fibers, co-exposure resulted the formation of abundant stress fibers (Fig 1. Phalloidin). The loss of E-Cad and ZO-1 with co-exposure was verified by western blot (Fig 1B). Additionally, an increase in the level of secreted MMP2 and MMP9 were detected in the conditioned medium of both LMP1 and TGF-β1 treated cells. The level of activated MMP9 was dramatically upregulated in the conditioned medium of co-exposed cells (Fig 1C).

Co-exposure to low dose TGF-β1 and LMP1 induced transcriptome changes A549 cells.

To determine if the loss of E-Cad and gain of MMP9 proteins were due to transcriptional regulation, mRNA was harvested at 24 hours post treatment and analyzed by qRT^2-PCR. TGF-β1 treatment resulted in a loss of E-Cad transcription while expression of LMP1 did not. Co-exposure resulted in a twofold loss in E-Cad mRNA level when compared to TGF-β1 exposure alone (Fig 2A). Similarly, while exposure to TGF-β1 or LMP1 increased MMP9 mRNA levels by 80-fold and 40-fold respectively, co-exposure resulted in an increase of 3400-fold (Fig 2C). The expression of PAI-1 increased with exposure to TGF-β1 or LMP1 independently and showed an additive increased with co-exposure (Fig 2B).

LMP1 enhanced cellular mobility in response to TGF-β1-induced EMT

The ability of LMP1 and TGF-β1 co-treatment to affect cell migration was assessed utilizing the FluoroBlok migration assay (Fig. 2D). Treatment with TGF-β1 alone increased migration by ~2 fold whereas LMP1 expression increased migration ~3 fold. Co-treatment induced a synergistic effect, increasing cell migration by ~ 9 fold.

Co-exposure to low dose TGF-β1 and LMP1 induced a gain of mesenchymal markers in A549 cells.

To determine if the loss of epithelial markers in A549 cells exposed to TGF-β1 and LMP1 was accompanied by a gain in mesenchymal markers, cells were exposed for 4 days and analyzed by western blot (Fig 2E). Exposure to TGF-β1 resulted in a gain in the mesenchymal markers N-Cadherin (Fig. 2G) and vimentin (Fig. 2H) as well as the fibrotic market fibronectin–EDA (Fn-EDA). Co-exposure with LMP1 resulted in an additive increase in both N-Cadherin and vimentin while the presence of LMP1 increased induction of Fn-EDA by TGF-β1 more than two-fold (Fig. 2F).

Immortalized primary cell response to LMP1 and TGF-β1 mirrored that of A549 cells.

To determine whether the phenotypic changes seen in A549 cells were cancer cell specific, the experiments were repeated using the immortalized primary cell line HPL1D. Consistent with the results seen in A549 cells, levels of ZO-1 and E-Cad were reduced in HPL1D cells exposed to LMP1 or TGF-β1 independently with a complete loss in co-exposed cells (Fig 3A) at 48 hours post treatment. Levels of Fn-EDA were increased with TGF-β1 treatment, which was enhanced by co-exposure. At the transcription level, treatment with TGF-β1 resulted in suppression of E-Cad that was synergistically enhanced by LMP1 expression (Fig 3B), while the increase in transcription of PAI-1 was found to be TGF-β1 dependent in HPL1D cells.

To determine the extent to which HPL1D cells might complete the transition to a mesenchymal phenotype, cells were co-exposed for 7 days. Co-treatment with TGF-β1 and LMP1 resulted in an increase the mesenchymal markers aSMA and N-Cad proteins (Fig 3D). Gelatin zymography analysis displayed a substantial increase in MMP9 expression with co-treatment (Fig 3E). Additionally, co-treatment with TGF-β1 and LMP1 resulted in the synergistic

upregulation of MMP9 mRNA similar to that seen in A549 cells (Fig 3I).

At the transcription level, 7 day exposure to TGF-β1 or LMP1 alone resulted in a loss of expression of the epithelial types II (AECII) marker surfactant protein-C (SP-C) while co-exposure resulted in an additive loss (Fig. 3F). Expression of the mesenchymal marker fibronectin (Fig. 3G) as well as the fibrotic markers Fn-EDA (Fig. 3H) and Collagen 1 (Fig. 3K) were increased by TGF-β1 treatment and enhanced with co-treatment with LMP1.

