UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Characterization of TGF-beta regulation during chronic infection with LCMV

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by

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The thesis of Ellen Lee is approved and is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2009

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ABSTRACT OF THE THESIS

**Characterization of TGF-beta regulation during chronic infection with LCMV**

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Master of Science in Biology

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Professor Elina I. Zuñiga, Chair

Transforming growth factor-β (TGF-β) has recently been identified as a critical inhibitory cytokine involved in the suppression of cytotoxic T cells during chronic viral infection with lymphocytic choriomeningitis virus (LCMV). Here, we provide further clarifications of its gene expression, protein production, activation, and signaling in the context of chronic infection. Our

investigations suggest TGF-β protein expression and bioavailability to cells is comparable during acute and chronic infection. However only chronically infected mice exhibit sustained TGF-β bioactivity and protein levels at day 30-31 post infection. This prolonged expression suggests the cytokine may play an important role in viral persistence. We further provide evidence highlighting upregulation of TGF-β gene expression in dendritic cells compared to other leukocyte populations during LCMV infection. More notably, we identify significant enhancements of uPAR, a key activator of TGF-β bioactivity, gene expression in DCs uniquely during chronic infection. These findings suggest TGF-β may be preferentially expressed and activated by chronically infected DCs through a distinctive pathway that can contribute to viral persistence. Altogether, our findings help to characterize TGF-β specifically in an immunosuppressive environment induced by chronic viral infections *in vivo*.

# INTRODUCTION

*Chronic viral infections are global health concerns*

Although there have been significant biomedical advancements in controlling chronic viral infections, microorganisms have evolved sophisticated mechanisms to subvert the immune response, and continue to affect hundreds of millions of people (1). The extent and severity of their impact around the world indicates that chronic viruses, in particular human immunodeficiency virus (HIV) and Hepatitis C virus (HCV), are global health concerns that must be addressed.

Several factors are critical in determining a virus's ability to persist in a host including the range of cells it can infect, the rate at which it replicates and perhaps most interestingly, it's ability to avoid, exhaust and deplete the host's immune responses (2). Several viruses, including HIV and HCV, have been shown to induce defective anti-viral CD8 T cell responses, causing diminished cytotoxic capacity and an inability to produce antiviral cytokines (3, 4). Importantly key inhibitory pathways of the immune system, including the programmed cell death-1 (PD-1/PD-L1) and interleukin-10 (IL-10/IL-10R) pathways, have been identified as partly responsible for the dysregulation of critical pathways necessary for proper clearance during chronic viral infection (5-7). This highlights the importance of advancing our knowledge of the

molecular factors that determine whether viral infection results in an acute or chronic infection. Understanding these immune mechanisms may help develop novel therapeutic strategies.

*Lymphocytic choriomeningitis virus*

Our understanding of chronic viral infections *in vivo* is limited by the lack of appropriate small animal hosts for infection with human viruses such as HCV and HIV. However, an outstanding model for studying viral infection and its complex interrelationship with the host immune system is lymphocytic choriomeningitis virus (LCMV) infection of mice (8, 9). LCMV is a negative-stranded RNA virus and a natural pathogen of mice. It is the archetypal member of the *Arenaviridae* family, and studies of this system could help us understand other arenaviruses including Lassa Fever and South American Hemorrhagic Fever viruses, which cause severe disease in humans (10). A number of fundamental findings have been made using this system including MHC restriction of T cells and viral mutational escape from host cytotoxic T cell (CTL) responses (11).

Another advantage of utilizing LCMV is the availability of two strains that can cause either acute or chronic infections of adult mice (12). Systemic infection with the parental Armstrong (ARM) 53b strain of LCMV causes an acute infection that is efficiently cleared by cytotoxic T lymphoctyes (CTLs)

within days 7-10 p.i (13). In direct contrast, inoculation of adult mice with the Clone 13 (Cl 13) variant, which differs from ARM by only 2 amino acids, results in CD4 and CD8 T cell exhaustion and dysfunction, and viremia that persists for up to 2-3 months (4, 14-17). Comparison of these two viruses has the unique advantage that the mutations do not affect any of the known LCMV specific T cell epitopes, meaning immune responses in the two infections can be easily compared (12, 15). It was in this system that the importance of the CTL response in clearing chronic infection was first described and where the importance of specific immune pathways in persistence (eg. PD-1, IL-10, IL-21) has been discovered (5-7). Many of these findings have subsequently been translated into human pathogens such as HIV and HCV (18-22).

Therefore, LCMV offers a unique way to compare the molecular determinants responsible for a robust immune response leading to successful viral clearance versus an exhaustive immune response contributing to persistent viral infection.

*Transforming Growth Factor (TGF-β)*

Several inhibitory pathways have been identified as causing host cells to shut down antiviral immunes response during chronic infection (6, 7, 23). One such factor is transforming growth factor-β (TGF-β). TGF-β is a potent cytokine that exerts a variety of functions on multiple cell types (5, 24). It is

produced as three isoforms (TGF-β 1-3), however TGF-β1 is the one predominantly found in hematopoietic cells and the one that plays a crucial role in the immune system (25). Specifically it is important in cell differentiation, proliferation, homeostasis, self-tolerance, and survival (26-28).

One of its major inhibitory roles is evident in TGF-β1 deficient mice, which exhibit an autoimmune phenotype and die within 3-4 weeks of age of multi-organ failure (26, 29, 30). This phenotype has been associated with the direct role of TGF-β in suppressing autoreactive T cells and maintaining peripheral regulatory T cells (31). Furthermore, TGF-β has been implicated as a powerful regulator of T cells as exclusive ablation of this cytokine receptor in this cell type leads to hyperproliferation, activation, and differentiation of T cells. These mice ultimately suffer from autoimmune disease similarly to TGF-$\beta1^{-/-}$ mice (26, 30).

*TGF-β in LCMV*

The Zuniga lab have recently discovered that TGF-β is a critical negative regulator during LCMV infection, responsible for the apoptosis of virus-specific $CD8^+$ T cell and enhancing persistence of LCMV Cl 13 (23). This study utilized a system whereby TGF-β signaling was exclusively attenuated on $CD4^+$ and $CD8^+$ T cells by expression of a dominant negative form of TGF-β receptor II under a modified CD4 promoter (CD4-dnTGFβRII) (32). These

mice generated a strong virus-specific T cell response during Cl 13 infection that was dramatically greater than the wild type control. Moreover, TGF-β was found to play a direct role in upregulating the propapoptotic protein Bim during Cl 13, but not ARM, infection of wild type mice (23). Thus modifications of TGF-β signaling prove to produce significant phenotypic effects on the host and understanding its regulation in a chronic viral infection may help to harness this cytokine for immunotherapy.

*TGF-β Activation*

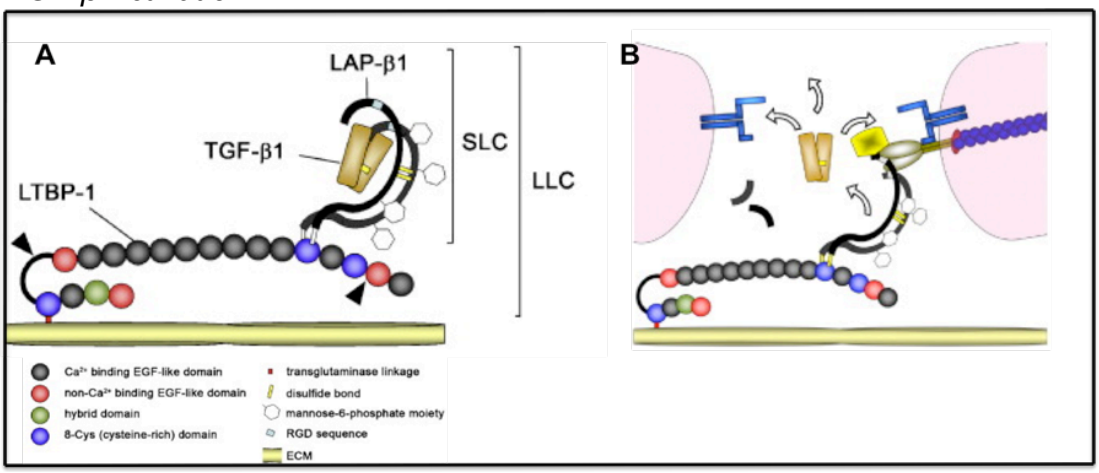


**Figure 1: Composition and Activation of TGF-β.** A) TGF-β associates with the latency associated protein (LAP) to form the small latent complex (SLC). This complex further binds to latent TGF-β-binding proteins (LTBP), which associates with extracellular matrix (ECM) proteins. B) Activation of TGF-β occurs when TGF-β is liberated from the SLC and LLC, which is achieved through proteolytic cleavage or mechanical disruption. TGF-β can freely diffuse, however it predominantly acts on a local autocrine or paracrine level due to strict localization of the activation process on the cell surface. Adapted from *Wipff et al., Eur J Cell Bio 2008*.

Due to the broad distribution of TGF-β and its receptors on various cell types, several regulatory mechanisms are enforced to regulate its production and signaling. TGF-β is synthesized in excessive amounts as an inactive molecule that forms the small latent complex (SLC) with the latent associated

protein (LAP) (33, 34). Latent TGF-β can be stored in various locations such as the extracellular matrix or the cell surface of immune cells. Thus, TGF-β activation, from the SLC, is a critical step in determining its ability to exert its biological effect on target cells (33). Conversion of latent TGF-β into its active form can be achieved by a variety of molecules depending on the cell type and physiological context (34, 35). Proteolytic cleavage is emerging to be the primary mechanism as numerous proteases such as plasmin, thrombin, elastase, and urokinase plasminogen activator system (uPA and uPAR) have been demonstrated to serve a direct role in TGF-β activation *in vitro* (36-39). Importantly, uPA binding to uPAR has been demonstrated to exert a negative regulatory effect on both acutely and chronically infected HIV cells (40). In addition, integrins can function as activators because they serve as docking points to bring TGF-β1 and proteases together for interaction (41, 42). This finding highlights the role of DCs in mediating TGF-β effects on T cell tolerance and development (42). Expression of integrin αvβ8 on DCs is of great interest as the loss of it leads to T-cell mediated multi-organ inflammation, similar to that observed in TGF-$\beta1^{-/-}$ mice (42). Interestingly, mice with uPAR deletion in T cells do not display the same phenotype as TGF-$\beta1^{-/-}$ mice, suggesting that it may be possible to control specific TGF-β1 activating factors, and enhance antiviral immunity, without inhibiting its beneficial effects on other cell types.

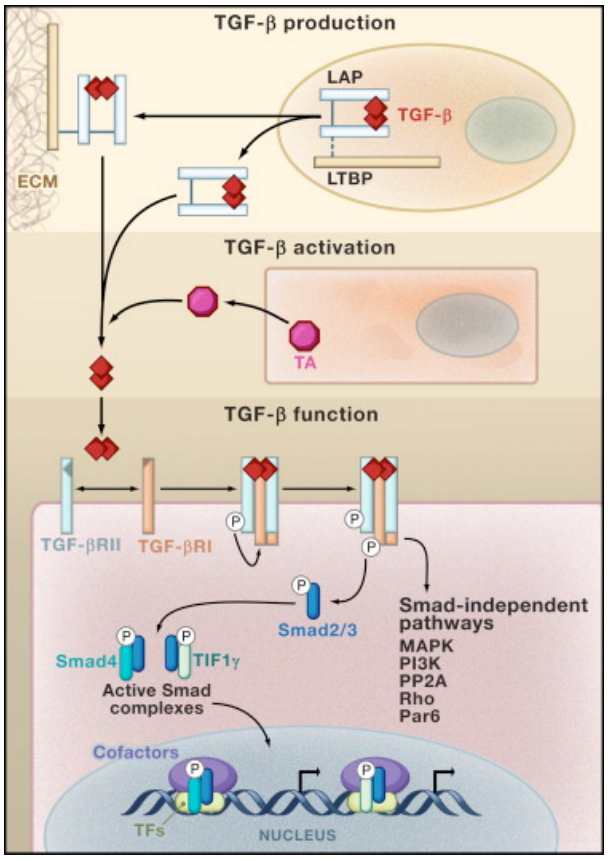


**Figure 2: TGF-β Cellular Regulation**
TGF-β is synthesized in an inactive form that associates with the latency-associated protein (LAP). This latent TGF-β can form a larger complex with latent-TGF-β-binding protein (LTBP). Interaction with TGF-β activating factors causes release of active TGF-β, which signals through TGF-β receptor II (TGF-βRII) and I (TGF-βRI) to begin a signaling cascade dependent on kinase activity of the receptors. Activated TGF-βRI phosphorylates the transcription factors Smad 2 and Smad 3, inducing translocation of Smad2/3 into the nucleus to combine with Smad 4. Smad complexes interact with additional transcription factors (TFs) and bind to regulatory sequences on target genes to regulate transcriptional activity. Adapted from Li et al., Cell 2008.

