ANNEXURE I

Tumor cells exhibit the property of immune evasion (Hanahan and Weinberg, 2000). One of the immune evasion strategies manifested by malignant cells is the downregulation of the Human Leukocyte Antigen (HLA). HLA Class I (HLA- A, -B, -C) present endogenous peptides including viral and tumor antigens to cytotoxic T lymphocytes for immune mediated destruction. Studies were carried out to determine the effect of EBV latency genes on HLA ABC expression which indicated the EBV LMP2A and not EBERs to be responsible for this HLA downregulation in gastric cancer cells. Our results further indicated the Sonic Hedgehog (Shh) pathway; primarily Gli1 to bring about the LMP2A mediated decrease in HLA expression. A detailed description of the results obtained is discussed below:

1. **EBV LMP2A decreases HLA ABC expression.**

Having known that EBV positive Korean gastric cancer cells do decrease HLA ABC expression [12], we next proceeded to determine the EBV latent gene which may be bringing about this effect. We targeted the two main genes detected in most gastric cancer samples viz; LMP2A and EBERs. Therefore, EBV negative gastric cancer cells AGS stably transfected with LMP2A, EBERs or doubly transfected with LMP2AEBERs were analysed for HLA ABC expression compared to AGS control cells. It was observed that while AGS-LMP2A and AGS-LMP2AEBERs displayed a decreased HLA class I expression, AGS-EBERs showed an expression similar to control cells (ANNEXURE 1.1.A). While SNU719, AGS-LMP2A and AGS-LMP2AEBERs show a median value of 3.43±0.74, 11.97±1.25, 8.82±0.2, AGS-EBERs shows a median of 23.71±2 compared to AGS control cells that display a median of 25.71 for HLA ABC expression (ANNEXURE 1.1.A). Furthermore RT-PCR analysis was performed to determine mRNA expression of HLA A, HLA B and HLA C and also β2 microglobulin in AGS-
LMP2A compared to control cells. HLA A, B and C showed a decreased mRNA expression in LMP2A expressing cells relative to AGS control (ANNEXURE 1.1.B). In order to further strengthen the fact that EBV LMP2A is indeed bringing about the HLA class I downregulation, LMP2A was subjected to time dependent siRNA mediated knockdown in gastric cancer cells SNU719 that constitutively express LMP2A. Transfection of LMP2A siRNA caused a time dependent increase in HLA ABC protein expression as well as in the individual HLA class I gene in SNU719 cells (ANNEXURE 1.1.C and 1.1.D).

1. 2: LMP2A activates the Sonic Hedgehog pathway.
EBV-LMP2A is known to activate the Sonic Hedgehog pathway in Nasopharyngeal carcinoma which imposes stem-like characteristics to these cells. Because immune evasion is one of the properties often associated with cancer stem cells [150, 151], we wanted to find out whether the Hedgehog pathway may also contribute to HLA class I downregulation. Sonic Hedgehog pathway activation in LMP2A expressing gastric cancer cells (SNU719 and AGS-LMP2A) was evaluated by determining the gene expression levels of Sonic Hedgehog genes in these cells compared to their respective vector controls. It was observed that LMP2A significantly upregulates the mRNA expression of Sonic Hedgehog (SHH), Patched (Ptc), Smootherned-1(Smo-1) and Gli1. Gli3 shows a decreased expression in AGS-LMP2A and SNU719 relative to AGS control cells (Annexure 1.2.A). Immunoblotting experiments also reveal an increased Gli1 and decreased Gli3 expression in LMP2A expressing cells (Annexure 1.2.B). To further confirm the Hedgehog pathway activation in AGS-LMP2A and SNU719, the transcript level of Gli1 and Gli3 was determined at 24, 48 and 72hrs post LMP2A knockdown. Indeed LMP2A siRNA decreases Shh, Ptc, Smo-1, Gli1 and increases Gli3 expression in a time dependent manner (Annexure 1.2.C).
1.1): EBV LMP2A decreases HLA ABC expression. (A) Flow cytometric analysis of protein expression of HLA ABC in SNU719, AGS-LMP2A, AGS-EBERs and AGS-LMP2AEBERs compared to AGS control cells. Quantitation of results performed by measuring median values using Cell Quest Pro software. (B) RT-PCR estimation of HLA A, HLA B, HLA C and β2 Microglobulin in SNU719 and AGS-LMP2A.(C) Calculation of median values representing levels of HLA ABC upon treatment of SNU719 cells with 20nM of LMP2A siRNA for 24, 48 and 72 hrs. histogram plot indicating increased HLA ABC expression upon siRNA mediated knockdown of LMP2A in SNU719 at 72 hrs is represented.(D) Quantitative Real Time PCR to determine relative transcript level expression of HLA A, B and C upon siRNA mediated inhibition of LMP2A in SNU719. Data represents an average of n=3 independent experiments. *, ** and *** denote P value <.05, <0.005 and < 0.001 respectively.
1.3: Decrease in HLA ABC expression upon LMP2A expression is via the Hedgehog pathway and is Gli1 dependent.