Effects of LMP1 expressed from the EBV genome mirrored those of LMP1 transient transfection and retroviral transduction in a dose dependent manner.

To investigate the effects of LMP1 expressed at biological levels and in the context of co-expression with the EBV latent proteome, A549 cells were infected with the recombinant EBV strain BX. Expression levels of LMP1 was determined by western blot (Fig. 3L) and cells from two separate infections differentially expressing LMP1 were exposed to 1 ng/ml TGF-β1 for 48 hours. EBV infected cells expressing LMP1 at low levels (2C) responded similarly to uninfected parental A549 cells (U) with equally lower E-Cad expression and increased expression of vimentin. TGF-b1 treatment of cells expressing a greater amount of LMP1(3A) resulted in a greater loss of E-Cad and gain in vimentin when compared to either the 2C cell line or uninfected parental cells (Fig. 3M).

LMP1 inhibited TGF-β1 activation of the Smad pathway

The canonical TGF-β1 signaling cascade involves phosphorylation of Smad2 and/or Smad3 by the TGF-β1 receptor [18]. To investigate the role of Smad phosphorylation in the phenotypic change seen with TGF-β1 and LMP1co-treatment, cells were transiently transfected with either the LMP1 expression or empty vector and exposed to 1 ng/ml TGF-β1 and the levels of phosphorylated Smad were assessed by western blot (Fig 4A). Exposure to TGF-β1 resulted in a robust increase in Smad 2 phosphorylation as early as 10 minutes that peaked at 60 minutes and diminished thereafter, while Smad 3 phosphorylation peaked at 30 min and was less robust. With co-exposure, however, Smad 2 phosphorylation peaked at 30 minutes at a greatly reduced level compared to TGF-β1 exposure alone. Smad3 phosphorylation was reduced with co-exposure and peaked at 60 minutes. To determine whether the reduction in Smad phosphorylation reduced expression of a Smad responsive gene, the Smad responsive, but LMP1 unresponsive, SMAD7 gene was analyzed by qRT^2-PCR. Expression of LMP1 alone did not affect expression of Smad7 while exposure to TGF-β1 increased expression of Smad7 by 2-fold at 24 hours in A549 cells (Fig 4B) and 12-fold in at 4 days in HPL1D cells (Fig 4C). Expression of LMP1 greatly reduced the TGF-β1 induced increase in Smad7 in both A549 and HPL1D cells.

The cytoplasmic isoform of the promyelocytic leukemia protein (cPML) has been shown to be essential for TGF-β1 signaling [19]. The levels of cPML were analyzed in A549 cells retrovirally transduced with either the empty vector or 2 separate transductions differentially expressing LMP1 (Fig. 4D). LMP1 was found to reduce the level of cPML in a dose responsive manner. To investigate whether the reduction in cPML affected the cPML/SMAD/SARA signaling complex, immunoprecipitation experiments were conducted (Fig 4E). Immunoprecipitation of SARA displayed a reduction in interaction between SARA and cPML in the presence LMP1.

LMP1 expression reduced expression of Twist but did not affect expression Snail, Slug or Zeb-1.

To investigate whether LMP1 alters expression of the classical E-Box binding inducers of EMT, Snail, Slug and ZEB1 expression were analyzed by qRT^2-PCR (Fig 5A). Treatment with TGF-β1 resulted in increased expression of all 3 gene products though expression was unaffected by LMP1 alone or when LMP1 was combined with TGF-β1. LMP1 has been reported to induce EMT in MDCK cells through the increased expression of Twist [14]. Cells were transiently transfected with either the LMP1 expression or empty vector and co-treated with TGF-β1 for 1, 2, and 3 days and Twist expression was analyzed by western blot analysis to investigate whether LMP1 affected the expression of Twist in A549 cells (Fig. 5B). While TGF-β1 exposure did not affect the expression of Twist at any time point, LMP1 reduced Twist protein in a time dependent manner. Stable expression of LMP1 by retroviral transduction resulted in a complete loss of Twist protein.

