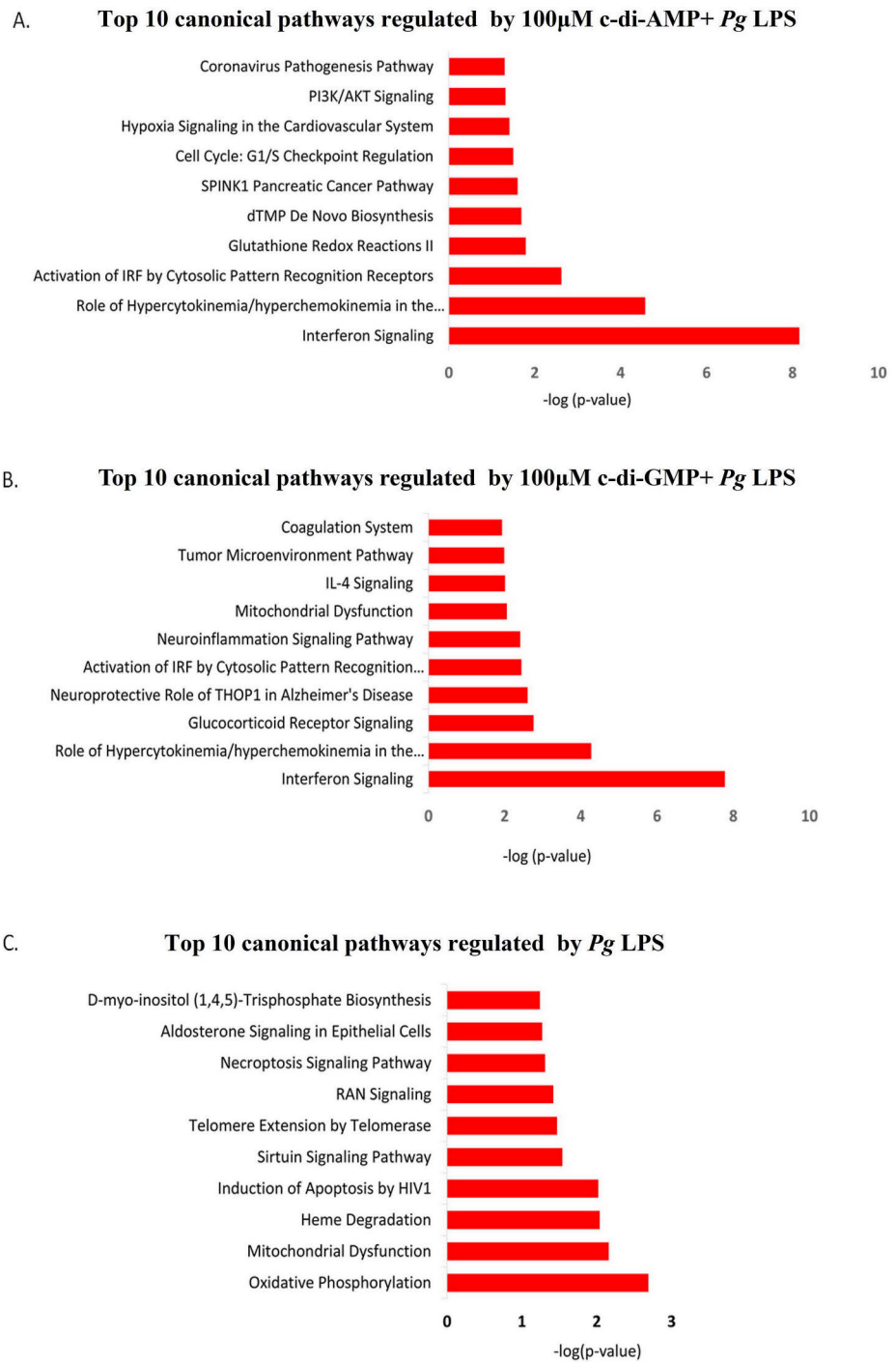

Figure 52. Top 10 ingenuity canonical pathways significantly regulated by (A) c-di-AMP+*Pg* LPS, (B) c-di-GMP+*Pg* LPS, (C) *Pg* LPS. A positive z-score of 2 represents two standard deviations above the mean. (From original publication IV).

