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PD-L1 is induced by the periodontal pathogen *Porphyromonas gingivalis* and can be blocked by small molecule gingipain inhibitors, including atuzaginstat

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Background

The periodontal keystone pathogen *Porphyromonas gingivalis* (*Pg*) has been linked to esophageal and other malignancies including HNSCC, gastrointestinal, and pancreatic cancer through epidemiology studies (Olsen et al, 2019). This gram-negative bacteria produces virulence factor proteases known as gingipains, which are critical for bacterial virulence and survival in vivo. *Pg* and the gingipains have been identified in esophageal cancer tissue and there is accumulating evidence that bacterial presence is correlated with worse disease prognosis (Qi et al, 2020).

Repeated low-level infection with *Pg* results in an increase in proliferation, invasion, and tumorigenic properties of cells (Geng et al, 2017). Xenograft studies with *Pg*-infected cell lines have demonstrated the bacteria produce an aggressive phenotype in models of oral squamous cell cancers including tongue and esophageal (Qi et al, 2020 and Kamaranjan et al, 2020).

In addition to direct tumorigenic effects, the bacteria are implicated in microbial dysbiosis and produce a dysregulated immune defense. Indeed, *Pg* has been shown to induce PD-L1 on the surface of infected cells, suggesting that the presence of *Pg* in esophageal cancer cells may contribute to PD-L1 expression and escape from immune surveillance (Groeger et al, 2017). Anti-PD-1 antibodies have shown some therapeutic success in esophageal cancer, but further understanding of the induction of PD-L1 in esophageal cells would identify additional potential treatment modalities (Mimura et al, 2018). One of several pathways known to induce PD-L1 expression is Wnt pathway activation resulting in β-catenin translocation to the nucleus (Martin-Orozco et al, 2019 and Wang et al, 2018). Prior studies have demonstrated that *Pg* infection can activate this pathway through a non-canonical mechanism, resulting in β-catenin nuclear localization (Zhou et al, 2015).

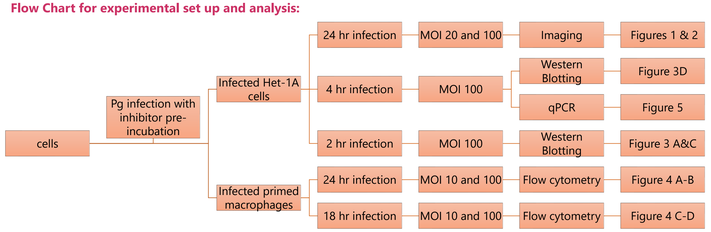
Atuzaginstat is a novel gingipain protease inhibitor being developed by for the treatment of Alzheimer's Disease (AD). The mechanism of action is based on the discovery of *Pg* in the AD brain with data demonstrating that oral infection of *Pg* results in hallmarks of AD pathology (Dominy et al, 2019). Atuzaginstat is currently being assessed in a Phase 2/3 trial in AD patients and has the potential for use in other indications in which *Pg* plays a causative role in disease pathology.

Study Aims

- **Aim 1**: Is increased PD-L1 membrane surface expression with *Pg* infection mediated by the gingipain protease activity?
- **Aim 2**: What is the mechanism by which *Pg* induces PD-L1 expression and can this inform us about possible responsive tumors?
- **Aim 3**: Is the induction of PD-L1 limited to tumors or also present in tumor-associated cells involved in immune surveillance and implicated in anti-PD-1 therapeutic response (such as macrophages, DCs)?
- **Aim 4**: Is PD-L1 the only immune evasion marker induced by *Pg* or are other markers of T-cell exhaustion also induced?

Experimental Design

Flow Chart for experimental set up and analysis:

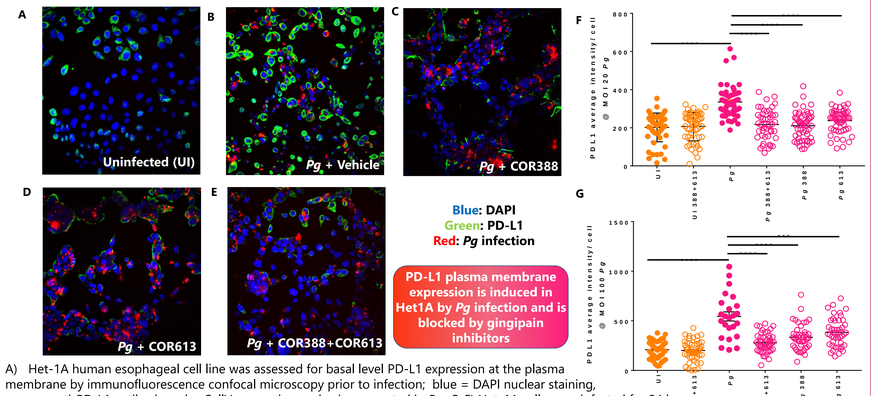


Statistical Analysis:
All statistical analysis were done in GraphPad Prism using a standard unpaired t-test. Significance is designated by standard asterisk convention where * is $p < 0.05$, ** is $p < 0.01$, *** is $p < 0.001$, and **** is $p < 0.0001$.