*TGF-β Signaling*

Once TGF-β is activated from the latent complex, the next step of regulation is through its signaling. TGF-β binds to a trans-membrane serine-threonine kinase receptor, TGF-β receptor II (TGF-βRII), which recruits TGF-β receptor I (TGF-βRI) to form a heterotetrameric receptor complex on the cell

surface (43). TGF-βRII then phosphorylates TGF-βRI kinase domain, which transmits the signal cascade by phosphorylating Smad proteins (44). The ensuing events are further regulated within the cell.

Downstream of TGF-β signaling, smad proteins are key players in transducing the receptor signals to target genes in the nucleus (43). They are organized into different classes with the three major ones being receptor-regulated Smad (R-smad), Co-mediator Smad (Co-smad), and the inhibitory Smad (I-smad) (44). R-Smad, include Smad 2 and Smad 3, which are unique in that they are the only ones to be phosphorylated by activated type I receptor kinases. They are of particular interest because only these respond to TGF-β subfamily signaling whereas the other R-smads respond to the bone morphogenetic protein (BMP) subfamily (44). Once phosphorylation occurs, Smad2/3 form heteromeric complexes with the Co-smad (Smad 4) and translocate into the nucleus to modulate target gene transcription with other nuclear co-factors (44). They encounter competition for Smad 4 interaction from I-Smad (Smad 6 and 7), which negatively regulate TGF-β signaling.

Control of these pathways and interactions is essential because dysregulation or mutations contribute to dysfunction of the immune system such as T-cell mediated self-reactivity (24). Mutation of Smad-2 phosphorylation sites has been found to block TGF-β-dependent gene

transcription (45, 46). Therefore, detection of phosphorylated Smad 2 (phospho-smad2) serves as a powerful indicator of TGF-β signaling on the cell. Furthermore, they serve as a direct link between the TGF-β receptor activation and transcriptional activity. Cell stimulation with TGF-β can lead to positive and negative expression of hundreds of genes. However it is likely that what determines Smad targeting of genes and recruitment of transcriptional co-factors is dependent on partner proteins specific for the cell-type or cell activation status (47). Therefore, it is also important to consider phospho-smad 2 production in particular cell subsets under specific conditions.

*Dendritic Cells*

A cell population of major interest is dendritic cells (DCs) because they provide a critical link between innate and adaptive immunity. DCs are crucial for innate recognition of invading pathogens because they mount the initial immune response with antiviral cytokine production. Detection for a wide array of microbial antigens is effectively achieved by its broad expression of pathogen recognition receptors including toll-like receptors (TLR) (48, 49). Furthermore, as potent antigen presenting cells (APCs), they determine the course of the adaptive immune response by directing the differentiation of naïve lymphocytes into effector cells (50). Due to their profound role in activating host immune response, pathogens have evolved ways to evade DC

detection and subvert their proper functions (51). For example vaccinia virus has been implicated in blocking TLR signaling which prevents DC maturation and causes immunosuppression of the host (52). Defective antigen presentation as seen in HIV can also lead to DC failure to stimulate T cells (53). Altogether, these mechanisms severely handicap the immune response during viral infections.

*Aims and objectives*

Considering the immunosuppressive and inhibitory role of TGF-β during LCMV Cl 13 infection, it is important to understand the cellular context in which TGF-β exerts detrimental effects by comparing its expression and activity in ARM and Cl 13 infections. We first investigated the total production of TGF-β in splenocytes during acute versus chronic infection. Next we sought to identify the relevant cellular source by isolating different cell populations and examining their mRNA expression of TGF-β. Finally we examined the levels of bioactive TGF-β available to the host during infection and the levels of signaling this induced on specific cell subsets.

## MATERIALS AND METHODS

*Mice and Viruses*

C57BL/6 mice or control littermates were used as WT controls. Mice were bred and maintained in a closed breeding facility and mouse handling conformed to the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of UCSD. Unless otherwise stated, mice (6–8 weeks old) were infected intravenously (i.v.) with $2 \times 10^6$ PFU of LCMV ARM or Cl 13. Viruses were grown, identified, and quantified as described (3, 54).

*SDS-PAGE and Immunoblotting*

Cells were lysed in a Ripa buffer (Thermo Scientific) containing protease and phosphatase inhibitors (Calbiochem). Protein homogenates were run on 4%–12% SDS-PAGE gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore) with a semidry transfer cell (Bio-Rad). Blots were blocked in blocking buffer (phosphate buffer saline [PBS] containing 0.1% tween-20 and 5% nonfat milk) and incubated with primary anti-pSmad-2, anti-Smad-2-3, anti-TGF-β1, or anti-phospholipase C γ (PLC-γ) mAbs (all from Cell Signaling; 1/1000 in blocking buffer) at 4°C overnight, or at room temperature for 2 hr. HRP-conjugated anti-rabbit IgG

(Cell Signaling; 1/5000 in blocking buffer) was then added for 45 min at room temperature and ECL (GE Healthcare) was used to visualize the proteins.

*Cell Purification*

Spleens were removed and incubated with collagenase D (1 mg/ml, Roche, Indianapolis, IN) for 20 min at 37°C. Single-cell suspension of splenocytes were enriched and purified as described previously (55). The following antibodies purchased from E-bioscience or BD-bioscience were used to stain spleen cells: anti-CD19-Percp-Cy5.5, anti-NK1.1-Percp-Cy5.5, anti-CD3- Percp-Cy5.5; anti-CD19-PE, anti-CD4-Percp-cy5.5, anti-CD8-FITC, anti-CD11b-Pe-Cy7).

*Flow Cytometry*

CD4 T cells (defined as $CD4^+$), CD8 T (defined as $CD8^+$) cells, B cells (defined as $CD19^+$), DCs (defined as $CD11c^+NK1.1^-CD19^-CD3^-$), and Macrophages (defined as $CD11b^+NK1.1^-CD19^-CD3^-$) were separated using a FACS-Aria Sorter (Becton Dickinson, San Jose, CA). Cells were acquired with the Digital LSR II flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometric data were analyzed with FlowJo software.

*Quantitative Real Time PCR*

Total RNA was extracted from splenocytes with RNeasy kits (QIAGEN), digested with DNase I (RNase-free DNase set; QIAGEN), and reverse transcribed into cDNA. cDNA quantification was performed with FAST SYBR Green PCR kits (Applied Byosistems) and a Real-Time PCR Detection System (ABI). The RNA levels of the TGF-β (forward: ccgcaacaacgccatctatg; reverse: ctctgcacgggacagcaat), Integrin αV (Forward: aga agg agg gca agt ttc tca; Reverse: gtg gta caa tgg agc aca gg), Integrin β8 (Forward: gcagc ctggg tattt tcact; Reverse: acatt tgcag agcca catct) uPA (Forward: ccc atg aat gga tca gga aat gtt a; Reverse: gaa gac caa atg gcc ccg c) uPAR (Forward: gga gct tga agg atg agg act aca c; Reverse: act ggg tgt agt tgc aac act tca g) were normalized to cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

*Mink Lung Epithelial Cell Co-Culture*

Splenocytes were co-cultured with mink lung epithelial cells (MLEC-clone 32) that have been stably transfected with the human plasminogen activator inhibitor-1 gene fused to the firefly luciferase reporter gene in a p19LUC-based vector. After 15 hours of culture, cells were removed and the adherent MLEC were lysed to quantify luciferase activity. Samples were co-cultured with and without 1D11 mAb (concentration of 2 μg/ml) that is specific for all isoforms of TGF-β. MLEC were a generous gift from Dr. Daniel Rifkin

(Department of Cell Biology, New York University Medical Center). This approach detects TGF-β activity regardless of the predominant isoform.

*$CD11c^+$ DC Enrichment*

Spleen mononuclear cells were stained with CD11c Microbeads and $CD11c^+$ DCs were isolated with positive selection using MS columns and MiniMACS separator from Miltenyi Biotec.

*Statistical Analysis*

Statistical differences were determined by Student t test or one way analysis of variance (ANOVA) with InStat 3.0 software (GraphPad, CA.). $p < 0.05$ were considered significant and indicated with 1 star (*), $p < 0.005$ (**), $p < 0.0005$ (***).

## RESULTS

*Total TGF-β production is significantly increased during LCMV ARM and Cl 13 infection.*

To first investigate the total TGF-β production at the protein level, C57BL/6J mice were infected with LCMV ARM or Cl 13 to compare acute versus chronic viral infection, respectively. Differential expression of total TGF-β in the spleen was then examined through Western blot at day 5 post infection (p.i) of ARM and Cl 13. This time point was of particular interest because ARM and Cl 13 virus infects several tissues (3). However only Cl 13 is spread throughout the spleen white pulp, which has been demonstrated to contribute to its viral persistence (3).

Both LCMV ARM and Cl 13 infected mice exhibit greater than a two-fold increase of total splenic TGF-β relative to PLC-γ at day 5 p.i compared to the uninfected controls (Figure 3B). Here we see that both acute and chronic viral infection in these mice induce relatively similar levels of TGF-β (Figure 3). LCMV Cl 13 continues to persist in the host with high levels of viremia in various tissues and extensive T cell exhaustion whereas ARM is effectively cleared with differentiation of T cells into memory cells (6, 14).

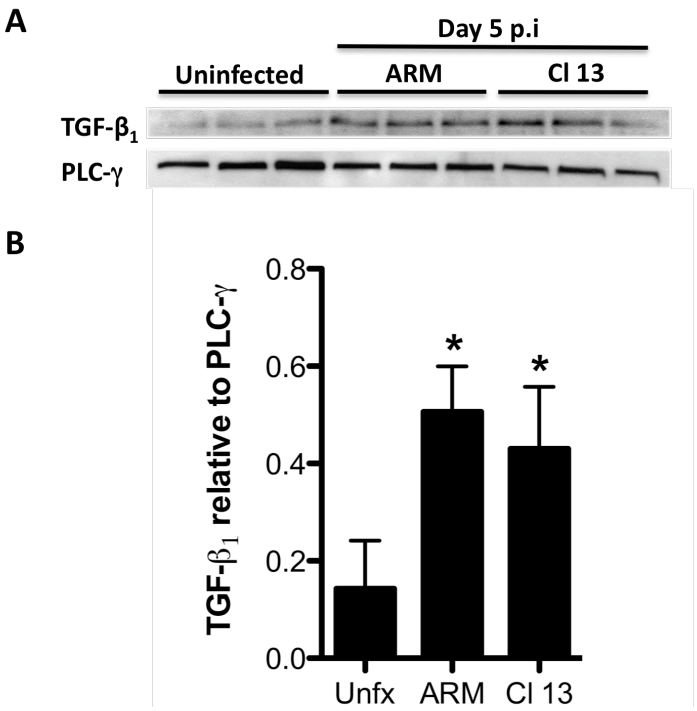


**Figure 3: TGF-β expression at day 5 after ARM or Cl13 infection.**
C57BL/6 mice 6-8 weeks of age were infected with LCMV ARM or Cl 13, and splenocytes were isolated at day 5 p.i. Splenocytes were processed for western blot using anti-TGF-β and anti-PLC-γ mABs. Levels of TGF-β were quantified and normalized against the loading control, phospholipase-γ (PLC-γ). (A) Each lane in the western blot corresponds to one individual mouse in the indicated group. (B) Bar graph shows the average values of $\pm$ SD of 3 mice per group. These results are representative of 2 independent experiments. $p < 0.05$ were considered significant and indicated with 1 star (*).

