The K1K2 Region of Lys-Gingipain of *Porphyromonas gingivalis* Blocks Induction of HLA Expression by Gamma Interferon

Peter L. Yun,^a Nan Li,^b Charles A. Collyer,^b and Neil Hunter^{a,c}

Institute of Dental Research, Westmead Millennium Institute and Centre for Oral Health, Westmead Hospital, Sydney, New South Wales, Australia^a; School of Molecular Bioscience, The University of Sydney, Sydney, New South Wales, Australia^b; and Faculty of Dentistry, The University of Sydney, Sydney, New South Wales, Australia^c

In the context of periodontal disease, cysteine proteinases or gingipains from *Porphyromonas gingivalis* have been implicated in the hydrolysis of cytokines, including gamma interferon (IFN- γ). This cytokine plays a crucial role in host defenses, in part, by regulating expression of major histocompatibility complex molecules. Our recent analysis has identified three structurally defined modules, K1, K2, and K3, of the hemagglutinin region of the lysine gingipain Kgp. These three structurally homologous domains have a common β -sandwich topology that is similar to that found in a superfamily of adhesins and carbohydrate binding domains. The three Kgp hemagglutinin modules are distinguished by variation in some of the loops projecting from the β -sandwich core. Recombinant products corresponding to both single and multidomain regions as well as native Kgp bound IFN- γ with similar affinities. Among the adhesin domain constructs, only the K1K2 polypeptide inhibited the upregulation of HLA-1 expression in a human erythroleukemia (K562) line induced by both recombinant and native IFN- γ . The K1K2 polypeptide also inhibited HLA-DR expression induced by IFN- γ in human umbilical vein endothelial cells. These effects were competitively inhibited by coinubation with sodium or potassium chloride solution. The N-terminal residues of IFN- γ were implicated in mediating the effect of K1K2, while antibody binding to loop 1 of K2 blocked the action of K1K2. The findings indicate the potential significance of structurally defined Kgp adhesin modules in the inactivation of IFN- γ but also the potential of K1K2 in locating the target for the catalytic domain of Kgp.

Periodontal diseases are characterized by the presence of inflammatory lesions and are customarily separated into infections affecting only the gingiva and those affecting the underlying structures of the periodontium (34). The transition from gingivitis to periodontitis is marked by the change in T-cell to B-cell predominance (9, 35). In this concept, it is clear that the balance of cytokines, bacterial factors such as protease production, and other host factors determine whether tissue destruction occurs or homeostasis is maintained. Among periodontal pathogens, most evidence points to a pathogenic role for *Porphyromonas gingivalis* (18, 37).

The mechanisms underlying the differences in virulence between *P. gingivalis* strains are not yet fully understood, but a group of cysteine proteinases, known as gingipains, play critical roles in hemagglutination, hemolysis, and disruption of cytokine networks (15, 21, 40, 41). Gingipains with either arginine (Arg-gingipain; RgpA and RgpB) or lysine (Lys-gingipain; Kgp) specificity are produced in large quantities by *P. gingivalis*. These proteases are likely to be in high concentration within tissue microenvironments where the organism has reached high biomass. Molecular analyses have revealed that Arg-gingipain is encoded by two genes, *rgpA* and *rgpB*, while Lys-gingipain is encoded by the single gene *kgp* (28, 29, 31). The predominant outer-membrane-associated high-molecular-weight forms of RgpA and Kgp form characteristic enzyme clusters comprising the catalytic domains linked to a number of hemagglutinin/adhesin modules (30). It is considered that the manner of secretion, processing, and attachment to the outer membrane utilizes the same pathway as for RgpB (26). Following extraction from *P. gingivalis*, the adhesin domains of the surface gingipains are observed to be proteolytically processed while remaining in tight molecular complexes. There is evidence that arginine gingipains play a key role in this processing (30), although catalytic activity of Kgp releases the hemoglobin recep-

tor HA2 from the structural domain K2 of Kgp (21). These observations have been widely interpreted to indicate a precise physiological processing of surface gingipains. However, if prior to extraction proteolytic activities of the gingipains are specifically inhibited, processing of the extracted products is incomplete. This is observed when using a monoclonal antibody to an adhesin domain epitope common to both RgpA and Kgp which detects a range of higher-molecular-weight fragments in extracts from pre-inhibited bacteria (36). This is interpreted to indicate either that processing of expressed gingipain is a continuous process during growth of the organism or that at least part of the autolytic/proteolytic processing results from the extraction process.

