# A role for cell free ervs in propagating ifn type-i responses

As previously mentioned, OC is the most lethal of the gynaecologic malignancy in the western world and this is due to poor prognosis and limited treatment efficacy (Coretz et al., 2018). In a meta-analysis Immunotherapy alone has been proven to not yield significant results in OC patients, requiring the need for a combinatorial therapy (Alipour et al., 2016). Until now, research has mostly focused on mechanisms of DNMTi including abnormal DNA methylation of tumor suppressor genes. However, focus has now shifted to ERVs suppression by hypermethylation, predominantly in somatic cells (Phillips, 2018). Work by Jones et al., 2019, has focused on viral mimicry mechanisms showing DNMTi treatment inducing transcription of ERVs within the cell. However, considering other mechanisms of ERVs apart from ‘ viral mimicry’ is crucial as OC tumours may rely on other mechanisms apart from DNA methylation to downregulate tumour suppressor genes and antigen presentation via the MHC-complex.

Interestingly, high plasma levels of the RNA of various HERV ENV genes (HERV-K, HERV-R, and HERV-H) have been found in primary cancer patients from various cancers, before and after treatment with Guadecitabine. These experiments conducted in Professor Ghaem-Maghami lab have hinted the possibility of ERVs in the plasma. Therefore, the presence of cell free HERV-H, the top candidate ERV from RNA-seq data, was investigated following Guadecitabine treatment of OC cell lines. Kuramochi and Ovsaho cell lines were utilised as they genetically resemble high grade serous OC which represents 80% of cases (Lisio et al., 2019). Data obtained demonstrated an increase in RNA expression of HERV-H in both Kuramochi and Ovsaho 5 days after treatment with SGI-110 as seen in *figure 3. 1A.* 1 uM and Kuramochi

yielded the highest HERV-H expression therefore was the most optimal effective dose to use for consecutive experiments. Although there was an observed increase, no statistical test had been applied due to limited technical repeats, thus it may not be significantly different. Figure 3. 1A indicates the presence of HERV-H in the supernatant in a dose-dependent manner as would be expected. Although the mechanism in which cell free RNA leaves the cell is unknown *, Jones et al., 2019* established that ERVs are reactivated into the cytosol as dsRNA following epigenetic drug exposure via MDA5, and translocate back into the nucleus through MAVS aggregation and IRF7 activation. However, research to date has not specifically explored the potential of a dsRNA escaping sensing by PRRs like MDA5, TLR3, RIG-1 and leaving the cell. The data mentioned above suggests Guadecitabine may activate ERVs, escape sensing and leave the cell either freely or through a mechanism of action not yet established. Although this data seems promising, expression fold change levels are below 5 and very variable. In order to optimise the HERV-H expression and reduce variability between replicates, RNA integrity needs to be established and improved, as RNA quantification results stated in Table 3. 1, showed that although there was sufficient RNA quantity, the A260-A280 values were controversial, potentially causing this issue. This can be rectified by measuring RNA integrity using an agarose gel electrophoresis on 1. 0% agarose gel containing GelRed™ (Biotium, Inc., Hayward, CA, USA) as performed in literature, in order to QC RNA quality used for qPCR. Treating OC cell lines with 5uM has shown minimal HERV-H expression potential as DNMTi at a high concentration may be cytotoxic and cause DNA damage in the cells. This elucidates why lower doses must be used considered clinically to avoid cytotoxicity in OC patients.

One can argue that HERV-H from inside the cell pellet is being detected as opposed to HERV-H within the supernatant. We attempted to decrease this possibility by spinning the media collected and removing the visible cell pellet, however, due to technical pipetting errors, there is the possibility of lcarry-over. This may have occurred during handling of the cells in tissue culture prior to RNA extraction. When calculating log fold change, CT values for PPIA housekeeping gene were quite variable, although standard deviation values were quite low. PPIA as opposed to GAPDH, has a higher stability and a good candidate for OC cell lines as stated by Jacobsen et al., 2014; however, the setup of the experiment and the SGI-110 treatment at 2 days may have interfered with PPIA causing instability. Additionally, RNA quantity may have been variable well to well, such as inconsistency in diluting RNA as cDNA enzymes and primers may have inhibited the reaction all due to technical errors.

Housekeeping genes involved in different biological processes may have varied stabilities so the DNMTi-treatment of OC cell lines could have influenced its stability as Yauhen et al., 2017 states that the enzymatic activity of PPIA may affect its stability. In addition, a study investigating reference genes in OC, PPIAs statistic stability remained the same despite different OC tissue conditions, however, other genes including RPL4, RPLP0, and HSPCB proved to be the most stable for qPCR in OC tissue, thus must be considered in future experiments in which DNMTi treatment does not affect its stability (Fu et al., 2010). Excluding variability, this data may indicate the presence of HERV-H in the supernatant although the mechanisms of action leaving the cell remains unknown.