Thus, we were curious to see whether this elevated TGF-β production was sustained at later time points when a chronic viral environment was established. Total TGF-β protein levels were examined by immunoblot at day 31 p.i and there was a dramatic increase observed during chronic infection compared to the uninfected controls (Figure 4). Moreover, the TGF-β expression after prolonged Cl 13 infection was statistically higher than the increase seen at day 5 p.i ($p<0.05$). Altogether these results indicate that

LCMV infection induces an enhanced TGFβ production in the spleen early on and is elevated at later time points during chronic infection.

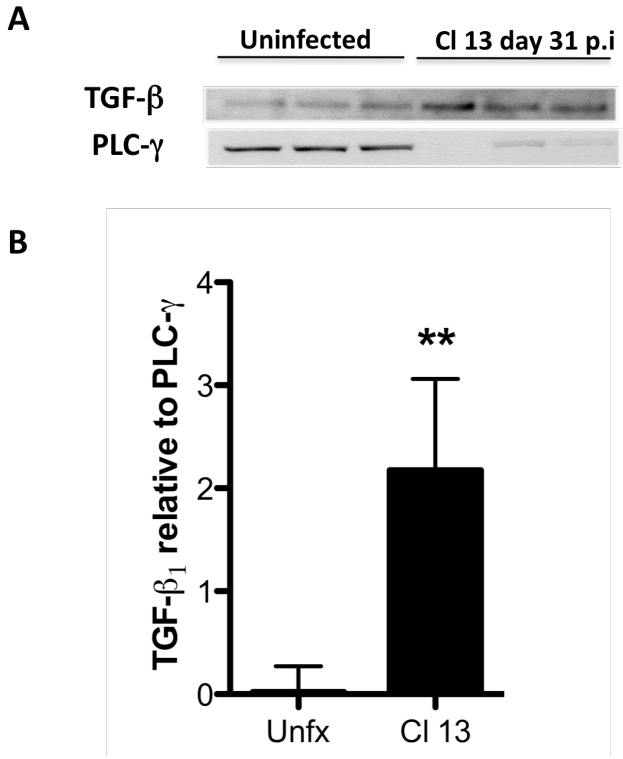


**Figure 4: TGF-β expression at day 31 after Cl 13 infection.**
Splenocytes were obtained from uninfected or Cl 13 infected mice at day 31 and analyzed by western blot using anti-TGF-β and anti-PLC-γ mABs. (A) Each lane in the western blot corresponds to one individual mouse in the indicated group. (B) Levels of TGF-β were quantified and normalized against PLC-γ. Bar graph shows the average values of ± SD of 3 mice per group. These results are representative of 1 experiment. $p < 0.05$ were considered significant and indicated with 1 star (*), $p < 0.005$ (**), $p < 0.0005$ (***).

*Potential cellular sources of TGF-β*

After discovering upregulation of TGF-β production in acute and chronic infection, we sought to identify the major producers of the cytokine. To achieve this aim, splenocytes were stained with fluorescently labeled antibodies and

FACS sorted at day 8 p.i from uninfected, ARM, and Cl 13 infected mice to isolate CD4 T cell ($CD4^+$), CD8 T cell ($CD8^+$), B cell ($CD9^+$), DC ($CD11c^+NK1.1^-CD3^-CD19^-$), and macrophage populations ($CD11b^+NK1.1^-CD3^-CD19^-$). This time point was studied because of the critical contrast in immune response between the two strains. ARM infected mice successfully mount a strong T cell response whilst Cl 13 infected mice have T cell exhaustion and dysfunction (4, 13).

FACS sorting of the individual cell populations in separate experiments yielded an average of greater than 98% purity (Figure 5 & 6). Gene expression of TGF-β, as measured by qPCR, was found to be surprisingly higher in the uninfected leukocyte populations than those in the ARM and Cl 13 (Figure 7). B cells and macrophages appear to express highest levels of TGF-β mRNA transcripts under uninfected conditions, which are subsequently dramatically reduced after LCMV ARM and Cl 13 infection. In comparing the other cell populations it seems that uninfected $CD4^+$ T cells, $CD8^+$ T cells also exhibit the same trend with at least a two fold increase in TGF-β mRNA levels compared to the same subsets during LCMV infection. However DCs are not substantially reduced after viral infection and remain elevated relative to the other populations. Additionally, it appears that gene regulation for total TGF-β is not significantly different between ARM and Cl 13 infection. It is worth noting that mRNA expression detect latent and active TGF-β levels and this cytokine

undergoes significant post transcriptional modifications (56). Collectively, the mRNA profiles from the different populations during infection indicate that gene regulation for TGF-β is not significantly altered during acute versus chronic infection and DCs express greater mRNA transcripts for the cytokine compared to other subsets during infection.

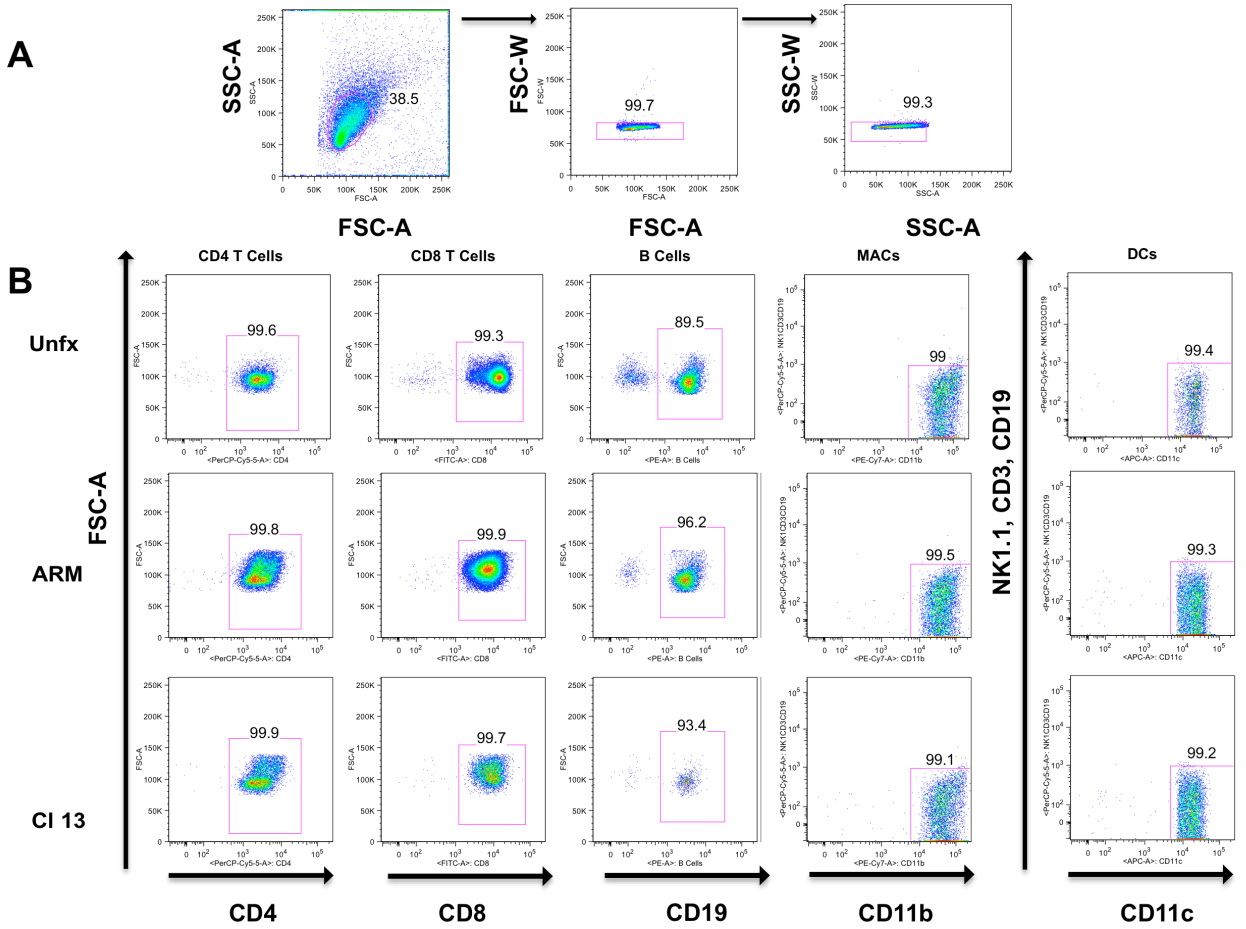


**Figure 5: Percent purity of sorted leukocyte populations at day 8 p.i (Sort I)**
WT mice were infected with LCMV ARM or Cl 13 and splenocytes were isolated at day 8 p.i. Total splenocytes were stained for CD4 T cells ($CD4^+$), CD8 T cells ($CD8^+$), B cells ($CD19^+$), Macrophages ($CD11b^-$), and DCs ($CD11c^+NK1.1^-CD3^-CD19^-$) and FACs sorted. (A) Gating for the live populations. (B) Subsequent gating from live populations in (A) for individual cell populations with percent purity. These results are representative of 1