Reagents and Inhibitors	Activity	Concentration
COR388 (Atuzaginstat, 388)	Lysine gingipain inhibitor	1 μM
COR613 (613)	Arginine gingipain inhibitor	1 μM
MG132	Proteasome inhibitor	10 μM
Wnt ligand (Wnt3a)	Wnt classical pathway activator	200 ng/mL
Lipopolysaccharide (LPS)	TLR ligand	20 mg/mL

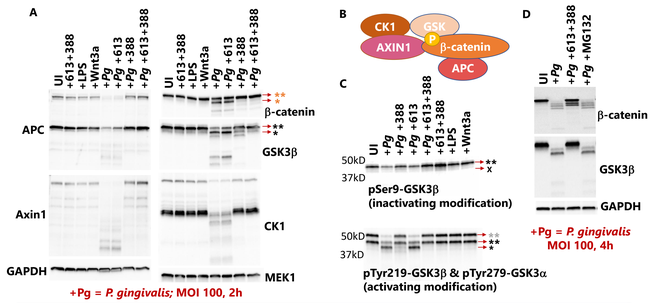
Results

Fig1. PD-L1 expression at the plasma membrane (PM) of Het-1A cells is blocked by gingipain inhibitors



A) Het-1A human esophageal cell line was assessed for basal level PD-L1 expression at the plasma membrane by immunofluorescence confocal microscopy prior to infection; blue = DAPI nuclear staining, green= anti-PD-L1 antibody, red = CellVue membrane dye incorporated in *Pg*. B-E) Het-1A cells were infected for 24 h with *Pg* at MOI 20 and treated during infection with B) DMSO vehicle, C) atuzaginstat, D) COR613, and E) atuzaginstat + COR613. PD-L1 expression at the plasma membrane was determined by staining as described for panel A. Expression of PD-L1 was quantitated over 70 fields in cells infected with *Pg* MOI = 20 (F) and *Pg* MOI = 100 (G) [MOI = multiplicity of infection]

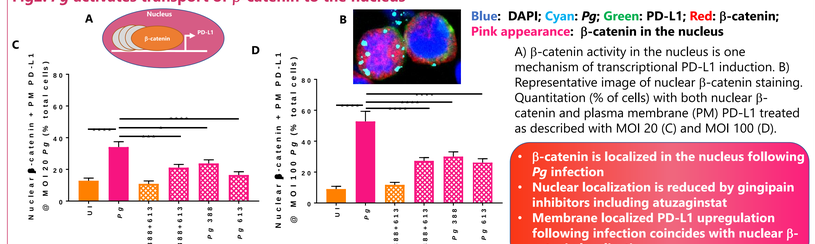
Fig3. Classical Wnt pathway β-catenin degradation complex is disrupted by lysine gingipain



Pg infection disrupts the β-catenin regulatory complex:
- AXIN, APC, and CK1, essential for negative regulation of β-catenin, are reduced
- GSK3β is activated by phosphorylation at Y219 site
- β- catenin in cleaved form is generated
- MG132 does not protect against β- catenin cleavage indicating effects are not proteasome-mediated
- Targeted pathway is specific, and no effects seen on GAPDH or MEK1
- Complex disruption is blocked by atuzaginstat

A) Immunoblot analysis of members of the β-catenin degradation complex of the classical Wnt pathway in Het-1A cells infected following *Pg* infection with the indicated therapeutics. LPS, TLR activation positive control and classical anti-microbial response. Wnt ligand (Wnt3a), Wnt pathway activator control. GAPDH and MEK1 normal loading controls. B) β-catenin degradation complex schematic. C) Activation of cleaved GSK3β following *Pg* infection assessed with anti-pSer9 antibody (GSK3β inactivating) and anti-pTyr219 (GSK3β activating). D) MG132, a proteasome inhibitor, does not block *Pg* effects.