LMP1 enhanceed TGF-β1 activation of ERK pathway

Activation of the ERK pathway has been reported to be essential for LMP1 induced EMT [11]. To investigate the possible co-operative activation of the ERK pathway by LMP1 and TGF-β1, cells were transiently transfected with either the LMP1 expression or empty vector and co-treated with TGF-β1. ERK phosphorylation was determined by western blot (Fig 6A, B). LMP1 expression increased ERK phosphorylation through the time course of the study. TGF-β1 exposure increased ERK phosphorylation as early as 15 minutes, peaked at 30 minutes, and remained above baseline through 48 hours. With co-treatment, ERK activation increased at 15 minutes, and when compared to LMP1 expression alone and was significantly increased at 15 and 30 minutes. Through 72 hours, co-treatment increased phosphorylation of p42 though the increase was subtle as determined by densitometric analysis (Fig S2).

Inhibition of ERK phosphorylation protected from LMP1 and TGF-β1 induced EMT

To investigate whether activation of the ERK pathway was essential to TGF-β1 and/or LMP1 induced EMT, the small molecule inhibitor of ERK phosphorylation, U0126 was utilized. A dose of 50μM U0126 was required to inhibit phosphorylation of ERK by either TGF-β1 or LMP1 alone, though this concentration did not completely abrogate ERK phosphorylation in response to co-exposure (Fig 6C). The reduction in ERK phosphorylation resulted in concomitant protection from loss of E-Cad expression. U0126 treatment blocked the TGF-β1 or LMP1 loss of expression but was insufficient to protect from the loss expression by co-treatment. Inhibition of ERK phosphorylation however was sufficient to protected against the TGF-β1 induced increase in expression of MMP2 and MMP9, while the upregulation of MMP9 with co-exposure was greatly diminished (Fig D, G). Inhibition of ERK phosphorylation prevented the loss of E-Cad expression associated with TGF-β1 exposure and TGF-β1/LMP1 co-exposure (Fig. 6E) though expression of Snail and Slug was not completely reduced to baseline (Fig. 6H, I). Expression of PAI-1 was unaffected by Inhibition of ERK phosphorylation (Fig.6F).

Inhibition of NF-κB activation protected against LMP1 induced EMT

To assess the relative contribution of NF-κB pathway activation in TGF-β1 and LMP1 induced EMT, LMP1 was co-expressed with a dominant active IκB construct (ΔAIκB). Inhibition of NF-κB activation had no effect on E-Cad expression with TGF-β1 or LMP1 individually or with co-exposure. The LMP1 effect of upregulating MMP9 and PAI-1,

alone or with TGF-β1co-exposure, was mitigated to either baseline or to that of TGF-β1 exposure alone (Fig. 7A, B, and C). The inhibition of NF-κB activation was verified by an NF-κB responsive luciferase assay (Fig. 13E). Expression of the ΔAIκB abrogated luciferase activity at baseline as well as with TNF-α stimulation.

Discussion

The clinical decline in patients afflicted with IPF is variable and may conceptually be modified by the lung microbiome. The model of epithelial injury and disregulated repair provides a central mechanism on how viral infection may modulate lung fibrogenesis [3,33]. The reported presence of Herpeviridae in the type II alveolar epithelial cells of patients with IPF suggests a role for herpes viral infection in the progression of IPF. The concept of epithelial cell damage by viral lytic phase replication has already been explored [2], but little is known regarding the effects of stable latent viral infection on the behavior of lung epithelial cells. Our data provides a molecular mechanism through which the presence of LMP1 in the lung epithelium predisposes cells to undergo EMT by enhancing signaling through the ERK pathway.

Treatment of cells expressing LMP1 with low dose TGF-β1 resulted in morphological changes, a loss of epithelial markers, a gain of mesenchymal markers, enhanced motility and increased expression of fibrotic markers. Co-treatment with TGF-β1 and LMP1 resulted in a disruption of adherent and tight junctions with loss of expression of E-Cad and ZO-1 as well as the loss of the type II pneumocyte marker SP-C. Subsequently, a gain in the mesenchymal markers N-Cad, vimentin and fibronectin was observed as well as an increase in expression of PAI1 and MMP9, and the formation of stress fibers. These data demonstrate a completion of the transition from epithelial phenotype to mesenchymal phenotype. Additionally, the induction of fibrotic markers, such as aSMA, Fn-EDA and Collagen 1 support a fibrogenic phenotype.