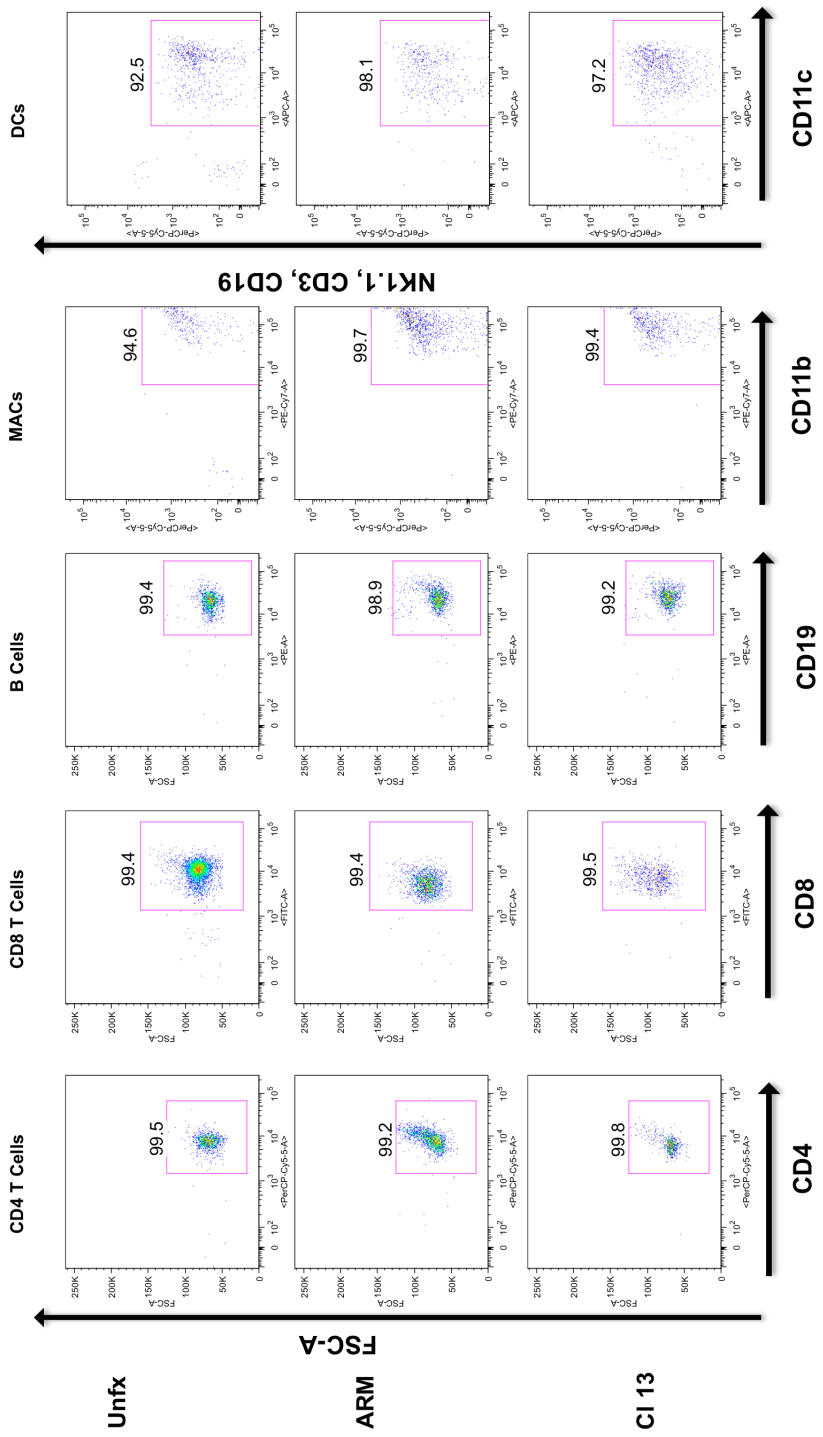


**Figure 6: Percent purity of sorted leukocyte populations at day 8 p.i (Sort II)**
WT mice were infected with LCMV ARM or Cl 13 and splenocytes were isolated at day 8 p.i. Total splenocytes were stained for CD4 T cells ($CD4^+$), CD8 T cells ($CD8^+$), B cells ($CD19^+$), Macrophages ($CD11b^+$), and DCs ($CD11c^+NK1.1^-CD3^-CD19^-$) and FACs sorted. The graphs show percent purity of indicated cell population. These results are representative of 1 independent experiment.

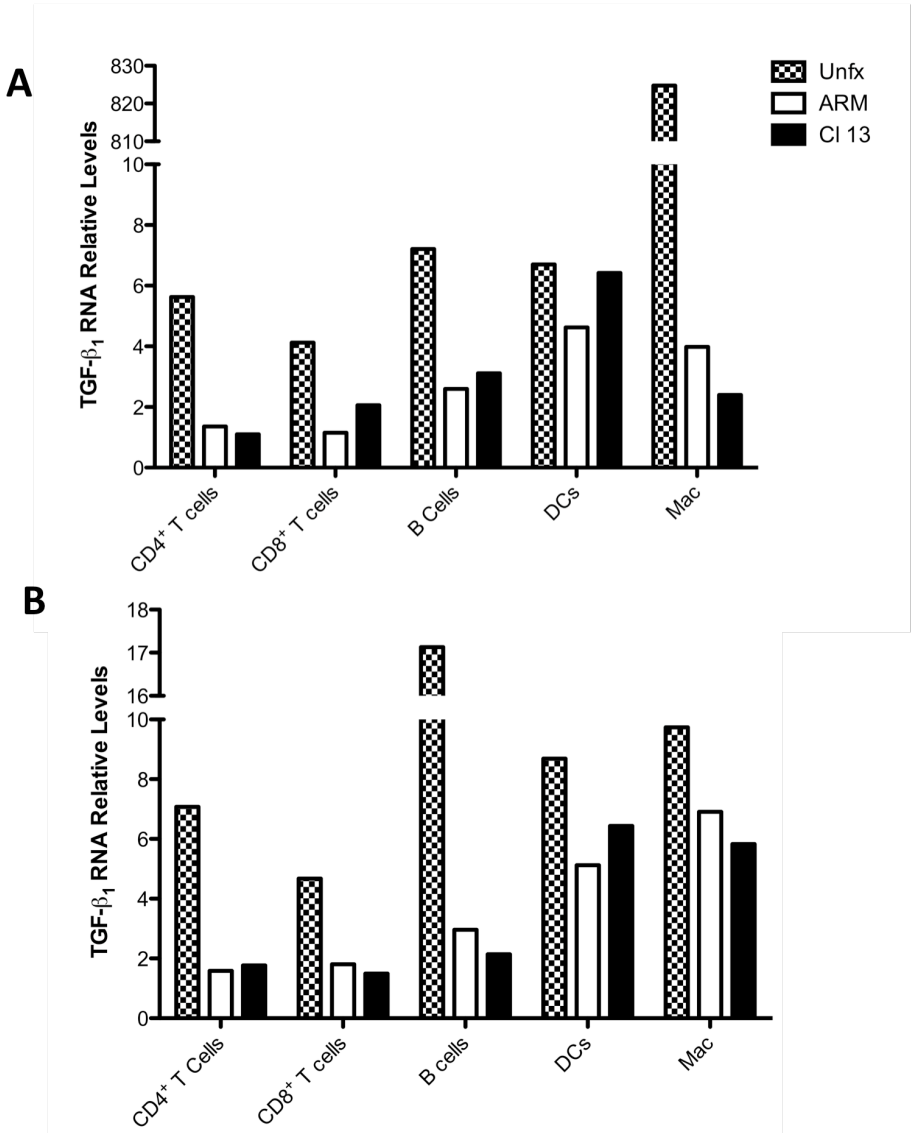


**Figure 7: TGF-β mRNA levels in sorted populations at day 8 p.i**
TGF-β expression of the sorted cell populations was measured by real-time PCR and is shown as average Ct Values normalized to GAPDH mRNA from Sort I (A) and Sort II (B) populations. These results are representative of 2 independent experiments.

*Sustained TGF-β bioactivity in LCMV Cl 13 infection*

Considering TGF-β is secreted in its latent format in notably large quantities and requires activation to exert its functions, and given mRNA expression was not increased by infection, it was pertinent to study the levels of active TGF-β during acute and chronic infection (33-35). To do this we used a transformed mink lung epithelial cell line (MLEC-clone 32), containing the human plasminogen activator inhibitor-1 (PAI-1) gene fused to the firefly luciferase reporter plasmid within a p19LUC-based vector (57). As TGF-β induces PAI-1 expression, this cell line serves as a useful bioassay for active TGF-β (Figure 8B) (57).

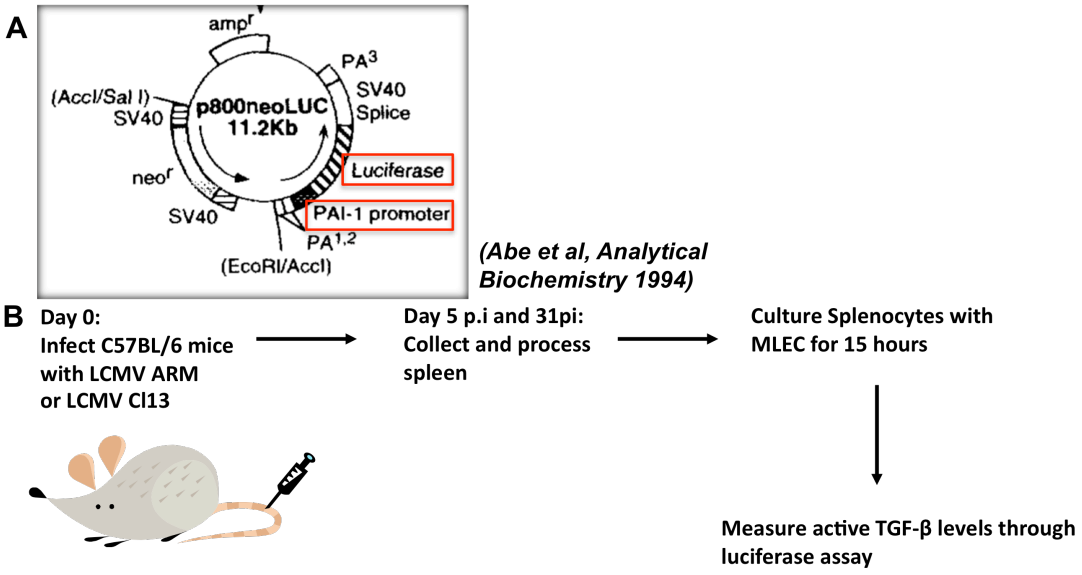


**Figure 8: Bioactive TGF-β Assay**
(A) Active TGF-β was measured using a mink lunge epithelial cell (MLEC) line stably transfected with a human plasminogen activator inhibitor-1 gene fused to the firefly luciferase reporter gene in a p19LUC-based vector. Adapted from (1).
(B) Schematic illustration of MLEC Co-culture Luciferase Assay to determine active levels of TGF-β in total splenocytes during acute versus chronic infection.

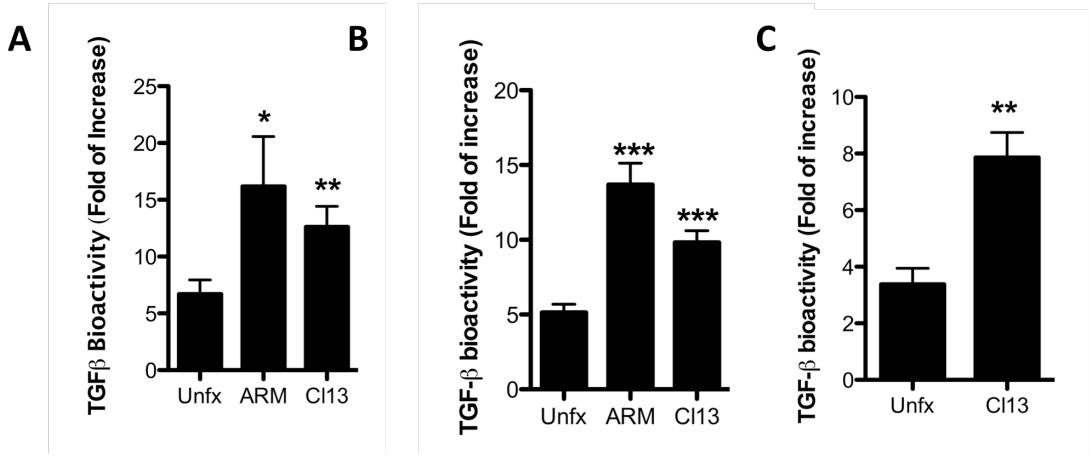


**Figure 9: TGF-β bioactivity during acute versus chronic infection**
Total spleen mononuclear cells from uninfected, ARM, or Cl 13 mice were co-cultured with MLEC to determine levels of biologically active TGF-β. Bar graphs from day 5 p.i (A) & (B) and from day 30 p.i (C) illustrate TGF-β bioactivity as folds of increase over blank controls. Results of (A) & (B) representative of 4 independent experiments and results of (C) are representative of 1 independent experiment with 3 mice per group. $p < 0.05$ were considered significant and indicated with 1 star (*), $p < 0.005$ (**), $p < 0.0005$ (***).

Again, we investigated total splenocytes at day 5 p.i and day 30 p.i. At day 5 p.i, we found that MLEC cultured with splenocytes from ARM and Cl 13 infected mice had approximately a 15- and 10-fold of increase over those MLECs with no cells added, respectively, in luciferase activity (Figure 9A,B). These levels were about 2-3 times higher than the uninfected group, demonstrating that the activity of TGF-β is elevated after LCMV infection. While ARM splenocytes displayed slightly higher levels of active TGF-β than those of Cl 13, the differences seen were not statistically significant. We continued the assessment on TGF-β bioactivity at day 30 p.i during chronic infection to determine if the elevated TGF-β protein production seen in the

immunoblots (Figure 4) corresponded to its heightened biological activity in the host. Cl 13 splenocytes continued to exhibit greater than twice the levels of TGF-β bioactivity, compared to uninfected splenocytes, at these later time points, confirming biological functional significance of the protein production (Figure 9C).