Enhanced type I IFN signalling

HERVs have previously been reported to promote tumour growth (Lemaître et al., 2017). However, current research focuses on to a new role of HERVs which include their ability to produce an anti-tumour response. As mentioned previously, DNMTi treatment induces an IFN response in OC cells following activation of MDA5, RIG-I and TLR-3, in which de-methylation of ERV expression, aids IRF3, NF-kB and IRF7 transcription leading to type- I IFN production. In this project, it was explored whether exposing untreated OC lines with cell free dsRNA, presumably HERV-H, upregulates IFN type-I response in OC cell lines. In *figure 3. 1B and C,* there is evident data possibly indicating the upregulation of IFN-α and IFN- β release in a dose-dependent manner, following exposure of supernatant from DNMTi-treated OC cells.

IFN-α and IFN- β both OC cell lines is increased in 24 hours which may due to cell death and debris being detected as an IFN response. This suggest that the OC cell lines may be over-confluent due to the DNMTi and inducing cell death. Cell free HERV-H expression was also assessed alongside type-I IFN responses and showed increase in expression compared to the vehicle, highlighting its influence on type 1 IFN response, as well as the potential ability to induce self-expression. IFN-α and IFN- β provide the first line of host defence against viruses in order to provide immune protection. However, they’ve also been implicated in the crosstalk between immune and tumour cells in which IFNs were previously known to drive inflammation within TME (Crow and Ronnblom, 2019). However, there is now more focus on type I possessing immune-stimulatory features on cells such as T -lymphocytes which will be discussed shortly. Recently, Buoncervello et al., observed in a

study focusing on colorectal cancer, that IFN-α potentiates the direct and immune-mediated antitumor responses following epigenetic drugs. Specifically, IFN-α has the potential to induce both anti-proliferative and pro-apoptotic features in vitro. It is now acknowledged to be vital to stimulate immunogenic cell death (ICD), strengthening the anti-tumour specific immune responses, enhancing its suitability as a target for novel therapeutics within epigenetic therapy for OC. Moreover, IFN-signalling has also been shown to cooperate with epigenetic drugs to inhibit tumour cell proliferation in vivo (Buoncervello et al., 2016). Specifically, Chiappinelli et al., 2015, have demonstrated that inhibiting DNA methylation induces IFN-signalling in OC via ERVs. Reversing abnormal DNA methylation on promotor site leads to re-expression of tumour suppressor genes, a key attribute in malignancy, as well as promoting apoptosis and changes in cell-cycle (Tsai et al., 2012).

In solid tumours, IFN- β is appreciated as an essential factor in viral defence signalling by up-regulating antigen presentation and immunogenicity by interacting with IFNAR1/2. This activates the JAK/STAT pathway, as JAK/STAT inhibitor strongly decreased ISG transcription. Following up-regulation of IFN-stimulated genes and production, apoptosis also occurs (Medrano et al., 2017). It was observed that IFN- β and viral defence genes (induced by DNMTi) are not hyper-methylated meaning that pathways upstream of these genes are stimulated following DNMTi treatment.

Data obtained from this project showing dose-dependent trend of IFN type-1 signalling of both IFN-α and IFN- β, by treating untreated tumour cells with cell free supernatant, corresponds with *Chiapenelli et al* data that

acknowledge DNMTi's inducing cytoplasmic sensing of dsRNA and stimulating type-1 IFN in OC cell lines. This suggests the possibility of cell-free RNA causing the induction of immunogenic type 1 IFN in neighbouring cells. If the mechanism of action of IFNs associated with anti-tumour effects can be established, this may lead to optimised combinatorial epigenetic therapy in the future for OC patients failing to response to monotherapies such as immunotherapy alone.

Cell free RNA, Tumour and T cells

γδ T cells, a key subset of " unconventional" T lymphocytes, have been widely used in immunotherapeutic research. γδ T cells are considered as good targets for therapies, optimising anti-tumour responses due to their distinct features, including their ability to recognise Ag's expressed by tumour cells without MHC-class presentation, as well as producing IFN-γ and TNF- α which possess strong cytotoxic features including stimulating DC and NK cells against neoplastic cells and inducing ADCC (Lo presti et al., 2018).