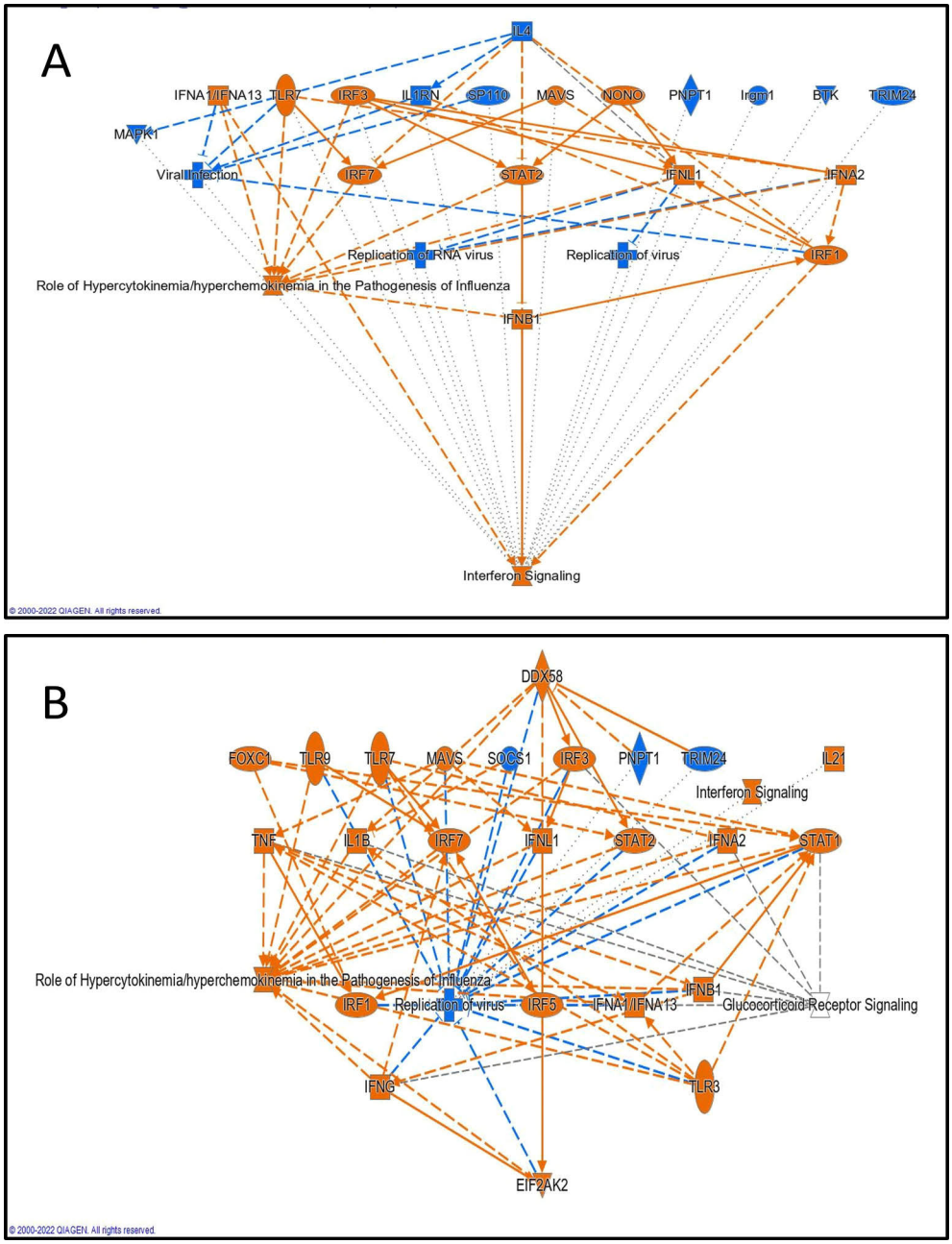

Figure 53. Summary of enrichment analysis of ingenuity pathways regulated by (A) c-di-AMP+*Pg* LPS and (B) c-di-GMP+*Pg* LPS. Direct relationships are represented by solid lines, while indirect relationships are represented by dotted lines. Orange lines or shapes indicate activation, and blue lines or shapes denote inhibition. The functional analysis and enrichment were generated using IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). (From the supplementary data of the original publication IV).

5.4 Cell proliferation (Unpublished data)

Viabilities of HMK cells and HGFs after 24 h of incubation with cyclic dinucleotides (c-di-AMP and c-di-GMP) at 100 µM, 10 µM, and 1 µM are represented in Figure 54. Cyclic dinucleotides did not affect the proliferation of HMK cells. However, increased proliferation was observed in HGFs incubated with 100 µM of c-di-GMP, while suppression occurred in HGFs cultured with 100 µM of c-di-AMP.

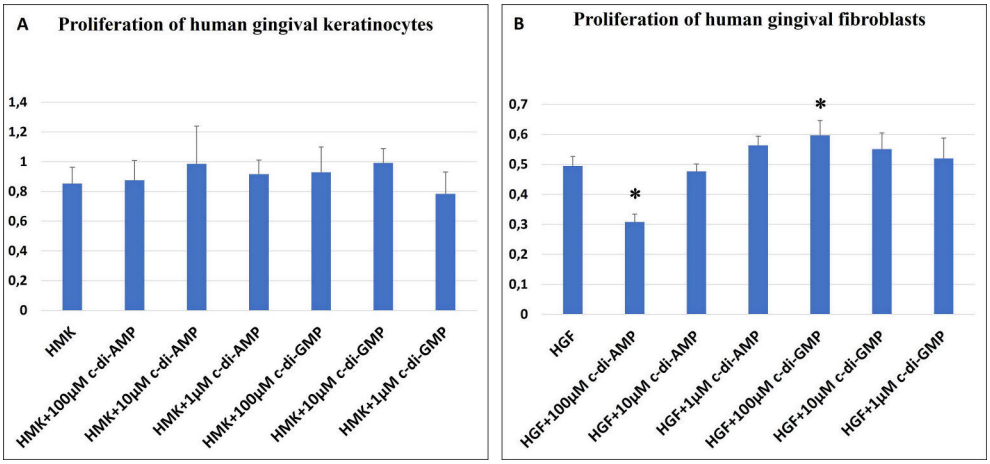

Figure 54. Viability levels of (A) HMK cells and (B) HGFs after 24 h of incubations with c-di-AMP, c-di-GMP. * Statistical difference ($p < 0.05$) with control. (Unpublished data).

6 Discussion

6.1 General discussion

Cyclic dinucleotides activate the immune response, indicating their potent immunostimulatory function in eukaryotic cells. The present PhD project's *in vitro* studies aimed to reveal the extent and magnitude of cyclic dinucleotide-regulated cellular response of gingival cells, either alone or together with *Pg* LPS.

The cellular response of HMK cells to cyclic dinucleotides and *Pg* LPS was assessed in study I, which focused on specific cytokines based on their roles in the host response. IL-1β, a pro-inflammatory cytokine, induces collagenase activation and osteoclastogenesis (Cheng et al., 2020). IL-1Ra inhibits the activities of IL-1α and IL-1β by binding to the IL-1 receptor (Dinarello et al., 2018). IL-8 is a chemoattractant cytokine that attracts and activates neutrophil migration to the site of infection and contributes to the differentiation of monocytes (Vilotić et al., 2022). MCP-1 is a mediator in the inflammatory response, and chemoattractant cytokine promotes the migration of monocytes (Deshmane et al., 2009). VEGF contributes to physiological functions such as wound healing and hematopoiesis, in addition to mobilizing endothelial cells to distant sites of neovascularisation (Johnson & Wilgus, 2014). Study I showed that depending on the type of cytokine, cyclic dinucleotides (c-di-AMP and, to a smaller degree, c-di-GMP) can act as either enhancers or inhibitors of the LPS response.