To ensure that the luciferase activity detected was specific for TGF-β, samples were next cultured with and without the monoclonal antibody 1D11, which recognizes all isoforms of TGF-β, at each time points. As a control the antibody tested a TGF-β concentration of 2500 pg/ml, which it successfully blocked (Figure 10A). When cultured in the presence of 1D11, the reduction in TGF-β activity with day 5 p.i splenocytes was not as pronounced as that seen with the top standard test and was even less effective with the day 30 p.i samples (Figure 10B,C). However TGF-β activity in the ARM and Cl 13 splenocytes at day 5 p.i was reduced to levels that were no longer statistically higher than the uninfected group (Figure 10B) indicating the increase in luciferase activity was specific for TGF-β. Together with the Western blot results, we concluded that not only was total TGF-β production enhanced at day 5 p.i of LCMV but it also contributed to dramatic elevations in its activity. The minor reduction in TGF-β activity in the presence of 1D11 raises the question on the specificity of the signal at this particular time point and/or the ability of 1D11 to block TGF-β signaling under these conditions.

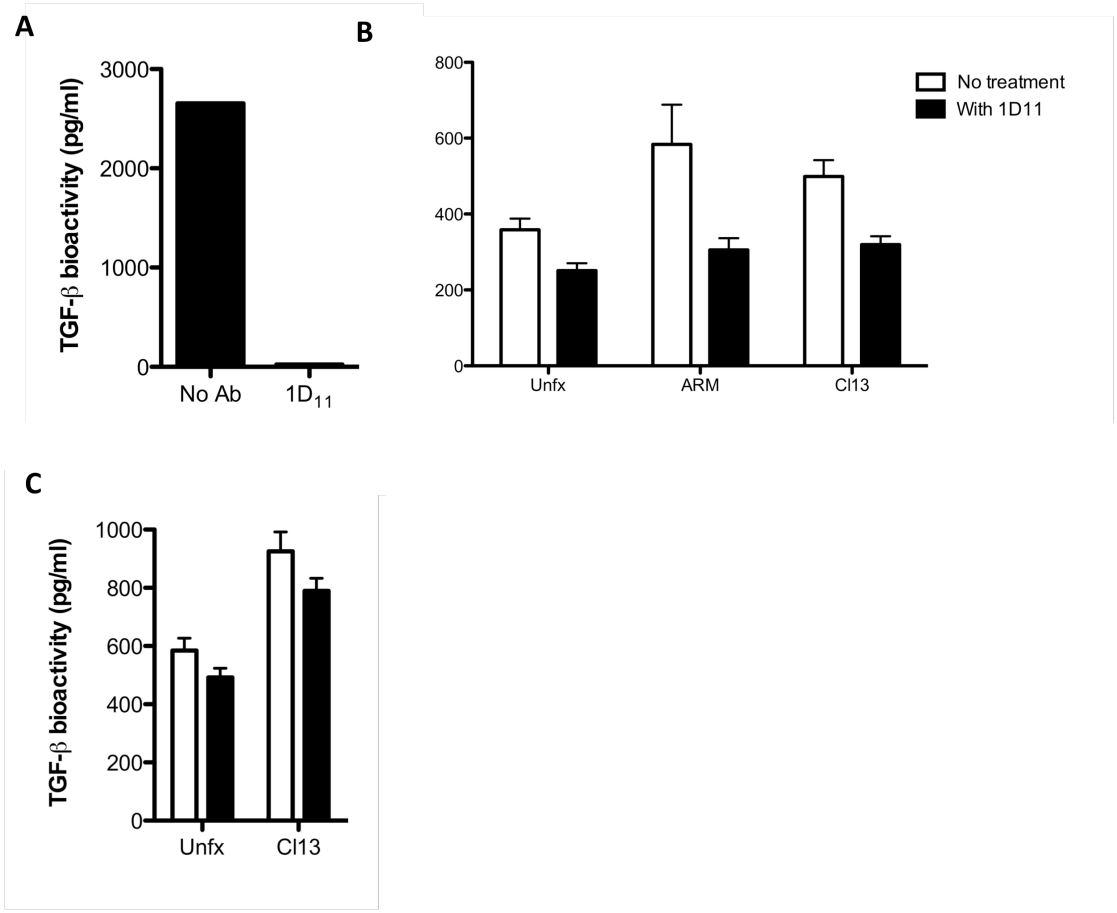


**Figure 10: Specificity of MLEC Co-culture for active TGF-β**
(A) Control test demonstrating blocking capacity of mAb 1D11 at a concentration of 2 μg/ml cultured without active for TGF-β.
(B) Bar graphs illustrate TGF-β bioactivity from splenocytes from experiment exhibited in Figure 7A cultured without (white bar) or with (black bar) 1D11.
(C) Bar graphs illustrate TGF-β bioactivity from splenocytes from experiment exhibited in Figure 7C cultured without (white bar) or with (black bar) 1D11.
These results are representative of 1 independent experiment.

*DCs preferentially upregulate uPAR gene expression during chronic infection*

We next sought to identify the potential factors that contribute to the increase in TGF-β bioavailability seen at day 5 p.i of ARM and Cl 13. We focused our analysis of integrins on integrin αV (itg αV), integrin β8 (itg β8), because DCs have been shown to control TGF-β bioavailability through

itgαvβ8 (58). We also looked at urokinase plasminogen activator (uPA) and its binding to its receptor (uPAR) because their roles in TGF-β activation are relevant within innate and adaptive immunity (40). Preliminary DC micorarray data also suggested their upregulation during viral infection (data not shown). Thus, DCs from ARM- and Cl 13-infected mice at various time p.i were FACS sorted and RNA extracted at day 8 p.i.

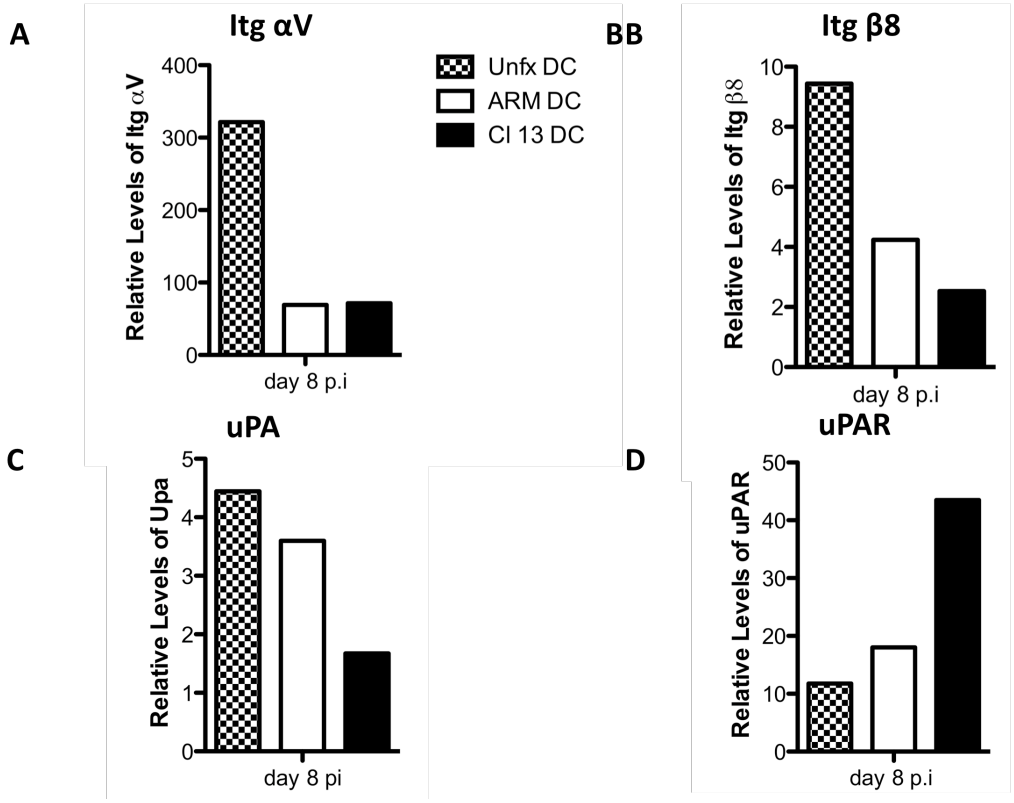


**Figure 11: Gene expression of Activating Factors in Sorted DCs**
FACs sorted dendritic cells from WT uninfected, ARM, and Cl 13 infected mice at day 8 p.i were purified for RNA and reverse transcribed. Itg αV (A), Itg β8 (B), uPA (C), and uPAR mRNA expression were quantified by real-time PCR and shown normalized to GAPDH RNA. Results from (A) and (D) are representative of 2 independent experiments and results from (B) and (C) are representative of 1 independent experiment.

Gene expressions for itg αV and itg β8 exhibit similar trends with the highest mRNA transcripts produced in uninfected controls and a 50-60% reduction in ARM and Cl 13 DCs (Figure 11A,B). uPA levels were also highest in the uninfected although its expression during the acute infection did not decrease significantly (Figure 11C). In contrast, the opposite was observed in uPAR expression with the lowest mRNA production seen in the uninfected controls and a striking upregulation of the receptor during infection with Cl 13 (Figure 11D). The results demonstrated that while production and bioactive levels of TGF-β were similar during acute and chronic infection, there may be a distinct difference in the mechanism by which activation occurs. The pronounced elevation of uPAR in DCs during Cl 13 infection prompted us to further analyze expression of these factors within ARM and Cl 13 infected mice at day 3 and day 5 p.i. The purity of the FACS sorted cells was not as high as previously achieved however both groups showed significant enrichment (Figure 12).

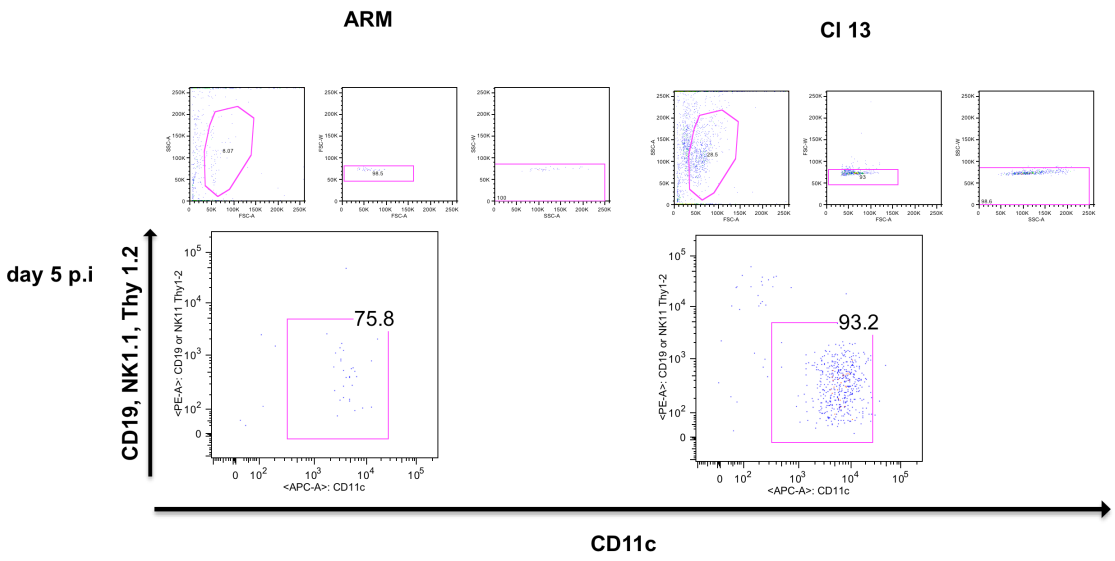


**Figure 12: Percent purity of sorted DCs at day 5 p.i**
WT mice were infected with LCMV ARM or Cl 13 and splenocytes were isolated at day 5 p.i. Total splenocytes were stained for DCs and FACs sorted. The graphs show percent purity and is representative of 1 independent experiment with 3-4 mice per group.