Work by Chen et al., 2019 has demonstrated that γδ T cells are implicated in tumorigenesis and metastasis when infiltrated in cancers including breast and colorectal cancers. However,

the ability of neoplastic cells to create immunosuppressive environment to evade immune surveillance is a set-back encountered in immune-oncology therapy. Specifically, there is

limited data in literature regarding γδ T cells in OC patients. It has been shown by Fisher et al., 2014, that they're a vital part of TILs in OC patients,

as well as Presti et al., 2018 working on a transcriptome study, established tumour-infiltrating γδ T cells as the most significant favourable prognostic mark. Their functions are often changed or abrogated by immunosuppressive signals originating from the TME. Due to γδ T cells unique features and their controversial implication in tumour progression, they were utilised in this project to assess the role of cell free RNA on tumour: T cell interaction.

Specifically, IFN-γ levels were measured to assess γδ T cell activation following exposure to cell free supernatant in the presence of tumour cells. Here, results displayed in *figure 3. 3B* showed a significant decrease in IFN-γ release in γδ T cells from DNMTi treated Kuramochi cells, in comparison to the vehicle.

IFN-γ is a key moderator or cell-mediated immune responses, specifically pro-inflammatory actions, however a recent publication by Bhat et al., 2017 has shown IFN-γ up-regulating anti-tumour and antiviral effects of CTLs. Therefore, the finding from *figure 3. 3B* showing a decrease in IFN-γ expression, was not expected as it was assumed there would be a higher IFN-γ release due to the presence of potential cell free ERV inducing T cell activation. However, this decrease in IFN-γ may be attributed to *T cell exhaustion* . Normally, a CD8+ T cell response is provoked in the presence of neoplastic cells, although a sustained stimulation pushes T cells into a non-functional state which decreases CTLs proliferative ability and hindering cytokine expression (Dogra et al., 2016)

This can be avoided in future experiments by collecting the supernatant of co-culture assay <24 hours to avoid prolonged exposure of γδ T cells with

the cell free RNA thus preventing T cell exhaustion. As well measuring IFN-γ, γδ T cells secrete pro-inflammatory cytokines, including TNF- α and IL-7, in response to tumour cells which can be measured by flow cytometry, in the future to establish γδ T cell activation in the presence of cell free RNA.

T-cell proliferation was assessed using the live/dead stain to investigate if DNMTi-treated tumour cells had an effect on γδ T cell proliferation. Indeed, it did as treating tumour cells with cell free RNA led to an increase in live percentage of γδ T cells at 48 hours compared to the vehicle.

Consequently, the data obtained verified T cell activation in the presence of DNMTi-treated tumour cells. Data from this project shows in the presence of DNMTi-treated tumour cells with ZA-stimulated γδ T cells, a significant decrease **, is observed in cell viability compared to the vehicle. This suggests that stimulated γδ T cells are involved in tumour cell killing, specifically in those treated with cell free supernatant. It has been established in the past that γδ T cells are implicated in tumour cell lysis (Handgretinger and Schilbach, 2018). γδ T cells interacts with IPP produced via mevalonate pathway. Bisphosphonates, such as ZA used in this project, inhibits FPPS leading to increase IPP and DMAPP in Kuramochi cells. γδ T cells detect HSPs and MICA/B or ULBP expressed through NKG2D on tumour cells (Gruenbacher and Thurnher, 2017). An increase in perforin is observed, as seen in *figure 3. 8* in the presence of DNMTi-treated cells, allowing granzyme B to enter through the pores and induce apoptosis in the Kuramochi cell lines. In addition, γδ T cells also releases IFN-γ as a bystander effect, which recruits other immune cells (Jarosz-Bie et al., 2019) and may be responsible for the killing, in which data in *figure 3. 8* encourages this rationale, as an

increase in both Perforin+ Granzyme B+ expression is seen in γδ T cells cocultured with DNMTi-treated tumour cells.

CD8+ T cell possess cytotoxicity functions very similar to γδ T cells, apart from recognising Ag’s via MHC-class 1 complex in order to induce anti-tumour response (Godfrey et al., 2018). Treating tumour cells with DNMTi and co-culturing with γδ T cells showed no apparent difference in CD8+ T cell activation. As previously mentioned, γδ T cells are also involved in priming CD8+ T cell responses, although it is not seen in *figure 3. 6B* , as well as inducing DC maturation, NK-cell mediated cytotoxicity, both contributing to indirect antitumor response (Srivastava et al., 2015). In addition, T-cell exhaustion as previously described may be the culprit in why the DNMTi-treated tumour cells are not activating CD8+ T cells it has been seen that DNMTi can work by avoiding the onset of exhaustion and reprogramming exhausted CTLs into effector phenotypes (Wherry and Kurachi, 2015).