The predominant model of EBV infected lung epithelium utilized in this study involved A549 cells transiently transfected with an LMP1 expression plasmid. LMP1 was expressed in the primary cell line HPL1D to validate this model. Expression of LMP1 with and without treatment with TGF-β1 in HPL1D cells mirrored that of LMP1 expression in A549 cells, though the response was less robust. This is consistent with the publish report of comparative TGF-β1 responsiveness in the HPL1D cell line [34]. To validate both the expression levels and function of LMP1 in the context of the EBV virus, A549 cells were infected with the BX-1 strain of EBV and exposed to TGF-β1. The EBV infected cells mimicked the LMP1 positive A549 cells in the response to TGF-β1 treatment with loss of E-Cadherin and increase in Vimentin expression in an LMP1 dose dependent manner. These data support the expression of LMP1 in A549 cells as an appropriate model of LMP1 positive EBV latency in lung epithelial cells in the context of TGF-β1 induced EMT. Though LMP1 and TGF-β1 treatment independently induce EMT at higher doses (TGF-β1) or longer time points (TGF-β1, LMP1), our goal was to investigate the possible cooperative effect of LMP1 and TGF-β1 in fostering EMT. The capacity of TGF-β1 to induce EMT [22] and the presence of EMT in IPF [21] have been reported. In this study, 1 ng/ml TGF-β1 treatment was insufficient to fully induce EMT in A549 cell within 72 hours as judged by morphological changes and loss of the epithelial cell marker E-Cad. LMP1 has

been reported to induce the formation of stress fibers [13] and EMT in epithelial cells [12,14]. In transient transfection experiments, expression of LMP1 was titrated so as not to fully induce EMT, though at longer time points where cells were retrovirally transduced and expressing very large amounts of LMP1, LMP1 did independently induce EMT, consistent with published reports.

In our model of latent EBV infection, LMP1 primes the cell for a TGF-β1 response. The dramatic change in cell morphology and protein expression in LMP1 positive cells treated with low dose TGF-β1 demonstrated a high level of synergy between LMP1 and TGF-β1. Phosphorylation and activation of the ERK pathway has been shown to be essential in TGF-β1 induced EMT in normal murine mammary gland epithelial cells and mouse cortical tubule epithelial cells [35], whilst LMP1 activation of the ERK pathway through the CTAR1 domain has been implicated in the regulation of cellular motility and invasion in a variety of epithelial cells [11]. In our system, LMP1 constitutively activated the ERK pathway and increased the activation of ERK in response to TGF-β1. In LMP1 positive cells, treatment with TGF-β1 resulted in more rapid and more robust ERK activation. This increase in ERK activation was maintained through the 72-hour time point. Inhibition of the ERK pathway resulted in protection from TGF-β1 induced EMT. Cells expressing LMP1 and pre-treated with the ERK specific inhibitor U0126 displayed protection from changes in morphology with TGF-β1 treatment (lab observation, not shown). Pre-treatment of LMP1 positive cells with U0126 resulted in complete protection from TGF-β1-induced loss of E-Cad mRNA and a 95% reduction in the induction of MMP9 mRNA. Inhibition of the ERK pathway reduced, but did not abrogate, the increase in PAI-1, Snail and Slug by TGF-β1. These data suggest that though ERK is a critical pathway for LMP1 and TGF-β1 to induce EMT in human lung epithelial cells, yet there are multiple pathways with varying contributions to EMT induction

A novel finding of our work is that the LMP1-mediated reduction of TGF-β1 induced Smad activation is attributable to the reduction in cytoplasmic PML and consequent disruption of the SARA/cPML/Smad activation complex. In this complex, cPML acts as a linking protein and has been shown to be essential to activation of Smad by TGF-β1 [19]. The reduction in cPML reduces the association of cPML and SARA and the resultant activation of Smad. Though TGF-β1 induced phosphorylation of Smad is reduced with LMP1 expression, the levels remained above that of cells not treated with TGF-β1. The Smad-dependent TGF-β1 upregulation of PAI-1 is involved in TGFβ–1-induced EMT and the progression of IPF, and was unaffected (HPL1D) or enhanced (A549) by the expression of LMP1, indicating that the Smad pathway remained at least partially activated.