Our recent analysis has disclosed that the hemagglutinin region of Kgp comprises three homologous structural domains (with the exception of Kgp from strain HG66, which contains only two adhesin domains) (19) characterized by loops extending from a β -strand core with a fold topology found in a superfamily of intercellular adhesins typified by the MAM domain and various carbohydrate binding modules (20, 21). The Kgp adhesin modules are linked in a tandem fashion, displaying their variable modular loop regions and presenting them for ligand binding and other interactions (20) (Fig. 1). There was no evidence to support proteolytic activity of any combination of adhesin domains (20).

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Address correspondence to Peter L. Yun, plwyun@gmail.com.

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FIG 1 Schematic diagram of the modular structure of the hemagglutinin/adhesin region of Kgp. The 100-kDa region is composed of a conserved domain of unknown structure and function (designated DUF2436) followed by three cleaved adhesin modules, K1, K2, and K3 (19). The observed crystal structures of K2 and K3 and the predicted homologous structure of K1 are represented as ribbon diagrams (20, 21). The K1K2 and K1K2K3 constructs used in this study are indicated. The variable loop region projecting from the core of each of the modules is indicated by a red circle. SAXS analysis of recombinant proteins K1K2 and K1K2K3 indicated that the variable loop regions are exposed on the surfaces of these proteins (20).

Similarly, adhesin modules/domains are predicted to comprise the hemagglutinin region of RgpA (19, 20). RgpB, which lacks the adhesin modules, is less effective than RgpA in hydrolyzing membrane tumor necrosis factor alpha (TNF- α), highlighting the significance of adhesin modules of the enzymes in mediating the effects of the gingipains on cell membrane proteins (25).

Gamma interferon (IFN- γ) is a type 1 four- α -helical bundle cytokine with an amphipathic structure containing a hydrophobic inner core (3). The IFN- γ chain is 143 amino acids long and is active as a homodimer; its structure reveals two intertwined polypeptides carrying globular N-terminal domains and a flexible C-terminal region. Human IFN- γ lacking the first 10 amino-terminal residues is devoid of biological activity (11, 23). IFN- γ induces dimerization of its receptor (7). Glycosylation may play a role in the ligand/receptor mechanism, as there is evidence that exogenous oligosaccharides affect essential biological functions of IFN- γ (10). Infection has been shown to downregulate IFN- γ receptor 1 (IFN- γ R1) expression (14, 17, 32), and defective natural resistance has been observed in mice that lack the IFN- γ receptor (13).

Cell-mediated immunity mediated by a Th1 response involves IFN- γ production (5). A pivotal role of IFN- γ in human immunity to *P. gingivalis* may lie in its ability to upregulate class I and II major histocompatibility complex (MHC) antigens (6). Several studies have reported a significant increase in the gingival crevicular fluid (GCF) concentration of IFN- γ in those subjects with severe periodontitis (8, 27, 38). In contrast, measurement of IFN- γ in cultures of peripheral blood mononuclear cells (PBMCs) from periodontitis and control subjects has shown that IFN- γ levels in leukocyte cultures from periodontitis patients are consistently low (24). In this context, we previously demonstrated that gingipains can degrade IFN- γ , and this may bias the immune response to a Th2 cytokine response (42).

Currently, the precise events relating to recognition of cytokine networks by *P. gingivalis* remain undetermined. The present study was conducted to determine the effects of hemagglutinin/adhesin domains of Kgp on IFN- γ -regulated responses in K562 cells. The data demonstrate selectivity in the action of adhesin domains of Kgp in relation to the inhibition of IFN- γ action. Among these adhesin polypeptides, prolonged exposure to K1K2 can decrease IFN- γ -regulated expression of HLA-1 and HLA-DR protein. Fur-

ther, binding of K1K2 to IFN- γ resulted in decreased surface expression of IFN- γ R1 and IFN- γ R2 protein in K562 cells.

MATERIALS AND METHODS

Reagents. Bovine submaxillary mucin, fetal calf serum (FCS), L-cysteine, sodium dodecyl sulfate (SDS), *N*- α -tosyl-L-lysyl chloromethyl ketone (TLCK), Trizma base, Tris hydrochloride (Tris-HCl), and Tween 20 were purchased from Sigma (St. Louis, MO). RPMI medium was obtained from ICN Biochemicals (Irvine, CA). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from Calbiochem (La Jolla, CA). Phosphate-buffered saline (PBS) and Trypticase soy broth were purchased from Oxoid (Basingstoke, United Kingdom). All reagents for electrophoresis and Western blotting were from Bio-Rad (Richmond, CA). Purified nontagged recombinant proteins K1, K2, K3, K1K2, and K1K2K3 corresponding to fragments of the expressed Kgp gene found in *P. gingivalis* strain W83 were prepared as previously described (20, 21).