Further research is necessary to understand how dosing and scheduling of these drugs in the clinical setting will modulate the immune response. It is important to mention that there is a counter balance in which epigenetic therapy can actually be immunosuppressive. As witnessed in in MDS and AML patients, the upregulation of PD1 in T cells by DNMTi has been shown to limit epigenetics therapy efficacy (Sun et al., 2018). Therefore, further work is required to classify the most effective approached to induce pro-immunogenic as opposed to immunosuppressive features.

Additionally, the TME can be classified as 3 states, firstly, inflamed state whereby T cells are actively participating & killing tumour cells, secondly, immune-excluded state whereby T cells cannot interact with tumour cells and lastly the immune-cold state in which T cells are absent. The inflamed state is linked with Th1 response which is a lineage of CD4+ helper cells (Spranger, 2016). In cancer, they promote cell-mediated immune responses for host defence against viral pathogens. Additionally, they are also a source of IFN-γ and an effective component within anti-tumour immunity. Their specific function has been shown to be supressed epigenetically in some models (Dobrzanski, 2013). However, a study conducted by *Tumes et al., 2013* , has shown using DNMTi stimulates an effector CD4+ response, specially a Th1 response. This was not seen in the data in *figure 3. 7B* in which values comparing vehicle and DNMTi-treated cells were not significantly different, possibly due to high variability or T-cell exhaustion , which is less likely as it is usually restricted to CD8+ subtype, however has been previously observed in certain chronic infections (Saeidi et al., 2018).

Experimental limitations

This study included investigating DNMTi-induced ERV expression in OC cell lines, characterised by the infection of cell free RNA. Ultimately, a higher number of repeats than was likely with the resources and time available for this project would be required to obtain consistent reproducible results. Alternately, detecting cell free ERVs using an ELISA would be possible in the future, although it is expensive and time-consuming, as well as not widely used in current papers. The main limitation in detecting cell free ERVs was examining if the dsRNA release into supernatant was indeed ERVs. In current

literature, ERVs are established to be within the nucleus and only reactivated and expressed as dsRNA within the nucleus. Issues surrounding its stability out of the cell, as RNA is highly unstable, as well as the mechanism it uses to leave the cell remains unknown and requires further work. Future experiment will need to focus on finding a solution for the high variability observed in the housekeeping gene, PPIA, which has been shown in literature to be very stable in OC cell lines. Although, it was mentioned that the incorrect dilution of RNA and even the treatment conditions themselves are sufficient to cause variable CT values. In addition, due to resource restraints, RNA integrity was not measured so the quality of RNA was not established, potentially causing variability among replicates. To increase RNA integrity in the future, treating RNA with DNase treatment may increase RNA isolation, although ThermoFisher Scientific states the isolation kits are efficient and do not usually require DNase treatment. However, using samples in qPCR very small or no introns require removal of potentially contaminating DNA. A260/A280 ratio indicates the level of protein contamination. Acceptable ratio for RNA is 1. 8-2. 0, however some of the samples had values <1. 8 or > 2. 0.

Further work is required in establishing the role of cell free RNA. In future experiments, this method needs to be optimised in order to produce values with higher CT values. IFN-γ was measured here as a T cell activation marker, although IFN-γ is also released by NK cells and does not only reflect T cell activation. Further, there was high variability in γδ T cell and PBMC numbers in each sample due to technical errors, attributing to diverse values during flow cytometric analysis. Due to a few controls having a low cell

count, it was difficult to set the gating for analysis for CD3+ cells which may have influenced the results for CD8+CD25+ and CD4+CD25+, as they were inconclusive and no significant effect was observed between the vehicle and DNMTi-treated tumour cells, and between PBMCs and γδ T cells. If there was more time available for this project, repeating it with caution would most likely produce consistent data.

Conclusion

The data seen in an ensemble of studies show that DNMTi can work synergistically with immunotherapies by acting on both the tumour cells and immune cells to enhance anti-tumour immune responses thus increasing immunogenicity and hopefully increases overall survival in OC patients. Epigenetics has been an area of focus in which inhibition of methylation in tumour suppressor genes by treating OC cells with DNMTi causes the activation of ERVs leading to an innate IFN response, increasing visibility of the tumour for immune surveillance. The data in this project has suggested that following DNMTi epigenetic treatment, cell free dsRNA is detected in the supernatant, possibly HERVs and may potentially cause the induction of type 1 IFN signalling. This data provides a promising direction in which the established mechanism of viral mimicry proposed by Jones et al., 2019, may not be the only mechanism of action of these therapeutic ERVs. This project also opened up the possibility of the cell free dsRNA interacting with tumour and immune cells within the TME, a potential new avenue to target in order to increase immunogenicity and thus the efficacy of combinatorial epigenetic and immunotherapy strategies in OC patients.

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