Hypermethylation of the E-Cad (CDH1) gene promoter has been proposed as the mechanism by which LMP1 suppresses E-Cad [36]. Treatment with TGF-β1 promoted the loss of E-Cad through transcriptional regulation, whereas LMP1 reduced E-Cad expression at the protein level only. However, the TGF-β1 induced suppression of E-Cad transcription more than doubled when LMP1 was present. Both the TGF-β1 induced suppression of E-Cad transcription and the LMP1 regulation of E-Cad protein were found to be ERK dependent as demonstrated by experiments employing U0126. Thus, for this marker of EMT, two

separate mechanisms dependent on a central signaling pathway were involved. Although the mechanism of E-Cad suppression at the protein level by LMP1 was inconsequential, under co-treatment conditions LMP1 primes the cells for the TGF-β1 response.

MMP9 is upregulated in cells undergoing EMT [37]. Moreover, elevated MMP9 expression has been implicated in the pathogenesis of IPF [38] and is associated with a poorer prognosis [39]. Activation of the ERK pathway has been implicated in LMP1-induced upregulation of MMP9 [40] and analysis of the MMP9 promoter region has shown 2 active AP1 sites and 1 active NF-κB site [41]. Inhibition of NF-κB eliminated the LMP1 component, but had no affect on TGF-β1 induction of MMP9 at the transcription level; blocking NF-κB reduced the co-treatment induction to that of TGF-β1 treatment alone. Similarly, inhibition of ERK activation abrogated TGF-β1 induction of MMP9, but had little effect on the LMP1 induction of MMP9 transcription. The mechanism of MMP9 upregulation by LMP1 and TGF-β1 displays two separate signaling pathways converging at the promoter region of the MMP9 gene to synergistically increase transcription. The significance of this finding is that MMP9 preferentially degrades type IV collagen, a major component of the basement membrane, and in turn disruption of the basement membrane is permissive for fibrogenesis. Thus, the synergistic upregulation of MMP9 by TGF-β1 in LMP1 positive cells may be a mechanism by which latent EBV infection exacerbates fibrogenesis.

Attempts to characterize the LMP1 and TGF-β1 synergy in mouse (C10, MLE12) and rat (RLE12) lung epithelial cells were unsuccessful, and unfortunately preclude our ability to look at LMP-1 and TGF-β1 in a murine model of lung fibrosis. Though LMP1 was expressed in cells transiently transfected, retrovirally transduced or infected by LMP1 adenovirus expression vectors in murine cells, the proteomics explored were essentially unchanged when compared to the control vector. More significant to this study, the response to TGF-β1 by LMP1 positive mouse or rat lung epithelial cells was not significantly different from the TGF-β1 response of parental control cells. This species specific phenomenon is consistent with the genetics of the Epstein-Barr virus in comparison to the Murid Herpes Virus 4 (MHV4). Though MHV 4 has been utilized as a model for gamma-herpeviridae infection of the lung, this virus does not encode a protein homologous to LMP1 [42]. Thus, MHV4 can be utilized as a tool for study of the deleterious effects of lytic herpes viral replication and subsequent cellular damage in lung epithelium, but not for studying the molecular mechanisms of latent EBV infection of the lung. The lack of an LMP1 homologue suggests differential signaling in mouse verses human epithelium. Though expression of LMP1 in transgenic mouse lines targeted to the B-cells promotes lymphomas [43] and mimics CD40 signaling [44], similar to the expression seen in humans, expression of LMP1 in murine pulmonary epithelial cells is not analogous to the signaling characteristics of LMP1 expression in human lung epithelial cells and does not provide an appropriate model for this study.