In order to estimate whether the Hedgehog pathway may also contribute to HLA class I downregulation, LMP2A expressing gastric cancer cells (SNU719 and AGS-LMP2A) were treated with hedgehog pathway inhibitor Forskolin. Forskolin increased HLA ABC expression in a dose and time dependent manner in both SNU719 (Annexure 1.3.A) and AGS-LMP2A (Annexure 1.3.B). Transcript expression of HLA A, B and C also showed increased expression when subjected to Forskolin treatment (Annexure 1.3.A and B). LMP2A has also been shown to activate other self renewal pathways such as the Notch pathway and the Wnt/β catenin pathway in epithelial cells [40, 44]. Interestingly, treatment of AGS LMP2A with Notch pathway inhibitor γ-secretase inhibitor (GSI) and Wnt/β catenin pathway inhibitor PNU74654 showed no indicative increase in HLA ABC expression in both SNU719 cells (Annexure 1.3.C) and AGS LMP2A cells (Annexure 1.3.D) thereby indicating the specificity of the Sonic Hedgehog pathway.
pathway in HLA Class Ia downregulation. The Hedgehog pathway activates downstream genes via its transcriptional activator Gli1. Silencing of Gli1 in LMP2A expressing cells using siRNA mediated approach showed an increased HLA ABC expression at both the protein and the RNA levels in SNU719 (Annexure 1.3.E) as well as in AGS-LMP2A cells (Annexure 1.3.F). Moreover silencing Gli3, another downstream mediator of the hedgehog pathway showed no significant change in HLA ABC expression both at the protein and the transcript levels (Annexure 1.3.E and F). Hence HLA ABC downregulation in LMP2A expressing gastric cancer cells is Gli1 dependent.

1.4: **LMP2A mediates Gli1 dependent decrease in Class Ia Transcription Factors.**

Literature highlights several Transcriptional Factors (TRFs) such as NFκB, CIITA and RFX5 to bind the HLA Class Ia promoter [152, 153] and thereby regulate its expression. We therefore proceeded to determine the expression levels of these TRFs in LMP2A expressing gastric cancer cells. Our results showed a decreased protein expression of NFκB, CIITA and RFX5 in LMP2A expressing gastric cancer cells SNU719 (figure 4A) and AGS LMP2A (figure 4B). Interestingly, treatment of SNU719 and AGS LMP2A cells with Forskolin and Gli1 siRNA increased the expression NFκB, CIITA and RFX5 proteins compared to untreated cells (figure 4C and 4D). Therefore, LMP2A mediated Gli1 dependent reduction in expression of HLA Class Ia TRFs may be responsible for a decreased HLA ABC expression and thereby an increased capacity of immune evasion in these cells.
1.3) Decrease in HLA ABC expression upon LMP2A expression is via the Hedgehog pathway and is Gli1 dependent: (A) Dose and time response of HLA ABC expression when SNU719 cells are treated with Forskolin compared to untreated cells. Flow cytometric analysis of SNU719 cells treated with Forskolin (30μM, 48 hrs). Quantitative RT-PCR of HLA -A, -B and -C mRNA expression of SNU719+Forskolin relative to untreated SNU719. (B) Dose and time response of HLA ABC expression when AGS-LMP2A cells is treated with Forskolin compared to untreated cells. Flow cytometric analysis of AGS-LMP2A cells treated with Forskolin (30μM, 48 hrs). Quantitative RT-PCR to determine the transcript expression of HLA -A, -B and -C in AGS-LMP2A+Forskolin cells relative to untreated AGS-LMP2A. (C) HLA ABC expression upon treating SNU719 cells with GSI and PNU74654. (D) Determination of HLA ABC expression when AGS LMP2A cells were subjected to GSI and PNU74654 treatment. (E) Time dependent response of HLA ABC expression when SNU719 cells are treated with 20nM Gli1/Gli3 siRNA relative to untreated cells. Flow cytometric analysis of SNU719 cells treated with Gli1//Gli3 siRNA for 72 hrs. Quantitative RT-PCR of HLA -A, -B and -C mRNA expression in SNU719+Gli1/Gli3 siRNA relative to untreated SNU719. (F) Time dependent response of HLA ABC expression when AGS-LMP2A cells are treated with Gli1/Gli3 siRNA relative to untreated cells. Flow cytometric analysis of AGS-LMP2A cells treated with Gli1/Gli3 siRNA.

1.4): LMP2A mediates Gli1 dependent decrease in Class Ia Transcription Factors. (A-B) Decreased protein expression of NFκB, CIITA and RFX5 in LMP2A expressing gastric cancer cells SNU719 (A) and AGS LMP2A (B). (C-D) Expression of NFκB, CIITA and RFX5 protein upon treating SNU719 (C) and AGS LMP2A (D) cells with Forskolin and Gli1 siRNA. All of the above data shown is an average of n=3 independent experiments. *, ** and *** denote P value <0.05, <0.005 and <0.001 respectively.