Study II examined the early and late IL and MMP responses of HGFs in response to cyclic dinucleotides and *Pg* LPS exposure. HGFs regulate tissue homeostasis and integrity (Herath et al., 2013; Wielento et al., 2023). ILs are significant players in immune cell activation and regulation. They mediate the activation, proliferation, and inflammatory response of T and B cells (Al-Qahtani et al., 2024; Luo et al., 2023). MMPs participate significantly in various biological processes by contributing to tissue remodeling and turnover of the periodontal tissue. Pathogens stimulate host cells to produce MMPs, an indirect pathological way to destroy the tissue in periodontal tissues (Checchi et al., 2020). Study II, which focused on targeted ILs (IL-8, IL-10, IL-34) and MMPs (MMP-1, MMP-2, MMP-3), were selected based on their contribution to inflammatory cascades. IL-10 can decrease the MMPs receptor activator for NF-kB ligand, and pro-inflammatory cytokines

(Garlet et al., 2006; Docherty et al., 2022). IL-34 contributes to osteoclastogenesis and the progression of periodontitis (Hwang et al., 2012; Boström & Lundberg, 2013). MMP-1, -2, and -3 have a role in the breakdown of connective tissue and modulate the expression of cytokines (Lagente & Boichot, 2010; Franco et al., 2017). Previous studies indicated that the HGF's cellular response against *Pg* LPS has time-dependency, meaning that the cellular response does not show a linear pattern (Tsai et al., 2014; Sakaki et al., 2004; Ara et al., 2009). Therefore, in study II, the cellular response of HGFs against the cyclic dinucleotides and *Pg* LPS was examined at two different time points, representing the early response at 2 h and the late response at 24 h. Time points have been suggested in previous studies; the early response of fibroblast mediators by messengers, such as the lipid mediators, appeared in minutes against lipids, and the early cellular response mediated by peptide messengers developed within hours. Hours to days are needed to create the late response regulated by two signal types: soluble signals, including growth factors, and insoluble signals, including ECM proteins (Jordana et al., 1994). The findings of study II presented that cyclic dinucleotides interact with *Pg* LPS to control the early response of gingival fibroblasts, while *Pg* LPS predominantly influences the late response. These findings indicated that certain MAMPs regulate the host's immune response against pathogens like *Pg* and sustain the homeostasis between the host and bacteria in the oral cavity.

On contrary to study II, in which the aim was to demonstrate the specific cellular responses of HGFs, studies III and IV aimed to present a general overview of HGFs' cellular responses when exposed to cyclic dinucleotides alone or together with *Pg* LPS. Unbiased global proteomics analysis was used to examine the effects of cyclic dinucleotides solely (III) or combined with *Pg* LPS (IV) on gingival fibroblasts' proteome response. Results of studies III and IV showed that interferon signaling and innate immune responses of HGFs could be regulated by bacterial cyclic dinucleotides, which widely influence various critical processes in human cells. Studies III and IV suggested that, with notable variations observed in individual exposures, the periodontal immune response is stimulated by the simultaneous influence of MAMPs by activating HGF's anti-microbial cellular responses.

The activation of STING in the immune and a few other cell types has been shown (Aryal et al., 2020; Ou et al., 2021), but it is silenced in some cell types. The current PhD project evaluated STING-suppression and cyclic dinucleotide-mediated activation of STING/TBK1/IRF3 pathway in HMK cells. The results fill in the gap of our understanding of cell types that harbor activated STING/TBK1/IRF3 pathway by demonstrating its activation in HMK cells and regulation by cyclic dinucleotides.

6.2 Cellular response of HMK cells against cyclic dinucleotides (I)

Cyclic dinucleotides and LPS, which act as MAMPs, can be recognized by eukaryotic PRRs (Danilchanka & Mekalanos, 2013; Ahn & Barber, 2019). The gingival cells can sense and recognize MAMPs through PRRs, which regulate the innate immune response through the production of IFNs and pro-inflammatory cytokines (Song et al., 2017). Study I showed that *Pg* LPS significantly decreases the extracellular levels of IL-8 and intracellular levels of IL-1β in HMK cells. In a previous study, *Pg* LPS in human oral keratinocytes inhibited the LPS-binding protein-induced IL-8 mRNA expression (Ding et al., 2017). Other studies revealed that *Pg* LPS stimulates the production of pro-inflammatory cytokines and causes disruption in the epithelial barrier of gingival epithelial cells (Li et al., 2021; Chen et al., 2020). In human gingival epithelial cells, mRNAs of IL-1β and IL-8 were elevated in response to *A. actinomycetemcomitans* (Uchida et al., 2001). *Pg* increased the IL-1β expression in macrophage cell lines (Hao et al., 2023). Although *Pg* stimulates innate immune response and induces the expression of inflammatory mediators, it disrupts host immune homeostasis (Xu et al., 2020). Various components of the host immune response can be modulated to avoid bacterial clearance, in addition to destroying the immunity by *Pg* LPS and some other virulence factors of *Pg* (Xu et al., 2020). Study I demonstrated that extracellular levels of VEGF and IL-8 were stimulated by c-di-AMP at a concentration of 100 μM. The inhibitory effect of *Pg* LPS on IL-8 was neutralized by c-di-AMP. The impact of c-di-AMP on levels of VEGF and IL-8 may indicate its contribution at high concentrations in vascularisation and neutrophil chemotaxis. Extracellular levels of MCP-1 had two different responses to c-di-AMP; at high concentrations, the level of cytokines did not change, but decreasing the concentration caused an increase in the expression of MCP-1 five-fold. Findings related to the expression of MCP-1 agreed with the phenomenon of the adaptive response of cells to moderate stress, known as hormesis, characterized by two different dose responses to agents, either by stimulation at a low dose or inhibition at a high dose (Mattson, 2008). Another study showed the same phenomenon when bacterial LPS of *Escherichia coli* and low concentrations of MAMPs stimulated monocytes, resulting in an elevation in the production of TNF-α and IL-6. In contrast, the production of these cytokines was inhibited at high concentrations (Ifrim et al., 2014). According to study I, no changes were observed in cytokine levels when cells were incubated with c-di-GMP. When cells were stimulated with *Pg* LPS and c-di-GMP simultaneously, this combination inhibited extracellular levels of IL-8 and intracellular levels of IL-1β, indicating that the suppression effect was from the effect of *Pg* LPS rather than that of c-di GMP. Previous studies have shown the impact of c-di-GMP on activating cytokine synthesis (Karaolis et al., 2007a; Karaolis et al., 2007b; Yan et al., 2009).