As seen at day 8 itg αV mRNA levels were comparable between the two groups on both day 3 and day 5 p.i. (Figure 13A). However, it appears that DCs from ARM infected mice initially upregulate itg αV at day 3 p.i but by day 5 p.i, its expression has declined again (Figure 13B). Gene expression of itg β8 in Cl 13 DCs was difficult to detect at these timepoints. Corresponding with our analysis at day 8 p.i, Cl 13 DCs exhibited higher levels of uPAR than their ARM counterparts with the greatest difference seen at day 5 p.i (Figure 13C). The data collected demonstrated that DCs from Cl 13 infected mice preferentially upregulate the expression of uPAR.

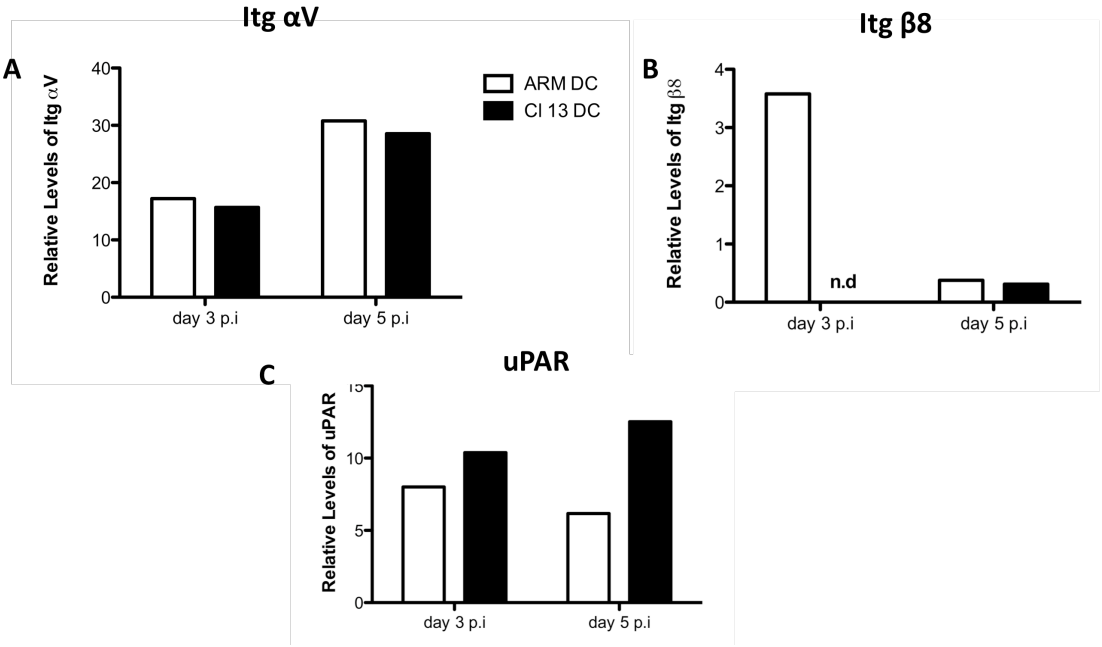


**Figure 13: Gene expression of Activating Factors in Sorted DCs at early time points post infection.**
FACs sorted dendritic cells from ARM, and Cl 13 infected mice at day 3 and 5 p.i were purified for RNA and reverse transcribed. Itg αV (A), Itg β8 (B), and uPAR (C) mRNA expression were quantified by real-time PCR and shown normalized to GAPDH RNA. These results are representative of 1 independent experiment.

*TGF-β signals directly on DCs during LCMV infection*

We then evaluated downstream signaling of TGF-β. As a receptor-regulated smad, phosphorylated smad 2 (p-smad 2) is of particular interest because it enters the nucleus with other nuclear co-factors to regulate gene transcription. Thus we measured the amount of p-smad 2 produced in dendritic cells at day 5 p.i. Total splenocytes were positively selected for $CD11c^+$ DCs using magnetic beads and then protein production of p-smad2 was analyzed through Western blot. The purity of these populations was lower

than FACs sorting as expected, however the enrichment was consistent (Figure 14).

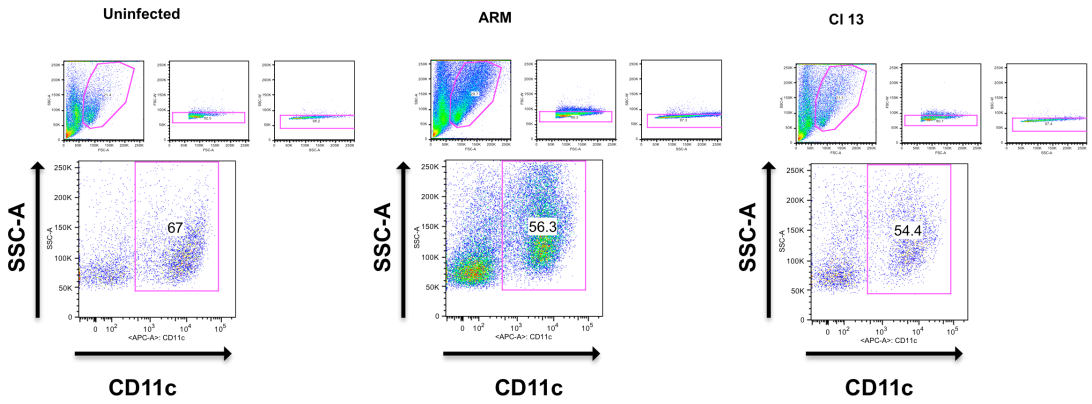


**Figure 14: Percent purity of sorted DCs at day 5 p.i**
WT mice were infected with LCMV ARM or Cl 13 and splenocytes were isolated at day 5 p.i. Total splenocytes were stained for with $CD11c^+$ Microbeads and isolated with Miltenyi Biotec MS columns. The graphs show percent purity for $CD11c^+$ DCs and is representative of 2-3 mice per group.

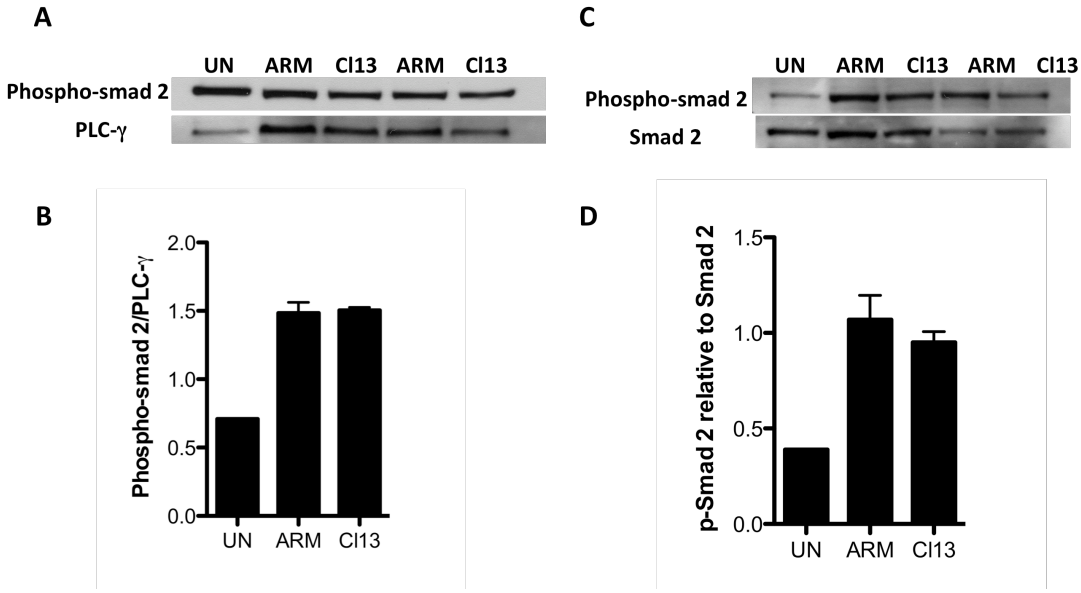


**Figure 15: Phospho-smad 2 expression in DCs at day 5 post ARM or Cl13 infection.** WT mice were infected with LCMV ARM or Cl 13, and splenocytes were isolated at day 5 p.i. Splenocytes were processed, enriched for $CD11^{+}$ cells, and processed for western blot using anti-phospho-smad 2 and anti-PLC-γ mABs. Levels of p-smad 2 were quantified and normalized against the loading control, phospholipase-γ (PLC-γ). (A,C) Each lane in the western blot corresponds to one individual mouse in the indicated group. (B,D) Bar graph shows the average values of $\pm$ SD of 1-2 mice per group. These results are representative of 2 independent experiments.

Here, we found that ARM and Cl 13 infection at this time point induced an increased p-smad 2 levels in DCs while uninfected controls yielded approximately 50% less phosphorylated protein (Figure 15C,D). These results indicated that bioactive TGF-β detected during acute and chronic infection was directly signaling on DCs. We further blotted for smad 2 in order to analyze the proportion of phosphorylated smad 2 in terms of its total availability within the cells. Western blot for these total smad 2 demonstrate ARM and Cl 13 DCs exhibit similar trends of p-smad 2 in relation to smad 2 production (Figure 15A,B). This suggests that the presence of p-smad in the DCs was indeed a

direct result of TGF-β signaling, rather than variations in smad 2 production. We can also conclude that at day 5 p.i DCs undergo similar TGF-β signaling during acute and chronic viral infection.

## DISCUSSION

In this study we have demonstrated that total TGF-β production is significantly upregulated shortly after LCMV ARM and Cl 13 infection. In the context of Cl 13, this remained the case long into chronic phase of infection. The enhanced expression of TGF-β in total splenocytes at day 5 p.i also correlated with an elevation in biologically active TGF-β, indicating that it is also being released from its latent form by activating factors. This finding is further supported by the increased expression of activator uPAR in the DCs of LCMV infected mice. Finally we showed, by increased levels of Smad-2 phosphorylation, that not only are DCs producing and potentially activating TGF-β, but also receiving increased TGF-β signaling after LCMV infection.

The ubiquitous nature of TGF-β in host immunity requires multiple levels of tight regulation for proper functions in the host. Others have found it serves a vital function in controlling autoreactive T cells because complete knockout of this gene leads to a multi-organ inflammatory phenotype and death within 3-4 weeks of age in mice (26, 29). Its immunosuppressive effects have also been clearly demonstrated because upregulation of TGF-β signaling in virus-specific CD8 T cells directly contributes to their increased apoptosis during chronic LCMV infection (23). Interestingly, limiting TGF-β signaling in this cell subset allows rapid viral infection instead of the persistence normally

seen in LCMV Cl 13 infected mice (23). Therefore whilst TGF-β can have a beneficial role in preventing autoimmunity or tolerance maintenance, it appears that in the context of some infections, it may be detrimental to the host. Our findings help to uncover the complexity of TGF-β functions and regulations on critical cell subsets within the context of a chronic infection.

The discoveries made by Tinoco *et al.* illustrate the inhibitory roles of TGF-β within the context of a chronic viral infection. Yet the comparative levels of TGF-β production and bioactivity shortly after infection with either acute or chronic infection suggest that the TGF- β function in CD8 T cell death may be a result of prolonged persistent infection/immune stimulation and is not a result of enhanced TGF-β activity early on. This is consistent with comparable levels of TGF-β signaling in CD8 T cells early after ARM and Cl 13 infection (23).