Cytokines and Abs. Nonglycosylated recombinant human gamma interferon (rIFN- γ) and glycosylated natural gamma interferon were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Monoclonal antibody (MAb) against IFN- γ (clone 25718) was obtained from R&D Systems (Minneapolis, MN). Fluorescein isothiocyanate (FITC)-anti human HLA-1 MAb (clone W6/32) was purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Monoclonal anti-human HLA class II DR Ab (clone TAL1B5) was purchased from Dako (Sydney). Polyclonal antibodies specific for amino acids 466 to 485 mapping at the COOH-terminal domain of the human IFN- γ R α chain (IFN- γ R1) or amino acids 318 to 337 mapping at the COOH-terminal domain for IFN- γ R β chain (IFN- γ R2) were obtained from Santa Cruz Biotechnology.

ELISA. Binding of adhesin domains to rIFN- γ or IFN- γ peptides was tested using an enzyme-linked immunosorbent assay (ELISA) method. IFN- γ peptide (peptide 1-14 or MKYTSYLAFLQICI [pI 8.8] at the N terminus) was synthesized by Mimotopes with purity of >80%. Briefly, 1 μ g of adhesin domains was used to coat the wells of a 96-well high-affinity flat-bottom ELISA plate (Sarstedt, Australia) overnight at 4°C. Thereafter, the wells were washed free of unbound material and blocking was performed with 1% skim milk in phosphate-buffered saline (PBS) for 1 h at 37°C. Subsequently, various levels of rIFN- γ or IFN- γ 1-14 peptide were added to wells, left overnight at 4°C, and washed, and mouse anti-IFN- γ monoclonal antibody at 1 μ g/ml in PBS–0.1% Tween 20 was added and left for 2 h at room temperature. Between each step, the plates were washed three times in PBS with 0.1% Tween 20. The ELISA was developed using an alkaline phosphatase-conjugated rabbit anti-mouse IgG (0.5 μ g/ml in PBS–0.1% Tween 20) and phosphatase substrate (Bio-Rad). Plates were analyzed spectrophotometrically using a Bio-Rad Benchmark microplate reader within 3 h (absorbance at 405 nm, the peak absorbance in the Soret region).

Flow cytometry. K562 (human myelogenous leukemia cell line) cells (22) were purchased from Sigma-Aldrich, Castle Hill, New South Wales, Australia. For time course and titration analyses, various levels of recombinant IFN- γ (rIFN- γ), native IFN- γ (nIFN- γ), or peptide IFN- γ (1-14) were pretreated with or without K1K2, K1K2K3, K1, K2, or K3 polypeptides at different molar ratios and time points at 4°C. After incubation, aliquots were added to the culture plates containing K562 cells in 10% FCS–RPMI medium and left for another 72 h at 37°C. After incubation, cells were stained with FITC–HLA-1 MAb and analyzed on a Becton Dickinson FACScan flow cytometer. Isotype-matched IgG1 was used as a control. For the staining of HLA-DR expression in human umbilical vein endothelial cells (HUVECs), K1K2, K1K2K3, K1, K2, or K3 polypeptide was pretreated with rIFN- γ (20 nM) at a molar ratio of 100:1 overnight at 4°C, and the mixtures were further incubated for 72 h at 37°C. Cells were stained with anti-HLA-DR MAb and then FITC-conjugated rabbit anti-mouse IgG (Dako, Sydney, Australia). For the staining of IFN- γ receptors in K562 cells, rIFN- γ or IFN- γ peptide 1-14 were pretreated overnight