In immature human dendritic cells, IL-8, MCP-1, and IFN-β levels were stimulated by c-di-GMP (Karaolis et al., 2007a). C-di-GMP contributes to the activation of macrophages and NK cells and increases the maturation of dendritic cells (Karaolis et al., 2007b; Yan et al., 2009). In addition, Karaolis et al. (2007a) demonstrated that c-di-GMP induces cytokine and chemokine expressions and activates the recruitment of monocytes and granulocytes in rodents. The variation in our results can be accounted for by differences in cell types and species tested, as the previous studies used mouse models; in contrast, study I tested the human gingival epithelial cell cytokine response. In human epidermal keratinocytes, secretion of IL-8 was significantly enhanced by c-di-GMP at concentrations of 1 μM, 5 μM, and 25 μM. In comparison, at the highest c-di-GMP concentrations of 125 μM, the cytokine level did not change; the highest level of IL-8 was at 5 μM (Gao et al., 2022). Findings of study I showed that depending on the type of cytokine, cyclic dinucleotides (c-di-AMP and, to a lesser degree) can regulate the cytokine response of keratinocytes by enhancing or inhibiting the LPS effect.

6.3 Early and late cellular response to cyclic dinucleotides in HGFs (II)

Gingival fibroblasts modulate the immune response against invading pathogens by producing cytokines and other inflammatory mediators to clear the invading bacteria and resolve the inflammation (Wielento et al., 2023). The results of study II showed an increase in IL-8 expression of HGFs when stimulated by *Pg* LPS, which is in line with previous studies that demonstrated that gingival fibroblasts express mRNA of IL-8 when exposed to *Pg* LPS in addition to an increase in IL-8 production (Mahanonda et al., 2007; Morandini et al., 2011; Tsai et al., 2014). Furthermore, in human periodontal ligament fibroblasts stimulated by *Pg* LPS for 24 h, mRNA IL-8 expression and MMP-1 and MMP-3 levels were significantly increased (Wu et al., 2020). In study II, no changes in the level of IL-8 were observed when the cells were incubated with c-di-AMP alone. In contrast, simultaneous exposure of HGFs to the highest concentration of c-di-AMP and *Pg* LPS for 2 h increased the levels of IL-8. Still, this effect was observed with all tested c-di-AMP concentrations in the presence of *Pg* LPS when the time of incubation was extended to 24 h. This observation indicates that the production of IL-8 by HGFs is mainly regulated by *Pg* LPS, not by c-di-AMP. As presented by Xia et al. (2018), c-di-AMP can stimulate the host immune response through NF-κB and increase the secretion of pro-inflammatory cytokines. Incubation of HGFs in the presence of c-di-GMP and *Pg* LPS produced a significant amount of IL-8 after 2 h and 24 h. Similarly, IL-10 levels showed a significant increase in HGFs treated with c-di-GMP and *Pg* LPS, but only after 2 h of incubation. In comparison, after increasing the incubation time to 24 h,

IL-10 levels increased only in the presence of c-di-GMP alone. Against our results, in dendritic cells, c-di-GMP did not induce expression of IL-10 (Karaolis et al., 2007a). In line with study II, the level of IL-10 did not show any changes in gingival fibroblasts treated with *Pg* LPS (Morandini et al., 2011). *In vivo*, c-di-GMP stimulates the production of anti-inflammatory cytokine, IL-10, essential in balancing the pro- and anti-inflammatory cytokine profile (Blaauboer et al., 2015). In another study, samples from patients with periodontitis revealed an increase in IL-10 levels in gingival crevicular fluid but a decrease in serum (Yemenoglu et al., 2024). Compared to healthy individuals, IL-34 levels were higher in gingival crevicular fluid, serum, and saliva of patients with periodontitis. IL-34 expression is induced by MAMPs and pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-10, IL-17A, and IFN-γ (Shang et al., 2023).

Regarding the MMP response, in study II, MMP levels of HGFs did not show any changes in response to *Pg* LPS alone. In another study, incubation of HGFs with 30 ng/ml LPS increased the expression of MMP-2 mRNA after 72 h, while MMP-1 mRNA expression increased markedly after 8 days when the cells were incubated with 3000 ng/ml LPS (Bozkurt et al., 2017). The controversy between the results of study II and the previous study may be due to the difference in the conditions of the experiment, such as the difference in exposure time and concentrations of LPS. In study II, no statistically significant difference was found in MMP-1 expression in c-di-AMP alone treated HGFs for 24 h. At the same time, this effect was significantly increased when HGFs were subjected to simultaneous exposure to c-di-AMP and *Pg* LPS. Also, concurrent exposure to c-di GMP and *Pg* LPS for 2 h increased the expression of MMP-2 levels; this enzyme was suppressed after 24 h of incubation, whereas the same combination, 10 μM and 1 μM of c-di GMP and *Pg* LPS, increased the expression of MMP-3 at two time points, 2 h, and 24 h. These results suggest that the simultaneous impact of MAMPs on the cellular response of HGFs differs from the individual effect of MAMPs. MMPs can boost or suppress inflammation by involving the post-transcriptional processing of inflammatory cytokines and chemokines (Kim et al., 2016).

Various inflammatory mediators can be degraded by *Pg*, which inhibits the cellular response of fibroblasts (Palm et al., 2013). Depending on the reciprocal relation between the MMPs and inflammatory cytokines, regulation of MAMPs or upregulation of pro-inflammatory cytokine expression may be attributed to the inhibition in the expression of MMP-2 (Moreau et al., 1999; Hong et al., 2017). These findings propose that the interaction of cyclic dinucleotides and *Pg* LPS can mediate the response of HGFs at an early phase. In contrast, only *Pg* LPS mediates the late cellular response, which refers to the multiple actions of MAMPs that can regulate the host immune response against bacteria such as *Pg* and preserve homeostasis in the oral cavity.