Fig2. *Pg* activates transport of β-catenin to the nucleus

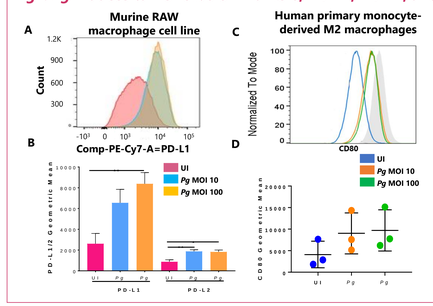


Blue: DAPI; Cyan: *Pg*; Green: PD-L1; Red: β-catenin; Pink appearance: β-catenin in the nucleus

A) β-catenin activity in the nucleus is one mechanism of transcriptional PD-L1 induction. B) Representative image of nuclear β-catenin staining. Quantitation (% of cells) with both nuclear β-catenin and plasma membrane (PM) PD-L1 treated as described with MOI 20 (C) and MOI 100 (D).

- β-catenin is localized in the nucleus following *Pg* infection
- Nuclear localization is reduced by gingipain inhibitors including atuzaginstat
- Membrane localized PD-L1 upregulation following infection coincides with nuclear β-catenin localization

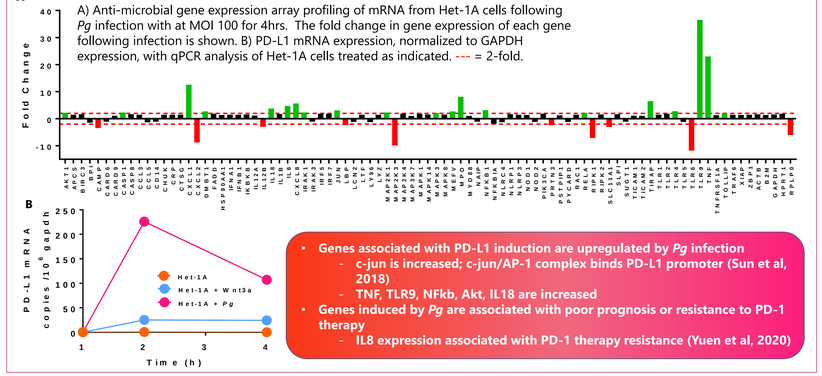
Fig4. *Pg* induces tumor evasion markers, PD-L1, PD-L2, and CD80 (CTLA4 ligand) on M2 primed macrophages



A) Primed M2 murine RAW cell line-derived macrophages were infected for 24 hrs with *Pg* MOI 10 or 100 analyzed for PD-L1 levels by flow cytometry. B) Quantitation of study in A, in triplicate for macrophages as indicated; includes analysis of of PD-L2 as well. C) Flow cytometry analysis of primed M2 human primary monocyte-derived macrophages infected for 18 hrs with *Pg* MOI 10 and MOI 100 and stained for CD80 detection. D) Quantitation CD80 expression of study in C from three donors.

- *Pg* infection of M2 macrophages induces expression of multiple T cell exhaustion markers
- Induction by *Pg* infection may play a role in the development of local T cell anergy and tumor immune evasion
- CD80 expression is a known target of the β-catenin transcriptional complex

Fig5. *Pg* infection modulates expression of several signaling proteins implicated in PD-L1 induction



A) Anti-microbial gene expression array profiling of mRNA from Het-1A cells following *Pg* infection with at MOI 100 for 4hrs. The fold change in gene expression of each gene following infection is shown. B) PD-L1 mRNA expression, normalized to GAPDH expression, with qPCR analysis of Het-1A cells treated as indicated. --- = 2-fold.

- Genes associated with PD-L1 induction are upregulated by *Pg* infection
  - c-jun is increased; c-jun/AP-1 complex binds PD-L1 promoter (Sun et al, 2018)
  - TNF, TLR9, NFkb, Akt, IL18 are increased
- Genes induced by *Pg* are associated with poor prognosis or resistance to PD-1 therapy
  - IL8 expression associated with PD-1 therapy resistance (Yuen et al, 2020)

Conclusions

- PD-L1 expression is increased by *Pg* infection in a dose-dependent manner in an esophageal cell line Het-1A and this induction is blocked by gingipain inhibitors including atuzaginstat.
- *Pg* infection results in nuclear β-catenin and disruption of the Wnt pathway complex regulating β-catenin localization and this is also blocked by gingipain inhibition.
- Tumor immune evasion markers PD-L1, PD-L2, and CTLA4 are induced by Pg infection on primed M2 macrophages, precursors to tumor associated macrophages (TAMs).
- *Pg* infection also results in the upregulation of several other genes implicated in PD-L1 expression resulting in a strong induction of PD-L1 mRNA.

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Acknowledgements

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