FIG 2 K1K2 polypeptide inhibits rIFN- γ -induced HLA-1 surface expression. (A) HLA-1 expression by K562 cells at 72 h after treatment with rIFN- γ alone at 24 nM or with rIFN- γ pretreated with increasing levels of K1K2 or K1K2K3. (B) HLA-1 surface expression on K562 cells at 72 h following either no treatment or addition of rIFN- γ (pretreated with or without adhesin domains [molar ratio, 1:100]). To assess the impact of additional domains, K1K2 at 2.4 μ M was pretreated with K3 for 6 h at 4°C at equal molar ratio before addition to rIFN- γ . (C) Panels i to vii show FACS histogram representations of HLA-1 expression at 72 h after rIFN- γ treatment. The interferon was pretreated with K1K2 for 6 h or 24 h or with K1K2K3, K1, K2, K3 for 24 h. The shaded areas represent the fluorescence levels of HLA-1 expression induced by IFN- γ after 3 days of culture. The broken lines in panels ii to vii represent background values, while the bold black lines represent IFN- γ pretreated with different adhesin polypeptides before the addition to culture medium. The results are representative of those from three experiments. Means $\pm$ SEM are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with rIFN- γ -treated cells).

with adhesin domains at a molar ratio of 1:100 at 4°C and the mixtures were further incubated for 72 h at 37°C. K562 cells were then stained for surface IFN- γ R1 and IFN- γ R2 expression for flow cytometric analysis.

Antibody blocking study. Loop 1 of K2 represents a unique characteristic for this particular adhesin domain (Fig. 1). To probe the contri-

bution of this loop to block IFN- γ activity, customized affinity-purified rabbit antibodies to the antigenic sequence ETFESSTHGEPAC (pI 3.8) of loop 1 were prepared by GenScript Corp as previously described (20). Monoclonal antibody (MAb) 5A1 recognizing the peptide ALNPDNYLIS KDVTG (pI 3.9) (4), which correlates to amino acid residues 1215 to 1229

FIG 3 Interaction of rIFN- γ with K1K2-, K1K2K3-, K1-, K2-, K3-, and TLCK-treated Kgp. Ninety-six-well ELISA plates were coated with K1K2-, K1K2K3-, K1-, K2-, K3-, and TLCK-treated Kgp proteins (1 μ g/well in PBS) and incubated overnight at 4°C. The wells were blocked with 100 μ l of 1% skim milk in PBS for 1 h. rIFN- γ was added to the plates in various concentrations. Thereafter, anti-IFN- γ MAb was added, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG. Color development was detected with alkaline phosphatase substrate. Data were fitted by nonlinear regression using GraphPad Prism software (GraphPad Inc., La Jolla, CA). Apparent K_d values were calculated from the fitted curves. The results are representative of those from three experiments. Means $\pm$ SEM are shown.

of K2, bridging the fifth beta strand and loop 5, was also used for comparison. This assay was established by preincubation of K1K2 at 2 μ M/well in PBS with or without dialyzed rabbit anti-K2 polyclonal antibody or 5A1 MAb up to 3 μ g/ml overnight at 4°C. K1K2 was also pretreated with control antibodies, including mouse anti-human IgG1 and rabbit anti-human IgG (Sigma-Aldrich), at the same concentration levels and temperature. PBS was also used as a medium control. The samples were then added to rIFN- γ at 20 nM and left for another 24 h at 4°C before the addition to wells, and the HLA-1 expression in K562 cells was determined after 72 h at 37°C by flow cytometry analysis.

Statistical analysis. All data were expressed as means $\pm$ standard errors of the means (SEM). Differences between groups were examined for

statistical significance using Student's paired t test for paired data. A P value of <0.05 denoted the presence of a statistically significant difference.