The pathology of UIP in IPF has been well characterized though the exact molecular mechanisms are proving to be more elusive. This study provides evidence for a model of disease progression attributable to viral influences. As a consequence of Epstein-Barr virus in the lung epithelium and expression of LMP1, the cells are primed for

exuberant expression and activation of MMP9 as well as for a pro-EMT response to TGF-β1. This synergistic overall effect is independent of the TGF-β1 mediated upregulation of Snail and Slug, however, the majority of the pro-EMT effect is co-operative. The upregulation of PAI-1, N-Cad, aSMA, Collagen1 and Vimentin by TGF-β1 and LMP1 is additive whereas the upregulation of MMP9 and loss of E-Cad are synergistic. The net effect on treatment of lung epithelial cells expressing LMP1 with low dose TGF-β1 is an augmentation of EMT.

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Figure Legends

Figure 1. LMP1 enhanced the TGF-β1-induced phenotypic change and loss of epithelial markers in A549 cells. A) Phase contrast and immunofluorescence staining of A549 cells treated with 1ng/ml TGF-β1 for 48 hours with and without LMP1. Adherent junctions are imaged by E-Cad staining; tight junctions are imaged by ZO-1 staining and filamentous actin of stress fibers are imaged by phalloidin staining. B) Western blot analysis of A549 cell lysates collected at the indicated time points post transfection and co-treatment with 1ng/ml TGF-β1. D) Gelatin zymography of A549 conditioned media obtained 48 hours post treatment with 1ng/ml TGF-β1 with and without LMP1.

Figure 2. LMP1 enhanced the TGF-β1 induced gain of mesenchymal markers. Real-time qRT^2-PCR analysis of expression of A) E-Cadherin, B) PAI-1 and C) MMP9 at 24 hours post treatment with 1ng/ml TGF-β1 with and without LMP1 expression. D) Migration assay of TGF-β1 treated A549 cells with and without LMP1 expression. E) Western blot of A549 cell lysates collected 4 days post transfection and co-treatment with 1ng/ml TGF-β1. F-H) Densitometric analysis of 3 separate western blots of which figure 1E is representative.

Figure 3. The response to LMP1 and TGF-β1 treatment in primary cell line mirrored that of A549 cells. A) Western blot analysis of HPL1D cell lysates collected 96 hours after infection with adenovirus (MOI 20) expressing either GFP or LMP1 and 48 hours co-treatment with

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Figure 4. LMP1 inhibits TGF-β1 signaling through the Smad pathway. A) Western blot of cell lysates collected at specified time points after treatment with 1ng/ml TGF-β1 with and without LMP1 expression. RT2-PCR of Smad7 mRNA expression levels in B) A549 cells 24 hours post treatment with 1ng/ml TGF-β1and 72 hours post infection with either GFP or LMP1 expression adenovirus (MOI20) or C) HPL1D cells 48 hours post treatment with 1ng/ml TGF-β1 and 96 hours post infection with either GFP or LMP1 expression adenovirus (MOI20). D) Western blots of cell compartmental fractions from A549 cells retrovirally transduced with either the empty vector or 2 separate transductions with the LMP1 expression vector expressing different levels of LMP1. E) Western blot analysis of SARA immunoprecipitation reactions from retrovirally transduced cells detailed in Fig 4D.

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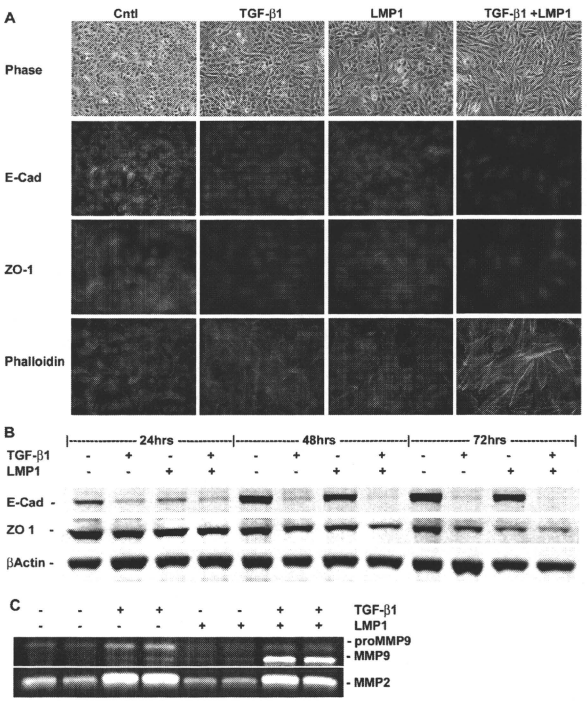

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C) Gelatin zymography of A549 conditioned media obtained 48 hours post treatment with 1ng/ml TGF-β1 with and without LMP1.