6.4 Effect of cyclic dinucleotides on MAPK levels (I)

As the MAPK pathways are molecular targets for anti-inflammatory therapy (Kaminska, 2005), detecting their levels in HMK cells treated with cyclic dinucleotides and *Pg* LPS is essential. LPS of gram-negative bacteria are potent activators of the host immune system by activating MAPKs to produce pro-inflammatory cytokines (Mazgaeen & Gurung, 2020). Expression of cytokines and chemokines, as well as cellular responses, can be regulated by MAPKs (Cargnello & Roux, 2011; Guo et al., 2020). In study I, although stimulation of ERK1/2 phosphorylation and inhibition of p38 phosphorylation were observed in HMK cells stimulated by *Pg* LPS, elevated phospho-p38 levels were observed when HMK cells were stimulated by the combination of *Pg* LPS and c-di-AMP. This finding is compatible with the impact of c-di-AMP on IL-8 levels and may indicate the regulation of IL-8 secretion by c-di-AMP through the phospho-p38 pathway in HMK cells. In line with the study I, other studies showed that phosphorylation of p38 is promoted by *Pg* LPS and by c-di-AMP (Miyabe et al., 2022; Mahmoud et al., 2020), and suppression of p38 inhibits the release of c-di-AMP-induced cytokines (Mahmoud et al., 2020).

The results of study I indicated that c-di-AMP, individually or combined with *Pg* LPS, stimulates the phospho-JNK pathway and MCP-1 levels in HMK cells. This result is in line with previous studies in human endothelial cells that showed MCP-1 expression was induced through the JNK and p38 MAPK pathways (Umebashi et al., 2023) and by the phospho-JNK pathway (Werle et al., 2002). The same result was shown in cultured astrocytes (Gao et al., 2009).

In study I, the suppression effect of c-di-GMP on phosphorylation of p38, JNK, and ERK1/2 was in the presence of *Pg* LPS. The effect of c-di-GMP on cytokine secretion was observed in HMK cells only when combined with *Pg* LPS; this combination suppressed IL-1β and IL-8 levels. This may be explained by the observation presented by Kjellerup et al. (2008); the secretion of pro-inflammatory cytokines in keratinocytes is regulated by the phosphorylation of p38, JNK, and ERK1/2. Another study showed that c-di-GMP has an effect on intracellular signaling by enhancing the activation of the p38 MAPK pathway in human dendritic cells and ERK phosphorylation in human macrophages (Karaolis et al., 2007a). It could be argued that c-di-GMP has a role in modulating the phosphorylation of MAPK pathways and, in turn, regulating the expression of ILs. ERK1/2 signaling pathway enhancing the expression of IL-1β-induced IL-6 mRNA was not excluded in canine dermal fibroblasts (Kitanaka et al., 2019). Guo et al. (2020) presented that ERK stimulates the expression of IL-8 and VEGF in tumor blood vessels, and ERK also stimulates the expression of VEGF in colorectal cancer.

6.5 Cyclic dinucleotides regulated STING/TBK1/IRF3 pathway activation in HMK cells (Unpublished data)

STING controls the inflammatory responses and shapes the immune response by mediating the cellular responses against invading pathogens (Ablasser & Chen, 2019). While in healthy gingiva, STING is weakly present, detectable levels of STING were observed in tissues with periodontitis and oral lichen planus (Elmanfi et al., 2021; Deng et al., 2022), indicating that the human gingival STING levels are related to infection-induced inflammatory conditions. In the current PhD work, STING levels were decreased in HMK cells when incubated with cyclic dinucleotides. STING signaling pathway mediates IRF3-dependent transcription of type I IFNs and NF-κB-dependent transcription of pro-inflammatory cytokines (Uemura et al., 2022). Cyclic dinucleotides can initiate a negative feedback control mechanism to prevent sustained innate immune gene transcription (Konno et al., 2013). In bone marrow-derived dendritic cells of mice, strongly decreased STING protein levels were observed in the presence of c-di-AMP or cGAMP as a result of proteasomal degradation followed by an inhibition in IFN-β secretion (Rueckert et al., 2017). The same finding was shown in peripheral human blood mononuclear cells treated with cGAMP (Rueckert et al., 2017). Cheng et al. (2022) suggested that c-di-AMP could stimulate the host immune response; however, this induction is controlled by regulatory mechanisms, as it is not continuous and is self-limited in aim to avoid an extra immune response induction by c-di-AMP that may cause immunopathologic diseases. The negative feedback mechanism and proteasomal degradation can explain the decreased STING levels in human gingival keratinocytes after incubation with cyclic dinucleotide.

In the current work, the TBK1 level was decreased in the presence of cyclic dinucleotide, which was in line with STING level. These similarities between the responses of TBK1 and STING have been shown in other studies (Konno et al., 2013; Ishikawa et al., 2009). Prolonged activation of STING correlates with a decrease in the degradation of phosphorylated TBK1 (Konno et al., 2013). STING is phosphorylated and degraded and causes proteolytic degradation of phosphorylated TBK1 after phosphorylating IRF3 (Konno et al., 2013). This could explain the unchanged level of IRF3 in the current study. It has been shown that to target IRF3, phosphorylated TBK1 might need a release from STING (Konno et al., 2013; Ishikawa et al., 2009). STING-TBK1 complex is required to activate TBK1, which activates the transcriptional factor IRF3 (Ishikawa et al., 2009; Ahn & Barber, 2019; Guimarães et al., 2021). Unmarked changes in IRF3 levels in human gingival keratinocytes after incubation with cyclic dinucleotides are in line with the study by Konno et al. (2013) using different cell lines, where IRF3 levels in human telomerase fibroblasts were maintained and did not show a rapid degradation. STING's role in

the activation of IRF3, which can trigger the production of inflammatory cytokines, could be inhibited by phosphorylation of STING-S366 and leads to blocking the activation of pro-inflammatory cytokines (Konno et al., 2013; Zhang et al., 2023). The results demonstrated the activation of STING/TBK1/IRF3 pathway in HMK cells and its regulation by cyclic dinucleotides.

6.6 Global proteomics profile of HGFs in response to microbe-associated molecular patterns (MAMPs) (III and IV)

Studies III and IV presented an unbiased view of the effect of cyclic dinucleotides solely (study III) or combined with *Pg* LPS (IV) on the proteomic profile of HGFs.

The results of studies III and IV showed a significant upregulation in proteins contributing to interferon signaling and innate immune responses in HGFs treated by cyclic dinucleotides individually or combined with *Pg* LPS. These proteins include ISG15, SAMHD1, OAS3, STAT1, MX1, IFIT 1, IFI T 3, and HLA-A.

Compared to the significant number of proteins in HGFs treated with c-di-AMP or c-di-GMP alone, the number of significantly upregulated proteins in HGFs treated with *Pg* LPS and c-di-AMP increased from 46 to 93. In contrast, in the presence of *Pg* LPS and c-di-GMP, the upregulated proteins did not demonstrate a marked change (77 and 72 proteins).