RESULTS

Induction of HLA-1 expression on K562 cells by rIFN- γ is inhibited by K1K2 polypeptide in a dose- and time-dependent manner. To analyze the interaction between rIFN- γ and the K1K2 polypeptide, K562 cells were treated with a single concentration of rIFN- γ (24 nM) in the presence of increasing concentrations of K1K2 polypeptide (Fig. 2A). Inhibition of HLA-1 expression on K562 cells by the K1K2 polypeptide (shown as percentage of positive cells using fluorescence-activated cell sorter [FACS] analysis) was most pronounced at higher concentrations of K1K2 (2.4 to 6.5 μ M, or in molar ratio of 100:1 to 200:1). This possibly reflects the impact of multiple potential alternative binding sites within the experimental cell system that also contained fetal bovine serum. That is, multiple alternative substrates likely provided competition, necessitating high molar ratios for maximum effect. Maximum inhibitory activity for the upregulation of HLA-1 expression by rIFN- γ was noted when 24 nM rIFN- γ was pretreated with K1K2 (6.5 μ M) for 24 h (Fig. 2A). Inhibition of HLA-1 was not observed when rIFN- γ was pretreated with a range of concentrations of K1K2K3. Addition of rIFN- γ resulted in a greater-than-6-fold increase in HLA-1 expression on K562 cells, compared to that with medium alone (Fig. 2B). K3 at 2.4 μ M did not modulate K1K2-rIFN- γ interaction. Only K1K2 at 2.4 μ M inhibited rIFN- γ (24 nM)-induced HLA-1 surface expression (Fig. 2B). Further, treatment of rIFN- γ with different adhesin polypeptide combinations, including K1K2 plus K3, K1 plus K2, K1K2K3, K1 plus K2 plus K3, K1, K2, or K3, did not affect the subsequent expression of HLA-1 on K562 cells (Fig. 2B). K1K2 alone did not affect HLA-1 surface expression on K562 cells after 3 days of culture (Fig. 2C, panel i). The time course study of rIFN- γ pretreated with recombinant adhesin domains in molar ratios of 1:200 showed that the degree of inhibition of HLA-1 expression by K1K2 ranged from 54 (Fig. 2C, panel ii) to 79% (Fig. 2C, iii), depending on the length of pretreatment, with maximum inhibition at 24 h. Treatment of rIFN- γ with K1K2K2, K1, K2, or K3 did not reduce HLA-1 expression on K562 cells (2C, panels iv to vi).

Binding affinity of K1K2 for rIFN- γ as a basis for selective action. It is possible that K1K2 blocked function because of a

FIG 4 K1K2 polypeptide downregulates native IFN- γ (nIFN- γ)-induced HLA-1 expression. K562 cells were grown and incubated with nIFN- γ pretreated overnight with K1K2, K1K2K3, K2, or TLCK-treated Kgp (KgpTL) in a molar ratio of 1:100, and the mixtures were further incubated for 72 h at 37°C. FACS histograms show the cell staining for surface expression of HLA-1 on K562 cells. The results are representative of those from three experiments.

FIG 5 K1K2 polypeptide reduces rIFN- γ -induced HLA-DR surface expression. HUVECs were grown and incubated with rIFN- γ (IFN- γ) pretreated overnight with K1K2, K1K2K3, K1, K2, or K3 in a molar ratio of 1:100, and the mixtures were further incubated for 72 h at 37°C. Histograms show the cell staining for surface expression of HLA-DR and mean fluorescence value (MFV) in HUVECs. Results are representative of three separate experiments. Means $\pm$ SEM are shown. *, $P < 0.05$; **, $P < 0.01$ (compared with rIFN- γ -treated cells).

higher binding affinity for IFN- γ than that of K1K2K3, K1, K2, or K3. Therefore, K1K2 and K1K2K3 as well as K1, K2, and K3 were compared for their relative binding affinities (K_d) for nonglycosylated rIFN- γ . The polypeptides bound rIFN- γ with small differences in affinities, yielding K_d values of 5.5 nM, 12.8 nM, 39.5 nM, 42 nM, and 45 nM for K3, K2, K1, K1K2, and K1K2K3, respectively (Fig. 3). From the data, K3, K2, and K1 showed higher binding affinity to rIFN- γ than K1K2, but those polypeptides did not block the biological activity of rIFN- γ . It therefore appeared that inhibition of rIFN- γ function by K1K2 was not due to a higher binding affinity but rather to the manner of binding.

K1K2 affects native IFN- γ -induced activity. K1K2 polypeptide at 1 μ M inhibited 50% induction of HLA-1 expression by 10 nM native glycosylated IFN- γ after 24 h of pretreatment (Fig. 4). Similar but less potent inhibition of native IFN- γ -regulated HLA-1 surface expression was detected following pretreatment with proteolytically inhibited Kgp. No blockade of native IFN- γ -induced HLA-1 activity was detected following pretreatment with K1K2K3 or K2 (Fig. 4) or with K1 or K3 (data not shown) for 24 h using the same molar ratio.

FIG 6 Effects of different salts on the binding of rIFN- γ with K1K2. rIFN- γ was pretreated with or without K1K2 (A to C) or K1K2K3 (A) in the presence or absence of different salts, including NaCl, KCl, and KBr, for 24 h at 4°C. After incubation, all mixtures were added to the K562 cells and left for 72 h at 37°C. Both NaCl and KCl can eliminate the K1K2 inhibition of rIFN- γ activities in a dose-dependent manner.