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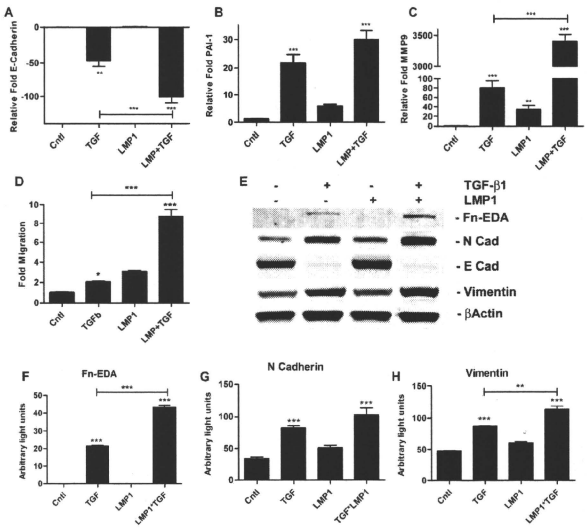

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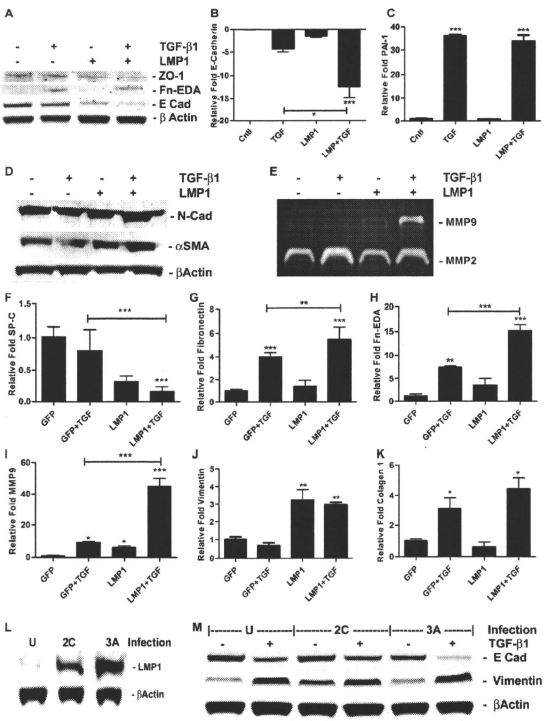

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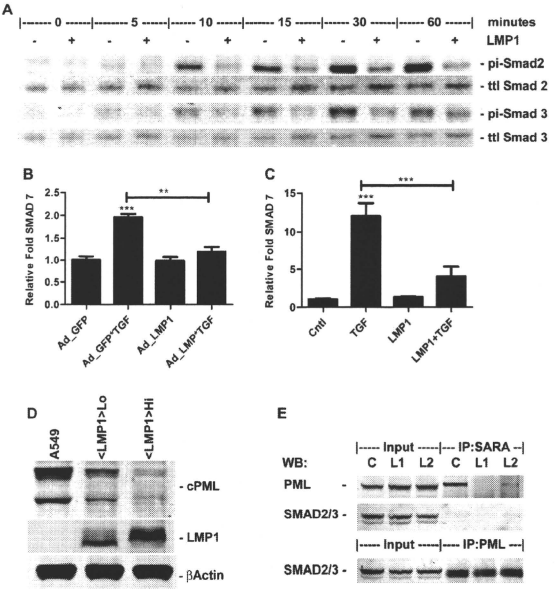

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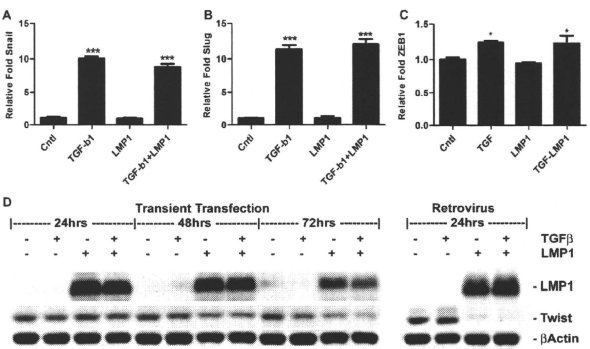