In gingival biopsies from individuals diagnosed with periodontitis, the gene expression analysis revealed an elevation in lymphocyte-related genes (Davanian et al., 2012a), which serve as indicators of adaptive immunity (Kinane et al., 2024). In addition, innate immunity gene markers were expressed in HGFs and response to *Pg* LPS (Xie et al., 2018). These findings align with our results, as proteins that contribute significantly to interferon signaling and innate immunity are upregulated when HGFs are treated with cyclic dinucleotides alone or combined with *Pg* LPS.

6.7 Regulated proteins in HGFs in response to cyclic dinucleotides (III)

Important innate immunity proteins were significantly upregulated by c-di-AMP and c-di-GMP include ISG15, SAMHD1, STAT, IRF-9, MX1, MX2, HLA-A, IFIT1, and IFIT3. For example, ISG15 can modulate immune responses and activate the type I IFN pathway (Dzimianski et al., 2019). A decrease in the level of ISG15 may indicate increased vulnerability to periodontal inflammation (Bostanci et al., 2020). STAT plays key roles in various processes, such as immune cell development and function (Awasthi et al., 2021). It contributes to the expression of genes associated with antibacterial function, cell survival, and response to pathogens, in addition to

mediating interferon signaling (Awasthi et al., 2021; Adámková et al., 2007; Tolomeo et al., 2022; Baran-Marszak et al., 2004). STAT1 is a significant transcription activator, and its regulation by cyclic dinucleotides has been shown (Mogensen, 2019). In addition, in gingival fibroblasts, activation of STAT by inflammatory cytokines such as TNF-α has been demonstrated by Davanian et al. (2012b). HLA-A has antigen presentation function, binds antigens and displays them to responding CD8+ T cells (Wieczorek et al., 2017). SERPINEB2, the second most upregulated protein by c-di-GMP, belonging to the serine protease inhibitors family, represents cytoprotective properties in cells and interacts with various components of the ubiquitin-proteasome system (Lee et al., 2015). Compared to wild-type cells, deletion of SERPINB2 significantly decreased the activity of the ubiquitin-proteasome system and perturbed autophagy (Lee et al., 2015). SERPINB2 specifically inhibits the proteolytic activity of *Pg* (Neilands et al., 2016) and in other conditions such as inflammatory, viral, and parasitic infections; SERPINB2 regulates the T helper 1/2 adaptive immune response (Schroder et al., 2010a; Major et al., 2013; Schroder et al., 2010b).

6.8 Regulated proteins in HGFs in response to cyclic dinucleotides and *Pg* LPS (IV)

In study IV, a situation highly like the infection was created, as the host is exposed simultaneously to various MAMPs, such as bacterial LPS and cyclic dinucleotides. For example, as various MAMPs simultaneously activate different signaling networks in periodontitis, HGFs were incubated with MAMPs for 24 h. Study IV aimed to study the crosstalk effect of these two MAMPs, *Pg* LPS and cyclic dinucleotides, on HGFs to evaluate their possible synergistic, additive, or antagonistic activity. The molecular signaling of these two MAMPs, *Pg* LPS and cyclic dinucleotides, produces key interferon products. Stimulation and binding of LPS to TLR4 trigger subsequent signals: early MyD88-dependent followed by later MyD88-independent (TRIF-dependent) (Pålsson-McDermott & O'Neill, 2004; Pereira & Gazzinelli, 2023). MyD88-dependent facilitates early and rapid activation of NF-κB and MAPK kinase pathways, while MyD88-independent involving adapters such as adapters TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adapter molecule (TRAM)) contributes to the production of inflammatory cytokines and IFNs (Pålsson-McDermott & O'Neill, 2004; Pereira & Gazzinelli, 2023). TLR4 activation contributes to TNF receptor-associated factor 6 (TRAF6) and TBK1 as the response progresses. TBK1 with IκB kinase (IKK) translocates and phosphorylates IRF3. TRAF6 activation later stimulates NF-κB by IKK α/β (Fitzgerald et al., 2003; Yamamoto et al., 2021). Both NF-κB and IRF3 stimulate the production of inflammatory cytokines and type I IFNs (Zhou et al.,

2020) (Figure 55). In both distinct signaling pathways, STING and MyD88-independent pathways, TBK1 is a crucial mediator required to transcribe type I IFNs. As in both signaling pathways, cyclic dinucleotides and LPS activate TBK1 to induce the production of type I IFNs, in addition to LPS control of the perinuclear translocation of STING and the nuclear translocation of IRF3. This could suggest intricate communication between two signaling pathways, explaining the interaction effect observed in study IV.

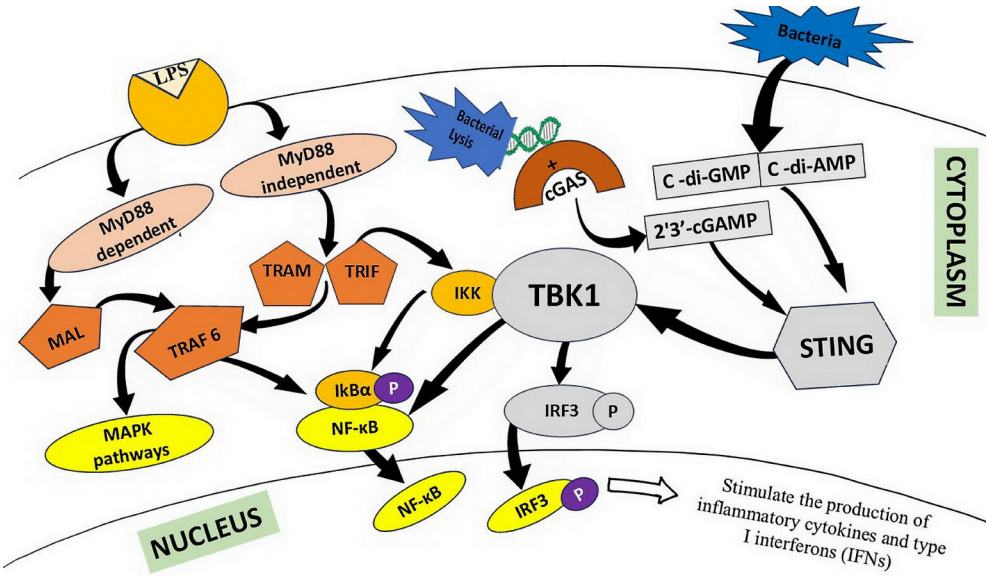

Figure 55. Signaling pathways stimulated by lipopolysaccharides (LPS) and cyclic dinucleotides. Both STING and MyD88-independent pathways feed into the expression of type I interferon through TANK-binding kinase1(TBK1). (Modified from original publication IV).