FIG 7 K1K2 polypeptide binds to and inhibits IFN- γ 1-14-induced HLA-1 surface expression. (A) ELISA plates were coated with K1K2-, K1K2K3-, K1-, K2-, K3-, and TLCK-treated Kgp proteins (1 μ g/well in PBS) and incubated overnight at 4°C. IFN- γ 1-14 peptide was added to the plates in various concentrations. Thereafter, anti-IFN- γ MAb was added, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG. (B) The percentage of HLA-1 expression in K562 cells at 72 h was shown by flow cytometry after treatment with IFN- γ 1-14 peptide at different concentrations or with K1K2/K1K2K3 at 2 μ M each pretreated with increasing levels of IFN- γ 1-14 peptide for 24 h. Results are representative of three separate experiments. Means and SE are shown. *, $P < 0.05$; **, $P < 0.01$ (compared with IFN- γ 1-14).

K1K2 blocks rIFN- γ -induced HLA-DR surface expression in endothelial cells. To determine whether pretreatment of rIFN- γ with K1K2 also blocks other inducible activity, rIFN- γ was pretreated with K1K2 or K1K2K3 and the ability of rIFN- γ to induce HLA-DR expression by endothelial cells was measured. The addition of K1K2 to rIFN- γ in a molar ratio of 100:1 reduced the percentage of HLA-DR-positive endothelial cells by 55%, whereas K1K2K3 had a weaker effect, reducing rIFN- γ -induced HLA-DR expression by 22% (Fig. 5). No significant changes were observed when rIFN- γ was pretreated with K1, K2, or K3 polypeptide. This finding suggested that the region where K1K2K3 binds to IFN- γ impacts HLA-II but not HLA-I expression.

K1K2-rIFN- γ interaction is affected in chloride-containing buffer but not in bromide-containing buffer. To explore possible ionic interactions as a mechanism for ligation of K1K2 to rIFN- γ , the impact of gradient concentrations of Cl⁻ and Br⁻ was studied. At higher concentration (68 mM) of NaCl, rIFN- γ -induced HLA-1 expression was reduced by 19%, indicating ionic interaction between rIFN- γ and its receptor (Fig. 6A). Concentrations of NaCl from 17 to 34 mM significantly reduced the ability of K1K2 to block rIFN- γ activity. The inhibitory effect of K1K2 on rIFN- γ activity was completely abolished by NaCl at above 34 mM. KCl reduced the inhibitory effect of K1K2 on rIFN- γ activity in a dose-dependent manner (Fig. 6B). However, KBr at up to 68 mM had no effect on the inhibitory effect of K1K2 on rIFN- γ activity (Fig. 6C), indicating the selective action of the higher charge density of the chloride anion.

K1K2 binds to an N-terminal fragment of IFN- γ . Using ELISA, we measured binding of recombinant adhesin domains to IFN- γ peptide 1-14. The apparent K_d value determined by ELISA for titrations of IFN- γ peptide 1-14 to immobilized adhesin domains was 14.7 nM for K1K2 (Fig. 7A), which was stronger than that for K1K2K3 (K_d = 21.5 nM). Binding of IFN- γ peptide 1-14 to *N*- α -tosyl-L-lysyl chloromethyl ketone (TLCK)-treated Kgp

was also observed, with a K_d value of 18.2 nM. Only low-affinity binding of IFN- γ peptide 1-14 to immobilized K1, K2, or K3 adhesin domains was detected.

K1K2 can block IFN- γ peptide 1-14 induced-HLA-1 expression in K562 cells. We next determined whether the binding of K1K2 to IFN- γ peptide 1-14 influenced inhibitory activity. IFN- γ peptide 1-14 was pretreated with K1K2 overnight at molar ratios of between 0 and 1:200. ELISA indicated strongest binding of the N-terminal peptide by K1K2 (Fig. 7A). Preincubation of increasing levels of IFN- γ peptide 1-14 with K1K2 reduced induction of HLA-1 expression in K562 cells as determined by flow cytometry (Fig. 7B). Further, preincubation of IFN- γ peptide 1-14 with K1K2K3 (Fig. 7B) or with K1, K2, or K3 (data not shown) adhesin domains did not block HLA-1 induction.