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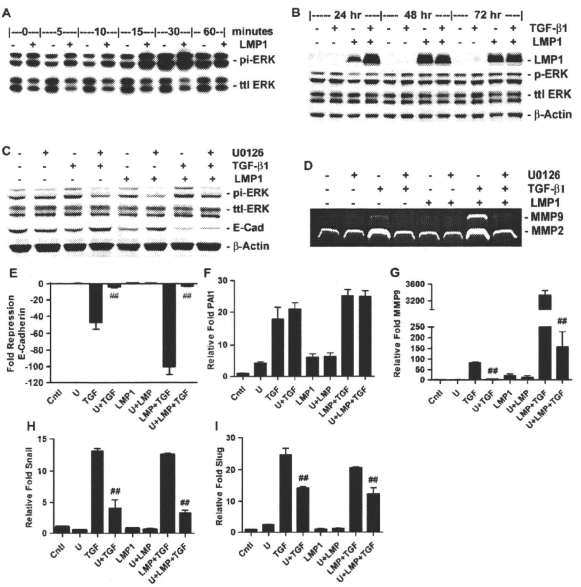

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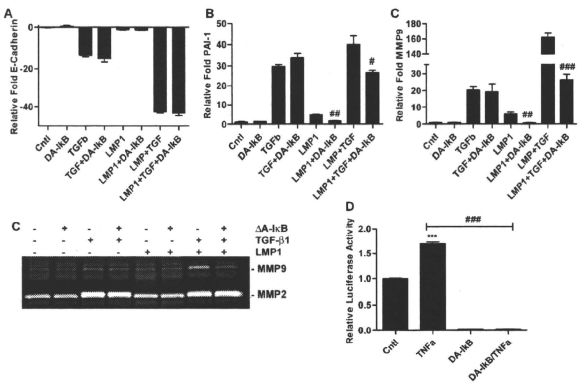

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Online Data Supplement

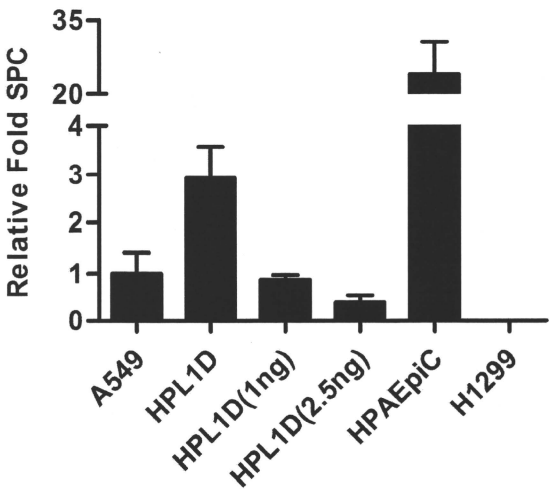
Mark D Sides[1,6], Ross C Klingsberg[1,6], Bin Shan[1], Kristin A Gordon[1], Hong T Nguyen[1], Zhen Lin [2,3,4], Takashi Takahashi[5], Erik K Flemington[2,3,4], Joseph A Lasky[1,7]*

Figure Legend

Figure S1. Comparison of expression of surfactant protein–C in cell lines utilized in this study. HPL1D cells were treated with the indicated amount of TGF-β1 for 24 hours and SP-C expression was analyzed by qRT2-PCR. SP-C levels were normalized to that of A549 expression. HPAEpiC cells are commercially available primary lung epithelial cells. H1299 cells were used a negative control.

Figure S2. LMP1 and TGF-β1 induce sustained ERK signaling in A549. Densitometric analysis of 6 separate western blots of which figure 6B is representative.

Figure S3. LMP1-TGF-β1 Signaling Map. TGF-β1 signals through both Smad and ERK. LMP1 signals through both NF-κB and ERK and inhibits TGF-β1 signaling through Smad. Signaling inhibitors are shown in black.



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