Interestingly in acute ARM infection, increased TGF-β production coincides with a decrease in natural (NK) cell proliferation (59). In this context the virus is still successfully cleared, predominantly by a high number of virus specific CD8 T cells, and it is likely that in acute infection TGF-β plays an important role in limiting lymphocyte overproliferation and preventing immunopathology. Whereas ARM infected mice clear virus before day 9 post infection, Cl 13 infected mice suffer from high viral titers in the blood, spleen,

brain, kidneys, and several other tissues (54). In this study we saw that TGF-β production and functional activity were still significantly increased at day 30-31 p.i, coinciding with the presence of exhausted immunity and it is likely that which contributes to the immunosuppression of the host (13, 60).

The TGF-β blocking antibody, 1D11, cultured with 2500pg/ml of the standard TGF-β effectively blocked TGF-β bioactivity in MLEC cultures. Interestingly, when splenocytes were co-cultured with MLECs, the TGF-β blocking antibody, 1D11, could not inhibit TFG-β activity, in spite of the fact that total TGF-β concentrations in these cultures were well below 2500pg/ml. This suggests that in a co-culture environment antibody treatment to prevent TGF- β signaling is ineffective. In a similar system with purified regulatory T cells co-cultured with MLECs, it was found that TGF-β signaled in direct cell-cell contact dependent manner (61). The same process could be in action in our system, meaning blocking antibody may not have sufficient time to access TGF-β prior to its activation and signal transduction. In the context of regulatory T cells, it was found that blockade of the TGF-β receptor on MLECs, rather than TGF-β itself, was able to prevent signaling. This approach could also be used in our system. What is particular promising about this hypothesis is that blockade was least effective when co-cultures were carried out with splenocytes taken at day 30 post infection with Cl 13, supporting our findings of not only increased production, but also activation in these mice,

and suggesting a contact based mechanism may be involved. Separation of the splenocytes and MLECs, by transwell could help assess relative contributions of contact dependent and independent TGF-β bioactivity.

Our findings on the presence of bioactive TGF-β in total spleen mononuclear cells, whilst important, do not indicate what the major TGF-β producing cell(s) are. Previous studies have discovered that T cell activation induces TGF-β mRNA production and activity (62). The sustained viral titers in several tissues during Cl 13 infection can also provide increased and continued antigenic stimulation on anti-viral T cells, preserving activation and maintaining TGF-β production for a prolonged period of time during viral infection. Alternatively, Cl 13 infection has been well demonstrated to directly cause lasting, intrinsic pDC dysfunction by deregulating its type-I interferon production specifically through the TLR pathway (63). Preferential tropism of Cl 13 in DCs strongly suggest that viruses take advantage of their critical role in imprinting the adaptive immune response and alter the genetic programming in favor of viral persistence. Here we looked at alterations in TGF-β production and signaling in DCs and found significant changes in gene expression of TGF-β activating factors in Cl 13 infected DCs.

Direct TGF-β-smad signaling on virus specific T cells in Cl 13 infected mice induces their upregulation of the pro-apoptotic protein Bim, thus causing

T cell deletion (23). Substantial $CD8^+$ T cell loss is characteristic of Cl 13 infection (3) and DCs have the ability to direct antiviral T cells. In this study we observed early TGF-β signaling on DCs, by elevated p-smad 2, suggesting chronic viral infection modulates TGF- β signaling within DCs, as well as activation and production. This could be another method in which chronic infection subverts the immune response, although this remains to be tested.

Interestingly whilst increased TGF-β protein and activity were consistently seen evaluation of gene expression revealed that, if anything, infection caused a downregulation in the transcription of TGF-β. These contrasting results could reflect the importance post-transcriptional regulation plays in determining the biological activity of TGF-β (64). For instance studies of the human TGF-β gene have identified a portion of the 5'-untranslated region (UTR) on the mRNA transcript that significantly hinders its expression by as much as 22-fold in cell type specific manner (56). Therefore it might be of interest to investigate whether the type of TGF-β transcript produced is somehow altered by infection. In the future it will also be of importance to look at TGF- β production and bioactivity on a cell specific level.

Even taking into account these contrasting findings, the cell specific TGF-β mRNA profile during LCMV infection suggests that DCs are primary contributors of TGF-β in ARM and Cl 13 mice, with a slightly greater

expression in the chronic state. As with other results this would need to be confirmed through immunoblot for total protein levels.

From our finding of increased TGF-β signaling in enriched DCs at day 5 p.i of both ARM and Cl 13 infected mice, it would be interesting to investigate if the comparable p-smad 2 production continues at day 8 p.i, when the immune responses begin to differ. This could potentially determine if TGF-β signaling on DCs is important in deregulating the immune response towards a chronic outcome as Tinoco *et al.* have proven to be important for specific $CD8^+$ T cells at this time point (23). Another approach would be the use of CD11c dnTGFβRII mice to investigate if intrinsic defects in DCs signaling can alter the state of chronic infection. This transgenic model expresses a dominant negative form of the TGF-β receptor II under the control of the CD11c promoter (65). In their steady state, they exhibit similar DC homeostasis and DC proliferation as their WT counterparts (65). Therefore, investigating whether or not attenuated TGF-β signaling on this subset affects virus specific T cell response and the overall immune response during LCMV infection would be of interest; furthering our understanding of the role of TGF-β activity within DCs in directing the adaptive immune response. In addition, use of TGF-β1 silencing in DCs, using CD11c-Cre TGF-$\beta^{loxp/loxp}$ mice, would help determine if DC specific TGF-β production is biologically relevant in chronic viral infection. If total TGF-β production is significantly altered in Cl 13 infected

mice, it would identify DCs as another major cell source of producing enhanced TGF-β secretion for modulating $CD8^+$ T cell exhaustion. This could be followed up with MLEC assays to determine whether production correlates with increased bioavailability.

One the most important regulatory step of TGF-β pathway occurs after its protein production, through its activation by various proteases and integrins (33, 34). As previously mentioned an example is integrin αVβ8 is primarily produced by DCs to activate TGF-β, which can in turn suppress autoreactive T cells (58). Comparison of TGF-β activating factors during acute and chronic infections provided insight on factors that are favored during chronic infection. Relatively similar levels of itg αV and β8 mRNA production in acute versus chronic infection suggest they probably do not result in different TGF-β activation at later time points of viral infection. Contrastingly, the consistent upregulation of uPAR mRNA at all three time points post infection of Cl 13 compared to ARM suggest this receptor could serve as a unique activation mechanism of TGF-β in a chronic viral environment. The comparison of infected and uninfected DCs at day 8 p.i among itg αv, itg β8, uPAR, and uPA highlights the changes that occur due to LCMV infection. The notably higher itg αV and itg β8 expression in uninfected DCs that is subsequently reduced in ARM and Cl 13 DCs, suggests itgαvβ8 is not the primary TGF-β activating factor during viral infection. It is possible that the greater expression of itg αV

compared to itg β8 in DCs is due to its role in combing with β5 to induce immature DC phagocytosis of apoptotic cells (66). Considering viral infection causes greater apoptosis of lymphocytes, particularly CD8+ T cells, there is a strong possibility itg αVβ5 is produced for that purpose (66). However, without measurement of integrin levels in sorted uninfected DCs at day 3 and day 5 p.i, it is difficult to determine if these are elevated due to viral infection

uPAR binding uPA and catalyzes the conversion of plasminogen to plasmin in the extracellular matrix. This reaction can subsequently lead to extracellular matrix (ECM) degradation, which others have shown contributes greater migration of lymphocytes, monocytes, and perhaps even cancer (40). It is possible that elevated expression of uPAR we see in DCs could allow for greater migration during infection and explain the elevation seen during LCMV ARM and Cl 13 infected mice. However, within the Cl 13 infection, it has been clearly shown that infection prevents the accumulation of DCs within the spleen and bone marrow (67). These previous findings support the notion that uPAR activity with uPA in DCs serves another purpose. The urokinase-type plasminogen activator system has also been strongly implicated in TGF-β activation, suggesting DCs are responsible for freeing TGF-β from its latent complex during chronic infection. Interestingly, uPAR expression is enhanced in various leukocytes, tissues, and fluids of patients affected by HIV-1. Moreover, it has been demonstrated *in vitro* and *in vivo* that high serum levels

of soluble uPAR (suPAR) strongly correlates with a worse prognosis for HIV-infected patients (68, 69). A hallmark of poorer outcomes in patients with elevated suPAR includes dramatic deletion of $CD4^+$ T cells. Enhanced serum suPAR is also an indication of up-regulated uPAR levels (69). These previous studies highlight the detrimental impact uPAR may have in inducing and/or sustaining chronic viral infection. Enhanced DC expression of uPAR together with decreased $CD4^+$ T cells in HIV patients with high suPAR levels support the likelihood that DC secretion of these particular TGF-β activating factors can contribute to $CD4^+$ T cell exhaustion (4). Studies of chronic infection in $uPAR^{-/-}$ mice would clarify its role in determining the host immune response. Additionally, while other activation factors have been vital for preventing autoimmunity, mice deficient in uPAR are healthy with no signs of generalized autoimmunity (58, 70). These observations suggest that it may be possible to therapeutically intervene in TGF-β signaling during infection in a pathway specific manner, therefore avoiding the detrimental effects seen when TGF-β is blocked entirely. Another approach would be to culture DCs with MLEC cell in the presence of latent TGF-β to measure the TGF-β activating ability. Use of DCs from $uPAR^{-/-}$, CD11c-dnTGFβRII, and/or $TGF\text{-}\beta^{-/-}$-infected mice, would help sort whether DC ability to produce uPAR, to receive TGF-β signaling, or to express TGF-β affects its effectiveness in activating TGF-β during chronic infection. Findings from these experiments would elucidate at what stage DCs are possibly important in TGF-β regulation in persistent viral infections.

Additionally, previous studies indicate that uPA-uPAR activity not only regulates TGF-β activity, but their expressions are also subject to TGF-β control (71). Together with our finding of upregulated p-smad 2 expression in the LCMV-infected DCs, it is likely that initial TGF-β signaling on DCs could function in an autocrine manner and consequently induce a greater expression of uPAR on its own surface. This could be confirmed by utilizing CD11c-dn TGF-βRII mice and studying their ability to produce bioactive TGF-β and TGF-β activating factors including uPAR during LCMV infection. It is important to note that uPAR can also be post transcriptionally regulated (71), so it would be worthwhile to determine if proteins are equally increased in chronically infected DCs.

In conclusion, whilst TGF-β remains a complicated protein, with characterization requiring extensive analysis at each step, this study has highlighted a number specific features such as uPAR and dendritic cells that could be critical to TGF-β's role in chronic infection. Due to the prevalence of its expression on several leukocyte populations, it is critical to examine its expression in individual subsets. Furthermore, TGF-β association with a latent complex emphasizes the importance of analyzing the amount of TGF-β that is biologically active in the host. In the future, however, it may be a good target for therapeutics against chronic infection.

## REFERENCES


1. Letvin, N. L., and B. D. Walker. 2003. Immunopathogenesis and immunotherapy in AIDS virus infections. *Nat Med* 9:861-866.

2. Virgin, H. W., E. J. Wherry, and R. Ahmed. 2009. Redefining chronic viral infection. *Cell* 138:30-50.

3. Borrow, P., C. F. Evans, and M. B. Oldstone. 1995. Virus-induced immunosuppression: immune system-mediated destruction of virus-infected dendritic cells results in generalized immune suppression. *J Virol* 69:1059-1070.

4. Brooks, D. G., L. Teyton, M. B. Oldstone, and D. B. McGavern. 2005. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. *J Virol* 79:10514-10527.

5. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439:682-687.

6. Brooks, D. G., M. J. Trifilo, K. H. Edelmann, L. Teyton, D. B. McGavern, and M. B. Oldstone. 2006. Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 12:1301-1309.

7. Ejrnaes, M., C. M. Filippi, M. M. Martinic, E. M. Ling, L. M. Togher, S. Crotty, and M. G. von Herrath. 2006. Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J Exp Med* 203:2461-2472.

8. Oldstone, M. B. 2002. Biology and pathogenesis of lymphocytic choriomeningitis virus infection. *Curr Top Microbiol Immunol* 263:83-117.