K1K2 polypeptide inhibits rIFN- γ -regulated IFN- γ R1 and IFN- γ R2 expression on K562 cells. IFN- γ R1 and IFN- γ R2 expression, measured by flow cytometry, increased following 72 h of IFN- γ treatment (Fig. 8A). rIFN- γ pretreated with K1K2 polypeptide during stimulation of K562 cells resulted in 31% and 32% decreases in IFN- γ R1 and IFN- γ R2 expression, respectively (Fig. 8A). Expression of both IFN- γ R1 and IFN- γ R2 receptor molecules was not affected by the K1K2 polypeptide or the other adhesin domains alone (data not shown). Further, IFN- γ R1 and IFN- γ R2 expression in K562 cells was reduced up to 25% by IFN- γ 1-14 peptide pretreatment with K1K2, whereas no inhibition was observed when IFN- γ 1-14 peptide was treated with K1K2K3 (Fig. 8B). These results indicate that K1K2 can bind to N-terminal IFN- γ and interfere with IFN- γ R1 and IFN- γ R2 expression by K562 cells.

K1K2 does not directly affect gamma interferon receptor expression. To assess whether K1K2 can interact with the IFN- γ receptor and inhibit IFN- γ -induced HLA-1 expression, K562 cells were pretreated with (Fig. 8C, thick line) or without (Fig. 8C, thin line) K1K2 (2 μ M) in serum-free RPMI medium at 37°C over-

FIG 8 K1K2 polypeptide modulates expression of IFN- γ R1 and IFN- γ R2 in K562 cells. (A and B) K562 cells were grown and incubated with rIFN- γ (A) or IFN- γ peptide 1-14 (B) pretreated overnight with recombinant adhesin domains as shown, and the mixtures were further incubated for 72 h at 37°C. FACS histograms show the cell staining for surface expression of IFN- γ R1 and IFN- γ R2 on K562 cells. The results are representative of those from three experiments. Means $\pm$ SEM are shown. *, $P < 0.05$; **, $P < 0.01$ (compared with rIFN- γ [A]- or IFN- γ peptide 1-14 [B]-treated cells). (C) K562 cells were pretreated with (thick line) or without (thin line) K1K2 at 37°C overnight. Cells were then cultured with rIFN- γ , and the HLA-1 expression on K562 cells at 72 h is shown.

night. Cells were then washed and further cultured with rIFN- γ at 20 nM for 3 days. The results indicated that preincubation of K562 cells with K1K2 did not affect HLA-1 expression subsequently induced by IFN- γ . The data suggested that K1K2 can affect HLA-1 expression only by the interaction with IFN- γ and not by interaction with the IFN- γ receptor.

Anti-K2 Ab interferes with K1K2-IFN- γ interactions. To explore the structural elements of K1K2 responsible for inhibition of biological activities of rIFN- γ , an affinity-isolated polyclonal antibody specific for loop 1 of K2 was utilized to determine possible blockade of K1K2-IFN- γ interactions. Anti-K2 Ab inhibited K1K2-IFN- γ interaction in a dose-dependent manner (Fig. 9). Anti-IFN- γ activity exhibited by K1K2 was completely eliminated in the presence of anti-K2 Ab at 3 μ g/ml. In contrast, K1K2 inhibition of IFN- γ -induced HLA-1 expression was not affected following preincubation with the same range of 5A1 antibody that recognized a different region of K2. The data suggest the involvement of loop 1 of K2 in binding of K1K2 to IFN- γ . Nonspecific effects of immunoglobulins were not involved, as the addition of irrelevant control antibodies at levels similar to those in the anti-K2 Ab (Fig. 9)- or 5A1 (data not shown)-treated cultures did not affect K1K2-IFN- γ interactions.

DISCUSSION

Perturbation of cytokine networks is considered a hallmark of the immunopathology characteristic of the pathogenesis of destructive periodontitis. It has become increasingly clear that immunity mediated primarily by MHC class II-restricted IFN- γ -producing CD4⁺ Th1 cells is deficient during the course of periodontal infection (1, 39). For instance, evidence is emerging that supports the failure of macrophages to become activated in destructive periodontal disease sites (2). Findings from the present study indicate an additional mechanism for inactivation of a key cytokine of this pathway, mediated by selective binding by a structurally defined gingipain construct.