Immune and interferon signaling-related proteins such as ISG15, SAMHD1, OAS3, STAT1, IRF-9, MX1, MX2, IFIT 1, IFIT 3, and HLA-A were markedly upregulated in HGFs exposed to cyclic dinucleotides combined with *Pg* LPS. These proteins were also upregulated in HGFs treated exclusively with cyclic dinucleotides. This suggests the response shown in study IV was primarily due to the impact of cyclic dinucleotides and may indicate minimal extensive crosstalk between bacterial cyclic dinucleotides and *Pg* LPS.

C-di-AMP+*Pg* LPS upregulated proteins involved in the inflammatory process as GNG12, that inhibit the LPS response while activating NF-κB signaling to modulate the immune response (Larson et al., 2010; Li et al., 2020). Furthermore, apart from the primary inflammatory proteins, other proteins were observed as

significantly upregulated by c-di-AMP+*Pg* LPS as DNA repair protein XRCC1. DNA repair protein XRCC1 is involved in the activity of DNA ligase III and DNA base excision repair (Demin et al., 2021). This regulation suggests that the combined impact of cyclic dinucleotides and *Pg* LPS may contribute to repairing damaged DNA.

In the realm of proteins related to innate and adaptive immune functions, the findings in studies III and IV indicated that c-di-GMP solely or c-di-GMP+*Pg* LPS increased the expression of NFKB2 and TNFRSF11B but were not upregulated by *Pg* LPS alone. NFKB2 shares in the activation of noncanonical NF-κB, which works as a complementary signaling pathway alongside the canonical NF-κB pathway, aiming this cooperation to modulate specific functions of the adaptive immune system (Beinke & Ley, 2004; Mise-Omata et al., 2014). The transcription of the immune response genes can be regulated by NF-κB, thereby inducing the production of IFNs and pro-inflammatory cytokine production (Yu et al., 2020), in addition to participation of NF-κB in various conditions such as apoptosis, tumorigenesis, inflammation, and many autoimmune diseases (Barnabei et al., 2021; Liu et al., 2017). TNFRSF11B serves as a suppressor of osteoclast activity (Makaram & Ralston, 2021). Cytokines such as IL-1β and TNF-α, which fibroblasts and inflammatory cells can produce in response to LPS, increased the TNFRSF11B mRNA expression (Sakata et al., 1999).

Several proteins were augmented by simultaneous exposure of c-di-GMP+*Pg* LPS, including interferon-induced guanylate-binding protein 1. Previous studies showed that intracellular defense mechanisms to counter microbes could be induced by a class of interferon-induced GTPases (guanylate-binding proteins) (Meunier & Broz, 2015; Rafeld et al., 2021). Additionally, in response to IFN-γ, these proteins can facilitate the migration and invasion of dental pulp stem cells (He et al., 2017) and periodontal ligament stem cells (Bai et al., 2018). The NF-κB essential modulator is a critical component of the IKK complex. This complex regulates the engagement of NF-κB signaling in various processes, such as inflammation, immunity, and cell survival (Liu et al., 2017). Compared to cyclic dinucleotides, the NF-κB essential modulator was upregulated in *Pg* LPS-treated HGFs.

6.9 MAMPs and signaling pathways (III and IV)

In HGFs, although c-di-AMP and c-di-AMP significantly upregulated 21 common pathways, 13 (~62%) of these pathways were immunity-related. The antigen presentation pathway is commonly regulated, aligning with the upregulation of HLA proteins and the downregulation of DGK, which are critical for modulating T-cell function (Yang et al., 2020; Jung et al., 2018). C-di-AMP in study III downregulated DGKs that convert diacylglycerol to phosphatidic acid and influence T helper cell

differentiation dose-dependently (Yang et al., 2020; Sakane et al., 2020). Study III demonstrated the expression of STING/TBK1/IRF3 pathway in HGFs treated with cyclic dinucleotides. The activation of STING has been presented in the current project's unpublished data in cyclic dinucleotides treated gingival keratinocytes. Furthermore, STING activation by these signaling molecules has been shown in other studies (Barker et al., 2013; Morehouse et al., 2020; Burdette et al., 2011; Zhao et al., 2023; Weiss et al., 2022).

Interferon signaling is a significant player in host responses against bacterial infections. It induces transcription of Janus kinases‑STAT signaling and expression of interferon-stimulated genes. Interferon signaling was the most regulated pathway by cyclic dinucleotides individually or combined with *Pg* LPS, while it stayed unregulated in HGFs stimulated solely by *Pg* LPS. This finding may suggest the contribution of cyclic dinucleotides in bolstering the host defense and combating bacterial activity.

Glucocorticoid receptors regulate the transcription of anti-inflammatory genes to inhibit inflammatory diseases (Desmet & De Bosscher, 2017). Exclusively, glucocorticoid receptor signaling, which mediates the expression of immune response-related genes, was upregulated by c-di-GMP+*Pg* LPS and was one of the pathways regulated solely by c-di-GMP. Cell cycle: G1/S checkpoint regulation pathway was upregulated in HGFs treated by c-di-AMP+*Pg* LPS. During cell division, this pathway prevents the accumulation and propagation of genetic errors by serving as a DNA surveillance mechanism. Cell cycle progression can be delayed or induce cell cycle exit or cell death in response to irreparable DNA damage or through the activation of checkpoints (Matthews et al., 2022). C-di-AMP+*Pg* LPS exclusively significantly regulated non-inflammatory pathways. Among these, the SPINK1 pancreatic cancer pathway acts as a protease inhibitor, which plays crucial roles in tissue differentiation, apoptosis, maintenance, and repair. Apart from its involvement in reproduction, it contributes to pathological processes and can serve as a diagnostic indicator, as its elevation in many cancers was observed (Itkonen & Stenman, 2014; Liao et al., 2022; Lin, 2021).