9. Zinkernagel, R. M. 2002. Lymphocytic choriomeningitis virus and immunology. *Curr Top Microbiol Immunol* 263:1-5.

10. Kunz, S., P. Borrow, and M. B. Oldstone. 2002. Receptor structure, binding, and cell entry of arenaviruses. *Curr Top Microbiol Immunol* 262:111-137.

11. Zinkernagel, R. M., and P. C. Doherty. 1997. The discovery of MHC restriction. *Immunol Today* 18:14-17.

12. Matloubian, M., T. Somasundaram, S. R. Kolhekar, R. Selvakumar, and R. Ahmed. 1990. Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. *J Exp Med* 172:1043-1048.

13. Byrne, J. A., and M. B. Oldstone. 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus in vivo. *J Virol* 51:682-686.

14. Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77:4911-4927.

15. Matloubian, M., S. R. Kolhekar, T. Somasundaram, and R. Ahmed. 1993. Molecular determinants of macrophage tropism and viral persistence: importance of single amino acid changes in the polymerase and glycoprotein of lymphocytic choriomeningitis virus. *J Virol* 67:7340-7349.

16. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. J. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188:2205-2213.

17. Salvato, M., P. Borrow, E. Shimomaye, and M. B. Oldstone. 1991. Molecular basis of viral persistence: a single amino acid change in the glycoprotein of lymphocytic choriomeningitis virus is associated with suppression of the antiviral cytotoxic T-lymphocyte response and establishment of persistence. *J Virol* 65:1863-1869.

18. Rigopoulou, E. I., W. G. Abbott, P. Haigh, and N. V. Naoumov. 2005. Blocking of interleukin-10 receptor--a novel approach to stimulate T-helper cell type 1 responses to hepatitis C virus. *Clin Immunol* 117:57-64.

19. Clerici, M., T. A. Wynn, J. A. Berzofsky, S. P. Blatt, C. W. Hendrix, A. Sher, R. L. Coffman, and G. M. Shearer. 1994. Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus. *J Clin Invest* 93:768-775.

20. Trautmann, L., L. Janbazian, N. Chomont, E. A. Said, S. Gimmig, B. Bessette, M. R. Boulassel, E. Delwart, H. Sepulveda, R. S. Balderas, J. P. Routy, E. K. Haddad, and R. P. Sekaly. 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nat Med* 12:1198-1202.

21. Urbani, S., B. Amadei, D. Tola, M. Massari, S. Schivazappa, G. Missale, and C. Ferrari. 2006. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 80:11398-11403.

22. Zhang, J. Y., Z. Zhang, X. Wang, J. L. Fu, J. Yao, Y. Jiao, L. Chen, H. Zhang, J. Wei, L. Jin, M. Shi, G. F. Gao, H. Wu, and F. S. Wang. 2007. PD-1 up-regulation is correlated with HIV-specific memory CD8+ T-cell exhaustion in typical progressors but not in long-term nonprogressors. *Blood* 109:4671-4678.

23. Tinoco, R., V. Alcalde, Y. Yang, K. Sauer, and E. I. Zuniga. 2009. Cell-intrinsic transforming growth factor-beta signaling mediates virus-specific CD8+ T cell deletion and viral persistence in vivo. *Immunity* 31:145-157.

24. Rubtsov, Y. P., and A. Y. Rudensky. 2007. TGFbeta signalling in control of T-cell-mediated self-reactivity. *Nat Rev Immunol* 7:443-453.

25. Govinden, R., and K. D. Bhoola. 2003. Genealogy, expression, and cellular function of transforming growth factor-beta. *Pharmacol Ther* 98:257-265.

26. Li, M. O., S. Sanjabi, and R. A. Flavell. 2006. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 25:455-471.

27. Massague, J. 2008. TGFbeta in Cancer. *Cell* 134:215-230.

28. Gorelik, L., and R. A. Flavell. 2002. Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* 2:46-53.

29. Kulkarni, A. B., C. G. Huh, D. Becker, A. Geiser, M. Lyght, K. C. Flanders, A. B. Roberts, M. B. Sporn, J. M. Ward, and S. Karlsson. 1993. Transforming growth factor beta 1 null mutation in mice causes

excessive inflammatory response and early death. *Proc Natl Acad Sci U S A* 90:770-774.

30. Marie, J. C., D. Liggitt, and A. Y. Rudensky. 2006. Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity* 25:441-454.

31. Marie, J. C., J. J. Letterio, M. Gavin, and A. Y. Rudensky. 2005. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 201:1061-1067.

32. Gorelik, L., and R. A. Flavell. 2000. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12:171-181.

33. Annes, J. P., J. S. Munger, and D. B. Rifkin. 2003. Making sense of latent TGFbeta activation. *J Cell Sci* 116:217-224.

34. Wipff, P. J., and B. Hinz. 2008. Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship. *Eur J Cell Biol* 87:601-615.

35. Jenkins, G. 2008. The role of proteases in transforming growth factor-beta activation. *Int J Biochem Cell Biol* 40:1068-1078.

36. Sato, Y., and D. B. Rifkin. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture. *J Cell Biol* 109:309-315.

37. Taipale, J., K. Koli, and J. Keski-Oja. 1992. Release of transforming growth factor-beta 1 from the pericellular matrix of cultured fibroblasts and fibrosarcoma cells by plasmin and thrombin. *J Biol Chem* 267:25378-25384.

38. Taipale, J., J. Lohi, J. Saarinen, P. T. Kovanen, and J. Keski-Oja. 1995. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J Biol Chem* 270:4689-4696.

39. Allison, R. S., M. L. Mumy, and L. M. Wakefield. 1998. Translational control elements in the major human transforming growth factor-beta 1 mRNA. *Growth Factors* 16:89-100.

40. Mondino, A., and F. Blasi. 2004. uPA and uPAR in fibrinolysis, immunity and pathology. *Trends Immunol* 25:450-455.

41. Munger, J. S., X. Huang, H. Kawakatsu, M. J. Griffiths, S. L. Dalton, J. Wu, J. F. Pittet, N. Kaminski, C. Garat, M. A. Matthay, D. B. Rifkin, and D. Sheppard. 1999. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* 96:319-328.

42. Sheppard, D. 2005. Integrin-mediated activation of latent transforming growth factor beta. *Cancer Metastasis Rev* 24:395-402.

43. Massague, J. 1998. TGF-beta signal transduction. *Annu Rev Biochem* 67:753-791.

44. Shi, Y., and J. Massague. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113:685-700.

45. Macias-Silva, M., S. Abdollah, P. A. Hoodless, R. Pirone, L. Attisano, and J. L. Wrana. 1996. MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* 87:1215-1224.

46. Derynck, R., W. M. Gelbart, R. M. Harland, C. H. Heldin, S. E. Kern, J. Massague, D. A. Melton, M. Mlodzik, R. W. Padgett, A. B. Roberts, J. Smith, G. H. Thomsen, B. Vogelstein, and X. F. Wang. 1996. Nomenclature: vertebrate mediators of TGFbeta family signals. *Cell* 87:173.

47. Massague, J., and D. Wotton. 2000. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 19:1745-1754.

48. Reis e Sousa, C. 2004. Toll-like receptors and dendritic cells: for whom the bug tolls. *Semin Immunol* 16:27-34.

49. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2:151-161.

50. Lee, H. K., and A. Iwasaki. 2007. Innate control of adaptive immunity: dendritic cells and beyond. *Semin Immunol* 19:48-55.

51. Rinaldo, C. R., Jr., and P. Piazza. 2004. Virus infection of dendritic cells: portal for host invasion and host defense. *Trends Microbiol* 12:337-345.

52. Bowie, A., E. Kiss-Toth, J. A. Symons, G. L. Smith, S. K. Dower, and L. A. O'Neill. 2000. A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. *Proc Natl Acad Sci U S A* 97:10162-10167.

53. Donaghy, H., J. Stebbing, and S. Patterson. 2004. Antigen presentation and the role of dendritic cells in HIV. *Curr Opin Infect Dis* 17:1-6.

54. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J Exp Med* 160:521-540.

55. Zuniga, E. I., D. B. McGavern, J. L. Pruneda-Paz, C. Teng, and M. B. Oldstone. 2004. Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection. *Nat Immunol* 5:1227-1234.

56. Kim, S. J., K. Park, D. Koeller, K. Y. Kim, L. M. Wakefield, M. B. Sporn, and A. B. Roberts. 1992. Post-transcriptional regulation of the human transforming growth factor-beta 1 gene. *J Biol Chem* 267:13702-13707.

57. Abe, M., J. G. Harpel, C. N. Metz, I. Nunes, D. J. Loskutoff, and D. B. Rifkin. 1994. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* 216:276-284.

58. Travis, M. A., B. Reizis, A. C. Melton, E. Masteller, Q. Tang, J. M. Proctor, Y. Wang, X. Bernstein, X. Huang, L. F. Reichardt, J. A. Bluestone, and D. Sheppard. 2007. Loss of integrin alpha(v)beta8 on dendritic cells causes autoimmunity and colitis in mice. *Nature* 449:361-365.

59. Su, H. C., K. A. Leite-Morris, L. Braun, and C. A. Biron. 1991. A role for transforming growth factor-beta 1 in regulating natural killer cell and T lymphocyte proliferative responses during acute infection with lymphocytic choriomeningitis virus. *J Immunol* 147:2717-2727.

60. Buchmeier, M. J., R. M. Welsh, F. J. Dutko, and M. B. Oldstone. 1980. The virology and immunobiology of lymphocytic choriomeningitis virus infection. *Adv Immunol* 30:275-331.

61. Oida, T., L. Xu, H. L. Weiner, A. Kitani, and W. Strober. 2006. TGF-beta-mediated suppression by CD4+CD25+ T cells is facilitated by CTLA-4 signaling. *J Immunol* 177:2331-2339.

62. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci. 1986. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* 163:1037-1050.

63. Zuniga, E. I., L. Y. Liou, L. Mack, M. Mendoza, and M. B. Oldstone. 2008. Persistent virus infection inhibits type I interferon production by plasmacytoid dendritic cells to facilitate opportunistic infections. *Cell Host Microbe* 4:374-386.

64. Grotendorst, G. R., G. Smale, and D. Pencev. 1989. Production of transforming growth factor beta by human peripheral blood monocytes and neutrophils. *J Cell Physiol* 140:396-402.

65. Laouar, Y., F. S. Sutterwala, L. Gorelik, and R. A. Flavell. 2005. Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. *Nat Immunol* 6:600-607.

66. Albert, M. L., S. F. Pearce, L. M. Francisco, B. Sauter, P. Roy, R. L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med* 188:1359-1368.

67. Sevilla, N., D. B. McGavern, C. Teng, S. Kunz, and M. B. Oldstone. 2004. Viral targeting of hematopoietic progenitors and inhibition of DC maturation as a dual strategy for immune subversion. *J Clin Invest* 113:737-745.

68. Stewart, C. E., and I. Sayers. 2009. Characterisation of urokinase plasminogen activator receptor variants in human airway and peripheral cells. *BMC Mol Biol* 10:75.

69. Sidenius, N., C. F. Sier, H. Ullum, B. K. Pedersen, A. C. Lepri, F. Blasi, and J. Eugen-Olsen. 2000. Serum level of soluble urokinase-type plasminogen activator receptor is a strong and independent predictor of survival in human immunodeficiency virus infection. *Blood* 96:4091-4095.

70. Bugge, T. H., T. T. Suh, M. J. Flick, C. C. Daugherty, J. Romer, H. Solberg, V. Ellis, K. Dano, and J. L. Degen. 1995. The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. *J Biol Chem* 270:16886-16894.

71. Aung, H., M. Wu, J. L. Johnson, C. S. Hirsch, and Z. Toossi. 2005. Bioactivation of latent transforming growth factor beta1 by Mycobacterium tuberculosis in human mononuclear phagocytes. *Scand J Immunol* 61:558-565.