The structural model obtained by small-angle X-ray scattering (SAXS) indicates that K1, K2, and K3 are relatively independent entities joined by flexible linker regions (Fig. 1). In this three-dimensional structural model, loop 1 of K2, which is directly implicated in binding to IFN- γ (Fig. 9), appears to be presented to binding partners independently of K1 (20). However, K2 alone is ineffective, suggesting a key role for K1 in mediating additional ligation of IFN- γ , and this is supported by the binding data. Further, the native structure of K1K2 is critical, as the addition of K1

FIG 9 Effects of anti-K2 Ab on the binding of rIFN- γ with K1K2. K1K2 was pretreated with anti-K2 Ab or control Ab overnight and then incubated with rIFN- γ for 24 h. In addition, 5A1 Ab was preincubated in the presence or absence of K1K2 before addition of rIFN- γ . After incubation, all mixtures were added to the K562 cells, and the HLA-1 expression at 72 h was determined. Results are representative of three separate experiments. Means $\pm$ SEM are shown.

and K2 polypeptides was ineffective in blocking IFN- γ -induced activity. Similarly, the lack of activity of the K1K2K3 construct could be mediated by competition by K3, which inhibits the binding of K2 to IFN- γ . K3 polypeptide added separately did not modulate K1K2-IFN- γ interaction, indicating that an intact K1K2K3 construct is needed for potential inhibition by K3 to occur. Such interactions within the hemagglutinin region of Kgp could also be mediated by proteolytic processing of this domain, as detected in Kgp extracted from *P. gingivalis* (20, 21). Profiles for IFN- γ binding to the adhesin domains (Fig. 3) suggest competition rather than cooperation. Thus, K2 and K3 are bound with higher apparent affinity than K1K2 or K1K2K3.

Native IFN- γ has three forms that vary in glycosylation: diglycosylated at Asn25 and Asn97 (2N), monoglycosylated at Asn25 (1N), and nonglycosylated (12). The results suggest that the glycan side chains do not prevent the binding of K1K2 to IFN- γ . Both native forms and recombinant nonglycosylated IFN- γ respond to K1K2 in an equivalent manner, implying that any binding of K1K2 to carbohydrate moieties is not central in mediating the observed effects.

Direct competition studies showed that the N-terminal α -helix spanning residues 3 to 11 of IFN- γ is involved in receptor binding (16). Data from the present study indicates that K1K2 binds strongly to the N-terminal IFN- γ 1-14 peptide. IFN- γ belongs to the group 1 cytokines, which have positively charged N-terminal sequences. Surface electrostatic potential analysis of K2 shows that flanking regions of this end of the β 2-barrel, formed by L1- β 2-L2, L3, and L4, are highly negatively charged as contributed by acidic residues Glu1170, Asp1179, Asp1181, Asp1183, Asp1196, and Asp1220 (21). Similar acidic residues are also found in the L1- β 2-L2, L3, and L4 of K1 or K3 polypeptide. The blockade of IFN- γ -induced activities by K1K2 could be mediated in part by ionic interactions between K1K2 and the N-terminal regions of

IFN- γ . In this work, we describe disruption of K1K2 inhibition of IFN- γ -induced HLA-1 expression by chloride anion. This is compatible with the high charge density of this anion effectively disrupting ionic interactions. In this regard, potential ionic interactions between loop 1 of K2 and the N-terminal peptide of interferon are of interest, given the opposite charges predicted at physiological pH for these two entities.

This is supported by the inhibitory effect of anti-loop 1 K2 Ab, suggesting the contribution of loop 1 of K2 to binding to the N-terminal region of IFN- γ . These residues of IFN- γ may be required for interaction with the IFN- γ receptor that is perturbed by binding of K1K2. In this regard, this adhesin moiety induces an altered conformational state in IFN- γ . Future site-directed mutational analysis could determine the precise targets.

IFN- γ exists predominantly as a homodimer of 17 kDa under physiological conditions, and IFN- γ signals are initiated by binding of the dimer to the IFN- γ R1 chain (33). The results show that while K1K2-IFN- γ interaction can inhibit the surface expression of IFN- γ R1, signaling through the IFN- γ receptor may also be affected. It remains to be determined whether the downregulation of IFN- γ R1 expression by K1K2 can affect the phosphorylation of IFN- γ R1, JAK1, JAK2, and STAT1.

Experimental studies have provided an extensive catalogue of evidence that the proteolytic actions of the gingipains provide a powerful mechanism for disruption of cytokine networks. Accordingly, gingipains hydrolyze IFN- γ efficiently (42). It is probable that selective interactions of the adhesin domains with cytokines are important in facilitating the activity of the catalytic domain. It is also postulated that the adhesin domains directly regulate the biological activities of cytokines, as demonstrated here for interferon. Refinement of the mechanism will provide insight into the potential to inhibit such interactions and also provide a focus for vaccine strategies aimed at preventing infection by *P. gingivalis*.

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