Findings of studies III and IV demonstrated that despite notable differences in individual exposures, the periodontal immune response is stimulated by the simultaneous influence of MAMPs by activating HGF's antimicrobial cellular responses. In addition, cyclic dinucleotides affect various critical processes in human cells.

6.10 Strengths and limitations

This PhD thesis is the first attempt to investigate the individual and combined impact of MAMPs, specifically cyclic dinucleotides and *Pg* LPS, on the behavior of gingival

cells regarding cellular response and proteomic overview. This investigation encompassed an analysis of cytokine response and MAPK pathway activation in human gingival keratinocytes, as well as the reaction of ILs and MMPs, alongside global proteomic analysis of HGFs. With some notable differences from individual exposures, gingival cells showed a significant response to MAMPs. The tested concentrations of cyclic dinucleotides used in the studies of this PhD project were based on their activity in binding bacterial receptors, thereby regulating motility and biofilm formation (Khan et al., 2023). Evidence indicates that c-di-AMP and c-di-GMP at concentrations less than 50 μM can permeate human cell membranes and trigger a cellular response (Karaolis et al., 2005a; Steinberger et al., 1999; Luteijn et al., 2019; Lahey et al., 2020; Ritchie et al., 2019; Cordova et al., 2021). In studies III and IV, cyclic dinucleotides were used at a concentration of 100 μM, as this concentration induces the highest IFN signal compared to the control, according to Sooreshjani et al. (2018) in human macrophages. The main strength of the studies of the current PhD thesis is that they examine the combined and independent actions of multiple MAMPs on gingival cells. However, certain limitations need to be acknowledged: 1) the cell culture was performed in monolayers. While this cell culture model has the significant advantage of attributing cellular responses and allowing for direct comparisons between host cells and bacterial species (Mountcastle et al., 2020), it does not mimic the characteristics of the oral environment (Almansoori et al., 2020; Bierbaumer et al., 2018). For instance, a monolayer cell culture environment offers a humid setting with steady oxygen levels and constant temperature. However, in the oral cavity, temperature, pH, humidity, and oxygen levels are subject to changes. Moreover, the culture media, often supplemented with growth factors, supports cell growth but does not fully replicate the complex nutrient profile of the oral cavity. Additionally, while the oral cavity harbors a diverse microbiome that interacts with oral cells, cell culture maintains sterile conditions to avoid contamination, thereby not replicating these microbial interactions (Segeritz & Vallier, 2017; Bierbaumer et al., 2018; Mountcastle et al., 2020; Akimbekov et al., 2022). Therefore, the results cannot lead to a definitive conclusion without being compared with clinical investigations. 2) Cells were stimulated with MAMPs instead of live bacteria that activate and suppress various eukaryotic pathways simultaneously. 3) Commercially *Pg* LPS, used in studies I and II, can induce TLR-2 response as they contain trace amounts of lipoproteins. However, live bacteria also contain other immune stimulants, such as peptidoglycan fragments and DNA (Dziarski & Gupta, 2010; Lipford et al., 1998), making it challenging to discern the individual or combined roles of LPS and cyclic dinucleotides if they were employed. Yet, to eliminate this limitation, ultrapure *Pg* LPS was used in studies III and IV. Finally, adjusted p-values or q-values were not utilized for all subsequent analyses following the initial analysis in Perseus software.

Although the initial identification of significantly upregulated or downregulated proteins in Perseus software used its inbuilt FDR-adjusted p-values for each treatment, these adjustments were not applied in further comparisons.

6.11 Future prospective

The studies presented in this PhD thesis were outlined to evaluate the regulatory role of cyclic dinucleotides and LPS of a key periodontal pathogen (*Pg*) on human gingival cells (keratinocytes and fibroblasts). However, before making any definitive conclusions, further investigations are necessary to examine the global proteome of gingival keratinocytes in response to MAMPs. Additionally, more studies are required to compare the effect of host-derived and bacterial-derived cyclic dinucleotides on gingival cells. Further research is needed to understand the impact of cyclic dinucleotides on the cellular and global proteomic levels of other significant cells in the oral cavity, such as osteoclasts and osteoblasts, that are responsible for maintaining bone remodeling (Bolamperti et al., 2022), in addition, osteoclasts mediating bone loss by increasing their resorptive activity in pathologic conditions (Kitaura et al., 2020).

Currently, an *in vitro* study is being conducted on human mesenchymal stem cells and osteoclast precursors obtained from bone marrow samples of healthy donors to evaluate the effect of host-derived and bacterial-derived cyclic dinucleotides on the differentiation and activities of osteoclasts and osteoblasts, which is in progress. In this study, cells were incubated with host-derived and bacterial-derived cyclic dinucleotides at two time points.

Finally, clinical studies are needed to comprehend how STING/TBK1/IRF3 pathway is regulated in human periodontal tissues and its potential contribution to the initiation and development of periodontitis.

7 Conclusions

Based on the results included in the current thesis, the following conclusions were drawn:

1. In keratinocytes, depending on the type of cytokine, cyclic dinucleotides (c-di-AMP and, to a lesser degree, c-di-GMP) enhance or inhibit the LPS effect to modulate the cytokine response.
2. In fibroblasts, the initial cellular response to ILs and MMPs is mediated by cyclic dinucleotides engaging with the LPS. In contrast, the subsequent later-stage response is controlled by LPS alone.
3. In the oral cavity, certain MAMPs maintain homeostasis between the host and bacteria by regulating the host's immune response against pathogens such as *Pg*.
4. Bacterial cyclic dinucleotides regulate the interferon signaling and innate immune responses of HGFs.
5. Beyond regulating cytokine production via STING, cyclic dinucleotides also widely influence various critical processes in human cells.
6. With distinct variations observed in individual exposures, the periodontal immune response is stimulated by the simultaneous influence of MAMPs (cyclic dinucleotides and *Pg* LPS) by activating HGFs' anti-microbial cellular responses.
7. STING/TBK1/IRF3 pathway operates within HMK cells, and bacterial cyclic dinucleotides can regulate the expression of STING and TBK1 proteins.
8. The regulatory role of cyclic dinucleotides in gingival cells presents evidence supporting their (or their analogues) potential use as an adjunctive tool in periodontal therapy.

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